

**Anti-inflammatory agents for smoking cessation? Focus on cognitive deficits associated with nicotine withdrawal in male mice**

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## **Abstract**

Nicotine withdrawal is associated with cognitive deficits including attention, working memory, and episodic memory impairments. These cognitive deficits are a hallmark of nicotine abstinence which could be targeted in order to prevent smoking relapse. The underlying mechanisms, however, are poorly understood. In this study, memory impairment was observed in mice 4 days after the precipitation of nicotine withdrawal by the nicotinic antagonist mecamylamine. The presence of cognitive deficits correlated with microglial activation in the hippocampus and the prefrontal cortex. Moreover, an increased expression of neuroinflammatory markers including IL1 $\beta$ , TNF $\alpha$  and IFN $\gamma$  was found in both memory-related brain regions. Notably, flow cytometric analysis also revealed an enhancement of TNF $\alpha$  and IFN $\gamma$  plasmatic levels at the same time point during nicotine withdrawal. Impaired neurogenesis, as shown by reduction in the expression of the endogenous cell proliferation marker Ki67 and the early neuron marker doublecortin, was also associated with nicotine abstinence. Treatment with the non-psychoactive cannabinoid cannabidiol abolished memory impairment of nicotine withdrawal and microglia reactivity, reduced the expression of IL1 $\beta$  and IFN $\gamma$  in the hippocampus and the prefrontal cortex, respectively, and normalized Ki67 levels. The nonsteroidal anti-inflammatory drug indomethacin also prevented cognitive deficits and microglial reactivity during withdrawal. These data underline the usefulness of anti-inflammatory agents to improve cognitive performance during early nicotine abstinence.

**Keywords:** nicotine, withdrawal, memory, microglia, cytokines, cannabidiol, indomethacin

## **1. Introduction**

Tobacco consumption remains a leading cause of preventable disease and mortality causing, worldwide, more than 5 million deaths per year (Gowing et al, 2015). Most of smokers who try to quit relapse within the first week of abstinence, pointing to early withdrawal as a critical window in maintaining tobacco abstinence (Ashare and Schmidt, 2014). Cessation from tobacco smoking leads to a withdrawal syndrome characterized by physical, affective and cognitive symptoms (Hall et al, 2015). Increasing attention has focused on cognitive impairments that emerge during early smoking abstinence which include alterations in attention, working memory and episodic memory (Wesnes et al, 2013; Hall et al, 2015). Indeed, these cognitive deficits seem to be involved in smoking relapse (Patterson et al, 2010), and therefore enhancement of memory performance during abstinence may represent a novel target for nicotine dependence pharmacotherapies (Ashare et al, 2014; Ashare and Schmidt, 2014). Available rodent models reveal the presence of cognitive deficits during nicotine withdrawal (Hall et al, 2015) providing an excellent preclinical model to investigate new pharmacological agents with cognitive-enhancing properties.

Many disorders of the central nervous system (CNS) characterized by cognitive deficits may have an inflammatory component underneath (Blank and Prinz, 2013). In these pathological conditions, microglia activation, release of inflammatory cytokines and altered neurogenesis seem to influence cognitive performance (Kohman and Rhodes, 2013). A potential link between smoking and inflammation has been widely established although these studies have mainly focused on the relationship between inflammatory markers and increased cardiovascular risk (Wannamethee et al, 2005; Lo Sasso et al, 2016). However, the potential role of neuroinflammation in the cognitive deficits associated with early nicotine withdrawal remains to be elucidated.

Cannabidiol is a non-psychoactive cannabinoid from *Cannabis sativa* plant with known anti-inflammatory and neuroprotective properties (Fernández-Ruiz et al, 2013; Burstein, 2015). Some studies using inflammation-based models reveal that cannabidiol administration can attenuate cognitive deficits including spatial learning and memory, recognition memory and associative learning impairments (Osborne et al, 2017). Interestingly, a potential usefulness of cannabidiol in tobacco addiction has been shown in recent clinical studies (Morgan et al, 2013; Hindocha et al, 2018a). Thus, cannabidiol administration reduces cigarette consumption in tobacco smokers (Morgan et al, 2013), and the salience and pleasantness of cigarette cues after overnight abstinence in dependent smokers (Hindocha et al, 2018a).

In this study, we investigated the role of neuroinflammation and neurogenesis in the cognitive deficits of the nicotine withdrawal syndrome in male mice. We also evaluated the effects of cannabidiol, a promising anti-inflammatory compound, in memory performance during nicotine withdrawal and the potential mechanisms involved in this response. The effects of cannabidiol were compared to those produced by the classical nonsteroidal anti-inflammatory drug (NSAID) indomethacin.

## **2. Materials and Methods**

### **2.1. Animals**

All experiments were performed in 8-weeks old male C57BL/6J mice (Charles River). Mice were housed 4 per cage in a temperature ( $21 \pm 1^\circ\text{C}$ ) and humidity ( $55 \pm 10\%$ ) controlled room with a 12-h/12-h light/dark cycle (light between 08:00 to 20:00). All experiments took place during the light phase. Food and water were available ad libitum. Animal procedures were conducted in accordance with the guidelines of the European Communities Directive 2010/63/EU regulating animal research and approved by the local ethical committee (CEEA-IMAS-UPF), and the statement of compliance with standards for use of laboratory animals for foreign institutions nr. A5388-01 approved by the National Institutes of Health. The observer was blind to treatment in all the experiments.

### **2.2. Drugs**

(-)-Nicotine hydrogen tartrate salt [(-)-1-methyl-2-(3-pyridyl)pyrrolidine (+)-bitartrate salt] (Glentham Life Sciences) was dissolved in physiological saline (0.9% NaCl) and administered at the dose of 25mg/kg/day by using subcutaneously (sc) implanted osmotic minipumps. Nicotine dose was calculated as (-)-nicotine hydrogen tartrate salt. Mecamylamine hydrochloride (2mg/kg, Sigma) was dissolved in physiological saline and administered sc. Cannabidiol (3, 10 and 30mg/kg, THC Pharm) and indomethacin (2mg/kg, Sigma) were diluted in 5% ethanol, 5% cremophor, 90% saline and administered intraperitoneally (ip). The effects of cannabidiol and indomethacin in the cognitive deficits of nicotine withdrawal were performed in different groups of mice. Animals were randomly assigned to the different drug conditions. All drugs were administered in a volume of 10ml per kg of body weight. Doses of nicotine,

mecamylamine, cannabidiol and indomethacin were based on previous studies (Ren et al, 2009; Ho et al, 2015; Saravia et al, 2017; Stern et al, 2017).

