The involvement of \(\mu\)-opioid receptors in different behavioral responses elicited by nicotine was explored by using \(\mu\)-opioid receptor knock-out mice. The acute antinociceptive responses induced by nicotine in the tail-immersion and hot-plate tests were reduced in the mutant mice, whereas no difference between genotypes was observed in the locomotor responses. The rewarding effects induced by nicotine were then investigated using the conditioning place-preference paradigm. Nicotine produced rewarding responses in wild-type mice but failed to produce place preference in knock-out mice, indicating the inability of this drug to induce rewarding effects in the absence of \(\mu\)-opioid receptors. Finally, the somatic expression of the nicotine withdrawal syndrome, precipitated in dependent mice by the injection of mecamylamine, was evaluated. Nicotine withdrawal was significantly attenuated in knock-out mutants when compared with wild-type mice. In summary, the present results show that \(\mu\)-opioid receptors are involved in the rewarding responses induced by nicotine and participate in its antinociceptive responses and the expression of nicotine physical dependence.

**Key words:** nicotine; opioid; knock-out mice; withdrawal; conditioning place preference; reward

Nicotine is one of the active components in tobacco smoke and appears to play a major role in tobacco addiction (Crooks and Dwoskin, 1997). This compound affects different aspects of behavior such as locomotion, nociception, anxiety, learning, and memory, and it produces several behavioral responses related to its addictive properties such as rewarding effects and physical dependence (Decker et al., 1995). The pharmacological effects of nicotine are mediated by the activation of nicotinic acetylcholine receptors (nACHRs), which are members of the superfamily of ligand-gated ion channels (Dani, 2001). nACHRs are located mainly at a presynaptic level and their activation increases the release of dopamine (Pontieri et al., 1996), noradrenaline (Clarke and Reuben, 1996), acetylcholine (Wilkie et al., 1993), glutamate (McGehee et al., 1995), and GABA (Yang et al., 1996).

The endogenous opioid system has been reported to participate in several central effects of nicotine (Balfour, 1982). Thus, the stimulation of nACHRs increases the synthesis and release of met-enkephalin in mouse striatum (Dhatt et al., 1995), and an enhancement on preproenkephalin mRNA levels in rat striatum and hippocampus has been also reported after acute nicotine and during nicotine withdrawal (Houdi et al., 1998). In addition, endogenous opioids have been implicated in the reinforcement of smoking because the administration of the opioid antagonists naloxone and naltrexone modulates cigarette consumption and the subjective pleasure derived from smoking (Karras and Kane, 1980; Wewers et al., 1998). However, the possible cross-reactivity of these antagonists with other receptors complicates the interpretation of these data (Almeida et al., 2000; Tome et al., 2001).

Furthermore, several behavioral and physiological effects, including antinociception, rewarding properties, and dependence (Decker and Meyer, 1999; Hildebrand et al., 1999; Watkins et al., 2000) are shared by nicotine and opioids. Three different opioid receptors \(\mu\), \(\delta\), and \(\kappa\), have been identified and cloned (Kieffer, 1999). \(\mu\)-Opioid receptors have been reported to be responsible for the addictive properties of opioids (Matthes et al., 1996; Hutcheson et al., 2001) and to be involved in the rewarding properties of other drugs of abuse, such as alcohol (Roberts et al., 2000) and cannabinoids (Ghoshland et al., 2002).

The present study was designed to evaluate the possible involvement of \(\mu\)-opioid receptors in several behavioral responses of nicotine by using \(\mu\)-opioid receptor knock-out mice (Matthes et al., 1996). For this purpose, we first studied locomotor and antinociceptive effects induced by acute nicotine administration in \(\mu\)-opioid receptor knock-out mice and their wild-type littermates. We also investigated the rewarding properties of nicotine by using the conditioning place-preference paradigm and the development of physical dependence after chronic nicotine treatment in both mutant and wild-type mice.

