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The Endogenous Cannabinoid System Modulates Nicotine Reward and Dependence

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Abstract

A growing body of evidence suggests that the endogenous cannabinoid system modulates the addictive properties of nicotine, the main component of tobacco that produces rewarding effects. In our study, complementary transgenic and pharmacological approaches were used to test the hypothesis that the endocannabinoid system modulates nicotine reward and dependence. An acute injection of nicotine elicited normal analgesic and hypothermic effects in cannabinoid receptor (CB₁) knockout (KO) mice and mice treated with the CB₁ antagonist rimonabant. However, disruption of CB₁ receptor signaling blocked nicotine reward, as assessed in the conditioned place preference (CPP) paradigm. In contrast, genetic deletion, or pharmacological inhibition of fatty acid amide hydrolase (FAAH), the enzyme responsible for catabolism of the endocannabinoid anandamide, enhanced the expression of nicotine CPP. Although the expression of spontaneous nicotine withdrawal (14 days, 24 mg/kg/day nicotine) was unaffected in CB₁ KO mice, acute administration of rimonabant (3 mg/kg) ameliorated somatic withdrawal signs in wild-type mice. Increasing endogenous levels of anandamide through genetic or pharmacological approaches exacerbated the physical somatic signs of spontaneous nicotine withdrawal in a milder withdrawal model (7 days, 24 mg/kg/day nicotine). Moreover, FAAH-compromised mice displayed increased conditioned place aversion in a mecamylamine-precipitated model of nicotine withdrawal. These findings indicate that endocannabinoids play a role in the rewarding properties of nicotine as well as nicotine dependence liability. Specifically, increasing endogenous cannabinoid levels magnifies, although disrupting CB₁ receptor signaling, attenuates nicotine reward and withdrawal. Taken together, these results support the hypothesis that cannabinoid receptor antagonists may offer therapeutic advantages to treat tobacco dependence.

Nicotine is the main addictive component in tobacco that acts on the brain to produce rewarding effects and aversive events upon cessation. When neuronal nicotinic acetylcholine receptors (nAChRs) are activated by nicotine, several neuro-transmitters are released (i.e., dopamine, norepinephrine, serotonin, and GABA), activating multiple neuronal systems that may regulate nicotine addiction (Wonnacott et al., 1989, 2005; Castañé et al., 2005). The endocannabinoid system has been implicated in addictive behavior and in the mechanism of action of several drugs of dependence, including nicotine. This system contains cannabinoid receptors (CB₁ and CB₂), the endocannabinoids anandamide (AEA) and 2-arachidonoyl-glycerol, and the enzymes involved in their synthesis and metabolism, for example, anandamide-e [i.e., fatty acid amide hydrolase (FAAH)] and 2-arachidonoyl-glycerol (i.e., monoacylglycerol lipase) (Rodríguez de Fonseca et al., 2005). Furthermore, reports have shown that AEA binds with highest affinity to CB₁ receptors on presynaptic neurons and activates the mesolimbic reward pathway, thereby

providing a common neurobiological substrate in nicotine addiction (Rodríguez de Fonseca et al., 2005). Recent studies have implicated endocannabinoids in the pharmacological and behavioral effects of nicotine. For example, chronic nicotine injections increased AEA levels in the limbic forebrain and brainstem but decreased levels in the hippocampus, striatum, and cerebral cortex (González et al., 2002). Moreover, a CB₁ receptor antagonist, rimonabant, decreased nicotine self-administration and conditioned place preference in rats (Le Foll and Goldberg, 2004; Cohen et al., 2005a,b), suggesting that endocannabinoid signaling may be involved in nicotine reinforcement and reward. In support of this idea, mice lacking CB₁ receptors failed to display nicotine-induced place preference (Castañé et al., 2005). In contrast, moderate doses of rimonabant failed to precipitate withdrawal in nicotine-dependent mice (Castañé et al., 2002; Balerio et al., 2004). Moreover, CB₁ knockout (KO) mice have shown no change in nicotine withdrawal intensity (Castañé et al., 2002, 2005). Altogether, these results suggest that the endocannabinoid system may be involved in modulating the rewarding properties of nicotine through a CB₁ mechanism, whereas modulation of chronic withdrawal is less evident.

AEA is synthesized on demand and may be derived by multiple biosynthetic pathways involving *N*-arachidonoylphosphatidylethanolamine and other possible lyso-*N*-arachidonoylphosphatidylethanolamines (Simon and Cravatt, 2006; Liu et al., 2008). Upon release, AEA is quickly degraded by FAAH into arachidonic acid and ethanolamine (Devane et al., 1992; Cravatt et al., 1996; Rodríguez de Fonseca et al., 2005). Hence, blocking FAAH leads to increased AEA levels and various CB₁ receptor-mediated phenotypes, including hypoalgesia and anxiolytic responses (Cravatt et al., 2001; Lichtman et al., 2004a,b; Moreira et al., 2008). Genetically engineered FAAH KO mice are severely impaired in their ability to metabolize fatty acid amides and exhibit 10- to 15-fold increased AEA levels in the brain (Cravatt et al., 2001). FAAH KO mice also show increased sensitivity to exogenous AEA, although overall behavior is similar to wild-type mice. URB597 has been shown to be an irreversible and selective inhibitor of FAAH activity that can produce a 3- to 5-fold increase in AEA brain levels as well as magnify the physiological responses of AEA in vivo without producing classic cannabinoid effects on its own (Fegley et al., 2005; Rodríguez de Fonseca et al., 2005; Piomelli et al., 2006; Zhang et al., 2007).

