Brief Definitive Report

Altered Hematopoiesis, Behavior, and Sexual Function in μ Opioid Receptor–deficient Mice

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Summary

The μ opioid receptor is thought to be the cellular target of opioid narcotics such as morphine and heroin, mediating their effects in both pain relief and euphoria. Its involvement is also implicated in a range of diverse biological processes. Using a mouse model in which the receptor gene was disrupted by targeted homologous recombination, we explored the involvement of this receptor in a number of physiological functions. Mice homozygous for the disrupted gene developed normally, but their motor function was altered. Drug-naive homozygotes displayed reduced locomotor activity, and morphine did not induce changes in locomotor activity observed in wild-type mice. Unexpectedly, lack of a functional receptor resulted in changes in both the host defense system and the reproductive system. We observed increased proliferation of granulocyte-macrophage, erythroid, and multipotential progenitor cells in both bone marrow and spleen, indicating a link between hematopoiesis and the opioid system, both of which are stress-responsive systems. Unexpected changes in sexual function in male homozygotes were also observed, as shown by reduced mating activity, a decrease in sperm count and motility, and smaller litter size. Taken together, these results suggest a novel role of the μ opioid receptor in hematopoiesis and reproductive physiology, in addition to its known involvement in pain relief.

Opioids exert their physiological effect by interacting with membrane-bound opioid receptors. Extensive studies in the past have established that there are three major types of opioid receptors, μ, δ, and κ, with somewhat overlapping, yet distinct pharmacology (1, 2). In recent years, molecular cloning has led to the identification of the genes for these opioid receptors (3), and subsequent studies using these clones have confirmed the pharmacological classification (3, 4). The μ opioid receptor is the major cellular target for most medically relevant opioid narcotics, including naturally occurring drugs such as morphine and codeine as well as synthetic compounds such as fentanyl and methadone. In addition to these μ-selective alkaloids, the μ opioid receptor is also activated by peptide ligands such as endorphins (endogenous) and [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]-enkephalin (synthetic).

The major clinical use of morphine and other opioids is for pain relief. Analgesic effects of opioids can be mediated by all three types of opioid receptors (2). A recent study using mice with a disrupted μ opioid receptor gene demonstrated the central role of this receptor in opioid-mediated pain relief (5). In addition to nociception, opioid receptor involvement has also been implicated in other physiological functions such as motor activity, gastrointestinal motility, and respiratory activity (6). To explore the role that the μ opioid receptor may play in diverse biological processes, we examined mice deficient in the μ opioid receptor gene. Here we report the findings of these studies.

Materials and Methods

Mice. The μ opioid receptor gene *Oprm* was disrupted in embryonic stem (ES) cells by replacing the coding exon 1 with a PGK-*neo* selection marker using the method of gene targeting as described (7, 8). Targeted ES cells were microinjected into C57/

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BL6 mouse blastocysts, and the resultant chimeric mice were mated to wild-type female mice of the Swiss black strain to obtain germline transmission of the targeted gene. Genomic Southern blotting and nested PCR were used to confirm the presence of the disrupted gene in the progeny mice. Mice were housed at 72–74°F and 60% humidity with 12-h light/dark lighting cycle. Water and food were available ad libitum.

Hematopoiesis Assays. Unseparated marrow and spleen cells were plated respectively at 5×10^4 and 5×10^5 per ml in 1.0% methylcellulose culture medium with 30% vol/vol fetal bovine serum (Hyclone Labs, Inc., Logan, UT) and 1 U/ml recombinant human erythropoietin (Amgen Biologicals, Thousand Oaks, CA), 50 ng/ml recombinant murine steel factor (Immunex Corp., Seattle, WA), 5% vol/vol pokeweed mitogen mouse spleen cell–conditioned medium, and 0.1 mM hemin (Kodak Co., Rochester, NY). Colonies deriving from hematopoietic progenitors were scored after 7 d of incubation in a humidified environment at 5% CO_2 and lowered (5%) O_2 as described (9–11). Absolute numbers of progenitors per organ were calculated based on the number of viable, unseparated nucleated cells per femur or spleen, and the number of colonies scored per number of cells plated. The percentage of progenitors in S-phase was estimated by the high specific activity tritiated thymidine kill technique (9–11).

