

A survey of acute and chronic heroin dependence in ten inbred mouse strains: Evidence of genetic correlation with morphine dependence

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ABSTRACT

Heroin and morphine exposure can cause physical dependence, with symptoms manifesting during their withdrawal. Inter-individual differences in symptom frequency during morphine withdrawal are a common finding that, in rodents, is demonstrably attributable to genotype. However, it is not known whether inter-individual differences characterize heroin withdrawal, and whether such variation can be similarly influenced by genotype. Therefore, we injected mice of ten inbred strains with acute and chronic heroin doses and compared their jumping frequencies, a common index of withdrawal magnitude, during naloxone-precipitated withdrawal. The data revealed significant strain frequency differences (range after acute and chronic heroin injection: 0–104 and 0–142 jumps, respectively) and substantial heritability ($h^2=0.94$ to 0.96), indicating that genetic variance is associated with heroin withdrawal. The rank order of strain sensitivity for acute and chronic heroin withdrawal jumping, and for the current heroin and previous morphine strain data, were significantly correlated ($r=0.75$ – 0.94), indicating their genetic and, ultimately, physiological commonality. These data suggest that the genetic liability to heroin dependence remains constant across a period of heroin intake, and that heroin and morphine dependence may benefit from common treatment strategies.

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1. Introduction

Opioids such as heroin and morphine remain widely abused drugs with a high physical dependence liability (Pouletty, 2002; Tarabar and Nelson, 2003). Manifested during a withdrawal syndrome comprised of behavioral and physiological signs, dependence is commonly assumed to result from chronic opioid use but in fact can be induced after even an acute opioid injection (Blasig et al., 1973; Kest et al., 2001, 2002; Klein et al., in press; Smits, 1975). In humans, the aversive character of opioid withdrawal symptoms in addicted populations is regarded as a causative factor in their continued use and abuse of opioids and is thought to engender their dangerous drug seeking behavior (Jasinski, 1977; Kenny et al., 2006).

The severity of opioid dependence in human populations has been reported to be subject to inter-individual variation. That is, whereas even minimal prior opioid exposure can cause dependence in some individuals, others are resistant to dependence despite chronic intake (Ikeda et al., 2005; Pasternak, 2004). These findings

may be attributable to variation in genotype. Indeed, variability in naloxone-precipitated withdrawal (NPW) magnitude in rodent subjects has been demonstrated to be associated with genetic variability (Han et al., 2004; Ikeda et al., 2005; Kest et al., 2002; Liang et al., 2006). For example, rat and mouse strains differ in both their sensitivity to undergo withdrawal per se and in the incidence of several withdrawal symptoms (Brase et al., 1977; Hoffmann et al., 1998; Liang et al., 2006). Substantial inter-strain variability is also reported for NPW jumping frequency (Brase et al., 1977; Kest et al., 2002; Liang et al., 2006; Suzuki et al., 1991), a sensitive and reliable index of opioid withdrawal magnitude (El-Kadi and Sharif, 1994; Kest et al., 2001; Klein et al., in press; Saelens et al., 1971; Smits, 1975). For example, substantial heritability estimates were obtained in a survey of 11 inbred (isogenic) mouse strains rendered dependent to morphine after both acute and chronic morphine treatment, and strain mean jumping frequency differences as great as 100-fold were observed in the most divergent responders (Kest et al., 2002). In addition to demonstrating a genetic contribution to response variability, strain surveys also identify strains with the most divergent responses to serve as progenitors in subsequent linkage analysis studies to identify genetic regions, and ultimately genes, contributing to genetic variability. This approach, referred to as quantitative trait locus (QTL) mapping, has already been successfully employed for

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several traits related to drug and alcohol abuse, including morphine dependence (Kest et al., 2004).