If endogenous cannabinoids play a role in the rewarding effects and dependence of nicotine, inhibition of FAAH should enhance the intensity of both effects. To test this hypothesis, we used FAAH KO mice and wild-type mice treated with URB597 to study the rewarding effects of nicotine, as assessed in the conditioned place preference paradigm. Furthermore, we evaluated whether FAAH blockade would alter the acute pharmacological effects of nicotine (analgesia and hypothermia). Finally, we examined FAAH-compromised mice in nicotine dependence by measuring somatic signs of withdrawal and affective withdrawal signs in the condition place aversion paradigm.

Materials and Methods

Animals

Adult male and female FAAH, CB₁ KO, and wild-type mice born in the National Institute on Drug Abuse Center Transgenic Colony at Virginia Commonwealth University (Richmond, VA) served as subjects. All the genetically modified mice used in these studies were backcrossed 13 generations onto a C57BL/6 background. The CB₁ KO mice were derived from CB₁ heterozygote parents, and the FAAH KO mice were derived from congenic FAAH KO parents (Varvel et al., 2007; Wise et al., 2007). Separate groups of adult male C57BL/6J mice obtained from The Jackson Laboratory (Bar Harbor, ME) were used for all drug experiments. All mice were housed either four or five per cage in a temperature-controlled (20–22°C) Association for Assessment of Laboratory Animal Care-approved facility with a 12-h light/

dark cycle and were given free access to food and water. The study was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Drugs

(-)-Nicotine hydrogen tartrate salt was purchased from Sigma-Aldrich (St. Louis, MO). URB597 was purchased from Cayman Chemical (Ann Arbor, MI). The selective CB₁ antagonist, rimonabant, was obtained from the National Institute on Drug Abuse (Rockville, MD). Mecamylamine was purchased from Sigma-Aldrich and dissolved in saline. Nicotine was prepared in saline and administered s.c. Rimonabant and URB597 were dissolved in a mixture of 1:1:18 [5% ethanol/5% Emulphor-620 (sanofi-aventis, Bridgewater, NJ)]/90% distilled water] and administered via the i.p. route of administration. All injections were given in a volume of 10 ml/kg. Nicotine doses are expressed as the free base.

Behavioral Analysis

Nicotine Acute Sensitivity—A control response was determined in CB₁ KO and wild-type mice for the tail-flick test, hot-plate test, and body temperature. The mutant and wild-type controls were treated with saline, mecamylamine (1 mg/kg), or various doses of nicotine (0.5, 1.0, 2.0, or 2.5 mg/kg s.c.). Additional naive C57BL/6J mice were pretreated with 1:1:18 vehicle or various doses of URB597 (0.3, 3, or 10 mg/kg i.p.) 30 min before receiving an acute dose of saline or nicotine (0.5, 1.0, 2.0, or 2.5 mg/kg s.c.). Doses of URB597 were chosen based on in vivo characterizations that maximally inhibit FAAH enzymatic activity (Alexander and Cravatt, 2005; Piomelli et al., 2006). Doses of nicotine were chosen based on established characterizations of acute nicotine administration in the mouse (Damaj et al., 2007). Behavioral assays were completed in the following manner ($n = 5\text{--}15$ per group).

Analgesia: Tail-Flick Test: Spinal antinociception was assessed by the tail-flick method of D'Amour and Smith (1941). Each animal was lightly restrained, whereas a radiant heat source was focused onto the upper portion of the tail. The control response (2–4 s) was determined for each mouse before treatment, and the test response was recorded 5 min after nicotine administration. To minimize tissue damage, a maximum latency of 10 s was imposed. The antinociceptive response was calculated as percent maximum possible effect (%MPE), in which $\%MPE = [(test\ latency - control\ latency)/(10 - control\ latency)] \times 100$.

Analgesia: Hot-Plate Test: Supraspinal antinociception was assessed using the hot-plate test as described previously (Damaj et al., 2007). The mice were placed on the hot-plate (thermostat apparatus maintained at 55°C) before any treatment to determine control responses (8–12 s). Approximately 5 to 8 min after nicotine injections, the test response was recorded. The latency to jump or lick a hind paw served as the dependent measure. A maximum latency of 40 s was imposed to minimize tissue damage. Antinociception was expressed as %MPE, where $\%MPE = [(test\ latency - control\ latency)/(40 - control\ latency)] \times 100$.

Body Temperature: Rectal temperature was measured using a thermistor probe (inserted 24 mm) and digital thermometer (YSI Inc., Yellow Springs, OH). Rectal temperatures were recorded before and 30 min after the s.c. injection of nicotine. The difference in rectal temperature before and after treatment was calculated for each mouse.

Nicotine-Conditioned Place Preference

Nicotine reward in the mouse was evaluated in an unbiased conditioned place preference paradigm as described previously (Walters et al., 2006). Adult wild-type, CB₁ KO, and FAAH KO male and female mice were used in this study due to the limited availability of mutants ($n = 6\text{--}12$ per strain, per group). The place-conditioning boxes consisted of three compartments

(MED Associates, St. Albans, VT), white and black compartments with distinct sides (20 × 20 × 20 cm) and flooring separated by a neutral gray compartment. Movement between the white and black compartments was accessible by two manual doors, which allow access to either side of the chamber. The entrances between the chambers were closed during acclimation and drug conditioning days.

Enrichment—Three to five days before the start of the place-conditioning procedure, all mice in the conditioned place preference studies were given cage enrichment devices to alleviate environmental stress (Reinhardt, 2004; Hutchinson et al., 2005).

Day 1: Preconditioning Phase—On day 1, animals were placed in the gray compartment for a 5-min acclimation period, followed by a 15-min exposure to all compartments, allowing them to roam freely from side to side. The time spent on each side was recorded in seconds (baseline measurements). These data were used to randomize the animals.

