Acquisition, Expression, and Reinstatement of Ethanol-Induced Conditioned Place Preference in Mice: Effects of Opioid Receptor-Like 1 Receptor Agonists and Naloxone

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ABSTRACT

The ability of the two opioid receptor-like receptor 1 (ORL1) agonists nociceptin (5 nmol i.c.v.) and synthetic (1S,3aS)-8-(2,3,3a,4,5,6-hexahydro-1H-phenalen-1-yl)-1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one hydrochloride (Ro 64-6198; 0.1, 0.3, and 1.0 mg/kg i.p.) and the opioid antagonist naloxone (0.1, 1.0, and 10.0 mg/kg s.c.) to modify ethanol-induced conditioned place preference was examined in NMRI male mice. The ORL1 agonists were found to significantly reduce the acquisition, expression, and ethanol-induced reinstatement of conditioned place preference. Unlike the ORL1 agonists, naloxone at the doses relevant for opioid receptor blockade failed to significantly influence the acquisition of ethanol-induced conditioned place preference. However, naloxone at 1.0 but not 0.1 mg/kg s.c. potently blocked the expression of ethanol-induced conditioned place preference and significantly inhibited ethanol-induced reinstatement of the conditioned place preference after extinction. Separate experiments indicated that nociceptin and Ro 64–6198 are both devoid of reinforcing or aversive properties. Naloxone, however, at 1.0 and 10.0 mg/kg, produced conditioned place aversion, indicating motivational properties of its own. Both nociceptin and Ro 64–6198 reduced locomotor activity after acute administration. However, tolerance developed very quickly to this effect and already after three i.c.v. (or i.p.) injections, there was no significant reduction of locomotor activity. It is concluded that ORL1 agonists can modulate the acquisition, expression, and reinstatement of the conditioned reinforcing effects of ethanol with no reinforcing or aversive properties of their own. This property might be a potential advantage in the treatment of alcoholism compared with nonselective opioid antagonist naltrexone.

Relapse to alcohol use after periods of abstinence is a common feature of alcoholism. Animal models have contributed to the identification of factors that may underlie the motivational aspects of ethanol and that contribute to relapse to ethanol taking. Drug-seeking behavior for ethanol can be initiated or maintained not only by the primary, reinforcing effects of the drugs themselves but also by secondary, drug-associated stimuli (for review, see O’Brien, 1975). The most common experimental model used to study relapse to drug seeking has been the reinstatement procedure. After training to make a response to self-administer a drug and the subsequent extinction of the acquired response, an acute noncontingent injection of test drug (or acute stress) results in the reinstatement of responding (Carroll and Comer, 1996). The conditioned place preference (CPP) procedure provides an alternative experimental approach to examine mechanisms underlying relapse. In this procedure a particular stimulus context or test environment is paired with the effects of the drug, without the animal having to learn to make an explicit response to obtain the drug. In the test trial, the animal is allowed to freely move between the test environment previously paired with the drug administration and the nondrug environment. By its behavior the animal can show which environment it prefers (for review, see Tzschentke, 1998). Recently, this learning paradigm was used to assess the relapse effect of a “priming” injection of a drug (e.g., cocaine; Mueller and Stewart, 2000). It was shown that a priming injection of the drug used to induce the conditioned place preference, given after extinction conditions, restored the salience or attractiveness of the environment previously paired with drug administration.

The neurochemical substrates for relapse to ethanol have been studied at some length. There is now evidence for a role

ABBREVIATIONS. CPP, conditioned place preference; ORL1, opioid receptor-like receptor 1; Ro 64–6198, (1S,3aS)-8-(2,3,3a,4,5,6-hexahydro-1H-phenalen-1-yl)-1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one hydrochloride; OFQ, orphanin FQ; CSF, cerebrospinal fluid; i.g., intragastric; ANOVA, analysis of variance; KO, knockout; WT, wild-type.
of the opioid systems in the risk for relapse due to their ability to mediate alcohol-induced euphoria. Naltrexone, a nonselective opioid antagonist, has a proven potential as a therapeutic agent for alcoholism. Naltrexone and its short-lasting analog naloxone display “antialcohol” properties in several behavioral paradigms (for review, see Froehlich and Li, 1994).

