Role of CB2 cannabinoid receptor in the rewarding, reinforcing and physical effects of nicotine

Running title: CB2 cannabinoid receptors and nicotine

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ABSTRACT

The present study was aimed to evaluate the involvement of CB2 cannabinoid receptors (CB2r) in the rewarding, reinforcing and motivational effects of nicotine. Conditioned place preference (CPP) and intravenous self-administration experiments were carried out in knock-out mice lacking CB2r (CB2KO) and in wild-type (WT) littermates treated with the CB2r antagonist AM630 (1 and 3mg/Kg). Gene expression analyses of tyrosine hydroxylase (TH), α3- and α4-nicotinic acetylcholine receptor subunits (nAChRs) in the ventral tegmental area (VTA) and immunohistochemical studies to elucidate if CB2r co-localized with α3- and α4-nAChRs in the nucleus accumbens (NAcc) and VTA were performed. Mecamylamine-precipitated withdrawal syndrome after chronic nicotine exposure was evaluated in CB2KO mice and in WT mice treated with AM630 (1 and 3mg/Kg).

CB2KO mice did not show nicotine-induced place conditioning and self-administered significantly less nicotine. In addition, AM630 was able to block (3mg/Kg) nicotine-induced CPP and reduce (1 and 3mg/Kg) nicotine self-administration. Under baseline conditions, TH, α3- and α4-nAChRs mRNA levels in the VTA of CB2KO mice were significantly lower compared with WT mice. Confocal microscopy images revealed that CB2r co-localized with α3- and α4-nAChRs. Somatic signs of nicotine withdrawal (rearings, groomings, scratches, teeth chattering and body tremors) increased significantly in WT but were absent in CB2KO mice. Interestingly, the administration of AM630 blocked the nicotine withdrawal syndrome and failed to alter basal behavior in saline-treated WT mice.

These results suggest that CB2r play a relevant role in the reinforcing and motivational effects of nicotine. Pharmacological manipulation of this receptor deserves further consideration as a potential new valuable target for the treatment of nicotine dependence.

KEYWORDS

Nicotine, cannabinoid, conditioned place preference, self-administration, withdrawal, mice.

INTRODUCTION

Tobacco consumption has been identified as a public health issue and one of the preventable causes of disease and death. Nicotine is the main psychoactive component of tobacco playing a major role in the development of dependence. Following cessation of chronic nicotine use a withdrawal syndrome occurs including a cluster of symptoms such as irritability, tremors, bradycardia and elevated anxiety. The withdrawal of nicotine promotes several neuroadaptive processes to counteract the negative state. Just as specific nicotinic acetylcholine receptor subunits (nAChRs) support the induction of nicotine addiction, other specific nAChRs underlie the withdrawal syndrome. For instance, α3-, α5-, and β4-nAChRs are all found in the same gene cluster, and all of these subunits seem to help regulate consequences of nicotine withdrawal (Salas *et al*, 2004).

Nowadays, the most commonly used treatment for smoking cessation is the nicotine replacement therapy that partially relieves withdrawal symptoms and nicotine craving (Benowitz, 2010; Benowitz *et al*, 1993). Other approved smoking cessation therapies are bupropion, an atypical antidepressant acting as a non-competitive antagonist of various nAChRs and inhibiting catecholamine reuptake (Dwoskin *et al*, 2006), and varenicline, an agonist on several nAChRs (Mihalak *et al*, 2006). The occurrence of serious side effects, including depression and suicidal behavior described in patients treated with bupropion or varenicline (Hays and Ebbert, 2010), justify all the efforts to develop improved therapeutic approaches.

Although diverse psychopharmacological effects contribute to the overall process of nicotine addiction, the endocannabinoid system is emerging as a critical piece mediating the reinforcing effects of nicotine, as well as relapse to nicotine-seeking behavior. The characterization of the crosstalk between nicotine addiction and the endocannabinoid system is fundamental to understand the neurobiological mechanisms underlying nicotine dependence, although the available results are controversial. An acute dose of Δ^9-THC reduced the incidence of precipitated nicotine withdrawal signs in mice (Balerio *et al*, 2004) while the co-administration of sub-threshold doses of Δ^9-THC and nicotine enhanced the somatic expression of nicotine withdrawal and nicotine-induced conditioned place preference (CPP) (Valjent *et al*, 2002). In addition, the CB1 cannabinoid receptor (CB1r) antagonist SR-141716A (rimonabant) reduced nicotine self-administration (Cohen *et al*, 2002), nicotine-induced CPP in rodents (Le Foll and Goldberg, 2004; Merritt *et al*, 2008), nicotine-induced dopamine release in the nucleus accumbens (NAcc) (Cohen *et al*, 2002) and failed to precipitate

withdrawal syndrome in nicotine-dependent mice (Castane *et al*, 2002). Moreover, CB1KO mice neither express nicotine-induced CPP (Castane *et al*, 2002) nor nicotine self-administration (Cossu *et al*, 2001) whereas no differences were found in nicotine withdrawal intensity between CB1KO and WT mice (Castane *et al*, 2005; Castane *et al*, 2002; Cossu *et al*, 2001).

In the last years, significant contributions have demonstrated the potential involvement of the CB2r in addiction. The first results showed increased CB2r gene expression in the brain of mice after chronic treatment with heroin or cocaine and decreased CB2r gene expression in striatum and ventral midbrain of mice after chronic ethanol intake (Onaivi *et al*, 2008a). In fact, mice that developed alcohol preference presented reduced CB2r gene expression and chronic treatment with JWH015, a CB2r agonist, enhanced alcohol consumption in stressed mice whereas the administration of the CB2r antagonist AM630 reduced alcohol intake in stressed mice (Ishiguro *et al*, 2007). Recently, it has been reported that activation of CB2r inhibited cocaine self-administration, cocaine-enhanced locomotion and cocaine-enhanced release of extracellular dopamine in the NAcc (Xi *et al*, 2011). Another study showed that activation of CB2r is involved in cocaine-induced reinstatement of cocaine seeking behavior, although its activity is not necessary for the rewarding effects of cocaine (Adamczyk *et al*, 2012). In addition, latest results from our group suggest that overexpression of CB2r (CB2xP mice) decreases cocaine motor responses and cocaine self-administration (Aracil-Fernandez *et al*, 2012).

The purpose of this study was to examine the role of CB2r on nicotine CPP, self-administration and nicotine withdrawal syndrome in mice. CB2KO mice and AM630-treated WT mice were used to study the rewarding effects of nicotine in the CPP, the nicotine reinforcing and motivational properties in self-administration, and the nicotine withdrawal syndrome. Specifics changes in the gene expression of tyrosine hydroxylase (TH) and functional α3- and α4-nAChRs in the ventral tegmental area (VTA) were measured by real time-PCR. The possibility of co-expression of CB2r with α3- and α4-nAChRs in cell bodies and terminals of the mesolimbic dopamine system was evaluated by confocal microscopy.

MATERIALS AND METHODS

Animals

Homozygotes male CB2KO mice on a CD1 background (n=82, for more details see Supplementary Information) and wild-type mice littermates (WT) (n=198) were used in all experiments. Mice were 2-3 months old and weighed 25-35g at the beginning of the experiments. Animals were maintained in a temperature-(23±2ºC) and humidity-(65±10%) controlled room with a light–dark cycle (lights on 0800-2000h) except for the CPP and self-administration studies where a reversed light–dark cycle (lights off 0800-2000h) was used. All studies were conducted in compliance with the guidelines of the European Council Directive 2010/63/UE regulating animal research and approved by the local ethical committees.

Drugs

(-)-Nicotine hydrogen tartrate salt (Sigma, Madrid, Spain) was dissolved in physiological saline (NaCl 0.9%) and administered 0.5-1mg/Kg (i.p.; 0.3ml), 0.03mg/Kg (i.v.; per infusion) or 25mg/Kg/day (s.c.;osmotic minipump). Mecamylamine hydrochloride (Sigma, Madrid, Spain) was dissolved in physiological saline and administered 2mg/Kg (s.c.; 0.3ml). AM630 (Biogen, Madrid, Spain) was dissolved in a mixture of DMSO, Tween 80 and distilled water (1:1:8 proportion) and administered 1-3mg/Kg (i.p.; 0.3ml). For surgery procedures, ketamine hydrochloride (100mg/kg) (Imalgène 1000; Rhône Mérieux, Lyon, France) and xylazine hydrochloride (20mg/kg) (Sigma) were mixed and dissolved in ethanol (5%) and distilled water (95%) and administered (i.p.; 0.3ml).

Conditioned Place Preference

Details of the apparatus and the procedure of nicotine CPP are described in the Supplementary Information.

Nicotine intravenous self-administration

Nicotine self-administration sessions were performed in accordance to protocols previously described (Burokas *et al*, 2012; Martin-Garcia *et al*, 2009; Soria *et al*, 2008; Soria *et al*, 2005). The details of the apparatus, surgery and the nicotine self-administration procedure are included in the Supplementary Information.