Despite the substantial abuse of heroin, there are no studies assessing the possible contribution of genotype to physical dependence induced by heroin. Since heroin is rapidly converted to morphine *in vivo* (Inturrisi et al., 1983) and both drugs preferentially bind and activate μ opioid receptors (Selley et al., 2001; Watson et al., 1996), heroin and morphine were logically thought to have a highly similar, if not identical, pharmacological profile. It is however becoming increasingly recognized that substantial mechanistic differences underlie heroin and morphine analgesia (Rossi et al., 1996) tolerance (Gilbert et al., 2004; Lange et al., 1980; Rossi et al., 1996), and, more recently, dependence (Bao et al., 2007; Klein et al., *in press*). For example, δ_1 opioid receptor blockade attenuates NPW jumping frequencies in mice subject to acute and chronic heroin treatment, but not those identically treated with equieffective doses of morphine (Klein et al., *in press*). Thus, extrapolating from one of these drugs to the other is done somewhat tenuously.

To determine whether, like for morphine, the magnitude of heroin dependence is also dependent on genotype, we compared NPW jumping frequencies of ten inbred mouse strains injected with heroin. Although acute as well as chronic opioid administration induces dependence (McLemore et al., 1997; Wiley and Downs, 1979), they do so by recruiting similar—but not identical—physiological substrates. This has recently been shown for heroin dependence as well (Klein et al., *in press*). Thus, separate groups of mice from each strain were subjected to either acute or chronic heroin injection protocols. The demonstration of a genetic correlation between two heritable traits among isogenic strains can be used as evidence of the existence of pleiotropic genes with a common influence on both traits (Hegmann and Possidente, 1981). If the traits have genes in common, then one can strongly infer their common physiology. Thus, NPW jumping frequencies obtained after acute and chronic heroin treatment were correlated. In addition, the inbred mouse strains tested here were previously rendered dependent to morphine and surveyed for NPW jumping using protocols virtually identical to those utilized in the present study (Kest et al., 2002). Thus, the strain data obtained here also afforded the opportunity to assess the genetic correlation between heroin and morphine dependence as well.

2. Materials and methods

2.1. Subjects

Male mice of the following inbred strains were obtained from The Jackson Laboratory (Bar Harbor, ME) and subject to acute and chronic heroin treatment: 129P3/J (*n*: acute, 6; chronic, 8), A/J (*n*: acute, 8; chronic, 8), AKR/J (*n*: acute, 8; chronic, 8), BALB/c/J (*n*: acute, 8; chronic, 11), C3H/He/J (*n*: acute, 8; chronic, 8), C57BL/6/J (*n*: acute, 8; chronic, 8), CBA/J (*n*: acute, 8; chronic, 8), DBA/2/J (*n*: acute, 8; chronic, 8), SJL/J (*n*: acute, 8; chronic, 8), and SWR/J (*n*: acute, 8; chronic, 8). Additional mice (*n*=6 / strain) served in control groups. All mice were housed four to a cage with same strain mates in the College of Staten Island Animal Facility. Mice were allowed free access to food (Purina chow) and water in a temperature-controlled (22 °C) environment maintained on a 12:12 h light/dark cycle (lights on at 07:00 h). All testing was performed following an acclimation period of at least 1 week after arrival and at 7–9 weeks of age. All experimental protocols were approved by the College of Staten Island Institutional Animal Care and Use Committee for the use of animal subjects.

2.2. Heroin treatment

Acute dependence was induced by a single subcutaneous 50 mg/kg heroin injection followed by a single naloxone dose

(50 mg/kg) 2 h later. In the chronic dependence condition, heroin was injected t.i.d. (09:00 h, 13:00 h and 17:00 h) for three days using a dosing schedule of 5, 10, and 20 mg/kg on Days 1, 2, and 3, respectively. On Day 4, a final 20 mg/kg heroin dose was injected, followed by a single naloxone dose (50 mg/kg) 1 h later. Control mice (*n*=6/strain) were similarly injected with naloxone but were previously treated with saline instead of heroin. The different heroin and naloxone doses, as well as heroin–naloxone intervals, used here to study acute and chronic heroin withdrawal have been previously shown to yield maximal NPW jumping frequencies in CD-1 mice following acute and chronic heroin treatment, respectively (Klein et al., *in press*).

2.3. Drugs

Both heroin hydrochloride, generously supplied by the Research Resources program of the National Institute on Drug Abuse (Rockville, MD), and naloxone hydrochloride (Sigma-Aldrich, St. Louis, MO) were dissolved in 0.9% physiological saline. Both drugs were injected via the subcutaneous route in a volume of 10 ml/kg.

