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Genetic correlates of morphine withdrawal in fourteen inbred mouse strains

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Abstract

Male mice from 14 standard inbred strains were exposed to morphine in a sustained released preparation injected subcutaneously. Five hours later withdrawal was precipitated by intraperitoneal injection of naloxone. Mice were tested from 0 to 15 minutes after naloxone for withdrawal jumping behavior, and then from minute 15–16 for other signs, including boli count, presence of soft stool, lacrimation, “wet dog” shakes, and air chewing. They were also assessed for change in body temperature 17 minutes after naloxone. Strains differed markedly in the severity of withdrawal for jumping, change in body temperature, and number of fecal boli. Strains also differed in percentage of animals displaying soft stool and air chewing behavior. The other two signs were seen at too low frequency for analysis. Correlations of strain mean withdrawal severity with other responses to morphine and other abused drugs showed that high morphine withdrawal jumping and low change in body temperature were both genetically related to high morphine consumption, but not generally to other measures of morphine withdrawal or morphine sensitivity.

Keywords

inbred mouse strains; pharmacogenetics; dependence; morphine; precipitated withdrawal; jumping

1. Introduction

Withdrawal from morphine in mice is characterized by jumping, changes in thermoregulatory set point, autonomic nervous system overactivity, increased gastrointestinal motility and stereotypy (Belknap, 1990; El-Kadi and Sharif, 1994; Ritzmann, 1981; Way et al., 1969). Of these, withdrawal-induced jumping precipitated by the antagonist naloxone is the most common index of opioid withdrawal reported in the literature (Belknap and O'Toole, 1991; Kest et al., 2002b; Marshall and Grahame-Smith, 1971). This behavior has been shown to be sensitive to genetic differences among inbred strains (reviewed by Belknap and O'Toole, 1991), and strain differences were larger than those across dose and procedural variations (Kest et al., 2002b). In mice and rats, opioid

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withdrawal is known to be associated with thermoregulatory dysfunction leading to hypothermia. This response can also serve as an index of withdrawal severity (Adler et al., 1988; Belknap, 1989; Wei et al., 1974). A third well-established opioid withdrawal sign in mice is increased gastrointestinal motility leading to increased fecal output and soft stool (Belknap, 1990), which can also serve to quantify withdrawal severity. In this study, we looked at all three types of opioid withdrawal signs among 14 standard inbred mouse strains.

Characterization of strain differences in withdrawal severity allows comparison of those differences with other behaviors. The correlation of inbred strain mean values on two traits serves as a rough index of the importance of influence of a common set of genes, as r^2 from this estimate of genetic correlation indicates the proportion of shared genetic variance (Hegmann and Possidente, 1981). We have successfully used this method to ascertain patterns of shared genetic influences of several sorts: across different doses of the same drug; across different behavioral responses (e.g., acute locomotor activity, acute thermal response, withdrawal severity, and voluntary drug consumption) to a single drug dose; and across different drugs for the same measure (e.g., locomotor activity changes after morphine, ethanol, diazepam, or pentobarbital; Belknap et al., submitted). We have also reported brain concentrations of these drugs 30 min after fixed i.p. doses in separate studies of all four drugs in these same inbred strains (Belknap et al., 1998; Belknap et al., 1993a; Belknap et al., 1993b; Crabbe, 1983; Crabbe et al., 1982; Crabbe et al., 1998; Crabbe et al., 1994; Crabbe et al., 1983; Crabbe et al., 2002; Metten and Crabbe, 1994; Metten and Crabbe, 1999; Metten and Crabbe, 2005).

Our goal in the present experiment was to evaluate genetic differences in drug sensitivity that were independent of pharmacokinetic differences. When exposed to the same dose of morphine, some strains might achieve higher blood (and brain) morphine concentrations than others, and therefore withdraw more severely because their effective dose was higher. There are a variety of methods for inducing morphine dependence (e.g., Belknap, 1990; Haghparast et al., 2008; Kest et al., 2002b). In this study, we sought to equalize exposure by injecting a very high dose of morphine in a sustained release preparation, which likely had saturated opioid receptors of the μ type, i.e., those implicated in morphine withdrawal in mice (Miyamoto and Takemori, 1993). Additionally, mice were injected with a sufficiently large dose of the specific competitive antagonist naloxone (12 mg/kg), to displace these receptors of morphine (Gutstein and Akil, 2001).

Naloxone-precipitated jumping behavior is a sensitive behavioral index of opioid physical dependence development in rodents. It occurs in a largely strain-specific fashion in inbred mouse strains following acute or chronic morphine injection and following morphine continuous infusion or following acute or chronic heroin injection (Kest et al., 2002b; Klein et al., 2008). Inbred strains also differ in acute drug withdrawal severity from sedative hypnotic drugs such as alcohol, pentobarbital and diazepam, and there are substantial genetic correlations ($.70 < r < .83$) among 15 strain withdrawal severity means among these three drugs (Metten and Crabbe, 1994; Metten and Crabbe, 1999). There is a large literature on the endogenous opioid system and its interactions with the brain reward and stress response systems suggesting a role in alcohol consumption and genetic predisposition to alcoholism (for example, see review by Gianoulakis, 2001). Early studies clearly demonstrated that naloxone could not precipitate withdrawal jumping in mice that had been made either tolerant to or dependent on ethanol (Goldstein and Judson, 1971; Way et al., 1969). However, in a subsequent study chronic naloxone treatment before and during ethanol withdrawal may have inhibited withdrawal (Blum et al., 1977). Thus, we also sought to explore the correlation of morphine withdrawal severity with that from ethanol, as well as the other two drugs from a different pharmacologic class.