Recent studies have also focused attention to the brain nociceptin system in relation to the rewarding effects of ethanol (Ciccocioppo et al., 1999, 2000; Martin-Fardon et al., 2000). Nociceptin, a 17-amino acid neuropeptide, was found to be a natural ligand of the G protein-coupled opioid receptor-like 1 receptor (ORL1) (Meunier et al., 1995; Reinscheid et al., 1995). Despite the structural similarity with dynorphin-A, nociceptin does not bind to opioid receptors and its pharmacological effects are not sensitive to naloxone treatment (Jenck et al., 2000). In general, nociceptin has an inhibitory role on synaptic transmission in the central nervous system and thereby may reduce the responsiveness to external (e.g., stress) or pharmacological (e.g., ethanol) stimuli (Calo et al., 2000). The peptide has also been reported to inhibit the activation of antinociceptive neurons in the nucleus raphe magnus and periaqueductal gray as well as hypothalamic β-endorphinergic neurons, projecting to the ventral tegmental area and nucleus accumbens (Wagner et al., 1998). Nociceptin, injected i.c.v., was found to modify ethanol intake in rats and to prevent the acquisition of ethanol-induced conditioned place preference in rats (Ciccocioppo et al., 1999). The peptide also had an inhibitory effect on ethanol-seeking behavior in rats trained for ethanol self-administration after exposure to foot shock (Ciccocioppo et al., 2000), and it significantly reduced the effects of foot shock stress on ethanol-seeking behavior (Martin-Fardon et al., 2000). In place conditioning experiments in rats, nociceptin did not affect place preference, suggesting that it is devoid of “motivational” properties of its own (Devine et al., 1996a; Ciccocioppo et al., 1999).

Studies on the functional role of the nociceptin receptor (ORL1) have been hampered by the lack of potent and selective ligands. Recently, the nonpeptide compound (1S,3αS)-8-(2,3,3α,4,5,6-hexahydro-LH-phenalen-1-yl)-1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one hydrochloride (mol.wt. 438.017) (2,3α,4,5,6-phenalen-1H-phenalen-1-yl)-1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one hydrochloride (mol.wt. 438.017) (Ro 64-6198), which penetrates well to brain after i.p. administration, has been described (Jenck et al., 2000; Wichmann et al., 2000). Ro 64-6198 was shown to be a full agonist with an affinity to the ORL1 receptor close to that of nociceptin itself (Jenck et al., 2000).

The aim of the present work is to investigate the effect of nociceptin and Ro 64-6198 on the acquisition, expression, and reinstatement of ethanol-induced conditioned place preference in mice and to compare their effects with those of naloxone (a nonselective opioid antagonist). In addition, the role of endogenous nociceptin for the effects of ethanol in the same experimental paradigm was studied in pronociceptin gene knockout mice.

Materials and Methods

Animals

Experiments with naloxone, nociceptin, and Ro 64-6198 were carried out in male NMRI mice (20–25 g; B&K Universal, AB, Sweden). Animals were kept under standard laboratory conditions with free access to food pellets (low-protein diet) and tap water. The animals were housed four to six per cage in a light-controlled room (12-h light/dark cycle, light on at 6:00 AM) at 21°C and 60% humidity. The experiments were approved by the Northern Stockholm Ethics Board of Animal Experimentation. All experiments were performed between 10:00 AM and 1:00 PM.

Nociceptin knockout mice were obtained from Dr. F. Jenck (Roche CNS Research, Basel, Switzerland). Briefly, a null mutation was introduced into the coding region of the OFQ peptide of the mouse ppOFQ gene by homologous recombination in embryonic stem cells of 129/OLA mice (Koster et al., 1999). Two clones were selected in which the correct disruption at one ppOFQ allele was confirmed and were injected into blastocysts of C57BL/6J embryos. Two male chimeric mice transmitted the mutated allele to their offspring and were mated with C57BL/6J female mice. Mice heterozygous for the ppOFQ mutation seemed normal, healthy, and fertile for at least 12 months on the mixed 129/Ola-C57BL/6J genetic background. Heterozygous mice (+) were intercrossed and the offspring were genotyped by Southern blot or polymerase chain reaction. Animals have not been backcrossed into a pure genetic background but +/- and +/-– littermates of the F2 or F3 generation with mixed 129-C57 background were cumulated in groups. Data comparing male –/– and male +/+ mice are presented.

Surgery and Cannulation

The mice were anesthetized with a combination of Ketalar (50 mg/ml ketamine hydrochloride, 1:10, 0.2 ml s.c.; Parke Davis, Barcelona, Spain) and Hypnorm (10 mg/ml fluanisom + 0.2 mg/ml fentanyl; 1:10, 0.2 ml i.p.; Janssen, Beersse, Belgium). The body temperature was maintained at 37°C using a thermostat-regulated heat pad (CMA/105; CMA/Microdialysis, Stockholm, Sweden). Permanent steel guide cannulae with an outer diameter of 0.4 mm (Plastics One, Roanoke, VA) were implanted into the right lateral ventricle using coordinates based on the stereotaxic plates (AP/bregma = ±2.5 mm; L, 3 mm; V, 4.25 mm; Franklin and Paxinos, 1997). Cannulae were fixed to the skull by dental carbobxyl cement (Durafon ESPE, Sollentuna, Sweden). The animals were allowed to recover for 2 to 3 days in the colony room (2–3 mice/cage) before the start of the experiment.

