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DISPARATE SPINAL AND SUPRASPINAL OPIOID ANTINOCICEPTIVE RESPONSES IN β-ENDORPHIN-DEFICIENT MUTANT MICE

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Abstract—The role of endogenous opioid systems in the analgesic response to exogenous opiates remains controversial. We previously reported that mice lacking the peptide neurotransmitter β-endorphin, although unable to produce opioid-mediated stress-induced antinociception, nevertheless displayed intact antinociception after systemic administration of the exogenous opiate morphine. Morphine administered by a peripheral route can activate opioid receptors in both the spinal cord and brain. However, β-endorphin neuronal projections are confined predominantly to supraspinal nociceptive nuclei. Therefore, we questioned whether the absence of β-endorphin would differentially affect antinociceptive responses depending on the route of opiate administration. Time- and dose–response curves were obtained in β-endorphin-deficient and matched wild-type C57BL/6 congenic control mice using the tail-immersion/withdrawal assay. Null mutant mice were found to be more sensitive to supraspinal (i.c.v.) injection of the μ-opioid receptor-selective agonists, morphine and D-Ala^2-MePhe^4-Gly-ol^5 enkephalin. In contrast, the mutant mice were less sensitive to spinal (i.t.) injection of these same drugs. Quantitative receptor autoradiography revealed no differences between genotypes in the density of μ, δ, or κ opioid receptor binding sites in either the spinal cord or pain-relevant supraspinal areas.

Thus we report that the absence of a putative endogenous ligand for the μ-opioid receptor results in opposite changes in morphine sensitivity between discrete areas of the nervous system, which are not simply caused by changes in opioid receptor expression.
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Key words: analgesia, knockout, morphine, μ receptor, transgenic mice.

β-Endorphin, one of several biologically active peptide products of the proopiomelanocortin (POMC) gene,[4] is a member of a family of endogenous opioid peptides that includes the products of the proenkephalin (e.g., met- and leu-enkephalin) and prodynorphin (e.g., dynorphin A) genes. Endogenous opioids are importantly involved in the modulation of pain[18] and a variety of physiological adaptations to stress. β-Endorphin is a potent analgesic when injected into the cerebrospinal fluid of mice and rats;[14,41] however, the identity of the opioid receptor subtype which mediates β-endorphin's effects on pain is unresolved. The peptide has equal affinity for both μ and δ receptors[28] and may have its own unique binding site called ε.[26]

The specific roles of each class of opioid peptide in pain modulation remain controversial, since a plethora of evidence has been collected supporting the importance of all three ligand classes. Intrinsic opioid neurons in antinociception-relevant loci of the brain express enkephalin or dynorphin, whereas these same regions receive afferent β-endorphinergic input from the arcuate nucleus and nucleus tractus solitarius.[4] A recent microdialysis study directly demonstrated the release of β-endorphin in the arcuate nucleus upon induction of a peripheral nociceptive stimulus.[47] The substantia gelatinosa of the spinal cord is also rich in enkephalin and dynorphin peptides, but has minimal expression of β-endorphin.

Although highly selective pharmacological reagents that distinguish among the different opioid receptor subtypes have been developed, no such drugs exist to clearly define the physiological actions of the cognate peptide ligands. An alternative genetic approach to investigate the functional role of individual opioid peptide precursors or opioid receptors in antinociception involves the generation and testing of null mutant mice lacking expression of the respective genes. Knockout mice lacking β-endorphin, enkephalins, dynorphin, μ-, δ and κ-receptors and ORL1 (orphanin FQ/nociceptin) receptors have been produced and characterized.[8,19]

A comparison of phenotypic alterations between β-endorphin-deficient (C57BL/6-*Pomc1*^{tm1Low}) and enkephalin-deficient (Hsd:ICR-*Penk2*^{tm1Pig}) mice illustrates the utility of the genetic approach. Opioid-mediated (i.e. naloxone-reversible) endogenous antinociception or stress-induced antinociception was abolished in the former[32] and preserved in the latter[12] animals. Together, these data indicate that β-endorphin, but not enkephalin, is a necessary neurochemical component of opioid-mediated stress-induced antinociception. Despite the complete absence of β-endorphin in C57BL/6-*Pomc1*^{tm1Low} mice, morphine antinociception after systemic (i.p.) administration was unaltered in two separate nociceptive assays (hot-plate and abdominal constriction tests) compared to wild-type littermates.[12] This finding

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Abbreviations: ACTH, adrenocorticotropic hormone; AD_{50}, half-maximal antinociceptive dose; ANOVA, analysis of variance; CI, confidence interval; DAMGO, D-Ala^2-MePhe^4-Gly-ol^5 enkephalin; DELT, D-Ala^2-deltorphin II; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; KO, knockout; MPE, maximal possible effect; OFQ/N, orphanin FQ/nociceptin; ORL1, OFQ/N receptor 1; PAG, periaqueductal gray; POMC, proopiomelanocortin; WT, wild-type.

suggested that the absence of one endogenous ligand for the μ-receptor did not alter any of the neurochemical substrates responsible for the antinociceptive response to systemically administered morphine.

We hypothesized that the absence of β-endorphin would alter exogenous opiate sensitivity in a region-selective manner not revealed by the systemic administration of morphine. Therefore, we constructed dose–response curves for morphine and D-Ala^2-MePhe^4-Gly-ol^5 enkephalin (DAMGO) administered selectively to the spinal and supraspinal compartments via i.t. and i.c.v. injection, respectively. Identical morphine injections were performed in the two progenitor strains of mice used to produce the β-endorphin mutants as a control for the influence of genetic background on opiate sensitivity. Since altered morphine antinociception has been observed in rodent populations with altered receptor density,[1,21,25] we also evaluated opioid-receptor binding sites of wild-type and knockout mice by quantitative autoradiography.

