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Retention of Supraspinal Delta-like Analgesia and Loss of Morphine Tolerance in δ *Opioid Receptor* Knockout Mice

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Summary

Gene targeting was used to delete exon 2 of mouse *DOR-1*, which encodes the δ opioid receptor. Essentially all 3H-[D-Pen^2,D-Pen^5]enkephalin (^{3}H-DPDPE) and 3H-[D-Ala^2,D-Glu^4]deltorphin (^{3}H-deltorphin-2) binding is absent from mutant mice, demonstrating that *DOR-1* encodes both δ_1 and δ_2 receptor subtypes. Homozygous mutant mice display markedly reduced spinal δ analgesia, but peptide δ agonists retain supraspinal analgesic potency that is only partially antagonized by naltrindole. Retained DPDPE analgesia is also demonstrated upon formalin testing, while the nonpeptide δ agonist BW373U69 exhibits enhanced activity in *DOR-1* mutant mice. Together, these findings suggest the existence of a second delta-like analgesic system. Finally, *DOR-1* mutant mice do not develop analgesic tolerance to morphine, genetically demonstrating a central role for DOR-1 in this process.

Introduction

Pharmacologically, three major classes of opioid receptors have been proposed, each of which is capable of modulating pain (Herz, 1993; Pasternak, 1993). Of these receptors, the δ receptor (DOR-1; also oprd1) was the first one cloned (Evans et al., 1992; Kieffer et al., 1992), followed soon afterward by the μ receptor (MOR-1; oprm1) and the κ receptor (KOR-1; oprk1) (see Kieffer, 1995, for review). These three classical opioid receptors are typical G protein receptors, with seven putative transmembrane regions, and are highly homologous to each other (Kieffer, 1995).

δ receptors have been associated with several physiological functions, including analgesia (Heyman et al., 1988), tolerance (Abdelhamid et al., 1991; Kest et al., 1996), and reproduction (Zhu and Pintar, 1998). The distribution of δ receptors in multiple regions of the adult nervous system, with high levels in the olfactory bulb, striatum, cortex, hippocampal formation, pons, spinal cord, and dorsal root ganglion (Mansour et al., 1993), reflects this potentially broad range of functions, while prenatal expression patterns (Zhu et al., 1998) suggest possible developmental roles as well. The ability of δ receptors to modulate pain perception in mice is illustrated by the analgesic activity of a number of δ-selective peptides as well as nonpeptide alkaloids (Heyman et al., 1988; Chang et al., 1993). Currently, most opiate analgesics used clinically act through μ receptors (Matthes et al., 1996), but their use is limited by side effects, such as respiratory depression, constipation, and dependence (Ellison, 1993). In contrast, δ receptor agonists can produce analgesia without physical dependence (Cowan et al., 1988; Maldonado et al., 1992), indicating that δ receptors remain potentially important therapeutic targets for the development of novel analgesic agents (Rapaka and Porreca, 1991). In addition to these functions, δ receptors also have been implicated in the production of morphine tolerance. For example, low doses of the δ receptor antagonist naltrindole can prevent tolerance to μ receptor agonists (Abdelhamid et al., 1991; Hepburn et al., 1997) without compromising the antinociceptive activity of morphine.

Pharmacological studies have suggested at least two δ receptor subtypes. The δ_1 receptor subtype is preferentially activated by the agonist [D-Pen^2,D-Pen^5]enkephalin (DPDPE) and antagonized by [D-Ala^2,Leu^5,Cys^6]enkephalin (DALCE), while the δ_2 receptor is preferentially activated by [D-Ala^2,D-Glu^4]deltorphin (deltorphin-2) (Mattia et al., 1991, 1992; Hiller et al., 1996) and antagonized by naltrindole-5′-isothiocyanate (5′-NTII). This classification is further supported by analgesic (Mattia et al., 1992; Tiseo and Yaksh, 1993), adenylyl cyclase (Olianas and Onali, 1995), and antisense approaches (Standifer et al., 1994; Rossi et al., 1997). However, the relationship of these two δ receptor subtypes to the cloned *DOR-1* gene remains unclear, and it is not known whether either or both δ_1 and δ_2 activities are derived from the *DOR-1* locus (Raynor et al., 1994).

Multiple components of the murine opioid system, including genes encoding MOR-1 (Matthes et al., 1996; Sora et al., 1997; Tian et al., 1997; Roy et al., 1998; Schuller et al., 1999), KOR-1 (Simonin et al., 1998), the preproenkephalin (pre-PENK) precursor (Konig et al., 1996), and the β-endorphin domain of the proopiomelanocortin (POMC) precursor (Rubinstein et al., 1996), have been disrupted by gene targeting. Analysis of these mutant strains of mice thus far suggests that each component has a distinct role in a wide range of physiological functions, including analgesia, stress responses, hematopoiesis, and reproduction. In this study, we have used gene targeting to produce δ receptor–deficient mice and explore δ receptor actions in these knockout (KO) mice.

Results/Discussion

The murine *DOR-1* gene was altered by replacing exon 2 with a *neomycin* resistance cassette (Figure 1a). The

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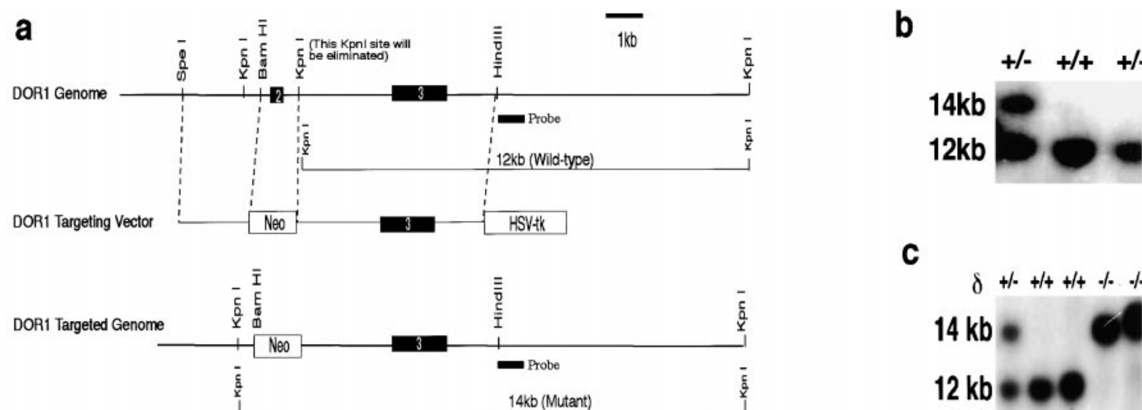

Figure 1. Gene Targeting of *DOR-1*

(a) Gene targeting construct for *DOR-1* disruption. A 2.1 kb SpeI–BamHI fragment derived from the intronic region 5′ to exon 2 and a 4.8 kb KpnI–HindIII fragment beginning in the intron region 3′ to exon 2 of the mouse *DOR-1* gene were cloned into the pBs–KO vector containing *neomycin* and thymidine kinase selection markers. Thus, in the targeting construct, exon 2 was replaced with the *neomycin* resistance gene. Genomic Southern blot was used to detect successful targeting events. When the *neomycin* gene was introduced into the endogenous *DOR-1* locus, the KpnI site located 3′ of exon 2 was deleted, which changed the size of the KpnI fragment from 12 kb to 14 kb, as detected by an external probe.