2.4. Naloxone-precipitated withdrawal

Immediately after naloxone injection, heroin and saline-treated control subjects were placed into individual Plexiglas observation cylinders (25×11 cm), and the frequency of jumps for each subject was tallied over the next 15 min. The jumping response—defined as the simultaneous removal of all four paws from the horizontal surface—is a reliable and sensitive index of opioid withdrawal intensity and the most widely used (El-Kadi and Sharif, 1994; Kest et al., 2001; Miyamoto and Takemori, 1993; Ritzmann, 1981; Smits, 1975; Saelens et al., 1971; Way et al., 1969). Furthermore, only jumping frequency has been shown to have a positive dose–response relationship with acute and chronic heroin withdrawal (Klein et al., *in press*). Thus, although symptoms such as diarrhea, ptosis, wet-dog shakes, and lacrimation were occasionally observed in the present study, they were excluded from analysis. Mean jump frequency per 15 min was used as the measure of dependence for each strain.

2.5. Data analysis

Jumping frequencies obtained in the acute and chronic dependence paradigms were analyzed separately using a two-way (strain×condition) ANOVA. Control and heroin treated mice were compared post-hoc within-strain only using Scheffe's test. Narrow-sense trait heritability was determined by comparing the between-strain variance to the total variance. Since animals are isogenic (i.e., genetically identical) within individual inbred strains, between-strain variance provides a measure of additive genetic (“allelic”) variation (V_A), whereas within-strain variance (“error variance”) represents environmental variability (V_E). An estimate of narrow-sense heritability (h^2) for each trait was obtained using the formula: $h^2 = V_A / (V_A + V_E)$ (Falconer and Mackay, 1996). Since these ten strains were initially chosen randomly, these values are likely accurate estimates (Hegmann and Possidente, 1981). To assess genetic correlation between acute and chronic heroin dependence, strains were also ranked from smallest to highest according to jumping frequencies and subject to Spearman's rank statistic (r_s), ensuring that correlation estimates were not unduly influenced by extreme scoring strains. Acute and chronic heroin data were also correlated in the identical manner with acute and chronic morphine dependence data, respectively (Kest et al., 2002). All correlations were subject to Bonferroni correction for multiple comparisons. For all statistical tests, the criterion for significance was $p < 0.05$.

3. Results

Analysis of NPW jumping frequencies in inbred mice subject to heroin or saline (i.e. control) injection revealed significant main effects of strain (acute: $F_{(9,118)}=43.2$, chronic: $F_{(9,123)}=18.7$; both $p<0.001$), treatment (acute: $F_{(1,118)}=151.8$, chronic: $F_{(1,123)}=80.9$; both $p<0.001$), and their interaction (acute: $F_{(9,118)}=31.4$, chronic: $F_{(9,123)}=14.5$; both $p<0.001$). As illustrated in Fig. 1, the range of mean frequency values obtained after both heroin treatment protocols was considerably broad. Specifically, whereas 129P3 mice did not respond after any heroin treatment, jumping frequencies as high as 104 and 142 were observed in SWR mice after acute and chronic heroin treatment, respectively. There were no significant strain differences in control mice (data not shown) nor were seizures evident in heroin treated subjects.

Narrow-sense heritability of acute and chronic heroin dependence was estimated as $h^2=0.98$ and $h^2=0.96$, respectively. These estimates are likely biased upward because of the extreme outlier status of the SWR strain. Even if this strain is excluded, however, heritability estimates remain high, at $h^2=0.96$ and 0.94, respectively.

The scatterplot matrix in Fig. 2 shows the regression of strain ranks for mean jumping frequencies after acute and chronic heroin and morphine treatment. The visual impressions of a correlation between responses are statistically confirmed by the r_s correlation coefficients provided in Table 1. All pairwise correlation coefficients