EXPERIMENTAL PROCEDURES

β-Endorphin mutant mice

The gene targeting vector, POMCX*4, and production of β-endorphin null mutant mice are described fully in Rubinstein *et al.*[32] In brief, a point mutation was introduced by site-directed mutagenesis into exon 3 of the POMC gene to generate a premature translational stop codon. The resultant truncated prohormone lacks the carboxyl-terminal 31 amino acids composing β-endorphin, but is expressed at normal levels and correctly processed to adrenocorticotropic hormone (ACTH), melanocyte stimulating hormones and γ-lipotropic hormone. The mutant allele was originally introduced into 129S2/SvPas-derived D3 embryonic stem cells and subsequently backcrossed for five successive generations to C57BL/6N mice (Simonsen, Gilroy, CA) and then an additional two or four generations to C57BL/6J mice (The Jackson Laboratories, Bar Harbor, ME). The β-endorphin wild-type (WT) and knockout (KO) mice used in the present experiments were derived at either the N7 or N9 generation on a C57BL/6 background from heterozygote mating pairs, WT mating pairs or KO mating pairs in roughly similar proportions. Mice were genotyped as described previously[32] or with an alternative polymerase chain reaction protocol using a combination of three oligonucleotide primers: 5′-ATG ACC TCC GAG AAG AGC CAG-3′ (POMC exon 3), 5′-GAG GAT TGG GAA GAC AAT AGC A-3′ (PGK-neo cassette, specific for the targeted allele), and 5′-GCT GGG GCA AGG AGG TTG AGA-3′ (3′ flanking sequence in POMC gene). The amplified products are approximately 300 bp and 100 bp for the WT and KO alleles, respectively.

Mice were either tested in Portland, OR, or transported from there to Champaign, IL, or Greenville, SC, and habituated to the new vivariums for at least four weeks before testing. Additional inbred mice were purchased from commercial suppliers: 129S6/SvEvTac (129S6; Taconic Farms, Germantown, NY) and C57BL/6J (C57BL/6; The Jackson Laboratories, Bar Harbor, ME). All mice were housed in a group of three to five per cage in a temperature-controlled environment, maintained on a 12 h light/dark cycle, and fed and watered *ad libitum*. All experimental protocols were approved by the appropriate institutional animal care and use committees at our institute, followed the guidelines in the Public Health Service Guide for the Care and Use of Laboratory Animals, and minimized both the suffering and number of animals used.

Nociceptive testing

Acute, thermal nociceptive thresholds were obtained using the tail-immersion/withdrawal assay. This assay was chosen because of its stability with repeated testing at high stimulus intensities, allowing the construction of full antinociceptive time–response curves. Mice were lightly restrained in a cloth/cardboard "pocket" entered voluntarily in virtually every case. The distal half of the tail was then dipped into a bath of circulating water thermostatically controlled at 49.0 ± 0.2°C. Latency to respond to the heat stimulus with vigorous, escape-directed flexion of the tail was measured to the nearest 0.1 s. Two separate withdrawal latency determinations (separated by 20 s) were averaged. Careful attention was paid to ensure that the ambient temperature was maintained at 22–23°C. A cut-off latency of 10 s was employed to avoid potential tissue damage. Each mouse was used for a single time–response curve. Both male and female mice were tested in equal numbers (± 1) for each injection route, injection dose and genotype.

Drug injections

Morphine sulfate and DAMGO were generously supplied by the Research Technology Branch of the National Institute for Drug Abuse (Rockville, MD). Drugs were dissolved in 0.9% physiological saline or artificial cerebrospinal fluid (140 mM NaCl, 3.36 mM KCl, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.38 mM glucose, 6.3 mM PO_4^{-3}, pH 7.4).

Systemic injections were made i.p. using an injection volume of 10 ml/kg; i.c.v. injections were made directly into the left lateral ventricle through the coronal suture following the method of Laursen and Belknap;[13] i.t. injections were made at L4–L5 following the method of Hylden and Wilcox.[10] In both cases, 2.5 μl of drug was injected under isoflurane/oxygen anesthesia, using a 10 μl Hamilton microsyringe attached to a 3 mm long, 27-gauge needle (for i.c.v. injections) or a 1 cm long, 30-gauge needle (for i.t. injections).

Experimental protocol

After establishing basal nociceptive thresholds, each mouse was briefly anesthetized, and injected (i.p., i.c.v., or i.t.) with vehicle or drug. Tail-withdrawal latencies were assessed 15, 30 and 60 min later. Drug doses were based on pilot experiments with strain 129S6 and C57BL/6 mice. The latter strain was found to be particularly insensitive to morphine antinociception (see Fig. 4 and Table 2), as has been reported previously.[23] Morphine doses used to construct dose–response curves ranged from 1 to 100 mg/kg (i.p.), 1 to 100 μg (i.c.v.), and 50 to 250 μg (i.t.). DAMGO doses used to construct dose–response curves ranged from 0.2 to 0.8 nmol (i.c.v.) and 1 to 10 nmol (i.t.). Sample sizes were $n = 5$–18/strain/drug/dose, based on animal availability.

Statistical analysis

Antinociception was expressed as the peak percentage of the maximum possible effect (%MPE) at any post-injection time-point. %MPE was calculated as follows: [(post-injection latency − baseline latency)/(cut-off latency − baseline latency) × 100%]. The use of %MPEs takes into account the cut-off latency and individual baseline latencies, so that these will not bias the quantification of antinociception. Peak %MPE data were subjected to two-way (sex × genotype; dose × genotype) analyses of variance (ANOVAs). Half-maximal antinociceptive dose (AD_{50}) estimates, potency ratios and corresponding 95% confidence intervals (CIs) were calculated using the method of Tallarida and Murray.[38] The criterion level of significance was chosen as $\alpha = 0.05$. Data were also analysed with respect to areas under the tail-withdrawal latency × time curve, and yielded qualitatively similar conclusions for each data set.