**MATERIALS AND METHODS**

**Animals.** The generation of mice lacking \(\mu\)-opioid receptors has been described previously (Matthes et al., 1996). Mice were housed five per cage in a temperature-controlled room (21 ± 1°C) with a 12 hr light/dark cycle (lights between 8 A.M. and 8 P.M.). Food and water were available ad libitum. Mice were habituated to their new environment and handled for 1 week before the experimental procedure was started. The mice (8–12 weeks old) used in this study were on a C57/BL6 genetic background. These were obtained from hybrid mutant mice originally created on a 129 SVJ-C57/BL6 background (Matthes et al., 1996) by backcrossing breeding over 10 generations. In each experimental group, mice were...
matched for age and sex. Animal procedures were conducted in accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and approved by the local ethical committee. The observer was blind to genotype and treatment in all the experiments.

Drugs. (−)-Nicotine hydrogen tartrate salt [(−)-3-methyl-2-[3-pyridyl] pyrrolidine] and mecamylamine hydrochloride (Sigma, Madrid, Spain) were dissolved in physiological saline (0.9%) and administered by subcutaneous route in a volume of 10 ml/kg.

Locomotor activity. The locomotor responses induced by nicotine hydrogen tartrate salt (0.5, 0.7, 1, and 3 mg/kg, s.c.) or saline administration were measured by using individual locomotor activity boxes (9 × 20 × 11 cm; Imetronic) as reported previously (Castañé et al., 2002).

Tail-immersion and hot-plate tests. The tail-immersion test was measured 15 min after nicotine hydrogen tartrate salt (1 and 3 mg/kg, s.c.) or saline administration as described previously (Simonin et al., 1998). The water temperature was maintained at 50 ± 0.5°C using a thermoregulated water-circulating pump (Clifton, North Somerset, UK). The trial was terminated once the animal flicked its tail. In the absence of tail flick, a 15 sec cutoff was used to prevent tissue damage.

The hot-plate test was performed as described previously (Simonin et al., 1998) 16 min after nicotine hydrogen tartrate salt (1 and 3 mg/kg, s.c.) or saline injection. The heated surface of the plate was kept at a temperature of 52 ± 0.1°C (Columbus Instruments, Columbus, OH). The nociceptive threshold evaluated was the jumping response. In absence of jumps, a 240 sec cutoff was used to prevent tissue damage.

The data obtained were expressed as absolute values (see Table 1) and as percentage of maximum possible effect (see Fig. 2) using the following equation (MPE %) = (test latency − control latency)/(cutoff time − control latency) × 100.

 Conditioning place preference. The rewarding effects of nicotine were evaluated by using the conditioning-place-preference paradigm as described recently (Castañé et al., 2002). The apparatus consisted of two main square conditioning compartments separated by a triangular central division. During the pre-conditioning phase, each mouse was placed in the middle of the central division and had free access to both compartments of the conditioning apparatus for 18 min, with the time spent in each compartment recorded. Treatments were counterbalanced between compartments to use an unbiased procedure. No initial place preference or aversion for the different compartments was observed in the experiment. For the conditioning phase, mice were treated during 8 d with alternate injections of nicotine hydrogen tartrate salt (0.5, 0.7, 1, and 1 mg/kg, s.c.) or saline. Mice were confined to the corresponding compartment immediately after injection for 20 min. Nicotine was administered on days 1, 3, 5, and 7, and saline was administered on days 2, 4, 6, and 8. Control animals received saline every day. The test phase was conducted as in the pre-conditioning phase, i.e., free access to both compartments for 15 min and the time spent in each compartment was recorded. A score was calculated for each mouse as the difference between test and pre-conditioning time spent in the drug-paired compartment.

Nicotine dependence and withdrawal. Nicotine dependence was induced by using Alzet osmotic minipumps (Model 2001; Alzet, Cupertino, CA) as reported previously (Castañé et al., 2002). These minipumps, implanted subcutaneously under brief ether anesthesia, contained saline or nicotine solutions and delivered a constant subcutaneous flow at a rate of 1 μl/hr. The concentration of nicotine was adjusted to compensate for differences in body weights of the mice. Thus, the average-weighted mice received a dose of ~10 mg·kg⁻¹·d⁻¹ nicotine hydrogen tartrate salt during 6 d. Nicotine withdrawal syndrome was precipitated 6 d after minipump implantation by injection of the nicotinic receptor antagonist mecamylamine (1 mg/kg, s.c.). The somatic signs of withdrawal were evaluated immediately after mecamylamine injection during a period of 30 min, as reported previously (Castañé et al., 2002). The number of wet dog shakes, front paw tremors, and scratches was counted. Body tremor, ptosis, teeth chattering, genial licks, and piloerection were scored 1 for appearance or 0 for nonappearance within each 5 min time. The locomotion was evaluated over 5 min periods was rated 0, 1, or 2 (0 for inactivity, 1 for low activity, and 2 for normal activity). A global withdrawal score was calculated for each animal by giving each individual sign a relative weight, as reported previously (Castañé et al., 2002).