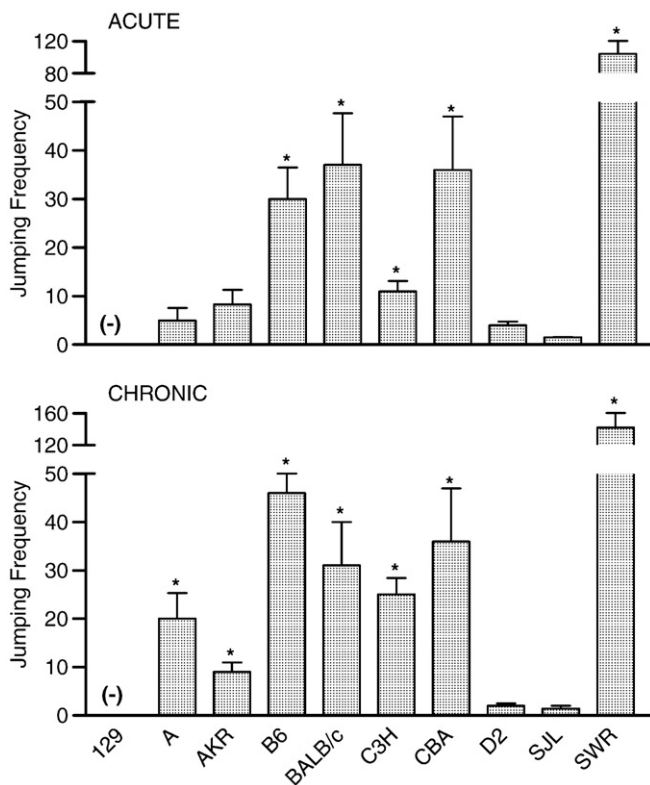


Fig. 1. Withdrawal jumping frequencies in 10 inbred mouse strains following acute and chronic heroin treatment. Mice undergoing acute heroin treatment (top figure) were injected once with heroin (50 mg/kg) and withdrawal precipitated 2 h later. Chronically treated mice (bottom figure) were injected repeatedly (t.i.d.) with heroin doses of 5, 10, and 20 mg/kg on Days 1, 2, and 3, respectively. Withdrawal was precipitated 1 h after a final 20 mg/kg heroin dose on Day 4. Withdrawal was precipitated by injecting a single naloxone dose (50 mg/kg). Jumping frequencies were tallied for the next 15 min and are presented as strain mean values. Except for 129P3 mice subject to acute heroin treatment ($n=6$), each strain was comprised of a minimum of 8 subjects per each treatment condition. (–) Indicates no response; * indicates a significant difference relative to within-strain controls.

Table 1

Spearman's rank correlation coefficients comparing the mean jumping frequencies of acute and chronic heroin dependence, acute heroin and morphine dependence and chronic heroin and morphine dependence in inbred strain of mice

	Acute heroin	Chronic heroin
Chronic heroin	0.94**	–
Acute morphine	0.77*	–
Chronic morphine	–	0.75*

Mice in the Acute heroin group were injected once (50 mg/kg) whereas those in the Chronic heroin group were injected t.i.d. for 3 days (5, 10, and 20 mg/kg on Days 1, 2, and 3, respectively) and received a final single injection (20 mg/kg) on Day 4. Withdrawal in both cases was elicited by a single naloxone injection (50 mg/kg) at the completion of heroin treatment. Morphine data were obtained from Kest et al. (2002). All correlation coefficients were significant ($P<0.05^*$, $<0.01^{**}$) after Bonferroni correction for multiple comparisons.

were significant after Bonferroni correction for multiple comparisons, and indicate a high degree of genetic correlation.

