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Morphine–nicotine interaction in conditioned place preference in mice after chronic nicotine exposure

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ABSTRACT

Previously we found that morphine's effects on locomotor activity and brain dopamine metabolism were enhanced in mice after cessation of 7-week oral nicotine treatment. In the present experiments we show that such chronic nicotine exposure cross-sensitizes NMRI mice to the reinforcing effect of morphine in the conditioned place preference paradigm. The nicotine-treated mice developed conditioned place preference after being conditioned twice with morphine 5 mg/kg s.c. whereas in control mice a higher dose (10 mg/kg) of morphine was required. Since the reinforcing effect of morphine is mediated via μ-opioid receptors we used [^{3}H]DAMGO autoradiography to study whether the number (B_{max}) or affinity (K_D) of μ-opioid receptors in the mouse brain are affected following chronic nicotine exposure. However, no changes were found in the number or affinity of μ-opioid receptors in any of the brain areas studied. Neither did we find alterations in the functional activity of μ-opioid receptors studied by [^{35}S]GTPγS-binding. In conclusion, chronic oral nicotine treatment augments the reinforcing effects of morphine in mice, and this cross-sensitization does not seem to be mediated by μ-opioid receptors.

1. Introduction

The endogenous opioid system has been suggested to be involved in several effects of nicotine, including reinforcement (Berrendero et al., 2002, 2005; Zarrindast et al., 2003). Such an involvement is also supported by the findings that the opioid agonist morphine elicits stronger effects in nicotine-pretreated animals than in controls. Thus, after chronic nicotine treatment mice are cross-sensitized to the locomotor activity enhancing properties of acute morphine (Biala and Weglinska, 2004; Vihavainen et al., 2006). Recently we reported that after cessation of chronic nicotine treatment, the nigrostriatal dopaminergic system responded to a small dose of morphine that failed to affect control mice (Vihavainen et al., 2006). The interaction of nicotine and opioids may be mediated by nicotine-induced alterations of the endogenous opioid system, as nicotine protects the μ-receptor from inactivation by the antagonist β-funaltrexamine (Davenport et al., 1990). Also, chronic nicotine treatment decreases, whereas withdrawal from nicotine increases, opioid peptide content and opioid peptide mRNA in the rodent brain (Dhatt et al., 1995; Houdi et al., 1998; Isola et al., 2002; Wewers et al., 1999).

Increase of extracellular dopamine in the striatal areas, especially in the nucleus accumbens, is considered to be critical for the effects of addictive substances (for review, see Wise, 2005), including opioids (Shippenberg et al., 1993; Spyraki et al., 1983). Indeed, morphine administration increases accumbal dopamine output (Di Chiara and Imperato, 1988) and produces conditioned place preference (Mucha et al., 1982; Mucha and Herz, 1985; Phillips and LePiane, 1980). The μ-opioid receptor is responsible for the morphine-induced reinforcement (Matthes et al., 1996; Piepponen et al., 1997) and the increase in extracellular dopamine (Piepponen et al., 1999; Spanagel et al., 1990). The dopamine increasing effects of μ-agonists are mediated via μ-receptors located on GABA-neurons in the ventral tegmental area (Johnson and North, 1992).

The purpose of this study was to examine whether mice after cessation of chronic oral nicotine treatment are cross-sensitized to the reinforcing effects of morphine, as is the case with its locomotor stimulatory effects. A conditioned place preference experiment with morphine was carried out using two doses of morphine (5 or 10 mg/kg s.c.) and two or four conditioning sessions to determine whether the nicotine-pretreated mice are conditioned to morphine using a smaller dose and if the conditioning occurs more rapidly than in control mice. Autoradiography experiments were conducted in brains of control and nicotine-treated mice to determine whether changes in μ-opioid receptor number, affinity or functional activity are involved in increased sensitivity to morphine. Time points (24 h, 8 days or 29 days after cessation of nicotine treatment) for autoradiography were chosen based on the results of our previous experiments on morphine's stimulatory effects on nicotine-pretreated mice. These results demonstrated that locomotor activity was enhanced at 24 h

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and at 8 days, but not at 29 days after cessation of nicotine treatment (Vihavainen et al., 2006). In autoradiography experiments [^{3}H]DAMGO was used to measure μ-opioid receptor number (B_{max}) and affinity (K_D), and DAMGO-stimulated [^{35}S]GTPγS was used to measure the functional activity of these receptors.