Days 2 to 4: Conditioning Phase—C57BL/6J mice were pre-treated with 1:1:18 vehicle or drug i.p. 30 min before saline and nicotine s.c. injections. Rimonabant-treated groups received doses of 0.3, 1.0, or 3.0 mg/kg prior to nicotine (Le Foll and Goldberg, 2004; Cohen et al., 2005a). URB597-treated groups received 0.3, 3.0, 5.0, or 10 mg/kg prior to nicotine. CB₁ and FAAH KO mice along with wild-type controls did not receive any pretreatment. Mice received unbiased conditioning of nicotine or saline for 20 min, with the control groups receiving saline in both sides of the boxes. Drug-treated groups received various doses of nicotine (0.1, 0.3, 0.5, 0.7, or 1.0 mg/kg) in one chamber and saline in the opposite chamber. Drug-paired sides were randomized among all groups. Animals received injections twice daily, receiving pretreatment at each dose (Walters et al., 2006). Mice were counterbalanced in terms of which side was paired with drug and order of treatment (saline versus drug at first injection), allowing exposure to nicotine in the drug-paired side only once per day over the course of 3 conditioning days. AM and PM doses were separated by a minimum of 4 h. At the end of conditioning, all drug-paired animals received a total of three drug and three saline conditioning trials, with alternating sequencing to yield unbiased results. Each group consisted of a sample size of six to 12 mice.

Day 5: Test Phase—On the test day, all animals were placed in the center gray compartment for a 5-min acclimation period and then allowed to roam freely for 15 min. Time spent on each side was recorded, and preference for the drug-paired chamber was expressed as time spent in the drug-paired side on test day (day 5) minus time spent in that chamber on the preconditioning day (day 1). A positive number indicates a preference for the drug-paired side, a negative number indicates an aversion to the drug-paired side, and a value of zero indicates no preference for either side.

Locomotor Activity—In a separate experiment, C57BL/6J mice were administered various doses of URB597 and were tested for activity on test day 5. All mice were placed into individual locomotor activity chambers (28 × 16.5 cm) for 20 min, and activity was measured using photocell beam breaks (MED Associates). Activity data were expressed as the total number of beam interruptions.

Nicotine Withdrawal Studies

Wild-type, CB₁ KO, and FAAH KO male and female mice were implanted with ALZET osmotic minipumps (Models 2004 and 1007D; Durect Corporation, Cupertino, CA) filled with either saline or nicotine (24 mg/kg/day) solutions. The mice were anesthetized using sodium pentobarbital (35 mg/kg i.p.), and the minipumps were surgically implanted s.c. in the back under sterile conditions. Wound clips were used to close the site of incision, and mice were

routinely monitored until recovery. Our laboratory has previously demonstrated that the severity of the nicotine withdrawal syndrome in the mouse is influenced by dose, duration of nicotine treatment, and whether a precipitated or spontaneous withdrawal paradigm is used (Damaj et al., 2003). Accordingly, different dependence procedures were appropriately used to examine the role of the endocannabinoid system in physical and affective signs of nicotine withdrawal. In particular, mice were infused with nicotine or saline for either 7 or 14 days to establish a mild (7-day) or moderate (14-day) magnitude of nicotine dependence, respectively (Damaj et al., 2003). At the end of day 7 or 14, the mice were anesthetized with ether, the pumps were removed by making a small incision at the location of the pump, and sutures were used to close the wound. The animals were allowed to recover overnight, and withdrawal was assessed the following day, approximately 15 to 20 h after mini-pump removal. After 14 days of nicotine infusion (24 mg/kg/day), CB₁ KO and wild-type mice were observed for signs of withdrawal. In a separate 14-day withdrawal model, additional C57BL/6J mice were challenged with 1:1:18 vehicle (i.p.) or rimonabant (3 mg/kg i.p.) and tested 10 min after injections (Balerio et al., 2004; Cohen et al., 2005b). In the 7-day nicotine withdrawal model (24 mg/kg/day), C57BL/6J mice were challenged with 1:1:18 vehicle (i.p.) or various doses of URB597 (3.0, 5.0, or 10 mg/kg i.p.) to observe any augmentation of nicotine withdrawal. Ten minutes after injections, the mice were observed for 20 min, during which time somatic withdrawal symptoms were scored. The subjects were then evaluated for 5 min on the elevated plus maze, followed by assessment in the tail-flick test, a 20-min assessment for locomotor activity, and finally in the plantar stimulator test (see below). Mutant and wild-type control groups did not receive any vehicle or drug challenges before testing on the day of withdrawal. Each group consisted of a sample size of 10 to 15 mice.

Somatic Signs—Animals were placed in individual, Plexiglas containers [28.5 cm (length) × 18 cm (width) × 13 cm (height)] and observed for 20 min for occurrences of head shakes, body tremors, involuntary paw tremors, retropulsion (involuntary backing), writhing, and jumping. For each animal, the total score for this assay was the sum of the individual measures.

Elevated Plus Maze—The elevated plus maze consisted of two closed arms and two open arms on a base raised 60 cm above the floor. The overhead lights were dimmed or removed before placing the mouse in the center of the maze. Each animal was allowed to roam freely between the open and the closed arms for 5 min. The duration of time that the mouse spent in the open arms was obtained through the use of photocell beams connected to a timing device. An increased amount of time spent in the open arms is considered to be an affective sign of withdrawal.

Plantar Stimulation—Subjects were placed in clear Plexiglas compartments [13 cm (length) × 6.5 cm (width) × 25.5 cm (height)]. A radiant heat source was applied to the rear right paw, and withdrawal latency was recorded (three to four measurements per animal). The intensity of the heat source was adjusted to yield withdrawal latencies between 9 and 12 s in experimentally naive C57BL/6J mice (data not shown). A cut-off time of 20 s was used to minimize tissue damage.

