CB1 cannabinoid receptors mediate cognitive deficits and structural plasticity changes during nicotine withdrawal

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Abstract

Background: Tobacco withdrawal is associated with deficits in cognitive function including attention, working memory and episodic memory. Understanding the neurobiological mechanisms involved in these effects is crucial because cognitive deficits during nicotine withdrawal may predict relapse in humans.

Methods: We investigated the role of CB1 cannabinoid receptors (CB1Rs) in memory impairment and spine density changes induced by nicotine withdrawal precipitated by the nicotinic antagonist mecamylamine. Drugs acting on the endocannabinoid system and genetically modified mice were used.

Results: Memory impairment during nicotine withdrawal was blocked by the CB1R antagonist rimonabant or the genetic deletion of CB1R in forebrain GABAergic neurons (GABA-CB1R). An increase of 2-arachidonoylglycerol (2-AG), but not anandamide, was observed during nicotine withdrawal. The selective inhibitor of 2-AG biosynthesis O7460 abolished cognitive deficits of nicotine abstinence, while the inhibitor of 2-AG enzymatic degradation JZL184 did not produce any effect in cognitive impairment. Moreover, memory impairment was prevented by the selective mTOR inhibitor temsirolimus and the protein synthesis inhibitor anysomicin. Mature dendritic spines on CA1 pyramidal hippocampal neurons decreased 4 days after the precipitation of nicotine withdrawal, when the cognitive deficits were still present. Indeed, a correlation between memory performance and mature spine density was found. Interestingly, these structural plasticity alterations were normalized in GABA-CB1R conditional knockout mice and after subchronic treatment with rimonabant.

Conclusions: These findings underline the interest of CB1R as a target to improve cognitive performance during early nicotine withdrawal. Cognitive deficits in early abstinence are associated with increased relapse risk.

Introduction

Tobacco consumption is one of the main public health problems worldwide and represents the leading cause of preventable premature mortality in the world. It is estimated that globally, 22.5% of adults (32% males, 7% females) currently smoke tobacco (1). Cessation from tobacco smoking produces numerous undesirable effects, including physical, affective and cognitive symptoms (2). Increasing attention has currently focused on cognitive impairments that emerge during smoking abstinence. Nicotine withdrawal alters a variety of cognitive processes in humans including impairments in attention, working memory and episodic memory (3,4), which can be partially restored by nicotine replacement therapy (4). These cognitive deficits peak within the first few days of tobacco cessation and are gaining importance as a core dependence phenotype and a target for medication development efforts (5). Indeed, cognitive impairment during early nicotine abstinence may promote short-term smoking relapse (6), and pharmacotherapies that increase cognitive performance during nicotine withdrawal may represent potential new drugs for smoking cessation (7). Consistent with this hypothesis, the partial agonist of the α4β2 nicotinic acetylcholine receptors varenicline, currently used for the treatment of tobacco dependence, enhances mood and cognitive function during early nicotine abstinence (8). These effects seem to be important for the efficacy of varenicline as a smoking cessation agent (8). According to human studies, withdrawal from chronic nicotine results in cognitive deficits in rodent models (9,10), providing an excellent tool to investigate the neural substrates that mediate the effects of nicotine abstinence on cognition.

The endocannabinoid system regulates a range of physiological functions including reward processing, learning and memory (11,12). An important role for the endocannabinoid system in the modulation of the addictive properties of nicotine has

been clearly established (13,14). Thus, CB1 and CB2 cannabinoid receptor (CB1R and CB2R) antagonism reduces nicotine self-administration in rodents (15,16), while an involvement of CB1R, but not CB2R, has been revealed in the processes driving to relapse to nicotine seeking behavior (17,18). Accordingly, CB1R blockade improved the ability of smokers to quit smoking in randomized clinical trials (19). However, the possible involvement of the endocannabinoid system in the neurobiological substrate underlying the cognitive deficits associated with nicotine withdrawal remains unexplored.

In this study, by using specific pharmacological agents acting on the endocannabinoid system and genetically modified mice, we investigated the role played by CB1R in the cognitive deficits and structural plasticity alterations associated with nicotine withdrawal.

