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ENDOGENOUS OPIOIDS MEDIATE BASAL HEDONIC TONE INDEPENDENT OF DOPAMINE D-1 OR D-2 RECEPTOR ACTIVATION

S. NARAYANAN,[a1] H. LAM,[a] L. CHRISTIAN,[a,b] M. S. LEVINE,[a,b] D. GRANDY,[c] M. RUBINSTEIN[d] AND N. T. MAIDMENT[a]*

[a]*Department of Psychiatry and Biobehavioral Sciences, University of California at Los Angeles Neuropsychiatric Institute, 760 Westwood Plaza, Los Angeles, CA 90024, USA*

[b]*Mental Retardation Research Center, University of California at Los Angeles Neuropsychiatric Institute, 760 Westwood Plaza, Los Angeles, CA 90024, USA*

[c]*Department of Physiology and Pharmacology, Oregon Health Sciences University, Portland, OR, USA*

[d]*Instituto de Investigaciones en Ingeneria Genetica y Biologia Molecular, CONICET, University of Buenos Aires, Buenos Aires, Argentina*

Abstract—Exogenously administered opiates are recognized as rewarding and the involvement of dopamine systems in mediating their apparent pleasurable effects is contentious. The aversive response to naloxone administration observed in animal studies suggests the presence of an endogenous opioid tone regulating hedonic state. We sought evidence for the requirement for dopamine systems in mediating this action of endogenous opioids by determining whether mice deficient in dopamine D-1 or D-2 receptors were able to display conditioned place aversion to naloxone. Mice received saline in the morning in one chamber and either saline or naloxone (10 mg/kg, s.c.) in the afternoon in another chamber, each day for 3 days. On the test day they were given free access to the testing chambers in the afternoon. Similar to their wild-type littermates, D-1 and D-2 receptor knockout mice receiving naloxone in the afternoon spent significantly less time on the test day in the compartment in which they previously received naloxone, compared with animals receiving saline in the afternoon. The persistence of naloxone-conditioned place aversion in D-1 and D-2 knockout mice suggests that endogenous opioid peptides maintain a basal level of positive affect that is not dependent on downstream activation of dopamine systems involving D-1 or D-2 receptors.

Key words: place conditioning, aversion, drug abuse, opioid, dopamine receptor, reward.

Accumulated evidence implicates the mesotelencephalic dopamine (DA) system as forming a common neurochemical/neuroanatomical substrate critical for the addictive potential of many different classes of abused drugs (for review see Koob 1992; Wise and Bozarth 1987). The concept of an endogenous reward circuitry in the brain mediating the pleasurable and motivating aspects of natural reinforcers that can be activated by drugs and thereby serve as a substrate for their abuse potential has proven an attractive basis for research in this area (see Kelley and Berridge, 2002). In further developments of this model, such circuitry is viewed as responsible for maintaining an "hedonic homeostasis" (used in the current context to describe what might be considered as a balanced affective, emotional and motivational state), and repeated activation of the system by exogenous drugs is proposed to alter its set point thereby producing hedonic homeostatic dysregulation or allostasis resulting in a drug-dependent state (Ahmed and Koob, 1998; Koob and Le Moal, 1997, 2001). The idea of the mesolimbic DA system within this circuitry as a mediator of the pleasurable/motivational effects of abused drugs has largely been supplanted by an involvement in associative learning and memory processes or in the attribution of incentive salience to conditions under which the rewarding effects of drugs are experienced (Di Chiara, 1998; Everitt et al., 2001; Robinson and Berridge 1993, 2000).