Nicotine-Conditioned Place Aversion

Adult wild-type and FAAH KO male and female mice were used in the nicotine-conditioned place aversion paradigm ($n = 5$ –10 mice per genotype). Adult C57BL/6J male mice were also used in this study for pharmacological treatment with URB597. All mice were surgically implanted with 14-day osmotic minipumps (Model 2004; Durect Corporation) filled with either saline or nicotine (36 mg/kg/day) solutions for 7 days before nicotine-conditioned place aversion testing. The daily nicotine dose was increased to increase the severity of nicotine dependence and establish conditioned aversion within a 1-week period (Damaj et al., 2003;

Jackson et al., 2008). Nicotine-conditioned place aversion conditioning began after 7 days of infusion. Saline and nicotine minipumps were not removed during conditioning to continue delivery of nicotine or saline during drug treatments. Conditioning for nicotine place aversion was conducted in the same apparatus previously described for place preference.

Day 1: Preconditioning Phase—After 7 days of saline or nicotine infusion, all mice were placed in conditioning chambers to obtain basal preference scores. Mice were placed in the gray compartment for a 5-min acclimation period, followed by a 15-min exposure to all compartments, allowing them to roam freely from side to side. The duration of time spent in each side was recorded (baseline measurements). Using a biased procedure, mice were drug-paired to their initially preferred compartment, as determined by basal preference scores. Nicotine-conditioned place aversion procedures were based on previous studies with nicotine aversion in rats (Suzuki et al., 1996) and concurrent mouse studies in our laboratory (Jackson et al., 2008).

Days 2 to 3: Conditioning Phase—Beginning at 8:00 AM, all mice received vehicle (i.p.) and were placed in the nonpreferred chamber for 30 min. Approximately 4 h later, the mice were given an i.p. injection of vehicle, mecamylamine (3.5 mg/kg), or URB597 (10 mg/kg) and were confined to the preferred chamber for 30 min. This dose of URB597 (10 mg/kg) was chosen in response to the most effective dose observed in somatic signs of nicotine withdrawal. The mice were conditioned in this manner for 2 days.

Day 4: Test Phase—All animals were placed in the center gray compartment for a 5-min acclimation period and then allowed to roam freely between all compartments for 15 min. Time spent on each side was recorded, and aversion was determined as time spent in the drug-paired side on test day (day 4) minus time spent in that chamber on the preconditioning day (day 1). Each group consisted of a sample size of five to 10 mice.

Statistical Analyses

All conditioning and behavioral scores were expressed as mean $\pm$ S.E.M. for each group. Statistical analyses of all conditioned place preference and aversion studies were analyzed using one- or two-way analyses of variance (ANOVA), followed by Newman-Keuls post hoc tests when appropriate. All other behavioral assessments were appropriately analyzed by Student's *t* tests or mixed-factor ANOVA followed by post hoc comparisons (Newman-Keuls test). *p* Values of less than 0.05 were considered to be significant.

Results

Endocannabinoid Modulation of Nicotine Reward Using the CPP Assay

Disruption of CB₁ Receptor Signaling Blocks the Rewarding Effects of Nicotine

—As shown in Fig. 1, genetic deletion (Fig. 1a) or pharmacological (Fig. 1b) inhibition of CB₁ receptors blocked the rewarding properties of nicotine CPP. Nicotine (0.5 or 0.7 mg/kg) produced rewarding effects in the conditioned place preference in wild-type mice; however, it failed to produce any preference response in CB₁ knockout mice, irrespective of dose. Likewise, rimonabant dose-dependently blocked nicotine CPP in C57BL/6J mice, without producing any effects on its own [$F(1,60) = 10.2$; $p < 0.05$].

Nicotine Reward Is Increased in FAAH KO Mice—To determine what influence elevated levels of AEA would have on the effects of nicotine, CPP was carried out in FAAH KO mice. Although a dose of 0.1 mg/kg nicotine was ineffective in wild-type mice, it significantly increased CPP in FAAH KO mice [Fig. 2a; $F(3,104) = 5.7$; $p < 0.05$]. In addition, FAAH KO mice continued to display CPP after increased doses of nicotine. Rimonabant

significantly blocked the rewarding properties of 0.1 mg/kg nicotine in CPP, indicating a CB₁ receptor mechanism of action [Fig. 2b; $F(7,58) = 1.5$; $p < 0.05$]. No sex differences were observed [FAAH KO, $F(3,41) = 0.1$; wild type, $F(3,51) = 0.6$; $p > 0.05$].

The FAAH Inhibitor URB597 Increases the Potency of Nicotine Reward—After establishing that FAAH KO mice displayed increased sensitivity to the rewarding properties of nicotine, studies were carried out to determine whether the FAAH inhibitor URB597 would similarly enhance nicotine-induced conditioned place preference in mice. Indeed, URB597 significantly enhanced the rewarding effects of 0.1 mg/kg nicotine [Fig. 3; $F(9,63) = 10.3$; $p < 0.05$]. It is interesting to note that increasing doses of URB597 treatment resulted in a U-shaped dose-response relationship with low doses (i.e., 0.3 and 3 mg/kg) but not high doses (i.e., 5 and 10 mg/kg) of URB597, eliciting significant increases in nicotine preference. URB597-induced enhancement of the effects of nicotine in the CPP occurred without a significant effect on the locomotor activity of the animals. In particular, when URB597 (0.3, 3.0, 5.0, and 10 mg/kg, i.p.) was administered before a low dose of nicotine (0.1 mg/kg s.c.), it failed to elicit any significant changes in locomotor activity (Table 1 [$F(9,45) = 1.0$; $p > 0.05$]).