### **2.3. Nicotine treatment and withdrawal**

Nicotine dependence was induced by using Alzet osmotic minipumps (Model 2002, Alzet®, Cupertino, USA). Minipumps previously filled with saline or nicotine solution were implanted sc in mice under brief isofluorane anesthesia. Minipumps delivered a constant sc flow in rate of 0.5µl/hour during 14 days. Nicotine concentration was adjusted to compensate differences in mice body weight. Thus, the average weighed mice received a dose of 25mg/kg/day of nicotine tartrate (equivalent to 8.12mg/kg/day nicotine free base). Fourteen days after minipump implantation, nicotine withdrawal syndrome was precipitated by the administration of the nicotinic receptor antagonist mecamylamine (2mg/kg). Systemic administration of mecamylamine in nicotine-treated rodents is a well-established model to study the neurobiological mechanisms involved in the different aspects that characterize the nicotine withdrawal syndrome (Muldoon et al, 2015; Qi et al, 2015; Parikh et al, 2016; Saravia et al, 2017), typically observed in abstinent smokers.

### **2.4. Evaluation of somatic signs of nicotine withdrawal**

Somatic signs of nicotine withdrawal were evaluated immediately after mecamylamine injection during a period of 25 minutes, as previously reported (Berrendero et al, 2005). The number of wet dog shakes, front paw tremors, and scratches were recorded. Body tremor, ptosis, teeth chattering, genital licks, and piloerection were scored 1 for appearance or 0 for nonappearance within each 5-minute period. The locomotor activity over 5-minute periods was rated 0, 1, or 2 (0 for inactivity, 1 for low activity, and 2 for

normal activity). A global withdrawal score was calculated for each animal by giving each individual sign a relative weight: 0.5 for each episode of wet dog shake, front paw tremor, and scratching; 1 for the presence of ptosis, genital licks, body tremor, piloerection, and teeth chattering, for each observation period of 5 minutes. The relative weight of locomotor activity for each 5 minute period was 0 for normal activity, 0.5 for low activity, and 1 for inactivity (Berrendero et al, 2005).

## **2.5. Object-recognition task**

In rodents, the object-recognition task is widely used to evaluate hippocampal-dependent learning, which is a trait of episodic memory (Ennaceur, 2010). We used this test to evaluate the presence of cognitive deficits during nicotine withdrawal, as previously reported (Borkar et al, 2017; Saravia et al, 2017). The novel object recognition test has been widely used to reveal memory impairment induced by different drugs of abuse including methamphetamine (Tran et al, 2018) and morphine (Morisot and Contarino, 2016). The object-recognition test was performed in a V-maze made of black plexiglas with 2 corridors (30 cm long x 4.5 cm wide) set in V with a 90° angle and 15 cm high walls. On the 13th day after minipump implantation mice were habituated to the V-maze for 9 minutes. On the 14th day, mice were placed back in the maze for 9 minutes where 2 identical objects were presented (training phase). Nicotine withdrawal was precipitated 20 minutes after training by the administration of mecamylamine (2mg/kg), allowing to test the influence of nicotine withdrawal on memory consolidation. To evaluate memory 24 hours after precipitation of withdrawal mice were placed in the V-maze on the 15th day for 9 minutes where one of the familiar objects used the day before was replaced by a novel object. Cannabidiol (10mg/kg) or vehicle were injected immediately after training, 20 minutes before mecamylamine injection in saline and nicotine-treated mice.

To evaluate memory 4 days after nicotine withdrawal, we used a similar methodology as the previously described except that mice had another training session on day 17 and the test was performed on day 18. Cannabidiol (3, 10 and 30mg/kg), indomethacin (2mg/kg), or the corresponding vehicles were injected once daily during four days (immediately after training on day 14, and on days 15, 16 and 17) in saline and nicotine-treated mice.

During the test session, the total time that mice spent exploring each of the 2 objects (novel, T<sub>n</sub> and familiar, T<sub>f</sub>) was recorded. Object exploration was defined as the orientation of the nose to the object at a distance of  $\leq 2$  cm. A discrimination index was calculated as the difference between the times spent exploring either the novel or familiar object divided by the total time exploring the 2 objects  $D.I. = [T_n - T_f] / [T_n + T_f]$ . A higher discrimination index is considered to reflect greater memory retention for the familiar object.

## **2.6. Immunofluorescence studies**

Four days after the precipitation of nicotine withdrawal, immediately after the object recognition test, saline and nicotine-treated mice were deeply anesthetized and intracardially perfused with 4% paraformaldehyde (PFA) solution. Brains were removed from skull and post-fixed in PFA for 24 hours at 4°C. Then, brains were transferred to a solution of 30% sucrose in PB 0.1M and kept at 4°C. Coronal sections of 30 $\mu$ m were obtained using a microtome and slices were stored in a 5% sucrose PB 0.1M solution. Brain areas coordinates relative to bregma were: hippocampus (from -1.34 to -2.46 mm), prefrontal cortex (PFC) (from 1.98 to 1.70 mm), and dorsal striatum (from 0.86 to 0.38 mm). Free floating slices were rinsed in PB 0.1M and after blocked in a solution containing 3% donkey serum and 0.3% Triton X-100 in PB 0.1M (DS-T-PB) during 2



hours at room temperature. Slices were incubated overnight in DS-T-PB at 4°C with the primary antibody to Iba1 (1:500, rabbit, Wako), Ki67 (1:150, mouse, Abcam) or doublecortin (DCX) (1:200, goat, Santa Cruz Biotechnology). Next day, after 3 rinses with PB 0.1M, slices were incubated with their corresponding secondary antibody AlexaFluor-555 donkey anti-rabbit (1:500), AlexaFluor-488 donkey anti-mouse (1:500) or AlexaFluor-555 donkey anti-goat (1:500) from Life Technologies at room temperature for 2 hours in DS-T-PB. Then, slices were rinsed three times and mounted with Fluoromount-G™ onto glass slides coated with gelatin.

