Absence of opioid stress-induced analgesia in mice lacking β-endorphin by site-directed mutagenesis

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ABSTRACT   A physiological role for β-endorphin in endogenous pain inhibition was investigated by targeted mutagenesis of the proopiomelanocortin gene in mouse embryonic stem cells. The tyrosine codon at position 179 of the proopiomelanocortin gene was converted to a premature translational stop codon. The resulting transgenic mice display no overt developmental or behavioral alterations and have a normally functioning hypothalamic–pituitary–adrenal axis. Homozygous transgenic mice with a selective deficiency of β-endorphin exhibit normal analgesia in response to morphine, indicating the presence of functional μ-opiate receptors. However, these mice lack the opioid (naloxone reversible) analgesia induced by mild swim stress. Mutant mice also display significantly greater nonopioid analgesia in response to cold water swim stress compared with controls and display paradoxical naloxone-induced analgesia. These changes may reflect compensatory upregulation of alternative pain inhibitory mechanisms.

Endogenous opioid peptides subserve a wide range of physiological adaptations to stress. Prominent among these functions are inhibition of reproduction (1), modulation of the hypothalamic–pituitary–adrenal (HPA) axis (2), and maintenance of homeostasis in response to autonomic challenge (3). Opioids, together with neurotransmitters in parallel nonopioid neural circuits, also mediate stress-induced analgesia (4). The existence of an intrinsic pain-inhibition system was first demonstrated by the induction of analgesia by electrical stimulation in periventricular, periaqueductal, and medial brainstem loci (5, 6). A possible opioid mechanism for central pain modulation was suggested by the ability of opioid antagonists to block (7) and opiate microinjection into the stimulation-sensitive locations to mimic (8) the analgesic effect of electrical stimulation. Structural components of the descending endogenous analgesia system include the periaqueductal central gray, raphé nuclei of the medulla, and substantia gelatinosa of the spinal cord. Each of these neuroanatomic areas is densely innervated by opioidergic neurons and contains a large number of specific opiate receptor binding sites (9, 10).

In rodents, "natural" environmental stressors including physical attacks by predative or conspecific aggressors and exposure to new situations can produce analgesic states (11–13) via activation of endogenous pain-inhibition mechanisms. For quantitative laboratory experiments, however, artificial stressors that can be applied uniformly to large numbers of subjects have been used, including forced swim, foot shock, and restraint (14). Parametric analyses have demonstrated repeatedly that the relative degree of opioid vs. nonopioid analgesia, assessed by opioid antagonist blockade and/or cross tolerance to the effects of exogenously applied opiates, is highly dependent on the severity of the stress (15–17) and both

the species and strain of rodent (18). The specific involvement in opioid analgesia of each of the identified endogenous opioid peptides and opiate receptor subtypes is less clear. There exists considerable pharmacological and neuroanatomic evidence to implicate β-endorphin, enkephalins, and dynorphins in pain control (19–21). Of the three classes of opioids, β-endorphin is particularly noteworthy because of its high potency and a nearly one-to-one correspondence between sites supporting electrical stimulation-produced analgesia and high concentrations of endorphinergic fibers in the human brain (22).

To further investigate the physiological roles of β-endorphin, we used a genetic approach of homologous recombination in embryonic stem cells to produce mice that are unable to synthesize β-endorphin. Because β-endorphin is posttranslationally processed from a larger multifunctional precursor, we introduced a point mutation into the proopiomelanocortin (POMC) gene that translates to a truncated prohormone lacking the entire C-terminal amino acid region encoding β-endorphin.

MATERIALS AND METHODS

Gene Targeting and Production of Mutant Mice. The gene targeting vector POMCX*4 encoding a truncated POMC prohormone caused by a point mutation in exon 3 and containing the phosphoglycerate kinase–neo and phosphoglycerate kinase–tk selection cassettes was constructed and electroporated into D3 embryonic stem cells as described (23). Correctly targeted embryonic stem cells from clone 76 were microinjected into embryonic day 3.5 blastocysts obtained from superovulated C57BL/6N mice. F_1 mice heterozygous for the POMCX*4 allele were obtained from the breeding of a single germ-line penetrant male chimera to C57BL/6N females and crossbred to obtain F_2 mice on a 129/Sv × C57BL/6N hybrid genetic background. Genotyping was performed on genomic DNA samples obtained from mouse tails by either Southern blot analysis of size separated, EcoRI digested DNA with a radiolabeled probe A as described (23) or by PCR. One set of oligonucleotides (5′ primer: GAAGTACGTCATGGGTCACT and 3′ primer: GACATGTTCATCTCTATACATAC) amplified a 1.6-kb product corresponding to the wild-type POMC allele. A second set of

