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Enhanced δ-opioid receptor-mediated antinociception in μ-opioid receptor-deficient mice

Chunyuan Qiu [a,b], Ichiro Sora [c], Ke Ren [a], George Uhl [c], Ronald Dubner [a,*]

[a] *Department of Oral and Craniofacial Biological Sciences, University of Maryland Dental School, Room 5E-08, 666 West Baltimore Street, Baltimore, MD 21201, USA*

[b] *Department of Anesthesiology, School of Medicine, University of Maryland, Baltimore, MD 21201, USA*

[c] *NIDA-IRP and Department of Neurology and Neuroscience, Johns Hopkins University School Medicine, Baltimore, MD 21224, USA*

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Abstract

Inflammatory hyperalgesia was induced in wild-type, heterozygous and μ-opioid receptor knockout mice after an intraplantar injection of complete Freund's adjuvant. μ-Opioid receptor knockout mice exhibited faster recovery from hyperalgesia as compared to heterozygous ($P < 0.05$) and wild-type ($P < 0.01$) mice. Naloxone restored hyperalgesia in all genotypes. Naltrindole (δ-opioid receptor-selective antagonist) partially restored the hyperalgesia only in μ-opioid receptor knockout mice ($P < 0.001$). Nor-binaltorphimine (κ-opioid receptor-selective antagonist) had no effect. The μ-opioid receptor-selective agonist, [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAMGO), reduced the hyperalgesia in heterozygous and wild-type but not in μ-opioid receptor knockout mice while U69,593 {(+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide, κ-opioid receptor-selective} produced similar effects in all mice. The δ-opioid receptor-selective agonists, [D-Pen^2, D-Pen^5]enkephalin (DPDPE) and deltorphin ([D-Ala^2]deltrophin-II), produced significantly greater antihyperalgesia in knockout mice ($P < 0.05$). The findings suggest that μ-opioid receptors may be involved in the persistence of inflammatory hyperalgesia and that a δ-opioid receptor-mediated compensatory mechanism in the absence of the μ-opioid receptor is activated by persistent hyperalgesia.

Keywords: Inflammation; μ-Opioid receptor; Knockout mice

1. Introduction

Endogenous opioid peptides and their receptors are integral components of neural circuits by which cognitive, emotional and attentional aspects of the pain experience modulate pain transmission at spinal levels (Fields and Basbaum, 1994). Opioids act through three classes of receptors referred to as the μ-opioid, δ-opioid and κ-opioid receptor types. The genes encoding these receptors have been cloned and all three receptors can mediate opioid-induced analgesia (Kieffer, 1999). Recently, mice lacking μ-opioid receptors have been generated by homologous recombination in several laboratories by disrupting exon 1 (Schuller et al., 1997; Sora et al., 1997b; Tian et al., 1997), exon 2 (Matthes et al., 1996) or exons 2 and 3 (Loh et al., 1998). In each of these knockout strains, selective μ-opioid receptor ligands were ineffective in modulating pain in tail flick and hot plate models of acute or transient pain. Most strains of μ-opioid receptor-deleted or knockout mice did not differ from their wild-type or heterozygous littermates in size, development, apparent fertility and locomotion in novel environments. No compensatory changes were seen in either δ- or κ-opioid receptor binding or distribution, G-protein activation or peptide message levels (Matthes et al., 1996, 1998; Sora et al., 1997b; Narita et al., 1999).

We have taken advantage of the known plasticity of the spinal cord in models of persistent inflammation and pain to study changes in opioid receptor function in mice with a μ-opioid receptor deletion. The injection of inflammatory agents such as complete Freund's adjuvant into the hindpaw of the rat produces an intense inflammation and hyperalgesia that is limited to the injected paw (Iadarola et al., 1988; Hylden et al., 1989). We successfully adapted this model to transgenic mice in order to study endogenous opioid mechanisms after inflammation and to examine the effects of selective opioid receptor ligands on opioid func-

* Corresponding author. Tel.: +1-410-706-0860; fax: +1-410-706-0865.