Statistical analysis. Results in all experiments were compared by using a between subjects two-way ANOVA (genotype and treatment as factors of variance). Individual treatment effects in each group (mutant and wild-type) were analyzed using one-way ANOVA between subjects. Post hoc comparisons were made when required by using Dunnett’s test after significant main effects of treatment by one-way ANOVA. Differences were considered significant if the probability of error was <0.05.

RESULTS

Nicotine decreased locomotion in wild-type and μ-opioid receptor knock-out mice

On days 1, 2, and 3, animals were exposed to the locomotor activity boxes to be habituated to the test environment (data not shown), and acute effects of nicotine (1 and 3 mg/kg, s.c.) were evaluated on day 4. Nicotine decreased locomotion in μ-opioid receptor knock-out mice and wild-type littermates (Fig. 1). Two-way ANOVA revealed a significant effect of treatment on the horizontal activity (F(2,54) = 41.19; p < 0.0001) as well as effect of genotype (F(1,54) = 32.97; p < 0.0001) and interaction between treatment and genotype (F(2,54) = 41.19; p < 0.0001). Subsequent one-way ANOVA (treatment) indicated a significant effect of treatment in wild-type (F(2,27) = 21.04; p < 0.0001) and knock-out mice (F(2,27) = 15.99; p < 0.0001). Post hoc analysis showed a similar decrease of horizontal activity when nicotine was administered in wild-type
(1 and 3 mg/kg; p < 0.01) and μ-opioid receptor knock-out mice (1 mg/kg; p < 0.05; 3 mg/kg; p < 0.01) (Fig. 1A).

Two-way ANOVA also revealed a significant effect of treatment on the vertical activity \( (F_{(2,54)} = 22.76; p < 0.0001) \), without effect of genotype \( (F_{(1,54)} = 2.58; \text{NS}) \) or interaction between these two factors \( (F_{(2,54)} = 1.08; \text{NS}) \). One-way ANOVA revealed significant effect of treatment in wild-type \( (F_{(2,27)} = 13.88; p < 0.0001) \) and knock-out mice \( (F_{(2,27)} = 11.21; p < 0.001) \). Post hoc comparisons showed a similar reduction of vertical activity in both genotypes at the doses of nicotine used (1 and 3 mg/kg; p < 0.01) (Fig. 1B).

In an additional experiment, locomotor effects induced by lower doses of nicotine were evaluated (data not shown). Nicotine administered at the dose of 0.7 mg/kg (s.c.) also induced a similar decrease of locomotor activity in wild-type and knock-out mice as revealed by two-way ANOVA on horizontal (treatment: \( F_{(2,54)} = 12.34, p < 0.01 \); genotype: \( F_{(1,54)} = 0.38, \text{NS} \); interaction: \( F_{(2,54)} = 0.15, \text{NS} \)) and vertical (treatment: \( F_{(1,54)} = 8.35, p < 0.01 \); genotype: \( F_{(1,54)} = 20.76, p < 0.0001 \)) measurements in wild-type and knock-out mice. Antinociceptive responses in the hot-plate \( (2,54) \) and tail-immersion \( (2,54) \) tests were measured 15 and 16 min, respectively, after nicotine administration (0, 1, and 3 mg/kg, s.c.). Data are expressed as mean ± SEM of percentage of maximum possible effect in wild-type \( (\text{white bars}) \) and knock-out \( (\text{black bars}) \) mice \( (n = 10 \text{ mice for each group}) \). **p < 0.01 when comparing with saline group of the same genotype. *p < 0.05; **p < 0.01 when comparing between genotypes (Dunnett test).