oligonucleotides (5' primer: GAGGATTGGGAAGACAAT-AGCA and 3' primer: GACATGTTCATCTCTATACATAC) amplified a 1.2-kb product corresponding to the short arm of the POMCX*4 allele. PCR conditions were standard (94°C for 1 min, 60°C for 1 min, 72°C for 1 min, 35 cycles).

RIA Analysis of POMC Peptides. Medial basal hypothalami, anterior pituitary, and neurointermediate pituitary lobes were homogenized individually in 10 vol of 2% acetic acid and equivalent aliquots from the three genotypes of mice were assayed with specific RIAs for β-endorphin-, α-melanocyte stimulating hormone (MSH)-, β-MSH-, and corticotropin (ACTH)-like immunoreactivity as described (24–26).

Immunohistochemistry. Immunohistochemistry was performed using free-floating 50-μm Vibratome sections and the Vectastain ABC immunoperoxidase method (Vector Laboratories) on brains obtained after perfusion fixation with buffered 4% paraformaldehyde as described (27). The specificities of the rabbit primary antisera were determined by RIA as described above. Antisera were used at a final dilution of 1:500 (anti-ACTH "Henri"/anti-β-endorphin "Nora"). The chromagen was diaminobenzidine and sections were counterstained with methyl green.

Analgesia Assays. The abdominal constriction assay was performed as described (28). Mice were placed in individual 30-cm diameter Plexiglas observation chambers for a 30-min acclimatization period prior to weighing, drug treatments, and/or swim stress. After treatment all mice were injected i.p. with 10 ml/kg 0.6% (wt/vol) acetic acid and returned to their observation chambers; abdominal constrictions were counted for the following 30-min period. Four mice were tested simultaneously by an experienced observer blinded to genotype and drug.

The hot-plate assay was performed as described (29) scoring hind-paw licks, flutter, jumping, or a cut-off latency of 60 s as the end point on a 55 ± 1°C aluminum plate. To determine the analgesic effects of morphine, hot-plate latency was measured immediately before and 20 min after injection. To determine the analgesic effects of swim stress, latency was measured before swimming and 2 min after the completion of a swim.

Statistical Analysis. Student's t test was used to compare non-paired groups for a single dependent variable. Two- and three-way ANOVAs were used to compare data sets with multiple dependent variables. Post hoc comparisons between selected groups were made with the Duncan New Multiple Range test. In all cases, a criterion level of $P < 0.05$ was considered significant.

RESULTS

Expression of the Truncated POMC Prohormone. We designed a replacement-type gene-targeting vector called POMCX*4 encoding a truncated prohormone as described (23). The codon for the amino-terminal tyrosine of β-endorphin was changed to a premature translational stop codon by site-directed mutagenesis. No other mutations were made in the known POMC promoter elements or coding sequences. The neo selection cassette, to be retained in the mutated locus after homologous recombination, was positioned in the short arm of the vector in the 3' flanking region of the POMC gene because previous experiments indicated that these sequences have no role in regulating POMC gene expression (30). Predicted structures of the resulting POMC prohormones, encoded peptides, and the location of epitopes to antisera used for analysis of the peptide phenotype are shown in Fig. 1A.

Southern blot analysis of genomic DNA obtained from the offspring of a heterozygous F_1 mating pair revealed Mendelian inheritance of the targeted allele in the F_2 generation (Fig. 1B). Additional restriction analysis and Southern blot analyses with a 5' flanking probe and PCR amplification of the region containing exon 3 showed that the long arm of the POMC gene was intact and the point mutation was retained in the genome
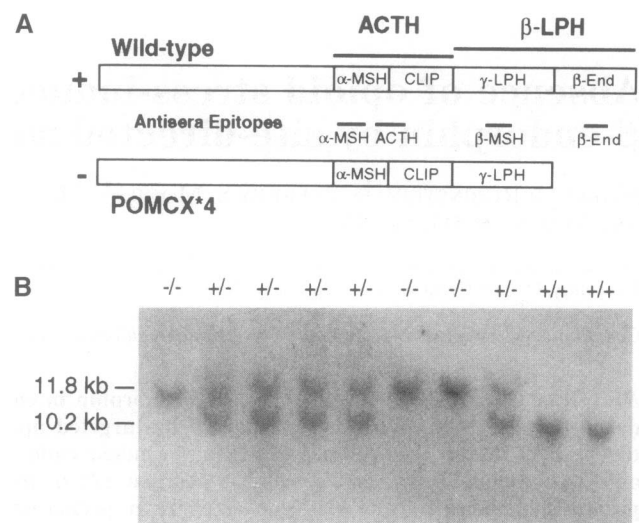