E-mail address: rnd001@dental.umaryland.edu (R. Dubner)

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tion. In contrast to previous studies in transient pain models, we demonstrated that μ-opioid receptors may be involved in mechanisms important for the persistence of inflammatory hyperalgesia and that compensatory changes in endogenous and exogenous antinociceptive mechanisms are present in μ-opioid receptor knockout mice as compared to their wild-type and heterozygous littermates. Some of these findings have been previously published in abstract form (Qiu et al., 1998).

2. Materials and methods

2.1. Animal preparation

Animals were bred under standard conditions in a 12 h dark–light cycle. The genetic background of μ-opioid receptor knockout animals was 1:1 hybrids from 129/SvEv and C57BL/6J mouse strains (Sora et al., 1997b) and F2 or F3 knockout, heterozygous and wild-type offspring of heterozygous intercrosses were used for behavioral and pharmacological testing. The animals were 12–16 weeks of age and equal numbers of males and females were used. All mice were genotyped by southern blot analysis (Sora et al., 1997b). In all the experiments described below, the genotypes were blinded to the experimenter until data analysis.

2.2. Induction of inflammation

Complete Freund's adjuvant suspended in an oil/saline (1:1) emulsion was used as the inflammatory agent. Mice received a subcutaneous injection of 0.005–0.01 ml (2.5–5 μg *Mycobacterium*) complete Freund's adjuvant. The same volume of 0.9% saline was injected in control animals. The animals were housed in cages in which the floor was covered with soft beddings to minimize the possibility of painful contact with a hard surface. The injection produces an intense tissue inflammation of the hindpaw characterized by erythema, edema and hyperalgesia. The inflammation was confined to the injected hindpaw. Animals with adjuvant-induced inflammation showed normal grooming behavior and normal levels of activity, but tended to limp and guard the inflamed paw. This mouse animal model has been approved by the University of Maryland Dental School Institutional Animal Care and Use Committee. The International Association for the Study of Pain ethical guidelines for the treatment of animals were adhered to in these experiments (Zimmerman, 1983).

2.3. Behavioral testing

Mice were tested for thermal hyperalgesia by a modified method described by Hargreaves et al. (1988). The mice were placed under a clear plastic chamber on an elevated glass surface and allowed to acclimate to their environment for 60 min. A heat stimulus was applied from underneath the glass floor with a high intensity projector lamp bulb (Osram 58-8007, 8 v, 50 W). The heat stimulus was directed on to the plantar surface of each hindpaw and the paw withdrawal latency to the nearest 0.1 s was determined using an electronic clock circuit and a microcomputer. If animals failed to withdraw their paws within 20 s, the stimulus was discontinued to avoid tissue damage. When normal paws are stimulated, the mice withdraw the paw at about 9 s when the temperature reaches approximately 45°C, the approximate pain threshold in humans. At peak inflammation-induced hyperalgesia in mice, the paws are withdrawn in about 2–4 s at 39–40°C temperatures. Paw withdrawal latencies are highly correlated with more integrative supraspinal behaviors such as guarding of the inflamed limb and exaggerated withdrawal duration. Therefore, we consider paw withdrawal latency a measure of nocifensive behavior. Since mice are not restricted, the stress related to the testing, environment could be considered mild. Paw edema was determined by measuring paw volume with a plethysmometer.

2.4. Intrathecal injection

All intrathecal injections were performed with disposable 30 gauge 1/2 in. needles mated to a 10 μl syringe (Hamilton, NV) (Hylden and Wilcox, 1980). A small caudal cutaneous incision (1 cm) was made 1–3 h before the injection under light halothane anesthesia. The mouse was held firmly by the pelvic girdle in one hand, while the syringe was held in the other hand at an angle of about 20° above the vertebral column. The needle was inserted into the tissue to one side of the L5 or L6 spinous process so that it slipped into the groove between the spinous and transverse processes. The needle was then moved carefully forward to the intrathecal space and a volume of 10 μl of a given solution was injected. The animals were examined for possible motor damage and behavioral tests were conducted 20 min later in those mice without motor changes.