2. Materials and methods

2.1. Animals

Male NMRI mice were used in all experiments. The mice were housed in groups of 4–7 under a 12-h light/dark cycle and had free access to food and drinking fluid. In the colony room the temperature was 21±2 °C and the humidity was 60%. The experimental protocols were approved by the Committee for Animal Experiments of the University of Helsinki.

2.2. Drugs and chronic oral nicotine treatment

Nicotine solutions were prepared in tap water using (-)nicotine (Fluka, Buchs, Switzerland); the pH was adjusted to 6.8–7.0 with 0.1 M HCl. The mice (age 4–5 weeks and weight 20–25 g at the beginning of treatment) received increasing concentrations of nicotine (base; 50–500 μg/ml) via drinking water for 7 weeks as described earlier (Pekonen et al., 1993). Briefly, the concentration of nicotine in the drinking solution was increased gradually in 3–4 day intervals from 50 to 350 μg/ml and thereafter in 7-day intervals from 350 to 500 μg/ml. The nicotine solution was the sole source of fluid for the nicotine-treated animals during the entire 7-week treatment; the control animals drank tap water. On day 50 the nicotine solution was replaced by tap water.

As previously reported, at the end of the 7-week drinking period the nicotine-treated mice weigh approx. 9% less than controls, probably due to hypodipsia induced by nicotine's antidiuretic properties (Pekonen et al., 1993). 24 h after cessation of nicotine treatment the weights of the nicotine mice reached the weights of the control mice (nicotine group: 39.9±0.4 g and controls: 40.2±0.6 g). The rapid gain of weight is due to the increased fluid intake after cessation of nicotine treatment (Pekonen et al., 1993).

Morphine-HCl was obtained from University Pharmacy (Helsinki, Finland) and dissolved in saline (0.9% NaCl solution). Injections were given in a volume of 10 ml/kg, s.c. The drug doses were calculated as free base.

2.3. Conditioned place preference

Conditioned place preference experiments were conducted using the Med Associates' (GA, USA) Activity test chamber ENV-515 with a place preference insert. The apparatus was controlled by a computer program, which recorded the infrared photo beam interruptions caused by a mouse in the apparatus. The apparatus consisted of two equal size (21×42×28 cm^3) compartments connected to each other with a guillotine door. The walls of one compartment were white and the floor was made of parallel metal bars. The walls of the other compartment were black and had a grid floor.

On the last two days of nicotine treatment the mice were habituated to the conditioned place preference apparatus for 60 min each day so that they could move freely between the compartments. On the third habituation day when the mice were still offered nicotine the initial preference (preconditioning) towards the compartments was measured for 15 min with the connecting door open. After measuring the preconditioning the nicotine drinking solution was replaced by tap water. The mice were randomized in experimental groups and conditioned with saline or morphine at 5 mg/kg or 10 mg/kg with a biased design, which is shown to be an appropriate method for place conditioning with morphine (Blander et al., 1984; Mucha and Iversen, 1984). Saline or morphine were given on days 1–2 and 4–5 after cessation of nicotine treatment. Thus, 33 nicotine-treated mice were conditioned with the testing drug to the dark side and 25 to the light side, and 37 water-treated mice to the dark and 19 to the light side of the apparatus according to their initial preference. All mice were given saline on the conditioning days before being placed individually in the initially preferred compartment for 60 min. Four hours after saline administration, the mice received either saline or morphine at 5 mg/kg or 10 mg/kg and were placed individually in the less-preferred compartment for 60 min. Postconditioning (no treatment) was measured for 15 min on days 3 (postconditioning 1) and 6 (postconditioning 2) after cessation of nicotine treatment. White noise was used during the experiment to hide any background noise.

2.4. [^{3}H]DAMGO autoradiography

Control mice and mice treated chronically with nicotine were decapitated 24 h, 8 days or 29 days after cessation of nicotine treatment. The brains were quickly removed, submerged in liquid isopentane in dry ice and thereafter frozen on dry ice. Brains were stored at −80 °C until sectioning. Fourteen-μm coronal sections were cut using a Leica cryostat at levels of 1.98 (prelimbic cortex, PrL), 1.10 (cingulate cortex, Cg; caudate putamen, CPu; nucleus accumbens core, NAcC and nucleus accumbens shell, NAcS), −1.58 (amygdala, Amy), −2.46 (thalamus, Thal), −3.16 (ventral tegmental area, VTA), −3.52 (substantia nigra pars reticulata, SNR and substantia nigra pars compacta, SNC) and −4.96 (periaqueductal gray, PAG) mm from the bregma (Franklin and Paxinos, 1997), thaw-mounted on SuperFrost Plus (Menzel-Gläser, Germany) slides, dried at room temperature and stored at −80 °C.

