

Confirmation of a Major QTL Influencing Oral Morphine Intake in C57 and DBA Mice Using Reciprocal Congenic Strains

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C57BL/6 (B6) and DBA/2 (D2) mice exhibit disparate behavior when tested for voluntary morphine intake in a two-bottle choice drinking paradigm with B6 mice consuming 10 times more drug than D2 mice. Previous genetic mapping studies identified a locus, *Mop2*, on the proximal part of chromosome 10 that explained over half of the genetic variance in this mouse model of opioid self-administration. We constructed a set of reciprocal congenic strains between B6 and D2 mice in which the proximal portion of chromosome 10 has been introgressed from one strain onto the background of the other. We tested mice from this pair of reciprocal strains together with progenitor B6 and D2 mice in a two-bottle choice drinking paradigm with morphine and quinine. The results showed that introgression of chromosome 10 alleles from the B6 strain onto a D2 genetic background increased voluntary morphine intake four-fold compared to progenitor D2 mice. Preference for morphine was also increased significantly in D2.B6-*Mop2* mice compared to progenitor D2 mice. Conversely, introgression of chromosome 10 alleles from the D2 strain onto a B6 genetic background decreased morphine intake by half compared to progenitor B6 mice in B6.D2-*Mop2* mice; however, high morphine preference was maintained in this congenic strain most likely due to strong quinine aversion. When quinine was eliminated from the control bottle, morphine preference in B6.D2-*Mop2* mice was decreased significantly relative to B6 and D2.B6-*Mop2* mice. Overall, these data confirm the existence of a gene(s) on chromosome 10 proximal to D10Mit124 that has a strong influence on the difference in morphine drinking behavior between B6 and D2 mice.

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INTRODUCTION

Compared to other inbred strains, C57BL/6 (B6) mice voluntarily drink large quantities of sweetened morphine solutions (Horowitz *et al*, 1977; Belknap *et al*, 1993). Oral morphine intake by B6 mice is attenuated following treatment with opioid antagonists (Berrettini *et al*, 1994a) suggesting that consumption of morphine solutions elicits opioid receptor-mediated phenomena in the brain. Quantitative trait locus (QTL) mapping studies indicate that a locus on the proximal end of chromosome 10 called *Mop2* harbors a gene(s) that determines a large part of the

difference in voluntary oral morphine consumption between B6 and DBA/2 (D2) mice (Berrettini *et al*, 1994b). The gene encoding the *Mu* opioid receptor (*Oprm*) is located on proximal chromosome 10 (Kozak *et al*, 1994; Giros *et al*, 1995) and is a compelling candidate for the *Mop2* QTL; however, the causative genetic variation between B6 and D2 mice has yet to be determined.

Although advances in technology and methodology have increased the potential for identifying genes that underlie specific QTLs (Abiola *et al*, 2003), many obstacles hinder success. Congenic strains have proven utility for helping to elucidate the functional effects of defined segments of the genome and recent work has documented success of this approach for dissecting genetic influences on drug abuse-related phenotypes in mice (Bennett *et al*, 2002). Once a QTL is captured in a congenic strain, simple breeding methods can be used to further refine its position (Ferraro *et al*, 2004). In some cases, genomic QTL intervals introgressed in congenic strains may be resolved into multiple genetic effects that are too weak to track

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individually (Legare *et al*, 2000; Legare and Frankel, 2000). In the present study, we report the creation of congenic strains of mice to confirm and further elucidate the effect of a gene(s) on proximal chromosome 10 that influences voluntary morphine intake. The results confirm a strong effect of the *Mop2* locus on both oral morphine consumption and preference and localize it to the proximal end of chromosome 10 between the acromere and D10Mit124.