2.5. Drugs

The drugs were provided by the National Institute on Drug Abuse (NIDA) unless otherwise indicated. Opioid receptor agonists: [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAMGO, 50 nM, i.t., μ-opioid-selective; RBI); [D-Pen^2,D-Pen^5]enkephalin (DPDPE, 50 nM, i.t., δ-opioid receptor-selective; RBI); [D-Ala^2]deltorphin-II (deltorphin, 50 nM, i.t., δ_2-selective); and U69,593 {(+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide, 100 nM, i.t., κ-opioid receptor-selective; RBI}. Opioid receptor antagonists: naloxone hydrochloride (non-selective, 2 mg/kg, i.p., RBI); naltrindole hydrochloride (1.0 nM, i.t., δ-opioid receptor-selective) and *nor*-binaltorphimine dihydrochloride (2 nM, i.t., κ-opioid receptor-selective). Doses were based on the

demonstration of selective effects in previous studies (Suh and Tseng, 1990; Horan and Porreca, 1993; Horan et al., 1992; Pugh et al., 1996).

2.6. Data analysis

Statistical comparisons were made by analysis of variance (ANOVA) (Fisher's Protected Least Significant Differences or Scheffe's *F*-test for post-hoc analysis). $P < 0.05$ was considered statistically significant. To compare the effects of opioid receptor agonists on the hyperalgesia in the three groups, raw latency data was converted to percentage of maximal possible effect according to the formula:

$$\%\,\text{maximal possible effect} = (\text{postdrug} - \text{predrug}) / (\text{cutoff} - \text{predrug})\,100\%$$

The time course of hyperalgesia data was also converted to percent of pre-complete Freund's adjuvant values to compare the time course in the three genotypes.

3. Results

3.1. Complete Freund's adjuvant-induced hyperalgesia in μ-opioid receptor knockout (−/−), heterozygous (+/−) and wild-type (+/+) mice

We first examined the effects of an intraplantar injection of the inflammatory agent, complete Freund's adjuvant, on baseline paw withdrawal latencies, a measure of nocifensive behavior. As shown in Fig. 1, baseline paw withdrawal latencies before complete Freund's adjuvant injection ranged from 8 to 9 s depending on the genotype. Baseline paw withdrawal latencies from male and female mice were separated and appeared to be similar depending on genotype (Fig. 1). A two factor ANOVA revealed significant differences between genotypes but no differences between males and females. The paw withdrawal latencies of μ-opioid receptor knockout male (8.08 ± 0.19 s; $n = 25$) and female (8.20 ± 0.23 s; $n = 28$) mice were significantly shorter than that of wild-type male(9.14 ± 0.32 s, $n = 29$, $P < 0.05$) and female (9.51 ± 0.28 s, $n = 27$, $P < 0.01$) mice. When these data from males and females were combined, the significant genotype differences were maintained. In view of the absence of sex differences, we have combined data in the remaining experiments from both sexes.

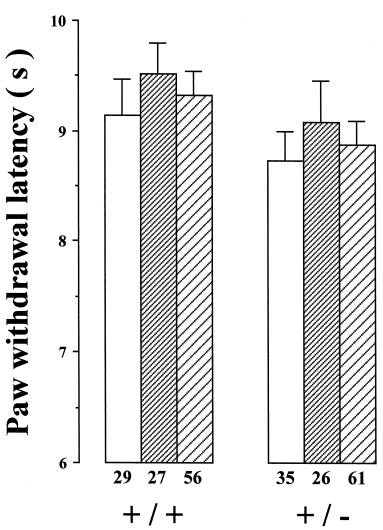

Fig. 1. Baseline paw withdrawal latencies. There were significant differences in paw withdrawal latencies between μ-opioid receptor knockout (−/−), heterozygous (+/−) and wild-type (+/+) mice when the data from males and females were separated or combined. μ-opioid receptor knockout males and females had significantly lower paw withdrawal latencies than wild-type mice. $^{**}P < 0.01$, $^{***}P < 0.001$; μ-opioid receptor knockout female mice and the combined group had significantly lower paw withdrawal latencies than heterozygous mice, $\#P < 0.05$. The numbers below the histogram bars indicate the number of mice per group.