Receptor autoradiography

Four adult WT and KO male mice were used for an autoradiographic survey of opioid binding sites. The mice were killed by CO_2 inhalation and brains were rapidly removed, embedded in OCT solution and frozen in a dry-ice, isopentane bath. Frozen, cryostat sections 15 μm thick were thaw mounted on poly-L-lysine coated slides, dried overnight in a desiccated vacuum chamber at 4°C and stored at −80°C. Immediately before use, the slides were brought to room temperature in a desiccated vacuum chamber and the tissue sections incubated with various tritiated opioid ligands in 50 mM Tris buffer (pH 7.5) for 60 min in a humid chamber at room temperature. The radiolabeled opioid concentrations used were 4.9 nM DAMGO at a specific activity of 55.3 Ci/mmol, 36 nM [D-Pen^2,D-Pen^5]enkephalin (DPDPE) at a specific activity of 36.0 Ci/mmol and 21 nM U69,593 at a specific activity of 47.5 Ci/mmol (New England Nuclear, Boston, MA). The tritiated ligands were drained off after the incubation period and the slides were washed in four consecutive changes of ice-cold 50 mM Tris (pH 7.5; 4°C), then rapidly dried with an unheated hair dryer. Autoradiography was performed in X-ray cassettes using Hyperfilm™-^{3}H (Amersham, Arlington Heights, IL) exposed to the slides

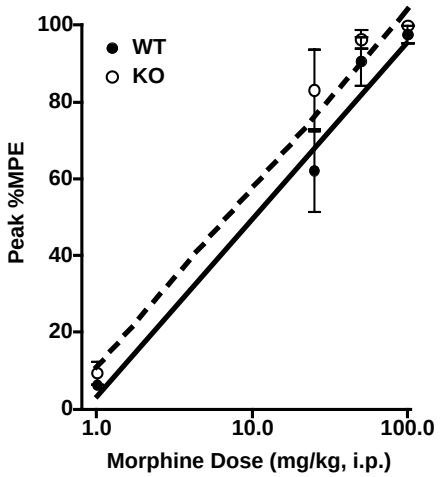

Fig. 1. Antinociception induced by systemic administration of morphine in WT and β-endorphin-deficient mice. Acute, thermal nociceptive thresholds were obtained using the tail-immersion/withdrawal assay and were assessed at baseline and 15, 30 and 60 min after administration of morphine (1, 25, 50 and 100 mg/kg; i.p.) in WT (WT, solid regression line) and β-endorphin-deficient (KO, dotted regression line) mice. Antinociception was expressed as the peak percentage of the maximum possible effect (%MPE, defined in Experimental Procedures) at any post-injection time-point. Symbols represent mean ± S.E.M. %MPE, $n = 5$–6/genotype/dose. There was no difference in sensitivity between genotypes ($F_{1,46} = 2.6$).

for three weeks for [^{3}H]DAMGO and [^{3}H]DPDPE and five weeks for [^{3}H]U69,593. The films were developed in Kodak D-19 (1 min, 19°C), rinsed in water for 30 s and fixed in Kodak Rapidfix (1 min, 19°C). The films were then washed in running water for at least 5 min and dried in a warm drying chamber.

For the purposes of standardization and quantification each autoradiography cassette contained a [^{14}C]microscale (Amersham), which was used to convert the labeled polymer activity to an estimated tissue equivalent. The developed autoradiograph films were scanned with a desktop laser scanner at 300 d.p.i. and two-times magnification and stored as TIFF files. NIH Image version 1.6 (available at http://rsb.info.nih.gov/nih-image/) was used to quantify scanned films by setting a calibration scale using values from the [^{14}C]microscale to convert densitometric values to nCi/g of wet tissue. Measurements were taken by carefully circumscribing specific anatomic regions based on a series of matched Nissl-stained slides and subtracting non-specific background density from a region known to be devoid of opioid receptors and where little or no binding was present (cerebellum for μ receptors). A minimum of 10 sections was counted from each animal for binding measurements in the dorsal horn of lumbar spinal cord and lateral and ventrolateral regions of the periaqueductal gray (PAG) corresponding to interaural coordinates spanning from 0.72 mm to −0.24 mm as given in the atlas of Franklin and Paxinos.[5] Measurements were averaged for each animal, then expressed as a mean ± S.E.M. of the four animals for each genotype.

RESULTS

Baseline tail-withdrawal latencies and effect of vehicle injection

Combining data from all experiments, a significant KO > WT difference in baseline 49°C tail-withdrawal latency was observed (3.0 ± 0.1 vs 2.7 ± 0.1 s, respectively; $t_{338} = 2.65$, $P < 0.005$). This small genotypic difference had not been seen previously; the present study, however, provided far greater statistical power owing to the very large number of total subjects. Indeed, the difference was significant in only one individual experiment in the present study (WT vs KO, i.c.v. DAMGO).

Repeated-measure ANOVAs performed on data from vehicle-treated mice (not shown) revealed that all routes of injection produced post-injection tail-withdrawal latency increases (i.p.: $F_{3,30} = 3.89$, $P < 0.05$; i.c.v.: $F_{3,15} = 7.61$, $P < 0.005$; i.t.: $F_{3,72} = 9.69$, $P < 0.001$). These increases ranged from approximately 0.5 s (i.p.) to 1.2 s (i.t.) and probably represent stress-induced analgesia produced by the injection and/or testing procedure, as we have previously demonstrated.[20] In no case, however, was there a significant interaction of genotype × repeated measure, indicating that the small increase was approximately the same in both genotypes and likely to be non-opioid.

Significant main effects of sex on baseline nociceptive sensitivity were found in some experiments (WT vs KO i.c.v. morphine, $F_{1,43} = 16.3$, $P < 0.001$; WT vs KO i.c.v. DAMGO, $F_{1,32} = 6.9$, $P < 0.05$; WT vs KO i.t. DAMGO, $F_{1,53} = 23.4$, $P < 0.001$). In addition, significant main effects of sex on opioid antinociception were found in one experiment (WT vs KO i.t. morphine, $F_{1,84} = 10.0$, $P < 0.005$). In no case, however, was a significant sex × genotype interaction noted, and the numbers of males and females of each genotype were precisely matched (within $n = 1$ of each other) in each experiment, so data from both sexes were pooled for the analyses reported below.