Methods & Materials

Animals

Experiments were performed in male C57BL/6J mice (Charles River) and in male CB1R constitutive knockout (KO) mice and conditional KO mice and their wild-type (WT) littermates (8 to 12 weeks old). Conditional KO mice lacking CB1R in forebrain GABAergic neurons (GABA-CB1R), cortical glutamatergic neurons (Glu-CB1R), in both type of neurons (GABA/Glu-CB1R) and their WT littermates were in a mixed genetic background, with a predominant C57BL/6N contribution (at least 7 generations of backcrossing) (20,21). See Supplement 1 for further details.

Drugs

(-)-Nicotine hydrogen tartrate salt [(-)-1-methyl-2(3-pyridyl) pyrrolidine] (Sigma) 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. The CB1 antagonist rimonabant (1mg/kg, Sanofi-Aventis Recherche) and the antagonist of the metabotropic glutamate receptor 5 (mGluR5) MTEP (10mg/kg, Tocris) were diluted in 5% ethanol, 5% cremophor, 90% saline and administered intraperitoneally (ip). The mTOR inhibitor temsirolimus (1mg/kg, ip, LC Laboratories) was dissolved in 2% ethanol, 8% cremophor and 90% saline. The protein synthesis inhibitor anisomycin (6mg/kg, ip, Sigma) was dissolved in physiological saline. The diacylglycerol lipase (DAGL) inhibitor O7460 (12mg/kg, ip) was kindly provided by the laboratory of Dr. Vincenzo Di Marzo (Pozzuoli, Italy) (22) and diluted in 10% DMSO and 90% saline. The monoacylglycerol lipase (MAGL) inhibitor JZL184 (8 and 20mg/kg, ip, Tocris)

was dissolved in DMSO. Corticosterone (3mg/kg, ip, Sigma) was dissolved in 10% ethanol and 90% saline. All drugs were administered in a volume of 10ml per kg of body weight, except for O7460 and JZL184 that were administered in 5ml/kg and 2ml/kg, respectively. Doses of the different drugs used were based on previous studies (22-25).

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. See Supplement 1 for details.

Object-recognition task

In preclinical rodent models, some components of episodic memory can be evaluated by an object-recognition task (26). We used this behavioral test to evaluate the presence of cognitive deficits during nicotine withdrawal. See Supplement 1 for details.

Endocannabinoid quantification

Endocannabinoid levels were measured as previously reported (24), with slights modifications. See details in Supplement 1.

Ballistic labeling with the fluorescent dye DiI

Four days after the precipitation of nicotine withdrawal, when the cognitive deficits were still present, we evaluated possible changes in dendritic spines density in CA1

pyramidal neurons of the hippocampus (HPC) and pyramidal medial prefrontal cortex (mPFC) neurons. See Supplement 1 for further details.

Immunoblot analysis

See Supplement 1

Data analysis

Data were analyzed using unpaired Student t test, one-way analysis of variance (ANOVA) with treatment as between group factor, or two-way ANOVA with nicotine and pretreatment/genotype as between factors of variation. Subsequent post-hoc analysis (Newman-Keuls) was used when required. The Pearson correlation coefficient was used to analyze the relationship between discrimination index values and mature spine density. Comparisons were considered to be statistically significant when the level of significance was $P < 0.05$.

