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NALOXONE FAILS TO PRODUCE CONDITIONED PLACE AVERSION IN μ-OPIOID RECEPTOR KNOCK-OUT MICE

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Abstract—There is growing evidence that tonic activity of the opioid system may be important in the modulation of affective state. Naloxone produces a conditioned place aversion in rodents, an effect that is centrally mediated. Previous pharmacological data using antagonists with preferential actions at μ-, δ-, and κ-opioid receptors indicate the importance of the μ-opioid receptor in mediating this effect. We sought to test the μ-opioid receptor selectivity of naloxone aversion using μ-opioid receptor knock-out mice. μ-Opioid receptor knock-out and wild-type mice were tested for naloxone (10 mg/kg, s.c.) aversion using a place conditioning paradigm. As a positive control for associative learning, knock-out mice were tested for conditioned place aversion to a κ agonist, U50,488H (2 mg/kg, s.c.). Naloxone produced a significant place aversion in wild-type mice, but failed to have any effect in μ-opioid receptor knock-out mice. On the other hand, both knock-out and wild-type mice treated with U50,488H spent significantly less time in the drug-paired chamber compared to their respective vehicle controls. We conclude that the μ-opioid receptor is crucial for the acquisition of naloxone-induced conditioned place aversion. Furthermore, in a separate experiment using C57BL/6 mice, the δ-selective antagonist naltrindole (10 or 30 mg/kg, s.c.) failed to produce conditioned place aversion.

Taken together, these data further support the notion that naloxone produces aversion by antagonizing tonic opioid activity at the μ-opioid receptor. © 2001 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: opioid, aversion, knock-out, antagonists, affect, locomotion.

The rewarding and reinforcing effects of opiates such as morphine and heroin are thought to be mediated primarily through an agonist action at the μ-opioid receptors (MOR). Concurrent administration of selective MOR antagonists reverses the actions of morphine and heroin in both self-administration and conditioned place preference (CPP) paradigms (Negus et al., 1993; Piepponen et al., 1997). Furthermore, a line of genetically engineered mice lacking the MOR fail to demonstrate morphine-induced CPP (Matthes et al., 1996). However, δ-opioid receptors (DOR) may also be important mediators of reward and reinforcing behavior since DOR-selective agonists are effective in both CPP (Stapleton et al., 1979; Phillips and LePiane, 1982; Phillips et al., 1983; Shippenberg et al., 1987; Bals-Kubik et al., 1990; Longoni et al., 1998) and self-administration paradigms (Glimcher et al., 1984; Goeders et al., 1984a,b; Devine and Wise, 1994).

There is growing evidence that tonic activity of the opioid system may be important for maintenance of 'hedonic homeostasis' (Koob and Le Moal, 1997). Opioid antagonists are known to produce dysphoria (Hollister et al., 1981; Martin del Campo et al., 1994) or tension/anxiety (Grevert and Goldstein, 1977a) in humans and to elicit aversion in animal models of affect (Downs and Woods, 1976; Grevert and Goldstein, 1977b; Mucha and Iversen, 1984; Mucha et al., 1985; Mucha and Herz, 1985; Mucha and Walker, 1987). Such aversion is centrally mediated as i.c.v. administration of a general opioid antagonist, naloxone (NLX), produces conditioned place aversion (CPA) in rats (Bals-Kubik et al., 1989). Moreover, methylated analogues of NLX, which do not readily penetrate the blood–brain barrier, do not produce CPA in mice when administered peripherally (Hand et al., 1988; Kuzmin et al., 1997).

It is not clear whether endogenous ligands act solely at the MOR to maintain a stable affective state or if the DOR may play a role. The preferential MOR antagonist, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2 (CTOP), administered either i.c.v. (Bals-Kubik et al., 1989) or locally into the ventral tegmental area (VTA) and nucleus accumbens (NAC) (Shippenberg and Bals-Kubik, 1995), produces CPA in rats whereas the prefer-

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Abbreviations: ANOVA, analysis of variance; CPA, conditioned place aversion; CPP, conditioned place preference; CTA, conditioned taste aversion; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2; DOR, δ-type opioid receptor; MOR, μ-type opioid receptor; MOR−/−, μ-opioid receptor knock-out; NAC, nucleus accumbens; NLX, naloxone; NTD, naltrindole; U50,488H, [*trans*-3,4-dichloro-*N*-methyl-*N*-(2-(1-pyrrolidinyl) cyclohexyl)-benzeneacetamide] methane-sulfonate salt; VEH, vehicle; VTA, ventral tegmental area; WT, wild-type.