Table 1. Antinociceptive sensitivity of wildtype and β-endorphin-deficient mice to systemic (i.p.) morphine, and supraspinal (i.c.v.) and spinal (i.t.) morphine and D-Ala^2-MePhe^4-Gly-ol^5 enkephalin

Drug	Route	Genotype	AD_{50} (CI)*	Potency ratio (CI)†
Morphine	i.p.	WT	9.3 (6.6–13.1)	
		KO	6.4 (5.0–8.4)	1.4 (0.8–2.2)
	i.c.v.	WT	24.9 (11.9–52.0)	
		KO	4.0 (2.5–6.3)	6.3 (2.6–14.9)‡
	i.t.	WT	60.3 (49.7–73.0)	
		KO	106.3 (76.5–147.8)	0.6 (0.4–0.8)§
DAMGO	i.c.v.	WT	0.48 (0.35–0.65)	
		KO	0.35 (0.27–0.45)	1.4 (0.9–2.1)
	i.t.	WT	1.5 (0.5–4.3)	
		KO	27.5 (10.5–72.3)	0.05 (0.01–0.23)§

Acute, thermal nociceptive thresholds were obtained using the tail-immersion/withdrawal assay and were assessed at baseline and 15, 30 and 60 min after treatment. Morphine doses used to construct dose–response curves ranged from 1 to 100 mg/kg (i.p.), 1 to 100 μg (i.c.v.), and 50 to 250 μg (i.t.). DAMGO doses used to construct dose–response curves ranged from 0.2 to 0.8 nmol (i.c.v.) and 1 to 10 nmol (i.t.). Sample sizes were $n = 5$–18/genotype/drug/dose. Antinociception was expressed as the peak percentage of the maximum possible effect at any post-injection time point. Half-maximal antinociceptive dose (AD_{50}) estimates, potency ratios and corresponding 95% confidence intervals (CIs) were calculated using the method of Tallarida and Murray.[38]

*Values are in mg/kg for i.p. morphine, μg/mouse for i.c.v. and i.t. morphine, and nmol/mouse for i.c.v. and i.t. DAMGO.

†Calculated as WT AD_{50}/KO AD_{50}.

‡Significantly greater than 1.0, $P < 0.05$.

§Significantly less than 1.0, $P < 0.05$.

Antinociceptive sensitivity to systemic morphine

In agreement with our previous findings using (129 × C57BL/6)F_2 mice on the hot-plate and abdominal constriction assays, incipient congenic C57BL/6 WT and KO mice were found to be equally sensitive to i.p. morphine inhibition of tail-withdrawal test nociception over a wide range of doses (see Fig. 1; Table 1). ANOVA revealed a main effect of dose ($F_{3,46} = 94.3$, $P < 0.001$); the main effects of genotype and dose × genotype interaction were not significant ($F_{1,46} = 2.6$; $F_{3,46} = 1.1$, respectively).

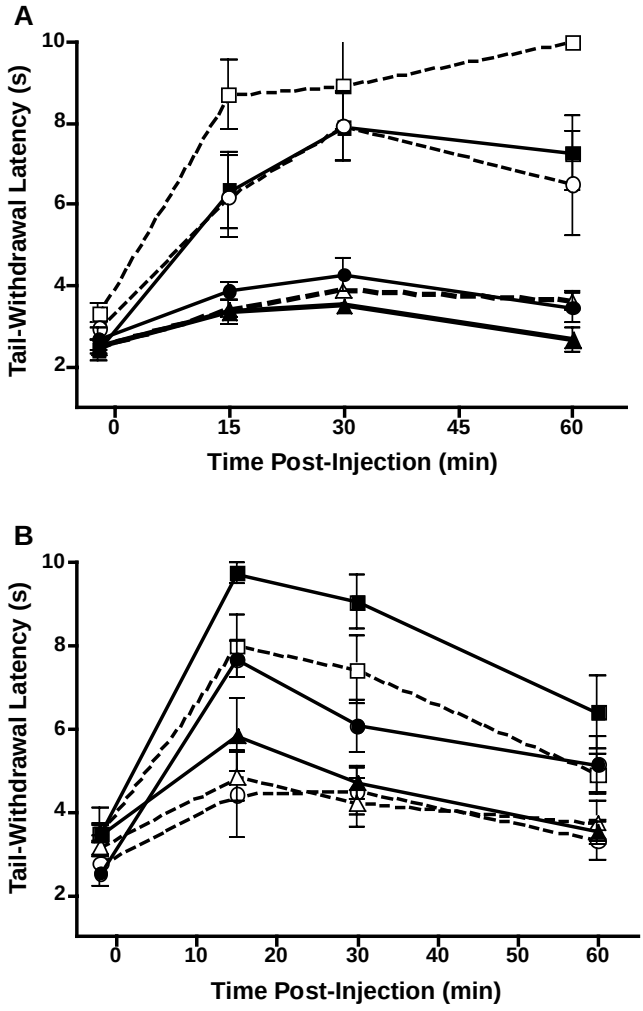

Fig. 2. Time-course of antinociception induced by supraspinal (i.c.v.) and spinal (i.t.) administration of morphine in WT and β-endorphin-deficient mice. Acute, thermal nociceptive thresholds were obtained using the tail-immersion/withdrawal assay and were assessed at baseline and 15, 30 and 60 min after administration of i.c.v. (A) or i.t. (B) morphine in WT (closed symbols; solid lines) and β-endorphin-deficient (open symbols; dotted lines) mice. Symbols represent mean ± S.E.M. tail-withdrawal latencies. For clarity, the responses to vehicle injection are not shown. (A) i.c.v. morphine doses were 1 μg, triangles; 10 μg, circles; and 100 μg, squares per mouse; $n = 6–8$/genotype/dose. (B) i.t. morphine doses were 50 μg, triangles; 100 μg, circles; and 200 μg, squares per mouse; $n = 8–10$/genotype/dose.

Antinociceptive sensitivity to supraspinal injection of morphine or D-*Ala*^{2}-*MePhe*^{4}-*Gly-ol*^{5} *enkephalin*

KO mice were found to be more sensitive to the antinociceptive effects of morphine (Figs 2A, 3A) and DAMGO (Fig. 3B) injected by the supraspinal route than WT mice. For i.c.v. morphine, ANOVA revealed significant main effects of dose ($F_{2,41} = 52.4$, $P < 0.001$) and genotype ($F_{1,41} = 26.6$, $P < 0.001$), and a significant dose × genotype interaction ($F_{2,41} = 6.8$, $P < 0.005$). For i.c.v. DAMGO, ANOVA revealed a significant main effect of dose ($F_{2,74} = 13.2$, $P < 0.001$). The main effect of genotype approached significance ($F_{1,74} = 3.5$, $P = 0.06$), whereas the dose × genotype interaction was not significant ($F_{2,74} = 1.2$). Morphine AD_{50} was shifted to the left in KO mice by a factor of 6.3; the corresponding 1.4-fold leftward shift for DAMGO approached significance (see Table 1).