Evaluation of CB₁ Receptors and FAAH Inhibition on the Acute Pharmacological Effects of Nicotine

Nicotine Produces Antinociception and Hypothermia Independently of CB₁ Receptors—To determine whether the role of the endocannabinoid system is unique to nicotine CPP, further studies were carried out to determine whether disruption of CB₁ receptor signaling would also affect the acute effects of nicotine. In these studies, antinociceptive responses were measured in spinal and supraspinal tail-flick and hot-plate tests, respectively, followed by evaluation of nicotine-induced hypothermia. As shown in Fig. 4, nicotine elicited significant dose-dependent effects in each of the three tests. No significant shifts in nicotine potency were observed between CB₁ KO and wild-type mice for all tests. Likewise, pharmacological blockade of CB₁ receptors failed to antagonize nicotine-induced antinociception and hypothermia [vehicle + vehicle = $0.5 \pm 0.3^\circ\text{C}$; vehicle + nicotine (2.5 mg/kg) = $-5.4 \pm 0.7^\circ\text{C}$; rimonabant (3 mg/kg) + nicotine (2.5 mg/kg) = $-5.3 \pm 0.8^\circ\text{C}$], although mecamylamine completely blocked the acute effects of nicotine (Fig. 5).

FAAH Inhibition Does Not Affect Nicotine-Induced Antinociception and Hypothermia—After evaluating the responses of CB₁ blockade in acute nicotine sensitivity, FAAH inhibition was investigated in acute nicotine responses. Table 2 depicts ED₅₀ values and 95% confidence limits of nicotine in tail-flick, hot-plate, and body temperature in mice pretreated with vehicle or URB597 (0.3, 3, or 10 mg/kg). The potency of nicotine was unaffected by all doses of URB597 evaluated.

Endocannabinoid Modulation of Spontaneous Nicotine Withdrawal

Effects of CB₁ Receptor Blockade on Nicotine Withdrawal—To investigate the involvement of the endocannabinoid system on nicotine withdrawal, studies were conducted in mice implanted with nicotine minipumps. Wild-type and CB₁ KO mice exhibited an equivalent magnitude of spontaneous withdrawal after removal of nicotine minipumps (Fig. 6a). In contrast, rimonabant (3 mg/kg) significantly reduced somatic signs of withdrawal compared with vehicle-treated C57BL/6J mice [Fig. 6b; $F(1,28) = 29.5$; $p < 0.05$; main effect of nicotine $F(1,28) = 118.2$; $p < 0.05$]. When individual signs were analyzed, the global decrease in somatic withdrawal signs by rimonabant was attributed to significant decreases in backing and body tremors [data not shown; backing, $T(26) = 2.5$; body tremors, $T(26) = 2.5$; $p < 0.05$]. Other somatic signs remained unchanged (i.e., paw tremors, head shakes, writhing).

FAAH Blockade Augments the Severity of Somatic Signs of Spontaneous

Nicotine Withdrawal—As shown in Fig. 7a, FAAH KO mice exhibited a 2-fold increase in global withdrawal scores compared with wild-type control mice [$F(1,55) = 28.2$; $p < 0.05$]. A breakdown of the somatic signs revealed that the FAAH KO mice had significantly more paw tremors, head shakes, and body tremors than the wild-type mice [paw tremors, $T(21) = -2.3$; head shakes, $T(21) = -4.4$; body tremors, $T(21) = -6.5$; $p < 0.05$]. On the other hand, neither FAAH KO mice nor wild-type mice infused with vehicle displayed any appreciable somatic signs.

Pharmacological inhibition of FAAH after removal of nicotine minipumps yielded a similar pattern of results as the FAAH KO mice. In particular, the high dose of URB597 significantly increased nicotine spontaneous withdrawal signs compared with the vehicle control [Fig. 8a; $F(1,32) = 10.3$; $p < 0.05$]. URB597 did not elicit any obvious effects in mice implanted with saline minipumps. In the nicotine-dependent mice, URB597 (10 mg/kg) elicited a significant increase in body tremors compared with the vehicle-treated group [$T(18) = -4.9$; $p < 0.05$]. Although paw tremors were significantly reduced in the URB597-treated mice compared with control mice, the magnitude of this effect was very small in the control group [$T(18) = 2.3$; $p < 0.05$].

Conditioned Place Aversion during Nicotine-Precipitated Withdrawal Is

Increased in FAAH KO Mice—In the final series of experiments, we used complementary genetic and pharmacological approaches to determine the impact of elevating endogenous cannabinoid levels in the affective signs of mecamylamine-precipitated nicotine withdrawal. In the nicotine-infused mice challenged with mecamylamine, FAAH KO mice exhibited a significant increase in conditioned place aversion compared with wild-type mice [Fig. 9a; $F(1,49) = 2.4$; $p < 0.05$]. No place aversion was displayed by saline-infused mice, with or without mecamylamine, regardless of genotype.

The highest effective dose of URB597 (10 mg/kg) observed in somatic signs of nicotine withdrawal was tested in nicotine CPA. The effects of URB597 (10 mg/kg) on nicotine-affective withdrawal signs are illustrated in Fig. 9b. URB597 (10 mg/kg) was administered before mecamylamine (3.5 mg/kg) and did not produce increases in nicotine-conditioned aversion compared with nicotine/mecamylamine treatment [$F(2,37) = 2.7$; $p = 0.7480$].

Discussion

The endocannabinoid system influences the rewarding properties of several drugs of abuse, including nicotine. In this study, we further analyzed the involvement of the endocannabinoid system in multiple aspects of nicotine dependence by using tools to increase endocannabinoids levels, in particular AEA or manipulating the CB₁ receptor. Previous studies in rats (Le Foll and Goldberg, 2004) have shown that CB₁ blockade abolishes nicotine CPP. In agreement with these results, we show that rimonabant blocks nicotine-induced CPP in the mouse and confirm other reports that CB₁ KO mice do not express nicotine preference (Castañé et al., 2002). These findings suggest that endogenous cannabinoids are tonically involved with either the expression or development of nicotine reward. Therefore, we hypothesized that direct manipulation of the endocannabinoid system would modulate nicotine reward through a CB₁ mechanism.