[^{3}H]DAMGO autoradiography was performed as described by Hyytiä et al. (1999), with exception that five concentrations of radioligand ([^{3}H]DAMGO; [D-ala^2,N-methyl-phe^4,-glyol^5][tyrosyl-3,5-^{3}H]Enkephalin, specific activity 43 nCi/mmol, Amersham Biosciences, UK) were used. Briefly, the sections were preincubated twice at room temperature for 15 min in 50 mM Tris–HCl (pH 7.4) containing 5 mM MgCl, 1 mg/ml bovine serum albumin and 100 mM NaCl. Then, the sections were rinsed twice for 5 min with the same buffer without NaCl to remove sodium ions. Thereafter, the sections were incubated in slide mailers in the presence of [^{3}H]DAMGO [10 nM, containing 3 nM of [^{3}H]DAMGO+7 nM of unlabelled DAMGO (Tocris); 3 nM; 1 nM; 0.3 nM and 0.1 nM] for 60 min at room temperature in 50 mM Tris–HCl (pH 7.4) containing 5 mM MgCl, 1 mg/ml bovine serum albumin (Sigma-Aldrich) and 100 μM phenylmethylsulphonylfluoride (Sigma, USA). The sections were then washed three times for 3 min in ice-cold 50 mM Tris–HCl (pH 7.4), dipped in distilled water and air-dried under a fan. Non-specific binding was defined in the presence of 1 μM naltrexone (RBI, USA). The sections were then exposed to Kodak Biomax MR film for 6–7 months together with [^{3}H]-standards (RPA 510, Amersham International). The films were developed, and the sections were quantified from the films with Dage MTI-apparatus (DAGE-MTI Inc.,USA) combined with MCID Elite M5+ version 4.0 (Imaging Research Inc., Canada) using the standard curve generated from [^{3}H]-standards. The specific binding values were determined by subtracting the nonspecific binding values from the corresponding binding values. The maximal binding capacity (B_{max}) and dissociation constant (K_D) for each sample were determined using non-linear regression analysis in GraphPad Prism 4.02 (GraphPad Software, Inc.).

2.5. [^{35}S]GTPγS autoradiography

A guanosine 5′-(γ-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding assay was performed as described by Hyytiä et al. (1999) on sections parallel to those used in [^{3}H]DAMGO autoradiography. Briefly, after thawing at room temperature the sections were preincubated for 20 min at room temperature in 50 mM Tris–HCl (pH 7.4) containing