To the extent that another single neurotransmitter/neuromodulator system might be expected to fulfill the general role of "hedonic mediator," the endogenous opioids are obvious candidates (Koob and Le Moal, 1997). Not only are exogenously applied μ opioid receptor agonists rewarding (see Di Chiara and North, 1992; van Ree et al., 1999) but, conversely, administration of the general opioid antagonists naloxone and naltrexone is aversive in rodents (Bals-Kubik et al., 1989; Grevert and Goldstein, 1977a; Mucha et al., 1982, 1985; Mucha and Iversen, 1984; Mucha and Walker, 1987) and produces dysphoria in humans (Grevert and Goldstein, 1977b; Hollister et al., 1981) suggesting the presence of an endogenous opioid tone maintaining a basal hedonic state. There is a body of evidence, however, indicating that the rewarding or motivating effects of exogenously administered opioids are purely secondary to activation of the mesolimbic DA system (Bozarth and Wise, 1981, 1983; Shippenberg et al., 1993; Spyraki et al., 1983; Wise and Bozarth, 1982), although the involvement of D-1 versus D-2 receptors is contentious (Maldonado et al., 1997; Shippenberg et al., 1993). Nevertheless, the absolute requirement for an intact DA system has been challenged and DA-independent mechanisms of opiate reward proposed (Dworkin et al., 1988ab; Ettenberg, 1989; Hubner and Koob, 1990; Mackey and van der Kooy, 1985; Pettit et al., 1984; Zito et al., 1985).

[1] Present address: Glenmark Pharmaceuticals Ltd., Glenmark Research Centre, Plot No. A-607, T.T.C. Industrial Area, MIDC, Mahape, Navi Mumbai 400 709, India.

*Corresponding author. Tel: +1-310-206-7767; fax: +1-310-825-7067.
E-mail address: nmaidmen@ucla.edu (N. T. Maidment).

Abbreviations: DA, dopamine; VTA, ventral tegmental area.

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A more pertinent question for the establishment of a role for the *endogenous* opioid system in hedonic homeostasis independent of DA systems is whether opiate antagonists retain their aversive properties in the face of disruptions in DA transmission. Previous studies have addressed this question using 6-hydroxydopamine lesions and DA receptor antagonists and imply that whereas an intact mesolimbic DA system may not be essential for naloxone's aversive action (Shippenberg and Bals-Kubik, 1995), activation of D-1 receptors, presumably elsewhere in the brain, is required (Shippenberg and Herz, 1988). The current study sought to determine the absolute requirement for changes in D-1 and D-2 receptor activation in mediating the aversive effect of naloxone by using mice deficient in one or other of these two receptors in conjunction with the place conditioning paradigm.

EXPERIMENTAL PROCEDURES

Animals

Male (six) and female (four) DA D-1 receptor knockout mice (Drago et al., 1994) and their male (nine) and female (two) wild-type (C57BL/6×129Sv) littermates (12–15 months of age balanced across treatments) and male (eight) and female (18) DA D-2 receptor knockout mice (Kelly et al., 1997) and their male (11) and female (13) wild-type (C57BL/6) littermates (2.5–16 months of age balanced across treatments) were housed at 22±1 °C and provided with food and water *ad libitum*. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering.

Genotyping

Genotyping of mice was performed at 3 weeks of age and again at the completion of the study. Tail biopsies (5 mm) were placed in 200 μl of lysis buffer (100 mM NaCl, 10 mM Tris–HCl, 20 mM EDTA, 1% SDS, 20 mg/ml proteinase K) and incubated at 55 °C for 24 h. DNA was precipitated using 400 μl cold absolute methanol and re-suspended in 70% ethanol followed by precipitation and re-suspension in 100% ethanol. PCR was performed using Hostar Tag DNA polymerase and appropriate primer sequences and run on 1% agarose gel.

Place conditioning protocol

Details of the conditioning apparatus were described previously (Skoubis et al., 2001). Briefly, a square arena was divided into three chambers: a neutral start chamber (gray walls and floor), and two conditioning chambers (black and white checkers and black and white cow patterns) which were accessible via the neutral chamber through guillotine doors. The two conditioning chambers were also distinguishable on the basis of odor: almond scent for the checkered chamber and lemon scent for the cow chamber. The assignment of the "drug-conditioning" chamber was balanced across groups. An unbiased CPA protocol was used as follows:

Day 1: Habituation for 15 min with free access to entire place-conditioning apparatus.