In our studies, we used FAAH KO mice and the FAAH inhibitor URB597 to increase endocannabinoid levels (Cravatt and Lichtman, 2003). Here, we show that FAAH inhibition by URB597 dose-dependently enhanced the rewarding properties of nicotine as measured by CPP, suggesting that increasing endocannabinoid levels yielded enhanced rewarding effects of low doses of nicotine. These results are in agreement with a previous report that showed

coadministration of low doses of nicotine and δ -9-tetrahydrocannabinol induced higher CPP in mice (Valjent et al., 2002). Furthermore, no significant changes in locomotor activity observed after nicotine conditioning with URB597 treatment were observed. Despite increases in endocannabinoid levels by URB597, these findings suggest that locomotor activity was not affected by nicotine CPP and that endocannabinoid enhancement of nicotine preference was not influenced by changes in animal motility. It is also interesting to note that enhancement of nicotine reward diminished at higher doses of URB597. Nicotine alone produces less rewarding effects at higher doses (Risinger and Oakes, 1995; Grabus et al., 2006), which is consistent with this finding.

Similar results were confirmed by CPP in FAAH KO mice in that enhancement of nicotine reward occurred at an inactive dose of nicotine. Therefore, it seems that enhancement of nicotine reward is limited to a narrow range of URB597 doses. Despite this limited enhancement of nicotine CPP, in FAAH KOs, we were able to show that pharmacological blockade of CB₁ receptors by rimonabant abolished the enhancement of nicotine reward (0.1 mg/kg). Altogether, these results further support the hypothesis that endocannabinoids influence the rewarding properties of nicotine through a CB₁ receptor mechanism.

One possible mechanism explaining endocannabinoid modulation of nicotine reward may be a potentiation of peak elevations of DA and a sustained duration of elevated DA in the nucleus accumbens (Solinas et al., 2006). Although increased AEA and low doses of nicotine may boost dopaminergic projections in the mesolimbic reward pathway, it is possible that the combination of high levels of endocannabinoids and high doses of nicotine causes an exhaustion of DA release. This hypothesis is further supported by a report showing that the cannabinoid receptor agonist WIN55,212-2 increased the frequency and amplitude of DA transients that were accompanied with a decrease in electrically evoked DA release in the nucleus accumbens, indicating that the amount of DA released for each pulse was diminished (Cheer et al., 2004). Indeed, our results show a 3- to 5-fold increase in nicotine CPP that may correlate with increasing AEA levels [3–5-fold and 10–15-fold increase by URB597 and FAAH KO mice, respectively (Cravatt and Lichtman, 2003)] as well as increasing the potency of nicotine CPP when pre-treated with low doses of URB597 (0.3 and 3.0 mg/kg). Alternatively, a culmination of nicotine and high doses of URB597 (5 and 10 mg/kg) in CPP eliminated preference. Therefore, an imbalance of direct and indirect activity on excitation and inhibition in DA signaling may induce higher preference or a lack of response in the rewarding properties of nicotine (Cohen et al., 2005a; Rodríguez de Fonseca et al., 2005).

Another possible mechanism for enhancing the rewarding effects of nicotine by endocannabinoids is an increase in the acute sensitivity to nicotine. Nicotine acutely activates several nAChR subtypes that are centrally mediated and induces several physiological and pharmacological effects. Previous reports have indicated that CB₁ KOs may enhance acute nicotine-induced antinociception in the tail immersion test with a lack of enhancement in the hot-plate and locomotor tests (Castañé et al., 2002). However, our data show that CB₁ deletion and CB₁ antagonism by rimonabant does not affect nicotine-induced antinociception in either the tail-flick or hot-plate tests, nor does it affect nicotine-induced hypothermia, clearly indicating that CB₁ receptors are not involved with acute nicotine expression under our experimental conditions. These inconsistencies may be due to differences in the genetic background of the mutant mice used in the two studies, the time at which measurements were taken, or the doses of nicotine used to elicit an effect. In addition, we show that FAAH inhibition by URB597 does not modulate nicotine potency in acute antinociception and hypothermia. Thus, it seems that acute sensitivity to nicotine is not a major contributor to endocannabinoid modulation of the rewarding effects of nicotine.

Previous reports have shown that CB₁ receptors are not directly involved in nicotine withdrawal. For example, somatic signs of nicotine withdrawal were unaffected in CB₁ KO mice (Balerio et al., 2004), and a moderate dose of rimonabant (3 mg/kg) failed to precipitate somatic signs in nicotine-dependent mice (Balerio et al., 2004). In agreement with these results, we observed that these nicotinic signs of withdrawal were unchanged in CB₁ KO mice. Other withdrawal signs such as anxiety-related behavior and hyperalgesia were also similar in CB₁ KO and wild-type mice (data not shown). However, although rimonabant (3 mg/kg) did not precipitate nicotine withdrawal in our study, it did significantly reduce the total withdrawal score for somatic signs in a nicotine spontaneous withdrawal model. These discrepancies between the KO and rimonabant data could be a result of compensation or molecular adaptations in the genetically altered mice or non-CB₁ actions of rimonabant.