(b and c) Southern blot analysis of KpnI-digested genomic DNA from electroporated ES cells (b) and mouse tail DNA from offspring of mice heterozygous for the mutated *DOR-1* allele (c). Abbreviations: neo, *neomycin* resistance gene; HSV-TK, *thymidine kinase* gene from the herpes simplex virus. Genotypes: +/+, wild-type; +/−, heterozygous; and −/−, homozygous.

targeting vector was introduced into CCE embryonic stem (ES) cells from the 129/SvEv mouse line, and three targeted ES cells lines were identified by Southern blotting (Figure 1b). Two targeted ES lines were injected into blastocysts and gave rise to germline-transmitting male chimeras. Heterozygotes for this mutated allele were mated, and mice homozygous for the altered allele were present in offspring (Figure 1c). Genotyping showed that the δ-deficient offspring were present in the expected Mendelian frequency (+/+, 26.2%; +/−, 49.7%; and −/−, 24.1%) (n = 370 offspring). No obvious developmental abnormalities were observed in the homozygous mutant mice. *DOR-1* mutant mice appear healthy and are similar in size to wild-type litter mates. Both male and female homozygous mice are fertile and produce litters of normal size, indicating that the high level of δ expression seen in the trophoblast giant cells (Zhu and Pintar, 1998) is not essential for the successful completion of pregnancy.

DOR-1 Immunoreactivity and δ_1 and δ_2 Binding Are Abolished in *DOR-1* Mutant Mice

To determine whether any *DOR-1*-derived protein, translated from low levels of residual *DOR-1* transcription (see below) could be detected in *DOR-1* mutant mice, we performed immunocytochemical experiments using a DOR-1 antiserum directed against exon 1–derived N-terminal sequences of DOR-1 (amino acids 3–17; Arvidsson et al., 1995). Immunostaining was absent throughout the brain of *DOR-1* KO mice (Figure 2a), indicating the absence of any detectable *DOR-1*-derived translation product, and was significantly reduced in heterozygous mice (data not shown).

δ receptor binding in brain membrane fractions from *DOR-1* (+/+), *DOR-1* (+/−), and *DOR-1* (−/−) mutant mice was analyzed using both the δ_1-preferring ligand ^{3}H-DPDPE and the δ_2-preferring ligand ^{3}H-deltorphin-2. Saturation analysis revealed little observable ^{3}H-DPDPE and ^{3}H-deltorphin-2 binding in brain membranes from homozygous mutant mice (Figure 2b). Autoradiographic analysis of ^{3}H-DPDPE binding also revealed a dramatic reduction of binding throughout the homozygous mutant mouse brain, with no area showing obvious binding above background (Figure 2c). These results indicate that the *DOR-1* locus encodes both of the pharmacologically defined receptors δ_1 and δ_2. Therefore, the different properties of the δ_1 and δ_2 receptor subtypes must arise from this locus and could result from differential post-translational modification of the DOR-1-encoded receptor protein, alterations in the molecular environment of the receptor protein, or possibly even splice variants, as suggested by *DOR-1* antisense mapping studies (Rossi et al., 1997).

Genetic disruption of DOR-1 did not significantly influence the expression of other opioid receptors or endogenous opioid peptide genes. No significant differences in the levels of binding among the wild-type, heterozygous, or homozygous mice were observed with μ (^{3}H-[D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin [^{3}H-DAMGO]) (+/+: K_d, 3.73 nM and B_{max}, 209.7 fmol/mg protein; −/−: K_d, 4.18 nM and B_{max}, 246.4 fmol/mg protein) or κ (^{3}H-D-(5α,7α,8β)-(+)-*N*-methyl-N-{7-1-pyrrolidinyl)-1-oxaspiro [4, 5]dec-8-yl}benzeneacetamide [^{3}H-U69,593]) (+/+: K_d, 1.16 nM and B_{max}, 23.29 fmol/mg protein; +/−: K_d, 1.19 nM and B_{max}, 22.66 fmol/mg protein; −/−: K_d, 1.09 nM and B_{max}, 25.55 fmol/mg protein) receptor ligands. In situ hybridization of the mRNA encoding all three opioid receptors and the endogenous opioid peptide precursor genes *PENK*, *POMC*, and *prodynorphin* (*PDYN*) were examined in adult brain (Figure 2d). As expected, exon 2 *DOR-1* transcripts were absent in the −/− mutant mice, although low residual levels (<15%+/+) of direct *DOR-1* exon 1 to *DOR-1* exon 3 transcripts were characterized by Northern and PCR analysis (Figures 2e and 2f). These transcripts do not appear to be translated