Nicotine antinociception was reduced in μ-opioid receptor knock-out mice

Nicotine-induced antinociceptive responses (1 and 3 mg/kg, s.c.) were decreased in μ-opioid receptor knock-out as compared with wild-type mice in the hot-plate and tail-immersion tests (Fig. 2, Table 1). The spontaneous nociceptive responses of both genotypes were similar in the tail-immersion test. However, the spontaneous latency of the jumping response in the hot-plate test was lower in mutant than in wild-type mice (Table 1), as reported (Matthes et al., 1998). In the hot-plate test, two-way ANOVA showed a significant effect of treatment \( (F_{(2,54)} = 20.76; p < 0.0001) \), genotype \( (F_{(1,54)} = 10.99; p < 0.01) \), and interaction between treatment and genotype \( (F_{(2,54)} = 6.93; p < 0.01) \). Subsequent one-way ANOVA revealed significant effects of treatments in wild-type \( (F_{(2,27)} = 13.72; p < 0.0001) \) and knock-out mice \( (F_{(2,27)} = 15.75; p < 0.0001) \). Nicotine induced an antinociceptive response at the dose of 3 mg/kg \( (p < 0.01) \) in both wild-type and knock-out mice as revealed by post hoc comparisons. Post hoc analysis also showed a reduction of nicotine-induced antinociception in μ-opioid receptor knock-out when compared with wild-type mice at the doses of 1 mg/kg \( (p < 0.05) \) and 3 mg/kg \( (p < 0.01) \) (Fig. 2A).

In the tail-immersion test, two-way ANOVA revealed a significant effect of treatment \( (F_{(2,54)} = 6.40; p < 0.01) \) and no effect of genotype \( (F_{(1,54)} = 2.36; \text{NS}) \) or interaction between treatment and genotype \( (F_{(2,54)} = 1.24; \text{NS}) \). However, subsequent one-way ANOVA (treatment) indicated a significant effect of treatment only in wild-type \( (F_{(2,27)} = 6.54; p < 0.01) \) and not in knock-out mice \( (F_{(2,27)} = 1.07; \text{NS}) \). Post hoc comparisons showed that nicotine induced antinociception only in wild-type mice at the dose of 3 mg/kg \( (p < 0.01) \) (Fig. 2B) when compared with the saline group.

Nicotine did not produce rewarding responses in the place-preference paradigm in μ-opioid receptor knock-out mice

A significant rewarding effect of nicotine (0.5 mg/kg, s.c.) was observed in the place-conditioning paradigm in wild-type but not in μ-opioid receptor knock-out mice (Fig. 3A). Thus, two-way ANOVA indicated treatment effect \( (F_{(1,53)} = 14.96; p < 0.001) \), no genotype effect \( (F_{(1,53)} = 0.0035; \text{NS}) \), and a significant interaction between these two factors \( (F_{(1,53)} = 6.424; p < 0.05) \). Subsequent one-way ANOVA revealed that nicotine produced a

### Table 1. Antinociceptive effects of acute nicotine in μ-opioid receptor knock-out and wild-type mice

<table>
<thead>
<tr>
<th>Test</th>
<th>Wild-type</th>
<th>Knock-out</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot-plate test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>35.62 ± 1.59</td>
<td>24.42 ± 2.39</td>
</tr>
<tr>
<td>Nicotine (1 mg/kg)</td>
<td>50.89 ± 3.53</td>
<td>29.55 ± 2.36</td>
</tr>
<tr>
<td>Nicotine (3 mg/kg)</td>
<td>120.40 ± 20.77</td>
<td>48.65 ± 4.44</td>
</tr>
<tr>
<td>Tail-immersion test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>0.81 ± 0.05</td>
<td>0.88 ± 0.05</td>
</tr>
<tr>
<td>Nicotine (1 mg/kg)</td>
<td>0.92 ± 0.06</td>
<td>0.95 ± 0.04</td>
</tr>
<tr>
<td>Nicotine (3 mg/kg)</td>
<td>1.11 ± 0.07</td>
<td>1.00 ± 0.08</td>
</tr>
</tbody>
</table>

Data (mean ± SEM) obtained in the hot-plate test (jumping response in seconds) and the tail-immersion test (tail withdrawal latency in seconds) are shown.