METHODS

Animals

Mice utilized in these studies were derived from C57BL/6 (B6) and DBA/2 (D2) mice that are maintained routinely in our animal colony at the Department of Veteran's Affairs Medical Center in Coatesville, PA. Genetic drift in these inbred strains is minimized by supplementing breeders with animals purchased from the Jackson Laboratory (Bar Harbor, ME) several times per year. In general, mice are weaned at 3 weeks of age and group-housed by gender until utilized for experimentation. They are maintained on a 12 h light/dark schedule (lights on at 0700) and have free access to food. Water is also available *ad libitum* except as noted below during the two-bottle choice paradigm. All studies were approved by the Animal Care and Use Committees governing the participating laboratories.

Mop2 Congenic Strains

The reciprocal pair of congenic strains used for these experiments was created in our animal colony over the past 5 years. We followed a conventional protocol (Flaherty, 1981) that began with a B6 × D2 intercross followed by backcross of the resulting F1 generation progeny to both parental strains. Backcross breeding was accomplished using a design in which both F1 males and females were mated with progenitor mice. We genotyped N2 backcross progeny using DNA extracted from tail snips acquired at the time of weaning and selected mice for subsequent rounds of backcross breeding based upon heterozygosity at a specific set of microsatellite DNA markers (Figure 1). A total of 10 serial backcross generations were produced in a reciprocal

manner. During creation of the strains, morphine consumption and preference were assessed in heterozygous congenic mice at generations N4 and N7 to insure capture of phenotypic effects. Initially, both of the reciprocal strains were developed using the marker panel spanning the interval between D10Mit75 and D10Mit186; however, a very small number of B6.D2-*Mop2* strain progeny were obtained at generation N8 and as a result it was not possible to identify a sufficient number of pups that inherited the entire introgression panel. The development of the B6.D2-*Mop2* strain then proceeded through the N9 and N10 generations using a smaller region of introgression defined by D10Mit75 proximally and D10Mit124 distally (Figure 1). At generation N10, heterozygous mice were intercrossed and DNA from N10F1 progeny was analyzed to identify homozygous congenic mice. Homozygous mice were brother × sister mated to propagate each of the reciprocal strains. B6.D2-*Mop2* N10F5–F7 and D2.B6-*Mop2* N10F10–F12 mice were used in morphine *vs* quinine experiments. B6.D2-*Mop2* N10F5–F9 and D2.B6-*Mop2* N10F10–F14 mice were used in morphine *vs* saccharin experiments.

Genotyping

DNA microsatellite markers were analyzed using PCR amplification followed by electrophoresis on polyacrylamide (Berrettini *et al*, 1994b) or agarose gels (Ferraro *et al*, 1998) as described previously. A B6/D2 strain polymorphism in the *Oprm* gene was typed as follows: approximately 100 ng of tail DNA was used in a PCR reaction with primers that amplified a 360 bp amplicon containing exon 10 of the *Oprm* gene. The PCR conditions were 1 × standard Perkin Elmer PCR buffer containing 125 μM dNTPs, 0.1 pmol/μl forward primer (5'-GGTTATGCCTCTCTGGAT TAG-3'), 0.1 pmol/μl reverse primer (5'-TCCATCGCTTA CATCTTACCA-3'), and 2.5 U of AmpliTaq DNA polymerase in a 20 μl reaction. PCR cycles were 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1 min, followed by a final extension step of 72°C for 5 min. A G-insertion in exon 10 of the B6 *Oprm* gene (data not shown) creates a *DdeI* restriction site. When the 403 bp amplicon from B6 is digested with *DdeI*, it creates two bands of 235 and 168 bp. For genotype analysis, 10 μl of the reaction was digested with the *DdeI* restriction enzyme (New England Biolabs, Beverly, MA) following the manufacturer's recommended conditions. Products were run on a 5% nondenaturing polyacrylamide gel and stained with ethidium bromide.

