Absence of Δ-9-Tetrahydrocannabinol Dysphoric Effects in Dynorphin-Deficient Mice

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The involvement of dynorphin on Δ-9-tetrahydrocannabinol (THC) and morphine responses has been investigated by using mice with a targeted inactivation of the prodynorphin (Pdyn) gene. Dynorphin-deficient mice show specific changes in the behavioral effects of THC, including a reduction of spinal THC analgesia and the absence of THC-induced conditioned place aversion. In contrast, acute and chronic opioid effects were normal. The lack of negative motivational effects of THC in the absence of dynorphin demonstrates that this endogenous opioid peptide mediates the dysphoric effects of marijuana.

Key words: cannabinoid; opioid; mice; mutation; withdrawal; addiction; place aversion

Pharmacological and genetic evidence suggest important functional interactions between the endogenous brain cannabinoid and opioid systems (Hine et al., 1975; Vela et al., 1995; Pugh et al., 1997; Tanda et al., 1997; Ledent et al., 1999; Manzanares et al., 1999; Valverde et al., 2000, 2001). Most of the behavioral effects of endogenous cannabinoids (arachidonyltyetanolamide, 2-arachidonoylglycerol, and 2-arachidonoylglycerol) and Δ-9-tetrahydrocannabinol (THC) are mediated by cannabinoid CB1 receptors (Ledent et al., 1999; Zimmer et al., 1999), whereas opioids (endorphin, enkephalins, and dynorphins) act on μ, δ, and κ receptors (Kieffer, 1999). Cannabinoids and opioids produce similar behavioral and physiological effects, such as antinociception, hypothermia, and reduced locomotor activity (Manzanares et al., 1999). Both systems seem to be functionally coupled in drug reward and addiction, because opioid withdrawal symptoms were alleviated by cannabinoids (Hine et al., 1975; Lichtman et al., 2001; Yamaguchi et al., 2001) and they were significantly reduced in CB1 receptor knockout mice (Ledent et al., 1999; Lichtman et al., 2001). CB1 receptor antagonists blocked heroin self-administration and morphine-induced place preference (Mas-Nieto et al., 2001; Navarro et al., 2001). Morphine-induced place preference was also abolished in CB1 receptor knockout mice (Martin et al., 2000). Conversely, THC withdrawal symptoms were significantly reduced in enkephalin-deficient animals (Valverde et al., 2000). The expression of opioid and cannabinoid receptors overlaps in many brain areas, including structures of the reward circuitry (Herkenham, 1992; Matsuda et al., 1993; Delfs et al., 1994; Mansour et al., 1995a,b). Although colocalization studies with cellular resolution have not been performed yet, it has been suggested that both receptor types may interact at the level of signal transduction (Manzanares et al., 1999).

Of particular interest is the potential role of cannabis as a gateway drug, which has been investigated primarily with epidemiological methods (Watson et al., 2000). Although most recreational users of cannabis experience a state of euphoria (high), some people also report dysphoria and anxiety after cannabis use (Gregg et al., 1976; Thomas, 1993; Grinspoon and Bakalar, 1997; Williamson and Evans, 2000). Both aspects can be revealed in rodents in which conditioned place aversion or place preference can be induced through different experimental protocols (Sanudo-Pena et al., 1997; Cheer et al., 2000; Valjent and Maldonado, 2000). Compounds that activate the μ- or δ-opioid receptors generally have positive motivational effects, whereas κ-opioid receptor agonists induce aversive effects (Shippenberg et al., 1987; Bals-Kubik et al., 1993). These opposing pharmacological responses may reflect an opposite modulation of the reward circuits by the different opioid receptors and could suggest that activation of κ receptors would counteract opioid rewarding effects. Opioids and cannabinoids both produce their motivational effects through the stimulation of the mesolimbic dopaminergic system (Koob, 1992; Manzanares et al., 1999). The neuronal mechanisms for the aversive effects of THC have not been ascertained yet. The elucidation of these mechanisms is crucial for a better understanding of the processes involved in the initiation of cannabinoid abuse. THC, but not the endogenous cannabinoid anandamide, stimulates the release of the endogenous κ agonist dynorphin, which may contribute to the antinociceptive effects of THC (Welch and Eads, 1999; Houser et al., 2000). To determine whether dynorphin is also involved in the regulation of motivational effects of THC and morphine, we investigated whether the genetic deletion of dynorphin influences several acute and chronic responses of these two drugs.