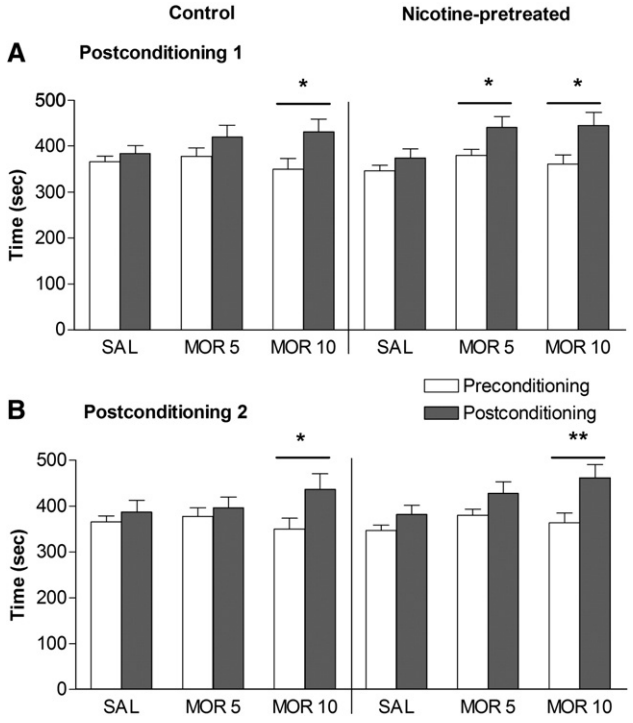

Fig. 1. Effect of nicotine pretreatment on morphine (5 mg/kg or 10 mg/kg s.c) -induced conditioned place preference after two (A) or four (B) conditioning days. Shown are the mean±S.E.M. times spent in the drug-associated compartment during the 15 min preconditioning and postconditioning sessions by mice conditioned with saline (SAL), morphine 5 mg/kg (MOR 5) or 10 mg/kg (MOR 10). The postconditioning tests were carried out one day after the two or four conditioning sessions. The number of animals in the groups were: Postconditioning 1: Control: SAL: 21; MOR 5: 20; MOR 10: 15 and nicotine: SAL: 21; MOR 5: 21; MOR 10: 16. Postconditioning 2: Control: SAL: 21; MOR 5: 19; MOR 10: 15 and nicotine: SAL: 21; MOR 5: 21; MOR 10: 15. Student's paired *t*-test revealed $*P<0.05$ and $**P<0.01$ as compared with corresponding preconditioning time.

1 mM EDTA, 100 mM NaCl and 5 mM MgCl. Thereafter, the sections were loaded with 2 mM guanosine diphosphate (GDP; Sigma, USA) in the same buffer for 60 min in the presence of 1 μM adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, Sigma-Aldrich). DPCPX was used to block activation of adenosine A_1-receptor dependent [^{35}S]GTPγS by endogenous adenosine (Laitinen and Jokinen, 1998). The final 90-min incubation with [^{35}S]GTPγS (54–68 pM; Perkin Elmer, USA) was performed in 50 mM Tris–HCl buffer (pH 7.4) with 2 mM GDP, 1 mM dithiotreitol (Sigma) and 1 μM DPCPX with or without (basal) 10 μM DAMGO in the presence and absence of 10 μM naltrexone. The sections were washed twice in racks for 5 min at 0 °C in 50 mM Tris–HCl (pH 7.4) containing 5 mM MgCl, dipped in cold distilled water and dried under a fan at room temperature. Non-specific binding was defined in the presence of 10 μM unlabelled GTPγS. The sections were then exposed to Kodak Biomax MR film for 6–7 days together with [^{14}C]-standards (GE Healthcare, UK). The sections were quantified as described above. GTP-stimulation is presented as percentage of basal values.

2.6. Data handling and statistics

All results are expressed as mean±S.E.M. The numbers of animals in each experiment are given in the legends for the tables and the figure. The preconditioning times of the conditioned place preference experiment were tested with one-way ANOVA. Conditioned place preference is defined as a change in preference measured as the time spent in the drug-associated side of the apparatus before and after drug administration. Thus, differences between preconditioning and postconditioning times were tested by Student's paired two-tailed *t*-test. The autoradiography data were analyzed using one-way ANOVA. Results were considered significant at $P<0.05$.

3. Results

3.1. Conditioned place preference

The preconditioning times in the conditioned place preference experiment between the groups were similar [ANOVA: $F(5,105)=0.762$, $P=0.5793$]. Morphine, 10 mg/kg s.c., induced significant place preference after two conditioning sessions in both control and nicotine-pretreated mice that received morphine 10 mg/kg (Fig. 1A, Postconditioning 1; $P=0.0331$ and $P=0.0217$, respectively, Student's paired *t*-test). The smaller dose of morphine, 5 mg/kg, induced significant place preference only in nicotine-pretreated mice ($P=0.0331$), but not in the corresponding control mice ($P=0.1834$). Saline treatment induced no differences between the preconditioning and postconditioning times in the control or nicotine-pretreated mice ($P=0.3552$ and $P=0.2369$, respectively).

After four conditioning sessions, morphine 10 mg/kg induced significant place preference in both control and nicotine-pretreated mice (Fig. 1B, Postconditioning 2; $P=0.0453$ and $P=0.007$, respectively). In mice that received morphine 5 mg/kg no significant differences were found between the pre- and postconditioning times (nicotine-pretreated: $P=0.1059$; control: $P=0.5338$). Saline treatment induced no differences between the preconditioning and postconditioning times in the control or nicotine-pretreated mice ($P=0.4663$ and $P=0.1302$, respectively).