Real time-PCR analyses

CB2KO and WT mice (basal conditions) were killed and brains were removed from the skull and frozen over dry ice. Coronal brain sections (500 µm) beginning at plates 19-20 (Paxinos and Franklin, 2001) were obtained in a cryostat (-10ºC). The VTA was microdissected according to a modification of the Palkovits method (Palkovits, 1983) as previously described (Navarrete *et al*, 2012). Total RNA was isolated from brain tissue micropunches using Trizol reagent (Invitrogen, Madrid, Spain) and subsequently retrotranscribed to cDNA. Quantitative analysis of the relative abundance of TH (Mm00447546_m1), α3-nAChRs (Mm00520145_m1) and α4-nAChRs (Mm00516561_m1) gene expressions was performed on the StepOne Sequence Detector System (Life technologies, Madrid, Spain). The reference gene used was 18S rRNA (Hs99999901_s1). Relative mRNA abundance was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Inmunohistochemistry

Floating coronal sections from WT mice containing NAcc and VTA (for more details see Supplementary Information) were incubated in parallel with goat anti-CB2r (1:250, Santa Cruz Biotechnology) combined with either rabbit anti-α3-nAChRs (1:25, Santa Cruz Biotechnology) or monoclonal mouse anti-α4-nAChRs (1:250, Santa Cruz Biotechnology). All sections were then incubated with monkey biotinylated antigoat antibody (1:800, Vector, Burlingame, CA) and avidin-BODIPY FL (1µg/ml, Molecular Probes, Eugene, OR) (CB2r, green) and with goat rhodamine red-X anti-rabbit (α3-nAChRs, red) or horse rhodamine red-X anti-mouse (α4-nAChRs, red) (1:150, Molecular Probes). Fluorescent sections were mounted using ProLong Gold (Molecular Probes), studied in a Leica confocal laser fluorescence microscope and processed using the LCS Lite software. CB2r antibody specificity was assessed with conventional immunohistochemistry (see Supplementary Information and Supplementary Figure S1).

Nicotine treatment and withdrawal

Nicotine dependence was induced by using Alzet osmotic minipumps (Model 2002) (Alzet, Cupertino, California). These minipumps, implanted subcutaneously under ketamine/xylazine mixture anesthesia, contained saline or nicotine solutions and delivered a constant subcutaneous flow in a rate of 0.5µL/hour during the whole experimental sequence. Animals received a dose of nicotine of

approximately 25mg/kg/day. Nicotine withdrawal syndrome was precipitated 14 days after minipump implantation by injection of the nicotinic receptor antagonist mecamylamine (2mg/kg;s.c.). Genetic (CB2KO mice) and pharmacological (AM630 administration) approaches were used in this set of experiments. For the pharmacological studies, mice received a systemic (1 and 3mg/kg,i.p.) injection of AM630 30 minutes before mecamylamine-precipitated nicotine withdrawal syndrome. The somatic signs of withdrawal were evaluated immediately after mecamylamine injection for 25 minutes in which animal were introduced in an open field arena. Rearings, groomings, scratches, teeth chattering and body tremors were counted.

Statistical analyses

Statistical analyses were performed using Student's t-test by comparing two groups and one-, two-, or three-way analyses of variance (ANOVA) when comparing three or more groups. When appropriate, post-hoc individual differences between groups were determined using the Student-Newman-Keuls test. Specific statistical analyses are described in Supplementary Information. Differences were considered significant when $p<0.05$. SigmaStat v3.11 and SPSS v17 software were used.

RESULTS

Nicotine conditioned place preference

The ANOVA of the data from the CPP procedure (Figure 1a) revealed a significant effect of the variable Days ($F_{1,58}$=6.021, p<0.01), as more time was spent in the drug-paired compartment in the Post-C test than in the Pre-C (p<0.01). The interaction Days x Genetics also showed an effect ($F_{1,58}$=3.253, p<0.05), since only WT mice developed nicotine-induced CPP (p<0.01) for the doses of 1 and 0.7mg/kg. No preference was obtained in CB2KO mice.

Effect of AM630 on nicotine conditioned place preference

The ANOVA of the effect of AM630 on the nicotine-induced CPP (Figure 1b) revealed a significant effect of the interaction Days x Nicotine dose ($F_{1,57}$=12.174, p<0.001), and Days x AM630 ($F_{1,57}$=3.338, p<0.05). More time was spent in the drug-paired compartment in the Post-C test than in the Pre-C only in those groups receiving nicotine (p<0.01). This effect was due to the groups conditioned with nicotine alone (p<0.01) or plus 1mg/kg of AM630 (p<0.001). In addition, the group conditioned only with 3mg/kg of AM630 developed aversion to the compartment associate with this CB2r antagonist (p<0.01). No CPP was observed in the group conditioned with 1mg/kg of nicotine plus 3mg/kg of AM630.

Acquisition and maintenance of nicotine self-administration in CB2KO and WT mice

Two-way ANOVA of the number of infusions during the 10 days of self-administration revealed significant main effects of genotype ($F_{1,28}$=77.23; p<0.001), day ($F_{9,252}$=4.74; p<0.001) and interaction between genotype and day ($F_{9,252}$=5.50; p<0.001). The number of infusions was significantly lower in CB2KO than in WT mice. On FR1 schedule, the acquisition criteria of the operant responding maintained by nicotine (0.03mg/kg/infusion) were achieved by 90% of WT and 0% of CB2KO mice. Active nose-poking responses were significantly lower in CB2KO than in WT mice (see table 1 for three-way ANOVA). Only WT mice discriminated between the active and the inactive holes during most of the whole period of training, except the first day, and the number of active nose-poking responses increased across days while the inactive nose-poking responses decreased over time (Figure 2a). The mean number of active nose-poking for nicotine reinforcement during the three last days of FR1 was 11.94±1.50 in WT and 2.84±0.45 in CB2KO.

In the PR schedule of reinforcement, the breaking point values were significantly decreased in CB2KO mice when compared to WT littermates ($F_{1,28}$=27.63; p<0.001) revealing a reduction in the motivation for nicotine in CB2KO mice (Figure 2b).

Pharmacological regulation of nicotine self-administration with AM630

WT mice were trained to self-administer nicotine during 7 days to evaluate the effects of AM630 on the regulation of the reinforcing effects of nicotine during self-administration (0.03mg/kg/infusion). One-way ANOVA of the number of infusions during the 7 days of self-administration training revealed significant main day effects ($F_{6,120}$=2.83; p<0.05). The acquisition criteria of the operant responding maintained by nicotine were achieved by 92% of mice. Two-way ANOVA revealed that mice discriminated between the active and the inactive holes ($F_{1,120}$=39.59; p<0.01) during the whole period of training, excepting the first day. The evolution across days was significant ($F_{6,120}$=2.95; p<0.05) showing an increase across days of the number of active nose-poking responses, while the inactive nose-poking responses decreased over time as revealed by the significant interaction between days and hole ($F_{6,120}$=6.04; p<0.001). The mean number of active nose-poking during the three last days of FR1 acquisition (sessions 5-7) was 11.91±1.90 (Figure 2c). After acquisition, mice were assigned to two different homogeneous groups as revealed by two-way ANOVA. Indeed no significant differences on active or inactive nose-poking were revealed between these two groups ($F_{2,18}$=0.26; n.s.) during the 7 days of self-administration training. After acquisition, the administration of AM630 (1 or 3mg/kg, ip) decreased the number of responses in the active hole when tested on day 8 (p<0.001) or day 12 (p<0.01) respectively (see table 2 for two-way ANOVA and Figure 2d). In the PR schedule of reinforcement, the breaking point values were significantly decreased in mice receiving AM630 (3mg/kg, i.p.) compared to mice treated with vehicle ($F_{1,19}$=4.78; p<0.05) revealing a reduction in the motivation for nicotine (Figure 2e). Additionally, one-way repeated measures ANOVA in active nose-pokes from session 7 to session 14 of mice injected with AM630 was performed to evaluate the effects of different doses on nose-poking responses and the recovery of basal levels after each injection. Results revealed significant effects of session ($F_{7,91}$=4.93; p<0.001) and post-hoc analyses (Student-Newman-Keuls) showed decreased active nose-poking response on session 8 when injected with AM630 (1 mg/kg, i.p.), compared to session 7 (p<0.01). The recovery to basal levels was then observed on session 10 (p<0.05) and 11 (p<0.01). Subsequently, active nose-poking decreased again

on session 12 when injected with AM630 (3 mg/kg, i.p.) ($p<0.05$) and basal levels were recovered on session 13 ($p<0.05$) and 14 ($p<0.05$). No significant differences between session 8 (AM630, 1 mg/kg, i.p.) and 12 (AM630, 3 mg/kg, i.p.) were found. In the vehicle group, one-way repeated measures ANOVA in active nose-pokes from session 7 to session 14 revealed no significant effects of session ($F_{7,42}$=1.46; n.s.) (See Figure S3).

Gene expression analyses of TH, α3- and α4-nAChRs between CB2KO and WT mice in VTA

Real time-PCR analyses showed that TH mRNA levels were significantly reduced in VTA of CB2KO mice compared with WT mice (Figure 3a, Student's *t*-test, *t*=-3.090, p=0.006, 18df). On the other hand, the evaluation of nAChRs gene expression revealed that there was a down-regulation of α3-nAChRs (Figure 3b, Student *t*-test, *t*=2.726, p=0.016, 18df) and α4-nAChRs (Figure 3c, Student *t*-test, *t*=3.269, p=0.005, 18 df) in VTA of CB2KO mice.

Double immunostaining CB2r/α3-nAChRs and CB2r/α4-nAChRs in NAcc and VTA cells.