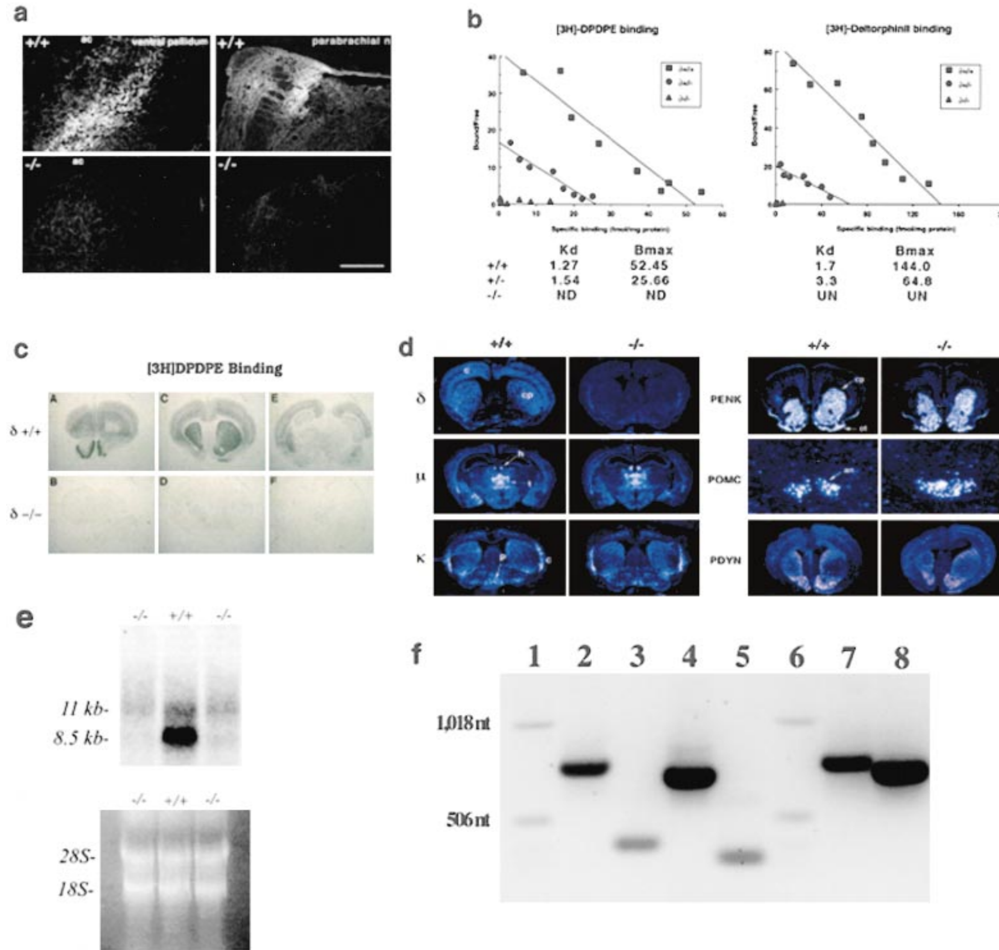

Figure 2. Characterization of Opioid Systems in *DOR-1* KO Mice

(a) Immunocytochemical analysis of *DOR-1* KO mice. Immunofluorescent confocal images of DOR-1 immunoreactivity in the pallidum (left) and parabrachial nucleus (right) from *DOR-1* (+/+) and *DOR-1* (−/−) mice, respectively. Abundant DOR-1 immunoreactivity is observed in wild-type mice but is virtually eliminated in sections from the homozygous *DOR-1* mutant. Scale bar, 200 μm.

(b) δ receptor binding sites in *DOR-1* (+/+), *DOR-1* (+/−), and *DOR-1* (−/−) mouse brain are shown in Scatchard plots of ^{3}H-DPDPE and ^{3}H-deltorphin-2 binding to brain membranes from mice of the indicated genotype. B_{max} (fmol/mg protein) and K_d (nmol) are indicated. Data are representative of four separate determinations for each ligand. See the Experimental Procedures for detailed procedures.

(c) Autoradiographic mapping of DPDPE binding to brain sections. Wild-type (A, C, and E) and homozygous *DOR-1* KO (B, D, and F) brains were coembedded and incubated with 5 nM ^{3}H-DPDPE. Results indicate that DPDPE binding is dramatically reduced throughout the neuroaxis, including regions such as the olfactory bulb (compare A and B), caudate-putamen (compare C and D), and cortex (compare A, C, and E with B, D, and F), which express relatively high levels of δ receptor binding.

(d) In situ hybridization analysis of δ, μ , and κ receptor and *PENK*, *POMC*, and *PDYN* genes in adult brains of *DOR-1* (+/+) and *DOR-1* (−/−) mice. In the left panel, dark-field images show that exon 2 *DOR-1* transcripts are absent in the mutant mice, while the distributions of μ and κ receptor mRNAs in the mutant are similar to those of the wild-type. In the right panel, dark-field images show that mRNA expressions of opioid peptide precursors PENK, POMC, and PDYN in *DOR-1* (−/−) mice brain are similar to those of *DOR-1* (+/+). Abbreviations: an, arcuate nucleus; cp, caudate-putamen; ot, olfactory tubercle.

(e) Northern analysis of δ receptor gene expression in adult brains of *DOR-1* (+/+) and *DOR* (−/−) mice. Upper panel shows that both 8.5 and 11 kb *DOR-1* transcripts are markedly decreased in *DOR* (−/−) brains (combined total is $<$15% of *DOR* (+/+), as estimated by NIH image). In the lower panel, ethidium bromide–stained gel shows that similar amounts of RNA were applied to each lane.

(f) RT-PCR of RNA isolated from *DOR-1* (+/+) and *DOR-1* (−/−) adult brain. RT-PCR using two different primer sets spanning *DOR-1* exon 1 and exon 3 show that *DOR-1* transcripts in *DOR-1* (+/+) brain contain exon 2 (lanes 2 and 4), whereas transcripts present in *DOR-1* (−/−) brain lack exon 2 (lanes 3 and 5). Lanes 1 and 6 show a molecular weight marker. *DOR-1* cDNA amplified with primer sets 1 and 2 served as a positive control (lanes 7 and 8, respectively).

Primer set 1 is expected to amplify a 758 nucleotide fragment if exon 2 is present (wild-type and cDNA), and a 408 nucleotide fragment if exon 2 is absent (758–350). Exon 1, 5′ primer: GGAGCCCGTAGTGCCTCGTCC (nucleotide position, 142–162) and exon 3, 3′ primer: CGATTGATGTCCACCAGCGTCC (nucleotide position, 878–899). Primer set 2 is expected to amplify a 706 nucleotide fragment if exon 2 is present, and a 356 nucleotide fragment if exon 2 is absent (706–350). Exon 1, 5′ primer: CTCGTCAACCTCTCGGACGCC (nucleotide position, 73–93) and exon 3, 3′ primer: CCTTGGAACCGGACAGCAGACG (nucleotide position, 757–778).

Abbreviations: c, cortex; cp, caudate-putamen; h, habenula; p, preoptic area; t, thalamus.