Table 1
μ-Opioid receptor binding (B_{max} in fmol/mg and K_D in nM; mean±SEM) in various brain regions of control mice and mice after cessation of chronic oral nicotine treatment

Brain region	H_2O		NIC 24 h		NIC 8 days		NIC 29 days		One-way ANOVA	
	B_{max}	K_D	B_{max}	K_D	B_{max}	K_D	B_{max}	K_D	B_{max}	K_D
PrL	353±17	0.66±0.06	380±43	0.67±0.14	337±40	0.60±0.08	413±44	0.55±0.05	$F(3,40)=0.953$, $P=0.4250$	$F(3,40)=0.334$, $P=0.8010$
Cg	237±13	0.56±0.05	252±33	0.52±0.06	223±30	0.77±0.12	287±33	0.54±0.07	$F(3,40)=1.127$, $P=0.3506$	$F(3,37)=2.101$, $P=0.1183$
CPu	301±23	0.70±0.04	298±53	0.68±0.06	309±56	0.71±0.13	328±45	0.67±0.08	$F(3,40)=0.098$, $P=0.9605$	$F(3,40)=0.074$, $P=0.9738$
NAcC	456±32	0.69±0.04	479±84	0.68±0.05	418±69	0.62±0.06	475±54	0.64±0.06	$F(3,40)=0.205$, $P=0.8926$	$F(3,40)=0.355$, $P=0.7856$
NAcS	517±38	0.72±0.05	468±97	0.72±0.06	532±98	0.58±0.10	542±72	0.59±0.06	$F(3,40)=0.183$, $P=0.9073$	$F(3,40)=1.361$, $P=0.2697$
Amy	385±18	0.51±0.03	457±35	0.60±0.11	380±37	0.46±0.03	439±41	0.50±0.03	$F(3,40)=1.605$, $P=0.2047$	$F(3,39)=0.993$, $P=0.4072$
Thal	316±13	0.89±0.08	341±24	0.81±0.09	302±38	0.72±0.05	343±33	0.84±0.02	$F(3,40)=0.588$, $P=0.6266$	$F(3,39)=0.743$, $P=0.5334$
VTA	318±25	0.86±0.08	339±59	0.70±0.07	348±36	0.79±0.04	364±38	0.80±0.07	$F(3,39)=0.346$, $P=0.7922$	$F(3,40)=0.590$, $P=0.6253$
SNR	124±9	0.93±0.08	144±15	0.77±0.17	116±10	0.75±0.07	122±11	0.95±0.20	$F(3,40)=0.889$, $P=0.4556$	$F(3,39)=0.660$, $P=0.5818$
SNC	264±15	0.90±0.06	302±27	0.85±0.13	264±16	0.87±0.04	266±13	0.95±0.11	$F(3,39)=0.696$, $P=0.5606$	$F(3,39)=0.170$, $P=0.9158$
PAG	350±19	0.68±0.05	353±42	0.56±0.04	362±34	0.59±0.04	385±42	0.60±0.04	$F(3,40)=0.244$, $P=0.8647$	$F(3,40)=1.025$, $P=0.3928$

H_2O=control; NIC 24 h=chronic oral nicotine treatment, 24 h after cessation of treatment; NIC 8 days=8 days after cessation of treatment; NIC 29 days=29 days after cessation of treatment. $n=6$–7 in NIC groups and 20–21 in control group. PrL=prelimbic cortex; Cg=cingulate cortex; CPu=caudate putamen; NAcC=nucleus accumbens core; NAcS=nucleus accumbens shell; Amy=amygdala; Thal=thalamus; VTA=ventral tegmental area; SNR=substantia nigra, pars reticulata; SNC=substantia nigra, pars compacta; PAG=periaqueductal grey.

Table 2
[^{35}S]GTPγS binding stimulated by 10 μM DAMGO (% of stimulation of basal binding; mean±SEM) and its blockade by naltrexone (NTX) in various brain regions of control mice and mice after cessation of chronic oral nicotine treatment

Brain region	H_2O (DAMGO+NTX)	H_2O	NIC 24 h	NIC 8 days	NIC 29 days	One-way ANOVA
PrL	114±11	244±17	286±55	228±33	237±51	$F(3,37)=1.038$, $P=0.3872$
Cg	113±6	219±14	246±33	169±18	188±11	$F(3,33)=1.277$, $P=0.2984$
CPu	104±7	248±22	310±34	265±50	243±28	$F(3,35)=0.783$, $P=0.5115$
NAcC	98±13	375±29	345±59	325±53	457±49	$F(3,37)=1.254$, $P=0.3042$
NAcS	96±5	324±24	343±55	273±31	423±78	$F(3,38)=1.602$, $P=0.2050$
Amy	92±6	203±13	203±24	220±19	185±5	$F(3,36)=0.464$, $P=0.7095$
Thal	87±3	235±17	274±15	241±18	197±13	$F(3,36)=1.764$, $P=0.1715$
VTA	96±10	189±9	211±16	192±17	177±15	$F(3,37)=0.774$, $P=0.5157$
SNR	103±7	126±4	133±7	125±8	136±5	$F(3,38)=0.871$, $P=0.4647$
SNC	101±7	172±7	206±9	165±12	178±11	$F(3,38)=2.782$, $P=0.0540$
PAG	84±3	160±9	131±7	153±12	175±25	$F(3,34)=1.542$, $P=0.2213$