Antinociceptive sensitivity to spinal injection of morphine or D-*Ala*^{2}-*MePhe*^{4}-*Gly-ol*^{5} *enkephalin*

KO mice were found to be less sensitive to the antinociceptive effects of both morphine (Figs 2B, 3C) and DAMGO (Fig. 3D) injected by the spinal route than WT mice. For i.t. morphine, ANOVA revealed significant main effects of dose ($F_{2,56} = 17.4$, $P < 0.001$) and genotype ($F_{1,56} = 10.1$, $P < 0.005$); the dose × genotype interaction was not significant ($F_{2,56} = 0.9$). For i.t. DAMGO, ANOVA revealed significant main effects of dose ($F_{2,47} = 5.4$, $P < 0.01$) and genotype ($F_{1,47} = 17.6$, $P < 0.001$); the dose × genotype interaction was not significant ($F_{2,47} = 0.2$). The AD_{50} of KO mice to i.t. morphine and i.t. DAMGO were significantly shifted to the right by factors of 1.7 and 20, respectively (see Table 1).

Antinociceptive sensitivity of 129*S*6 *and C*57*BL*/6 *strains to morphine*

Dose–response curves were compiled from these strains in order to evaluate the possible contribution of genetic background to the aforementioned findings. For i.c.v. morphine, ANOVA revealed significant main effects of dose ($F_{3,48} = 10.4$, $P < 0.001$) and strain ($F_{1,48} = 10.4$, $P < 0.005$), and a significant dose × strain interaction ($F_{3,48} = 3.0$, $P < 0.05$). For i.t. morphine, ANOVA revealed significant main effects of dose ($F_{3,57} = 32.8$, $P < 0.001$) and strain ($F_{1,57} = 22.5$, $P < 0.001$), and a significant dose × strain interaction ($F_{3,57} = 4.3$, $P < 0.01$). As shown in Fig. 4 and Table 2, the C57BL/6 strain displayed significantly lower antinociceptive sensitivity to morphine after both i.c.v. and i.t. injection compared to 129S6 mice by factors of 38 and 20, respectively.

Receptor autoradiography

Several different CNS regions were examined for binding to the μ receptor-specific ligand, [^{3}H]DAMGO. Qualitatively, the distribution of μ receptors throughout the brain was similar to that reported by other groups in mouse brain.[2,11,33] The PAG and the dorsal horn of the spinal cord are two regions that are important for the relay of ascending nociceptive information, and regions known to contain μ receptors that modulate this information. We did not find any quantitative differences between the two genotypes for receptor binding in these two regions or in amygdala, nucleus accumbens and thalamus (Table 3). An example of [^{3}H]DAMGO binding at the level of the PAG and in the dorsal horn of the spinal cord is shown in Fig. 5. We also examined radioligand binding to other opioid receptors in the PAG and in the spinal cord. [^{3}H]DPDPE binding (not shown) was identical between the two genotypes in both the PAG (WT: 18.1 ± 0.8 nCi/g; KO: 17.8 ± 3.9 nCi/g) and in the dorsal horn of the spinal cord (WT: 47.8 ± 2.3 nCi/g; KO: 49.2 ± 2.2 nCi/g). [^{3}H]U68,598H binding (not shown) was also identical between the two genotypes in both the PAG (WT: 17.8 ± 2.4 nCi/g; KO: 19.8 ± 2.1 nCi/g) and in the dorsal horn of the spinal cord (WT: 10.3 ± 0.8 nCi/g; KO: 10.2 ± 0.9 nCi/g).

DISCUSSION

In this study we demonstrate reciprocal alterations in supraspinal and spinal opioid antinociception in null mutant mice lacking β-endorphin. KO mice were found to be more