Measurements of Motor Activity and Sexual Function. Locomotor activity measurements were recorded in a quiet room under dim light by video cameras, and the tapes were analyzed off-line. Individual mice placed in a round plastic chamber were habituated for 15 min before the activity was recorded. Horizontal activity was scored as breaking either one of the two center lines (perpendicular to each other). Vertical activity was scored as the animal raised its head and/or stood up along the side wall. Morphine (in 0.9% saline) was injected intraperitoneally at a 2.3 mg/kg, the ED_{50} dosage of analgesia in mice (12). U62066 (dissolved in ethanol and diluted in saline) was injected intraperitoneally at a dose of 3 pmol/kg.

Mating behavior was tested essentially as described (13). Sperm from the right-side epididymis was flushed out in 5 ml of phosphate-buffered saline, and sperm number counted both by Coulter cell counter and by warmed Neubauer counting chamber (14). Sperm motility was the percentage of sperm showing forward progressing, linear motion.

Statistics. Data of heterozygotes and homozygotes were analyzed for statistically significant differences from that of wild-type mice by Student's *t* test. An asterisk (*) indicates significant difference ($P < 0.05$) from the wild type. All data are shown as mean ± SEM.

Results and Discussion

Normal Growth but Altered Motor Activity in μ Opioid Receptor–deficient Mice. The μ opioid receptor gene *Oprm*, a single copy gene on mouse chromosome 10 (15), was disrupted in ES cells by gene targeting (16, 17) for generation of μ opioid receptor–deficient mice. Analysis of genomic DNA from progeny mice confirmed the presence of the disrupted gene (data not shown). Deficiency in the μ opioid receptor does not appear to cause major changes in mouse development, as mice of all three genotypes grew with comparable body weight (Fig. 1), without noticeable changes in neuroanatomy or histology (data not shown). When tested for opioid-mediated antinociception using the tail flick latency assay, we observed similar results to those

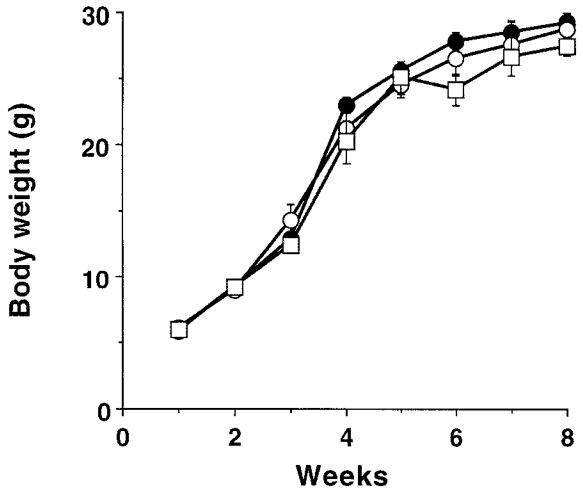

Figure 1. Normal growth of μ opioid receptor–deficient mice. Body weight after birth of wild-type ($Oprm^{+/+}$, *solid circles*, $n = 14$), heterozygous ($Oprm^{+/-}$, *open circles*, $n = 23$), and homozygous ($Oprm^{-/-}$, *open squares*, $n = 18$) mice. Data were expressed as mean ± SEM. There are no significant differences among the three genotypes (Student's *t* test).

reported by Matthes et al. (5), i.e., a lack of morphine-induced increase of tail flick latency in mice homozygous for the deficient receptor gene ($Oprm^{-/-}$; data not shown).