## **2.7. Microglia morphological analysis**

Images from Iba1 stained cells were acquired using a confocal microscope (Leica TCS Sp5 STED) with an oil immersion lens 40x/1.3 and 1x zoom. A 30µm z-stack image with 0.29µm depth intervals was obtained from every slice. Microglial cell architecture was examined using ImageJ software on flattened images. The perimeter of microglial soma was measured using the tool “Freehand line” and the option “Analyze and Measure”. Four images per brain area of a minimum of 4 animals per group were analyzed.

## **2.8. Ki67 and doublecortin analysis**

Images from Ki67 and DCX labeling were obtained using a confocal microscope (Leica TCS Sp5 STED) with 10x/0.40 dry lens and 2x zoom. A 30µm z-stack image with 1.01 µm depth intervals was obtained from every slice. All DCX images were acquired using the same laser gain and intensity. For Ki67 and DCX analysis z-stack images were flattened. Quantification of Ki67+ cells in the subgranular zone (SGZ) of the hippocampus was performed using the ImageJ tool “Multi-point” to mark and count

Ki67+ cells. SGZ area was the result of its length (measured using the “Segmented line” tool) x its wide (25µm). Density of Ki67+ cells was calculated as Ki67+ cells divided per SGZ area. Four images per brain area of a minimum of 4 animals per group were analyzed. DCX fluorescence intensity in the granular cell layer was measured using the ImageJ software. Slices for all experimental groups were processed simultaneously to avoid difference in staining. Additionally, microscope settings were tailored to obtain images without pixel saturation but maintaining the same gain intensity. Images were adjusted to a dark background and processed to a binary image. Using the “Segmented line” tool and “Analyze and Measure” option, we measured the integrated density of the granular cell layer. Integrated density represents the mean grey value x area. To reduce bias, the integrated density of a single slice was the mean of 3 different measurements. Relative DCX integrated density (as evaluated in Walker et al, 2007) is represented as a percentage of the control treatment. Four images per brain area of a minimum of 4 animals per group were analyzed.

## **2.9. RNA extraction and reverse transcription**

Hippocampus and PFC tissues from control and nicotine-treated mice were extracted 4 days after the precipitation of nicotine withdrawal and were immediately frozen and store at -80°C. Isolation of total RNA was performed using an RNeasy Mini kit (QIAGEN) for hippocampus or the RiboPure™ RNA Purification Kit (Invitrogen) for PFC according to the manufacturer’s instructions. The quality of the total RNA was assessed by the spectrophotometric ratio of A260/A280 (1.9:2.1). Total RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed with 0.3 µg of total RNA from each animal to produce cDNA in a 20µl reaction with 200 units of SuperScript III Reverse Transcriptase (Invitrogen)

and 500-ng oligo(dT) primers. Reverse transcriptase reactions were carried out at 25°C for 10 minutes, then at 42°C for 50 minutes, and at 70°C for 15 minutes. The resulting cDNAs from these reactions were stored at –20°C until use.

## **2.10. Quantitative real-time PCR analysis**

Quantitative real-time PCR was carried out with a QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems) using the SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. All the samples were tested in triplicate, and the relative expression values were normalized to the expression value of GAPDH. The primers sequences used for IL1 $\beta$ , TNF $\alpha$ , IL10, IL6, IFN $\gamma$ , COX1, COX2, BDNF and GAPDH are indicated in Supplemental Table S1. Samples were analyzed by the  $\Delta\Delta C_t$  method.  $\Delta\Delta C_t$  values were calculated as the  $\Delta C_t$  of each test sample (different pharmacological treatments) minus the mean  $\Delta C_t$  of the calibrator samples (saline-vehicle group) for all the genes analyzed. The fold change was calculated using the equation  $2(-\Delta\Delta C_t)$ .

## **2.11. Flow cytometric analysis**

Blood samples from saline and nicotine-treated mice were collected at 2 different time points: 4 and 8 days after the precipitation of nicotine withdrawal. All blood samples were collected into 1.5mL EDTA coated tubes containing 10ul of EDTA 0.5M. The samples were centrifuged at 3000 rpm for 15 min at 4°C and plasma was stored at –80°C. A multiplex bead-array assay was used to quantify cytokines (TNF $\alpha$ , IL10 and IFN $\gamma$ ) in plasma samples. These cytokines were chosen according to mouse antibodies availability and previous analysis of mRNA expression in hippocampus and PFC.

The method is based on the binding of cytokines to different capture beads. This capture beads exhibit defined fluorescence properties and they can be analyzed by standard fluorescence cytometry. The MACSplex Cytokine kit (Miltenyi Biotec) for mouse was used according to the manufacturer's instructions to detect TNF $\alpha$  (MACSplex TNF $\alpha$  Reagents Kit, mouse), IL10 (MACSplex IL10 Reagents Kit, mouse) and IFN $\gamma$  (MACSplex IFN $\gamma$  Reagents Kit, mouse) (Miltenyi Biotec). Plasma samples were thawed at room temperature and then placed on wet ice for duration of analysis. One vial of mixed standards was freshly reconstituted in 0.2 mL of assay diluent, and then was serial diluted. The concentrations of standards for each cytokine were 0, 3.2, 16, 80, 400, 2000, and 10000 pg/mL. Three types of cytokine capture beads were freshly mixed in equal amounts (2 $\mu$ L of each cytokine capture beads per test) and fill up to 20 $\mu$ L of assay diluent. To perform the assay, 20 $\mu$ L of the mixed beads were incubated with 50 $\mu$ L of standards or plasma samples in a MACSplex filter plate at room temperature for 2 hours. After 3 washes, the beads were resuspended in 100 $\mu$ L of the mix (2 $\mu$ L of each cytokine detection reagent per test) and were incubated at room temperature for 1 hour. The measurement was performed by means of the MACSQuant 10 Analyzer (Miltenyi Biotec) and the data were automatically analyzed using the respective tool from the MACSQuantify software.