4. Discussion

4.1. Genetic variability

The present study surveyed NPW jumping frequencies in ten inbred mouse strains after acute and chronic heroin treatment. In mice, jumping frequency is the most widely used measure of opioid withdrawal and is a sensitive and reliable index of heroin and morphine withdrawal magnitude (El-Kadi and Sharif, 1994; Kest et al., 2001; Klein et al., in press; Saelens et al., 1971; Smits, 1975). All strains considered, jumping frequency was greater following chronic relative to acute heroin administration, consistent with previous quantitative comparisons of opioid dependence after acute and chronic opioid treatment in various species including humans (Bickel et al., 1988; Eisenberg, 1982; Heishman et al., 1989; Jasinski, 1977; Kest et al., 2001; Martin and Eades, 1964). More relevant to the aims of this study, the data revealed an array of jumping frequency values and yielded substantial h^2 estimates (acute: 0.96; chronic: 0.94) even when an extreme responding strain (i.e., SWR) was excluded from analysis. Since all the mice in the present study were subject to identical handling/treatment protocols, the data demonstrate that the magnitude of acute and chronic heroin dependence is associated with genetic variability and is subject to substantial heritability. Identical conclusions have been reported with regards to morphine dependence in mice (Kest et al., 2002). The actual genes contributing to the strain differences in heroin dependence may be any one or more of those whose proteins regulate the pharmacodynamic (opioid receptors and their intracellular messengers) and/or pharmacokinetic (enzymes that regulate heroin metabolism) effects of heroin, as well as those genes that specifically underlie opioid dependence. Furthermore, the genetic basis for these strain differences will almost certainly vary with the two particular strains being compared. That is, it is highly unlikely that a single mechanism will completely account for the distribution of strain mean jumping frequencies reported here. Therefore, even if the background information of every strain tested here were at hand, it would be beyond the scope of the present report (and likely erroneous) to speculate which possible substrates underlie frequency differences for each pairwise comparison. The identity of genes contributing to heroin dependence and withdrawal symptoms thus remain to be uncovered by subsequent QTL mapping. We are however intrigued by the finding that whereas heroin analgesia in some strains is mediated by the μ -opioid receptor subtype, it is mediated by the δ -opioid receptor subtype in others (Rady et al., 1991, 1994, 2000). These data indicate that genetic factors contribute to the opioid receptor selectivity of heroin in mice. Since dependence caused by μ -opioid receptor activity is more severe than that caused by δ -opioid receptor activity (Cowan et al., 1988;

Maldonado et al., 1990), it is possible that differences in withdrawal magnitude between some strains may result from differences in heroin opiate receptor type selectivity. This is an interesting possibility that warrants study.

4.2. Genetic correlations

By using a sufficient number of randomly-chosen strains, the present study design should provide a valid estimate of the genetic correlation of acute and chronic dependence traits (Hegmann and Possidente, 1981). To ensure that correlation estimates were not unduly influenced by extreme scoring strains such as 129P3 and SWR, values subject to correlation were analyzed using Spearman's rank statistic. Based on these considerations, the covariation of naloxone-precipitated jumping among strains after acute and chronic heroin injection ($r=0.94$) suggests the contribution of common genes and common (although not necessarily identical) physiological substrates. To date, we have indeed found a highly similar, but not identical, contribution of various opioid and excitatory amino acid receptors to heroin dependence (Klein et al., in press). The remarkably high degree of genetic correlation between acute and chronic heroin dependence provides for the following additional implications. First, it attests to the resiliency of the putative mechanistic relationship between acute and chronic heroin dependence despite the inherent differences in treatment duration (single injection vs. 3 daily injections for 3 days, respectively) and cumulative heroin dosing (50 mg/kg and 125 mg/kg, respectively). Second, it engenders confidence in our assessment of the inter-strain variability in dependence after chronic heroin injection since the acute heroin treatment protocol is not exposed to the potentially confounding contribution of strain differences in contextual learning which has clearly been shown to affect dependence liability (Azorlosa et al., 1994; Deffner-Rappold et al., 1996). Third and lastly, the obtained significant correlation implies that NPW jumping frequencies after a single heroin exposure very likely reflect the development of physical dependence and are not artifactual motor responses (Ritzmann, 1981).

The data also reveal a strong genetic correlation between acute heroin and acute morphine dependence, and between chronic heroin and chronic morphine dependence. Heroin is rapidly converted to 6-monoacetylmorphine (6-MAM) and then morphine in vivo (Inturrisi et al., 1983). Thus, it is possible that the impressive strain rank order correlation coefficients for withdrawal jumping frequencies after heroin and morphine treatment actually resulted from morphine activity in each case. A role for morphine in dependence caused by chronic heroin injection is an intriguing possibility since withdrawal is not precipitated soon after heroin administration and there is ample time over the several treatment days for the conversion of heroin to morphine and an increase in central morphine levels. This supposition must however be tempered with the caveat that, despite the conversion of heroin to morphine, the effects of chronic heroin treatment are not simply mediated by morphine. For example, chronic administration of these two opioids cause different neurochemical changes in brain μ -opiate receptor function (Bolger et al., 1988) and opiate dependence that is differentially regulated by hippocampal function (Bao et al., 2007) and δ -opiate receptors (Klein et al., in press; Suzuki et al., 1997). The possibility that morphine mediates dependence after acute systemic heroin injection is similarly tenuous since the heroin–naloxone interval utilized (2 h) may not be of sufficient duration for morphine levels to appreciably accumulate. Specifically, 6-MAM levels are significantly increased while morphine levels are