In WT mice, double immunolabeled cells with CB2r/α3-nAChRs (Figure 4A1-B3) and CB2r/α4-nAChRs (Figure 4C1-D3) antibodies were seen in several brain regions, and in particular in NAcc and VTA. As in previous studies where different brain regions were analyzed (Aracil-Fernandez *et al*, 2012; Onaivi *et al*, 2008a), CB2r immunolabeling was mostly found in the soma of neurons, and in the soma and processes of glial cells (Figure S1). The α3-nAChRs immunolabeling was much widely distributed in the cytoplasm of immunolabeled cells (mostly neurons; Figure 4A2). In the neocortex, α3-nAChRs Ab labeled long apical dendrites segments of pyramidal neurons (Figure S2). In contrast, α4-nAChRs immunolabeling was mostly found in the cell bodies and in the proximal processes of few cells.

In NAcc, all α3- and α4-nAChRs immunolabeled cells were CB2r immunopositive (Figure 4A3,B3). In VTA, scarce α3- and α4-nAChRs immunolabeled cells were, however, CB2r immunonegative (Arrow in figure 4B2 and arrowhead in figure 4D2).

Nicotine withdrawal syndrome evaluation in WT and CB2KO mice

Mecamylamine-precipitated withdrawal syndrome in chronically nicotine-treated mice was evaluated in video recordings. The behavior of nicotine-treated CB2KO mice did not significantly differ from saline-treated littermates whereas nicotine-treated WT mice showed a marked withdrawal

syndrome compared with saline-treated WT mice (Figure 5). The somatic signs evaluated include the number of rearings (Figure 5a, Two-way ANOVA, genotype: $F_{1,39}$=12.182, p=0.001; treatment: $F_{1,39}$=11.179, p=0.002; genotype x treatment interaction: $F_{1,39}$=19.579, p<0.001), groomings (Figure 5b, Two-way ANOVA, genotype: $F_{1,39}$=39.502, p<0.001; treatment: $F_{1,39}$=7.942, p=0.008; genotype x treatment interaction: $F_{1,39}$=22.952, p<0.001), scratches (Figure 5c, Two-way ANOVA, genotype: $F_{1,39}$=45.715, p<0.001; treatment: $F_{1,39}$=44.367, p<0.001; genotype x treatment interaction: $F_{1,39}$=54.352, p<0.001), teeth chattering (Figure 5d, Two-way ANOVA, genotype: $F_{1,39}$=235.698, p<0.001; treatment: $F_{1,39}$=175.402, p<0.001; genotype x treatment interaction: $F_{1,39}$=168.491, p<0.001) and body tremors (Figure 5e, Two-way ANOVA, genotype: $F_{1,39}$=38.955, p<0.001; treatment: $F_{1,39}$=46.015, p<0.001; genotype x treatment interaction: $F_{1,39}$=32.482, p<0.001).

Pharmacologic regulation of nicotine withdrawal with AM630

The effects of CB2r acute blockade on nicotine withdrawal syndrome were evaluated in WT mice by the administration of AM630. Mecamylamine-precipitated withdrawal was significantly higher in the nicotine-vehicle group in comparison with saline-vehicle group (p<0.001). On the other hand, AM630 treatment did not produce any significant change in saline-treated animals (p>0.05), whereas AM630 significantly reduced the number of rearings (Figure 5f, Two-way ANOVA, genotype: $F_{1,59}$=8.779, p=0.005; treatment: $F_{2,59}$=1.708, p=0.192; genotype x treatment interaction: $F_{2,59}$=1.383, p=0.261), groomings (Figure 5g, Two-way ANOVA, genotype: $F_{1,59}$=25.425, p<0.001; treatment: $F_{2,59}$=3.773, p=0.030; genotype x treatment interaction: $F_{2,59}$=7.811, p=0.001), scratches (Figure 5h, Two-way ANOVA, genotype: $F_{1,59}$=45.121, p<0.001; treatment: $F_{2,59}$=14.328, p<0.001; genotype x treatment interaction: $F_{2,59}$=12.768, p<0.001), teeth chattering (Figure 5i, Two-way ANOVA, genotype: $F_{1,59}$=133.631, p<0.001; treatment: $F_{2,59}$=17.270, p<0.001;genotype x treatment interaction: $F_{2,59}$=18.501, p<0.001) and body tremors (Figure 5j, Two-way ANOVA, genotype: $F_{1,59}$=120.028, p<0.001; treatment: $F_{2,59}$=17.020, p<0.001; genotype x treatment interaction: $F_{2,59}$=17.002, p<0.001).

DISCUSSION

This study provides the first evidence about the role of CB2r in the rewarding, reinforcing, motivational and physical effects induced by nicotine administration. This assumption is supported by the following observations: 1) deletion of CB2r or pharmacological blockade with AM630 inhibited the rewarding effects of nicotine in the CPP, 2) nicotine-self administration was attenuated in CB2KO mice or in WT mice treated with AM630, 3) deletion of CB2r reduced TH, α3 and α4-nAChRs gene expression in VTA, 4) CB2r co-localization with α3- and α4-nAChRs both in NAcc and VTA, and 5) the somatic signs of nicotine withdrawal were significantly reduced in CB2KO mice or in WT mice treated with AM630.

Previous studies suggest a relevant role of CB2r on opiates, cocaine and ethanol addiction behavior (Adamczyk *et al*, 2012; Aracil-Fernandez *et al*, 2012; Onaivi *et al*, 2008b). In order to explore the role of CB2r in the rewarding properties of nicotine, CB2KO mice were exposed to the CPP following an unbiased procedure. Nicotine induced a reliable preference in WT mice, in agreement with previous results (Jackson *et al*, 2012), whereas no CPP was obtained in CB2KO mice at any of the nicotine doses used. In addition, a lower percentage of acquisition of intravenous nicotine-self administration was observed in CB2KO mice. The deficits in nicotine-self administration observed in CB2KO mice are not due to possible unspecific learning or motor deficits produced by the lacking of CB2r as both genotypes similarly acquired and maintained stable operant responding for water (unpublished data).

To further explore the role of CB2r in mediating nicotine actions, pharmacological studies employing the selective CB2r antagonist AM630 were performed in WT mice. AM630 blocked nicotine-induced CPP with the highest dose (3mg/kg) and significantly reduced nicotine self-administration at FR1 (1 and 3mg/Kg) and PR (3mg/Kg) schedules.

It is possible that the blockade of nicotine CPP could be produced by the aversive properties of AM630 (3mg/Kg) in saline-treated mice. It is also important to consider that the action of AM630 may depend on the state of the endocannabinoid tone that should be different between nicotine- and saline-treated mice. Further studies are needed to determine the precise molecular action of AM630 in these two different conditions.

Taken together, the present results revealed that the deletion or the pharmacological blockade of CB2r reduced the rewarding, reinforcing and motivational properties of nicotine. However, these

results are in contrast with those reported by Gamaleddin and colleagues (Gamaleddin *et al*, 2012) in which the administration of AM630 did not produce any significant effect on nicotine reinforcing properties. The discrepancies between the two studies may be due, at least in part, to the following different experimental conditions: 1) these authors studied the effects of AM630 in long Evans rats instead of in CD1 mice; 2) the nicotine self-administration protocol used by Gamaleddin and colleagues also differs from that used in the present study (see (Gamaleddin *et al*, 2012), for instance they evaluated AM630 effects under FR5, PR and reinstatement; instead of under FR1 and PR schedules; and 3) the doses of AM630 employed by Gamaleddin and coworkers (1.25, 2.5 and 5mg/kg) were slightly different from that used in the present study (1 and 3mg/Kg). On the other hand, the results obtained in the present study are opposite in comparison with those found with cocaine motor-sensitization, CPP and self-administration in mice overexpressing CB2r (Aracil-Fernandez *et al*, 2012) or with the administration of CB2r agonists (Xi *et al*, 2011). This could be explained by the different brain effects between both drugs since nicotine reward properties are related with the activation of several nAChRs, whereas cocaine produces a blockade of the dopamine transporter. The different molecular mechanisms underlying the addictive properties of both drugs may justify, at least in part, why genetic and pharmacological manipulation of CB2r results in opposite effects between cocaine and nicotine actions.