## **2.12. Data analysis**

Data were analyzed using unpaired Student t test, or two-way ANOVA with nicotine and cannabidiol or indomethacin treatments as between factors of variation. Subsequent post hoc analysis (Newman-Keuls test) was used when required. The Pearson correlation coefficient was used to analyze the relationship between discrimination index values and microglial perimeter soma. All data were analyzed using Statistica (StatSoft) software.

Comparisons were considered to be statistically significant when the level of significance was  $p < 0.05$ .

### **3. Results**

#### **3.1. Microglial activation and impaired neurogenesis correlate with the cognitive deficits during nicotine withdrawal**

The object-recognition task was used to assess the presence of cognitive deficits during nicotine withdrawal. Nicotine abstinence was induced by mecamylamine administration (2mg/kg) 20 minutes after the first training session, and memory was evaluated 4 days after withdrawal precipitation (Fig. 1A). Memory impairment and structural plasticity changes during nicotine withdrawal were previously reported at this time point (Saravia et al, 2017). Mecamylamine does not affect memory by itself at this dose (Saravia et al, 2017). Nicotine-treated mice exhibited a lower discrimination index compared with controls ( $p < 0.01$ ) (Fig. 1B), as previously reported (Saravia et al, 2017). Memory performance differences were not due to locomotor activity alterations since both experimental groups showed similar times of exploration (Fig. 1C).

Cognitive deficits have been associated with inflammatory responses in many CNS pathologies (Zhao et al, 2013; McKim et al, 2016). Under normal conditions, microglia is found in a surveillance state as indicated by a ramified morphology with small soma. However, in response to a pathological stimulus, microglia morphology shifts to a reactive state characterized by retracted ramifications and larger amoeboid soma (Kohman and Rhodes, 2013). To evaluate the presence of neuroinflammation during nicotine withdrawal, we analyzed possible changes in microglia morphology 4 days after the precipitation of withdrawal. A significant increase in the perimeter of microglia soma was observed in nicotine abstinent mice in the stratum pyramidale ( $p < 0.01$ ) and stratum

radiatum ( $p < 0.05$ ) of the CA1 region of the hippocampus (Fig. 1D), and in the granular cell layer ( $p < 0.01$ ) and hilus ( $p < 0.01$ ) of hippocampal dentate gyrus (Fig. 1E). This effect was also found in the PFC ( $p < 0.01$ ), whereas microglial reactivity remained unaltered in the dorsal striatum (Fig. 1F). Moreover, chronic nicotine treatment did not modify microglia morphology in the CA1 region of the hippocampus (stratum pyramidale and stratum radiatum) and hippocampal dentate gyrus (granular cell layer and hilus) (Fig. S1), suggesting that microglial activation was due specifically to the withdrawal syndrome. These results reveal that nicotine withdrawal involves changes in microglial reactivity which are associated with the presence of cognitive deficits. Indeed, a significant negative correlation between microglial activation (soma perimeter) and memory performance (discrimination index) was found in the granular cell layer (Fig. 1G) and hilus (Fig. 1H) of the hippocampal dentate gyrus, and the PFC (Fig. 1I) in control and nicotine-withdrawn mice. A clear tendency ( $p = 0.07$ ) was also observed in the stratum pyramidale of the CA1 region of the hippocampus (Fig. S2A).

We next investigated hippocampal neurogenesis in the subgranular zone (SGZ) of the dentate gyrus, since an inflammatory environment may alter this neurobiological process (Kohman and Rhodes, 2013). Moreover, adult neurogenesis has a role in cognition, learning and memory (Yau et al, 2015). A significant decrease of the endogenous cell proliferation marker Ki67 was observed in nicotine-treated mice 4 days after withdrawal precipitation ( $p < 0.05$ ) (Fig. 1J and L). Immunoreactivity for the early neuron marker DCX was also significantly lower in nicotine-treated group ( $p < 0.05$ ) (Fig. 1K and L) in comparison with control mice. Overall, our results indicate that enhanced microglial reactivity and altered neurogenesis are related to the cognitive deficits of nicotine abstinence.

### **3.2. Cannabidiol prevents the cognitive deficits associated with nicotine withdrawal through modulation of microglial reactivity**

As previously described, cognitive deficits of nicotine withdrawal course with an inflammatory phenotype of microglia. We next studied the effect of cannabidiol, a non-psychoactive cannabinoid with anti-inflammatory properties (Burstein, 2015), in the memory impairment and microglial reactivity during nicotine withdrawal (Fig. 2A). Subchronic treatment with cannabidiol was administered at the doses of 3, 10 and 30 mg/kg (once daily during 4 days) (Fig. 2A). Cannabidiol prevented the memory impairment of the nicotine withdrawal syndrome at the doses of 10 and 30 mg/kg in the object recognition test, while this compound was ineffective at the dose of 3 mg/kg (Fig. 2B). Thus, two-way ANOVA showed an interaction between cannabidiol and nicotine ( $F_{3,60}=4.96$ ,  $p<0.05$ ). Post hoc analysis revealed the improvement of memory induced by the doses of 10 and 30mg/kg ( $p<0.01$ ) of cannabidiol. Total exploration time was not modified in these experiments (Fig. 2C).

We next evaluated whether the improvement of cannabidiol on memory processing during nicotine abstinence could be due to the modulation of microglia reactivity. For this purpose, we used the lowest effective dose of cannabidiol (10 mg/kg). Four days after withdrawal precipitation, subchronic treatment with cannabidiol reduced microglial active phenotype in the CA1 field and the dentate gyrus of the hippocampus, and in the PFC. Thus, the enhanced perimeter of microglia soma observed during nicotine withdrawal was reversed in mice treated with cannabidiol in the stratum pyramidale ( $p<0.01$ ) and in the stratum radiatum ( $p<0.01$ ) (cannabidiol x nicotine interaction:  $F_{1,16}=4.89$ ,  $p<0.05$  and  $F_{1,16}=7.56$ ,  $p<0.05$ , respectively) (Fig. 2D, E and F). Cannabidiol also prevented microglial activation in the granular cell layer ( $p<0.01$ ) and in the hilus ( $p<0.01$ ) of the hippocampal dentate gyrus (Fig. 2 G and H) (nicotine effect:  $F_{1,16}=23.76$ ,

$p < 0.001$ , cannabidiol effect:  $F_{1,16} = 18.52$ ,  $p < 0.001$  and nicotine effect:  $F_{1,16} = 15.32$ ,  $p < 0.001$ , cannabidiol effect:  $F_{1,16} = 29.29$ ,  $p < 0.001$ , respectively), although no significant interactions were found in these areas. The same effect was observed in the PFC ( $p < 0.01$ ), as shown by two-way ANOVA (nicotine x cannabidiol interaction  $F_{1,12} = 20.00$ ,  $p < 0.001$ ) (Fig. 2I). These results suggest that cannabidiol prevents memory deficits during nicotine abstinence through the modulation of microglia reactivity in key brain regions related to cognitive processes.