ential DOR antagonists, naltrindole (NTD) and ICI 174,864, fail to induce significant CPA (Shippenberg et al., 1987; Bals-Kubik et al., 1989; Menkens et al., 1992; de Vries et al., 1995). However, a clear trend toward aversion was observed with ICI 174,864 at higher doses (Shippenberg et al., 1987; Bals-Kubik et al., 1989). Furthermore, NTD is effective in the rat conditioned taste aversion (CTA) paradigm (Froehlich et al., 1998; Hutchinson et al., 2000), as is the δ2-selective antagonist, naltriben (Reid et al., 1996). Since the selectivity of antagonists for receptor types is never absolute, we assessed the ability of the general opioid antagonist, NLX, to induce CPA in mice lacking the MOR (MOR−/−). As a positive control for associative learning in these animals, MOR−/− mice were tested for CPA to the κ agonist, [*trans*-3,4-dichloro-*N*-methyl-*N*-(2-(1-pyrrolidinyl) cyclohexyl)-benzeneacetamide] methane-sulfonate salt (U50,488H). In a separate experiment, the effectiveness of NTD to produce a CPA in C57BL/6 mice was also investigated.

EXPERIMENTAL PROCEDURES

Subjects

Experiment 1: MOR−/− and wild-type (WT) controls bred from the same F_2 generation were used in this study (Matthes et al., 1996). Adult male mice (31 ± 3 g) were handled and weighed daily for 5 days prior to the start of the experiment.

Experiment 2: Adult male C57BL/6 mice obtained from Charles River (Wilmington, MA, USA) (weighing 27 ± 1 g) were similarly handled and weighed. Subjects were housed in groups of four or five in transparent (27×17×13 cm) plastic cages and received water and standard laboratory rat chow *ad libitum*. The colony was maintained on a 12-h light/dark cycle, lights on 8.00–20.00 h at an ambient temperature of 20–21.1°C.

Animals were treated in accordance with the Guide for Care and Use of Laboratory Animals established by the National Institutes of Health in the USA and with the Animal Care and Use Guidelines established by the Chancellor's Animal Research Committee at the University of California, Los Angeles. All efforts were made to reduce the number of animals used and their suffering.

Apparatus

The conditioning apparatus allowed for automated recording of subject location (time spent in each chamber) and locomotor activity (Coulbourn Instruments, Allentown, PA, USA). The square arena of the apparatus was divided into three chambers by a Plexiglas partition. Two chambers, identical in size, shape (trapezoid), and texture (smooth), were used as the conditioning chambers. One of these chambers was decorated on all four walls and floor with black and white checkered (2-cm squares) contact paper and contained almond extract (McCormick and Co., Hunt Valley, MD, USA) as an olfactory cue (200 μl on a strip of filter paper hung from the top corner of the chamber). The other chamber was decorated on all four walls and floor with black and white cow print contact paper (RubberMaid, 'Wholly Cow' pattern) and was accentuated with lemon extract (McCormick and Co.) presented in the same fashion. The visual cues offered approximately equal amounts of white and black. A smaller triangular shaped chamber decorated with gray contact paper on all three walls and floor was designated the 'start' chamber. This neutral chamber remained odorless. Two removable guillotine doors allowed access from the start chamber to each of the conditioning compartments. Animals were placed directly into this start chamber during habituation and test sessions.

Habituation

On day 1, subjects were placed in the start chamber and permitted free access to the entire apparatus for 15 min. The time spent in each of the chambers was recorded to measure any initial bias.

Conditioning

On days 2–4, in the morning, animals received placebo injection of saline s.c. (referred to below as 'placebo' treatment) and were confined to the 'placebo-paired' chamber for 30 min and subsequently returned to the home cage. Four hours later, subjects received a s.c. injection of one of the test drugs or saline, and were confined to the 'drug-paired' chamber for 30 min. The drug-paired chamber was randomized across subjects. Locomotor activity was recorded during each of the six conditioning sessions.

Test

On day 5, 24 h after the last drug treatment, subjects, in a drug-free state, were allowed to freely explore the apparatus with the doors removed for 15 min. The time spent in each chamber was recorded.