Studies of genetic correlation among phenotypic responses using inbred strains allow interpretation of common genetic, or predisposing, risk for any particular pair of phenotypes. Many studies have found a significant negative correlation between severity of withdrawal from ethanol and voluntary consumption of oral 10% ethanol under two-bottle choice (alcohol vs water) conditions indicating that those strains predisposed to severe ethanol withdrawal, but without prior ethanol experience, will choose to consume little to no ethanol (Chester et al., 2003; Metten et al., 1998; Metten and Crabbe, 2005). Thus, we examined whether any of the morphine withdrawal signs were correlated with new morphine data in inbred strains that have been published for a variety of other traits (e.g., morphine consumption, analgesic tolerance, thermoregulatory responses, activity, etc. [Belknap et al., 1993a; Belknap et al., 1993b; Kest et al., 2002a]).

Kest et al. (2002b) assessed morphine dependence in eleven standard inbred mouse strains using three different methods to induce physical dependence, all by the subcutaneous (s.c.) route. These were: a single large injection of morphine (acute model), three injections per day over four days (chronic model), and a chronic infusion model using a 7-day exposure via osmotic implants (Minipumps®). In all three regimens, withdrawal was precipitated by naloxone. Strain mean (genetic) correlations were quite high among all three methods (all $r > 0.80$), indicating that the three models were closely similar in their underlying genetic determinants. The method we used in this study most closely resembles their acute model, although procedural differences remain. We compared our results with the Kest et al. (2002b) data as well.

2. Materials and Methods

2.1 Animals and husbandry

Adult male mice of the following inbred strains were obtained at 4–6 weeks of age from The Jackson Laboratory (Bar Harbor, ME): 129P3/J (129), A/HeJ (A), AKR/J (AKR), BALB/cJ (BALB), C3H/HeJ (C3H), C57BL/6J (B6), C57BR/cdJ (C57BR), C57L/J (C57L), CBA/J (CBA), DBA/1J (DBA/1), DBA/2J (DBA/2 or D2), PL/J (PL), SJL/J (SJL), SWR/J (SWR). Mice were housed 2–4 per polycarbonate or polysulfone cage with others of the same strain for 7–22 days (average of 14) before testing. Mice were tested between 43 and 59 days old (mean $\pm$ SEM: 53.8 ± 0.3 overall; strain mean range $49.0 - 57.8$). Body weights ranged from 16.9 – 31.9 g, with the range of means being 20.9 ± 0.2 (PL/J) to 28.7 ± 0.8 (AKR/J). A total of 146 mice (10–11/strain) were exposed to morphine and tested. Food (Purina 5001) and water were available *ad libitum*, and lights were on from 0600 – 1800 hr in colony rooms maintained at 22 ± 2 °C. All procedures were approved by the Portland VA Institutional Animal Care and Use Committee in accordance with USDA and USPHS guidelines and were conducted in accordance with the *Guide for the Care and Use of Laboratory Subjects* as adopted and promulgated by the US National Institutes of Health.

2.2 Drug Preparation

Morphine was prepared in the sustained release preparation originally used by Collier et al. (1972) as modified by Ben-Eliyahu et al. (1992). A 35 mg/ml morphine sulfate (RBI) in saline (McGaw) solution was thoroughly and vigorously vortexed with a 6:1 light mineral oil (Sigma):mannide monooleate (Arlacel A, Sigma) mixture in a 10:7 ratio of morphine:oil solution. This results in a viscous white emulsion that must be vortexed within a few minutes of each injection. The preparation was administered subcutaneously at a dose of 200 mg/kg morphine sulfate using a 21 gauge needle in a volume of 8 ml/kg body weight in colony rooms maintained at 22 ± 2 °C (0.2 ml for a 25 g mouse). Based on preliminary studies, duration of demonstrable morphine effects was about 12 hours based on Straub tail and elevations of hot plate response latencies (unpublished observations).

Naloxone (Sigma) was prepared as 12 mg/kg in 0.9% physiological saline (McGaw) and was injected intraperitoneally at a volume of 0.01 ml/g body weight.

2.3 Morphine dependence induction

Mice were removed to a procedure room and allowed to acclimate for 30–60 minutes. Ten mice per day were injected with morphine as described above, returned to the home cage, and allowed to remain in the room undisturbed for about 5 hours after morphine injection. Mice were tested on one of 17 days, each day representing several inbred strains, over a period spanning 1 month. For each strain, mice from multiple cages (3–4) from each strain were used, and data from each strain were collected on at least three separate days.

2.4 Withdrawal testing

Rectal temperatures were taken at approximately 5 hours after morphine administration. Immediately upon injection of naloxone, all mice were scored for number of jumps during the first 15 minutes, and in the next minute for presence or absence of soft stool, wet dog shakes, air chewing, and lacrimation. Temperatures were taken again at the end of the one-minute observation period. Lastly, boli were counted. The platform was cleaned between mice with a 10% solution of isopropyl alcohol in water.

2.5 Genetic correlations

If inbred strains have been maintained and tested in very similar environments, the extent to which a common set of genes influences two traits can be estimated by correlating the inbred strain means (Hegmann and Possidente, 1981; Crabbe et al., 1990). With few strains (genotypes), it is somewhat difficult to achieve the usual levels of statistical significance (with 14 strains, a correlation of $|r| = 0.53$ is required to achieve $P < .05$). We used the traditional $P < .05$ criterion, but did not correct for the multiple traits studied. For this reason, the results presented here should be considered as hypothesis generating rather than hypothesis testing data. Future studies will undoubtedly be needed to confirm many results reported below.

We attempted to look at all morphine traits reported in the literature for which inbred strain data exist for at least 7 strains identical to those reported here. We also included data and report some analyses that ignored substrain differences.

3. Results

3.1 Withdrawal-induced Jumping

Table 1 shows the morphine withdrawal-induced mean jumping counts by strain during the 15-min test period. Mean $\pm$ SEM jumping counts by strain ranged from 0 ± 0 (strains 129P3/J and PL/J) to 27.9 ± 3.5 (strain C57L/J). Another C57-derived substrain, C57BR/cdJ, also had very high jumping scores (21.3 ± 2.1), while the C57BL/6J substrain showed mean jumping scores of 8.3 ± 1.4 . One-way ANOVA by strain showed that strains differed significantly ($F_{13,132} = 22.31$, $p < 0.0001$), indicating that strain controlled 68.7% of the variance (R^2) in this withdrawal response.