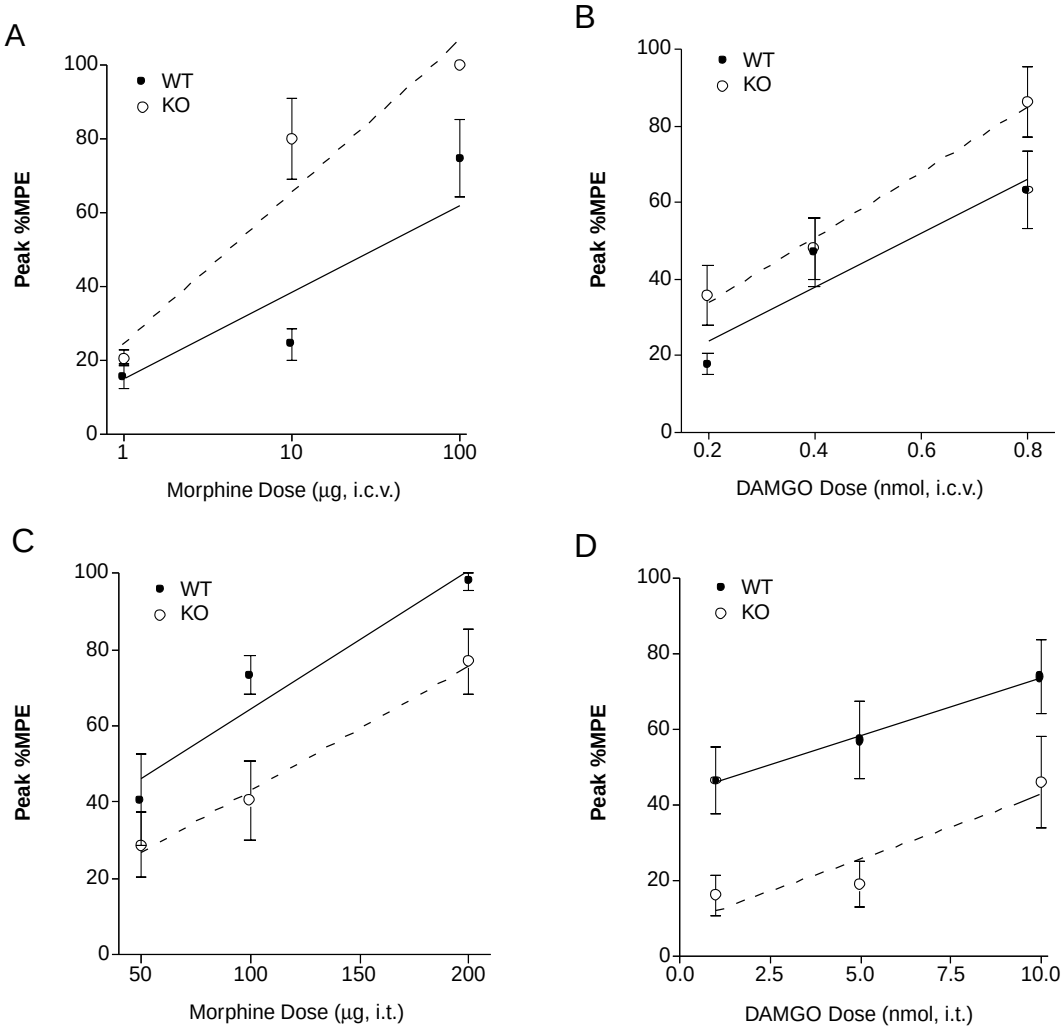

Fig. 3. Antinociception induced by supraspinal (i.c.v.) and spinal (i.t.) administration of μ-opioid receptor agonists in WT and β-endorphin-deficient mice. Acute, thermal nociceptive thresholds were obtained using the tail-immersion/withdrawal assay after administration of morphine (A, C) or DAMGO (B, D) by either i.c.v. (A, B) or i.t. (C, D) injection in WT (WT, closed circles, solid regression lines) and β-endorphin-deficient (KO, open circles, dotted regression lines) mice. Antinociception was expressed as the peak percentage of the maximum possible effect (%MPE, defined in Experimental Procedures) at any post-injection time-point (15, 30 or 60 min). Symbols represent mean ± S.E.M. %MPE, $n = 6–18$/genotype/drug/dose. Note the differing *x*-axis scales in each graph. KO mice showed higher supraspinal antinociception to morphine ($F_{1,41} = 26.6$, $p < 0.001$) and DAMGO ($F_{1,74} = 3.5$, $P = 0.06$) but lower spinal antinociception to morphine ($F_{1,56} = 10.1$, $P < 0.005$) and DAMGO ($F_{1,47} = 17.6$, $P < 0.001$) than WT mice.

and less sensitive, respectively, than their WT counterparts to antinociception from i.c.v. and i.t. morphine. Although morphine, especially at high doses, can bind to δ- and κ-opioid receptors,[36] the phenomenon described presently was shown to be selectively related to μ antinociception since parallel results were obtained with the μ-specific ligand, DAMGO. Consistent with our previous observations that β-endorphin deficiency does not significantly affect antinociceptive sensitivity to systemically administered morphine on the hot-plate and abdominal constriction tests, we demonstrate here that WT and KO mice display equivalent sensitivity to i.p. morphine on the tail-withdrawal test. A parsimonious explanation of these findings is that the opposing supraspinal and spinal alterations in KO mice counteract each other when morphine is injected systemically.

Low antinociceptive potency of opioid agonists in wild-type mice

The reported AD_{50} values of morphine and DAMGO in these experiments are higher than those commonly encountered in the literature. This discrepancy is due largely to the genetic background of the subjects used. The WT and KO mice tested here are in the process of being backcrossed to a congenic C57BL/6 background and are predicted to retain <1% strain 129 alleles at any given genetic locus that is not tightly linked to the *Pomc1* gene. C57BL/6 mice are well known for their insensitivity to opioid antinociception compared to other inbred strains (but most commonly DBA/2).[22] Virtually all of this previous work has been conducted using systemic administration; we demonstrate presently that the C57BL/6 strain is even more deficient in this trait following supraspinal and spinal administration. It can be seen from Tables 1 and 2 that the morphine AD_{50} values in WT mice are similar to those of C57BL/6 mice, as would be expected given the preponderance of C57BL/6 alleles in the WT (and KO) incipient congenic background.

However, the insensitivity of this background strain precluded a meaningful comparison of δ-opioid antinociception in WT and KO mice. Since β-endorphin has

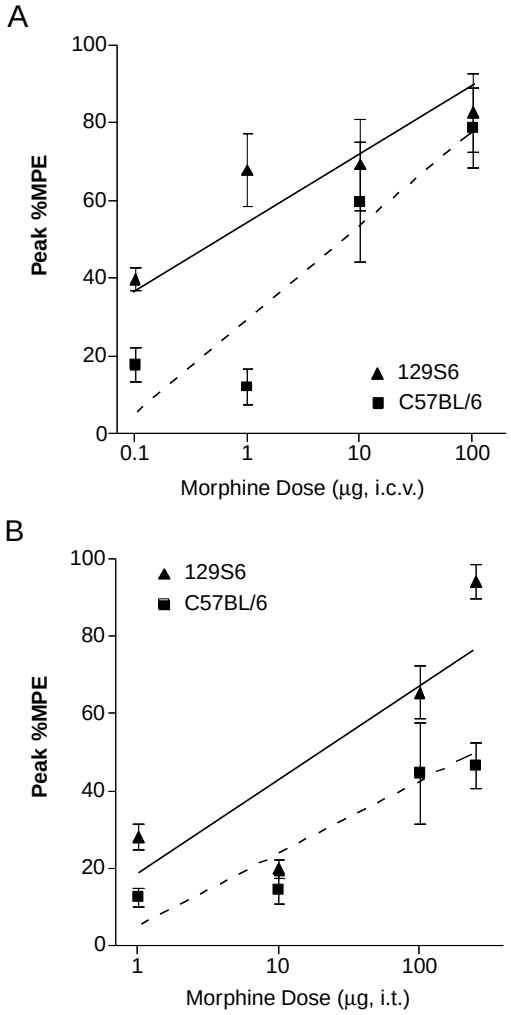

Fig. 4. Antinociception induced by supraspinal (i.c.v.) and spinal (i.t.) administration of morphine in two inbred strains of mice. Acute, thermal nociceptive thresholds were obtained using the tail-immersion/withdrawal assay after administration of morphine by either i.c.v. (A) or i.t. (B) injection in 129S6 (closed triangles, solid regression lines) and C57BL/6 (closed squares, dotted regression lines) mice. These two inbred strains represent the progenitor strains for the β-endorphin-deficient mice. Antinociception was expressed as the peak percentage of the maximum possible effect (%MPE, defined in Experimental Procedures) at any post-injection time point (15, 30 or 60 min). Symbols represent mean ± S.E.M. %MPE, $n = 6$–8/genotype/dose. 129S6 mice were markedly more sensitive to morphine adminstered either i.c.v. ($F_{1,48} = 10.4$, $P < 0.005$) or i.t. ($F_{1,57} = 22.5$, $P < 0.001$) than C57BL/6 mice.