MATERIALS AND METHODS

Mice. A gene targeting vector was constructed in a pPNT-M1-lox vector using a 7 kb Smal-XbaI fragment from a prodynorphin genomic BAC clone (Research Genetics) containing exons 1 and 2 and a PCR-amplified 1.2 kb fragment from a genomic 129/SV lambda GEM 11 clone contain-
ing the 3’ end of exon 4. This targeting construct was electroporated into MPIII embryonic stem (ES) cells (a gift from A. Voss and P. Gruss, Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany). Five ES cell clones with the expected homologous recombination event were isolated and injected into C57BL/6J mouse blastocysts. The resulting chimeras were bred to C57BL/6J mice. Heterozygous offspring were intercrossed to obtain homozygous mutants. Mice used in behavioral studies were between 10 and 16 weeks old. Animals were housed in groups of four to five animals per cage. The light cycle was 8:00 A.M. lights on and 8:00 P.M. lights off. Food and water was provided ad libitum. Both sexes were equally represented. All animal procedures met the guidelines of the National Institutes of Health detailed in the Guide for the Care and Use of Laboratory Animals and the European Communities directive 86/609/EEC regulating animal research and were approved by the Local Ethical Committees.

**Ratelin**. Mice brains (five for each genotype) were homogenized in 3 ml of 2N acetic acid at 4°C and clarified at 15,000 × g for 30 min at 4°C. Duplicate aliquots of 10, 50, and 100 μl were dried under vacuum in polystyrene tubes (Falcon 35 2052), resuspended in 100 μl of RIA (Peninsula Laboratories, San Carlos, CA) buffer, and assayed following the protocol for the RIK 8730 Dynorphin A (Porcine) Radioimmunoassay kit from Peninsula Laboratories. The range for the standard curve was between 10–400 pg/ml.

**Drugs.** Morphine-HCl, naloxone-HCl, U50,488H, nor-binaltorphimine (NBI), and THC were provided by Sigma (St. Louis, MO). THC was diluted to a working solution in 10% ethanol (NBI), and THC was evaluated during 30 min as reported previously (Maldonado et al., 1997). The apparatus consisted of two main squared conditioning chambers (15 × 15 × 15 cm) separated by a triangular central division. The light intensity within the conditioning chambers was 20 ± 5 lux. The movement and location of the mice were monitored by computerized monitoring software (Videotrack: View Point, Lyon, France) with images relayed from a camera placed above the apparatus. During the preconditioning phase, drug naive mice were placed in the middle of the central division and had free access to both compartments of the conditioning apparatus for 20 min, with the time spent in each compartment being recorded. For morphine experiments, the condition- ing procedure consisted of two main squares (15 × 15 cm) and three pairings with saline for a 30 min conditioning time (Maldonado et al., 1997). For THC experiments, the conditioning phase consisted of five pairings with THC (5 mg/kg, i.p.) and five pairings with vehicle for a 45 min period (Valjent and Maldonado, 2000). Mice were injected with vehicle or drug and then immediately confined to the conditioning compartment. The drug-assigned compartment could be either the most or the least preferred. Treatments were counterbalanced as closely as possible between compartments. Control animals received vehicle every day. The test phase was conducted exactly as the preconditioning phase, i.e., free access to each compartment for 20 min. The time in the central area was proportionally shared and added to the time value of each conditioned compartment, as described previously (Maldonado et al., 1997). A place conditioning score was calculated for each animal as the difference between time spent in the drug-paired compartment during the test and preconditioning phases. Experiments with NBI (Sigma) were performed in CD1, mice (22–24 gm). Ninety minutes after pretreatment with NBI (0, 5, and 10 mg/kg, s.c.), mice were injected intraperitoneally with vehicle or THC (5 mg/kg, i.p.) and placed in the conditioning compartment. Six groups of mice were formed: saline–vehicle, 5 mg/kg NBI-vehicle, 10 mg/kg NB1-vehicle, saline–THC, 5 mg/kg NBI–THC, 10 mg/kg NBI–THC.

**Statistical analysis.** At least 10 animals were used for each behavioral test. Acute effects and global withdrawal scores were compared by using two-way ANOVA (genotype and treatment) between subjects, followed by one-way ANOVA for individual differences. Values of tolerance...
studies were compared by using three-way ANOVA (genotype and treatment as between-group factors and day as within-group factor), followed by corresponding two-way and one-way ANOVA and post hoc comparisons when required. For place conditioning studies, score values were compared by using two-way ANOVA between subjects (genotype and treatment) followed by one-way ANOVA to ensure use of an unbiased procedure. Individual comparisons of time spent in the drug-paired compartment during pre-conditioning and test phases were made with paired two-tailed Student’s t test.