In an additional experiment, we investigated whether cannabidiol could affect the severity of nicotine physical dependence (Fig. S3A). For this purpose, acute cannabidiol injection (10 mg/kg) was administered immediately after the training session, 20 minutes before the precipitation of nicotine withdrawal with mecamylamine. Somatic signs of nicotine withdrawal were evaluated during 25 minutes after withdrawal precipitation (Fig. S3A). The severity of nicotine abstinence (nicotine effect,  $F_{1,33} = 32.84$ ,  $p < 0.01$ ) was not significantly altered in mice pretreated with cannabidiol (cannabidiol x nicotine interaction:  $F_{1,33} = 4.56$ , NS) (Fig. S3D). Individual signs of withdrawal are shown in Supplemental Table S2. In contrast, similar cannabidiol treatment prevented the cognitive deficits observed 24 hours after nicotine withdrawal precipitation in the object-recognition test, as shown by two-way ANOVA (cannabidiol x nicotine interaction:  $F_{1,30} = 13.84$ ,  $p < 0.01$ ) and post hoc analysis ( $p < 0.01$ ) (Fig. S3B). Total exploration time was not modified in this experiment (Fig. S3C). These data suggest that different neurobiological mechanisms seem to be involved in the somatic signs and cognitive deficits of nicotine abstinence, as previously reported (Saravia et al, 2017).



### 3.3. Expression of inflammatory markers is enhanced during nicotine withdrawal

Reactive microglia has been described as a sustained source of inflammatory molecules (Kohman and Rhodes, 2013), which have been directly implicated in hippocampus-dependent learning and memory (Blank and Prinz, 2013). Therefore, we studied the expression of some inflammatory cytokines 4 days after nicotine withdrawal precipitation and its regulation by cannabidiol subchronic treatment (10mg/kg, once daily during 4 days) in the hippocampus and the PFC. Quantitative analysis of the mRNA of inflammatory markers in hippocampal homogenates showed an enhanced expression of IL1 $\beta$ , TNF $\alpha$ , IL10 and IFN $\gamma$  (Fig. 3A). Two-way ANOVA analysis showed a significant effect of nicotine treatment in the expression of TNF $\alpha$  ( $F_{1,23}=10.57$ ,  $p<0.01$ ) and IL10 ( $F_{1,23}=17.30$ ,  $p<0.001$ ). Two-way ANOVA also revealed a significant interaction between nicotine and cannabidiol treatment in the mRNA levels of IL1 $\beta$  ( $F_{1,23}=9.65$ ,  $p<0.01$ ) and IFN $\gamma$  ( $F_{1,23}=5.77$ ,  $p<0.05$ ). Interestingly, post hoc analysis showed a normalization of the expression of IL1 $\beta$  ( $p<0.05$ ) by cannabidiol, while this compound did not affect the increase of IFN $\gamma$  induced by nicotine abstinence ( $p=0.32$ ) (Fig. 3A). Neither nicotine abstinence nor cannabidiol administration altered the expression of COX1, COX2, IL6 and BDNF in the hippocampus (Fig. S4). Similarly to the effects observed in the hippocampus, nicotine withdrawal increased the expression of IL1 $\beta$  (nicotine treatment:  $F_{1,26}=9.76$ ,  $p<0.01$ ), and IFN $\gamma$  (nicotine treatment:  $F_{1,26}=6.84$ ,  $p<0.05$ ) in the PFC (Fig. 3B). No effect was found in IL10 mRNA levels (nicotine treatment:  $F_{1,25}=0.06$ , NS). Two-way ANOVA also revealed a significant interaction between nicotine and cannabidiol treatment in the expression of TNF $\alpha$  ( $F_{1,26}=7.35$ ,  $p<0.01$ ). Cannabidiol reversed the increase of this cytokine induced by nicotine withdrawal, as indicated by post hoc analysis ( $p<0.05$ ) (Fig. 3B). In contrast to the result obtained in the hippocampus, cannabidiol did not alter IL1 $\beta$  expression in the PFC (Fig. 3B). These results suggest that cannabidiol

could improve memory performance during nicotine abstinence normalizing the expression of IL1 $\beta$  and TNF $\alpha$  in the hippocampus and the PFC, respectively. However, since alterations in mRNA levels do not necessarily translate into changes in synthesis and release of cytokines, the evaluation of protein levels of these cytokines in the hippocampus and PFC will require special attention in future experiments.

We next investigated the role of peripheral pro-inflammatory cytokines as potential biomarkers of the cognitive deficits associated with early nicotine abstinence, given the increased expression of several of these molecules in the hippocampus and the PFC. Notably, flow cytometric analysis revealed a significant increase of plasmatic levels of TNF $\alpha$  ( $p < 0.05$ ) and IFN $\gamma$  ( $p < 0.01$ ), but not IL10, 4 days after the precipitation of nicotine withdrawal (Fig. 3C). Under similar experimental conditions, mice completely recovered from cognitive impairment 8 days after abstinence precipitation (Saravia et al, 2017). Interestingly, differences in cytokine plasmatic levels were not observed at this time point (Fig. S5), suggesting that cognitive deficits of nicotine withdrawal are associated with an enhancement of peripheral inflammatory cytokines.