Days 2–4: Conditioning with vehicle in the morning followed, 5 h later, by conditioning with vehicle or naloxone (10 mg/kg s.c) in the afternoon. Each session was 30 min in duration with the animals being confined to the "vehicle conditioning chamber" in the morning and the "drug conditioning chamber" in the afternoon.

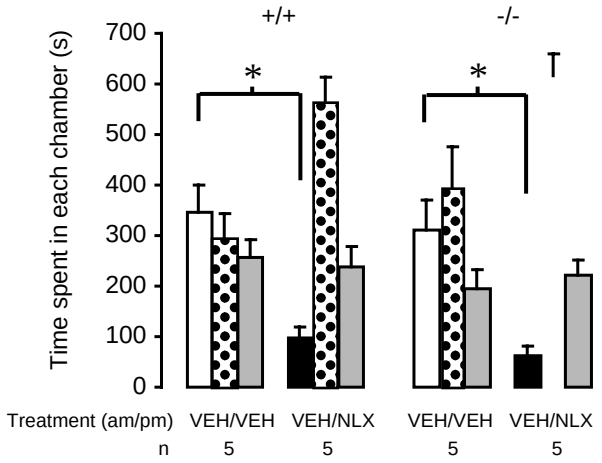

Fig. 1. Naloxone aversion in D-1 knockout mice. Time spent in each of the three chambers during the 15 min test session on the afternoon of day 5 for D-1 knockout mice and their wild-type counterparts. The "drug-conditioned" chamber is shown in black for naloxone-treated animals and white for vehicle-treated animals. Stippled bars represent the chamber in which all animals received vehicle in the morning of the conditioning sessions and gray bars represent the neutral chamber in each case. Animals treated with naloxone in the afternoon on days 2–4 spent significantly less time in the "drug-conditioned" chamber on the test day compared with animals that received vehicle in this chamber. This was the case for both wild-type and D-1 receptor knockout mice. * $P<0.05$.

Day 5: Test for 15 min with free access to the entire CPA apparatus after placement in the gray "neutral chamber."

The time spent in each chamber on the test day was recorded using a photobeam apparatus consisting of two intersecting arrays of photobeams directed across the floor of the arena (True Scan; Coulbourn Instruments, Allentown, PA, USA). (The base of the chamber walls were clear Plexiglas permitting passage of the photobeams.) The position of the animal at specific time intervals was revealed by the two-dimensional coordinates of the interrupted beams. The locomotor activity of the animals during the test session was also determined from this information in terms of distance traveled, measured in centimeters.

Drugs

Naloxone HCl was generously provided by National Institute on Drug Abuse and was dissolved in 0.9% saline immediately prior to s.c. injection in a volume of 0.1 ml/10 g body weight.

Statistical analysis

Place conditioning data are represented as mean±S.E.M. of time spent in each of the three chambers on the test day. The time spent in the drug-paired chamber on the test day was compared using a two-factor ANOVA (genotype versus drug treatment) followed by Bonferroni post hoc tests. Locomotor activity on the test day was similarly analyzed by two-factor ANOVA. Linear regression analysis was conducted to test for effects of gender and age on outcome measures. $P<0.05$ was considered significant.

RESULTS

Naloxone-induced aversion in D-1 knockout mice

Naloxone-treated animals spent significantly less time in the "drug-conditioning chamber" on day 5 compared with their vehicle-treated counterparts in both genotypes: wild-