The reduced behavioral expression occurring in CB2KO compared with WT mice after nicotine administration could be associated with functional alterations in different key elements regulating nicotine addiction. This drug exerts its reinforcement properties through the activation of nAChRs (Stolerman and Jarvis, 1995) expressed at high levels in the mesolimbic reward dopamine (DA) system (Champtiaux *et al*, 2003; Jones and Wonnacott, 2004; Pidoplichko *et al*, 1997). The activation of nAChRs by nicotine increases DA release and TH expression in VTA (Liu *et al*; Rahman *et al*, 2003). These molecular changes in DA signaling are crucial for the development of nicotine dependence (David *et al*, 2006; Ferrari *et al*, 2002; Ikemoto *et al*, 2006; Laviolette and van der Kooy, 2004; Rahman *et al*, 2003). Intra-VTA infusion of nicotine receptor antagonists blocked the effect of systemic nicotine injections on DA release in NAcc (Nisell *et al*, 1994) and disrupt nicotine self-administration (Corrigall *et al*, 1994). Several evidences suggested that nicotine receptors containing the α3- and α4-nAChRs are involved in the reinforcement effects of nicotine (Glick *et al*; Liu *et al*; Pons *et al*, 2008). Considering the key role of nAChRs mediating the rewarding effects of nicotine, it is

tempting to speculate that the lack of nicotine reinforcement properties observed in CB2KO may be related to functional alterations occurring in nAChRs. Interestingly, α3- and α4-nAChRs gene expression was significantly reduced in the VTA of CB2KO. In addition, these alterations were accompanied by a significant reduction of TH gene expression in the VTA of CB2KO mice. This fact may explain, at least in part, the lack of nicotine reinforcing effects and the impairment in the acquisition of nicotine self-administration and CPP observed in CB2KO mice. The results of this study also revealed that CB2r are found in neurons and glial cells (mostly astrocytes) of WT mice. In addition, in NAcc and VTA of WT mice, almost all α3- and α4-nAChRs immunolabeled cells were also CB2r immunopositive, which strongly suggest that CB2r and both α3- and α4-nAChRs are located in the same neurons. There is no information available regarding the functional cooperation between the CB2r and α3- and α4-nAChRs. However, Jafari and colleagues revealed that the activation or blockade of nAChRs modulates the analgesic effect induced by the selective CB2r agonist JWH133 (Jafari *et al*, 2007). Taken together, these results may provide a possible cellular mechanism to understand the important cooperative role of α3- and α4-nAChRs and CB2r in the behavioral responses associated to the acquisition of nicotine-seeking behavior. Nevertheless, functional alterations in additional key elements closely related with the reward system, such as GABA and/or glutamate may contribute to the low nicotine sensitivity phenotype observed in CB2KO mice. Additional studies are needed to explain the exact nature of the interaction between the CB2r and the nAChRs, and the molecular mechanisms responsible of these functional alterations in CB2KO mice.

The role of CB2r in the development of withdrawal signs was also evaluated. The experimental design used to precipitate a nicotine withdrawal syndrome was previously reported by others (Biala and Weglinska, 2005; Damaj *et al*, 2003; Plaza-Zabala *et al*, 2012; Stoker *et al*, 2008). In this study, the administration of mecamylamine in WT mice significantly increased the most prominent somatic signs observed during nicotine withdrawal (rearings, groomings, scratches, teeth chattering and body tremors). Interestingly, the lack of CB2r completely blocked the somatic expression of the nicotine withdrawal syndrome. To further evaluate whether the CB2r is specifically involved in the regulation of nicotine withdrawal syndrome, the effects of AM630 were examined in the same experimental design in WT mice. Indeed, the acute administration of AM630 significantly attenuated the somatic expression of nicotine withdrawal syndrome, obtaining a behavioral response similar to that observed in CB2KO mice.

In conclusion, the results presented here revealed that CB2r plays a pivotal role in the regulation of the rewarding and reinforcing effects of nicotine and in the behavioral expression of the nicotine withdrawal syndrome. The lack or pharmacological blockade of CB2r resulted in impairments in the CPP, the acquisition of nicotine self-administration and nicotine withdrawal syndrome. Changes in TH, α3 and α4-nAChRs gene expression were found in VTA of CB2KO mice that may be involved, at least in part, in the distinct behavioral responses to nicotine. In addition, the results pointed out that pharmacological manipulation of the CB2r deserves further consideration as a potential new valuable target for the treatment of nicotine dependence. Further studies are needed to explore this hypothesis.

Conflict of interest statement

Neither of the other authors have relevant financial interests to disclose, nor a conflict of interest of any kind.

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Acknowledgements

This work was supported by the following research grants: Ministerio de Ciencia e Innovación, (SAF2011-23420 to Jorge Manzanares; SAF2009-10689 to Pere Berbel and SAF2011-29864 to Rafael Maldonado); Ministerio de Economía y Competitividad, Dirección General de Investigación (PSI2011-24762) to Jose Miñarro; Generalidad Valenciana, Consejería de Educación (PROMETEO/2009/072) to Jose Miñarro; Gobierno Catalán (SGR2009-00131) to Rafael Maldonado; ICREA Academia-2008 to Rafael Maldonado; and Instituto de Salud 'Carlos III' (FIS), RETICS, Red de Trastornos Adictivos (RD06/0001/1004, RD12/0028/0019 to Jorge Manzanares; RD06/001/0016, RD12/0028/005 to Jose Miñarro; RD06/001/001, RD12/0028/0023 to Rafael Maldonado).

We thank Patricia Rodríguez, Analía Rico and Roberto Cabrera for invaluable technical assistance and collaboration.

FIGURE LEGENDS

Figure 1: Nicotine-induced CPP in CB2KO and WT mice, and effects of AM630. (a, b) The bars represent the time in seconds spent in the drug-paired compartment during the pre-conditioning test, and the post-conditioning test. * p<0.01, significant difference in the time spent in the drug-paired compartment in pre-conditioning vs post-conditioning tests.

Figure 2: Acquisition of nicotine self-administration and motivation for nicotine in CB2KO and WT mice, and effects of AM630. (a) Mean number of active and inactive nose-pokes during FR1 schedule of reinforcement to obtain nicotine at the dose of 0.03 mg/kg/infusion i.v. in 1 h daily sessions during 10 days; (b) mean breaking point in a session of progressive ratio that was conducted once and lasted 3 h for WT and CB2KO mice; (c) mean number of active and inactive nose-pokes in WT mice during FR1 schedule of reinforcement to obtain nicotine at the dose of 0.03 mg/kg/infusion i.v. in 1 h daily sessions during 7 days; (d) after acquisition, the administration of AM630 (1 or 3 mg/kg, ip) decreased the number of responses in the active hole when tested on day 8 or day 12 respectively; and (e) mean breaking point in a session of progressive ratio that was conducted on day 15 and lasted 3 h for mice administered with vehicle or AM630 (3 mg/kg, ip). The number of mice was 12 for the WT group and 18 for the KO group in plots (a) and (b). The total number of subjects was 21 in plot (c). The number of mice was 7 for the vehicle group and 14 for the AM630 group in plots (d) and (e). Data are expressed as mean ± SEM. ★P < 0.05; ★★ P < 0.01; ★★★ P < 0.001 comparison between groups (Student-Newman-Keuls or one-way ANOVA).

Figure 3: Real time PCR studies of TH and α3-/α4-nAChRs in the VTA of WT mice. Evaluation of TH and α3- and α4-nAChRs gene expression in the VTA under baseline conditions. Columns represent the means and vertical lines ± SEM of $2^{-(\Delta\Delta Ct)}$ of relative TH (a), α3-nAChRs (b) and α4-nAChRs (c) gene expression in WT and CB2KO mice. * values from CB2KO mice that are significantly different (Student´s *t*-test, p<0.05) from WT mice.

Figure 4: Immunolabeling for CB2r and α3-/α4-nAChRs in the NAcc and VTA of WT mice. Confocal photomicrographs showing immunolabeling for CB2r (green cells in A1, B1, C1 and D1) and either for α3- (red cells in A2 and B2) or α4-nAChRs (red cells in C2 and D2) in NAcc and VTA of WT

mice. In NAcc, double labeling (yellow cells in A3 and C3) indicates that α3- and α4-nAChRs co-localize with CB2r immunoreactive cells. In VTA, scarce α3- and α4-nAChRs immunoreactive cells are not labeled for CB2r (arrow in B2 and arrowhead in D2). Arrows in D1 points two CB2r labeled cells which are immuno-negative for α4-nAChRs.

Figure 5: Somatic signs of mecamylamine-precipitated nicotine withdrawal in WT and CB2KO or WT mice treated with AM630. Columns represent the means and vertical lines ± SEM of number of rearings (5a, 5f), groomings (5b, 5g), scratches (5c, 5h), teeth chattering (5d, 5i) and body tremors (5e, 5h). *, values from WT nicotine-treated mice that are significantly different from WT saline-treated mice ($p<0.05$). &, values from CB2KO nicotine-treated mice that are significantly different from WT nicotine-treated mice. #, values from WT nicotine- and vehicle-treated mice that are significantly different from WT saline- and vehicle-treated mice ($p<0.05$). Φ, values from WT nicotine- and AM630-treated mice that are significantly different from WT nicotine- and vehicle-treated mice ($p<0.05$).

Table 1 Operant responding maintained by nicotine during acquisition.