Results

CB1R mediates the cognitive deficits associated with nicotine withdrawal

The object-recognition test was used to evaluate the presence of cognitive deficits during nicotine withdrawal. Nicotine abstinence was precipitated by mecamylamine injection (2mg/kg) 20 minutes after the training phase, and memory was evaluated 24 hours later. Mecamylamine did not produce any effect on memory by itself (1 and 2 mg/kg) (Figure S1). Nicotine-treated animals showed lower discrimination index after the precipitation of withdrawal compared with the saline-treated group ($p<0.01$) (Figure 1A). To exclude an effect of the chronic nicotine treatment on memory, we performed the object-recognition task after 13 days of minipump implantation. We found no differences between animals chronically treated with nicotine or saline (Figure 1B), thus corroborating that the reduced discrimination index previously observed is only due to the precipitation of nicotine withdrawal. The administration of a low dose of the CB1R antagonist rimonabant (1mg/kg), 20 minutes before the precipitation of nicotine withdrawal blocked the memory impairment. Thus, two-way ANOVA showed an interaction between treatment and pretreatment ($F_{1,20}=10.77$, $p<0.01$), and post hoc analysis revealed the effect of the CB1R antagonist ($p<0.01$) (Figure 1C). In agreement, nicotine withdrawal did not induce memory deficits in CB1R KO mice ($p<0.05$) (interaction treatment x genotype: $F_{1,17}=5.74$, $p<0.05$) (Figure 1D). To further elucidate the neuronal type expressing CB1R responsible for this effect, we used CB1R conditional KO mice lacking CB1R primary from cortical glutamatergic neurons (Glu-CB1R) or forebrain GABAergic neurons (GABA-CB1R) (20,21). Interestingly, memory impairment was prevented in GABA-CB1R KO mice ($p<0.01$) and in double GABA/Glu CB1R KO mice ($p<0.01$) (treatment effect: $F_{1,47}=6.41$, $p<0.05$; genotype effect: $F_{3,47}=3.15$, $p<0.05$). In contrast, memory deficits were still present in Glu-CB1R

KO mice (Figure 1E). Total exploration time was not altered in any of the different experimental groups, except a reduction observed in the case of Glu-CB1R KO animals (Figure S2), as previously reported (27,28). Basal anxiety-like behavior is not modified in these mutant mice (29), and it is suggested that the presence of a stressful stimulus (i.e. new object) is required to allow observable phenotype differences between Glu-CB1R KO and WT mice (29). All together, these data suggest that activation of CB1R in GABAergic neurons is necessary to reveal memory deficits during nicotine withdrawal. Nevertheless, we cannot rule out the participation of other subpopulations of CB1R in these cognitive defects since drugs in the pharmacological experiments were peripherally injected.

2-arachidonoylglycerol is involved in the cognitive deficits associated with nicotine withdrawal

We next evaluated the possible endocannabinoid responsible for the memory deficits of nicotine abstinence. A liquid chromatography-mass spectrometry analysis revealed that 2-arachidonoylglycerol (2-AG), but not anandamide (AEA), levels increased in whole brain homogenates extracted 10 minutes after the precipitation of nicotine withdrawal ($p<0.05$) (Figure 2A-C). Protein expression levels of MAGL were reduced at this time point in nicotine abstinent animals under our experimental conditions (Figure 2D), while DAGL-α protein levels were not modified. These results suggest that a decrease of degradation could explain the observed enhancement of 2-AG. This endocannabinoid only exhibited a tendency to increase 30 minutes after the precipitation of this syndrome (Figure S3). Activation of mGluR5 can induce the synthesis and release of endocannabinoids, mainly 2-AG (30). Administration of the mGluR5 antagonist MTEP (10mg/kg) reversed the cognitive deficits induced by nicotine abstinence, as revealed by

two-way ANOVA (interaction treatment x pretreatment: $F_{1,25}$=13.80, p<0.01) and post hoc analysis (p<0.01) (Figure 2E). 2-AG is synthesized by DAG lipases, while is mainly degraded by the hydrolytic enzyme MAGL (31). Interestingly, the administration of the specific DAGL inhibitor O7460 (12mg/kg) (22) abolished the memory impairment associated with nicotine abstinence (p<0.01), as shown by two-way ANOVA (interaction treatment x pretreatment: $F_{1,33}$=13.15, p<0.01) (Figure 2F). In contrast, the injection of the MAGL inhibitor JZL184 (8 and 20mg/kg) did not significantly alter these cognitive deficits (interaction treatment x pretreatment: $F_{2,45}$=2.71, NS) (Figure 2G). Indeed, JZL184 treatment in control mice tended to decrease the discrimination index at the dose of 20mg/kg (Figure 2G), suggesting that the enhancement of 2-AG levels can impair learning and memory performance, as recently reported (32). Taken together, these results point to a relevant role for 2-AG in the appearance of cognitive deficits during nicotine withdrawal.