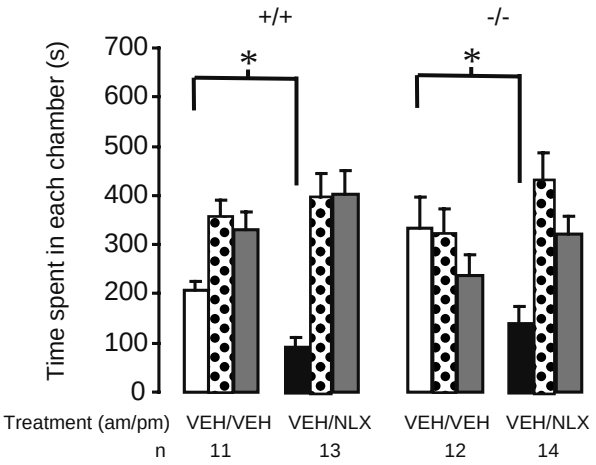

Fig. 2. Naloxone aversion in D-2 knockout mice. Time spent in each of the three chambers during the 15 min test session on the afternoon of day 5 for D-2 knockout mice and their wild-type counterparts. The chambers are depicted as in Fig. 1. Similar to the case for the D-1 receptor, naloxone-treated D-2 receptor knockout mice and their wild-type counterparts spent significantly less time in the "drug-conditioned" chamber compared with vehicle-treated controls.

types and knockouts (treatment effect $F_{1,16}=33.93$, $P<0.05$; Fig. 1). There was no significant genotype$\times$ treatment interaction ($F_{1,16}=0.0003$, $P>0.05$) indicating that there was no difference in the aversion produced by naloxone between the two genotypes. Post hoc analysis revealed significant differences in time spent in the "drug-conditioning" chamber for naloxone versus vehicle groups in both wild-type and knockout animals. The time "lost" to the naloxone-paired chamber was compensated by additional time in the vehicle-paired chamber in both wild-type and knockout mice.

Naloxone-induced aversion in D-2 knockout mice

Similarly, naloxone produced a significant aversion across both genotypes - wild-types and knockouts (treatment effect $F_{1,46}=16.49$, $P<0.05$; Fig. 2). There was no genotype$\times$treatment interaction ($F_{1,46}=1.12$, $P>0.05$), again indicating that there was no difference in the degree of aversion produced by naloxone between the genotypes. Post hoc analysis revealed significant differences in time spent in the "drug-conditioning" chamber for naloxone versus vehicle groups in both wild-type and knockout animals. In this case, the time "lost" to the naloxone-paired chamber was compensated by additional time in both the vehicle-paired chamber and the neutral chamber for the D-2 knockouts and their wild-type littermates, rather than vehicle-paired chamber alone. (There was a significant overall effect of genotype [$F_{1,46}=5.40$, $P<0.05$], arising from a tendency for vehicle-treated wild-type animals to spend less time in the "drug-conditioning" chamber on the test day. The reason for this is unclear. However, as noted above, this did not abrogate naloxone's further aversive effect in these animals).

Linear regression analysis showed neither age nor gender to be factors significantly influencing genotype or treatment effects on time spent in the drug-conditioned chamber for either D-1 or D-2 knockout experiments.

Locomotor activity in D-1 knockout mice

Total distance traveled during the test session was unaffected by prior drug treatment in both D-1 knockouts (vehicle 2,035$\pm$255 cm; naloxone 1,854$\pm$182 cm) and their wild-type counterparts (vehicle 1,867$\pm$164 cm; naloxone 2,355$\pm$262 cm). Statistical analysis showed no effect of treatment ($F_{1,16}=0.48$, $P>0.05$) or genotype ($F_{1,16}=0.57$, $P>0.05$) and no interaction between treatment and genotype ($F_{1,16}=2.31$, $P>0.05$).

Locomotor activity in D-2 knockout mice

Total distance traveled during the test session was unaffected by prior treatment ($F_{1,46}=0.03$, $P>0.05$) in both D-2 knockouts (vehicle 1,205$\pm$199 cm; naloxone 1,257$\pm$176 cm) and their wild-type counterparts (vehicle 2,878$\pm$193 cm; naloxone 2,751$\pm$265 cm).

D-2 knockout mice showed significantly less locomotor activity compared with wild type mice littermates ($F_{1,46}=56.52$, $P<0.05$) as previously described (Kelly et al., 1998). There was no interaction between treatment and genotype ($F_{1,46}=0.18$, $P>0.05$).