Therefore, to rule out a direct involvement of endocannabinoids, we investigated nicotine withdrawal in FAAH KO mice and after pharmacological inhibition of the enzyme by URB597. For that, we reduced the duration of nicotine infusion (7 days, 24 mg/kg/day) to detect possible enhancements of withdrawal caused by elevated endocannabinoid levels. After acute treatment, only a high dose of URB597 (10 mg/kg) resulted in a significant increase in nicotine withdrawal intensity. This increase in somatic signs was mainly due to a large increase in body tremors. It is interesting to note that this dose of URB597 did not show any increase in nicotine-induced CPP, indicating that FAAH inhibition by URB597 enhances the potency of nicotine reward (3–10 times lower dose of URB597). However, it is possible that at such a high dose, URB597's actions could possibly be an effect of off-target proteins other than FAAH or elevated levels of other fatty acid amide signaling molecules (such as oleamide, *N*-acyl-ethanolamines), causing differential effects that are not directly related to increased levels of AEA (Cravatt and Lichtman, 2003; Zhang et al., 2007). Nevertheless, when FAAH KO mice were tested, somatic signs were 2-fold higher compared with wild-type controls. The increase in somatic signs for FAAH KO mice was also increased in multiple signs, showing a generalized enhancement of nicotine withdrawal.

Taken together, nicotine withdrawal results suggest that endocannabinoids modulate the somatic signs of nicotine withdrawal through non-CB₁ receptors. These data further suggest that FAAH inhibition may worsen some of the negative aspects of nicotine dependence by enhancing the physical signs of nicotine withdrawal. In addition, the affective signs of nicotine withdrawal (i.e., conditioned place aversion) were enhanced in FAAH KO mice compared with wild-type mice. However, FAAH inhibition by a high dose of URB597 (10 mg/kg) given with mecamylamine failed to significantly enhance aversion compared with the mecamylamine control group. It should be noted, that compensatory changes may have occurred in the FAAH KO mice as a result of the genetic deletion. Therefore, the differences noted in our withdrawal studies using the FAAH KO mice and the FAAH inhibitor URB597 could be a result of compensation mechanisms in FAAH KO mice or drug interactions (mecamylamine-URB597) during conditioning. Although we cannot completely rule out effects on FAAH, these findings indicate that the observed enhancement of nicotine withdrawal may involve other enzymatic systems and is not solely due to inhibition of FAAH activity. It is possible that increased levels of endocannabinoids could modulate multiple aspects of nicotine dependence by inhibiting selective nAChR subtypes. Indeed, recent studies indicate that AEA directly inhibits the function of expressed (Spivak et al., 2006) and native (Butt et al., 2008) $\alpha 4\beta 2$ nAChRs in a CB₁ receptor-independent manner.

In conclusion, we have shown that the endocannabinoid system modulates various aspects of nicotine dependence in a differential way. Indeed, FAAH inhibition dramatically enhances nicotine reward through a CB₁ mechanism that is most likely due to elevated levels of AEA. Moreover, our findings indicate that endogenous cannabinoid tone indirectly modulates the

development of nicotine addiction by affecting the balance between the rewarding and aversive properties of nicotine.

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ABBREVIATIONS

nAChR, nicotinic acetylcholine receptor; CB, cannabinoid receptor; AEA, anandamide; FAAH, fatty acid amide hydrolase; KO, knockout; WIN55,212-2, *R*-(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazinyl]-(1-naphthalenyl) methanone mesylate; URB597, 3'-carbamoyl-biphenyl-3-*y*-cyclohexylcarbamate; %MPE, percentage of maximum possible effect; CPP, conditioned place preference; DA, dopamine..

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Fig. 1.

Disruption of CB₁ receptor signaling blocks nicotine place preference. a, CB₁ KO mice fail to exhibit normal nicotine CPP compared with wild-type mice at 0.5 and 0.7 mg/kg nicotine s.c. b, nicotine preference is observed in the control group given vehicle (i.p.) 30 min before nicotine 0.5 mg/kg s.c. Thirty-minute pretreatment with the CB₁ antagonist, rimonabant (1 and 3 mg/kg i.p.), blocks nicotine preference. *, $p < 0.05$ compared with saline controls (a and b). #, $p < 0.05$ compared with respective wild-type controls (b). Data are expressed as means $\pm$ S.E.M. of six to eight mice per group.

Fig. 2.

FAAH deletion enhances nicotine preference. This figure shows nicotine CPP at different doses after s.c. administration in male and female FAAH KO and wild-type mice. a, FAAH KO mice exhibit an increase in 0.1 mg/kg nicotine CPP and do not show any further differences at higher doses of nicotine. b, enhancement of nicotine CPP (0.1 mg/kg) is blocked by pretreatment of rimonabant (3 mg/kg). *, $p < 0.05$ compared with respective saline controls. #, $p < 0.05$ compared with respective wild-type controls (a) or compared with rimonabant/nicotine treatment (b). Data are expressed as means $\pm$ S.E.M. of 10 to 12 mice per group. Sal, saline; Veh, vehicle; Nic, nicotine.

Fig. 3. URB597 enhances nicotine CPP in mice. Pretreatment with the FAAH enzyme inhibitor URB597 or vehicle, given 30 min before an active dose of 0.1 mg/kg nicotine s.c., significantly enhances nicotine preference at 0.3 and 3.0 mg/kg URB597 i.p. *, $p < 0.05$ compared with respective saline control and vehicle/nicotine control. #, $p < 0.05$ compared with all treatment groups. Data are expressed as means $\pm$ S.E.M. of 10 to 12 mice per group.

Fig. 4.

Effects of acute nicotine administration in CB₁ KO mice. This figure displays dose-response curves for nicotine-induced antinociception in the tail-flick test (a), the hot-plate test (b), and hypothermia (c) in wild-type (●) and CB₁ KO (□) mice. Mice were treated with acute doses of nicotine (s.c.) and tested 5 min later for antinociception and 30 min after injection for body temperature measures. Data are expressed as means ± S.E.M. of six to eight mice per group.

Fig. 5.