Morphine Consumption

Morphine consumption and preference were quantified using a two-bottle choice paradigm as described previously (Berrettini *et al*, 1994b). Briefly, mice (male, 8–12 weeks of age) were housed individually in cages with two water bottles for 1 week prior to the start of the 12-day test paradigm. On days 1–4 of the test, both drinking bottles contained water. Fluid intake was measured by weighing the bottles between 0900 and 1000 daily. On days 5–8, the water in one bottle was replaced with a solution of morphine sulfate (0.4 mg/ml) in 0.2% saccharin and the water in the other (control) bottle was replaced with a solution of

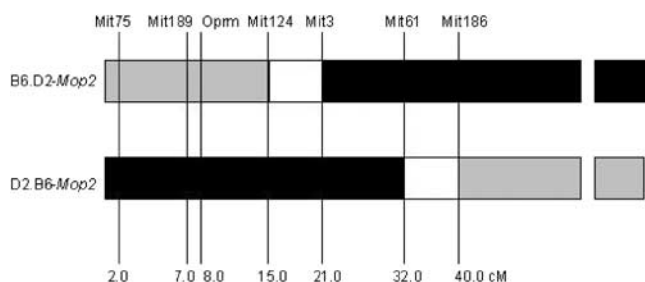


Figure 1 Schematic diagram of chromosome 10 in B6.D2-*Mop2* (top) and D2.B6-*Mop2* (bottom) congenic mice. Lightly shaded regions reflect genomic contribution from the D2 strain and darkly shaded regions reflect genomic contribution from the B6 strain. White segments represent intervals of undetermined origin. DNA microsatellite markers used for genomic introgression are shown above the chromosomes. Numbers below represent the genetic map position (<http://www.informatics.jax.org/>) and are given in cM.

quinine (0.2 mg/ml) in 0.2% saccharin. Mice were allowed continuous access to both bottles. To minimize effects related to learning, the position of the bottles in the cage was alternated at the time of daily bottle weighing. On days 9–12, the concentration of morphine was increased to 0.7 mg/ml and the concentration of quinine was increased to 0.4 mg/ml. Again mice were allowed continuous access. Body weights of the mice were measured every other day throughout the test paradigm. We also used a modified version of the test in which quinine was eliminated from the control bottle while all other parameters were maintained as described above.

Data Analysis

The study was conducted in three parts. In the first part, six mice from each of the four strains (B6, D2, B6.D2-*Mop2*, and D2.B6-*Mop2*) were tested simultaneously in the two-bottle choice paradigm with morphine and quinine. In the second part, which took place approximately 8 weeks after the first, the same protocol was repeated using naïve mice. Strain data from the two experiments were first compared by *t*-test and, with no significant differences noted, were then combined for further analysis. In the third part, additional mice from each of the four strains ($n=4$ per strain) were tested in the paradigm without quinine. Daily drinking values were entered into a Microsoft Excel workbook and statistical analyses were conducted with the DOS-based Truepistat software package (Truepistat, Richardson, TX). One-way ANOVA was used to evaluate the influence of genetic background on morphine, quinine and saccharin consumption (mg/kg/day) and also on morphine preference (percent of total fluid intake). Analyses were based on drinking values for each mouse averaged over the last 4 days (days 9–12) of the two-bottle test paradigm. Between-group comparisons were conducted *post hoc* with the Neuman–Keuls test. Total fluid intake measures were analyzed by comparing the average daily value over days 1–4 with the average daily value over days 9–12 for each strain using a two-way ANOVA and Neuman–Keuls *post hoc* test.

RESULTS

Data shown in Figure 2 confirm the large difference between B6 and D2 mice with respect to oral morphine intake in the two-bottle choice paradigm. In this study, B6 mice consumed about 150 mg/kg/day on average, whereas D2 mice consumed about 20 mg/kg/day. As predicted from mapping studies (Berrettini et al, 1994b), B6.D2-*Mop2* mice exhibited significantly reduced morphine consumption compared to parental B6 mice with about one-half of total morphine consumption in B6 mice explained by the *Mop2* locus on chromosome 10. Also in keeping with the prediction, D2.B6-*Mop2* mice consumed significantly more morphine (about four-fold) than progenitor D2 mice, demonstrating the large effect of the B6 *Mop2* locus when isolated on a D2 genetic background. This locus seems to function in a dominant or semidominant mode since effects of similar magnitude were observed in heterozygous congenic mice at generations N4 and N7 (data not shown). With respect to quinine, D2 mice consumed over two times