Morphine and other opioids are known to enhance motor activity in rodents. Locomotor activity is defined as two types of behavior: horizontal (the extent of the mouse's running around in observation cage) and vertical (the frequency of the animal's upward movement, either as rearing or standing up against the side of the cage). When observed for spontaneous horizontal activity, wild-type mice habituated over a period of time after being placed in the observation cage (Fig. 2 *A*). In homozygous mice ($Oprm^{-/-}$) that had not been exposed to morphine, the spontaneous horizontal activity was greatly reduced compared with that of the wild type (Fig. 2 *A*). This suggests that even in naive animals that have not been exposed to exogenously administered opioids, the μ opioid receptor may play a role in modulating the basal tone of spontaneous locomotor activity.

When morphine was injected, wild-type mice exhibited increased locomotor activity (Fig. 2 *B*), a phenomenon commonly observed in mice (18). The homozygous mice, on the other hand, were not affected by morphine injection, and their spontaneous horizontal activity stayed relatively constant over the course of the test (Fig. 2 *B*). This difference between wild-type and homozygous mice is significant both for the 10-min segments during recording (Fig. 2 *B*, *left*), and over the entire recording time of 60 min (Fig. 2 *B*, *right*). Interestingly, heterozygous mice displayed intermediate levels of horizontal activity that did not change after morphine injection (Fig. 2 *B*).

When the vertical activity was examined, there was no significant difference among three genotypes of mice (Fig. 2 *C*). Upon morphine injection, the vertical activity in wild-type mice was greatly reduced (Fig. 2 *D*), in agreement with

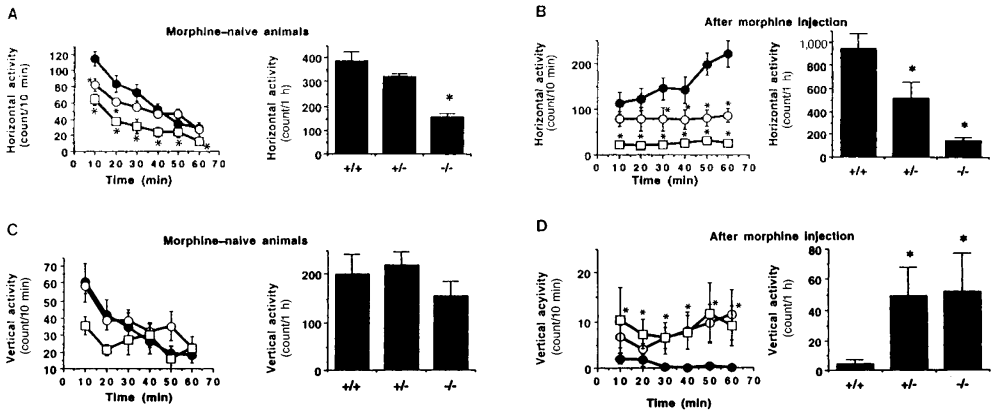

Figure 2. Locomotor activity. Locomotor activity was measured in mice of the wild-type (*Oprm*$^{+/+}$, *solid circles*, $n = 6$), heterozygous (*Oprm*$^{+/-}$, *open circles*, $n = 5$), and homozygous (*Oprm*$^{-/-}$, *open squares*, $n = 6$) genotypes. Movement counts are shown both in 10-min intervals (*A–D*, *left*) and for the total 1-h testing period (*A–D*, *right*). Data are shown as mean ± SEM. (*, significant difference [$P < 0.05$, Student's *t* test] from the wild type.) (*A*) Spontaneous horizontal activity in naive mice not exposed to morphine. Homozygotes showed reduced locomotor activity. (*B*) Horizontal activity after injection of morphine. Wild-type mice displayed hyperactivity, while homozygotes and heterozygotes showed little change. (*C*) Spontaneous vertical activity in naive mice not exposed to morphine. There are no significant differences between the groups. (*D*) Vertical activity after injection of morphine. Wild-type mice showed dramatic reduction in activity upon morphine injection, and there was little change in either the homozygotes or the heterozygotes.

previous studies (18). In homozygous mice, morphine did not change the level of vertical activity during the test period (Fig. 2 *D*), indicating a lack of μ opioid receptor for mediating the morphine effect. Just as for horizontal activity, vertical activity in heterozygous mice was not affected by morphine (Fig. 2 *D*), suggesting that one normal allele of the μ opioid receptor gene is not sufficient to overcome the deficit of the other mutated allele in this respect.