Groups

Experiment 1: MOR−/− and WT mice were each divided into three drug treatment groups to produce a total of six groups. MOR−/− mice were treated with vehicle (VEH) ($n=16$), NLX (10 mg/kg; $n=9$), or U50,488H (2 mg/kg; $n=8$). WT mice were similarly grouped, VEH ($n=7$), NLX ($n=8$), and U50,488H ($n=5$).

Experiment 2: C57BL/6 mice were divided into three drug treatment groups: VEH, NTD (10 mg/kg), or NTD (30 mg/kg) with $n=8$ in each group.

Statistical analysis

Experiment 1: Time spent in the drug-paired chamber was analyzed by two-way analysis of variance (ANOVA) (genetic background×drug treatment) followed by Fisher's protected LSD post-hoc one-tailed tests. Locomotor activity during the three conditioning sessions was similarly analyzed by two-way ANOVA and Fisher's LSD post-hoc one-tailed test ($P \leq 0.05$ was considered statistically significant).

Experiment 2: CPA and locomotor data were similarly analyzed, however, one-way ANOVAs (drug treatment) were employed.

Drugs

Naloxone HCl (generously provided by NIDA), U50,488H (Sigma, St. Louis, MO, USA), or naltrindole HCl (Sigma, St. Louis) were dissolved in 0.9% filtered (Sterile Acrodisc, 0.2 μm, Gelman Sciences) saline and injected in a volume of 10 ml/kg.

RESULTS

Experiment 1: Analysis of habituation data using a two-factor ANOVA (genotype×chamber) showed a slight preconditioning bias for the checkered conditioning chamber across all animals ($F_{1,104}=6.31$; $P<0.05$), but showed no interaction with genotype ($F_{3,102}=2.50$; $P>0.05$) demonstrating no significant difference in ini-

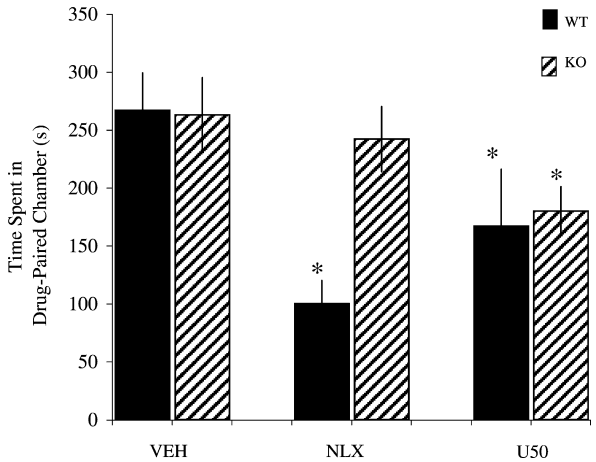

Fig. 1. NLX (10 mg/kg, s.c.) produced a significant CPA in WT mice, but had no effect in MOR−/− mice. U50,488H (2 mg/kg, s.c.) produced CPA in both WT and MOR−/− mice. Bars represent mean ± S.E.M. time spent in drug-paired chamber. *$P < 0.05$ compared to genotype-matched VEH group.

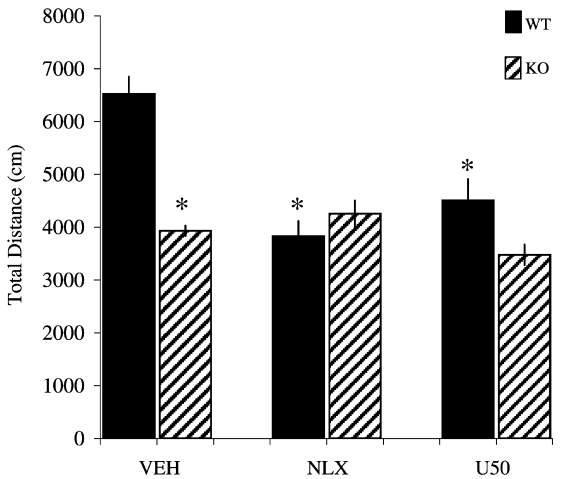

Fig. 2. NLX (10 mg/kg, s.c.) and U50,488H (2 mg/kg, s.c.) decreased horizontal locomotor activity in WT mice, but had no effect in MOR−/− mice. Locomotion was reduced in MOR−/− mice treated with VEH compared to their WT counterparts. Data are mean ± S.E.M. total distance traveled over the three drug conditioning sessions. *$P < 0.05$ compared to WT VEH control.

tial chamber preference between WT and MOR−/− mice (data not shown). Moreover, after random allocation to drug treatment groups, time spent in the future drug-paired chamber during the habituation period was balanced across the three treatment groups (VEH, NLX, U50,488H) ($F_{2,50} = 0.726$; $P > 0.05$) and there was no interaction with genotype ($F_{5,47} = 0.369$; $P > 0.05$).