	Three-way ANOVA	
	Acquisition FR1	
	F-value	*P*-value
Genotype	$F_{(1,28)} = 58.33$	$P < 0.001$
Hole	$F_{(1,28)} = 58.40$	$P < 0.001$
Day	$F_{(9,252)} = 6.79$	$P < 0.001$
Hole × Day	$F_{(9,252)} = 9.17$	$P < 0.001$
Genotype × Hole	$F_{(1,28)} = 49.01$	$P < 0.001$
Genotype × Day	$F_{(9,252)} = 2.04$	$P < 0.05$
Genotype × Hole × Day	$F_{(9,252)} = 5.95$	$P < 0.001$

Table 2 Pharmacological regulation of nicotine self-administration with AM630

	Two-way ANOVA		Two-way ANOVA	
	Session 8 FR1		Session 12 FR1	
	F-value	*F*-value	*F*-value	*F*-value
Group	$F_{(1,19)} = 11.77$	$P < 0.01$	$F_{(1,19)} = 5.25$	$P < 0.05$
Hole	$F_{(1,19)} = 45.40$	$P < 0.001$	$F_{(1,19)} = 31.08$	$P < 0.001$
Group × Hole	$F_{(1,19)} = 11.03$	$P < 0.01$	$F_{(1,19)} = 9.47$	$P < 0.01$

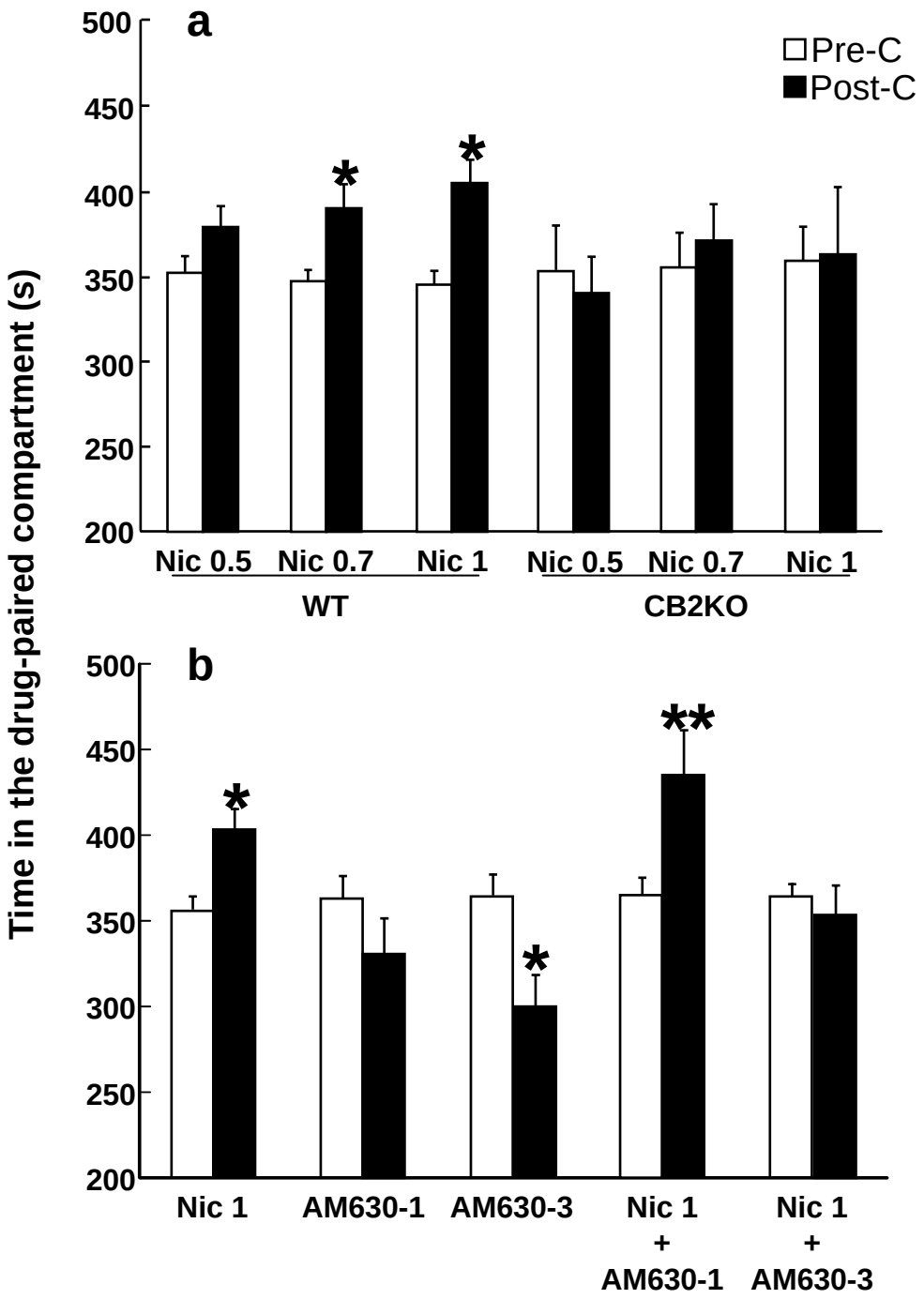
a
Pre-C
Post-C
Time in the drug-paired compartment (s)
500
450
400
350
300
250
200
Nic 0.5
Nic 0.7
Nic 1
Nic 0.5
Nic 0.7
Nic 1
WT
CB2KO
b
Nic 1
AM630-1
AM630-3
Nic 1 + AM630-1
Nic 1 + AM630-3

Figure 1

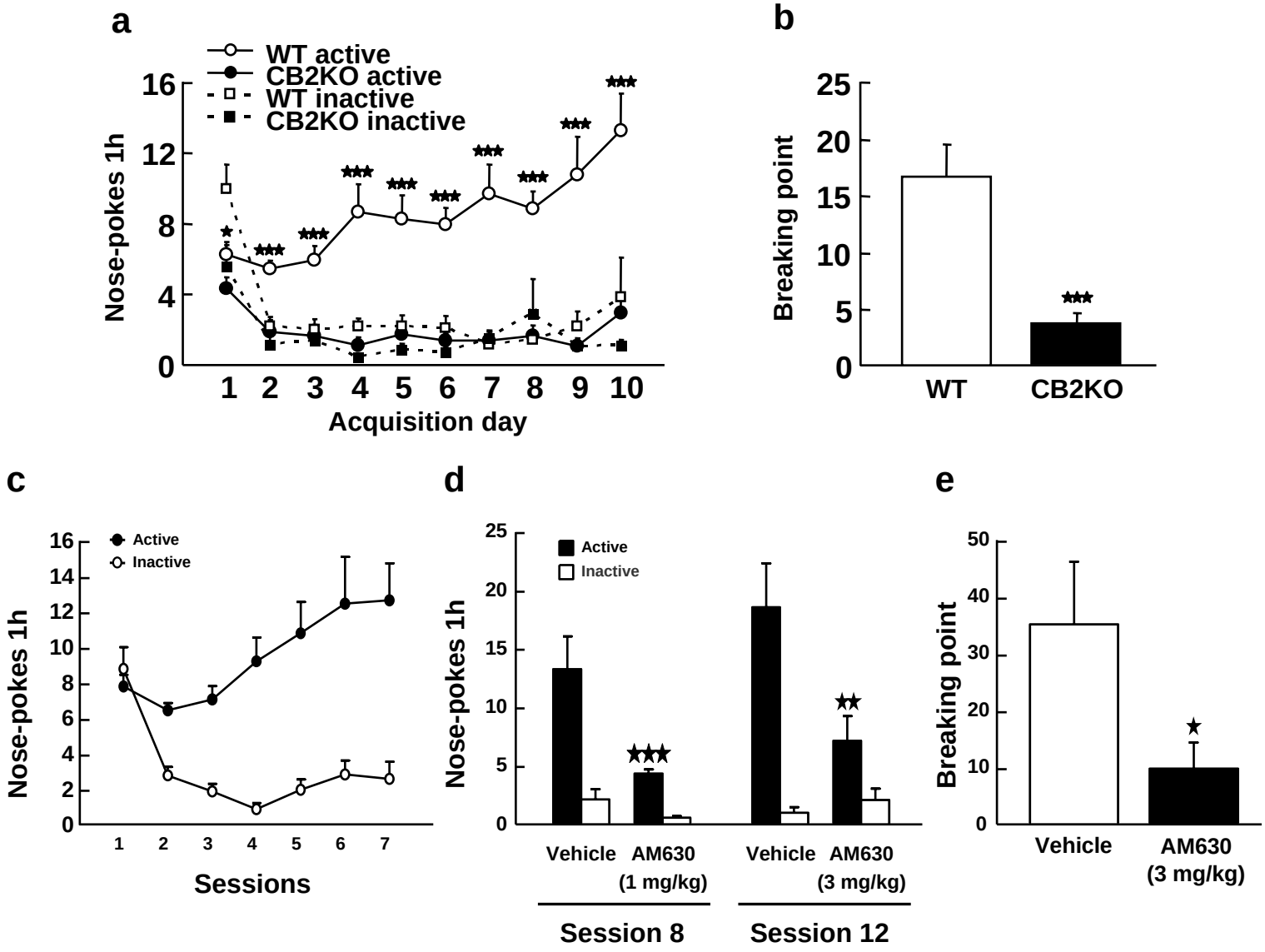
a
WT active
CB2KO active
WT inactive
CB2KO inactive
Nose-pokes 1h
Acquisition day
b
Breaking point
WT
CB2KO
c
Active
Inactive
Nose-pokes 1h
Sessions
d
Active
Inactive
Nose-pokes 1h
Vehicle
AM630 (1 mg/kg)
Vehicle
AM630 (3 mg/kg)
Session 8
Session 12
e
Breaking point
Vehicle
AM630 (3 mg/kg)