These results are consistent with previous observations that in normal (wild-type) mice, morphine increases horizontal locomotor activity and reduces vertical locomotor activity (18). Our data demonstrate that the μ opioid receptor plays a central role in morphine modulation of locomotor activity, since in both homozygous and heterozygous mice, the morphine effects were largely abolished.

Hematopoiesis. Animal studies support the role of opioids in modulating the host defense system (19, 20). For example, both β-endorphin and enkephalins, endogenous opioids that have high affinity for μ and δ opioid receptors, affect mature cells of the hematopoietic system, i.e., monocytes, macrophages, and neutrophils (19). However, no studies have thus far linked the μ opioid receptor system to blood cell production, especially for progenitor cells of myeloid origin that give rise to granulocytes, monocytes/macrophages, erythrocytes, and platelets. Hematopoiesis, the production of blood cells, is regulated by cytokines, which include mem-

Table 1. *Hematopoietic Progenitor Cells*

	Femur			Spleen		
	+/+	+/−	−/−	+/+	+/−	−/−
Nucleated cells ($\times 10^{-6}$)	18.5 ± 1.1	18.2 ± 1.6 [1.0]	16.9 ± 0.9 [0.9]	152.6 ± 15.8	141.1 ± 11.4 [0.9]	131.6 ± 10.7 [0.9]
Progenitors $\times 10^{-3}$ per femur or spleen						
CFU-GM	32.5 ± 4.3	34.5 ± 1.5 [1.1]	*45.5 ± 4.1 [1.4]	11.2 ± 3.2	11.2 ± 2.0 [1.0]	19.9 ± 6.3 [1.8]
BFU-E	8.2 ± 1.5	11.9 ± 0.9 [1.5]	*12.5 ± 1.6 [1.5]	9.8 ± 3.2	8.8 ± 2.6 [0.9]	10.9 ± 3.3 [1.1]
CFU-GEMM	2.9 ± 0.5	2.8 ± 0.3 [1.0]	*6.4 ± 0.6 [2.2]	1.2 ± 0.4	1.0 ± 0.3 [0.8]	2.1 ± 1.1 [1.8]
Cycling status (percent of progenitors in S-phase)						
CFU-GM	4 ± 2	*17 ± 7 [4.3]	*64 ± 2 [16.0]	5 ± 2	*35 ± 4 [7.0]	*63 ± 4 [12.6]
BFU-E	6 ± 3	*35 ± 6 [5.8]	*64 ± 3 [10.7]	4 ± 2	*29 ± 5 [7.3]	*61 ± 4 [15.3]
CFU-GEMM	7 ± 5	*45 ± 5 [6.4]	*73 ± 2 [10.4]	7 ± 4	*42 ± 5 [6.0]	*68 ± 6 [9.7]

Nucleated cellularity and numbers and cycling status of myeloid progenitor cells in the femur and spleen of μ opioid receptor *Oprm*$^{+/+}$, *Oprm*$^{+/-}$, and *Oprm*$^{-/-}$ mice. Results are shown as the mean ± SEM for 14 mice (from a total of three experiments) each for *Oprm*$^{+/+}$ and *Oprm*$^{-/-}$ and for 4 mice (from one experiment) for *Oprm*$^{+/-}$ in which each mouse was assessed individually. Numbers in brackets signify fold change from *Oprm*$^{+/+}$ cells. *Significant difference ($P < 0.05$, Student's *t* test) from wild-type value.

bers of the chemokine family (21–24). Chemokine receptors, for example the interleukin-8 receptor, are members of the G protein-coupled receptor family like the μ opioid receptor, and the μ opioid receptor displays moderate levels of sequence homology to both the interleukin-8 receptor and C-C chemokine receptors (25). Given these structural parallels and implied functional interactions, it was of interest to examine the μ opioid receptor–deficient mice for potential changes in hematopoietic processes.