FIG. 1. Gene targeting of the POMC locus in mice by homologous recombination. (A) Structure of the wild-type POMC prohormone (+), the carboxyl-truncated prohormone lacking β-endorphin sequences encoded by the targeted POMCX*4 allele (−), and the location of epitopes recognized by the panel of antisera used to characterize POMC peptides in the resulting mice. (B) Southern blot of genomic DNA from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) POMCX*4 mice within an F_2 litter demonstrating Mendelian inheritance of the targeted allele.

of embryonic stem-cell derived mice (data not shown). The absence of unpredicted mutations or deletions as a result of homologous recombination was further confirmed by analysis of POMC gene expression in the homozygous mutant mice as described below.

In situ hybridization using an exon 2 oligonucleotide probe as described previously (30) showed normal distribution and levels of POMC mRNA in the hypothalamus and pituitary of homozygous mice (data not shown). This finding suggests that the point mutation in exon 3 and the inclusion of the phosphoglycerate kinase–neo cassette downstream of the POMC transcriptional unit had no effect on expression of the mutant POMC allele. Total levels of β-endorphin- and β-MSH-like immunoreactivity were measured in extracts of hypothalamus and pituitary from sibling matched wild-type, heterozygous, and homozygous POMCX*4 mice (Table 1). Heterozygotes had ≈50% of the β-endorphin content in each tissue compared with wild-type, suggesting that the two POMC alleles have equal transcriptional activity and that no upregulation of the normal allele occurred in response to the loss of β-endorphin expression from the mutant allele. Homozygous mice had no detectable β-endorphin in hypothalamus or pituitary. There was no difference in β-MSH content among the three genotypes confirming that the truncated POMC prohormone was translated normally from the mutated allele.

Immunohistochemistry of serial brain sections from wild-type mice showed that the distribution of ACTH- and β-endorphin-like immunoreactivity was identical in cell bodies within the arcuate nucleus of the hypothalamus (Fig. 2 A and B) and throughout the extensive fiber projections to the forebrain, midbrain, and hindbrain (data not shown). Homozygous mice retained the same set of POMC-expressing neurons and fiber projections revealed by the ACTH antisera (Fig. 2C) but had no detectable β-endorphin (Fig. 2D). Apparently the expression of β-endorphin is not required for maintenance of the POMC neurons and their projections in the adult brain. Immunohistochemical analysis demonstrated a normal distribution and expression of dynorphin and enkephalin in the homozygous mice (data not shown).

Table 1. POMC peptides and the HPA axis in β-endorphin-deficient mice

Sample	Measurement	Genotype		
		+/+	+/−	−/−
Hypothalamus	β-Endorphin (pmol/g)	55.8 ± 7.0	34.5 ± 7.0*	0
	β-MSH (pmol/g)	10.1 ± 0.6	11.1 ± 1.6	8.3 ± 1.8
Pituitary				
Neurointermediate lobe	β-Endorphin (pmol/lobe)	546 ± 72	371 ± 26*	0
	β-MSH (pmol/lobe)	31.1 ± 9.7	20.7 ± 2.0	16.8 ± 3.5
Anterior lobe	β-Endorphin (pmol/lobe)	5.4 ± 2.2	2.0 ± 0.4*	0
	β-MSH (pmol/lobe)	9.5 ± 0.9	9.7 ± 0.4	8.0 ± 0.7
Adrenal glands	Wet weight (mg)			
Male		3.7 ± 0.4	3.7 ± 0.1	4.4 ± 0.2
Female		5.1 ± 0.5†	5.9 ± 0.4†	5.7 ± 0.6†
Basal diurnal serum	Corticosterone (ng/ml)			
Male		11 ± 4	10 ± 3	13 ± 3
Female		24 ± 9	21 ± 4†	19 ± 6
Restraint stress serum				
Male		139 ± 25‡	145 ± 24‡	134 ± 27‡
Ether stress serum				
Male		168 ± 14‡	ND	200 ± 13‡

Hormones were measured by specific RIAs as described in *Materials and Methods.* Values are the mean ± SEM. *n* varies from 5 to 28. ND, not determined.
*$P < 0.05$ by Student's *t* test, compared with +/+.
†$P < 0.05$ by Student's *t* test, compared with male. No genotype differences.
‡$P < 0.0001$ by Student's *t* test, compared with basal. No genotype differences.