3.2 Body Temperature

Previous data from our laboratory from four different studies on these same inbred strains showed that basal body temperatures (no prior treatment) measured in the same way as in this study differ to a relatively small extent; overall strain means ($N > 110$ mice per strain) ranged from 37.1 (DBA/1, C57BR, C3H) to 37.8°C (SWR, SJL, 129), with SEM per strain always less than 0.1°C (Belknap et al., 1998; Crabbe et al., 1994, 1998, 2002). Morphine

markedly reduced body temperatures in this experiment (range 32.5 – 36.8°C) and temperatures differed significantly among strains ($F_{13,132} = 10.45$, $p < 0.0001$). Naloxone-precipitated withdrawal effects on mean body temperature by strain ranged from 32.2 to 37.3°C, with 5 strains showing increased body temperatures of 0.8 – 2.1°C and the rest showing mild to moderate reductions of 0.2 – 1.7°C compared to pre-naloxone temperatures (Table 1). Mean difference scores (pre- minus post-naloxone) ranged by strain from $-1.7 \pm 0.3^\circ\text{C}$ (strain C57BL/6J) to $2.1 \pm 0.3^\circ\text{C}$ (strain SWR/J). One-way ANOVA by strain showed that strains differed significantly using either measure ($F_{13,132} = 9.08$, $p < 0.0001$), suggesting that strain controlled 47–56% of the variance in this withdrawal response.

3.3 Other Withdrawal Signs

Boli counts differed significantly by strain, as shown in Table 1 ($F_{13,132} = 4.62$, $p < 0.0001$), but was significantly phenotypically ($r = 0.25$, $p = 0.002$, across all 146 mice) and genetically ($r = 0.74$, $p = 0.003$) correlated with body weight. We also analyzed defecation as the residual from linear regression of boli count on body weight to correct for body weight differences. This measure also differed significantly by strain ($F_{13,132} = 2.63$, $p < 0.003$); both measures were highly intercorrelated ($r > 0.95$, $p < 0.0001$).

Table 1 also shows the morphine withdrawal-induced incidence of soft stool and air chewing during the 1 minute test following the jumping observation period. The frequency histograms of these measures indicated that approximately 50% of animals regardless of strain showed soft stool and air chewing behaviors. We examined the proportion of animals within strain displaying soft stool or air chewing behavior using Kruskal-Wallis one-way ANOVAs. Strains differed significantly in the presence of air chewing and soft stool (K–W test statistics = 38.2, $p = 0.001$, $df=13$). Fewer than 20% of animals showed lacrimation or wet dog shakes. The low incidence of these latter behaviors precluded further analyses; however, it is interesting to note that of the animals that showed lacrimation ($N=18$), 5 were PL/J and 6 were C57BR/cdJ. Nine animals showed wet dog shakes, with an incidence of no more than 3 mice per strain.

3.4 Genetic Correlations with Other Traits

3.4.1 Comparison of measures of withdrawal within the experiment—Strain mean jumping counts in this experiment were not significantly genetically correlated with any other withdrawal measures reported in this study ($-0.19 \leq r \leq 0.18$, all $ps > 0.50$). This was true whether we used Pearson's r or Spearman rank order correlations (Metten and Crabbe, 2005). We discuss Pearson's r here (see Table 2 for both). Examination of scatterplots of all pair-wise comparisons within this data set revealed one cluster of withdrawal-related variables. Body temperature after morphine (pre-withdrawal) trended toward a significant positive correlation with the number of boli produced after naloxone ($r = 0.48$, $p = 0.08$), and was significantly correlated with presence of soft stool ($r = 0.66$, $p < 0.05$). The correlation between thermal and alimentary dysregulation was further supported by the tendency for the temperature change index to be correlated with occurrence of soft stool ($r = -0.54$, $p = 0.05$). Finally, vacuous (air) chewing was significantly correlated with the temperature change measure ($r = 0.78$, $p < 0.01$).

3.4.2 Comparison of the current data with those from other laboratories—Kest et al. (2002b) reported strain mean jumping counts following acute or chronic injection or chronic infusion of morphine precipitated by naloxone using 9 strains identical to those we tested plus a highly related substrain (A/J vs. A/HeJ), for a total of ten common strains out of eleven. Despite several procedural differences, their results tended to correlate with the present values (Spearman's rank correlation coefficients = 0.50–0.63; see Figure 1). In their “acute” study, SWR/J showed extremely high jumping scores (approximately 65 jumps, 2–3

fold greater than their second highest scoring strain, and about 50-fold greater than our SWR/J mean jumps; see Table 1). Exclusion of SWR/J resulted in Spearman rank correlation coefficients = 0.78–0.97 when correlated with the present strain mean ranks. In contrast, BALB/cJ and C57BL/6J ranked higher in our study relative to the other strains than they did in the Kest et al. study (2002b).

3.4.3 Correlations with other responses to Morphine—It would be predicted that morphine sensitivity might share some of the same genetic mechanisms with the withdrawal phenotype. For example, lines of mice bred for high levorphenol analgesia showed more severe naloxone-precipitated withdrawal jumping than mice bred for low analgesia, suggesting the action of genes on withdrawal and analgesic sensitivity (Kest et al., 1998). Examination of the present withdrawal data with other behaviors after morphine treatment tested in most of these same inbred strains showed generally that morphine withdrawal jumping was genetically unrelated with other measures of morphine sensitivity, including acute sensitivity to morphine induced changes in activity and body temperature. Withdrawal variables were also uncorrelated with brain concentrations of morphine 30 minutes after i.p. injection of either 16 or 32 mg/kg morphine (all $|r| \leq 0.35$; with brain concentration data from Belknap et al., 1998), suggesting that the behavioral indices we report here do not represent differences in strain pharmacokinetics. Two withdrawal signs were significantly genetically correlated with morphine consumption in a two-bottle choice test versus water (Belknap et al., 1993a). Morphine withdrawal jumping was significantly positively correlated ($r = 0.59$, $p = 0.03$) with morphine consumption, while a negative correlation ($r = -0.55$, $p = 0.04$) was observed between morphine withdrawal-induced change in body temperature and morphine consumption (Belknap et al., 1993a).