Drugs

Naloxone HCl (EndoLab, Wilmington, PA) was dissolved in saline and injected s.c. in a 5-ml/kg volume. Nociceptin (1–17, peptide free base, mol.wt. 1809; Tocris, Bristol, UK) was dissolved in artificial CSF just before injection. Artificial mice CSF contained 7.20 g/l NaCl, 1.96 g/l NaHCO3, 0.18 g/l KCl, 0.068 g/l K2HPO4, 0.16 g/l CaCl2, 0.17 g/l MgCl2⋅6H2O, 0.07 g/l Na2SO4, and 1.0 g/l glucose. The pH of the solution was adjusted to 7.1 with 0.01 N NaOH and HCl. After i.c.v. injection, the needle remained in place for 3 to 5 s.

Ro 64-6198 was a gift of Dr. Juergen Wichmann (F. Hoffmann-La Roche, Pharma Preclinical Research, Basel, Switzerland). The drug was freshly dissolved in saline and was administered i.p. (5-ml/kg injection volume).

Ethanol solutions (ethanol 95%, density 0.9 g/ml) were prepared with tap water (v/v) to the required concentration and were administered intragastrically through a specially designed Teflon-coated needle (AgNTho’s, Lidingo, Sweden) in a volume of 0.2 ml/mouse.

Conditioned Place Preference

Apparatus.

Place preference experiments were performed in plastic shuttle boxes divided by guillotine doors into two compartments of equal size (25 × 25 × 25 cm) with different degrees of illumination (30 and 110 lux) and color (black and white, respectively). Floor texture (solid) was equal in both compartments. The location of the animal in the box was monitored with a video camera-based computerized imaging system (Videomot; TSE GmbH, Hamburg, Germany).
Experimental Design. Each experiment consisted of a preconditioning, conditioning, and postconditioning phase (Fig. 1). During the preconditioning phase mice were placed in the center of the white compartment facing the opened door between the compartments. This 10-min procedure was repeated 1 h later. Time spent in the white compartment during the second preconditioning session was defined as the baseline level of preference. The experimental conditions were chosen (see above) to result in a balanced preference for both compartments during the preconditioning test. In addition, on the basis of the results from preconditioning test we excluded from the experiments mice with a tendency for unbalanced preference (more than 60% of time in one of the compartments). Although the criteria for exclusion were relatively strict, most of the mice were found to fulfill them (60–80% depending on the group).

The conditioning phase consisted of two 20-min daily sessions on four consecutive days (total eight sessions: four with ethanol and four with water). On each of the four days of conditioning mice received water i.g. (5 min before the first daily conditioning session) and placed randomly to one of the compartments for 20 min. Thereupon, they received an i.g. ethanol solution (5, 10, and 20%, 0.2 ml/mouse, corresponding to 0.4, 0.8, and 1.6 g/kg ethanol) and were placed into the opposite compartment for 20 min with the door between compartments closed. The control group received water i.g. in both compartments with the random starting placement (i.e., half of mice in each group started from the white compartment and half from the black).

After the completion of two 20-min trials, the animals were returned to their home cages. To avoid possible influence of intragastric food on ethanol absorption, mice were food deprived 1 h before the conditioning procedure.

The postconditioning test was conducted 48 h after the last conditioning session. During the postconditioning test, mice in a drug-free state (except in the experiments with the expression of conditioned place preference) were placed in the apparatus with open doors for 10 min and the time spent in the white compartment and the number of crossings between compartments were automatically recorded in the same manner as during the preconditioning test. Due to the degree of illumination the video tracking system detected mice only in the white compartment. Time spent in the opposite black part was calculated as a difference between the total time of the experiment and the time spent in the white compartment. The difference of time spent in the ethanol-associated compartment during post- versus preconditioning test was taken as the measure of drug-induced place preference. In control experiments (conditioning with water in both compartments) the shift of time was calculated with reference to the compartment used for the second conditioning with water. The boxes were cleaned and deodorized with 3% H2O2 solution after each experiment.

Effects of Nociceptin/Ro 64-6198/Naloxone on the Acquisition of Ethanol-Induced Place Conditioning. The experiments were performed as described above but during the conditioning period mice were pretreated 2 min before the second conditioning trial (0.8 g/kg ethanol or water administration) with nociceptin (5 nmol in 2 μl i.c.v.) or Ro 64-6198 (0.3, 1.0, and 3.0 mg/kg i.p.) or naloxone (0.1, 1.0, and 10.0 mg/kg s.c.). Animals in the control group received i.c.v. injections of artificial cerebrospinal fluid (2 μl) or i.p./s.c. injection of saline, respectively.

Influence of Nociceptin/Ro 64-6198/Naloxone on the Reinstatement of Ethanol-Induced Place Preference by a Priming Injection of Ethanol Solution. The acquisition of ethanol-induced CPP (ethanol dose) place preference was performed as already described. After being tested for place preference, mice were subjected to 4 days of extinction trials (one trial per day) during which the mice were allowed to investigate both compartments for 20 min. During the last extinction trial the preference for the compartments was again recorded (last 10 min of the trial).