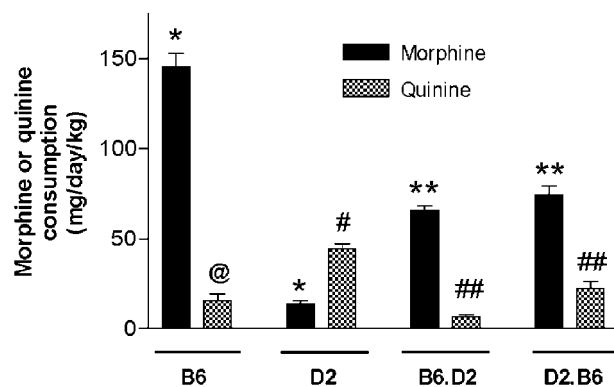


Figure 2 Morphine and quinine consumption in parental and *Mop2* congenic strains. Mice were tested in the two-bottle choice paradigm with morphine plus saccharin vs quinine plus saccharin as described in Methods. The top of each bar represents the mean (+ SEM) consumption over days 9–12 ($n=12$). * $P<0.01$ vs all other morphine groups; ** $P<0.01$ vs B6 and D2 morphine; # $P<0.01$ vs all other quinine groups; ## $P<0.01$ vs D2 and reciprocal congenic strains; @ $P<0.01$ vs D2 quinine.

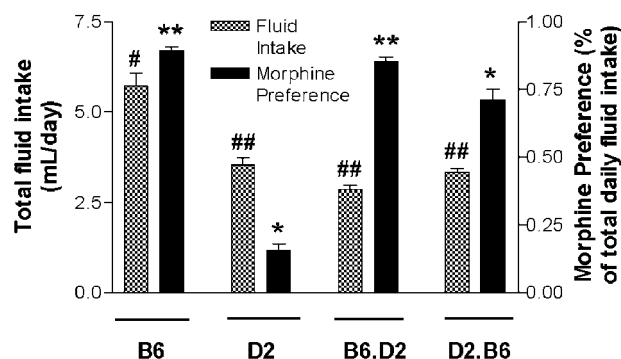


Figure 3 Morphine preference and total fluid intake in parental and *Mop2* congenic strains. Mice were tested in the two-bottle choice paradigm with morphine plus saccharin vs quinine plus saccharin as described in Methods. The top of each bar represents the mean (+ SEM) preference or intake value over days 9–12 ($n=12$). * $P<0.01$ vs all other group preferences; ** $P<0.01$ vs D2 and D2.B6 preference; # $P<0.01$ vs all other total fluid intake values; ## $P<0.01$ vs B6 total fluid intake.

more than B6 mice, an effect that was partially abrogated in D2.B6-*Mop2* mice, as the latter exhibited increased morphine consumption relative to D2 in association with decreased quinine consumption. On the other hand, there was no significant change in quinine consumption in B6.D2-*Mop2* mice compared to B6 mice in spite of a significant reduction in morphine consumption.

When drinking data were expressed as preference scores, a slightly different picture emerged (Figure 3). Compared to preference scores for B6 mice, which were greater than 0.9 (90% of daily fluid from the morphine bottle), scores in B6.D2-*Mop2* congenic mice were not significantly different. Related to this finding, it is noteworthy that total fluid consumption increased by about 50% in B6 mice specifically during the morphine/quinine phase of the two-bottle choice test (days 5–12). Thus, during days 1–4 of the test, when both bottles contained water, all four strains drank the same amount of daily fluid, about 4 ml (data not shown). This value increased to about 6 ml/day in B6 mice during