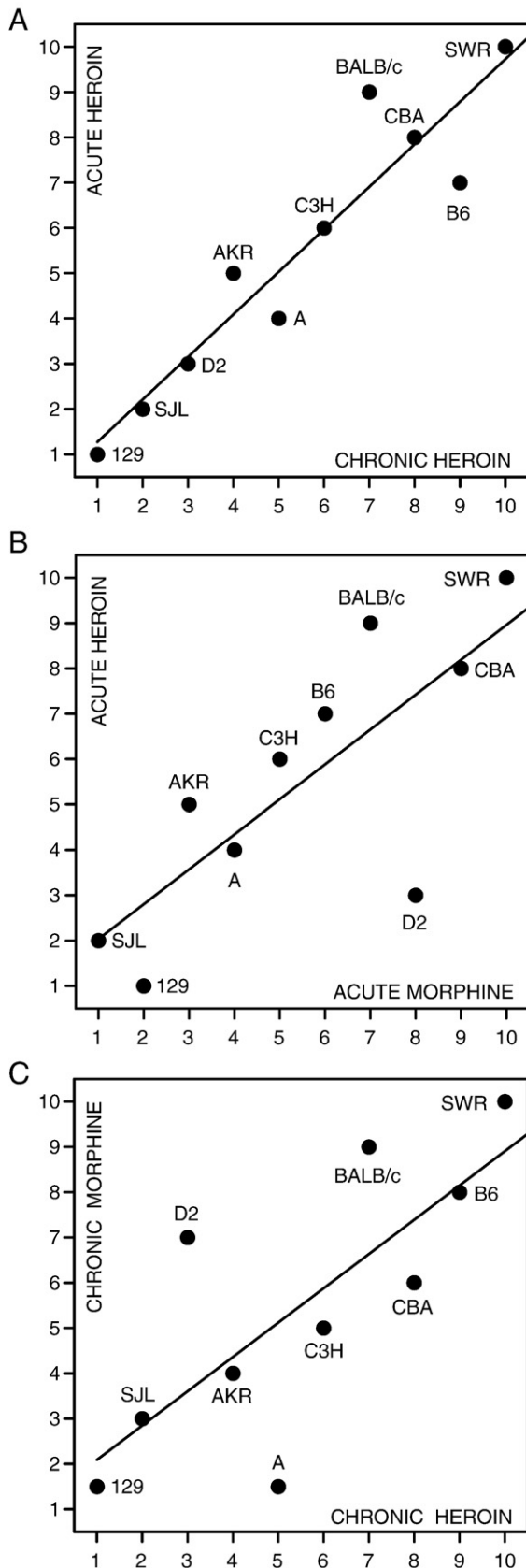


Fig. 2. Genetic correlation of acute and chronic heroin and morphine dependence. Symbols represent 10 inbred mouse strains arranged by their rank order of naloxone-precipitated withdrawal jumping frequency (1, lowest frequency; 10, highest frequency). Except for 129P3 mice subject to acute heroin treatment ($n=6$), each strain was comprised of a minimum of 8 subjects per each treatment condition. As indicated by the labels appearing on the inside of the x and y axis, data are for the correlation between acute and chronic heroin injection (A), acute heroin and acute morphine injection (B), and between chronic morphine and chronic heroin injection (C). The corresponding Spearman rank coefficients ($r_s=0.75-0.94$; Table 1) indicate a significant correlation for each pairwise comparison. Some strain names are abbreviated as follows: 129, 129P3; B6, C57BL/6; C3H, C3H/He; D2, DBA/2.