For experiments with nociceptin i.c.v. administration, the day after the last extinction trial the mice were operated and cannulae were implanted into the lateral ventricles. Three days after the operation (mice were left undisturbed) the reinstatement experiment was initiated. Mice were treated i.g. with either a 0.4-g/kg ethanol dose or with water. Five minutes after ethanol/water administration the mice were treated with nociceptin (5 nmol/2 μl i.c.v.) or CSP (2 μl i.c.v.) and allowed to explore the shuttle boxes. The time spent in the white compartment and the number of crossings between compartments were recorded as described above.

The experiments with Ro 64-6198/naloxone administration were run similarly to the nociceptin study, e.g., 3 days after the last extinction trial. In the reinstatement experiment, separate groups of mice received, 5 min after being treated i.g. with either ethanol (0.4 g/kg) or with water, Ro 64-6198 (0.3 mg/kg i.p.), naloxone (1.0 mg/kg s.c.), or saline (i.p.) and were then allowed to explore the shuttle boxes. The time spent in the ethanol-associated compartment and the number of crossings between compartments were recorded as described above.
Effects of Ro 64-6198 on Locomotor Activity

Mice were individually tested in a dimly lit sound-controlled area ventilated by fans. They were removed from their home cages and placed in the middle of an activity monitor (standard transparent A3 (42 × 26 × 20 cm) Macrolon cage B&K Universal, AB, Sweden with 50 ml of wood shavings on the floor) and the data-collecting system was immediately activated. In experiments with nonhabituated animals, mice were treated with the drug just before placement in the activity box and different parameters of motor activity were recorded during 6 × 10-min intervals.

Motor activity was measured in eight animals simultaneously by means of a multicage red and infrared-sensitive motion detection system (Ogren et al., 1986). The system is fully computerized and uses beams of red and infrared lights in combination with vertical (infrared light-sensitive photocells in the walls of the apparatus) and horizontal (red light-sensitive photocells in the floor of the apparatus) arrays. The distance between photocells is 4 cm. Rearing was measured by counting the number of times an animal stands on its hind legs and interferes with any of the six invisible infrared beams passing horizontally through the cages. The height of these photocells was adapted to the size of the animal. Motility was measured as all movements of a distance of 4 cm or more detected by 48 vertical photocells, and represents a measurement of general activity. Locomotion was measured by counting the number of times an animal had covered eight horizontal photocells and moved from one side of the test cage to the other side (a distance of at least 32 cm).

Effects of Subchronic Treatment with Ro 64-6198 on the Locomotor Inhibitory Effect of Ro 64-6198

Drug and experimentally naive mice were treated twice a day (10:00 AM and 6:00 PM) i.p. for 4 days with either saline or 1 mg/kg Ro 64-6198. On day 5, mice were randomly divided into three treatment groups (n = 8):

1. Subchronic treatment with saline + acute treatment with Ro 64-6198 (1 mg/kg).
2. Subchronic treatment with Ro 64-6198 + acute treatment with Ro 64-6198 (1 mg/kg).
3. Subchronic treatment with saline + acute treatment with saline.

Immediately after treatment mice were placed into the “activity” boxes and their rearing, motility, and locomotion were recorded for 6 × 10-min periods.

Dose-dependent effects of Ro 64-6198 on rearing, motility, and locomotion in mice were analyzed by ANOVA for repeated measures, the factors being treatment (drug doses and saline) and time (6 × 10-min recording). This was followed by a post hoc Newman-Keuls test for every time period recorded. The whole study was designed as a between-subjects (independent groups) experiment (i.e., each animal was used only once).

Results

Dose-Dependent Effects of Ethanol in the CPP Paradigm

Measurement of the natural preference in the preconditioning test indicated that the mice displayed no preference for the particular compartment. One-way ANOVA revealed no significant differences between the experimental groups either in time spent in the white compartment or in the number of crossings between the compartments during the 10-min experiment. The mean number of crossings was about 17 to 25 in 10 min.

In animals with water conditioning (i.e., administration before placement in both compartments), a slight tendency for an increase in the time spent in the white compartment was found. No changes in the exploratory activity (number of crossings) were found after conditioning with water in both compartments.

Ethanol produced a significant place conditioning effect in NMRI mice (Fig. 2). ANOVA revealed a significant effect with respect to the shift of time spent in the ethanol-paired compartment [F(3,20) = 6.5, p < 0.01]. Post hoc test revealed that the 0.8-g/kg ethanol group differed significantly from the water group (p < 0.01) and the mean increase in time spent in ethanol paired compartment was 95.3 s.