Dysregulation of mTOR signaling has been related to neurodevelopmental and neuropsychiatric disorders, including those related to intellectual disability (33). Moreover, amnesic effects of Δ^9-tetrahydrocannabinol (THC), the main psychoactive constituent of *Cannabis sativa*, are mediated through the activation of this pathway (23). Notably, the mTOR pathway inhibitor temsirolimus (1mg/kg) blocked the cognitive deficits associated with nicotine withdrawal, as shown by two-way ANOVA (interaction treatment x pretreatment: $F_{1,28}$=19.07, p<0.01), and post hoc comparisons (p<0.01) (Figure 2H). mTOR signaling affects memory processes through its involvement in protein synthesis (34). Although it has been established that long-term memory formation requires the synthesis of new proteins, an aberrant increase in protein synthesis can also lead to memory impairment (23,35). Interestingly, the injection of a non-amnesic dose of the protein synthesis inhibitor anisomycin (6mg/kg)

(Figure 2I) rescued memory impairment ($p<0.01$) of nicotine abstinence, as revealed by two-way ANOVA (interaction treatment x pretreatment: $F_{1,25}=8.79$, $p<0.01$) (Figure 2J). Total exploration time was not modified in any of the different experiments performed (Figure S4). These results suggest that activation of the mTOR pathway and an excess of protein synthesis could negatively affect cognition during nicotine withdrawal.

Structural plasticity alterations during nicotine withdrawal are normalized by CB1R blockade and in GABA-CB1R knockout mice

We next evaluated the duration of the memory impairment associated with nicotine withdrawal by using the object-recognition task at several time points in different cohorts of mice (Figure 3A). A significant decrease in the discrimination index was observed at day 1 ($p<0.01$), 2 ($p<0.01$) and 4 ($p<0.01$) after the precipitation of withdrawal with mecamylamine. Mice recovered completely from the cognitive impairment 8 days after the precipitation of nicotine abstinence (Figure 3A). Subchronic treatment with rimonabant (1mg/kg, once daily during 4 days) prevented the memory impairment 4 days after withdrawal precipitation ($p<0.05$) (interaction treatment x pretreatment: $F_{1,39}=8.79$, $p<0.01$) (Figure 3B), confirming the role played by CB1R in this behavior. Total exploration time in the object-recognition task was not altered in these experiments (Figure S5). Given the involvement of protein synthesis in the cognitive deficits and the duration of this effect, we investigated whether possible changes in structural plasticity could underlie the memory impairment observed during nicotine withdrawal. Dendritic spine density and morphology were analyzed in key areas related to cognitive processes, such as the HPC and mPFC. Four days after the precipitation of nicotine withdrawal, when the cognitive deficits were still present, animals subchronically treated with vehicle or rimonabant (1mg/kg) were sacrificed and

their brains processed for ballistic delivery to label whole neurons with the dye DiI. Total dendritic spine density was not significantly altered in CA1 hippocampal pyramidal neurons (Figure 3C). Dendritic spines are classified into different groups depending on their morphology (stubby, mushroom, thin, branched and filopodia) and can undergo remodelling, that modifies their functionality (36). A decrease in the density of mushroom (mature) spines (interaction treatment x pretreatment: $F_{1,26}$=7.54, $p<0.01$) was observed in nicotine abstinent mice ($p<0.01$) (Figure 3D). Notably, this reduced density of mature spines was normalized after a subchronic treatment with rimonabant ($p<0.01$), suggesting that this spine morphology alteration is regulated through CB1R (Figures 3D, E). Dendritic spine density and morphology of pyramidal mPFC neurons was not altered in nicotine abstinent mice (Figure S6). As previously shown, CB1R specifically located in GABAergic neurons are involved in the memory impairment related to nicotine abstinence. We next evaluated whether the activation of CB1R in GABAergic neurons also mediate the structural plasticity alterations observed in nicotine abstinent mice. As expected, there was no difference in total spine density in saline or nicotine treated animals after the precipitation of withdrawal in CA1 hippocampal pyramidal neurons (Figure 3F). In agreement with the pharmacological experiments, a decrease of mushroom spine density was found in nicotine withdrawn WT animals in CA1 pyramidal neurons ($p<0.01$) (interaction treatment x pretreatment: $F_{1,18}$=4.47, $p<0.05$). Interestingly, this low density of mature spines was completely reversed in GABA-CB1R KO mice ($p<0.01$) (Figures 3G, H). AMPA and NMDA glutamate receptors are involved in the regulation of synaptic plasticity (37,38). A decrease of GluR2 expression was observed in the HPC during nicotine abstinence while no differences were observed in the expression of GluR1, GluR3, NR2A and