To explore a potential role of μ opioid receptors in hematopoiesis, myeloid blood cell production in mice was examined using bone marrow, spleen, and blood. We found that the absolute numbers of femoral bone marrow granulocyte-macrophage (CFU-GM), erythoid (BFU-E), and multipotential (CFU-GEMM) progenitor cells in bone marrow were significantly higher in homozygotes ($Oprm^{-/-}$) compared to wild-type mice (Table 1), whereas there were no apparent differences in nucleated cellularity in the bone marrow and spleen (Table 1) or in the leukocytes, erythrocytes, and platelet counts in the blood (data not shown). Also, although myeloid progenitors in the bone marrow and spleen were in a slowly cycling state in wild-type mice, these progenitors were in rapid cell cycle in homozygous mice as determined by the percentage of progenitors in S-phase (Table 1). The cycling status of myeloid progenitors in bone marrow and spleen of heterozygous mice ($Oprm^{+/-}$) was intermediate between that of wild type ($Oprm^{+/+}$) and homozygotes ($Oprm^{-/-}$). Also, the absolute numbers of CFU-GM, BFU-E, and CFU-GEMM in spleen were increased in homozygotes compared to wild-type mice, although these differences were not significant.

Cells of the myeloid lineage, such as monocytes, macrophages, and neutrophils, are among the body's first line of defense against infections, and serve important functions such as antigen presentation, phagocytosis of foreign materials, and production/release of stimulatory and suppressing cytokines active on myeloid and lymphoid cell production and function (21–24). Endogenous opioids in circulation could potentially activate the μ opioid receptor on these cells, which may modulate hematopoiesis. Our finding of enhanced production of the myeloid progenitors in μ opioid receptor–deficient mice thus provides strong evidence of this receptor's involvement in blood cell production, most likely as a negative regulatory influence. The exact link remains to be determined, and may be directly or indirectly mediated at the level of hematopoietic stem and progenitor cells. The enhanced proliferation status and number of myeloid progenitors in bone marrow and spleen without a subsequent increase in mature blood cell number suggests involvement of additional cell–cytokine mediated events between premature and mature blood cell regulation.

Sexual Function. During breeding, we noticed that it took homozygous male mice ($Oprm^{-/-}$) a significantly longer time to impregnate a female than wild-type male mice (Ta-

Table 2. *Mating Behavior and Reproductive System in Male Mice*

Genotype	+/+	+/−	−/−
Mating behavior			
Mounting			
Latency (min)	5.3 ± 2.3 (5)	16.3 ± 7.1 (5)	*29.5 ± 10.5 (5)
Appropriate (count)	25.2 ± 6.7 (5)	18.3 ± 1.4 (5)	*4.4 ± 3.9 (5)
Inappropriate (count)	3.0 ± 1.4 (5)	2.8 ± 0.4 (5)	4.6 ± 3.0 (5)
Total contact time (min)	52.2 ± 15.1 (5)	52.7 ± 9.5 (5)	41.7 ± 13.5 (5)
Intromission (count)	12.3 ± 8.8 (5)	3.3 ± 0.5 (5)	*0.8 ± 0.8 (5)
Ejaculating during the test	40% (5)	*0% (5)	*0% (5)
Overnight plug	60% (5)	40% (5)	*20% (5)
Parameters of male reproductive system			
Sperm count ($\times 10^6$ epididymis)	10.9 ± 1.4 (4)	12.1 ± 0.6 (4)	*7.0 ± 2.3 (4)
Sperm motility (%)	78.4 ± 2.2 (4)	74.0 ± 1.3 (4)	*54.5 ± 7.4 (4)
Ventral prostate weight (mg/g weight)	0.46 ± 0.13 (4)	0.47 ± 0.10 (4)	0.47 ± 0.05 (4)
Seminal vesicle weight (mg/g body weight)	6.8 ± 0.6 (4)	9.3 ± 1.5 (4)	8.2 ± 0.8 (4)
Time to pregnancy (d)	1.3 ± 0.2 (6)	3.2 ± 0.8 (15)	*5.1 ± 0.5 (22)
Offspring litter size	9.3 ± 0.6 (8)	8.1 ± 0.5 (23)	*6.0 ± 0.3 (34)