Kest et al. (2002a) examined 11 inbred strains for tolerance to morphine-induced analgesia. Those data were reported as mean $\pm$ SEM in tables in their paper, so we were able to calculate Pearson's r on their variables with our morphine withdrawal data with 9 identical strains plus one closely related substrain (A/J vs. A/HeJ). Their study presented two tolerance measures for morphine-induced analgesia: the “latency change,” defined as chronic morphine treatment's reduced effect in changing latency to withdraw the tail from a hot water bath and the “potency shift,” defined as the change after chronic treatment in the half-maximal dose of morphine to effect tail withdrawal. They found the potency shift measure of tolerance to be highly correlated with withdrawal jumping (Kest et al., 2002b). While most variables from the present study did not correlate significantly with either of these tolerance measures, the incidence of soft stool in our study was significantly negatively correlated with the latency change measure ($r = -0.79$, $p < 0.01$) and the number of boli trended in the same direction with this measure ($r = -0.61$, $p = 0.06$). Another trend was observed in the correlation of *morphine*-induced body temperature changes with their potency shift measure ($r = 0.56$, $p = 0.09$), although this association was positive. As expected, examination of scatterplots and use of Spearman rank correlations confirmed these relationships (correlation coefficients $-0.81 \leq r_s \leq 0.66$).

3.4.4 Correlations with withdrawal from other drugs—There was a general lack of association of the present morphine withdrawal jumping scores with chronic withdrawal severity from ethanol, or with acute withdrawal from ethanol, pentobarbital, or diazepam ($-0.11 \leq r \leq 0.37$ using the same 13 – 14 strains). This finding could be predicted since the behavioral indices of withdrawal are quite different (handling-induced convulsions vs. jumping). Similarly, none of the other morphine withdrawal scores were significantly associated with withdrawal from these sedative-hypnotics ($-0.07 \leq r \leq 0.44$ using the same 13 – 14 strains).

4. Discussion

4.1 Genetic characterization of morphine withdrawal

4.1.1 Jumping—The narrow-sense heritability of withdrawal induced jumping was estimated from the ANOVA by strain (R^2) as $h^2 = 0.68$, which suggests that about two-thirds of the total variability among individual animals is attributable to genetic influences. The extreme-scoring strains in the present study were 129P3/J and PL/J (both with no jumps) and C57L/J and C57BR/cdJ (each strain with an average of over 20 jumps). Despite the fact that the phenotype we examined was not identical to those used by Kest et al. (2002b), the present findings are consistent with their conclusion that a substantial proportion of variance in morphine withdrawal-induced jumping is under genetic control (their heritabilities ranged from 0.53 – 0.70) and extends the observations to an additional five strains (Kest et al., 2002b and present data). Jumping following naloxone-precipitated heroin withdrawal was genetically correlated with morphine withdrawal jumping in both laboratories (Klein et al., 2008; present data [$r_s = 0.55$]).

Some laboratories have reported very mild jumping behavior observed in vehicle-treated mice upon injection of naloxone (i.e., a non-morphine dependent control; Belknap, 1989; Marshall and Grahame-Smith, 1971). Under our conditions, naïve mice do not show the characteristic jumping behavior (all four feet off of the platform) indicative of morphine withdrawal. Therefore, we are confident that our frequency counts for this behavior represent a withdrawal response.

4.1.2. Thermal and Gastrointestinal Responses—Narrow-sense heritabilities for these traits (h^2) ranged from 0.31 to 0.56 (Table 1), indicating that these traits are also highly influenced by genotype. The relationships among withdrawal-induced change in body temperature and gastrointestinal motility (as indexed by boli count and proportion of animals within strain having soft stools; Table 2) would suggest that these traits may be influenced by some genes in common.

We examined boli count data from 6 – 13 inbred strains in common with those reported in the present study that were downloaded from the Mouse Phenome Database (Grubb et al., 2004). Seven separate indices of boli counts were available as well as two reports of food intake (<http://www.jax.org/phenome>, projects Brown1, Flaherty1, Seburn1, Tordoff2, and Wahlsten1, downloaded 07/09/08). All boli count data were from naïve animals and were obtained during tests such as anxiety-like behavior on several apparatus (e.g., light/dark box, elevated plus maze, open field activity). None of these data sets correlated well with morphine withdrawal-induced boli production ($-0.10 < r_s < 0.45$, $p_s > 0.30$), although they were well-correlated with each other ($0.62 < r_s < 0.92$, $p_s < 0.06$). Furthermore, food intake measures standardized by body weight (g food per 30 g body weight) were also generally not correlated with morphine-withdrawal induced boli production ($-0.37 < r < -0.29$, $p_s > 0.23$). Together, these findings suggest that withdrawal-induced gastromotility is a valid and quantifiable measure of morphine withdrawal independent of “baseline” or anxiety-related defecation, is not dependent on quantity of food consumption, and is sensitive to genetic differences.