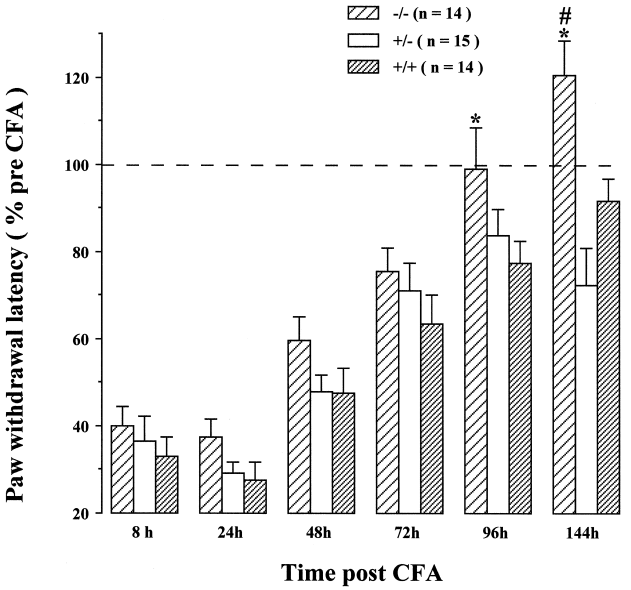

Fig. 2. Complete Freund's adjuvant-induced hyperalgesia in μ-opioid receptor knockout (−/−), heterozygous (+/−), and wild-type (+/+) mice. Paw withdrawal latencies are normalized to percentage of pre-complete Freund's adjuvant level that is indicated by a horizontal dashed line. Note that the recovery in paw withdrawal latencies occurred earlier in −/− than in +/− and +/+ mice. $^{*}P < 0.05$, knockout vs. wild-type mice; $\#P < 0.001$, knockout vs. heterozygous mice.

The rat model of complete Freund's adjuvant-induced inflammation was successfully adapted to the mouse. Following intraplantar injection of complete Freund's adjuvant, the inflammation induced a dramatic decrease in paw withdrawal latencies in all animals. Peak hyperalgesia occurred at 6–24 h and persisted for 3–6 days or more depending on the genotype (Fig. 2). The time course of complete Freund's adjuvant-induced hyperalgesia was shorter in μ-opioid receptor knockout mice than in the heterozygous and wild-type mice, although the peak hyperalgesia appeared to be similar in all genotypes. There were no significant differences in paw withdrawal latencies

among the three genotypes until 96 h after complete Freund's adjuvant-induced inflammation when the paw withdrawal latencies of μ-opioid receptor knockout mice ($n = 14$) had returned to pre-complete Freund's adjuvant levels while the paw withdrawal latencies of heterozygous ($n = 15$) and wild-type ($n = 14$) mice were about 80% of their pre-complete Freund's adjuvant levels (knockout vs. wild-type: $P < 0.05$). At 144 h after inflammation, the paw withdrawal latencies of knockout mice were still significantly longer than that of the heterozygous ($P < 0.001$) and wild-type ($P < 0.05$) mice whose paw withdrawal latencies had still not returned to pre-complete Freund's adjuvant levels.