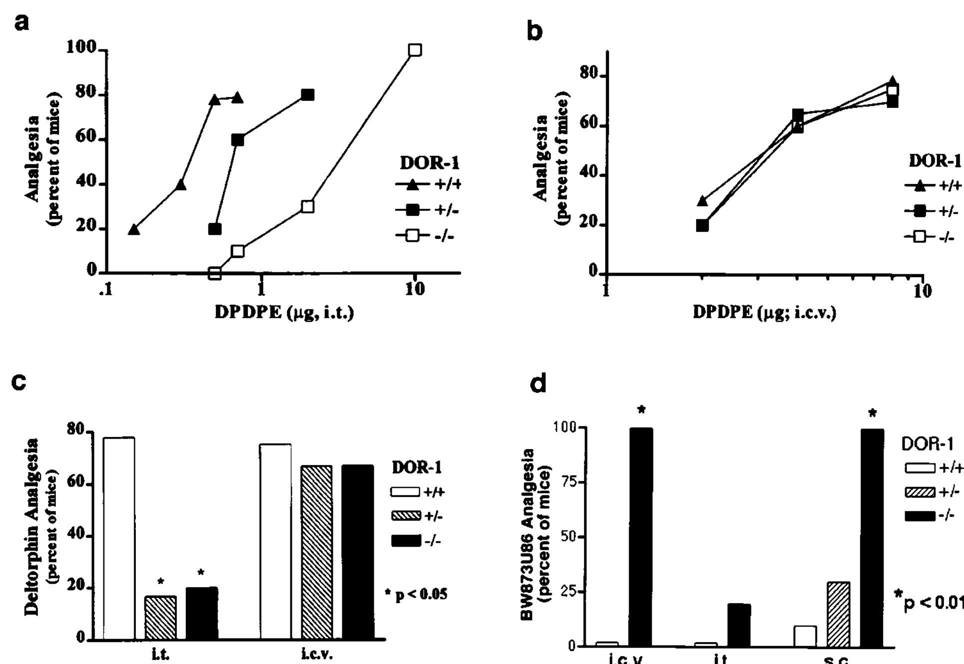

Figure 3. Delta-Related Analgesia in DOR-1-Deficient Mice

(a and b) DPDPE analgesia in *DOR-1* KO mice. Cumulative dose–response curves were generated in groups of mice ($n \geq 20$) given DPDPE administered either i.t. (a) or i.c.v. (b). Tailflick latencies were determined prior to drug administration and 15 min afterward. The ED_{50} value for DPDPE following i.t. administration in the wild-type mice was 300 ng (95% confidence interval, 208–434 ng) for +/− mice, and the ED_{50} was 820 ng (510 ng–1.32 μg); for −/− mice, the ED_{50} was 1.83 μg (99 ng–3.38 μg). The rightward shift was 6.1-fold ($p < 0.05$).

(c) Deltorphin-2 analgesia in DOR-1-deficient mice. Groups of mice ($n = 15$) received deltorphin-2 either i.t. (15 μg) or i.c.v. (20 μg) and were tested for analgesia 15 min later. Statistical significance was determined by the Fischer exact test.

(d) BW373U86 analgesia in *DOR-1* KO mice. Groups of mice received BW373U86 (60 ng, i.c.v. [n = 6], 60 μg, i.t. [n = 6], and 30 mg/kg, s.c. [n = 10]), and analgesia was assessed 5, 10, or 30 min later, respectively, in the radiant heat tailflick assay. The response in the KO group was significantly greater than that of the wild-type group following both i.c.v. and s.c. administration ($p < 0.01$).

(Figure 1a) and, even if translated to a small degree, would encode a completely altered protein structure (see Experimental Procedures). In contrast, the distributions of all of the other mRNAs were qualitatively and semiquantitatively similar (Figure 2d). Thus, DOR-1 expression does not appear to exert any regulatory control on the transcription of genes encoding other opioid receptors and endogenous ligands. Independent regulation of opioid system components has also been noted following disruptions of the μ (Matthes et al., 1996; Schuller et al., 1999) and κ (Simonin et al., 1998) receptor genes. This effect on only δ receptor expression thus provided the opportunity to characterize δ receptor functions in *DOR-1* KO mice that retain generally normal expression of many other opioid system components.

Spinal δ Analgesia Is Lost and Supraspinal Analgesia Retained in *DOR-1* Mutant Mice

We next explored analgesia in *DOR-1* mutant mice. In initial experiments, no differences in baseline tailflick latencies were observed among the wild-type, heterozygous, and homozygous groups using either the standard lamp intensity, with latencies between 2 and 3 s, or a reduced lamp intensity that produced latencies of ~8 s (data not shown). We then explored the action of several δ agonists. δ receptor–selective peptides, such as DPDPE and deltorphin-2, are effective analgesics both spinally and supraspinally (Heyman et al., 1987, 1988). The insensitivity of DPDPE analgesia to μ-selective antagonists (Heyman et al., 1987) and its reduction by only antisense probes targeting *DOR-1* (Standifer et al., 1994; Rossi et al., 1997) initially indicated the importance of *DOR-1*-derived receptors in this response. We thus tested the responses to these compounds following both intrathecal (i.t.) and intracerebroventricular (i.c.v.) administration. *DOR-1* homozygous mice were markedly less sensitive to the δ agonist DPDPE given i.t., although analgesia could be elicited at high-DPDPE doses, with an ED_{50} >6-fold higher than in wild-type mice (Figure 3a). Spinal deltorphin-2 analgesia was also markedly diminished in the KO mice (Figure 3c), indicating a major contribution of DOR-1 to spinal analgesia. However, both DPDPE (Figure 3b) and deltorphin-2 (Figure 3d) retained their analgesic activity, with no reduction in potency, following supraspinal i.c.v. administration in the *DOR-1* KO mice. Thus, these data indicate that there are analgesic systems for classical δ agonists that can be mediated through receptors other than DOR-1.

We next assessed the activity of a nonpeptide δ analgesic, BW373U86 (Chang et al., 1993). Given i.c.v., virtually no analgesic response was elicited in wild-type mice, as had previously been shown (Wild et al., 1993). In contrast, robust analgesia was observed in *DOR-1* KO, with all mice tested not only exhibiting analgesia (Figure 3d) but also reaching maximal analgesic cut-off times. When administered i.t., BW373U86 did not elicit

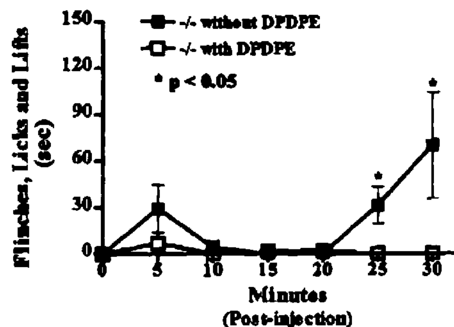

Figure 4. Formalin Testing in *DOR-1* KO Mice

Formalin was administered via hindpaw injection to *DOR-1* KO mice following i.c.v. injection of either DPEPE (8 μg) (n = 4) or saline (n = 4) 15 min earlier. The response was monitored for 30 min and indicates that the second phase of the formalin response is significantly reduced by DPDPE in the *DOR-1* mutant mice. Significance was assessed by ANOVA.

analgesia in either wild-type mice or *DOR-1* homozygous mutants, even following a 60 μg injection (Figure 3d). Thus, both peptide and nonpeptide δ agonists elicit analgesia in *DOR-1* KOs following i.c.v., but not i.t., administration. We finally tested the response of wild-type and mutant mice to systemic BW373U86 administration. Given systemically, this compound also had little effect in wild-type mice and elicited a maximal analgesic response of only 10% at 30 mg/kg. Heterozygous mice were more sensitive, with 30% responding, while homozygous mutant mice developed significantly more analgesia than wild-type mice (Figure 3d).