Figure 2

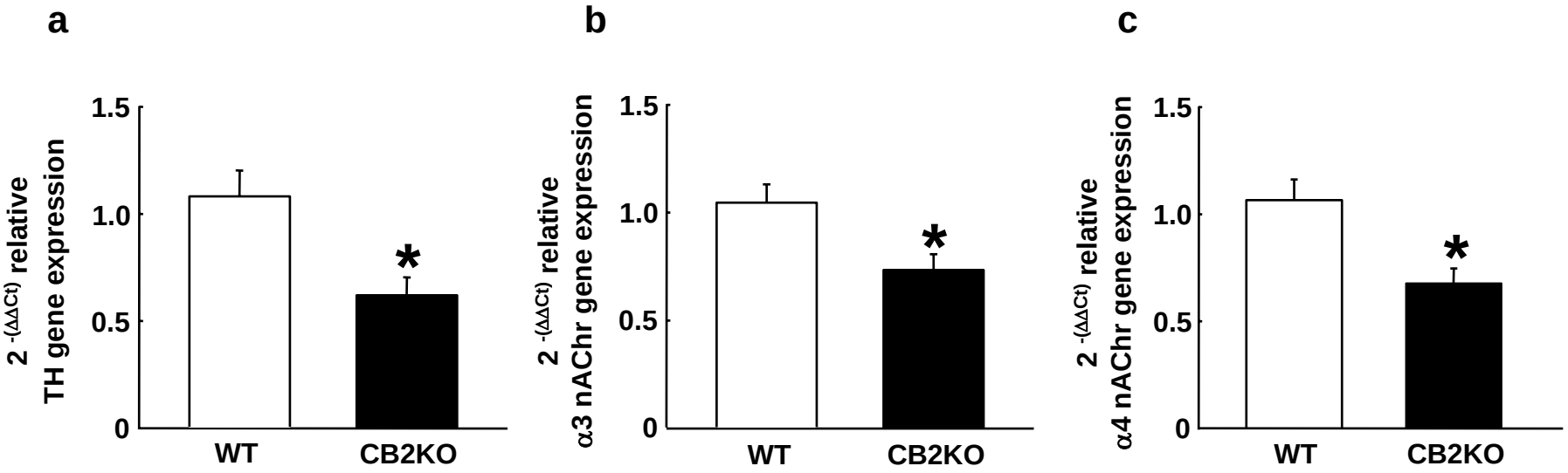
a
b
c
2 -(ΔΔCt) relative TH gene expression
2 -(ΔΔCt) relative α3 nAChr gene expression
2 -(ΔΔCt) relative α4 nAChr gene expression
1.5
1.0
0.5
0
WT
CB2KO
*

Figure 3

Confocal microscopy image
(CB2 and alpha3/alpha4 nAChRs co-localization in ACC and VTA)

Figure 4

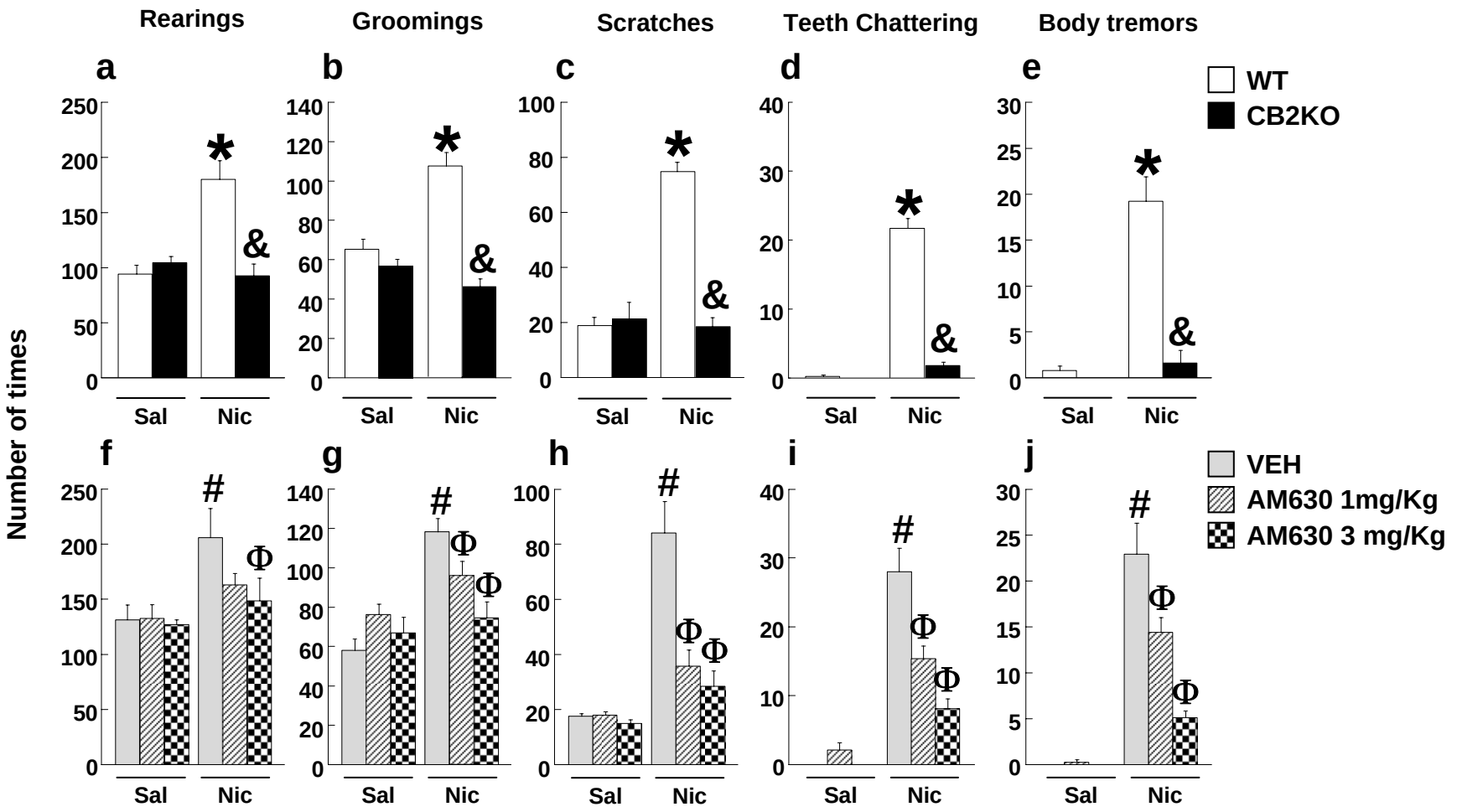
Rearings
Groomings
Scratches
Teeth Chattering
Body tremors
a
b
c
d
e
f
g
h
i
j
Number of times
Sal
Nic
WT
CB2KO
VEH
AM630 1mg/Kg
AM630 3 mg/Kg

Figure 5

SUPPLEMENTARY INFORMATION

MATERIAL AND METHODS

Animals

Male CB2KO mice on a C57BL/6J congenic background (kindly provided by Nancy E. Buckley, Cal State Polytechnic University, Pomona, CA, USA) were used. CB2KO founders were crossed with outbred CD1 (Charles River, France) background (Buckley *et al*, 2000). For immunohistochemical analyses to evaluate CB2 antibody specificity, male mice overexpressing CB2r (CB2xP) on a CD1 congenic background were used. These mice were prepared in our laboratory as described elsewhere (Racz *et al*, 2008).

Conditioned Place Preference

Apparatus

For place conditioning, eight identical Plexiglas boxes with two equal size compartments (30.7 cm length x 31.5 cm width x 34.5 cm height) separated by a gray central area (13.8 cm, length x 31.5 cm, width x 34.5 cm height) were used. The compartments have different colored walls (black vs white) and also distinct floor textures (fine grid in the black compartment and wide grid in the white one). Four infrared light beams in each compartment of the box and six in the central area allowed the recording of the position of the animal and its crossings from one compartment to the other. The equipment was controlled by two IBM PC computers using MONPRE 2Z software (CIBERTEC, SA, Spain).

Procedure

The place conditioning, consisting of three phases, was carried out during the dark cycle following a procedure unbiased in terms of initial spontaneous preference (Manzanedo *et al*, 2011). During the first phase or pre-conditioning (Pre-C) mice were given access to both compartments of the apparatus for 15 min (900 s) only one day. Animals showing strong unconditioned aversion (<33% of the session time) or preference (>67%) for any compartment were discarded. In each group, half the animals received the drug or vehicle in one compartment and the other half in the other compartment. After assigning the compartments, an

ANOVA showed that there were no significant differences between time spent in the drug-paired and the vehicle-paired compartments during the Pre-C phase. In the second phase (conditioning), animals were conditioned with nicotine or saline through six pairings with the respective compartment. Mice received two pairings each day: an injection of physiological saline before being confined in the vehicle-paired compartment for 30 min, and after an interval of 4 h received 1.0, 0.7 and 0.5 mg/kg of nicotine immediately before being confined in the drug-paired compartment for 30 min. In the second experiment, AM630 was injected 30 minutes before conditioning and nicotine was injected immediately prior to conditioning. The central area was made inaccessible during conditioning by lowering the guillotine doors. During the third phase or post-conditioning (Post-C) on day 8, the guillotine doors separating the two compartments were removed and the time spent by the untreated mice in each compartment during a period of 900 seconds was recorded (Post-C tests were performed between 10:00 and 14:00 hours). The difference in seconds between the time spent in the drug-paired compartment in the Post-C test and that spent in the same compartment in the Pre-C test is a measure of the degree of conditioning induced by the drug. If this difference is positive, then the drug has induced a preference for the drug-paired compartment whereas the opposite indicates the induction of an aversion.