still relatively low during the first hour after heroin injection (Way et al., 1960, 1965). By the time morphine levels are increased relative to 6-MAM heroin injection 2 h after heroin injection, mice are already undergoing NPW. Furthermore, while it is not known how, or even if, 6-MAM induces dependence, it is interesting to note that the pharmacological profile of 6-MAM in the production of analgesia is distinct from that of morphine but identical to that of heroin (Brown et al., 1997; Rady et al., 1994, 1991, 2000; Schuller et al., 1999). Further work is needed to address the possible contribution of 6-MAM and morphine to dependence following acute and chronic heroin treatment. Nevertheless, the present data suggest that heroin and morphine dependence liability are genetically identical. Collectively, the present data correlation data indicate that the genetic liability to heroin dependence is independent of the magnitude or duration of heroin exposure, and that the same treatment strategy could be equally effective throughout the course of heroin intake. Furthermore, the impressive genetic association between heroin and morphine dependence suggests that highly similar treatments protocols might be effective in both morphine and heroin dependence. Indeed, selective blockade of δ_2 opioid receptors as well as the NMDA and AMPA subtypes of excitatory amino acid receptors attenuates NPW jumping frequencies in mice rendered opioid dependent after both acute and chronic treatment with heroin or morphine (Kest et al., 1996; Klein et al., in press; Suzuki et al., 1997; McLemore et al., 1997; Trujillo and Akil, 1991).

4.3. Dependence-resistant and sensitive strains

The present data identifies 129P3 mice as being absolutely refractory to jumping during heroin withdrawal. As noted above, only jumping frequency alone among withdrawal measures has been shown to have a positive dose–response relationship with withdrawal from opioids (El-Kadi and Sharif, 1994; Kest et al., 2001; Marshall and Weinstock, 1971; Smits, 1975) including heroin (Klein et al., in press). Although 129P3 mice may thus be regarded as a genetic model of insensitivity to opioid dependence, it is certainly possible that evidence of dependence in this strain will be manifest when monitoring other NPW symptoms. In contrast to 129P3 mice, SWR mice were much more sensitive to NPW from heroin than any other strain, with jumping frequencies ~3-fold greater than the strain with next highest tally after either acute or chronic heroin injection. Since this is the first report of heroin withdrawal in these two strains, we are unable to compare and validate the responses obtained here with those of other studies. However, their responses during heroin withdrawal are similar to those observed in a survey of acute and chronic morphine dependence in 11 inbred strains (Kest et al., 2002). Specifically, 129P3 NPW jumping frequencies were also minimal (<7 jumps/15 min) and among the lowest of all other strains, whereas the response frequency in SWR mice were at minimum 2- to 2.5-fold greater than any other strain.

Among inbred strains, the SWR mouse has not been the particular focus of many studies. Consequently, there are few if any descriptions in this strain of physiological systems, particularly those with relevance to dependence, which might differ from other mouse strains that might afford insight into their relatively increased sensitivity to heroin and morphine dependence. We do, however, believe that some comment regarding the dependence resistance of 129P3 mice is possible by referring to neurobiological characterizations of 129 S6 mice (previously referred to as 129/SvEv), another 129 substrain. Such a comparison may be of heuristic value since they are highly related (although not identical; Simpson et al., 1997) genetically. There is evidence that 129 S6 mice are deficient in GM1 ganglioside-regulated excitatory opioid function (Crain and Shen, 2000). GM1 ganglioside has been shown to block the translocation and activation of protein kinase C from cytosol to neuronal membranes, a biochemical pathway critical in the development of

opioid dependence (Crain and Shen, 2000; Mayer et al., 1995). In another study, 129 S6 mice were shown to possess deficiencies in the NMDA excitatory amino acid receptor system, and/or the biochemical cascade activating nitric oxide synthase consequent to its activation (Kolesnikov et al., 1998). The importance of the NMDA/ nitric oxide signaling system in dependence to opioids such as morphine (Elliott et al., 1995; Herman et al., 1995) and heroin (Klein et al., in press) has been clearly demonstrated as well. Further studies will determine if the compromised GM1 ganglioside and NMDA/nitric oxide signaling in the 129 S6 mouse contribute to the ability of 129P3 mice to resist heroin dependence. However, even if this signaling deficiency were demonstrated in 129P3 mice and determined to underlie their resistance to heroin dependence, the genetic basis of this deficiency may not be identical to those contributing to the genetic variability between this inbred strain and any other. Those genes may be identified in future gene mapping studies.

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