NR2B subunits. Glutamate receptor expression was not altered in the mPFC of nicotine withdrawn mice (Figure S7).

As a whole, these results indicate that nicotine withdrawal involves changes in the density of mature spines in HPC, which are associated with the presence of cognitive deficits. Indeed, a significant correlation between memory performance (discrimination index values) and mushroom spine density in control and nicotine withdrawn mice (Figure 3I) was observed. Moreover, these alterations are mediated through CB1R specifically located in GABAergic neurons.

Different neurobiological mechanisms mediate somatic signs and cognitive deficits during nicotine withdrawal

As previously shown, 2-AG levels are increased after the precipitation of nicotine withdrawal contributing to the cognitive impairment associated with this syndrome. We next studied the consequences of the pharmacological modulation of 2-AG levels in the somatic signs of nicotine abstinence. Interestingly, the administration of the MAGL inhibitor JZL184 decreased the severity of withdrawal, as recently reported (25), while the DAGL inhibitor O7460 exacerbated these symptoms (Figure 4A). Thus, two-way ANOVA showed an interaction between treatment and pretreatment ($F_{3,50}$=13.63, $p<0.05$). Subsequent post hoc analysis revealed a decrease of the global withdrawal score by JZL184 at the dose of 20mg/kg ($p<0.05$), and an enhancement of this score by O7460 (12mg/kg) ($p<0.05$). These results suggest that 2-AG is released after nicotine withdrawal to attenuate somatic signs of withdrawal producing simultaneously cognitive deficits, which would persist during longer time than physical symptoms. Moreover, these data indicate that different neurobiological mechanisms would be involved in the appearance of physical signs and memory impairment during nicotine

abstinence. Indeed, the mTOR inhibitor temsirolimus (1mg/kg) and the mGluR5 antagonist MTEP (10mg/kg) abolished cognitive deficits (Figure 2D,G), but did not modify the severity of the somatic signs of withdrawal (interaction: $F_{1,25}$=0.44, NS, and $F_{1,25}$=3.16, NS, respectively) (Figure 4B,C). Individual signs of withdrawal in the different experiments are shown in Table S1.

Discussion

Our data show a pivotal role for CB1Rs, specifically those located in GABAergic cells, in the memory impairment and structural dendritic morphology alterations associated with the nicotine withdrawal syndrome. Moreover, we reveal that 2-AG is involved in this effect since an increase of this endocannabinoid was observed after the precipitation of withdrawal and the inhibition of its biosynthesis prevented cognitive deficits of nicotine abstinence. These data suggest that CB1R could be targeted to normalize cognition during early abstinence, which could have a clear therapeutic interest given that cognitive impairment may predict smoking relapse (6).

Despite the negative consequences of smoking, only approximately 3-5% of smokers who attempt to quit on their own remain abstinent at 6 months (39), and around 50-75% of smokers relapse during the first week of a quit attempt (5). Cognitive deficits that appear during early nicotine withdrawal seem to be involved in smoking relapse (6,40), and therefore drugs improving withdrawal-related cognitive deficits could represent a strategy of treatment or serve as adjunctive pharmacotherapy for smokers most likely to experience these problems (7).