Phenotype of β-Endorphin-Deficient Mice. Homozygous mice had normal birth weights, growth and development into adulthood, and normal brain weights. However, after the onset of puberty the homozygous mice attained 10–15% greater body weight than wild-type mice (unpublished data). Brains and pituitary glands were structurally normal based on Nissl stains of serial sections. There was no obvious abnormality in exploratory, grooming, or rearing behaviors based on close observation of mice in an open field. Activity levels measured by interruption of photocell beams in environmentally isolated cages were identical between wild-type and homozygous POMCX*4 mice. Homozygous mutant male and female mice had normal fertility based on the onset of puberty, average litter size, and the number of consecutive litters in established mating pairs.

Because exogenous opiates and endogenous opioid peptides can modulate activity of the HPA axis (2), we analyzed several parameters of this system in the β-endorphin-deficient mice. It was also important to determine if the mice had alterations in glucocorticoid production that could have profound secondary effects on brain development and behavior. No significant changes in corticotropin-releasing hormone mRNA in the paraventricular nucleus of the hypothalamus were found by *in situ* hybridization (data not shown). Adrenal gland weight, an indicator of chronic integrated ACTH secretion, was identical among wild-type, heterozygous, and homozygous mice (Table 1). Basal and stimulated corticosterone levels in response to both restraint- and ether-induced stress were also normal (Table 1).

Analgesia in β-Endorphin-Deficient Mice. To determine whether the opiate receptors relevant to analgesia were normally functional in the mutant mice, we quantified analgesic responses to morphine sulfate. In two independent nociceptive tests, the abdominal constriction assay and the hot-plate assay,
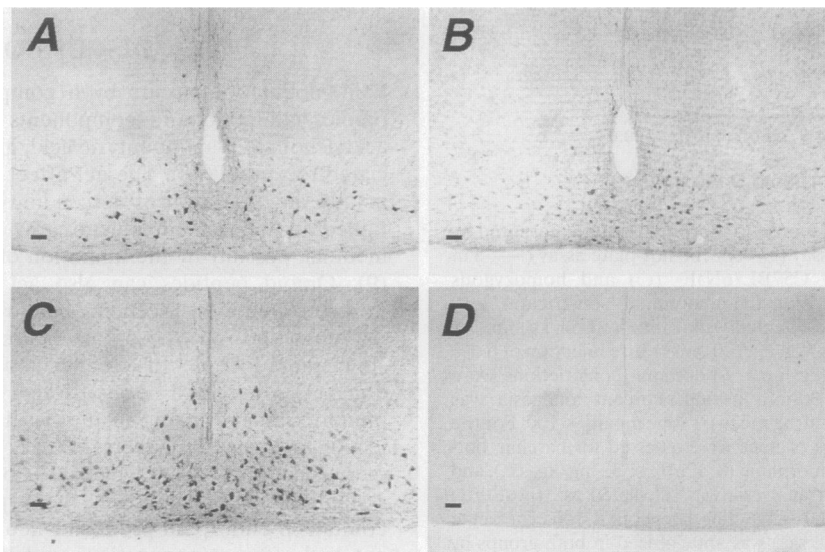

FIG. 2. Immunohistochemistry of POMC-derived peptides in the arcuate nucleus of the hypothalamus. (*A*) ACTH-like and (*B*) β-endorphin-like immunoreactivity in a heterozygous mouse. (*C*) ACTH-like and (*D*) β-endorphin-like immunoreactivity in a homozygous POMCX*4 −/− mouse. Sections from the two mice are at different rostrocaudal levels in the hypothalamus. (Bar = 50 μm.)

a linear relationship between morphine dose and the percentage analgesia in their measurable dose ranges was revealed (Fig. 3). There were no differences in the regression curves and ED_{50} between wild-type and homozygous POMCX*4 mice. Naloxone (5 mg/kg) completely blocked the analgesia produced by morphine (32 mg/kg) in both groups on the hot-plate test. These data suggest that analgesic μ-opiate receptor systems are intact in the mutant mice and are neither upregulated nor desensitized by the lack of endogenous β-endorphin. This finding may not be surprising in light of the dissociation of supraspinal mechanisms underlying morphine and β-endorphin analgesia (31). Since β-endorphin shows good affinity to δ- as well as μ-opiate receptors (21), it remains possible that δ-receptor analgesia has been altered in these mice.