Ethanol-Induced CPP in Nociceptin Knockout (KO) Mice

Nociceptin KO male mice and their wild-type (WT) controls were tested for the acquisition of ethanol-induced conditioned place preference comparing 0 (water), 0.4-, 0.8-, and 1.6-g/kg ethanol doses (Fig. 3). Two-way ANOVA of the shift of time spent in the ethanol-paired compartment revealed a significant ethanol dose-related effect [F(3,35) = 8.2, p < 0.01] but no genotype effect [F(1,35) = 3.0, p = 0.08] and no genotype × dose interaction [F(3,35) = 0.3, p = 0.8]. In general, no genotype differences were found but the nociceptin KO mice tended to show a stronger response to ethanol (Fig. 3A). No significant genotype differences were found with respect to the number of crossings between white and black compartments (20–25 crossings in 10-min experiment) in post-test (Fig. 3C). However, KO mice exhibited higher exploratory activity in the pretest (Fig. 3B), which was indicated by a significantly higher number of crossings between compartments [genotype effect F(1,35) = 5.2, p < 0.05]. Thus, it was concluded that KO mice in comparison with WT mice displayed higher exploratory activity in the novel environment and tended to show a higher sensitivity to the conditioning effects of ethanol.

Effects of Nociceptin/Ro 64-6198 on the acquisition of ethanol-induced CPP

Nociceptin (5 nmol/2 μl/mouse) administration blocked (Fig. 4A) the acquisition of ethanol-induced (0.8 g/kg) condi-
tioned place preference \([F(3,35) = 6.7, p < 0.01]\). The post hoc test revealed a significant \((p < 0.01)\) reduction in the shift of time spent in the ethanol-paired compartment in the group pretreated with nociceptin before ethanol administration. However, the groups treated with CSF and nociceptin and received conditioning with water in both compartments, did not differ. No significant reduction of the number of crossings in groups treated with nociceptin during the post-test was found.

Administration of Ro 64-6198 (0.1, 0.3, and 1.0 mg/kg i.p.) reduced ethanol-induced conditioned place preference \([F(3,28) = 33.5, p < 0.01]\) with a significant effect even at the lowest dose tested \((0.1 \text{ mg/kg}, p < 0.01\) compared with the vehicle-treated group). It is interesting that the combined administration of ethanol and Ro 64-6198 at the 0.3-mg/kg dose produced a significant aversion for the compartment associated with the combination \((-87.1 \pm 9.7\) s). There was a significant reduction in the number of crossings between the compartments in the group that received Ro 64-6198 at the dose of 0.3 mg/kg \((p < 0.05)\) but not at the 1.0-mg/kg dose. In experiments with water conditioning, similar to nociceptin, Ro 64-6198 produced neither place preference nor place aversion, and there were no differences between groups in the shift of time spent in the compartment associated with drug treatment \([F(3,28) = 0.11, p = 0.9]\).

**Effects of Naloxone on the Acquisition of Ethanol-Induced CPP**

Administration of naloxone (0.1, 1.0, and 10.0 mg/kg s.c.) reduced ethanol-induced conditioned place preference \((F(3,20) = 9.8, p < 0.01)\). However, a significant effect was found only at the highest dose tested \((10 \text{ mg/kg}, p < 0.01\) compared with the saline-treated group). It is important to note that mice treated with the naloxone \((10 \text{ mg/kg})\) displayed reduced spontaneous locomotor (significant reduction of the number of crossings, \(p < 0.01\)) activity, high level of grooming, and generalized reaction to external stimuli with jumping and screaming. All this indicates that naloxone at the 10-mg/kg dose causes nonspecific effects probably unrelated to opioid systems. Importantly, the combined administration of naloxone \((10 \text{ mg/kg})\) and ethanol produced a significant aversion for the compartment associated with the combination \((-41.5 \pm 19.7\) s). In experiments with water conditioning, naloxone at the 0.1-mg/kg dose produced neither place preference nor place aversion. However, the higher doses of naloxone \((1.0\) and \(10 \text{ mg/kg})\) produced place aversion \((-12.2 \pm 8.5\) and \(-40.5 \pm 21.3\) s, respectively) and a significant reduction of motor activity as shown previously with the 10-mg/kg dose.
Effects of Nociceptin/Ro 64-6198 on the Expression of Ethanol-Induced CPP

In this set of experiments mice received a single administration of nociceptin or Ro 64-6198 just before the postconditioning test (Fig. 5). In the experiments with nociceptin administration (Fig. 5A) ANOVA revealed a significant group effect with respect to the shift of time in the ethanol-paired compartment \([F(3,32) = 9.2, p < 0.01]\) and the number of crossings in the postconditioning test \([F(3,32) = 10.4, p < 0.01]\). Nociceptin administration blocked the expression of the ethanol-induced CPP and reverted it to place aversion \([\text{mean shift of time } -54.3 \pm 22.1, n = 9]\). In contrast, administration of nociceptin to the animals, which received conditioning with water, failed to produce place aversion \([\text{mean shift of time } 34.0 \pm 22.2, n = 8]\). Nociceptin treatment also significantly reduced the number of crossings between compartments \(p < 0.01\) in both groups (e.g., water-conditioning group and ethanol-conditioning group).