The time spent in the drug-paired chamber on the test day was averaged across all subjects in each group (Fig. 1). Statistical analysis revealed a significant interaction between genetic background and drug treatment ($F_{5,47} = 4.18$; $P < 0.05$). NLX (10 mg/kg, s.c.) produced a significant place aversion in WT mice ($P < 0.05$), but failed to have any effect in MOR−/− mice. On the other hand, both MOR−/− and WT mice treated with U50,488H spent significantly less time in the drug-paired chamber ($P < 0.05$) compared to their respective VEH controls (Fig. 1).

Locomotor activity is presented as the total distance traveled during the three drug conditioning sessions (Fig. 2). There was a significant genotype vs. treatment interaction ($F_{5,46} = 3.005$, $P < 0.05$). Post-hoc analysis revealed that, as expected, NLX decreased locomotor activity in WT mice ($P < 0.05$). As previously reported (Matthes et al., 1996; Filliol et al., 2000), MOR−/− mice displayed reduced locomotor activity under basal (saline) conditions compared to WT mice ($P < 0.05$). In MOR−/− mice, NLX did not further decrease locomotor activity ($P > 0.05$). Furthermore, U50,488H significantly decreased locomotor activity in WT mice ($P < 0.05$), but, as with NLX, U50,488H had no effect on locomotor activity in MOR−/− mice.

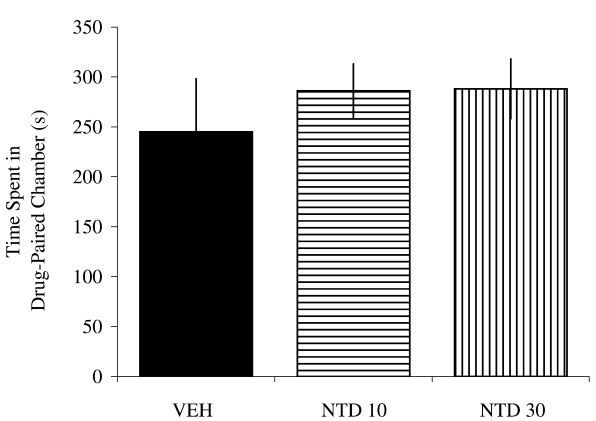

Fig. 3. NTD (10 or 30 mg/kg, s.c.) had no effect on place conditioning behavior in C57BL/6 mice. Bars represent mean ± S.E.M. time spent in drug-paired chamber.

Experiment 2: Analysis of habituation data using a one-factor ANOVA (chamber) showed no preconditioning bias for either conditioning chamber across all treatment groups ($F_{1,46} = 0.143$; $P > 0.05$) (data not shown). Moreover, after random allocation to drug treatment groups, time spent in the future drug-paired chamber during the habituation period was balanced across the three treatment groups (VEH, NTD 10, NTD 30) ($F_{2,21} = 0.293$; $P > 0.05$) (data not shown). The time spent in the drug-paired chamber on the test day was averaged across all subjects in each group (Fig. 3). Statistical analysis failed to reveal a significant main effect of drug treatment ($F_{2,21} = 1.009$; $P > 0.05$).

Locomotor activity is presented as the total distance traveled during the three drug conditioning sessions (Fig. 4). There was a significant main effect of drug treatment ($F_{2,20} = 2.947$, $P < 0.05$). Post-hoc analysis revealed that NTD decreased locomotor activity at the highest dose tested (30 mg/kg) ($P < 0.05$).