#### **3.4. Effects of cannabidiol on neurogenesis during nicotine withdrawal**

As previously described, cognitive deficits of nicotine withdrawal are associated with impaired neurogenesis in the SGZ of the hippocampal dentate gyrus. To evaluate the effect of cannabidiol on neurogenesis, mice underwent a subchronic cannabidiol treatment (10mg/kg, once daily during 4 days). Interestingly, cannabidiol showed a clear tendency to increase Ki67+ cells 4 days following nicotine abstinence precipitation as shown by two-way ANOVA (nicotine x cannabidiol interaction:  $F_{1,20} = 10.07$ ,  $p < 0.01$ ) and subsequent post hoc analysis ( $p = 0.06$ ) (Fig. 4A and B). Regarding DCX

immunoreactivity, cannabidiol did not modify ( $p=0.20$ ) the expression of this marker of young neurons in spite of a significant interaction between nicotine and cannabidiol treatment ( $F_{1,20}=6.60$ ,  $p<0.05$ ) (Fig. 4C). These results indicate that the improvement of cognitive function induced by cannabidiol administration may involve a promotion of cell proliferation in the hippocampus.

### **3.5. The nonsteroidal anti-inflammatory drug indomethacin counteracts cognitive deficits and microglia reactivity associated with nicotine withdrawal**

We next evaluated the effects of indomethacin, a NSAID, on the cognitive impairment associated with nicotine abstinence. This study was based on the fact that modulation of the pro-inflammatory phenotype seems to be a key element to prevent withdrawal-related cognitive deficits. Interestingly, subchronic treatment with indomethacin (2mg/kg, once daily during 4 days) reversed memory deficiency 4 days following precipitation of nicotine abstinence, as revealed by two-way ANOVA (nicotine x indomethacin interaction:  $F_{1,45}=4.95$ ,  $p<0.05$ ), and post hoc analysis ( $p<0.01$ ) (Fig. 5A). Total time of exploration was similar in the different experimental groups (Fig. 5B). Indomethacin treatment ameliorated the effects of nicotine withdrawal on microglial reactivity. Thus, two-way ANOVA revealed a significant interaction between nicotine and indomethacin in the stratum pyramidale ( $F_{1,12}=9.19$ ,  $p<0.01$ ) and the stratum radiatum ( $F_{1,12}=6.70$ ,  $p<0.05$ ) of the CA1 hippocampal region. Subsequent post hoc analysis showed that pretreatment with indomethacin reduced microglial soma perimeter in nicotine abstinent mice in both regions ( $p<0.01$ ) (Fig. 5C and D). Similarly, indomethacin reduced the activation of microglia in the granular cell layer ( $p<0.01$ ) and hilus ( $p<0.05$ ) of the hippocampal dentate gyrus, as shown by two-way ANOVA (nicotine x indomethacin interaction:  $F_{1,12}=8.04$ ,  $p<0.05$  and  $F_{1,12}=5.88$ ,  $p<0.05$ , respectively). These data suggest

that the modulation of inflammation is a potential novel target for alleviating cognitive deficits of nicotine abstinence.

#### **4. Discussion**

Our results identify new mechanisms involved in the cognitive deficits associated with nicotine abstinence and support novel possible therapeutic strategies. Microglial activation and increased expression of pro-inflammatory cytokines were found in the hippocampus and the PFC 4 days after nicotine withdrawal in male mice, when the cognitive deficits are still present. Impaired neurogenesis was also observed in the SGZ of the dentate gyrus in the hippocampus during nicotine withdrawal. Notably, cannabidiol and indomethacin prevented memory impairment of nicotine withdrawal and most of these neurobiological alterations. These data suggest the usefulness of anti-inflammatory agents to normalize cognition during early abstinence, which could have a therapeutic interest since these cognitive deficits are related to increased risk of relapse to tobacco consumption in humans.

Around 50% to 75% of smokers relapse during the first week of a quit attempt (Ashare et al, 2014). Increasing attention has focused on cognitive impairments that emerge during smoking abstinence given that these deficits may predict relapse (Patterson et al, 2010; Loughhead et al, 2015). FDA-approved medications, such as varenicline and bupropion, can reverse abstinence-induced working memory deficits as revealed in abstinent smokers tested during medication in comparison with placebo (Loughhead et al, 2010; Perkins et al, 2013). Although the mechanisms that mediate nicotine withdrawal-induced cognitive impairments are not clear, different studies have suggested a role for CB1 cannabinoid receptors (Evans et al, 2016; Saravia et al, 2017), neuropeptide CART (Borkar et al, 2017),  $\alpha 4\beta 2$  nicotinic acetylcholine receptors (Yildirim et al, 2015) or noradrenaline (Davis and Gould, 2007) in these deficits.

The precipitation of nicotine withdrawal with mecamylamine after the training phase revealed a memory consolidation impairment in the object-recognition task, which was present at least during 4 days, as previously reported (Saravia et al, 2017). In agreement,

several studies have shown similar cognitive deficits in other hippocampal-dependent tasks in rodents such as the spatial object-recognition and contextual fear conditioning during spontaneous (Wilkinson et al, 2013; Yildirim et al, 2015) and precipitated nicotine withdrawal (Raybuck and Gould, 2009). Under similar experimental conditions, chronic nicotine exposure did not modify memory performance (Saravia et al, 2017) suggesting that the cognitive deficits observed were specific of the withdrawal period. In agreement, chronic nicotine administration does not affect memory performance in rodents (Raybuck and Gould, 2009; Davis et al, 2005).

Microglia cells play crucial roles in normal development, plasticity and maintenance of neural circuits (Wake et al, 2013). Under conditions of disturbed brain homeostasis, microglia structure and function are rapidly altered. Although morphological and functional changes are essential for coping with pathogenic challenges (Yirmiya et al, 2015), these changes can also compromise and interfere with the normal brain physiological function (Wake et al, 2013). Memory impairment during nicotine withdrawal was associated with microglial activation in key brain regions regulating cognitive processes. Thus, amoeboid morphology characterized by an enlargement of the soma perimeter was found in the CA1 field and dentate gyrus of the hippocampus, and in the PFC. This alteration was not observed in other brain areas such as the dorsal striatum. The stressful condition occurring during nicotine withdrawal could be responsible for this microglial activation since ample evidence demonstrates how stress exposure can induce changes in microglia structure and function leading to cognitive deficits (Yirmiya et al, 2015; Tay et al, 2017). Activation of microglia in the PFC and hippocampus has been related to the cognitive impairments that characterize stress-related conditions (Hinwood et al, 2013; McKim et al, 2016). Corticotropin-releasing factor is involved in the dysphoria and anxiety-like behavior observed during nicotine withdrawal (Bruijnzeel,