To evaluate the effects of AM630, CD1 WT animals were divided into 5 groups for the conditioning phase: Nic 1 (Nicotine 1 mg/kg, n=13), AM630 1 (AM630 1 mg/kg, n=10), AM630 3 (AM630 3 mg/kg, n=10), Nic 1 + AM630 1 (Nicotine 1 mg/kg + AM630 1 mg/kg, n=15), Nic 1 + AM630 3 (Nicotine 1 mg/kg + AM630 3mg/kg, n=14).

Nicotine self-administration

Apparatus

Drug self-administration training and testing occurred in operant chambers (Model ENV-307A-CT, MED Associates, Inc., Georgia, VT, USA) equipped with two holes, one randomly selected as the active hole and the other as the inactive. Pump noise and stimuli lights (cues), one located inside the active hole and the other above it were paired with the delivery of the reinforcer. Chambers were made of aluminum and clear acrylic, had grid floors and were housed in sound- and light-attenuated boxes equipped with fans to provide ventilation and

ambient noise. When mice responded on the reinforced hole, the stimulus light went on and a drug infusion was delivered. Nicotine was infused via a syringe that was mounted on a microinfusion pump (PHM-100A, MED Associates, Inc., Georgia, VT, USA) and connected via Tygon tubing (0.96 mm o.d., Portex Fine Bore Polythene Tubing, Portex Limited, Hythe, Kent, UK) to a single channel liquid swivel (375/25, Instech Laboratories, Plymouth Meeting, PA, USA) and to the mouse intravenous (i.v.) catheter. The swivel was mounted on a counterbalanced arm above the operant chamber.

Surgery

Mice were anesthetized with a ketamine/xylazine mixture (20 ml/kg of body weight) and then implanted with indwelling i.v. silastic catheters (Soria et al. 2005). Briefly, a 6 cm length of silastic tubing (0.3 mm inner diameter, 0.6 mm outer diameter) (Silastic®, Dow Corning, Houdeng-Goegnies, Belgium) was fitted to a 22-gauge steel cannula (Semat, Herts, UK) that was bent at a right angle and then embedded in a cement disk (Dentalon Plus, Heraeus Kulzer, Wehrheim, Germany) with an underlying nylon mesh. The catheter tubing was inserted 1.3 cm into the right jugular vein and anchored with suture. The remaining tubing ran subcutaneously to the cannula which exited at the midscapular region. All incisions were sutured and coated with antibiotic ointment (Bactroban, GlaxoSmithKline, Madrid, Spain). After surgery, animals were allowed to recover for 3 days prior to initiation of self-administration sessions. The catheter was flushed daily with a heparinized saline (30 USP units/ml). The patency of intravenous catheters was evaluated after the PR session and whenever drug self-administration behavior appeared to deviate dramatically from that observed previously by infusion of 0.1 ml thiopental sodium (5 mg/ml) through the catheter. If prominent signs of anesthesia were not apparent within 3 s of the infusion, the mouse was removed from the experiment. The success rate for maintaining patency of the catheter (mean of duration of 11 days) until the end of the nicotine self-administration training was 83%.

Procedure

Acquisition and maintenance of nicotine self-administration in CB2KO and WT mice

CB2KO and WT mice were trained for acquisition and maintenance of nicotine self-administration. Responding was maintained by nicotine (0.03 mg/kg per injection, i.v.) delivered in 23.5 µl over 2 s. Mice were given 1-h daily self-administration sessions during 10 consecutive days. Nose-poking on the active hole resulted in the delivery of a reinforcer (nicotine) while nose-poking on the inactive hole had no consequences. The side of active and inactive hole was counterbalanced between animals. The house light was on at the beginning of the session for 3 s and off during the remaining time of the session. No extra house light was turned on during session. Each daily session started with a priming injection of the drug. Mice were trained under a fixed ratio 1 (FR1) schedule of reinforcement during 10 consecutive daily sessions. After the 10 FR sessions, animals were tested in a PR schedule where the response requirement to earn the reinforcer escalated according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. The maximum duration of the PR session was 3 h or until mice did not respond on any hole within 1 h, and was performed only once. The stimuli light together with the pump noise (environmental cues) signaled delivery of the nicotine infusion. The timeout period after infusion delivery was 10 s. During this 10 s period, the cue light was off and no reward was provided after nose-poking on the active hole. Responses on the inactive hole and all the responses elicited during the 10 s timeout period were also recorded. The session was terminated after 50 reinforcers were delivered or after one hour, whichever occurred first. As previously described (Burokas *et al*, 2012; Martin-Garcia *et al*, 2009; Soria *et al*, 2008; Soria *et al*, 2005), the criteria for self-administration behavior was achieved when all of the following conditions were met: 1) mice maintained a stable responding with less than 20% deviation from the mean of the total number of reinforcers earned in three consecutive sessions (80% of stability); 2) at least 75% responding on the active hole, and 3) a minimum of 4 reinforcers per session.

Pharmacological regulation of nicotine self-administration with AM630

A different group of CD1 wild-type mice were trained for acquisition and maintenance of nicotine self-administration and the procedure was similar to the described above except for the following details. After 7 days of self-administration training, animals were divided in two homogeneous groups corresponding to each pharmacological treatment (AM630 or vehicle) in

order to evaluate the effects of AM630 on nicotine reinforcement. On day 8, mice received an injection of AM630 at the dose of 1 mg/kg, or vehicle i.p., 30 minutes before session. On day 12, after 3 days of recovery to basal levels of nicotine self-administration, mice received an injection of AM630 at the dose of 3 mg/kg, i.p., or vehicle 30 minutes before session. Finally on day 15, after 2 days of recovery to basal levels of nicotine self-administration, mice received an injection of AM630 at the dose of 3 mg/kg, i.p., or vehicle 30 minutes before session, and animals were tested in a PR schedule to assess the effect of the CB2r antagonist on motivation for nicotine. In summary, mice of the AM630 group received a first injection of AM630 at the dose of 1 mg/kg, i.p., on day 8 (FR1), a second injection of AM630 at the dose of 3 mg/kg, i.p., on day 12 (FR1) and a third injection of AM630 at the dose of 3 mg/kg, i.p., on day 15 (PR). Mice of the vehicle group received a total of three injections only with vehicle on days 8 (FR1), 12 (FR1) and 15 (PR).

Immunohistochemistry

WT, CB2xP and CB2KO mice were perfused with 4% paraformaldehyde, 0.002% CaCl2 in 0.1M phosphate buffer (PB; pH 7.3–7.4) for 15 min without post-fixation. Coronal sections (60 µm-thick) were obtained at 0.98–1.70mm from bregma, containing NAcc, and at -2.54 to -2.92 from bregma, containing VTA, using a vibratome. Sections were distributed in eight parallel series. Selected sections from WT mice were subsequently used for confocal double immunolabeling. The remaining sections were post-fixed in the same fixative as used for the perfusion during 4h and stored in 0.05% azide in PBS (PB saline) at 4ºC. For antigen retrieval, WT, CB2xP and CB2KO post-fixed sections were incubated in parallel with Target Retrieval Solution (ref. S1700; Dako Diagnósticos, S.A., Sant Just Desvern, Barcelona, Spain) during 10 min at 95ºC and then rinsed in PBS at room temperature. Series 2 was first incubated with goat anti-CB2r (1:250, Santa Cruz Biotechnology), followed with monkey biotinylated antigoat antibody (1:800, Vector). Then, with Vectastain ABC kit (1:200, Vector) and 0.05% 3,3'diaminobenzidine (DAB; Sigma-Aldrich Co.), mounted on gelatinized slides, air dried during 24 h, dehydrated in ethanol, cleared in xylol and coverslipped. Additional sections were immunostained as previously described, avoiding the incubation with the primary antibody and

were used as negative controls. No immunolabeled cells were seen in these sections (data not shown).

Statistical analyses

For conditioned place preference, the time spent in the drug-paired compartment during pre- and post-conditioning was analyzed by means of a mixed ANOVA with two between variables – Genetics/Dose of AM630 and Dose of nicotine- one within variable -Days-, and two levels -Pre and Post conditioning test-. For the acquisition and maintenance of nicotine self-administration in CB2KO and WT mice, analysis of the data of number of infusions obtained during the acquisition phase from nicotine intravenous self-administration was conducted using two-way ANOVA of repeated measures with day as within-subjects factor and genotype as between-subjects factor. Post-hoc analysis (Newman-Keuls) was performed when required. Data of active and inactive nose-poking responses during the FR1 period of acquisition phase was conducted using three-way ANOVA of repeated measures with day and hole (active/inactive) as within-subjects factors and genotype as between-subjects factor. Post-hoc analysis (Student-Newman-Keuls) was also performed when required. To evaluate the motivation for nicotine, one-way ANOVA of the breaking point obtained on the progressive ratio with genotype as between-subjects factor was performed. For the pharmacological experiments with AM630 on nicotine self-administration, analysis of the number of infusions during the first 7 days of self-administration training was conducted using one-way ANOVA of repeated measures with day as within-subjects factor. Post-hoc analysis (Student-Newman-Keuls) was performed when required. Data of nose-poking responses during the FR1 period of acquisition phase (7 days) was conducted using two-way ANOVA of repeated measures with day and hole (active/inactive) as within-subjects factors. Post-hoc analysis (Student-Newman-Keuls) was performed when required. To evaluate the effects of AM630 on the reinforcing effects of nicotine, two-way ANOVA of nose-poking responses with hole (active/inactive) as within-subjects factor and pharmacological treatment (AM630 or vehicle) as between-subjects factor was performed separately on day 8 and day 12. To evaluate the effects of AM630 on the PR schedule, one-way ANOVA of the breaking point with pharmacological treatment (AM630 or vehicle) as between-subjects factor was performed. To evaluate the effects of different doses of AM360

and the recovery of basal response after each injection, one-way repeated measures ANOVA of active nose-pokes of mice injected with AM630 or vehicle from session 7 to session 14 was performed. Real time PCR data analysis was performed with a Student's t-test to compare relative gene expression between WT and CB2KO mice. The somatic signs of nicotine withdrawal were analyzed by a Two Way ANOVA with nicotine treatment and genotype or AM630 treatment as between-subjects factors. Post-hoc analysis (Student-Newman-Keuls) was performed when required. All results are expressed as mean ± S.E.M. Differences were considered significant at $p<0.05$. The statistical analysis was performed using the Statistical Package for Social Science program SPSS® 15.0 (SPSS Inc, Chicago, USA) and SigmaPlot v11.0 (Systat Software Inc., Chicago, IL).