H_2O=control; NIC 24 h=chronic oral nicotine treatment, 24 h after cessation of treatment; NIC 8 days=8 days after cessation of treatment; NIC 29 days=29 days after cessation of treatment. n=5–7 in NIC groups and 18–21 in control group. See Table 1 for abbreviations.

3.2. [^{3}H]DAMGO autoradiography

Quantitative analysis of [^{3}H]DAMGO-autoradiography data showed the highest levels of μ-opioid receptor binding in the nucleus accumbens core, nucleus accumbens shell and amygdala, moderate binding in the prelimbic cortex, cingulated cortex, caudate putamen, thalamus, ventral tegmental area, substantia nigra pars compacta and periaqueductal gray, and the lowest binding in the substantia nigra pars reticulata (Table 1). Since there were no statistical differences between the control groups, the data from the controls were pooled (H_2O group in Table 1). No significant differences were found in the μ-opioid receptor B_{max} or K_D in any of the brain areas studied at 24 h, 8 days or 29 days after cessation of chronic oral nicotine treatment as compared with controls. For detailed statistical analysis, see Table 1.

3.3. DAMGO-stimulated [^{35}S]GTPγS autoradiography

The μ-opioid receptor agonist DAMGO (10 μM) widely stimulated GTP-binding in the brain (Table 2). This G-protein activation was prevented in the presence of naltrexone (data for control group shown in Table 2). The highest DAMGO-stimulated [^{35}S]GTPγS-bindings were found in the nucleus accumbens core (375% of basal binding in the control group) and nucleus accumbens shell (324%) and lowest in the substantia nigra pars reticulata (126%) and substantia nigra pars compacta (172%). Since the control groups did not differ in G-protein activation, the data were pooled (H_2O group in Table 2). Nicotine treatment and different periods of abstinence did not significantly alter G-protein activation in the studied brain areas. For detailed statistical analysis, see Table 2.

4. Discussion

Nicotine and morphine induce conditioned place preference in experimental animals (Fudala et al., 1985; Phillips and LePiane, 1980). In the present study a possible cross-sensitization between the two addictive substances was studied in mice using this paradigm. Mice pretreated for seven weeks with nicotine via drinking water developed conditioned place preference after two conditioning sessions with morphine 5 mg/kg, whereas control mice showed place preference only after conditioning with the higher dose (10 mg/kg) of morphine. After four conditioning sessions, no difference was observed. Thus, a smaller dose of morphine caused place preference with only a very short conditioning suggesting cross-sensitization of nicotine-pretreated mice to the reinforcing effects of morphine. Shippenberg et al. (1996), as well as Biala and Weglinska (2004), have found parallel results in conditioned place preference experiments with rats; a 5-day nicotine pretreatment enhanced conditioned place preference induced by morphine. Together the previous and present findings indicate that nicotine pretreatment can enhance the positive reinforcing properties of morphine. Interestingly, in mice treated with tolerance-inducing high doses of either morphine or nicotine, neither nicotine nor morphine was able to induce conditioned place preference (Zarrindast et al., 2003), indicating the development of cross-tolerance and suggesting common pathways for the effects of morphine and nicotine. Development of tolerance might also explain why the nicotine-pretreated mice conditioned four times with morphine no longer showed place preference at the 5 mg/kg dose.

The reinforcing effects of morphine are well known (Phillips and LePiane, 1980). It induces conditioned place preference with a large range of doses and even after only a few administrations (Mucha et al., 1982). Therefore, it is quite surprising that previous nicotine exposure is still able to enhance the reinforcing effect of morphine. This finding is in agreement with our previous findings — that morphine's locomotor activity and dopamine metabolism enhancing effects are increased in nicotine-pretreated mice (Vihavainen et al., 2006). The dopaminergic system is likely involved in the cross-sensitization, as both nicotine and morphine increase dopamine firing in the ventral tegmental area (Grenhoff et al., 1986; Nowycky et al., 1978).