We also explored the activity of DPDPE in the formalin test in *DOR-1* KO mice. The appearance of the first and second phases of enhanced licking and flinching that follow hindpaw formalin administration were present in the *DOR-1* KO mice and were not significantly different from those seen in littermate wild-type mice (data not shown). Since the formalin response can be reduced by exogenous DPDPE (Calcagnetti et al., 1988), the analgesic properties of this compound in conjunction with formalin administration were tested in *DOR-1* KO mice. DPDPE (8 μg) given i.c.v. virtually eliminated the second phase of the formalin response (Figure 4). Thus, DPDPE analgesic activity in the *DOR-1* KO mice can also be demonstrated in an analgesic model distinct from thermal nociception.

We next chose to investigate in detail one of the delta-like responses—i.c.v. DPDPE analgesia—exhibited by *DOR-1* mutant mice. We first explored the sensitivity of the retained i.c.v. DPDPE analgesia to opioid receptor antagonists. The residual supraspinal DPDPE analgesia was effectively blocked in all genotypes by naltrexone, a general opioid receptor antagonist (Figure 5a), confirming that the analgesia is opioid in nature. Conversely, neither the μ-selective antagonist β-funaltrexamine (β-FNA) nor the κ-specific drug norbinaltorphimine (nor-BNI) blocked DPDPE supraspinal analgesia in any group (Figure 5a). As expected, the δ-selective antagonist naltrindole (Portoghese et al., 1988) significantly reduced DPDPE analgesia in wild-type mice. However, although naltrindole partially reduced DPDPE analgesia in the *DOR-1* KO mice, it was not as effective as in the wild-type animals (Figure 5a). Similar results were observed when the hot plate test was instead used to monitor analgesia (Figure 5b). Again, naltrexone reversed the analgesic response in all groups, while naltrindole significantly blocked the response in wild-type mice but not in littermate heterozygous or homozygous mice.

Antisense approaches also distinguished the residual supraspinal DPDPE analgesia from that normally elicited in wild-type mice. Thus, a *DOR-1* antisense probe targeting exon 3, which had previously been shown to reduce δ ligand binding (Standifer et al., 1994), significantly blocked supraspinal DPDPE analgesia in wild-type mice but not in heterozygous or homozygous *DOR-1* mutant mice (Figure 6), indicating that any residual transcription of *DOR-1* exon 3 is not contributing to this analgesia. While these findings confirm a role for DOR-1 in supraspinal DPDPE analgesia in wild-type mice, they also indicate that the residual supraspinal DPDPE analgesia seen in the KO animals is due to a receptor other than DOR-1, a conclusion also consistent with the partial, rather than complete, sensitivity of the residual analgesia to naltrindole. Moreover, the enhanced activity described above of i.c.v. and peripheral BW373U86 in *DOR-1* mutant mice suggests that the absence of DOR-1 increases the sensitivity of this novel secondary δ analgesic system, which can both mediate continued DPDPE and deltorphin-2 supraspinal analgesia and dramatically enhance the efficacy of at least one non-peptide δ agonist. These observations indicate that non-peptide agonists, such as BW373U86, likely act predominantly on this secondary system though we cannot at present distinguish whether this novel δ system is unmasked or upregulated following *DOR-1* ablation.

Although the *DOR-1* KO mice contained no observable ^{3}H-DPDPE or ^{3}H-deltorphin-2 binding under the traditional binding conditions used for these studies, we did observe low levels of ^{3}H-naltrindole binding with an affinity (K_d, 7.1 nM) ~10-fold lower than that of traditional δ receptor binding in wild-type mice (K_d, 0.54 nM) (data not shown). This low-affinity ^{3}H-naltrindole binding site may correspond to the receptor responsible for the residual δ analgesia.

To summarize, several lines of evidence indicate that a novel naltrexone-reversible mechanism which is not blocked by subclass-specific opioid antagonists mediates retained DPDPE and deltorphin-2 analgesia, as well as enhanced BW373U86 analgesia, in *DOR-1* KO mice. These include differential sensitivity to naltrindole, a heightened response to BW373U86, and differential sensitivity to an exon 3 oligonucleotide that does not suppress DPDPE analgesia in *DOR-1* KO as it does in wild-type mice. Based on these data, we suggest that δ analgesia in wild-type mice is mediated predominately through *DOR-1* systems, consistent with its sensitivity to both naltrindole and antisense probes based upon *DOR-1*. In contrast, an upregulated (or unmasked), secondary δ system is responsible for the residual δ analgesia in *DOR-1* KO mice, which explains its insensitivity toward the same antisense probe and the distinct patterns of response to both δ agonists (such as BW373U86) and antagonists (such as naltrindole), compared with wild type.

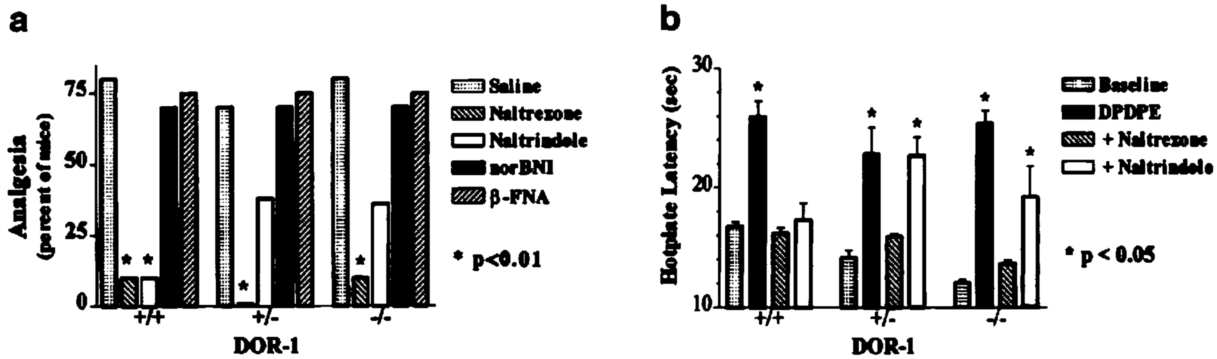

Figure 5. Sensitivity of DPDPE Supraspinal Analgesia to Selective Opioid Receptor Antagonists

(a) In the tailflick assay, groups of mice (n = 20) received a fixed dose of DPDPE (8 μg i.c.v.) alone or following administration of the opioid antagonists β-FNA (40 mg/kg, s.c.), naltrexone (1 mg/kg, s.c.), naltrindole (20 mg/kg, s.c.), or nor-BNI (10 mg/kg, s.c.). All antagonists were administered 15 min prior to DPDPE, with the exception of β-FNA, which was administered 24 hr prior to treatment. Analgesia was expressed as the percentage of mice responding. Statistical significance was determined by the Fischer exact test.