DISCUSSION

The main findings of this study are that: (1) the general opioid antagonist, NLX, failed to induce CPA in MOR−/− mice and (2) the predominantly DOR antagonist, NTD, failed to induce CPA in C57BL/6 mice. Considered together, these data suggest a role for the MOR, but not the DOR in homeostatic mechanisms

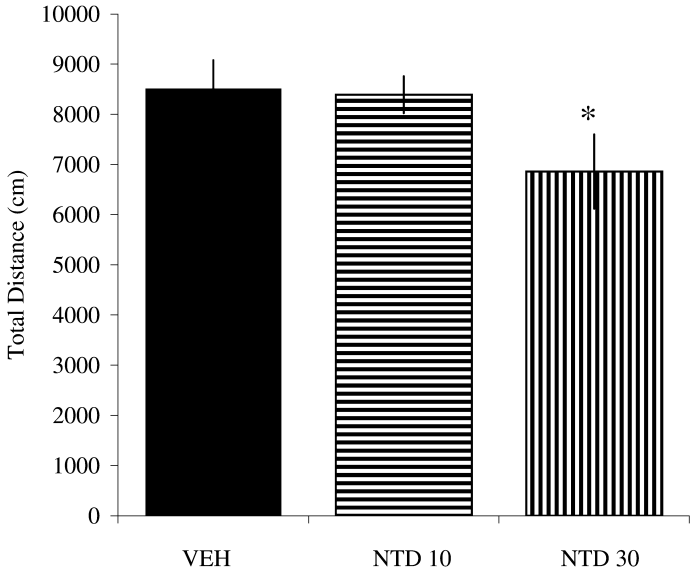

Fig. 4. NTD decreased horizontal locomotor activity in mice when administered at the higher dose (30 mg/kg, s.c.), but had no effect at the lower dose (10 mg/kg, s.c.). Data are mean ± S.E.M. total distance traveled over the three drug conditioning sessions. $*P < 0.05$ compared to VEH control.

regulating the 'hedonic status' of the organism. The failure of NLX to induce aversion in MOR−/− mice implies that NLX exerts its aversive effect by blocking tonic activity of endogenous opioid ligands acting solely at the MOR since the total number and distribution of the DOR and κ-opioid receptors are consistent across WT and MOR−/− mice (Matthes et al., 1996).

However, it is important to consider an alternative interpretation of this finding. That is, rather than lacking an aversion to NLX, MOR−/− mice are incapable of learning Pavlovian associations required to demonstrate CPA to any unconditioned stimulus. For instance, the phenotype of decreased spontaneous locomotor activity in MOR−/− mice could potentially interfere with exploration of the conditioning environments and exposure to conditioning cues. We minimized such potential effects by incorporating olfactory cues in the conditioning apparatus. Such cues permeate to reach subjects regardless of their activity levels. Furthermore, we conducted a positive control for associative learning using a paradigm identical to that of our main study. We demonstrated that although these MOR−/− mice did not learn a CPA to NLX, they were capable of learning a CPA to the κ agonist, U50,488H. Therefore, a general deficit in associative learning cannot be responsible for the lack of CPA in these mice. Moreover, U50,488H produced a CPA in MOR−/− mice at a dose that did not affect locomotor activity in these animals, supporting the idea that the CPA presented here is not simply a byproduct of distress created by a drug-induced decrease in locomotor activity.

Since only one, high, dose of NLX was used, we cannot rule out the possibility that the failure of NLX to produce CPA in MOR−/− mice may reflect a shift to the left of a bell-shaped dose–response curve. However, this is unlikely given that there are no data in the literature suggesting such a biphasic dose–response curve for NLX in aversion paradigms. Doses as high as 45 mg/kg i.v. have been shown to produce CPA (Mucha et al., 1982).

The high dose of NLX used is probably sufficient to act as an antagonist at the DOR as well as the MOR (Robson et al., 1983). The complete lack of aversion in MOR−/− mice treated with NLX suggests that the DOR is not involved in mediating NLX aversion. The likelihood of an involvement of the DOR in hedonic homeostasis is further reduced given the result of the second experiment in which a high dose of NTD failed to produce a CPA in C57BL/6 mice, a dose that was effective in reducing locomotor activity. This is consistent with previous data from rats in which the selective DOR antagonists, ICI 174,864 or NTD, did not condition significant place aversion in rats (Shippenberg et al., 1987; Bals-Kubik et al., 1989; Menkens et al., 1992; de Vries et al., 1995).