2017), while blockade of corticosterone effects abolishes acute or chronic stress-induced microglial proliferation and activation (Frank et al, 2012; de Pablos et al, 2014). Congruent with microglial activation, we found an increased mRNA expression of several inflammatory markers such as IL1 $\beta$ , TNF $\alpha$ , and IFN $\gamma$  in both the hippocampus and the PFC during nicotine withdrawal. Overexpression of pro-inflammatory cytokines in the CNS has been associated with several neuropsychiatric disorders including depression, Alzheimer's disease and Parkinson's disease (Borsini et al, 2015). Notably, an elevation of plasmatic levels of TNF $\alpha$  and IFN $\gamma$  was also observed 4 days after nicotine withdrawal precipitation suggesting that these cytokines could be investigated as biomarkers of the cognitive deficits present during tobacco abstinence. Indeed, cytokine levels in plasma were normalized by the 8<sup>th</sup> day of withdrawal when mice are recovered from memory impairment under our experimental conditions (Saravia et al, 2017). Inflammatory cytokines including IL1 $\beta$ , TNF $\alpha$ , and IFN $\gamma$  are also key modulators of neurogenesis (Borsini et al, 2015), which has a critical role in mediating human brain functions including memory formation and cognition (Kohman and Rhodes, 2013). Interestingly, cognitive deficits of nicotine withdrawal were associated with reduced expression of cell proliferation and young neuron markers in the SGZ of the hippocampus. Taken together, these results suggest that inflammation-induced deficits in cognitive performance during nicotine withdrawal could be related to the reductions in hippocampal neurogenesis.

A dramatic increase in the interest of the non-psychoactive cannabinoid cannabidiol has been revealed in recent years. This compound has anti-inflammatory and neuroprotective properties (Fernández-Ruiz et al, 2013; Burstein, 2015), with potential benefits for the treatment of motivational disorders such as drug addiction, anxiety and depression (Zlebnik and Cheer, 2016). Subchronic treatment with cannabidiol prevented the memory impairment in the object-recognition task and the activation of microglia in the

hippocampus and the PFC observed 4 days after nicotine withdrawal. In agreement, cannabidiol has been shown to improve object-recognition memory in preclinical models that course with cognitive impairment, including schizophrenia (Gomes et al, 2015), Alzheimer's disease (Cheng et al, 2014), brain ischemia (Pazos et al, 2012) and cerebral malaria (Campos et al, 2015). Cannabidiol also normalized the increased expression of IL1 $\beta$  and TNF $\alpha$  in the hippocampus and the PFC, respectively, observed during nicotine withdrawal. Although present data about possible regulation of neuroinflammatory markers by cannabidiol in preclinical models of cognitive impairment are sparse and controversial (Osborne et al, 2017), some studies suggest a possible involvement of TNF $\alpha$  in the mechanisms underlying the ability of cannabidiol to improve cognition (Osborne et al, 2017). In addition, cannabidiol treatment tended to promote cell proliferation during nicotine withdrawal in the SGZ of the hippocampal dentate gyrus. Accordingly, an increase of hippocampal neurogenesis following cannabidiol administration was observed in a rat model of Alzheimer's disease (Esposito et al, 2011). As a whole, these data indicate that cannabidiol might improve cognitive performance during nicotine withdrawal through the modulation of inflammation and cell proliferation. Interestingly, preliminary findings in humans show that cannabidiol reduces cigarette consumption (Morgan et al, 2013), and pleasantness of cigarette cues after overnight abstinence (Hindocha et al, 2018a) in tobacco smokers. In contrast, a recent study has shown that acute administration of a single dose of cannabidiol did not improve memory performance in tasks previously shown to be impaired during cigarette abstinence (Hindocha et al, 2018b). The effects of repeated administration of different doses of cannabidiol on cognition should be evaluated in dependent smokers (Hindocha et al, 2018b), considering the bell-shaped dose response effects widely reported for this compound and higher effectiveness usually revealed after repeated administration (Zuardi et al, 2017).



We next investigated the effects of the NSAID indomethacin in order to verify whether the improvement of memory performance by cannabidiol could be generalized to other anti-inflammatory agents. Therefore, although interesting, no direct comparison between the effects of cannabidiol and indomethacin was made. Notably, indomethacin administration rescued memory impairment during nicotine withdrawal and prevented concurrent microglial activation in the CA1 field and dentate gyrus of the hippocampus. Congruent with this observation, indomethacin abolished recognition memory deficits reveal during intermittent ethanol intoxication (Pascual et al, 2007), and in a mouse model of Alzheimer's disease (Balducci et al, 2017), but not in a model of social defeat stress (Duque et al, 2017). Indomethacin is a broad spectrum NSAID that inhibits cyclooxygenase (Cox)-1 and -2 activity, although hippocampal mRNA expression of Cox-1 and -2 was not modified under our experimental conditions.

The use only of male mice is a limitation of this study since several reports show evidences for sex differences in the central effects of nicotine in rodent models (Flores et al, 2017; Peartree et al, 2017). Although social factors are crucial in smoking dependence in humans, differences in the pharmacokinetic properties of nicotine or the effect of gonadal hormones may underlie some of the sex differences observed (Pogun and Yararbas, 2009). Moreover, recent studies show that sex could modulate the effects of tobacco smoke on inflammation (Ashare and Wetherill, 2018). A pilot study in humans also suggested the presence of cognitive differences between sexes during tobacco abstinence (Merrit et al, 2012). Therefore, the investigation about how males and females might differ in the effects of tobacco abstinence on memory and cognition will require special attention in the future. **On the other hand, the evaluation of the neurobiological mechanisms described here under spontaneous nicotine withdrawal would be also**

interesting since these experimental approach might add translational value to the results obtained in our study.

In summary, our work reveals for the first time an inflammatory process associated with the cognitive deficits that characterize early nicotine abstinence. Moreover, these findings underline the efficacy of anti-inflammatory agents to improve cognitive deficits during nicotine withdrawal. Given that the presence of cognitive alterations are associated with increased smoking relapse risk, our results identify anti-inflammatory drugs as new potential therapeutic strategies for nicotine dependence.