RESULTS

We inactivated the prodynorphin gene (Pdyn) by deleting exon 3 and part of exon 4 (Fig. 1A,B). This genetic mutation deletes the translation initiation codon of the dynorphin gene to generate a null allele. Analysis of whole brain extracts by radioimmunoassays showed that, indeed, mice homozygous for the Pdyntm1zim mutation (henceforth referred to as Pdyn−/−) did not produce any dynorphin peptides (Fig. 1C). Pdyn−/− mice were obtained with the expected Mendelian frequency. They did not show any apparent developmental defects, were fertile, and raised their offspring.

Locomotor activity of dynorphin-deficient mice was evaluated in an open-field system. Horizontal and vertical movements were similar in Pdyn+/+ and Pdyn−/− mice, indicating that dynorphin is not essential for normal motor functions. As has been reported previously (Wang et al., 2001), we also did not find any differences between wild-type and dynorphin knock-out mice in tests for thermal pain sensitivity.

First, we evaluated the responses of dynorphin-deficient mice to opioids. Injection of the k agonist U50,488H (7.5 mg/kg, i.p.) reduced locomotor activity in both genotypes (Fig. 2A), whereas morphine (5 mg/kg, s.c.) induced a similar increase in horizontal locomotion in Pdyn+/+ and Pdyn−/− mice (Fig. 2B) compared with saline injection. Thus, locomotor effects of opioids also appeared to be unchanged in Pdyn−/− mice. Injection of U50,488H (7.5 mg/kg, i.p.) also produced similar analgesia in Pdyn+/+ and Pdyn−/− mice at intraperitoneal doses of 2 and 5 mg/kg. We also found no differences in morphine-induced hyperactivity or in the conditioned place preference test. Morphine withdrawal symptoms, elicited in chronically treated mice through naloxone administration, were also unchanged by the lack of dynorphin. All values are expressed as means ± SEM.

Figure 1. Generation of Pdyn−/− mice. A, Map of the wild-type Pdyn locus, the targeting construct, and the Pdyn−− locus. The 3’ external probe used for Southern blot analysis and the expected restriction fragments are indicated. K, KpnI; X, XbaI. B, Genotyping of offspring from Pdyn−−×Pdyn−− crosses by Southern blot analysis of mouse tail DNA. C, Analysis of dynorphin peptide levels from whole brain extracts of Pdyn+/+ and Pdyn−/− mice. Shown are the average values (means ± SEM) from five animals per genotype.

Figure 2. Effects of U50,488H and morphine. A, Acute antinociceptive effects of the k-selective ligand U50,488H and vehicle were determined on the tail-immersion test. Maximum percentage of analgesia was calculated as [(postinjection latency − baseline latency)/(cutoff time − baseline latency)] × 100. Locomotor effects were evaluated in activity boxes, by measuring horizontal and vertical beam breaks (rears). No significant differences were observed in these tests between Pdyn+/+ and Pdyn−/− mice. B, Morphine antinociception, also evaluated by determining tail-immersion analgesia, was not significantly different in Pdyn+/+ and Pdyn−/− mice at intraperitoneal doses of 2 and 5 mg/kg. We also found no differences in morphine-induced hyperactivity or in the conditioned place preference test. Morphine withdrawal symptoms, elicited in chronically treated mice through naloxone administration, were also unchanged by the lack of dynorphin. All values are expressed as means ± SEM.
**Pdyn** 

Pdyn 

Pdyn mice also showed no significant differences in morphine abstention symptoms (Fig. 2B), suggesting that dynorphin does not participate in the development and expression of morphine dependence.

Next, we evaluated the effects of the dynorphin deletion on acute responses induced by THC (20 mg/kg, i.p.). THC analgesia was similar in both genotypes in the hot-plate test, but the tail-immersion test revealed a significantly reduced analgesia in Pdyn 

In contrast to THC analgesia, THC-induced hypoactivity and reduction in body temperature were not affected by the dynorphin mutation (Fig. 3A).

We next explored the motivational effects of THC in Pdyn mice in a place conditioning paradigm. THC has dysphoric properties in mice under most experimental conditions and produces a conditioned place aversion (Manzanares et al., 1999).