(b) In the hot plate assay, groups of mice (n = 10) received a fixed dose of DPDPE (8 μg, i.c.v.) alone or with naltrexone (1 mg/kg, s.c.) or naltrindole (20 mg/kg, s.c.) 15 min prior to treatment. Naltrexone significantly antagonized DPDPE analgesia in all cohorts. However, naltrindole, a selective δ receptor antagonist, significantly blocked DPDPE analgesia only in the wild type. Statistical significance was determined by two-tailed t-test, with a 95% confidence interval.

Characterization of Non-Delta Analgesia and Development of Tolerance in *DOR-1* KO Mice

The disruption of DOR-1 did not alter the analgesic effectiveness of the other classes of opioid analgesics. The μ agonists morphine (Figure 7a) and morphine-6β-glucuronide, the κ1 agent U50,488H, and the κ3 drug nalaxone benzoylhydrazone (Figure 7b) all maintained their analgesic activity in the *DOR-1* KO mice. These data demonstrate that δ expression is not required for either the development or maintenance of circuitry underlying their analgesic responses.

Finally, we examined the role of δ receptors in morphine tolerance (Figures 7c and 7d). In contrast to wild-type animals, in which the analgesic response to a fixed morphine dose was lost within 5 days, *DOR-1* KO mice failed to demonstrate tolerance following daily administration of morphine (5 mg/kg, subcutaneously [s.c.]) for 8 days (Figure 7c). After 10 days days of chronic morphine dosing, cumulative dose–response curves revealed a significant 2.8-fold shift to the right of the morphine ED_{50} in the wild-type mice. In contrast, the potency of morphine in the *DOR-1* KO mice was unchanged following chronic morphine (Table 1) and thus was identical to that observed in naive, wild-type mice. These results confirm and extend in a genetic model the importance of DOR-1 in morphine tolerance implied by earlier studies in which morphine tolerance was blocked by either low doses of naltrindole, which selectively antagonized δ receptors (Hepburn et al., 1997), or by antisense targeting of *DOR-1* (Kest et al., 1996). In both prior cases, however, the complete blockage of tolerance development that was evident in the *DOR-1* KO model was not observed.

Since supraspinal DPDPE analgesia was exhibited by *DOR-1* KO mice (see above), we also examined whether tolerance to DPDPE developed in these mice. The analgesic response to chronic DPDPE in wild-type mice, like the response to morphine, was lost completely within 4 days, indicating the development of tolerance. In contrast, the ability of DPDPE to elicit analgesia in homozygous *DOR-1* KO mice remained unchanged for at least 6 days (Figure 7d), while some heterozgyous mice became tolerant over this period. The lack of tolerance development is similar to that seen with morphine, although the conclusions are less clear since the ability of the receptor mediating DPDPE analgesia in the *DOR-1* KO mice to exhibit tolerance under the present dosing paradigm is unknown. Nonetheless, these data indicate that DOR-1 may be critical to the development of tolerance to opioid agonists besides morphine. The importance of DOR-1 circuitry in the development of tolerance in vivo is further indicated by analysis of a novel KO mouse line lacking met-enkephalin peptides, derived from PENK, that constitute the major DOR-1 ligand in vivo. In these mice, the ability to develop tolerance to both morphine and DPDPE is also completely abolished, indicating that an enkephalin–DOR-1 ligand–receptor system is likely an integral component of the pathway(s) mediating tolerance (A. G. P. S. et al., unpublished data).

Taken together, these studies provide genetic evidence for a unique role for δ receptors in the central

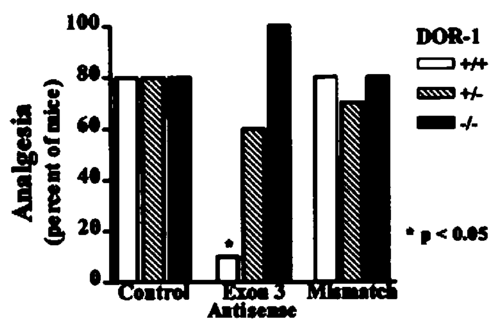

Figure 6. *DOR-1* Antisense Effect on DPDPE i.c.v. Analgesia

Groups of mice (n ≥ 10) received saline, a *DOR-1* exon 3–directed oligodeoxynucleotide, or a mismatch oligonucleotide (5 μg, i.c.v.) on days 1, 3, and 5. On day 6, all mice were tested with DPDPE (8 μg, i.c.v.), and analgesia was assessed. The exon 3 antisense probe was effective in *DOR* (+/+) mice but did not reverse DPDPE analgesia in *DOR-1* KOs. Significance was analyzed by the Fisher exact test.