4.2 Relationships with other responses to Morphine

4.2.1. Morphine consumption and dependence—Consistent with reports that ethanol consumption and ethanol withdrawal share some common genetic determination, and presumably also mechanisms, the present data show genetic correlations of morphine withdrawal-induced jumping and change in body temperature with morphine consumption in a two-bottle choice experiment (Belknap et al., 1993a; Metten et al., 1998; Metten and

Crabbe, 2005). The propensity to consume ethanol voluntarily is *negatively* correlated with ethanol withdrawal severity in inbred mice. This negative association for ethanol is also corroborated in selectively bred lines of mice and rats (Chester et al., 2003; Metten et al., 1998). It is also apparent in strains drinking or withdrawing from diazepam (Belknap et al., submitted). For example, mice bred for higher withdrawal drink less, while mice bred to drink little alcohol display more severe withdrawal when administered alcohol without choice. In contrast, morphine withdrawal-induced jumping was positively associated with morphine consumption, indicating that the shared genetic influences do not confer protective effects against this index of potential abuse. We have no explanation for why genes support a negative relationship for ethanol and diazepam, but a positive one for morphine. We found no studies that examined both morphine consumption and morphine withdrawal in the same selectively bred lines. However, the present finding that the change in body temperature upon withdrawal from morphine is negatively correlated with morphine consumption suggests that strains at risk for severe thermoregulatory disruption during morphine withdrawal do not voluntarily consume much morphine even without prior morphine experience.

4.2.2. Morphine analgesic tolerance and dependence—Kest et al. (2002a; 2002b) concluded that morphine withdrawal jumping was genetically correlated with morphine analgesic tolerance using the potency shift data in their studies. However, in a gene mapping study using an F2 intercross of 129P3/J and C57BL/6J mice, they determined that none of the chromosomal regions identified as containing genes influencing morphine withdrawal-induced jumping showed any evidence of linkage with morphine analgesic tolerance using a separate F2 population of the same cross (Kest et al., 2004). In pharmacological studies, morphine-induced analgesic tolerance has been shown to be independent of morphine-induced hyperalgesia, thus suggesting that there are multiple mechanisms to be identified (Juni et al., 2006). Perhaps not surprisingly then, our withdrawal jumping severity rank data correlated less well with potency shift ($r_s = 0.49$) and was negatively correlated with the latency change measure ($r_s = -0.44$), while gastrointestinal symptoms of withdrawal (boli counts and incidence of soft stool) were associated with latency shift (see Results).

4.3 Gene Mapping Implications

Most inbred strain studies of morphine responses in the literature used only one or two strains. Studies involving a much larger number of strains offer a number of advantages, such as allowing a more accurate assessment of the heritability, which is the proportion of the observed variability that is genetically determined. Genetic correlations can be assessed, which indexes the degree to which two traits show common genetic influences (Crabbe et al., 1990). Moreover, they allow the identification of genotypes (strains) that possess useful and/or interesting characteristics, such as those exhibiting very high or very low jumping behavior. Extreme scoring high and low strains are often good choices for creating two-strain crosses for gene mapping efforts and other genetic analyses. This approach has been used successfully for morphine withdrawal jumping and morphine analgesic tolerance (Kest et al., 2004) and morphine analgesia (Smith et al., 2008; Bergeson et al., 2001).

It is interesting that the C57BR/cdJ and C57L/J substrains showed such extreme withdrawal jumping compared with the C57BL/6J strain (see Table 1). Although related ancestrally and thought to be highly genetically similar, use of such similar strains in crosses to create F2 populations, recombinant inbred, congenic, or recombinant congenic strains for gene mapping and mechanistic studies has proven fruitful in several areas. For example, we have used an F2 intercross between the highly similar DBA/1J and DBA/2J inbred strains, which differ significantly in withdrawal severity from sedative hypnotic drugs including ethanol,

for fine-mapping of a region on mouse chromosome 11 that influences these traits (Hood et al., 2006).

Another cross that has proven fruitful includes the CXBK recombinant inbred strain which has reduced morphine sensitivity and a low level of μ -opioid agonist binding relative to the progenitor strains, C57BL/6ByJ and BALB/cByJ. The CXBK strain has been used to identify a deficiency in μ -opioid receptor mRNA levels due to a difference in size of an untranslated region of the μ -opioid receptor gene (Ikeda et al., 2001; Han et al., 2006). Novel gene regions harboring genes influencing ethanol consumption and preference also are being identified using a combination of backcross and intercross strategies with inbred and recombinant inbred strains to create congenics for fine mapping (Vadasz et al., 2007). Another study has examined the correlation of gene expression in the striatum with morphine self-administration and morphine analgesia in both recombinant inbred strains and four inbred strains (Korostynski et al., 2006). The identification of overlapping gene expression probe sets among these two populations has led to a very small number of candidates for genes influencing each phenotype (Korostynski et al., 2006).

Finally, standard inbred strains can now be useful for gene mapping studies directly because of the large number of single nucleotide polymorphism (SNP) marker loci that are now available for most standard inbred strains, although the greater degree of shared pedigree, the lower effective number of strains (over 8 million SNPs have been genotyped across 15 strains [Mouse Phenome Database, MPD, 2008; Wang et al., 2005; Wiltshire et al., 2003]). The fine haplotype structure mapping this new method provides can be valuable for increasing map resolution of already mapped QTLs (e.g., Fehr et al., 2002; Wiltshire et al., 2003; Cervino et al., 2005, 2007). Combinations of these various mapping approaches and gene expression microarray analyses are being successfully used to map effective specific gene loci because the genes whose expression is studied are known (Letwin et al., 2006; Mulligan et al., 2008; Mulligan et al., 2006).

4.4 Summary and Conclusions

There is growing evidence that different phenotypic responses, including drug withdrawal responses, typically thought to belong to the same “behavioral domain,” are under the control of largely different genetic mechanisms. For example, the behavioral domain of ethanol-induced motor incoordination was recently systematically examined in a series of experiments examining different dose, time, and apparatus parameters in inbred strains (summarized in Crabbe et al., 2005). The primary conclusion from these studies was that the ataxic response to ethanol in these tests (e.g., balance beam, grid test, loss of righting reflex, observer-rated ataxia, etc.) showed remarkably little evidence for underlying common genetic substrates among two or more tests (Crabbe et al., 2005).

For ethanol withdrawal, and other sedative-hypnotic drugs, the primary signs investigated are handling-induced convulsions (in mice) and anxiety-related responses (in rats and mice as well as other species). Recently, we identified another sign of ethanol withdrawal: motor learning, as measured by acquisition of ability to remain on a constantly accelerating rotarod (Philibin et al., in press). This withdrawal sign shows robust treatment differences, but does not correlate with withdrawal handling-induced convulsion severity in selected lines bred for the latter trait (Philibin et al., in press).