Ro 64-6198 (0.1, 0.3, and 1.0 mg/kg i.p.) also blocked the expression of ethanol-induced CPP (Fig. 5B) in a dose-related way \([\text{dose effect: } F(3,18) = 16.4, p < 0.01]\) and reversed it to a clear-cut place aversion at the dose of 1 mg/kg (mean shift of time \(-51.7 \pm 13.8, n = 8\)). Interestingly, the same dose of Ro 64-6198 failed to produce place aversion in the group conditioned with water (mean shift of time \(15.3 \pm 6.1, n = 8\)). Moreover, no significant differences between groups were observed \([\text{dose effect: } F(3,12) = 0.48, p = 0.6]\). Treatment with Ro 64-6198 (1 mg/kg) produced a significant reduction in the number of crossings between compartments in the postconditioning test.

Effects of Naloxone on the Expression of Ethanol-Induced CPP

Treatment with naloxone (0.1, 1.0, and 10.0 mg/kg s.c.; Fig. 5C) blocked the expression of ethanol-induced CPP in a dose-related way \([\text{dose effect: } F(3,20) = 12.2, p < 0.01]\) and reversed it to a clear-cut place aversion at the dose of 10 mg/kg \([\text{mean shift of time } -52.8 \pm 22.7, n = 8\]). As in the acquisition experiments mice treated with the 10-mg/kg dose of naloxone displayed behavioral inhibition and a significant reduction of the number of crossings between compartments in post-test. Administration of the opioid antagonist to the groups, which had received conditioning with water, failed to produce significant place aversion, although some tendency for the avoidance of the white compartment was observed in mice treated with 10 mg/kg naloxone regardless of the starting compartment in conditioning trials. No significant differences between groups were observed \([\text{dose effect: } F(3,20) = 0.72, p = 0.6]\) in mice with water conditioning.

Effect of Nociceptin, Ro 64-6198 and Naloxone on the Ethanol-Induced Re reinstatement of CPP after Extinction

Figure 6 shows the results of the experiments in which animals first acquired CPP and then were given four daily extinction trials, and then received a single priming i.g. administration of ethanol (0.4 g/kg i.g.). The data were analyzed using a two-way repeated measures ANOVA with water versus ethanol conditioning as the first between group factor and drug treatment (nociceptin, CSF, saline, Ro 64-6198, and naloxone) as the second between group factor. Three data blocks (post-test 1, post-test 2, and post-test 3; Fig. 1) were used as the repeated measurement factor. ANOVA revealed a significant conditioning effect \([F(1,80) = 63.9, p < 0.01]\) as well as a significant interaction between conditioning and treatment \([F(4,80) = 2.7, p < 0.05]\) and conditioning and time \([F(2,160) = 37.4, p < 0.01]\). Treatment effect and treatment \(\times\) time interaction were also significant \([F(4,80) = 6.0, p < 0.01]\) and \([F(8,160) = 6.5, p < 0.01]\), respectively.

It can be seen that time spent in the ethanol-paired chamber diminished after the extinction trials (Fig. 6) and it did not differ from the time spent in the water-paired chamber. Analysis of the shift of time in the three testing blocks (e.g., acquisition, extinction, and priming) revealed a significant shift of time spent in the ethanol-paired compartment \([F(2,20) = 7.62, p < 0.01]\) in the control groups (i.e., saline or i.p. saline), which received conditioning with ethanol solution (Fig. 6). This indicates that the time spent in the ethanol-paired compartment significantly increased after acquisition and after priming with ethanol \(p < 0.01\), compared with the values obtained in pretest) but did not differ from pretest values after extinction trials. In contrast, animals that received only water administration (Fig. 6), displayed no significant shift of preference \([\text{compared with pretest values}\) after acquisition, extinction and priming with 0.4-g/kg ethanol dose. The priming injection of ethanol (0.4 g/kg i.g.), which by itself was ineffective to establish a significant CPP
before the test, resulted in complete reinstatement of CPP in the group that had acquired a significant place preference reaction after conditioning with 0.8-g/kg ethanol dose. However, in animals primed with tap water (i.g.) there was no increase in time spent in the compartment previously associated with ethanol. Both nociceptin (i.c.v), Ro 64-6198 (0.3 mg/kg i.p.), and naloxone strongly inhibited the “priming” effect of the noncontingent administration of ethanol ($p < 0.01$ for nociceptin and Ro 64-6198 and $p < 0.05$ for naloxone, comparison between post-test 1 and post-test 3). In all groups treated with nociceptin and Ro 64-6198, a significant reduction ($p < 0.05$) of the number of crossings between compartments was observed (compared with the corresponding control groups treated with CSF/vehicle). Treatment with naloxone failed to influence the motor activity of mice.