3.2. The effects of opioid receptor antagonists on the paw withdrawal latencies of μ-opioid receptor knockout, heterozygous and wild-type mice after complete Freund's adjuvant-induced inflammatory hyperalgesia

We next tested the hypothesis that the differences in time course of recovery of behavioral hyperalgesia in the three genotypes were due to endogenous opioid modulatory mechanisms. In a separate group of transgenic mice, we demonstrated the effects of administering nonselective and selective opioid receptor antagonists at the 96 h post-complete Freund's adjuvant time point and comparing the paw withdrawal latencies with those found pre-complete

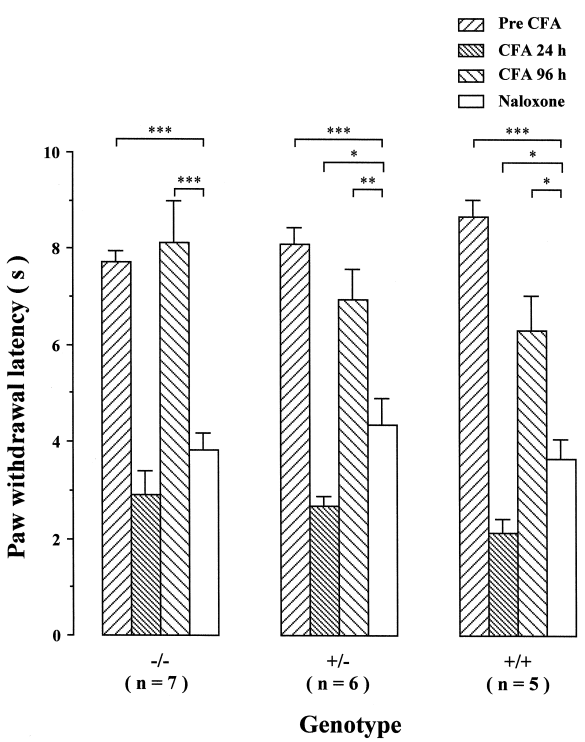

Fig. 3. Restoration of hyperalgesia by naloxone at 96 h after inflammation. Significant decreases in paw withdrawal latencies were observed in all genotypes of mice treated with naloxone (2 mg/kg, i.p.). Note that only in μ-opioid receptor knockout mice naloxone restored the hyperalgesia that is not significantly different from peak hyperalgesia (24 h post complete Freund's adjuvant). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

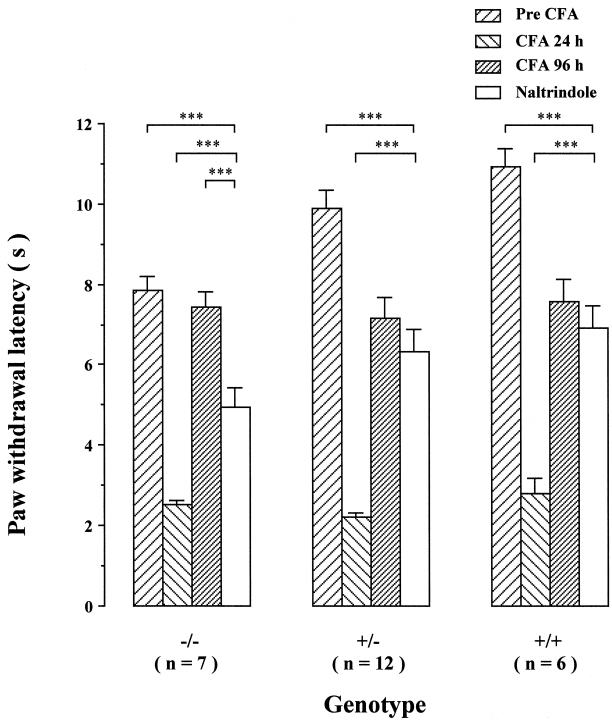

Fig. 4. Restoration of hyperalgesia by naltridole at 96 h after inflammation. Naltrindole partially restored the hyperalgesia only in μ-opioid receptor knockout mice. $^{***}P < 0.001$.