However, it should be noted that studies using the CTA paradigm show aversive effects of NTD (Froehlich et al., 1998; Hutchinson et al., 2000). One possible explanation for this discrepancy is the dose dependence of the findings. CTA developed to NTD at a dose of 18 mg/kg, but not at 10 mg/kg. It is possible, but unlikely, that our chosen dose, 30 mg/kg, may have exceeded the optimal dose window for CPA. A more likely explanation is that the two aversion paradigms rely on different sensory processing and are inherently different with respect to the parameters they reflect (Yamamoto et al., 1994; Hernadi et al., 1997; Sakai and Yamamoto, 1998, 1999).

It is also important to note that the DOR antagonists NTD and naltriben have been reported to produce CPA in morphine-dependent rats at doses as low as 0.1 mg/kg s.c., a dose that is unlikely to affect the MOR (Funada et al., 1996). It is therefore possible that both the MOR and the DOR mediate hedonic homeostasis in a positive

manner, but that the MOR effect predominates. Thus, under normal conditions, selective loss of DOR function can be compensated by increased MOR activity (explaining the ineffectiveness of NTD in our experiments), but the converse is not true (such that the general opioid antagonist, NLX, is ineffective in our experiments). Only when the system is chronically activated, as in the morphine-dependent state, is the role of the DOR revealed by NTD.

While our data show that the MOR is necessary for mediation of NLX aversion in opiate-naive animals, they do not prove that the MOR is sufficient. Even though apparent selective blockade of the MOR does produce CPA (Bals-Kubik et al., 1989; Shippenberg and Bals-Kubik, 1995), whereas selective DOR blockade does not (Menkens et al., 1992; de Vries et al., 1995), the presence of the DOR may nevertheless be necessary for NLX's effect via the MOR. Functional interactions between MOR and DOR have been implicated by pharmacological data (e.g., Traynor and Elliott, 1993) and further suggested by the observation that DOR-mediated analgesia may be modified in MOR−/− mice (Sora et al., 1997, 1999; Matthes et al., 1998; Qiu et al., 2000) (although see Loh et al., 1998). It is all the more necessary to consider the possibility of interactions between these two receptors in light of mounting biochemical, pharmacological, and functional evidence for MOR/DOR heterodimerization (Jordan and Devi, 1999; Allouche et al., 2000; Jordan et al., 2000; Gomes et al., 2000). Further CPA studies using DOR−/− mice are planned to address this question.

The parsimonious explanation for NLX's aversive action is that it is blocking the effect of a tonically released opioid peptide ligand involved in mediating reward. Just as these 'reward pathways' are proposed to be activated by exogenous drugs of abuse to induce euphoria, it is conceivable that blockade of basal opioid ligand activity within this circuitry would produce dysphoria or aversion. Perhaps the most likely candidate peptide, at this time, is β-endorphin, since lesions of the arcuate nucleus of the hypothalamus apparently prevent NLX-induced CPA (Shippenberg et al., 1988). However, the enkephalins and endomorphins (Zadina et al., 1997) are also possible mediators. It is also conceivable that NLX is effective in producing CPA by virtue of its ability to block constitutive activity of the MOR receptor (Cruz et al., 1996) in the absence of endogenous ligand (Wang et al., 1994; Sadee and Wang, 1995). Furthermore, the DOR has also been shown to exhibit constitutive activity (Neilan et al., 1999; Befort et al., 1999). Our failure to demonstrate a CPA with the neutral antagonist, NTD, in WT mice does not preclude the possibility that constitutive activity of the DOR is involved in maintaining hedonic status.

Our data do not speak to NLX's site of action in the brain for inducing CPA. However, on the basis of previous studies, it appears that NLX may act at multiple loci to exert this effect. Regions so far identified include the VTA, NAC (Shippenberg and Bals-Kubik, 1995), and ventral pallidum (unpublished data, in preparation) in drug-naive animals. Additionally, studies using methylnaloxonium have also implicated the medial dorsal thalamus, periaqueductal gray, and amygdala as sites mediating NLX-induced CPA in morphine-dependent rats (Stinus et al., 1990; Maldonado et al., 1992; Heinrichs et al., 1995).

CONCLUSION

In summary, these data demonstrate that the MOR is necessary for mediation of the aversive effect of NLX and support the hypothesis that tonic activity of endogenous peptides acting at the MOR is involved in the maintenance of hedonic homeostasis.

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