**Effects of Acute and Subchronic Treatment with Ro 64-6198 on Spontaneous Locomotor Activity in Mice**

**Effects of Acute Treatment with Ro 64-6198 (Fig. 7, left column).** In animals treated with vehicle ($n = 8$), there was a gradual decrease of motor activity over time and an almost complete elimination of rearing, motility, and locomotion after 1 h of habituation to the activity cages. Ro 64-6198 caused a significant influence on rearing [$F$(3,39) = 3.8, $p < 0.05] and motility [$F$(3,39) = 4.7, $p < 0.01]. A significant time effect for rearing, motility, and locomotion [$F$(5,195) = 61.7, $p < 0.0001; 143.5, $p < 0.001$ and 83.6, $p < 0.001$, respectively] and a significant dose $\times$ time interaction for all parameters [$F$(15,195) = 1.9, $p < 0.05$, 1.7, $p < 0.05$ and 2.1, $p = 0.01$, respectively] were also found. The post hoc Newman-Keuls test, analyzed for each time point separately, revealed that there was a significant ($p < 0.01$) lower level of rearing 20 and 30 min after treatment with Ro 64-6198 at the dose of $1 \text{ mg/kg}$ (with respect to vehicle-treated group) motility and locomotion 20, 30, and 40 min after treatment with $1 \text{ mg/kg}$ Ro 64-6198.

**Effects of Subchronic Treatment with Ro 64-6198 on the Locomotor Inhibitory Effect of Ro 64-6198 (Fig. 7, right column).** One-way ANOVA revealed significant group $\times$ time interaction for rearing, motility, and locomotion [$F$(10,105) = 2.4, $p < 0.05$; 3.7, $p < 0.01$; and 2.1, $p < 0.05$, respectively]. Subsequent post hoc analysis revealed that only the group that received subchronic treatment with saline and acute administration of Ro 64-6198 (sal-ro group) displayed significantly lower rearing, motility and locomotion at 10 to 40 min after treatment ($p < 0.05$, compared with group sal-sal). Subchronic treatment with Ro 64-6198 (1 mg/kg i.p.) did not cause a significant inhibition (compared with the control group sal-sal) of the locomotor activity.
**Discussion**

The present experiments have demonstrated that 1) intragastric administration of ethanol, paired with a distinct environment, can produce CPP in mice. The CPP can be extinguished by repeated exposure to the place conditioning environment in an ethanol-free state. 2) A priming administration of a low dose of ethanol, which in an alcohol-naive animal failed to induce significant place preference, reinstated the previously acquired and extinguished ethanol-induced CPP. 3) Nociceptin blocked the acquisition of ethanol-induced CPP in line with previous findings, a property shared by the synthetic nociceptin agonist Ro 64-6198. For the first time it was demonstrated that both nociceptin and the synthetic ORL1 agonist could block the expression and ethanol-primed reinstatement of CPP. 4) The nonselective opioid antagonist naloxone failed to block the acquisition of ethanol-induced CPP but inhibited the expression and ethanol-induced priming of the acquired CPP at the dose relevant for opioid receptor blockade.

There is a large body of experimental reports demonstrating reliable ethanol-induced CPP in inbred and outbred mice (Cunningham et al., 1991; Crabbe et al., 1992; Cunningham, 1995; Risinger and Oakes, 1996; Bormann and Cunningham, 1997). In the present study, ethanol at the dose of 0.8 g/kg produced maximal place conditioning effect in outbred NMRI mice. This dose is somewhat lower than those previously reported to induce of CPP in mice. Cunningham et al. (1992) reported a maximal conditioning effect with the doses 3 and 4 g/kg using DBA/2J mice, whereas Risinger et al. (1996) demonstrated significant CPP in Swiss-Webster mice with ethanol doses 1 and 2 g/kg. The higher potency of ethanol in our study compared with the previous reports might be explained by the shorter conditioning trials used in the present study (20 min) compared with 30 and 60 min in the cited study (Risinger and Oakes, 1996).

Comparing pronociceptin gene KO and WT mice we failed to observe significant differences in the acquisition of ethanol-induced CPP. This suggests that during basal conditions nociceptin systems probably play a minor role in modulating the motivational effects of ethanol. However, the increased reactivity of KO mice (as indicated by the increased number of crossings between compartments in the pretest) was noted, which supports previous observations about the higher reactivity to novelty in nociceptin-deficient mice (Reinscheid et al., 1999).

In animals, administration of the originally self-administered drug was shown to be an effective cue for reinstatement of drug-taking behavior (Stewart and Wise, 1992). Here, it was found that repeated daily testing in the absence of unconditioned stimuli lead to a reduction of ethanol-induced CPP, supporting earlier observations obtained in mice with cocaine-induced CPP (Mueller and Stewart, 2000). However, after a priming administration of a low dose of ethanol, the animals chose the environment associated with the ethanol-related stimuli indicative of the reinstatement of the extinguished place preference by the priming dose of ethanol.