Freund's adjuvant, 24 h post-complete Freund's adjuvant and at 96 h post-complete Freund's adjuvant just before injection of the receptor antagonist. We first administered naloxone, a non-selective opioid receptor antagonist, systemically(i.p., 2 mg/kg), to test for general opioid sensitivity. All animals showed a dramatic restoration of the hyperalgesia (reduction in paw withdrawal latencies) (Fig. 3). The behavioral testing was conducted 15–30 min after injection of naloxone when levels of the drug peak in the brain (Misra et al., 1976). Although there were no differences in final paw withdrawal latency values among the three genotypes, μ-opioid receptor knockout mice ($P < 0.001$) ($n = 7$) exhibited a greater reduction in paw withdrawal latencies than heterozygous($P < 0.01$) ($n = 6$) or wild-type ($P < 0.05$) ($n = 5$) mice. Among the three genotypes, only in μ-opioid receptor knockout mice did naloxone restore the hyperalgesia to levels that were not significantly different from peak hyperalgesia (Fig. 3), suggesting that there was more complete restoration of hyperalgesia in the knockout mice than in the other genotypes.

We next tested whether the restoration of complete Freund's adjuvant-induced hyperalgesia was mediated at the spinal level by intrathecally administering two selective opioid receptor antagonists. The δ-opioid receptor-selective antagonist, naltrindole (1 nM, i.t.), partially restored the hyperalgesia only in μ-opioid receptor knockout mice (Fig. 4). The κ-opioid receptor-selective opioid receptor antagonist, nor-binaltorphimine (2 nM, i.t.), did not restore the hyperalgesia in any genotypes (not shown).

3.3. The effects of intrathecal injection of selective opioid receptor agonists

The differential effects of a selective δ-opioid receptor antagonist on the time course of hyperalgesia prompted us to examine the effects of selective opioid receptor agonists on the three genotypes at the peak of the hyperalgesia at 24 h after complete Freund's adjuvant injection. The intrathecal μ-opioid receptor agonist, DAMGO (50 nM), the κ-opioid receptor agonist, U69,539 (100 nM), and the δ-opioid receptor agonists, DPDPE (50 nM) and deltorphin (50 nM), all produced antihyperalgesia that primarily depended on the genetic background of the animal (Fig. 5). DAMGO had no analgesic effect on μ-opioid receptor knockout mice ($n = 5$) as expected, but it produced a 78% increase in paw withdrawal latency in heterozygous mice ($n = 4$) and a 100% increase of paw withdrawal latency in wild-type mice ($n = 5$). DPDPE and deltorphin produced antihyperalgesia in all three genotypes (Fig. 5). However, δ-opioid receptor-selective receptor agonists produced a significantly greater antihyperalgesic effect in knockout mice as compared to wild-type mice ($P < 0.05$). U69,593 produced a greater, but not statistically significant antihyperalgesic effect in wild-type as compared to μ-opioid receptor knockout mice.

3.4. Complete Freund's adjuvant-induced peripheral edema in μ-opioid receptor knockout, heterozygous and wild-type mice

Hindpaw volume was measured before and after complete Freund's adjuvant-induced inflammation. Complete Freund's adjuvant induced significant edema in all three genotypes, with a peak at 24 h after complete Freund's adjuvant stimulation. There were no noticeable differences in hindpaw edema between the knockout, heterozygous and wild-type mice.