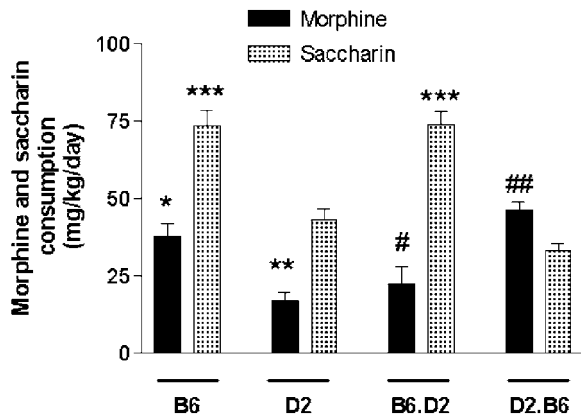


Figure 4 Morphine and saccharin consumption in parental and *Mop2* congenic strains. Mice were tested in the two-bottle choice paradigm with morphine plus saccharin vs saccharin as described in Methods. The top of each bar represents the mean (\pm SEM) consumption over days 9–12 ($n=4$). * $P<0.01$ vs D2, 0.05 vs B6.D2; ** $P<0.01$ vs B6, D2.B6; # $P<0.01$ vs D2.B6, 0.05 vs B6; ## $P<0.01$ vs D2, B6.D2; *** $P<0.01$ vs D2, D2.B6.

days 5–12 ($P<0.01$ vs days 1–4), whereas the other strains showed slight decreases that were not statistically significant compared to intake on days 1–4. Lack of decrease in preference score of B6.D2-*Mop2* congenic mice relative to B6 mice in spite of a 50% reduction in morphine consumption reflects this difference in total fluid consumption. On the other hand, preference scores for D2.B6-*Mop2* mice were significantly increased (about four-fold) compared to scores for D2 mice, which were about 0.15 with similar levels of total fluid intake.

Elimination of quinine from the control bottle had several effects on drinking behavior. Most notably, morphine intake decreased in all strains due to the natural preference of mice for sweet tasting substances (Figure 4). In spite of the fact that B6 mice exhibited a sharp reduction in morphine intake (levels were reduced to about one-third of those observed when quinine was included) and also significantly greater saccharin intake than D2 mice, morphine intake was still significantly greater than that exhibited by both D2 and B6.D2-*Mop2* mice (Figure 4). Furthermore, the level of morphine intake was significantly lower in D2 compared to D2.B6-*Mop2*. With regard to morphine preference, although omission of quinine decreased slightly the high preference level exhibited by D2.B6-*Mop2* mice, this level was significantly greater than that exhibited by B6 mice (Figure 5). On the other hand, morphine preference level for B6.D2-*Mop2* mice was decreased significantly relative to B6 mice. This latter result stands in sharp contrast to that obtained when quinine was included in the drinking paradigm.

DISCUSSION

The robust difference between B6 and D2 mice in voluntary morphine self-administration by the oral route was proposed several decades ago as a genetic model of opioid addiction (Horowitz *et al*, 1977). Since then, a number of laboratories have pursued studies of this mouse model. Confirmation of the strain difference between B6 and D2

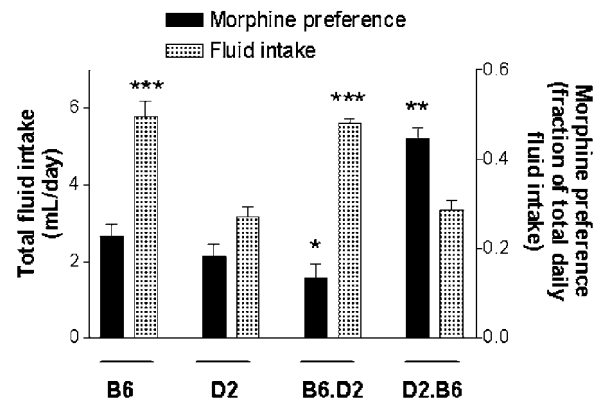


Figure 5 Morphine preference and total fluid intake in parental and *Mop2* congenic strains. Mice were tested in the two-bottle choice paradigm with morphine plus saccharin vs saccharin as described in Methods. The top of each bar represents the mean (\pm SEM) preference or intake value over days 9–12 ($n=4$). * $P<0.01$ vs D2.B6, 0.05 vs B6; ** $P<0.01$ vs all other groups; *** $P<0.01$ vs D2, D2.B6.