Linear regression analysis showed neither age nor gender to be factors significantly influencing genotype or treatment effects on distance traveled for either D-1 or D-2 knockout experiments. The apparent lower activity of the D-1 wild-type versus D-2 wild-type mice is most likely a reflection of the their different genetic backgrounds, the 129Sv component of the D-1 wild-type background contributing to reduced activity in comparison to the C57BL6 background of the D-2 wild-types (Murphy et al., 2001).

DISCUSSION

The rewarding effects of exogenous opiates and endogenous opioid peptides acting at μ opioid receptors is well established (see Di Chiara and North, 1992; van Ree et al., 1999). The additional observation that administration of the general opioid antagonist, naloxone, produces aversion in rodents (Bals-Kubik et al., 1989; Grevert and Goldstein, 1977a; Mucha et al., 1982, 1985; Mucha and Iversen, 1984; Mucha and Walker, 1987) and dysphoria in humans (Grevert and Goldstein, 1977a; Hollister et al., 1981) has led to the concept of an endogenous opioid tone maintaining hedonic homeostasis (Koob and Le Moal, 1997). There is considerable evidence in favor of a role for the mesolimbic DA system as a channel through which increases in opioid transmission are translated into reward and reinforcement of drug-seeking behavior. For example, local injection of opiates into the ventral tegmental area (VTA), the site of mesolimbic DA neuron cell bodies, is able to sustain self-administration (Bozarth and Wise 1981; Phillips and LePiane 1980, 1982; Philips et al., 1993) and supports a conditioned place preference (Bozarth, 1987). Moreover, μ opioid receptor agonists enhance dopaminergic neuronal activity in the VTA (Johnson and North, 1992) and evidence for the DA elevating effects of μ agonists has

been provided by several microdialysis studies (Di Chiara and Imperato, 1988; Leone et al., 1991; Spanagel et al., 1992). Furthermore, DA antagonists are reported to attenuate i.v. opiate self-administration and place preference (Bozarth and Wise, 1981, 1983; Schwartz and Marchok 1974; Shippenberg et al., 1993; Shippenberg and Herz, 1988; Spyraki et al., 1983; Wise and Bozarth, 1982). Results of the use of subtype selective DA receptor antagonists favor the involvement of D-1 rather than D-2 receptors in this regard (Acquas et al., 1989; Acquas and Di Chiara, 1994; Bals-Kubik et al., 1993; Leone and Di Chiara, 1987; Shippenberg and Herz, 1988; Shippenberg et al., 1993). However, a crucial role for the D-2 receptor was indicated by a report of the loss of morphine conditioned place preference in mice deficient in D-2 receptors (Maldonado et al., 1997) although this was subsequently challenged by Dockstader et al. (2001) (see below). More recently, such mice were reported not to self-administer morphine (Elmer et al., 2002).

The present experiments were designed to address the question of the role of D-1 and D-2 receptors in *endogenous* opiate-mediated hedonic homeostasis by determining if the conditioned aversion produced by blockade of endogenous opioid tone with naloxone requires the presence of these receptors. The controversial nature of the D-1 versus D-2 dependence of opiate reward notwithstanding, we argued that if either of these receptors were important mediators of endogenous opioid effects, the aversive effect of opiate receptor blockade should be compromised in their absence. The dose of naloxone used in this study was previously determined to be a maximally effective dose for the development of conditioned place aversion in mice and to be dependent on an action at μ opioid receptors, since the effect is absent in μ receptor knockout mice (Skoubis and Maidment, 2001). Our data show that neither the D-1 nor the D-2 receptor is essential for acquisition and expression of naloxone conditioned place aversion.