A parametric analysis of swim stress-induced analgesia was performed to determine the optimum conditions for detection of the opioid component of stress-induced analgesia in the hybrid genetic background of mice used in our study (Fig. 4). Three minute swims at 10°C produced the most profound analgesic response on the hot-plate assay. β-Endorphin-deficient mice displayed significantly more analgesia than wild-type controls at this temperature. Pretreatment with naloxone revealed that the analgesia was completely non-opioid in both groups. Swims in 20°C water produced proportionately less analgesia in both groups, also naloxone-insensitive. Only at 30°C, which produced minimally detectable analgesia on the hot-plate assay, was an apparently naloxone-reversible, opioid component demonstrated in wild-type mice. Under the same conditions, the β-endorphin-deficient mice did not develop measurable analgesia. The small magnitude of analgesia displayed at this temperature on this assay severely limited the power of statistical analyses to detect significant strain and drug effects, however.

Therefore, to characterize further the endogenous analgesia induced by a mild swim stress, we used the abdominal constriction assay. As demonstrated above (Fig. 3), this assay is at
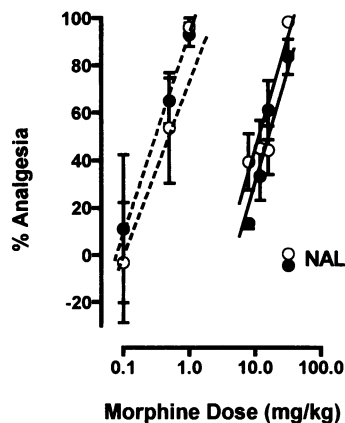

FIG. 4. Parametric analysis of swim stress-induced analgesia measured by the hot-plate assay. Adult wild-type (129/Sv × C57BL/6N)F2 and homozygous POMCX*4 male mice were weighed, injected with naloxone (NAL; 5 mg/kg, i.p.) or saline vehicle (10 ml/kg, i.p.), and placed in individual holding cages for 20 min. After assessment of baseline hot-plate latency, each mouse swam individually in water at the indicated temperature for 3 min, was allowed to dry for 2 min, and then was retested on the hot plate. Separate groups of mice did not swim, but instead merely were retested 5 min after baseline assessment; in both strains a modest and nonsignificant hyperalgesia was observed (not shown). Bars represent mean ($\pm$ SEM) % analgesia: [(post-swim latency − baseline latency)/(60 − baseline latency)] × 100 ($n = 20$ for all 10°C and 30°C groups and $n = 10$ for all 20°C groups). Data were analyzed by two-way ANOVA for each swim temperature. The asterisk (*) represents a significant main effect of genotype, $P < 0.05$. The genotype × drug interaction for 30°C analgesia approached significance ($P < 0.10$).

least 10-fold more sensitive than the hot-plate assay to inhibition by analgesic manipulations. Under these experimental conditions, a 45-s swim in 20°C water produced significant and long-lasting analgesia in the wild-type but not the β-endorphin-deficient mice (Fig. 5). Neither saline nor naloxone injections alone had a significant effect on the nociceptive sensitivity of either group of mice. However, naloxone (10 mg/kg) reversed the analgesia induced by the forced swim in wild-type (129/Sv × C57BL/6)F2 mice, demonstrating its opioid nature. Instead of reversing analgesia in the POMCX*4 homozygous mice, naloxone in combination with the swim stress actually induced a highly significant analgesia. The magnitude of this paradoxical analgesia was equivalent to that produced by swim stress alone in the wild-type mice.