In our place conditioning experiment nicotine administration was discontinued one day before starting the administration of morphine. Neither nicotine nor cotinine can be found in mouse plasma at 24 h after cessation of chronic treatment (Pietilä, 1998), although nicotine concentration in mouse plasma after 7-week oral nicotine treatment has been reported to be as high as 54±19 ng/ml (Pekonen et al., 1993). As nicotine is present in the plasma neither at the time of morphine administration nor testing, the question is whether the mice had withdrawal symptoms during the conditioning and/or postconditioning, as withdrawal may affect the motivational state of the experimental animals (Koob, 2003).

Nicotine withdrawal in rodents can be measured as physical (somatic signs, hyperalgesia or changes in locomotor activity) and affective signs (anxiety, elevated reward threshold, fear conditioning or place aversion) (see e.g. Jackson et al., 2008; Koob and Le Moal, 2006). The methods for measuring affective signs may tell about different aspects of withdrawal (Koob and Le Moal, 2006). In our mice, locomotor activity was decreased at 12–14 h after cessation of 7-week oral nicotine treatment, but no longer at 24 h after cessation (Gäddnäs et al., 2000; Vihavainen et al., 2006). In addition, a neurochemical marker of withdrawal, hypothalamic MOPEG concentration, was increased at 12–14 h after cessation of chronic nicotine indicating increased noradrenaline turnover, but neither hypothalamic, cortical nor accumbal MOPEG were changed at 24 h after cessation (Gäddnäs et al., 2000; Vihavainen et al., 2006). According to these results, we conclude that our mice may experience physical withdrawal symptoms at around 12–14 h, but likely no longer at 24 h (or later) after cessation of nicotine treatment. No differences between nicotine-treated and control mice were found in elevated plus-maze test at

12 h, 24 h nor 72 h after cessation of nicotine treatment (Soininen N., Gäddnäs H. and Ahtee L.; unpublished results). However, as no other criterion for affective signs of nicotine withdrawal than anxiety has been measured, we cannot exclude the possibility of changes in the motivational state in our mice at the time of conditioning and/or postconditioning which could affect the results.

The reinforcing effects of morphine are mediated via μ-opioid receptors (Matthes et al., 1996) and therefore an obvious hypothesis for the mechanism of nicotine–morphine cross-sensitization is that chronic nicotine increases the number of μ-opioid receptors in the brain. [^{3}H]DAMGO autoradiography was conducted on 11 brain areas, and among these the highest [^{3}H]DAMGO-binding was found in the nucleus accumbens core and the lowest in the substantia nigra pars reticulata. These findings agree with those reported by Lesscher et al. (2003). We did not find any changes in the number or affinity of the μ-opioid receptors in mice after cessation of chronic nicotine treatment as compared with controls. Results from previous studies concerning μ-opioid receptor binding or autoradiography after nicotine treatment have been diverse. Nicotine treatment has been suggested to increase, decrease and not to induce changes in the density of μ-opioid receptors (Galeote et al., 2006; Marco et al., 2007; Walters et al., 2005; Wewers et al., 1999). Whether changes have been found, and the directions in which these changes occurred, seems to depend on the duration of the treatment and the abstinence, the dose of nicotine used, and possibly the age and sex of the experimental animals. In regard to the dose of nicotine, our chronic oral treatment has been shown to resemble human smoking (Pekonen et al., 1993). Furthermore, the length of our treatment was multifold as compared to the studies mentioned above, and only male mice were used. Thus, it seems that chronic seven-week nicotine treatment and 24-h, 8-day or 29-day abstinence after it do not affect the number or affinity of μ-opioid receptors.

As the cause of increased agonist response may well be a consequence of changes in the receptors' functional activity, DAMGO-stimulated [^{35}S]GTPγS-autoradiography was conducted to observe possible changes in the functional activity of μ-receptors in mice after cessation of chronic oral nicotine treatment. In our [^{35}S]GTPγS-binding experiment, DAMGO stimulated the binding most in the nucleus accumbens core and least in the substantia nigra pars reticulata, agreeing with previous studies (Soini et al., 2002). No significant differences between the groups were found in any of the studied brain areas, but a tendency for increased μ-receptor functional activity was found in the substantia nigra pars compacta of the mice 24 h after cessation of chronic nicotine. Besides our [^{35}S]GTPγS-binding experiment, only one (Galeote et al., 2006) other study has been published where μ-receptor functional activity has been studied after nicotine administration. In that study the functional activity of μ-receptors was found to be increased in the spinal cord, but no changes in [^{35}S]GTPγS-binding were found in several studied brain regions. Thus, both these studies indicate that even intense nicotine treatment has minor effects on μ-opioid receptor functional activity in the mouse brain.