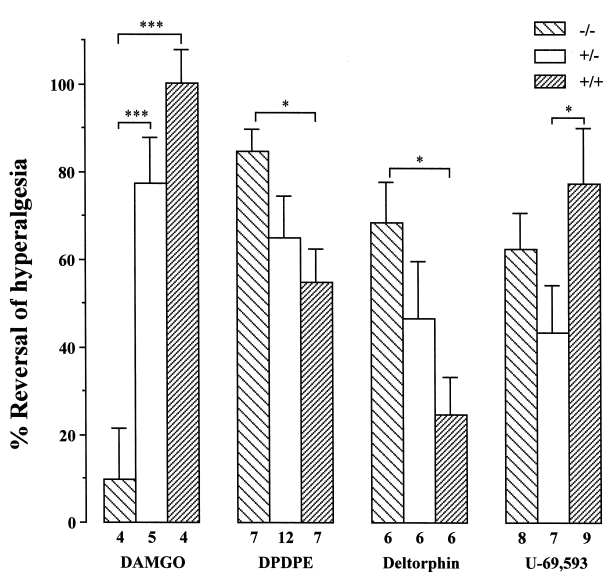

Fig. 5. The effects of intrathecal μ-, δ- and κ-opioid receptor agonists on complete Freund's adjuvant-induced hyperalgesia. Wild-type (+/+) and heterozygous (+/−) mice are significantly sensitive to DAMGO, a μ-opioid receptor-selective agonist; whereas μ-opioid receptor knockout (−/−) mice are more sensitive to DPDPE and deltorphin, two δ-opioid receptor-selective agonists. $^{*}P < 0.05$, $^{***}P < 0.001$. The numbers below the histogram bars indicate the number of mice per group.

4. Discussion

Our major findings indicate that the life-long deletion of μ-opioid receptors leads to different types of phenotypic change in μ-opioid receptor knockout mice in response to acute or transient pain as compared to the murine adaptation of the complete Freund's adjuvant-induced inflammation model of persistent pain (Hargreaves et al., 1988; Iadorala et al., 1988). μ-Opioid receptor knockout mice without inflammation were hyperalgesic in response to transient painful stimuli, as previously reported (Sora et al., 1997b). We have also revealed a faster recovery from complete Freund's adjuvant-induced hyperalgesia and identified an apparent δ-opioid receptor-selective adaptation in these knockout mice. The δ-opioid receptor-selective antagonist, naltrindole, restored inflammatory hyperalgesia only in μ-opioid receptor knockout mice, while δ-opioid receptor agonists produced significantly greater antihyperalgesic effects in these mice. These results suggest that a δ-mediated compensatory mechanism has developed as a result of persistent inflammation and hyperalgesia.

Studies of transgenic mice need to be interpreted in light of the life-long lack of the targeted gene product. In theory, such long lasting alterations could either result in a loss of function, or maximally engage available compensatory mechanisms (Mogil and Grisel, 1998). Despite this theoretical possibility, we and other laboratories have failed to reveal any dramatic compensatory phenotypic changes in the expression of other opioid transmitter and receptor genes: there were no differences in either δ or κ-opioid receptor binding or distribution, G-protein activation, or peptide message levels (Matthes et al., 1996, 1998; ; Sora et al., 1997b; Narita et al., 1999). Only a small fraction of the genes whose expression can be monitored in these mice are altered in the knockout (Liu et al., 1999). Some changes in behavioral phenotype in the μ-opioid receptor knockout mice have been observed including diminished δ-opioid receptor-induced analgesia [DPDPE and deltorphin II in the hotplate and tail flick test (Matthes et al., 1998; Sora et al., 1999) and SNC 80 {(+)-4-[(αR)-α-((2*S*,5*R*)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide} in the writhing test (Sora et al., 1999)], and the absence of deltorphin-induced respiratory depression (Matthes et al., 1998).

The shorter time course of inflammatory hyperalgesia in μ-opioid receptor knockout mice suggests a requirement for the μ-opioid receptor in order to produce the normal time course. Functional interactions between different opi-