The precipitation of withdrawal with mecamylamine in nicotine-dependent mice after the training phase decreased the discrimination index in the object-recognition task, revealing a deficit in memory consolidation. Similar cognitive deficits have been shown in rodents in other hippocampal-dependent tasks such as the spatial object-recognition and contextual fear conditioning during spontaneous (41,42) and precipitated nicotine withdrawal (43). Chronic nicotine treatment did not alter memory performance in C57BL/6J mice as previously reported (43,44), suggesting that the cognitive deficits observed were specific of the withdrawal period. Pharmacological or genetic deletion of CB1R prevented memory impairment induced by nicotine abstinence. Moreover, these

cognitive deficits were abolished in conditional KO mice lacking CB1R in forebrain GABAergic neurons, the neuronal population in which CB1Rs are more abundantly expressed in the HPC. In agreement, the amnesic-like effects of THC, the main psychoactive component of *Cannabis sativa*, were demonstrated to be mediated by CB1Rs located in these particular neurons by using the same paradigm (23).

We identified the 2-AG as the endocannabinoid responsible for the cognitive deficits associated with the nicotine withdrawal syndrome. An increase of 2-AG, but not AEA, was observed 10 minutes after the precipitation of withdrawal. Notably, the selective inhibitor of DAG lipases O7460 (22), which are the enzymes in charge of 2-AG biosynthesis, totally prevented memory deficits. In contrast, the administration of JZL184 which inhibits MAGL, the enzyme responsible for 2-AG degradation, did not modify this effect. Indeed, JZL184 induced a cognitive impairment by itself at 20mg/kg, consistent with the observation that 2-AG hydrolysis blockade impairs learning and memory performance (32). Taken together, these data indicate that 2-AG and activation of CB1Rs in GABAergic neurons are required for the appearance of nicotine withdrawal-induced memory impairment.

mTOR signaling regulates many integrated physiological functions of the nervous system including neuronal development, synaptic plasticity, memory storage, and cognition (45), and perturbation of this cascade appears to be a common pathophysiological feature of human neurological disorders including those related to cognitive alterations (34). In agreement, activation of mTOR signaling was revealed to be necessary for the amnesic-like effects of THC (23). Interestingly, the administration of the mTOR inhibitor temsirolimus (46), as well as the protein synthesis inhibitor anysomicin, abolished the cognitive deficits present during nicotine abstinence. Thus, mTOR activation and excessive protein synthesis could also underlie the behavioral

deficit induced by nicotine withdrawal. In this sense, studies using animal models have revealed an aberrant increase in protein synthesis in other conditions characterized by memory impairment such as the fragile X syndrome (35) or the administration of THC (23).

We next investigated the duration of the cognitive deficits associated with nicotine withdrawal and the possible existence of changes in structural plasticity during this syndrome. Memory impairment was still present 4 days after the precipitation of withdrawal and mice recovered from this deficit by the 8^{th} day of withdrawal. In agreement, a previous study showed a similar duration of these cognitive deficits in the hippocampal-dependent contextual fear conditioning test in C57BL/6J mice (47). Interestingly, morphological analysis of dendritic spines in the CA1 region of the HPC revealed a decrease of mushroom (matures) spines 4 days after the precipitation of nicotine withdrawal, when cognitive impairment was still observed. Accordingly, the number and shape of synapse-bearing spines in the hippocampal formation are dynamic (48,49) and are regulated by several factors including stress (50). The stressful condition of nicotine withdrawal could be responsible for the reduced density of mature spines, probably leading to synaptic dysfunction and cognitive deficits. However, the neurobiological mechanisms described in our study should not be generalized to other types of stressful conditions such as acute corticosterone injection. Thus, the administration of compounds affecting the endocannabinoid system such as MTEP and O7460 before an acute amnesic dose of corticosterone did not prevent memory impairment in the object-recognition test (Figure S8). On the other hand, the decrease of GluR2 expression observed in the HPC of nicotine withdrawn mice could be related to the reduced number of mature spines. Thus, AMPA glutamate receptors including GluR2 subunits (51) modulate synaptic strength, and are abundant in mushroom spines,

but sparsely distributed in thin and filopodia spines of CA1 hippocampal pyramidal neurons (52). In line with our study in mice, cortical neuroplasticity in cigarette smokers was also altered during early withdrawal as revealed by using transcranial direct current stimulation (53). This effect on neuroplasticity might be relevant for the high probability of relapse in heavy smokers (53). However, under our experimental conditions, no modifications of structural plasticity were observed in pyramidal neurons of mPFC.