Rimonabant does not block acute nicotine responses in mice. Pretreatment with mecamlamine (1 mg/kg s.c., 10 min) significantly blocks acute nicotine-induced antinociception (2.5 mg/kg s.c.) as measured in the tail-flick (a) and hot-plate (b) tests in C57BL/6J mice. Rimnabant (3 mg/kg s.c.) given 30 min before acute nicotine does not alter the analgesic response of nicotine in the tail-flick (a) and hot-plate (b) tests. *, $p < 0.05$ compared with respective saline control. #, $p < 0.05$ compared with other nicotine groups. Data are expressed as means $\pm$ S.E.M. of six to eight mice per group (VEH, vehicle; SAL, saline; MEC, mecamlamine).

Fig. 6.

Effects of CB₁ deletion and antagonism in nicotine withdrawal. a, male CB₁ KO and wild-type mice are implanted with saline or nicotine minipumps (24 mg/kg/day) for 14 days and then tested for somatic signs of spontaneous withdrawal. b, effects of an acute dose of vehicle or rimónabant (3 mg/kg) on the expression of somatic signs are shown in graph b. Minipumps are removed at day 14; 15 to 20 h later, mice received injections with vehicle or rimónabant. They are observed for somatic signs of withdrawal 10 min after injections. Rimónabant significantly decreases the total withdrawal score of somatic signs compared with the vehicle/nicotine control group. *, $p < 0.05$ compared with respective saline controls. #, $p < 0.05$ compared with nicotine controls. Data are expressed as means $\pm$ S.E.M., eight to 10 mice per group.

Fig. 7.

Somatic signs of nicotine withdrawal in FAAH KO mice. FAAH KOs and wild-type controls receive saline or nicotine minipumps (24 mg/kg/day) for 7 days. Minipumps are removed at day 7; 15 to 20 h later, the mice are observed for somatic signs of withdrawal. a, FAAH KOs show a 2-fold increase in the expression of withdrawal signs, compared with wild type. b, FAAH KOs do not elicit new somatic signs but showed significant increases in paw tremors, head shakes, and body tremors. *, $p < 0.05$ compared with the saline controls. #, $p < 0.05$ compared with wild-type controls. Data are expressed as means $\pm$ S.E.M., 10 to 15 mice per group.

Fig. 8.

Effects of FAAH inhibition by URB597 on somatic signs of nicotine withdrawal. Somatic signs are observed in spontaneous withdrawal model mice exposed to saline or nicotine minipumps (24 mg/kg/day) for 7 days. Minipumps are removed at day 7; 15 to 20 h later, mice received injections with vehicle or URB597 (3, 5, or 10 mg/kg). They are observed for somatic signs of withdrawal 10 min after injections. All nicotine groups shown are significantly higher than their saline controls. Groups treated with 3 and 5 mg/kg URB597 are not significantly different from the vehicle/nicotine control. *, $p < 0.05$ compared with respective saline controls. #, $p < 0.05$ compared with vehicle controls groups. Data are expressed as means $\pm$ S.E.M., 10 to 15 mice per group.

Fig. 9.

FAAH inhibition and deletion potentiates the affective signs of aversion in nicotine withdrawal. Male mice are implanted with saline or nicotine minipumps (36 mg/kg/day) for 7 days and then conditioned with vehicle or mecamylamine (3.5 mg/kg) for 2 days. a, FAAH KO mice exhibit higher aversion in nicotine withdrawal compared with wild-type controls. b, C57BL/6J male mice treated with URB597 (10 mg/kg) 30 min before mecamylamine fail to enhance nicotine/mecamylamine controls. *, $p < 0.05$ compared with the saline controls (a and b). #, $p < 0.05$ compared with wild-type controls (b). Data are expressed as means $\pm$ S.E.M., five to 10 mice per group. SAL, saline; NIC, nicotine; M.P., minipump.

TABLE 1**FAAH inhibition by URB597 does not affect locomotor activity in nicotine CPP**

Pretreatment with URB597 or vehicle (milligrams per kilogram i.p.), given 30 min before an inactive dose of 0.1 mg/kg nicotine (s.c.), does not significantly decrease locomotor activity in C57BL/6J mice. $p > 0.05$ Compared with respective saline and vehicle/nicotine controls. Data are expressed as average beam interruptions $\pm$ S.E.M. for each group.

Pretreatment	Treatment	
	Saline	Nicotine (0.1)
<i>mg/kg i.p.</i>		<i>mg/kg s.c.</i>
Vehicle	1065 $\pm$ 64	1072 $\pm$ 61
URB597 (0.3)	1065 $\pm$ 75	987 $\pm$ 72
URB597 (3)	1097 $\pm$ 88	1003 $\pm$ 69
URB597 (5)	1097 $\pm$ 52	970 $\pm$ 76
URB597 (10)	1019 $\pm$ 53	864 $\pm$ 87

TABLE 2**Potency of nicotine-induced antinociception and hypothermia**

Following 30-min pretreatment with vehicle and various doses of URB597 (milligrams per kilogram i.p.), nicotine potency was expressed as ED₅₀ values [milligrams per kilogram ($\pm$ confidence limits)].

Pretreatment	Treatment		
	Tail Flick	Hot Plate	Rectal Temperature
<i>mg/kg i.p.</i>		<i>mg/kg s.c.</i>	
Vehicle	2.0 (1.7–2.4)	1.9 (1.6–2.2)	1.2 (1.2–1.4)
URB597 (0.3)	1.4 (1.2–1.8)	1.4 (1.1–1.7)	1.4 (1.1–1.8)
URB597 (3)	2.1 (1.4–3.2)	2.4 (1.5–3.8)	1.2 (1.0–1.4)
URB597 (10)	1.3 (1.0–1.7)	1.7 (1.1–2.5)	1.3 (1.1–1.5)