Figure 2. Antinociceptive effects of acute nicotine in μ-opioid receptor knock-out and wild-type mice. Antinociceptive responses in the hot-plate (A) and tail-immersion (B) tests were measured 15 and 16 min, respectively, after nicotine administration (0, 1, and 3 mg/kg, s.c.). Data are expressed as mean ± SEM of percentage of maximum possible effect in wild-type (white bars) and knock-out (black bars) mice (n = 10 mice for each group). **p < 0.01 when comparing with saline group of the same genotype. *p < 0.05; **p < 0.01 when comparing between genotypes (Dunnett test).
conditioned place preference for the nicotine-assigned compartment in wild-type mice \((p < 0.01)\), whereas no effect was observed in knock-out mice. Nicotine administered at the dose of 0.7 mg/kg (s.c.) also produced conditioned place preference in wild-type but not in \(\mu\)-opioid receptor knock-out animals, as indicated by two-way ANOVA (treatment: \(F_{(1,49)} = 1.24, \text{NS}\); genotype: \(F_{(1,49)} = 0.20, \text{NS}\); interaction: \(F_{(1,49)} = 5.76, p < 0.05\) (Fig. 3B). One-way ANOVA revealed a significant rewarding effect of nicotine (0.7 mg/kg, s.c.) in wild-type mice \((p < 0.01)\) and no effect in knock-out mice. The administration of a higher dose of nicotine (1 mg/kg, s.c.) did not induce rewarding responses in any genotype, as revealed by two-way ANOVA (treatment: \(F_{(1,36)} = 0.19, \text{NS}\); genotype: \(F_{(1,36)} = 0.128, \text{NS}\); interaction: \(F_{(1,36)} = 0.08, \text{NS}\) (Fig. 3C).

**DISCUSSION**

The present results clearly demonstrate the involvement of \(\mu\)-opioid receptors in several behavioral responses induced by nicotine and strongly support a functional interaction between nicotine and the opioid system. Thus, antinociception and nicotine withdrawal symptoms were reduced in mice lacking \(\mu\)-opioid receptors. Moreover, nicotine did not produce rewarding effects in these mutant mice.

Nicotine antinociception was evaluated in the tail-immersion and hot-plate tests. However, a more intense and reliable nicotine

**Figure 3.** Rewarding effects of nicotine in \(\mu\)-opioid receptor knock-out and wild-type mice. Data are expressed as mean \pm SEM of score values in wild-type (white bars) and knock-out (black bars) mice \((n = 10–15\) mice for each group). Nicotine was administered subcutaneously at doses of 0.5 \((A)\), 0.7 \((B)\), and 1 mg/kg \((C)\) immediately before each conditioning session. \(^{**} p < 0.01\) when comparing with saline group of the same genotype (one-way ANOVA).