Although it was originally thought that morphine withdrawal-induced jumping reflected the same neural mechanism as other withdrawal signs (Way et al., 1969), it is becoming clear that these signs are under at least partially separate genetic control. For example, el-Kadi and Sharif (1994) found that the dose-response curves for naloxone-precipitated morphine withdrawal differed greatly between jumping and “wet dog” shakes suggesting that these

traits utilize different biological substrates. In the present studies using inbred strains, the general lack of strong correlation among morphine withdrawal signs clearly indicates that the traits we measured are largely under separate genetic control, and suggests that identification of genes underlying each trait will prove to lead to independent targets. Continued exploration of these and other behavioral and neurobiological phenotypes in standardized strain panels will increasingly reveal the biological effectors of genetic differences.

Reference List

- Adler MW, Geller EB, Rosow CE, Cochin J. The opioid system and temperature regulation. *Annu Rev Pharmacol Toxicol.* 1988; 28:429–449. [PubMed: 2837979]
- Belknap JK. Components of the opioid withdrawal syndrome in mice are thermoregulatory responses. *Pharmacol Biochem Behav.* 1989; 34(2):241–245. [PubMed: 2622979]
- Belknap JK. Physical dependence induced by the voluntary consumption of morphine in inbred mice. *Pharmacol Biochem Behav.* 1990; 35:311–315. [PubMed: 2320638]
- Belknap JK, Crabbe JC, Riggan J, O'Toole LA. Voluntary consumption of morphine in 15 inbred mouse strains. *Psychopharmacology.* 1993a; 112:352–358. [PubMed: 7871041]
- Belknap JK, Crabbe JC, Young ER. Voluntary consumption of ethanol in 15 inbred mouse strains. *Psychopharmacology.* 1993b; 112:503–510. [PubMed: 7871064]
- Belknap JK, Metten P, Beckley EH, Crabbe JC. Common and drug-specific genetic influences on responses to four drugs of abuse: Multivariate analyses of mouse inbred strain responses to ethanol, morphine, and pentobarbital. submitted.
- Belknap, JK.; O'Toole, LA. Studies of genetic differences in response to opioid drugs. In: Harris, RA.; Crabbe, JC., editors. *The Genetic Basis of Alcohol and Drug Actions*. Plenum, NY: 1991.
- Belknap JK, Riggan J, Cross S, Young ER, Gallaher EJ, Crabbe JC. Genetic determinants of morphine activity and thermal responses in 15 inbred mouse strains. *Pharmacol Biochem Behav.* 1998; 59:353–360. [PubMed: 9476981]
- Ben-Eliyahu S, Marek P, Vaccarino AL, Mogil JS, Sternberg WF, Liebeskind JC. The NMDA receptor antagonist MK-801 prevents long-lasting non-associative morphine tolerance in the rat. *Brain Res.* 1992; 575:304–308. [PubMed: 1533339]
- Bergeson SE, Helms ML, O'Toole LA, Jarvis MW, Hain HS, Mogil JS, Belknap JK. Quantitative trait loci influencing morphine antinociception in four mapping populations. *Mamm Genome.* 2001; 12(7):546–553. [PubMed: 11420618]
- Cervino A, Li S, Edwards J, Zhu C, Laurie C, Tokiwa G, Lum PY, Wang S, Castellini LW, Lusi AJ, Carlson S, Sachs AB, Schadt EE. Integrating QTL and high-density SNP analyses in mice to identify *Insig2* as a susceptibility gene for plasma cholesterol levels. *Genomics.* 2005; 86:505–517. [PubMed: 16126366]
- Cervino A, Darvasi A, Fallahi M, Mader CC, Tsinores NF. An integrated in silico gene mapping strategy in inbred mice. *Genetics.* 2007; 175:321–333. [PubMed: 17028314]
- Chester JA, Blose AM, Froehlich JC. Further evidence of an inverse genetic relationship between innate differences in alcohol preference and alcohol withdrawal magnitude in multiple selectively bred rat lines. *Alcohol Clin Exp Res.* 2003; 27:377–387. [PubMed: 12658103]
- Collier HOJ, Francis DL, Schneider C. Modification of morphine withdrawal by drugs interacting with humoral mechanisms: Some contradictions and their interpretation. *Nature.* 1972; 237:220–223. [PubMed: 4261153]
- Crabbe JC. Sensitivity to ethanol in inbred mice: genotypic correlations among several behavioral responses. *Behav Neurosci.* 1983; 97(2):280–289. [PubMed: 6849690]
- Crabbe JC, Gallaher EJ, Cross SJ, Belknap JK. Genetic determinants of sensitivity to diazepam in inbred mice. *Behav Neurosci.* 1998; 112:668–677. [PubMed: 9676982]
- Crabbe JC, Gallaher ES, Phillips TJ, Belknap JK. Genetic determinants of sensitivity to ethanol in inbred mice. *Behav Neurosci.* 1994; 108:186–195. [PubMed: 8192844]