Morphine Dose (mg/kg)

FIG. 3. Analgesic dose–response to morphine sulfate measured by the abdominal constriction assay (- - -) or the hot-plate assay (——) in adult wild-type (129/Sv × C57BL/6N)F2 (○) and homozygous POMCX*4 (●) male mice. For the abdominal constriction test, separate groups of mice were injected with saline (10 ml/kg, i.p.) or morphine (0.1, 0.5, and 1.0 mg/kg, i.p.) 20 min before injection of 0.6% (wt/vol) acetic acid (10 ml/kg, i.p.). Abdominal constrictions were counted for the following 30-min period. Percent analgesia was calculated as: [(saline mean − drug mean)/saline mean] × 100. For the hot-plate test, separate groups of mice were assessed for baseline hot-plate sensitivity, injected with morphine (8, 12, 16, or 32 mg/kg, s.c.), and retested 20 min later. Percent analgesia was calculated as: [(post-drug latency − baseline latency)/(60 − baseline latency)] × 100. The acute effect of morphine (32 mg/kg, s.c.) was antagonized in both groups by administration of naloxone (NAL; 5 mg/kg, i.p.) 10 min after morphine injection. Linear regression analyses of each data set produced significant r^2 values ranging from 0.87 to 0.98. There were no significant differences in regression slopes or ED_{50}s ($n = 10$–12 per group).
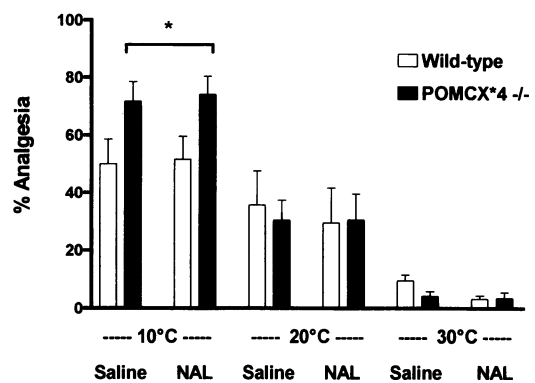
DISCUSSION

Pain modulation occurs by a complex interaction of both opioid and nonopioid components including monoamines, acetylcholine, γ-aminobutyric acid, glutamate, and their associated receptors (20). The net effect of supraspinal opioids is mainly the activation of descending projection neurons contained in the periaqueductal gray and the rostral ventral medulla, possibly by the inhibition of inhibitory interneurons (9). Opioid peptides can also act spinally by directly or indirectly inhibiting ascending pain transmission neurons (32). Although the intrinsic opioid neurons in analgesia-relevant supraspinal loci are all either enkephalinergic or dynorphinergic (33), there is a heavy afferent innervation by β-endorphinergic fibers arising in the basal hypothalamus and projecting to the periaqueductal gray (22). In addition, the nucleus of the tractus solitarius, which is the only other central nervous system nucleus apart from the hypothalamic arcuate that contains β-endorphin-producing neurons, has extensive afferent and efferent connections with the periaqueductal gray, nucleus raphé magnus, and the spinal cord (34, 35) and has been postulated to serve as a central relay coordinating autonomic reflexes and pain inhibition (35). Our data dem-

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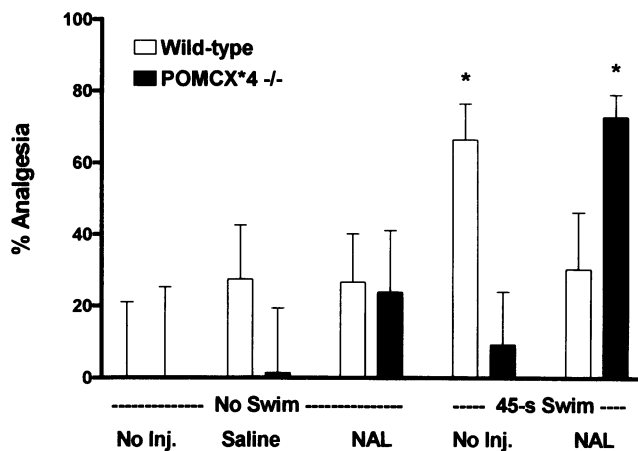
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FIG. 5. Mild swim stress-induced analgesia measured by the abdominal constriction assay. Following a 30-min acclimation to observation chambers, adult wild-type (129/Sv × C57BL/6N)F_2 and homozygous POMCX*4 male mice were weighed, and some groups were injected with naloxone (NAL; 10 mg/kg, s.c.) or saline vehicle (10 ml/kg, s.c.). Mice were subjected to a 45-s forced swim in 20°C water or kept in individual holding cages for an equivalent period of time. All mice were then injected with 0.6% (wt/vol) acetic acid (10 ml/kg, i.p.), returned to the observation chambers, and abdominal constrictions were counted for the following 30-min period. Bars represent mean (± SEM) % analgesia scores compared to the corresponding no swim/no injection (No Inj.) group mean. The data were analyzed by a three-way ANOVA (including all groups except the unmatched no swim/saline groups), which revealed a significant condition × drug × genotype interaction. *, $P < 0.05$ by Duncan new multiple range post hoc test, vs. corresponding no swim/no injection group ($n = 10$–12 per group).