Examination of the motivational effects of nociceptin and the synthetic nociceptin receptor agonist Ro 64-6198 revealed that both substances are devoid of either reinforcing or aversive properties in mice, supporting previous observations in rats (Devine et al., 1996a; Ciccocioppo et al., 1999). However, when nociceptin and Ro 64-6198 were tested versus the effect of ethanol in the place-conditioning paradigm, they significantly reduced both the acquisition, expression, and ethanol-induced reinstatement of CPP. Moreover, the combined administration of nociceptin/Ro 64-6198 and ethanol produced a clear-cut place aversion. The fact that nociceptin/Ro 64-6198 can modulate the expression and reinstatement of the conditioned reinforcing effects of ethanol is of special importance. The conditioned positive motivational responses are thought to underlie “craving” in humans, and possibly contribute to relapse to drinking in abstinent alcoholics (Stewart et al., 1984). It could be argued that the effects of nociceptin receptor ligands seem not to be due to unspecific changes in behavior. Acute nociceptin is known to suppress locomotor activity in rats (Devine et al., 1996b) and mice (Reinscheid et al., 1995), although at low doses (lower than 1 nmol/mouse) nociceptin is reported to increase locomotor activity, probably via an “anxiolytic” action (Jenck et al., 1997; Florin et al., 1996). In our experiments, we confirmed that both nociceptin (5 nmol/mouse) and Ro 64-6198 (1 mg/kg) reduced locomotor activity after acute administration. However, tolerance developed very quickly to this effect and already after three injections we failed to observe a significant reduction of locomotor activity as indicated by the number of transitions between the compartments. This observation is in line with the previous data obtained in rats (Devine et al., 1996b; Ciccocioppo et al., 1999).

At present, the neurochemical mechanisms underlying the action of nociceptin and Ro 64-6198 on the rewarding effects of ethanol and reinstatement of ethanol-induced CPP are unknown. Nociceptin has been shown to decrease basal (Murphy and Maitland, 1999) and morphine-stimulated (Di Giovanni and Pieretti, 2000) extracellular dopamine in the nucleus accumbens. This action of nociceptin is probably due to an activation of GABA-ergic interneurons, which exert inhibitory control of mesolimbic dopaminergic neurons. It is also possible that nociceptin can modulate the enkephalinergic fibers known to project to the VTA and synapse with GABA neurons (Sesack and Pickel, 1995). In addition, nociceptin has been shown to decrease glutamate release in cortical slices (Nicol et al., 1996).

Because the endogenous opioids seem to be essential for initiation and maintenance of excessive alcohol consumption it was of interest to include the opioid antagonist naloxone in the present study. Naloxone failed to inhibit acquisition of ethanol-induced CPP at the doses that are relevant to the opioid receptor blockade (0.1 and 1.0 mg/kg), consistent with previously published data (Cunningham et al., 1995). However, at the highest dose tested (10 mg/kg) naloxone completely blocked acquisition of CPP. The disturbances of the mice behavior with the 10-mg/kg dose of naloxone are indicative of unspecific toxic effects of naloxone, probably mediated via nonopioid mechanisms. At doses 1 mg/kg and higher naloxone produced conditioned place aversion, in line with previous observations (Cunningham et al., 1995). In our experiments, we unexpectedly found that naloxone could block the expression of ethanol induced CPP at the doses 1 mg/kg and higher. These doses of naloxone are known to nonselectively block all types of opioid receptors (Pitts et al., 1996) and this probably indicates that multiple opioid systems are involved in the acquisition and expression of ethanol-induced CPP. This observation contrasts with some previous reports (Cunningham et al., 1995, 1998; Bormann and Cunningham, 2000).
318 Kuzmin et al.

1997) but is in line with the findings of Middaugh and Bandy (2000). The discrepancies in the results using naloxone might be explained by differences in experimental approaches (biased versus unbiased procedure, different conditional cues, different duration of the conditioning trials as well as different strains of mice used). In our experiments naloxone (1 mg/kg) also strongly inhibited the reinstatement of ethanol-induced CPP primed by the low dose of ethanol (0.4 g/kg). This is, to our knowledge, the first demonstration of the “antirelapse” activity of an opioid antagonist in experiments with mice.

The results of these experiments can also be interpreted in terms of a learning impairment or a retrieval deficit after treatment with nociceptin agonists. In fact, nociceptin has been found to impair hippocampal-dependent spatial learning (Higgins et al., 2002). Thus, it seems possible that nociceptin and its synthetic agonist can block the formation of the learned association between the effects of ethanol and environmental cues. In the reinstatement experiments, nociceptin agonists may prevent retrieval of the priming dose of ethanol. Stimulation of nociceptin receptors may prevent the reactivation of memory circuits by subthreshold stimuli (e.g., low doses of ethanol or conditional environmental stimuli). Needless to say, this hypothesis requires experimental confirmation.

In conclusion, the present results support the hypothesis that stimulation of nociceptin (ORL1) receptors reduces the reinforcing and motivational effects of ethanol in mice as well as reinstatement of preference for place induced by ethanol.

This suggests that drugs targeted to this receptor may have therapeutic potential in the treatment of alcohol-seeking behavior and relapse.

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References


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