- Crabbe JC, Janowsky JS, Young ER, Kosobud A, Stack J, Rigter H. Tolerance to ethanol hypothermia in inbred mice: genotypic correlations with behavioral responses. *Alcohol Clin Exp Res.* 1982; 6:446–458. [PubMed: 6758616]
- Crabbe JC, Metten P, Cameron AJ, Wahlsten D. An analysis of the genetics of alcohol intoxication in inbred mice. *Neurosci Biobehav Rev.* 2005; 28:785–802. [PubMed: 15642621]
- Crabbe JC, Metten P, Gallaher EJ, Belknap JK. Genetic determinants of sensitivity to pentobarbital in inbred mice. *Psychopharmacology.* 2002; 161:408–416. [PubMed: 12073169]
- Crabbe JC, Phillips TJ, Kosobud A, Belknap JK. Estimation of genetic correlation: interpretation of experiments using selectively bred and inbred animals. *Alcohol Clin Exp Res.* 1990; 14(2):141–151. [PubMed: 2190477]
- Crabbe JC, Young ER, Kosobud A. Genetic correlations with ethanol withdrawal severity. *Pharmacol Biochem Behav.* 1983; 18(Suppl.1):541–547. [PubMed: 6685310]
- el-Kadi AO, Sharif SI. The influence of various experimental conditions on the expression of naloxone-induced withdrawal symptoms in mice. *Gen Pharmacol.* 1994; 25(7):1505–1510. [PubMed: 7896067]
- Fehr C, Shirley RL, Belknap JK, Crabbe JC, Buck KJ. Congenic mapping of alcohol and pentobarbital withdrawal liability loci to a <1 centiMorgan interval of murine chromosome 4: identification of Mpdz as a candidate gene. *J Neurosci.* 2002; 22(9):3730–3738. [PubMed: 11978849]
- Grubb SC, Churchill GA, Bogue MA. A collaborative database of inbred mouse strain characteristics. *Bioinformatics.* 2004; 20:2857–2859. [PubMed: 15130929]
- Gutstein, HB.; Akil, H. Opioid analgesics. In: Hardman, JG.; Limbird, LE., editors. *Goodman & Gilman's the Pharmacological Basis of Therapeutics.* 10th ed.. McGraw-Hill, NY: 2001. p. 569–619.
- Haghighparast A, Khani A, Lashgari R, Fallahian S. Reducing the time and dose of morphine application used in Marshall and Grahame-Smith method for induction of morphine tolerance and dependence in mice. *Drug Alcohol Depend.* 2008; 93(1–2):185–9. Epub 2007 Oct 25. [PubMed: 17961932]
- Han W, Kasai S, Hata H, Takahashi T, Takamatsu Y, Yamamoto H, Uhl GR, Sora I, Ikeda K. Intracisternal A-particle element in the 3' noncoding region of the mu-opioid receptor gene in CXBK mice: a new genetic mechanism underlying differences in opioid sensitivity. *Pharmacogenet Genomics.* 2006; 16:451–460. [PubMed: 16708053]
- Hegmann JP, Possidente B. Estimating genetic correlations from inbred strains. *Behav Genet.* 1981; 11:103–114. [PubMed: 7271677]
- Hood HM, Metten P, Crabbe JC, Buck KJ. Fine mapping of a sedative-hypnotic drug withdrawal locus on mouse chromosome 11. *Genes Brain Behav.* 2006; 5(1):1–10. [PubMed: 16436183]
- Ikeda K, Kobayashi T, Ichikawa T, Kumanishi T, Niki H, Yano R. The untranslated region of μ -opioid receptor mRNA contributes to reduced opioid sensitivity in CXBK mice. *J Neurosci.* 2001; 21:1334–1339. [PubMed: 11160404]
- Kest B, Hopkins E, Palmese CA, Adler M, Mogil JS. Genetic variation in morphine analgesic tolerance: a survey of 11 inbred mouse strains. *Pharmacol Biochem Behav.* 2002a; 73(4):821–8. [PubMed: 12213527]
- Kest B, Palmese CA, Juni A, Chesler EJ, Mogil JS. Mapping of a quantitative trait locus for morphine withdrawal severity. *Mamm Genome.* 2004; 15(8):610–617. [PubMed: 15457340]
- Kest B, Palmese CA, Hopkins E, Adler M, Juni A, Mogil JS. Naloxone-precipitated withdrawal jumping in 11 inbred mouse strains: evidence for common genetic mechanisms in acute and chronic morphine physical dependence. *Neuroscience.* 2002b; 115(2):463–9. [PubMed: 12421612]
- Kest B, McLemore GL, Sadowski B, Mogil JS, Belknap JK, Inturrisi CE. Acute morphine dependence in mice selectively-bred for high and low analgesia. *Neurosci Lett.* 1998; 256(2):120–122. [PubMed: 9853718]
- Korostynski M, Kaminska-Chowanec D, Piechota M, Przewlocki R. Gene expression profiling in the striatum of inbred mouse strains with distinct opioid-related phenotypes. *BMC Genomics.* 2006; 7:146–163. [PubMed: 16772024]
- Marshall I, Grahame-Smith DG. Evidence against a role of brain 5-hydroxytryptamine in the development of physical dependence upon morphine in mice. *J Pharmacol Exp Ther.* 1971; 173:634–641. [PubMed: 4257807]