Mating was measured over a 2-h period. Mounting latency was the time from when an estrous female mouse was introduced into the cage to when the male first attempted mounting. Appropriate mounting, mounting attempt oriented toward the rear of the female; inappropriate mounting, attempt oriented elsewhere (side, front, etc.); total contact time, accumulative time when the male was investigating the female mouse, including mounting and sniffing of body and genital areas. Ejaculation was determined at the end of the test by inspection of the female's vagina for the presence of a seminal plug. Overnight plug was determined by overnight housing of individual male mice after the testing with an estrous female mouse and scoring the presence of a seminal plug. Reproductive system parameters were measured as described in Materials and Methods.
The numbers in parentheses represent data shown as mean ± SEM. *Significant difference ($P < 0.05$, Student's t test) from the wild-type value.

ble 2), and the offspring litter size by homozygous males was smaller than that by wild type (Table 2). Two factors could contribute to such a change: a reduction in mating activity and a change in reproductive physiology. To examine whether the μ opioid receptor plays a role in the sexual behavior in mice, we measured the mating activity in male mice. When an estrous wild-type female mouse was placed in a cage with a male mouse, the wild-type male would start to investigate the female and, within a few min, initiate sexual activity by mounting the female mouse. The homozygous males (*Oprm*$^{-/-}$), on the other hand, showed a significantly lengthened period before they initiated sexual activity with the female mouse (Table 2). Also, whereas the majority of the mounting attempts by a wild type male were appropriate (mounting from the rear of the female), the proportion of appropriate mounting by a homozygous male was much reduced (Table 2). In addition, the number of intromissions during the test session was significantly reduced in homozygous males as compared with the wild type. These results indicate that sexual activity is reduced in the homozygous male mice compared with that in the wild type. The reduction of sexual activity in homozygous mice does not appear to be caused by reduced locomotor activity, because during mating tests there was no significant difference among the wild-type, heterozygous, and homozygous male mice in the total contact time, determined as the accumulative time when the male was investigating the female mouse, including mounting and sniffing of body and genital areas (Table 2).

When physiological parameters of the male reproductive system were examined, they also appeared to be altered by disruption of the μ opioid receptor gene. As shown in Table 2, even when a homozygous male initiated intromission, there was little ejaculation (a lack of seminal plugs) during the 2-h test. This is not due to a complete inability to ejaculate, as homozygous males were able to form seminal plugs when left overnight in the cage with female mice (Table 2). However, the percentage of overnight plugs was reduced compared with that of the wild-type male mice.

The male reproductive system in homozygous male mice appears to be functional, as the prostate and seminal vesicle weights were of similar values among different genotypes (Table 2). However, sperm motility and sperm counts in homozygous mice were significantly reduced compared to those in wild-type mice (Table 2). These data suggest that the development of sperm may be affected by disruption of the μ opioid receptor gene even though the gross anatomy of the male reproductive system in homozygous male mice is not. Taken together, these results indicate that the μ opioid receptor may play an important role in the sexual behavior and reproductive physiology in mice. Further studies are needed to address whether the μ opioid receptor acts directly on the reproductive organs or functions by modulating a central mechanism.

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