approximately equal affinity for μ and δ receptors,[28] it is possible that δ antinociception in addition to μ antinociception has been altered in KO mice. However, we could not obtain reliable antinociception in WT, KO or C57BL/6 mice from any δ agonist. For example, DPDPE doses up to 1000 nmol were injected both i.c.v. and i.t., and failed to affect tail-withdrawal latencies in WT mice even though the same maximal dose was lethal in more sensitive strains (129S6 and DBA/2). Similarly, D-Ala^2-deltorphin II (DELT) doses up to 50 nmol and SNC 80 doses up to 400 nmol were ineffective by both routes in WT mice. Solubility and availability limitations prevented an investigation of higher doses.

Since the phenotypic effect of a null mutation is dependent on epistatic interactions with other genes, it remains to be determined whether the functional alterations in opioid

Table 2. Antinociceptive sensitivity of 129S6 and C57BL/6 mice to supraspinal (i.c.v.) and spinal (i.t.) morphine

Drug	Route	Genotype	AD_{50} (CI)*	Potency ratio (CI)†
Morphine	i.c.v.	129S6	0.20 (0.03–1.18)	
		C57BL/6	7.7 (2.7–21.8)	38.5 (4.9–333.3)‡
	i.t.	129S6	19.0 (11.1–32.6)	
		C57BL/6	379.0 (118.7–1211)	20.0 (5.5–71.4)‡

Acute, thermal nociceptive thresholds were obtained using the tail-immersion/withdrawal assay and were assessed at baseline and 15, 30 and 60 min after treatment. Morphine doses used to construct dose–response curves ranged from 0.1 to 100 μg (i.c.v.) and 1 to 250 μg (i.t.). Sample sizes were $n = 8$–10/genotype/dose. Antinociception was expressed as the peak percentage of the maximum possible effect at any post-injection time point. Half-maximal antinociceptive dose (AD_{50}) estimates, potency ratios and corresponding 95% confidence intervals (CIs) were calculated using the method of Tallarida and Murray.[38]

*Values are in μg/mouse.

†Calculated as C57BL/6 AD_{50}/129S6 AD_{50}.

‡Significantly different from 1.0, $p < 0.05$.

Table 3. Quantitative autoradiography of [^{3}H]DAMGO binding in wildtype and β-endorphin-deficient mice in several μ-receptor-dense regions of the nervous system

Region	WT	KO
Amygdala	47.3 ± 2.6	54.5 ± 5.5
Dorsal horn, spinal cord	22.0 ± 1.1	21.8 ± 0.7
Nucleus accumbens	77.3 ± 13.7	72.1 ± 11.1
Periaqueductal gray	47.1 ± 1.4	49.4 ± 3.0
Thalamus	86.0 ± 6.0	80.9 ± 5.2

Mean specific binding ($n = 4$) of the μ-receptor selective ligand [^{3}H]DAMGO in nCi/g tissue ± S.E.M. with regional determinations made from both right and left sides of sections. There are no significant differences between genotypes in any region.

sensitivity observed after ß-endorphin inactivation are specific to the present genetic background or will generalize widely. Because of the "floor effects" exhibited by the C57BL/6 strain, we have argued that the phenotypically average DBA/2 strain may be a useful alternative for nociceptive testing.[24]

Effect of targeted mutation or background genotype?

Another potential caveat to the present findings derives from the "hitchhiking donor gene" confound. That is, the possibility exists that the present results reflect allelic differences between 129 and C57BL/6 mice rather than the direct effects of the targeted mutation.[6,23] In this case, such a conclusion is rendered unlikely by the demonstration that 129S6 mice are more sensitive to both supraspinal and spinal opioid antinociception than are C57BL/6 mice, unlike the β-endorphin-deficient mice (having 129-derived alleles at genes in close proximity to *Pomc1*) that are differentially more and less sensitive than WT mice (having C57BL/6-derived alleles at these linked genes), respectively. Therefore, although one might postulate that the supraspinal increase in antinociception exhibited by KO mice could be secondary to a hitchhiking 129 allele, one cannot logically use this argument to explain the KO decrease in spinal antinociception.

Effect of β-endorphin deficiency on basal nociception

Injection of the opiate antagonists, naloxone and naltrexone,

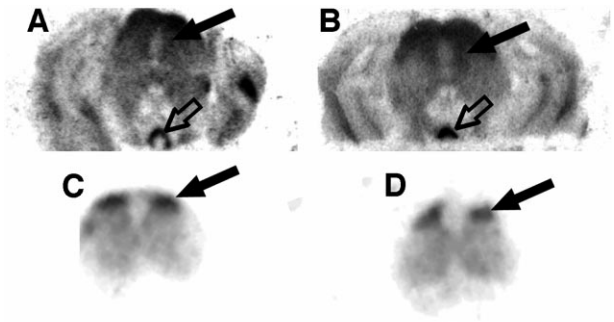

Fig. 5. μ-Opioid receptor binding sites in the midbrain and spinal cord of wild-type and β-endorphin-deficient mice. Autoradiographic analysis of [^{3}H]-DAMGO binding was performed on four mice of each genotype. Representative images at the level of the colliculus and periaqueductal gray (A, B) and thoracic spinal cord (C, D) from WT (A, C) and β-endorphin-deficient (B, D) mice are shown. Solid and open arrows in panels A and B point to the periaqueductal gray and interpeduncular nucleus, respectively. The arrows in panels C and D point to the dorsal horn of the spinal cord. The distribution and intensity of radiolabeled μ-receptor binding sites is qualitatively similar between the two genotypes.

produces hyperalgesia under some circumstances,[3] indicating the possible existence of a tonically active opioid antinociceptive "tone". This phenomenon may be mediated by μ receptors, since μ receptor KO mice display relative hyperalgesia on the tail-withdrawal test[34] (but see Ref. 15). Mice lacking enkephalins display lowered nociceptive latencies on the supraspinally mediated hot-plate test, but no significant alterations in the tail-withdrawal test.[12] The present demonstration that β-endorphin KO mice have minimally increased baseline tail-withdrawal latencies does not support the notion of a tonic β-endorphinergic tone, since it would be predicted that KO mice should display *lowered* latencies.