- Metten P, Crabbe JC. Common genetic determinants of severity of acute withdrawal from ethanol, pentobarbital and diazepam in inbred mice. *Behav Pharmacol*. 1994; 5:533–547. [PubMed: 11224305]
- Metten P, Crabbe JC. Genetic determinants of severity of acute withdrawal from diazepam in mice: commonality with ethanol and pentobarbital. *Pharmacol Biochem Behav*. 1999; 63:473–479. [PubMed: 10418790]
- Metten P, Crabbe JC. Alcohol withdrawal severity in inbred mouse (*Mus musculus*) strains. *Behav Neurosci*. 2005; 119(4):911–925. [PubMed: 16187819]
- Metten P, Phillips TJ, Crabbe JC, Tarantino LM, McClearn GE, Plomin R, Erwin VG, Belknap JK. High genetic susceptibility to ethanol withdrawal predicts low ethanol consumption. *Mamm Genome*. 1998; 9:983–990. [PubMed: 9880664]
- Miyamoto Y, Takemori AE. Sites of action of naloxone in precipitating withdrawal jumping in morphine-dependent mice: investigations by the ED50 value and CNS content of naloxone. *Drug Alcohol Depend*. 1993; 32(2):163–167. [PubMed: 8389690]
- Ritzmann RF. Opiate dependence following acute injections of morphine and naloxone: the assessment of various withdrawal signs. *Pharmacol Biochem Behav*. 1981; 14(4):575–577. [PubMed: 7195040]
- Smith SB, Marker CL, Perry C, Liao G, Sotocinal SG, Austin JS, Melmed K, Clark JD, Peltz G, Wickman K, Mogil JS. Quantitative trait locus and computational mapping identifies *Kcnj9* (*GIRK3*) as a candidate gene affecting analgesia from multiple drug classes. *Pharmacogenet Genomics*. 2008; 18(3):231–241. [PubMed: 18300945]
- Vadasz C, Saito M, Gyetvai BM, Oros M, Szakall I, Kovacs KM, Prasad VVTS, Morahan G, Toth R. Mapping of QTLs for oral alcohol self-administration in B6.C and B6.I quasi-congenic RQI strains. *Neurochem Res*. 2007; 32:1099–1112. [PubMed: 17273929]
- Way EL, Loh HH, Shen F-H. Simultaneous quantitative assessment of morphine tolerance and physical dependence. *J Pharmacol Exp Ther*. 1969; 167(1):1–8. [PubMed: 5814495]
- Wang J, Liao G, Usuka J, Peltz G. Computational genetics: from mouse to human? *Trends Genet*. 2005; 21:527–532.
- Wei E, tseng LF, Loh H, Way EL. Similarity of morphine abstinence signs to thermoregulatory behavior. *Nature*. 1974; 247:398–400. [PubMed: 4856350]
- Wiltshire T, Pletcher MT, Batalov S, Barnes SW, Tarantino LM, Cooke MP, Wu H, Smylie K, Santrosyan A, Copeland NG, Jenkins NA, Kalush F, Mural RJ, Glynne RJ, Kay SA, Adams MD, Fletcher CF. Genome-wide single-nucleotide polymorphism analysis defines haplotype patterns in mouse. *Proc Natl Acad Sci U S A*. 2003; 100:3380–5. [PubMed: 12612341]

Figure 1.

Rank-ordered withdrawal severity in the current experiment (Jumping, ordinate), plotted versus comparable data from the Kest et al., 2002b "acute" withdrawal experiment. Each point represents a strain rank value. Substrain designation in plain font is for the current data, while bold font designation gives the substrain used by Kest et al., 2002b, where a different substrain was used.

TABLE 1

Inbred strain morphine withdrawal data

Strain	N	Body Weight (g)	Jumping	Boli Count	Body Temperature (°C)		Soft Stool	Air Chewing
					After Morphine	After Naloxone		
						Change		(% Incidence)
129P3/J	10	21.6±0.6	0±0	0.70±0.4	35.96±0.30	36.75±0.25	0.79±0.25	30±15
A/HeJ	10	23.0±0.6	2.2±0.9	0.60±0.2	32.45±0.51	32.24±0.60	-0.21±0.60	20±10
AKR/J	10	28.7±0.8	1.7±0.5	3.70±0.5	36.73±0.12	36.15±0.27	-0.58±0.30	70±13
BALB/cJ	10	23.2±0.3	10.2±3.6	1.40±0.5	35.45±0.28	34.81 ±0.40	-0.64±0.47	60±16
C3H/HeJ	11	22.2±0.7	1.8±1.0	1.45±0.5	36.38±0.64	34.73±0.58	-1.65±0.80	55±16
C57BL/6J	11	22.4±0.3	8.3±1.4	0.45±0.2	36.11±0.18	34.43±0.31	-1.68±0.34	45±16
C57BR/cdJ	11	23.8±0.6	21.3±2.1	2.09±0.9	35.26±0.23	34.69±0.30	-0.57±0.36	64±15
C57L/J	10	23.4±0.6	27.9±3.5	2.20±0.3	34.53±0.51	35.44±0.27	0.91±0.46	40±16
CBA/J	10	24.8±0.5	2.4±1.4	3.10±0.7	36.76±0.16	36.53±0.19	-0.23±0.15	90±10
DBA/1J	10	22.3±0.4	4.3±2.8	1.40±0.4	36.10±0.41	35.96±0.29	-0.14±0.41	60±16
DBA/2J	11	25.2±0.7	1.9±1.8	1.45±0.5	35.10±0.37	36.42±0.31	1.32±0.28	64±15
PL/J	10	20.9±0.2	0±0	2.10±0.4	36.38±0.38	34.76±0.37	-1.62±0.25	90±10
SIL/J	11	22.5±0.3	0.1±0.1	0.91±0.4	34.13±0.18	35.64±0.24	1.51±0.23	36±15
SWR/J	11	20.9±0.5	1.4±0.8	0.36±0.2	35.22±0.31	37.34±0.26	2.12±0.34	9±9
Total	146							100±8
Overall Means		23.2±0.2	6.0±0.8	1.5±0.1	35.5±0.1	35.4±0.1	-0.04±0.1	53±4
% Variance accounted for by Strain		58.6%	68.7%	31.3%	50.7%	56.5%	47.2%	

Table 2

Genetic (strain mean) correlations among withdrawal measures

Measure*	Jumping	Boli	Soft Stool	MORTemp	TempChg	Air Chewing
Jumping	—	0.17	0.11	-0.13	-0.19	-0.09
Boli	0.18	—	0.82	0.44	-0.21	-0.00
Soft Stool	-0.06	0.86	—	0.64	-0.47	-0.23
MORTemp	-0.18	0.48	<i>0.66</i>	—	-0.65	-0.15
TempChg	-0.02	-0.27	-0.53	-0.43	—	0.70
Air Chewing	-0.09	0.08	-0.18	-0.02	0.78	—

Correlation values below the diagonal are Pearson's r ; significance was determined using $d.f. = 12$. Correlations above the diagonal are Spearman's rank order calculations. **Bold font** indicates Pearson's correlations significant at $p < 0.01$, ($|r| = 0.66$). *Italic font* indicates Pearson's correlations significant at $p < 0.05$, ($|r| = 0.54$). Abbreviations: MORTemp: Body temperature 5 hours after morphine treatment; TempChg: Body temperature 17 minutes after naloxone injection minus MORTemp. For description of variables, see text.