Our present and previous (Vihavainen et al., 2006) experiments show that morphine more readily induces/enhances dopamine related behaviors, place preference and locomotor activity, as well as enhances dopamine turnover/metabolism in the nicotine-pretreated mice than in control mice. There is strong evidence for the role of accumbal dopamine in reinforcement and reward (Koob and Le Moal, 2001), and dopamine-dependent synaptic plasticity in the caudate putamen has been suggested to have a remarkable role in learning processes of addiction, such as habit formation (Gerdeman et al., 2003). No significant differences were found in the number, affinity or functional activity of μ-opioid receptors between control and nicotine-treated mice suggesting that these receptors are not responsible for the enhanced effects of morphine after chronic nicotine treatment. The alterations in the regulation of striatal/accumbal dopamine neurons after chronic nicotine treatment might be due to changes in their neuronal inputs. Thus, cholinergic receptors may play a role in this phenomenon, since muscarinic or nicotinic receptor blockade by atropine or mecamylamine, respectively, in ventral tegmental area (Rezayof et al., 2007), basolateral amygdala (Zarrindast et al., 2005) or dorsal hippocampus (Rezayof et al., 2006) inhibited morphine-induced conditioned place preference in rats. The implication of nicotinic receptors is not specific to morphine-induced conditioned place preference, as also cocaine reinforcement in mice was attenuated by mecamylamine (Zachariou et al., 2001). However, it is to be noted that learning and memory have a major role in the formation of conditioned place preference, and that antagonists of cholinergic receptors may attenuate these cognitive functions (Hiramatsu et al., 1998).

Recently interesting findings concerning the role of nicotinic α3β4-receptor, densely localized in the interpeduncular nucleus and medial habenula, have been published. Blockade of this receptor by 18-methoxycoronaridine was shown to attenuate dopamine sensitization to morphine (Taraschenko et al., 2007b) and to reduce morphine self-administration (Glick et al., 1996). Acute morphine administration to rats decreases acetylcholine release in striatum (Taguchi et al., 1993), cortex (Osman et al., 2005), and in the core and shell of nucleus accumbens (Fiserova et al., 1999). However, acute morphine at 5 mg/kg, a dose relevant in addiction studies, increases (while 20 mg/kg decreases) acetylcholine release in interpeduncular nucleus of rat (Taraschenko et al., 2007a). A cholinergic pathway projects from medial habenula to interpeduncular nucleus, and this habenulo-peduncular pathway has connections to VTA and nucleus accumbens (Sutherland, 1982). Chronic nicotine administration is known to induce nicotinic receptor upregulation (for review, see Gentry and Lukas, 2002), and this phenomenon has been shown also using our method of chronic nicotine administration via drinking fluid (Nuutinen et al., 2005; Pietilä et al., 1998). Thus, it seems likely that together the upregulation of nicotinic receptors and acetylcholine released by acute morphine administration are responsible for the increased sensitivity to morphine after chronic oral nicotine treatment, and we speculate that the location of the nicotinic receptors responsible for our results may be medial habenula and/or interpeduncular nucleus. As tobacco smoking increases the nicotine binding sites in human brain (Gentry and Lukas, 2002), it is possible that human smokers, similar to the mice in our experiment, are cross-sensitized to the reinforcing effects of morphine. Indeed, tobacco smoking increases the craving for opioids and other drugs (Spiga et al., 1998; Taylor et al., 2000).

In conclusion, mice pretreated with chronic oral nicotine were conditioned to morphine with a smaller dose than that required by control mice, suggesting a cross-sensitization between nicotine and morphine. These findings give further support for the existence of common pathways in the mechanisms of action of nicotine and opioids. No significant differences were found in the number, affinity or functional activity of μ-opioid receptors between control and nicotine-treated mice suggesting minor role for these receptors in the enhanced effects of morphine after chronic nicotine treatment.

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