Supplementary figures legends

Figure S1. Immunolabeling for CB2r in NAcc of WT, CB2xP and CB2KO mice.

Photomicrographs show immunolabeling for CB2r in NAcc of WT (A), CB2xP (B) and CB2KO (C) mice. Both in WT and CB2xP mice, immunolabeled cells are mostly neurons, although CB2r immuno-positive glial cells were also seen. Some of these glial cells look like microglia (arrow in B). Arrowheads point to the anterior commissure. Note the increase of immunolabeling in CB2xP mice compared to WT mice. In CB2KO mice immunolabeling was dramatically reduced. Same scale for A, B and C.

Figure S2. Double immunolabeling of CB2r and α3-nAChR in the frontal cortex of WT mice. Confocal photomicrographs showing immunolabeling for CB2r (green labeling in A) and for α3-nAChR (red labeling in B) in the frontal cortex of WT mice. CB2r immunolabeling is found in the soma of neurons (A) while α3-nAChR immunolabeling is located in the soma and apical dendrites of pyramidal cells (B). Note several astrocytes CB2r immunolabeled (A; arrows). CB2r-positive neuron (A and C; arrowheads) and astrocyte (C; arrow) not labeled for α3-nAChR are shown.

Figure S3. Acquisition of nicotine self-administration during 14 sessions in mice injected with vehicle and AM630. (A) Mean number of active and inactive nose-pokes during FR1 schedule of reinforcement to obtain nicotine at the dose of 0.03 mg/kg/infusion i.v. in 1 h daily sessions during 14 days. After acquisition, arrows indicate the administration of AM630 (1 or 3 mg/kg, ip) or vehicle on day 8 and day 12. The number of mice per group was 7 for the vehicle group and 14 for the AM630 group. Data are expressed as mean ± SEM. ★★ $P < 0.01$; ★★★ $P < 0.001$ comparison between groups (Student-Newman-Keuls). (B) Schematic representation of the procedure of the study of the pharmacological regulation of nicotine self-administration with AM630.

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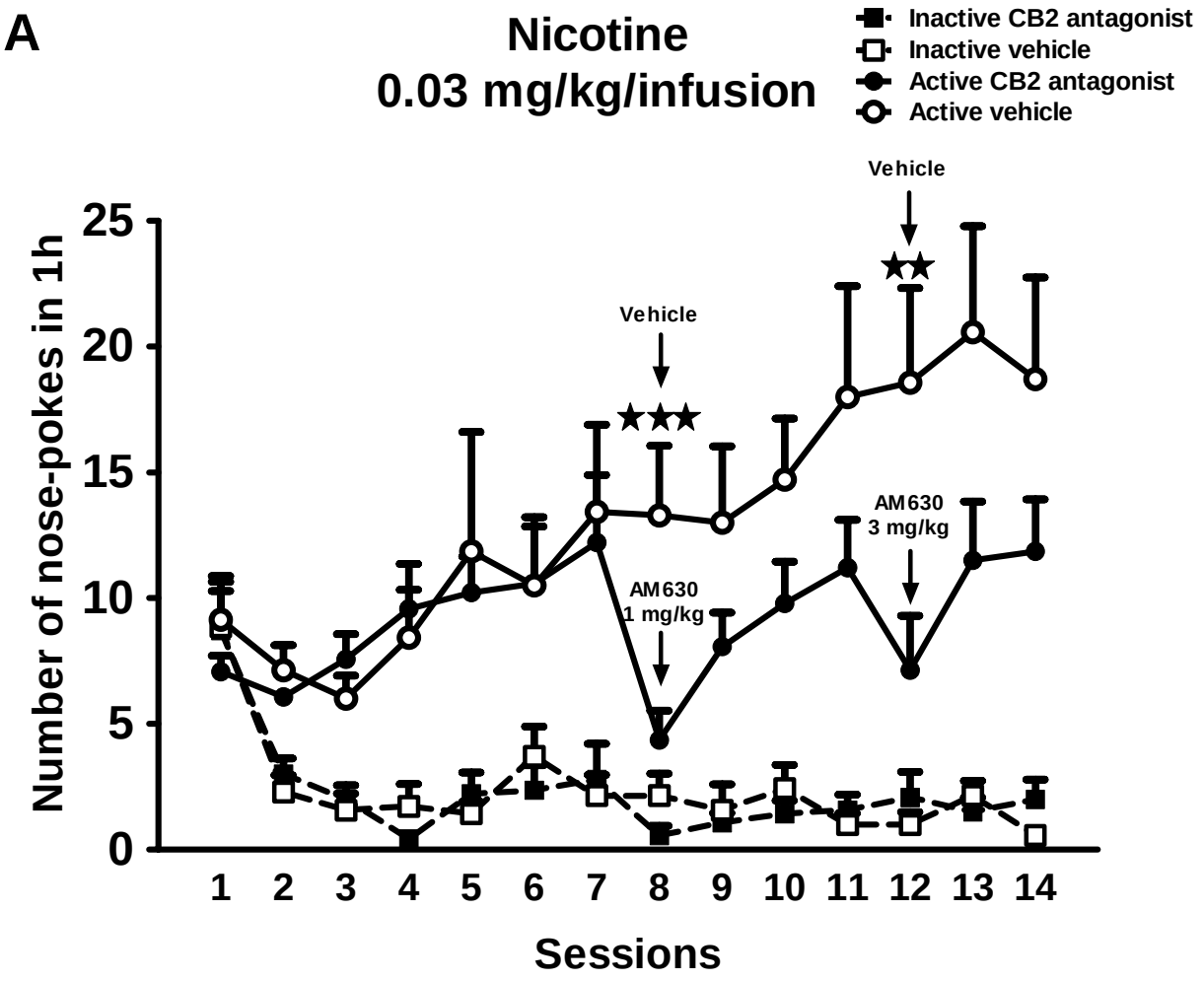
A
Nicotine
0.03 mg/kg/infusion
Inactive CB2 antagonist
Inactive vehicle
Active CB2 antagonist
Active vehicle
Vehicle
Vehicle
AM630
3 mg/kg
AM630
1 mg/kg
Number of nose-pokes in 1h
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Sessions

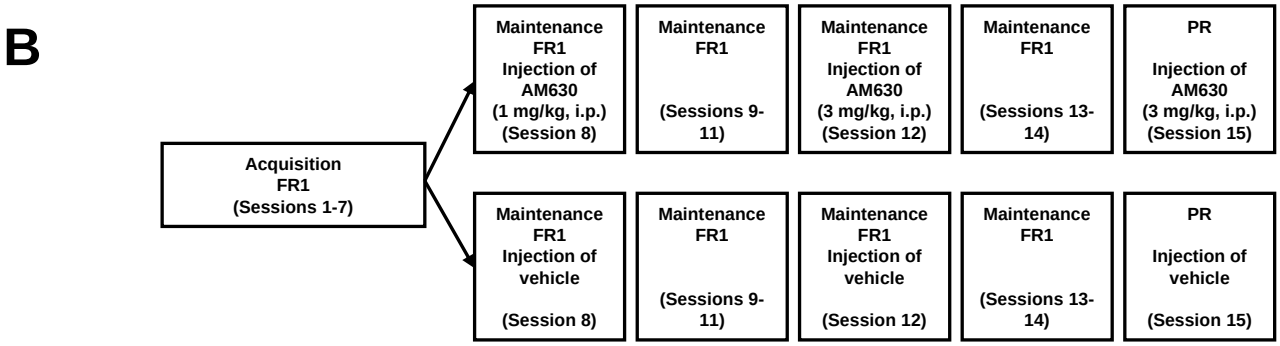
B
Acquisition
FR1
(Sessions 1-7)
Maintenance
FR1
Injection of
AM630
(1 mg/kg, i.p.)
(Session 8)
Maintenance
FR1
(Sessions 9-
11)
Maintenance
FR1
Injection of
AM630
(3 mg/kg, i.p.)
(Session 12)
Maintenance
FR1
(Sessions 13-
14)
PR
Injection of
AM630
(3 mg/kg, i.p.)
(Session 15)
Maintenance
FR1
Injection of
vehicle
(Session 8)
Maintenance
FR1
(Sessions 9-
11)
Maintenance
FR1
Injection of
vehicle
(Session 12)
Maintenance
FR1
(Sessions 13-
14)
PR
Injection of
vehicle
(Session 15)

Figure S3