The parsimonious explanation of these data is that the action of endogenous opioids in maintaining positive hedonic state is not mediated through an increase in DA activity at D-1 or D-2 receptors. The absence of an effect of D-1 receptor ablation is at odds with previous pharmacological data showing blockade of naloxone-conditioned place aversion by simultaneous treatment with selective D-1 receptor antagonists (Acquas et al., 1989; Acquas and Di Chiara, 1994; Shippenberg and Herz, 1988). There are several possible explanations for this apparent discrepancy. The selectivity of such drugs is not absolute and the possibility that their effect results from actions at other receptors cannot be ruled out. For example, SCH23390 is reported to be an agonist at certain serotonin receptors (see Millan et al., 2001). This drug also has aversive properties of its own (Shippenberg and Herz, 1988) which has the potential to confound the behavioral measure, although chronic infusions have been used in an attempt to circumvent this issue (Shippenberg and Herz, 1988). The possibility of a species difference must also be considered since previous studies were carried out in rats. Finally, as with all unconditional knockout models, the possibility that compensatory mechanisms during development replace a normal function of D-1 receptors in mediating naloxone's effect cannot currently be ruled out.

The lack of effect of D-2 receptor ablation on naloxone aversion is particularly interesting in view of previous reports that morphine place preference and morphine-self administration are both absent in D-2 receptor knockout mice (Elmer et al., 2002; Maldonado et al., 1997). Such a differential dependence upon D-2 receptors of opiate reward versus naloxone aversion runs counter to the simple idea that these two opioid effects represent points at opposite ends of a spectrum of hedonic states mediated by a single circuit ultimately acting through the DA system. However, it should be noted that Dockstader et al. (2001) reported that morphine conditioned place preference remained intact in D-2 knockout mice that were backcrossed to a C57BL/6 background (the same line of mice used in the present study) in contrast to the data of Maldonado et al. (1997). Only when the animals were conditioned in a morphine-dependent and withdrawn state was the development of morphine conditioned place preference absent in these D-2 knockout mice (Dockstader et al., 2001). Under these conditions the aversive effect of naloxone was also D-2 receptor-dependent (Dockstader et al., 2001). Thus, in the opiate dependent and withdrawn state, DA D-2 systems do appear to be important in mediating the change in hedonic state brought about by both increases and decreases in opioid receptor activation.

Our data do not, of course, rule out a role for DA systems per se in mediating endogenous opioid-based hedonic tone in the opiate-free state since other DA receptors may be involved. Indeed, further studies of mice deficient in other DA receptor subtypes are planned. It is also possible that knockout of multiple DA receptors will be required to reveal DA's role in this behavior. For instance, it is possible that D-2 receptors substitute for D-1 receptors in D-1 receptor knockout mice and vice versa. That endogenous opioid peptide systems may indeed regulate hedonic state independent of DA transmission is not without pretext, however. Countering arguments for a critical role of mesolimbic DA in opiate reinforcement is the persistence of morphine and heroin self-administration following 6-hydroxydopamine lesions of the nucleus accumbens (Dworkin et al. 1988a; Pettit et al. 1984). Moreover, such lesions also failed to block naloxone-induced conditioned place aversion (Shippenberg and Bals-Kubik, 1995; but see Spyraki et al., 1983). A major output of the nucleus accumbens, that projecting to the ventral pallidum, is rich in enkephalin (Zahm et al., 1985) and recent studies in our laboratory have shown the ventral pallidum to be a sensitive site for mediating naloxone conditioned place aversion (Skoubis and Maidment, 2003). Naloxone may therefore be acting at a point downstream from DA in hedonic circuitry. Indeed, earlier studies have pointed to the importance of the ventral pallidum as a possible site of convergence mediating the reinforcing effects of opiates and psychostimulants (Dworkin et al., 1988b; Hubner and Koob, 1990; Koob, 1992; Zito et al., 1985).

In conclusion, our data show that naloxone retains its aversive qualities in mice deficient in either D-1 or D-2 receptors and therefore suggest that endogenous opioid peptide release maintaining a basal positive hedonic state does so through mechanisms that do not depend entirely on D-1 or D-2 receptor activation.

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