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The authors declare no competing interests.

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## Figure legends

### Figure 1

Microglial activation and impaired neurogenesis are associated with the cognitive deficits during nicotine withdrawal. (A) Schematic representation of the experimental design for (B-C) behavioral procedures. Memory and perimeter of microglia soma were evaluated 4 days after the precipitation of nicotine withdrawal by mecamylamine administration (2mg/kg). (B) Discrimination index and (C) total exploration time obtained in the novel object-recognition paradigm (n = 7 mice per group). (D-F) Perimeter of microglia soma in the (D) stratum pyramidale and stratum radiatum of the CA1 region of the hippocampus, the (E) granular cell layer and hilus of the hippocampal dentate gyrus, the (F) dorsal striatum and the prefrontal cortex (n = 4-7 mice per group). (D-F) Representative images of microglial cells obtained via confocal microscopy after direct labeling with rabbit polyclonal antiserum to Iba-1 in nicotine abstinent and control mice. Scale bar represents 20 $\mu$ m. (G-I) Correlation between memory performance (discrimination index values) and microglial activation (soma perimeter values) in the (G) granular cell layer and the (H) hilus of the hippocampal dentate gyrus, and (I) prefrontal cortex. (J-L) Neurogenesis in the SGZ of the hippocampus assessed by the (J) density of Ki67+ cells (n = 5-6 mice per group) and the (K) percentage of control doublecortin (DCX) immunoreactivity integrated density (n = 6 mice per group). (L) Representative images of both Ki67 and DCX markers in nicotine abstinent and control mice. Scale bar represents 100 $\mu$ m. Data are expressed as mean  $\pm$  SEM. \* $p$ <0.05, \*\* $p$ <0.01 compared with the saline group. DG, dentate gyrus; SGZ, subgranular zone; DCX, doublecortin. Arrows indicate Ki67+ cells.

## Figure 2

Cannabidiol prevents the cognitive deficits of nicotine abstinence through the modulation of microglial reactivity. (A) Schematic representation of the experimental design for (B-C) behavioral procedures to evaluate the effects of subchronic treatment with cannabidiol on memory impairment during nicotine withdrawal. (B-C) Cannabidiol was administered immediately after the training session 20 minutes before the precipitation of nicotine withdrawal. Cannabidiol (3, 10 and 30mg/kg) was injected once daily during 4 days and discrimination index (B) and total exploration time (C) was obtained 4 days after the training ( $n = 8-10$  mice per group). (E-J) Soma perimeter of microglial cells in the (E) stratum pyramidale and (F) stratum radiatum of the CA1 region of the hippocampus, the (H) granular cell layer and (I) hilus of the hippocampal dentate gyrus, and (J) prefrontal cortex ( $n = 4-5$  mice per group) (G) Representative images of microglial cells from the CA1 area of the hippocampus obtained via confocal microscopy after direct labeling with rabbit polyclonal antiserum to Iba-1. Scale bar represents  $20\mu\text{m}$ . Data are expressed as mean  $\pm$  SEM.  $**p < 0.01$  compared with the saline-treated groups in (B);  $**p < 0.01$  compared with the saline-vehicle group,  $##p < 0.01$  compared with the nicotine-vehicle group;  $\$p < 0.05$  compared with the saline- vehicle group. CBD, cannabidiol; DG dentate gyrus.

## Figure 3

Cannabidiol modulates the expression of neuroinflammatory markers during nicotine withdrawal. (A-B) Expression of inflammatory markers IL1b, TNF $\alpha$ , IL10 and IFN $\gamma$  in homogenates of (A) hippocampus and (B) prefrontal cortex of nicotine abstinent and control mice 4 days after the precipitation of nicotine withdrawal. Mice were treated with cannabidiol (10mg/kg, once daily during 4 days) or vehicle ( $n = 6-9$  mice per group). (C)

Plasmatic levels of TNF $\alpha$ , IL10 and IFN $\gamma$  in nicotine abstinent or control mice 4 days after the precipitation of withdrawal ( $n = 9$  mice per group). Data are expressed as mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$  compared with the saline group; # $p < 0.05$  compared with the nicotine-vehicle group. HPC, hippocampus; PFC, prefrontal cortex.

#### **Figure 4**

Cannabidiol promotes hippocampal cell proliferation during nicotine withdrawal. (A) Density of the endogenous cell proliferation marker Ki67 and (C) Percentage of control DCX immunoreactivity expressed as integrated density in the SGZ of the dentate gyrus ( $n = 6$  mice per group) 4 days after nicotine withdrawal precipitation in mice treated with vehicle or cannabidiol (10mg/kg, once daily during 4 days). (B) Representative images of Ki67 labeling in the SGZ of the dentate gyrus. Arrows indicate Ki67+ cells. Scale bar represents 100 $\mu$ m. Data are expressed as mean  $\pm$  SEM. \* $p < 0.05$ , compared with the saline-vehicle group. DG, dentate gyrus; SGZ, subgranular zone; DCX, doublecortin.

#### **Figure 5**

Nonsteroidal anti-inflammatory drug (NSAID) indomethacin abolishes the cognitive deficits associated with nicotine withdrawal. Memory was evaluated 4 days after nicotine withdrawal precipitation in mice treated with indomethacin (2mg/kg, once daily during 4 days) or vehicle. (A) Discrimination index and (B) total exploration time obtained in the novel object-recognition paradigm ( $n = 12-14$  mice per group). (C-G) Soma perimeter of microglial cells of the (C) stratum pyramidale and (D) stratum radiatum of the CA1 hippocampal area, and (F) granular cell layer and (G) hilus of the hippocampal dentate gyrus ( $n = 4$  mice per group). (E) Representative images of microglial cells from the CA1 area obtained via confocal microscopy after direct labeling with rabbit polyclonal

antiserum to Iba-1. Scale bar represents 20 $\mu$ m. Data are expressed as mean  $\pm$  SEM. \* $p$  < 0.05, \*\* $p$  < 0.01 compared with the saline-vehicle group; ## $p$  < 0.01 compared with the nicotine-vehicle group.