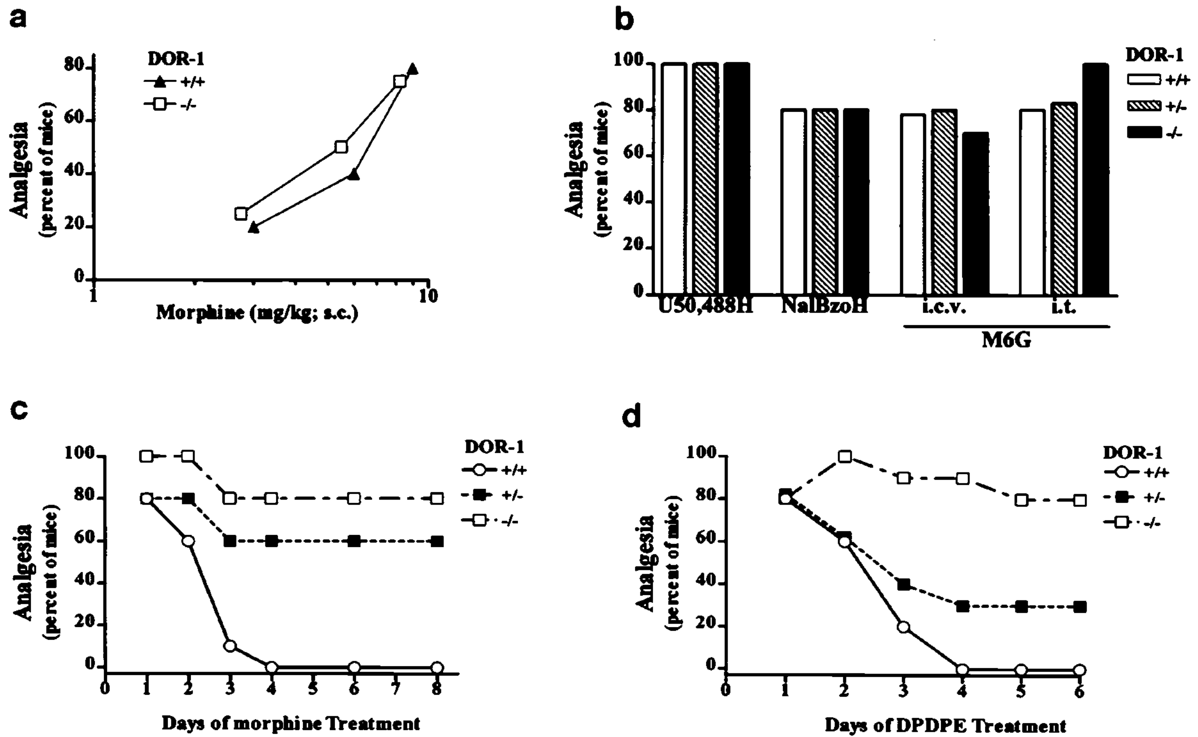

Figure 7. Analgesic Action of Non-Delta-Selective Opiates and Opioid Tolerance in *DOR-1* KO Mice

Groups of mice (n = 10) received morphine at indicated doses (a) or M6G (12.5 ng, i.c.v. or 12.5 ng, i.t.), U50,488 (5 mg/kg, s.c.), or NalBzoH (50 mg/kg, s.c.) (b). Mice receiving M6G were tested for analgesia 15 min later, and mice treated with morphine, U50,488, or NalBzoH were tested for analgesia 30 min later. Significance was analyzed by the Fisher exact test.

(c) Groups of mice (n = 20 per group) received morphine (5 mg/kg, s.c.) daily for 10 days. Analgesia was tested at the indicated times. After 10 days of morphine treatment, the wild-type mice exhibited a significant decrease in morphine analgesia, whereas the homozygote group exhibited no change in analgesia.

(d) Groups of mice (n = 10 per group) received DPDPE (8 μg, i.c.v.) daily for 5 days. Analgesia was tested at the indicated times. DPDPE analgesia in wild-type mice was lost by the fourth day, while the response in the *DOR-1* homozygous mice was unaltered.

nervous system. Our results indicate that *DOR-1* encodes both the δ_1 and δ_2 subtypes and significantly extend antisense studies implicating DOR-1 in δ analgesia (Standifer et al., 1994; Rossi et al., 1997). Equally important, characterization of the *DOR-1* KO mice has also uncovered a novel δ analgesic system not previously observed. Finally, these investigations have documented the critical importance of δ receptors in the development of opioid tolerance. These observations provide new perspectives for understanding the pharmacology of opioid receptors and the central role of the δ receptor in in vivo action of the opioid system.

Table 1. Effects of Chronic Morphine Administration in Wild-Type and *DOR-1* KO Mice

	Morphine ED_{50} (mg/kg, s.c.) (95% Confidence Limits)		
	Naive Mice	Chronically Treated Mice	Shift
Wild-type (+/+)	4.8 (3.9, 5.9)	13.4 (11.6, 15.4)	2.8
DOR-1 knock-out (−/−)	4.3 (3.0, 6.0)	3.7 (2.9, 4.7)	0.9

Groups of mice (n = 10) were used to determine ED_{50} values in wild-type or *DOR-1* knockout mice. Groups were then treated daily with morphine (5 mg/kg, s.c.), and ED_{50} values were determined again after 10 days with a cumulative dose–response paradigm. Significance was determined by the lack of overlap of 95% confidence limits. The shift in the wild-type mice was significant, while that of the knockout mice was not. The experiment was repeated twice.

Experimental Procedures

Gene Targeting and Production of Mutant Mice

A *DOR-1* genomic clone was isolated from a Swiss Webster 129/ReJ mouse genomic library after screening with a full-length (1.8 kb) *DOR-1* cDNA (a gift from Dr. Chris Evans, University of California, Los Angeles) probe generated by the random primed digoxigenin DNA labeling method (Boehringer Mannheim). This clone contained exons 2 and 3 of *DOR-1*. A 2.1 kb SpeI–BamHI fragment corresponding to the 5′ untranslated region right before exon 2 of *DOR-1* and a 4.8 kb KpnI–HindIII fragment corresponding to the 3′ region outside exon 2 of *DOR-1* were subcloned into a targeting construct containing the *neomycin* resistance cassette and the *HSV-TK* gene (*thymidine kinase* gene from the herpes simplex virus). The latter fragment contains exon 3 of *DOR-1*. Deletion of exon 2 eliminates sequences encoding amino acids extending from the beginning of intracellular loop 1 through transmembrane 4 (TM4) and introduces a frameshift into the *DOR-1* coding region that would completely disrupt the sequence downstream of Arg-76 and eliminate additional TM domains and G protein binding sites if any translation occurred of residual transcripts containing direct splicing of *DOR-1* exon 1 to *DOR-1* exon 3. The targeting vector was linearized and introduced into ES cells by electroporation. ES cells were selected for homologous recombination by culture in medium containing G418 and 2 μM gancyclovir on days 2–7 following electroporation. DNA extracted from the selected clones was digested with KpnI and screened by Southern blotting using an external 0.6 kb HindIII–DraII

fragment 3′ to the region included in the targeting construct. Screening of all of the available ES cell clones isolated following electroporation identified three correctly targeted clones, which were subsequently injected into C57B16/J blastocysts to generate chimeras. Germline transmitters derived from two targeted ES cell lines were identified and used to establish lines of *DOR-1* mutant mice. Offspring of germline-transmitting chimeras were genotyped as described above. Heterozygous mice were crossed to C57Bl6/J mice, and littermate males, generally from matings of F2 and F3 generation heterozygous mice, were used in essentially all behavioral experiments. A few more recent experiments have used littermates from heterozygous/heterozygous matings expanded from F4 and F5 generation backcrosses to C57Bl6/J.

Immunocytochemistry

Mice were perfused and processed for immunohistochemistry as previously described (Arvidsson et al., 1995). After perfusion fixation (4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 6.9), the brains were removed and placed in 10% sucrose overnight. Cryostat sections (14 μm) were thaw mounted onto gelatin-coated slides. Incubation in primary antiserum (anti-mouse DOR-1 3–17 [1:1000]; Arvidsson et al., 1995) was carried out at 4°C overnight followed by incubation in secondary antisera (cyanine 3.18 [1:200]; Jackson Immunoresearch) for 1 hr at room temperature. All images were acquired with a BioRad MRC1000 confocal microscope equipped with an argon laser.