The reduction of mushroom-type spines in CA1 hippocampal neurons associated with nicotine withdrawal was normalized by a subchronic treatment with a low dose of rimonabant and in GABA-CB1R KO mice. Accordingly, the same CB1R antagonist reversed cognitive and hippocampal dendritic spine deficits in a model of fragile X syndrome (54). We did not observe modifications in the average spine density in CA1 pyramidal neurons in GABA-CB1R KO mice under basal conditions, although a reduction of this density was shown by a recent study (55). Different methodology used (apical versus apical and basal dendrites analysis) could explain this discrepancy. Taken together, these results suggest that CB1R activation in GABAergic neurons induces modifications in the morphology of hippocampal dendritic spines leading to the appearance of cognitive deficits during early nicotine withdrawal. An important role for this population of CB1Rs has been recently shown in other behavioral responses such as voluntary exercise performance (56), food intake (21), cocaine addiction (57), or anxiety (29).

2-AG seems to play divergent functional effects during nicotine abstinence. Thus, the pharmacological modulation of 2-AG levels induced opposite effects on the somatic signs and memory impairment revealed during withdrawal. The MAGL inhibitor JZL184 reduced the severity of nicotine physical dependence, as recently reported (25),

while the inhibition of DAG lipases by O7460 (22) exacerbated somatic signs of withdrawal. These data suggest that 2-AG might be released during nicotine withdrawal to alleviate physical signs producing simultaneously cognitive deficits, which seem more persistent in time, and may be therefore more relevant for early relapse than physical symptoms. Several reports suggest that stress exposure increases 2-AG levels to counteract many of the negative effects of this response (58). Different neurobiological mechanisms seem to mediate these two aspects of nicotine abstinence. Thus, the mTOR inhibitor temsirolimus and the inhibitor of mGluR5 MTEP blocked memory impairment without affecting somatic signs. In agreement, no changes in the physical severity of nicotine abstinence were observed in a previous study using the mGluR5 antagonist MPEP (59). Although the administration of MTEP should reduce 2-AG levels after nicotine withdrawal and therefore worsen somatic signs, other biochemical processes could compensate or mask the effect of MTEP in this response. Indeed, mGluR5 blockade is a very general mechanism that could lead to these compensatory mechanisms beyond the modulation of the endocannabinoid system. O7460 increased nicotine physical severity probably because this compound directly inhibits the enzyme in charge of 2-AG synthesis.

All together, our work reveals the crucial involvement of CB1Rs located in GABAergic cells in the cognitive impairment and neuronal plasticity changes in the HPC occurring during nicotine withdrawal. This subpopulation of CB1Rs could be targeted to prevent smoking relapse by increasing cognitive performance during early nicotine withdrawal syndrome.

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The authors have no conflicts of interest to declare.

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Figure legends

Figure 1

CB1R mediates the cognitive deficits associated with nicotine withdrawal. **(A)** Nicotine withdrawal was precipitated with mecamylamine (2mg/kg) 20 minutes after the training session, and discrimination index was obtained 24 hours after the training (n = 11 mice per group). **(B)** Discrimination index obtained after chronic treatment with saline or nicotine during 13 days (n = 6-7 mice per group). **(C)** Rimonabant (1mg/kg) was administered immediately after the training session 20 minutes before the precipitation of nicotine withdrawal, and discrimination index was obtained 24 hours after the training (n = 5-7 mice per group). **(D-E)** Nicotine withdrawal was precipitated with mecamylamine (2mg/kg) 20 minutes after the training session in **(D)** WT and CB1R KO mice (n = 5-6 mice per group), and in **(E)** WT and GABA-CB1R KO, Glu-CB1R KO and GABA/Glu CB1R KO mice (n = 5-8 mice per group), and discrimination index was obtained 24 hours after the training. Data are expressed as mean ± SEM. ★★p<0.01 (compared with saline), #p<0.05 (comparison between genotypes) ##p<0.01 (comparison between pretreatments or genotype). WT, wild-type mice; KO, knockout mice.