Dissociations between supraspinal and spinal opioid analgesia

It is well known that endogenous opioids are present in both the supraspinal and spinal compartments. The antinociceptive actions of exogenously administered opioids such as morphine are known to result from synergistic interactions between supraspinal and spinal mechanisms.[31,46] Any explanation of the present findings must consider why the response to β-endorphin deficiency is different, and indeed opposite, in these two regions of the CNS. Although we cannot yet provide a precise mechanism to reconcile our data, we note that spinal and supraspinal opioid mechanisms can be dissociated in a number of ways.

First, it should be noted that fundamental differences exist in the nature of spinal versus supraspinal opioid antinociception. Opioids in the spinal cord suppress nociceptive transmission directly, whereas supraspinal opioids are thought to activate (through disinhibition) descending antinociceptive mechanisms.[43,44] The two opioid mechanisms are clearly independent as well. Morphine administered i.c.v. is largely insensitive to antagonism by i.t. naloxone,[40] and the antinociception from i.c.v. and i.t. morphine summates supra-additively.[31,46]

Secondly, there exists pharmacological evidence for the putative involvement of different μ receptor subtypes at each level (μ_1 supraspinally, μ_2 spinally). For example, Paul and colleagues[29] demonstrated that i.c.v. DAMGO antinociception was sensitive to reversal by the μ_1-specific antagonist, naloxonazine, but i.t. DAMGO antinociception was resistant to such antagonism. The recombinant inbred CXBK mouse strain shows a paucity of μ_1, but not μ_2, binding sites in the CNS.[25] This strain has been shown to display correspondingly deficient systemic and i.c.v. morphine and DAMGO antinociception, but unaltered i.t. morphine antinociception.[22]

Similarly, pharmacological evidence has been gathered suggesting the existence of δ receptor subtypes (δ_1 and δ_2) that may play differential roles in supraspinal and spinal mechanisms of antinociception. Although it is well established that both subtypes are involved in antinociception, Ossipov *et al.*[27] observed in rats that the δ_2-specific agonist, DELT, was active in the supraspinally organized hot-plate test but not the spinal reflexive tail-flick test. In the Swiss Webster mouse, by contrast, the antinociceptive effects of i.c.v. enkephalins were selectively antagonized by the δ_1-specific antagonist, 7-benzylidenenaltrexone (BNTX), whereas i.t. enkephalin antinociception was selectively blocked by the δ_2-specific antagonist, naltriben.[37] Mattia and colleagues,[16] working also with mice, came to the same conclusion based on the inability of the δ_1-preferring antagonist, DALCE, to block the antinociception from i.t. DPDPE or DELT.

Very compelling data suggests that the actions of the orphan opioid-like peptide, orphanin FQ/nociceptin (OFQ/N), are dissociable at the supraspinal and spinal levels, although the specific actions at both levels are subject to ongoing controversy. After supraspinal injection, OFQ/N produces hyperalgesia[17,30] and/or anti-opioid actions.[9,20,45] OFQ/N does not, however, possess anti-opioid activity when injected i.t.,[7] and in fact can potentiate i.t. morphine antinociception.[39] There is some consensus that OFQ/N produces spinal antinociception and anti-hyperalgesic effects.[9,45] Thus, if OFQ/N levels and/or functioning have been altered in response to the absence of β-endorphin, it is conceivable that the effect on exogenous opioid antinociception might be specific to the level of the CNS activated. Preliminary data, however, demonstrated identical tissue content of OFQ/N in the hypothalamus of WT and KO mice (D. Quigley, R. Allen and M. J. Low, unpublished observations).

Finally, a potentially important difference concerns the differential action of β-endorphin in supraspinal versus spinal pathways of pain modulation. According to the model of Tseng and colleagues,[35,40,42] β-endorphin binds supraspinally to ε receptors, which when activated cause the release of met-enkephalin, which facilitates antinociception by binding to spinal μ receptors. β-Endorphin can also produce antinociception when administered to the spinal cord by acting on μ receptors, although this situation would not occur endogenously. If this model is correct, then the loss of supraspinal β-endorphin might indirectly affect spinal μ receptors, but have no effect on supraspinal μ receptors. Conversely, if β-endorphin does bind to supraspinal μ receptors, then the absence of β-endorphin would only directly affect these μ receptors since the peptide is not found in the spinal cord. Either way, it is conceivable that the alterations in μ receptor functioning seen presently arise from a change in one compartment only, followed by a compensatory change in the other.

The exact nature of any such change remains speculative at present. The simplest possibility, that supraspinal and spinal μ receptor numbers are up- and down-regulated, respectively, in KO mice, was not supported by the present autoradiography data. Nor were there any changes in δ- or κ-receptor

density in the PAG or spinal cord. It remains possible that subtle receptor density or affinity changes in restricted nociception-relevant loci have occurred. Such effects have recently been noted in transgenic mice lacking μ receptor, κ receptor and preproenkephalin expression.[2,11,33] Alternatively, the functional alterations in nociceptive responses noted presently may reflect changes in receptor-effector coupling, or signal transduction mechanisms.

CONCLUSIONS

Although the antinociceptive actions of systemically administered morphine were unaltered in mice lacking β-endorphin, opposing effects were seen in the mutant mice when morphine was specifically injected into the supraspinal or spinal compartments. Relative to WT mice of the same C57BL/6 congenic background, KO mice displayed increased antinociceptive sensitivity to i.c.v. morphine, but decreased sensitivity to i.t. morphine. Both phenomena are apparently related to the μ-opioid receptor, since the μ-specific ligand DAMGO yielded similar results. These data suggest the existence of CNS locus-dependent interactions between the μ receptor and one of its putative endogenous ligands, β-endorphin. The mechanism responsible for the functional changes in mice as a result of the constitutive absence of β-endorphin is likely to be more complex than alterations in μ receptor density.

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