oid receptors have previously been suggested (Rothman and Westfall, 1982; Jiang et al., 1990; Sutters et al., 1990; Traynor and Elliott, 1993), particularly between systems expressing μ-opioid receptors and δ-opioid receptors. Our present findings of increased δ-opioid receptor and δ agonist sensitivity in μ-opioid receptor knockout mice after complete Freund's adjuvant-induced inflammation support this hypothesis. Taken together with previous observations of stable δ-opioid receptor binding, these results suggest an increment in δ-opioid receptor function. This apparent upregulation of the stable population of δ-opioid receptors could be primarily responsible for the increased endogenous pain control in μ-opioid receptor knockout mice after inflammation. Previous studies of δ-opioid receptor-mediated analgesia in μ-opioid receptor knockout mice have reported it to be substantially reduced (Sora et al., 1997a, 1999), more modestly reduced (Matthes et al., 1996), or little changed (Loh et al., 1998). Under at least some circumstances, then, μ-opioid receptors may be necessary to obtain full δ-opioid receptor mediated responses in intact mice in response to transient noxious stimuli. Our findings suggest that the interaction of these receptors may be very different after inflammation. In this circumstance, the absence of the μ-opioid receptor appears to result in a release of endogenous δ-opioid receptor activation and increased sensitivity to δ-specific ligands. These novel effects of persistent noxious and stressful inputs on phenotypic switches in opioid systems have precedent. Mice with β-endorphin knockouts display greater nonopioid stress analgesia and a lack of opioid analgesia induced by stress (Rubinstein et al., 1996).

The effect of the μ-opioid receptor deletion on basal nociceptive sensitivity has been debated. Matthes et. al (1996) reported no change in thresholds on tail flick and hot plate tests in their first publication, but reported higher sensitivity of μ-opioid receptor knockout mice to noxious thermal stimuli in a subsequent publication (Matthes et al., 1998). Sora et al. (1997b) found reduced latencies in the tail-flick and the 55°C hot plate test in the strain of mice studied here. The current findings of decreases in baseline paw withdrawal latencies in male and female μ-opioid receptor knockout mice as compared to wild-type littermates support the findings that pain thresholds are reduced in response to transient noxious stimuli in μ-opioid receptor knockout mice (Sora et al., 1997b; Matthes et al., 1998). This finding suggests that endogenous μ-opioid systems are tonically active in intact mice. The lack of δ-opioid receptor-mediated increases in analgesia found by others (Sora et al., 1997a; Matthes et al., 1998) supports our conclusions that compensatory changes in other opioid receptor systems recruited during persistent pain and inflammation are less dramatic during development or in response to transient noxious stimulation.

Our findings of the restoration of hyperalgesia by a selective δ-opioid receptor antagonist can also be explained, in part, by an increase in preproenkephalin mRNA induced by inflammation and hyperalgesia. Previous studies have shown that inflammation of the rat hindpaw in intact animals results in increased preproenkephalin mRNA (Iadarola et al., 1988; Noguchi et al., 1992). Increases in the enkephalin peptide product coded by this gene could enhance the antihyperalgesia found in the present study. Actions of this peptide on δ-opioid receptors could be those that are reversed by naltrindole. Conceivably, compensatory changes in μ-opioid receptor knockout mice could occur in other functionally related neurotransmitter systems. The actions of excitatory amino acids in the spinal dorsal horn are enhanced presynaptically and postsynaptically after inflammation by the neuropeptides substance P and calcitonin-gene-related peptide (Murase et al., 1989; Kangrga and Randic, 1990; Dougherty and Willis, 1991). The increased activity of δ-opioid receptors or enkephalin could lead to a suppression of substance P and glutamate release and a reduction in spinal dorsal horn excitability (Suarez-Roca and Maixner, 1992a,b).

Opioid receptors are also present on peripheral sensory nerves and in some of the white blood cells that can be recruited into the sites of inflammation (Stein et al., 1997). Opioid peptides are expressed in immune cells within inflamed tissue and when released appear to bind to the receptors on peripheral nerves where they can play a role in inhibition of pain and the release of substances that contribute to the inflammatory process. In the present study, we did not find differences in peripheral edema in the three genotypes suggesting that peripheral inflammatory processes were not detectably altered in the μ-opioid receptor knockout mice. However, the measurement of hindpaw volume provides insufficient resolution to draw any firm conclusions and future studies are needed to examine the role of peripheral opioids in pain sensitivity and inflammation in mice with the μ-opioid receptor deletion.

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