Figure 2

2-arachidonoylglycerol is involved in the cognitive deficits associated with nicotine withdrawal. **(A)** Schematic representation of the experimental design for **(B,C)** endocannabinoids measure. Levels of **(B)** 2-arachidonoylglycerol and **(C)** anandamide in whole brain homogenates extracted 10 minutes after the precipitation of nicotine withdrawal, measured by liquid chromatography-mass spectrometry (n = 8 mice per group). (D) Protein levels of DAGL-α and MAGL in whole brain homogenates 10

minutes after the precipitation of nicotine withdrawal (n = 6 mice per group). (E,F,G,H,J) MTEP (10mg/kg), O7460 (12mg/kg), temsirolimus (1mg/kg), and anysomicin (6mg/kg) were administered immediately after the training session 20 minutes before the precipitation of nicotine withdrawal. JZL184 (8 and 20mg/kg) was administered immediately after the training session 2 hours before the precipitation of nicotine withdrawal. Discrimination index was obtained 24 hours after the training (n = 7-11 mice per group). **(I)** Anysomicin (4, 6, 8, 12 and 18mg/kg) was administered immediately after the training session, and discrimination index was obtained 24 hours after the training (n = 5-12 mice per group). Data are expressed as mean ± SEM. ★p<0.05 (compared with vehicle); ★★p<0.01 (compared with saline), $^{\#\#}p$<0.01 (comparison between pretreatments).

Figure 3

Structural plasticity changes associated with nicotine withdrawal are normalized by CB1R blockade and in GABA-CB1R knockout mice. **(A)** Schematic representation of the experimental design used to evaluate the duration of the cognitive deficits associated with nicotine abstinence. Nicotine withdrawal was precipitated with mecamylamine (2mg/kg) 20 minutes after the training session, and discrimination index was obtained 1, 2, 4 and 8 days after the training in different cohorts of mice (n = 9-12 mice per group). **(B)** Schematic representation of the experimental design used to evaluate the effects of subchronic treatment with rimonabant in the cognitive deficits observed 4 days after the precipitation of nicotine withdrawal. Rimonabant was injected at 1mg/kg, once daily during 4 days (n = 9-12 mice per group). **(C)** Overall dendritic spine density, **(D)** Analysis of spine morphology and **(E)** Representative DiOlistics staining of CA1 hippocampal pyramidal neurons of mice subcronically treated with vehicle or

rimonabant (1 mg/kg, once daily during 4 days) evaluated 4 days after the precipitation of nicotine withdrawal (n = 7-8 mice per group). **(F)** Overall dendritic spine density, **(G)** Analysis of spine morphology and **(H)** Representative DiOlistics staining of CA1 hippocampal pyramidal neurons of WT and GABA-CB1R knockout mice evaluated 4 days after the precipitation of nicotine withdrawal (n = 4-7 mice per group). Scale bar = 2 μm. Arrows indicate mushroom (mature) spines. **(I)** Correlation between memory performance (discrimination index values) and mushroom spine density 4 days after the precipitation of nicotine withdrawal. Data are expressed as mean ± SEM. ★★p<0.01 (compared with saline), $^{\#}p$<0.05 (comparison between pretreatments), $^{\#\#}p$<0.01 (comparison between pretreatments or genotypes). WT, wild-type mice; KO, knockout mice.

Figure 4

Different neurobiological mechanisms underlie cognitive deficits and somatic signs of nicotine withdrawal. Mecamylamine (2mg/kg) was administered to precipitate withdrawal in nicotine dependent mice. O7460 (12mg/kg), temsirolimus (1mg/kg) and MTEP (10mg/kg) were injected 20 minutes before mecamylamine. JZL184 (8 and 20mg/kg) was injected 2 hours before mecamylamine. A global withdrawal score was calculated by giving each individual sign a relative weight in **(A)** vehicle, JZL184 and O7460 pretreated mice (n = 6-10 mice per group), in **(B)** temsirolimus pretreated mice (n = 7-8 mice per group), and in (C) MTEP pretreated mice (n = 7-8 mice per group). Data are expressed as mean ± SEM. ★★p<0.01 (compared with saline), $^{\#}p$<0.05 (comparison between pretreatments).