mice in oral morphine preference (Belknap, 1990; Belknap *et al*, 1993) was followed by a quantitative genetic study that mapped a major locus for the trait to chromosome 10 (Berrettini *et al*, 1994b). This QTL for oral morphine preference, termed *Mop2*, was confirmed in a follow-up study (Alexander *et al*, 1996) and support was generated for the concept that it had a broader influence with regard to the pharmacological effects of morphine being involved also in determining morphine-induced analgesia and the density of [3 H]naloxone binding sites in the brain (Belknap *et al*, 1995). Subsequent mapping of the *Mu* opioid receptor gene (*Oprm*) to the same chromosomal location as the *Mop2* QTL (Kozak *et al*, 1994; Giros *et al*, 1995) focused attention on this gene as a strong candidate for mediating differences in opioid-related effects between B6 and D2 mice. In spite of intensive study, the causative genetic variation that underlies *Mop2* has yet to be identified. *Oprm* knockout mice on the B6 genetic background exhibit lack of morphine-induced analgesia, locomotor activity, and conditioned place preference (Hall *et al*, 2003). *Oprm* knockout mice have also been shown to lack robust responsiveness in intracerebroventricular morphine self-administration studies (Becker *et al*, 2000); however, this latter study did not control for genetic background and did not test responsiveness of knockout mice in a voluntary drinking paradigm. Thus, the role of *Oprm* and the *Mu* opioid receptor in mediating the differential effects of morphine in B6 and D2 mice is still unresolved.

The present study was carried out to map *Mop2* to a defined genomic interval and also to isolate it for further characterization. The results of experiments with reciprocal congenic strains reported here confirm that a gene(s) with a large effect on oral morphine consumption and preference lies between the centromere (acromere) and D10Mit124 on chromosome 10. This conclusion is supported by data generated using the D2.B6-*Mop2* strain in which both morphine consumption and preference were increased relative to the D2 background strain. On the other hand, comparison of the results between B6.D2-*Mop2* and B6 mice supports the existence of a gene that influences morphine

consumption on proximal chromosome 10, but not one that influences preference. Given the difference in the size of the introgressed interval between the two reciprocal congenic strains (Figure 1), it is possible that the 'preference gene(s)' is distinct from the 'consumption gene(s)' and is located between D10Mit124 and D10Mit186. A more likely explanation for high morphine preference in B6.D2-Mop2 mice relative to progenitor B6 mice involves taste effects associated with quinine in the control bottle. Morphine consumption depends on the rewarding properties of the drug; however, preference is influenced by the degree of quinine aversion. Thus, it is possible that aversion to the bitter taste of quinine results in maintenance of relatively high levels of fluid consumption from the morphine bottle in spite of a presumed reduction in the rewarding effect of the drug. This interpretation is supported by the results of the experiment in which quinine was omitted from the control bottle. Under this condition, not only did the chromosome 10 region of introgression influence morphine intake in a reciprocal manner, but it also influenced preference similarly. This interpretation is also supported by mapping results in which preference scores in the morphine/quinine two-bottle choice paradigm were shown to be influenced strongly by a locus on chromosome 6 (Berrettini et al, 1994b; Lush, 1984).

In summary, we have created a reciprocal pair of congenic mouse strains to confirm the existence of the Mop2 QTL on chromosome 10. The results indicate that Mop2 is located proximal to D10Mit124 and that it is a major determinant of the difference in both morphine consumption and morphine preference between B6 and D2 mice. Further reduction of the Mop2 critical interval may be achieved using a strategy that involves identifying recombinant progeny and propagating new interval specific congenic strains for behavioral testing. This systematic approach to dissecting the Mop2 critical interval may lead to the identification of the causative genetic variation that underlies significant differences in the pharmacology of opioids between B6 and D2 mice.

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