DISCUSSION

In this study, we generated mice with a targeted deletion of the prodynorphin gene and analyzed the effects of this mutation on opioid and cannabinoid drug responses. The antinociceptive effects of the k-selective ligand US5,488H were unaltered. In addition, morphine antinociception, reward, and naloxone-precipitated morphine withdrawal were also unaffected by the dynorphin mutation. These findings were unexpected because the recent analysis of k receptor knock-outs (Siminon et al., 1998) suggested an involvement of the k-opioid system in the modulation of morphine depen-
Most strikingly, dynorphin-deficient mice do not show any manifestation of THC-conditioned place avoidance. Furthermore, pretreatment of mice with NBI also completely abolished the development of THC place aversion in wild-type animals. Together, these results strongly indicate that the dynorphin–κ-opioid receptor system is crucial for the manifestation of cannabinoid aversive effects. A mechanism for the interaction between opioid and cannabinoid systems in motivational drug effects could involve the increased synthesis and release of opioid peptides in brain regions that regulate drug reward after THC treatment. This hypothesis is supported by studies demonstrating the release of dynorphin after THC administration at the level of the spinal cord (Mason et al., 1999; Houser et al., 2000) and the induction of opioid peptide gene expression in the spinal cord (Corchero et al., 1996, 1997). CB1 receptors are implicated in THC and stress responses (Weidenfeld et al., 1994; Rodriguez de Fonseca et al., 1996, 1997). CB1 receptors are involved in THC and stress responses (Weidenfeld et al., 1994; Rodriguez de Fonseca et al., 1996, 1997). CB1 receptors are involved in THC and stress responses (Weidenfeld et al., 1994; Rodriguez de Fonseca et al., 1996, 1997). However, recent studies have shown that the aversive effects of the κ agonist U50,488H are also blunted in cannabinoid CB1 receptor knock-out mice (Ledent et al., 1999), which indicates that dynorphin is not a simple downstream regulator of the aversive effects of THC. It rather appears that the cannabinoid–dynorphin–κ-opioid systems act in concert in the modulation of negative motivational drug effects.

Our findings do not exclude the possibility that other systems, such as the corticotropin releasing factor (CRF), are involved in the modulation of THC dysphoric effects as well. CRF has been implicated in THC and stress responses (Weidenfeld et al., 1994; Rodriguez de Fonseca et al., 1996, 1997). CB1 receptors are expressed in CRF-containing neurons in the hypothalamus and limbic structures, including the medial prefrontal cortex, hippocampus, and amygdala (Herkenham et al., 1991). Long-term cannabinoid administration alters CRF function in the limbic system (Rodriguez de Fonseca et al., 1997).

The selectivity of the observed THC-motivational effects cannot be defined in the present study. Indeed, stimulation of κ-opioid receptors through endogenous dynorphins in response to other dysphoric–aversive stimuli has been reported (Staley et al., 1997a, 1999), and periaqueductal gray (Manzanares et al., 1998). This hypothesis is supported by studies demonstrating the release of dynorphin after THC administration at the level of the spinal cord (Mason et al., 1999; Houser et al., 2000) and the induction of opioid peptide gene expression in the spinal cord (Corchero et al., 1996, 1997). CB1 receptors are implicated in THC and stress responses (Weidenfeld et al., 1994; Rodriguez de Fonseca et al., 1996, 1997). CB1 receptors are involved in THC and stress responses (Weidenfeld et al., 1994; Rodriguez de Fonseca et al., 1996, 1997). CB1 receptors are involved in THC and stress responses (Weidenfeld et al., 1994; Rodriguez de Fonseca et al., 1996, 1997). However, recent studies have shown that the aversive effects of the κ agonist U50,488H are also blunted in cannabinoid CB1 receptor knock-out mice (Ledent et al., 1999), which indicates that dynorphin is not a simple downstream regulator of the aversive effects of THC. It rather appears that the cannabinoid–dynorphin–κ-opioid systems act in concert in the modulation of negative motivational drug effects.

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In summary, our results demonstrate for the first time that the endogenous opioid peptides generated from the dynorphin gene are crucial for the negative motivational effects of THC and participate in the spinal analgesia induced by this compound. This is in agreement with the idea that the activation of cannabionid receptors stimulates the production and release of endogenous opioid peptides. The disappearance of the dysphoric effects of THC after repeated exposure to the drug (Valjent and Malondo, 2000) could be essential for the initiation of cannabionid abuse. The present results clearly indicate that endogenous dynorphin controls this important cannabionid motivational response.

REFERENCES