In Situ Hybridization

Cryostat brain sections were prepared from both wild-type and homozygous *DOR-1* mutant mice and hybridized as described (Zheng and Pintar, 1995). ^{33}P-UTP-labeled single-stranded RNA probes were synthesized and purified in vitro from the plasmid vectors harboring the appropriate cDNA sequences. The *DOR-1* probe was synthesized from an exon 2–derived 282 bp BalI–SmaI cDNA fragment. The μ receptor probe was synthesized from a 346 bp fragment (nucleotides 237 to 108) of the μ receptor cDNA (a gift from Dr. Christopher Evans, University of California, Los Angeles). The *KOR-1* probe was synthesized from a 376 bp PstI–EcoRI fragment (nucleotides 172 to 548) of the κ receptor cDNA (a gift from Dr. Graeme I. Bell). The *PENK* RNA probe was synthesized from a 520 bp PstI fragment of *PENK* cDNA. The *POMC* probe was synthesized from a 220 bp *POMC* cDNA fragment (exon 1 and exon 2). The *PDYN* probe was synthesized from a *PDYN* cDNA fragment (nucleotides 292–1900) (provided by Dr. James Douglass, Vollum Institute, Portland, OR).

Ligand Binding on Brain Homogenates

Wild-type, heterozygous, and homozygous δ receptor mutant mouse brains were homogenized in 60 vol of 50 mM Tris HCl (pH 7.4) at 4°C. The resulting homogenates were centrifuged at 30,000 × g for 15 min at 4°C, and the pellets were resuspended in 60 vol of the same buffer. The homogenates were centrifuged at 30,000 × g for 15 min. The resulting pellets were homogenized in 60 vol of the same buffer and then incubated for 30 min at 37°C in a water bath in order to dissociate endogenous ligands from the receptors. After a third centrifugation, the pellets were resuspended in 60 vol of 50 mM Tris HCl (pH 7.4). Protein concentrations were determined by the Lowry procedure (Sigma).

Aliquots (∼0.3 mg protein) of freshly prepared homogenates were incubated with eight concentrations of ^{3}H-DPDPE (120 min at 25°C), ^{3}H-naltrindole (30 min at 25°C), ^{3}H-deltorphin-2 (45 min at 35°C), ^{3}H-DAMGO (45 min at 4°C), and ^{3}H-U69,593 (45 min at 25°C) in 50 mM Tris HCl (pH 7.4). Concentrations of ^{3}H-DPDPE, ^{3}H-naltrindole, ^{3}H-U69,593, and ^{3}H-DAMGO ranged from 0.1 to 10 nM, while those for ^{3}H-deltorphin-2 were between 0.25 and 12 nM. All radioligands were purchased from New England Nuclear (Boston, MA). Nonspecific binding was assessed by inclusion of 10 nM naloxone, an opioid antagonist. Free radioligand was separated from radioligand bound to receptor by filtration under reduced pressure through Whatman GF/B filters with a Brandel cell harvester. Filters were washed with two (for DPDPE and DAMGO) or three (for U69,593, naltrindole and deltorphin-2) aliquots of 50 mM Tris HCl (pH 7.4) at 4°C and assayed by liquid scintillation spectrometry. Binding affinities and capacities were determined by Scatchard analysis using the LIGAND program of Munson and Rodbar (Munson and Rodbar, 1980).

Receptor Autoradiography

DORs were visualized in brain sections following receptor autoradiography (Unterwald, 1994). Briefly, brains from *DOR-1* (+/+) and *DOR-1* (−/−) mice were coembedded in OTC and fresh frozen, and 10 μm sections were prepared. Sections were preincubated for 30 min in 50 mM Tris HCl (pH 7.4) at room temperature. Sections were then incubated in the same buffer containing 5 nM ^{3}H-DPDPE for 2 hr at 21°C. Adjacent sections were incubated under the same conditions in a solution containing radiolabeled ligand in the presence of 10 μM naloxone to assess nonspecific binding. Sections were then washed in ice-cold 50 mM Tris HCl (6 × 20 s), dried under a stream of cold air, and stored at room temperature for 24 hr. Labeled tissue sections were then exposed to tritium-sensitive Hyperfilm-^{3}H for 12 weeks and subsequently developed using D-19.

Analgesia Testing

Deltorphin-2 was synthesized by the Core Facility at Memorial Sloan-Kettering Cancer Center and purified by high-pressure liquid chromatography, and its structure was verified by mass spectroscopy. Naltrexone, β-FNA, nor-BIN, morphine sulfate, morphine-6β-glucuronide, DPDPE, and U50,488 were gifts from the Research Technology Branch of the National Institute on Drug Abuse. Naloxone benzoylhydrazone (NalBzoH) was synthesized as described previously (Luke et al., 1988). Naltrindole was purchased from Research Biochemicals International. Male mice from heterozygous/heterozygous matings of F2 and F3 generation *DOR-1* (+/−) mice were used in essentially all analgesic tests. Drugs were administered i.c.v. (Haley and McCormick, 1957) or i.t. via lumbar puncture (Hylden and Wilcox, 1980). Analgesia of male mice was assessed by either the tailflick or the hot plate assay. The radiant heat tailflick assay utilized a light intensity that produced baseline latencies ranging from 2 to 3 s. A 10 s cutoff was imposed to minimize tissue damage. Analgesia or antinociception was defined as a doubling or greater of the baseline latency for an individual mouse. Group comparisons were performed by the Fischer exact test. The hot plate was set at 52°C, producing baseline latencies ranging from 16 to 18 s. A 40 s cutoff was imposed to minimize tissue damage. Statistical differences were determined by a two-tailed t-test with a 95% confidence interval. ED_{50} values with 95% confidence limits were determined with the Bliss program, as previously described (Rossi et al., 1997). For formalin testing, mice were injected with 20 μl of 5% formalin into the hindpaw. After formalin injection, time spent in licking, flinching, and rearing events was recorded for 30 min and plotted in 5 min time intervals. Statistical significance was determined by ANOVA. The *DOR-1* exon 3 antisense oligodeoxynucleotide was based on the mouse *DOR-1* sequence (Evans et al., 1992; Kieffer et al., 1992) and corresponded to nucleotide positions 662–681 (AACACG CAGATCTTGGTCAC). The mismatch antisense probe was CGAGC GCAACAGCTGCAT. These oligodeoxynucleotides were synthesized by Midland Certified Reagent (Midland, TX), purified, and dissolved in 0.9% saline. Mice received i.c.v. injections of oligonucleotides (5.0 μg in 2.0 μl) on days 1, 3, and 5 and were tested on day 6, as previously described (Standifer et al., 1994; Rossi et al., 1997).

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