**Figure 4.** Mecamylamine-precipitated nicotine withdrawal in \(\mu\)-opioid receptor knock-out and wild-type mice. Abstinence was precipitated by acute mecamylamine administration (1 mg/kg, s.c.) after 6 d of nicotine perfusion \((10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1})\) by using subcutaneous minipumps. A global withdrawal score was calculated for each animal by giving each individual sign a relative weight. Data are expressed as mean \pm SEM in wild-type (white bars) and knock-out (black bars) mice \((n = 20–25\) mice for each group). \(^{**} p < 0.01\) when comparing with saline group of the same genotype. \(^{\ddagger} p < 0.01\) when comparing between genotypes (one-way ANOVA).
antinociceptive effect was observed in the hot-plate test. The spontaneous latency of the jumping response in the hot-plate test was lower in control mutant than in wild-type mice, indicating a higher sensitivity of μ-opioid receptor-deficient mice to painful stimuli in these experimental conditions, as reported previously (Matthes et al., 1998). Nicotine-induced antinociception in the hot-plate test was significantly reduced in μ-opioid receptor knock-out mice, suggesting that this receptor is recruited to produce nicotine antinociception. The reduced jumping response observed in knock-out mice cannot be attributed to a motor impairment. Indeed, the spontaneous locomotion and nicotine-decreased locomotion were similar in both genotypes. In agreement with our results, several studies have reported that the opioid system could play a role in modulating nicotine antinociception. Thus, naloxone decreased nicotine-induced antinociception in the formalin test (Zarrindast et al., 1997) as well as the potentiation of this effect elicited by the coadministration of morphine (Matthes et al., 1995; Picciotto et al., 1998). In addition, the activation of μ-opioid receptors to compensate for the reduction of other drugs of abuse such as morphine (Matthes et al., 1996), ethanol (Roberts et al., 2000), and Δ⁹-tetrahydrocannabinol (Ghozland et al., 2002). However, μ-opioid receptor knock-out mice did not present a general impairment in the performance for rewarding stimuli. Indeed, the same line of μ-opioid receptor knock-out mice showed an appropriate learning to respond for food reward in an operant paradigm (Roberts et al., 2000). A possible interaction on dopaminergic mesolimbic activity could explain the present findings because nAChRs and μ-opioid receptors are highly expressed in these areas (Mansour et al., 1995; Picciotto et al., 1998). In addition, the activation of both μ-opioid receptors and nAChRs induces release of dopamine in the shell of the nucleus accumbens (Pontieri et al., 1996). However, the pharmacological approach failed to reveal this nicotine/opioid interaction when using the self-administration paradigm (Corrigall and Coen, 1991).

Finally, nicotine abstinence was precipitated by mecamylamine in chronic nicotine-treated mice. Previous studies have characterized the behavioral manifestations of nicotine withdrawal in rodents (Hildebrand et al., 1999; Castañé et al., 2002). Some common mechanisms underlying opioid and nicotine dependence have been suggested recently (Malin, 2001). Thus, the opioid antagonist naloxone is able to precipitate withdrawal after chronic nicotine treatment (Malin et al., 1993), whereas morphine attenuates spontaneous nicotine withdrawal (Malin et al., 1993). Conversely, nicotine also reduces naloxone-precipitated morphine withdrawal (Zarrindast and Farzin, 1996). In addition, the opioid antagonist naltrexone has been shown to reduce the tobacco consumption rate and satisfaction with smoking in humans (Wewers et al., 1998). In our experimental conditions, we have observed an attenuation of the somatic expression of nicotine

<table>
<thead>
<tr>
<th>Wild-type mice</th>
<th>Knock-out mice</th>
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<tbody>
<tr>
<td>Saline</td>
<td>Nicotine</td>
</tr>
<tr>
<td>Locomotor activity</td>
<td>6.45 ± 0.51</td>
</tr>
<tr>
<td>Body tremor</td>
<td>4.45 ± 0.39</td>
</tr>
<tr>
<td>Genital licks</td>
<td>1.05 ± 0.22</td>
</tr>
<tr>
<td>Ptosis</td>
<td>0.35 ± 0.22</td>
</tr>
<tr>
<td>Wet dog shakes</td>
<td>0.95 ± 0.31</td>
</tr>
<tr>
<td>Teeth chattering</td>
<td>0.40 ± 0.13</td>
</tr>
<tr>
<td>Paw tremor</td>
<td>3.35 ± 0.69</td>
</tr>
<tr>
<td>Scratches</td>
<td>1.25 ± 0.43</td>
</tr>
<tr>
<td>Piloerection</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>GWS</td>
<td>11.80 ± 0.70</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. Two-way ANOVA revealed a significant effect of nicotine treatment for locomotor activity, body tremor, wet dog shakes, teeth chattering, paw tremor, piloerection, global withdrawal scores (GWS) (p < 0.01), and ptosis (p < 0.05).
withdrawal in the absence of μ-opioid receptors, indicating that these receptors could modulate nicotine physical dependence. Although further experiments will be necessary to elucidate the nature of this interaction, the release of endogenous opioids by nAChR stimulation and subsequent activation of μ-opioid receptors could explain at least some of these findings.

In conclusion, our data clearly demonstrate an involvement of the opioid system, through μ receptors, in modulating some behavioral responses induced by nicotine and improve the understanding of the neurobiological bases of nicotine addiction.

REFERENCES