onstrating that β-endorphin-deficient mice fail to exhibit opioid swim stress-induced analgesia are consistent with the neuroanatomic localization of β-endorphin. Indeed, electrical- or monosodium glutamate-induced lesions of the arcuate nucleus in both neonatal and adult rats have been shown to attenuate opioid stress-induced analgesia (36, 37). These data further call into question the role of the remaining opioids, enkephalins and dynorphins, in this particular form of analgesia.

The pituitary gland is the major source of peripherally circulating β-endorphin, and its role in analgesia remains uncertain. Although most β-endorphin produced in the intermediate lobe is acetylated at the amino-terminal tyrosine and is therefore inactive at opiate receptors, a significant amount of desacetyl β-endorphin is produced in the mouse anterior lobe and cosecreted with ACTH in response to stress activation of the HPA axis. The adrenal gland is also a source of peripheral enkephalins. However, previous studies have shown that hypophysectomy or adrenalectomy do not abolish (38), and may in fact enhance (39), most types of opioid stress-induced analgesia. Therefore, it is likely that the effects we have documented on analgesia in mutant mice are due to the loss of central rather than pituitary β-endorphin.

It is well known that separate opioid and nonopioid mechanisms of pain inhibition exist and can be independently or jointly activated by different types of stress or different parameters of a common stressor (4). These alternate pathways are known to interact; in the initial demonstration the antagonism of morphine analgesia by the irreversible opiate antagonist, naloxazone, was found to potentiate nonopioid swim analgesia (40). Additional data have shown that opioid and nonopioid systems are mutually inhibitory, and this reciprocal interaction has been termed collateral inhibition. We propose that the lack of a crucial endogenous opioid ligand in homozygous POMCX*4 mice throughout development may have caused the compensatory upregulation of a nonopioid analgesic system. That is, the higher levels of nonopioid analgesia exhibited by mutant mice may represent the effects of a

permanent, developmentally organized form of opioid/nonopioid collateral inhibition that will be amenable to future mechanistic studies. The observed results could also reflect the loss of both the opioid agonist, β-endorphin 1–31, and putative anti-opioid peptides (e.g., β-endorphin 1–27) processed from the intact β-endorphin (41).

Considerable controversy surrounds the neurochemical basis of paradoxical naloxone-induced analgesia. Naloxone-induced analgesia can be produced by very low doses of naloxone (42), repeated administration in combination with a stressor (43, 44), induction of morphine tolerance (45), or induction of diabetes by streptozotocin (46). One hypothesis suggests that naloxone-induced analgesia is really a form of stress-induced analgesia, supported by the finding that naloxone-induced analgesia is augmented by the anxiogenic benzodiazepine antagonist, Ro 15–1788 (47). Furthermore, it has been proposed that naloxone-induced analgesia is a nonopioid (or at least, non-μ receptor) phenomenon, since it does not appear to develop tolerance (43), does not show cross-tolerance with morphine (48), and can be elicited without repeated pairings in streptozotocin-induced diabetic mice (46) which are known to have hyporesponsive supraspinal μ-receptor analgesia (49). The naloxone-induced analgesia exhibited by β-endorphin-deficient mice may thus be due to the same compensatory changes responsible for their supranormal nonopioid analgesic response to cold water swim.

Remarkably, no obvious changes in endocrine function have been documented in the β-endorphin-deficient mice despite the large experimental data base concerning opiate effects in the hypothalamus (1–3). While it is possible that β-endorphin in fact plays no role in the regulation of the HPA and hypothalamic–pituitary–gonadal axes, a more likely explanation in light of our analgesia studies is that subtle compensatory mechanisms have appeared to maintain normal functioning of these critical neuroendocrine systems.

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