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ORIGINAL INVESTIGATION

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Transgenerational consequences of adolescent morphine exposure in female rats: effects on anxiety-like behaviors and morphine sensitization in adult offspring

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Abstract *Rationale and objective:* Opiate abuse in adolescent girls has increased in the past decade; however, few animal studies have examined the potential consequences of opiate use occurring at this time. The purpose of the present study was to determine whether exposing female rats to morphine during the peripubertal period can alter the adult behavior of their offspring. *Methods:* Beginning at 30 days of age, female rats were injected subcutaneously (s.c.) twice daily with either morphine sulfate or saline. The initial morphine dose of 2.5 mg/kg was increased by 2.5 mg/kg daily for a total of 20 days. Ten days after the final drug treatment, all subjects were mated. Their subsequent offspring were then tested as adults on the elevated plus maze, in a novel environment or were examined in a morphine locomotor sensitization paradigm. *Results:* Adult female offspring of dams exposed to morphine during puberty spent less time in the open arms of the elevated plus maze and displayed decreased exploration in a novel environment. Female offspring also demonstrated a more rapid induction of morphine sensitization. Finally, male offspring demonstrated a significant enhancement in the expression of morphine sensitization. *Conclusions:* Chronic morphine exposure during adolescence can have significant transgenerational effects on adult offspring. Future studies will be needed to determine how these changes are transferred to the offspring and whether these effects are specific to drug exposure that occurs during the peripubertal period.

Keywords Anxiety · Elevated plus maze · Novel environment · Tolerance · Maternal environment

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Introduction

The profile of the typical opiate abuser has been changing during the past decade to include many younger users. This change has been due in part to the introduction of inexpensive heroin pure enough to be sniffed or smoked rather than injected (National Institute on Drug Abuse 2000). In addition, the use of other opiates, such as Oxycontin and Vicodin, also appears to be increasing in adolescents (Johnson and Gerstein 1998; Johnston et al. 2004a,b). While in the past, substance abuse disorders were more prevalent in adolescent boys than girls, recent epidemiological data indicate a trend toward similar rates of use for several drugs, including opiates (Greenfield and O'Leary 1999; Greenfield et al. 2003). This is especially true at younger ages (eighth and tenth grade), suggesting that early and middle adolescence may be a period of increased vulnerability to substance abuse in girls (National Center on Addiction and Substance Abuse at Columbia University 2003). Perhaps not surprisingly, this period of increased vulnerability coincides with what is often a turbulent period of development, adolescence. The impact of drug use, particularly opiate use, during a time when the activation and regulation of hormone functions are being established, has not been determined. Indeed, there is very little information regarding the potential long-term consequences of opiate abuse that may be unique to the adolescent female abuser.

We have recently begun using an animal model of adolescent opiate abuse to examine the potential impact of prior drug exposure on reproductive outcomes in female rats. Our initial studies found that chronic morphine exposure during puberty had no effect on subsequent maternal behavior latencies in adulthood. There was, however, a significant reduction in suckling-induced prolactin secretion during the early postpartum period (Byrnes 2005). These findings indicate that pubertal opiate exposure can have long-lasting effects on some aspects of maternal physiology. As even subtle variations in dam/pup interactions can have significant effects on adult behavioral phenotypes (Francis et al. 1999; Meaney 2001), it is possible

that some long-term effect of morphine exposure during adolescence on the dam may ultimately impact the development of subsequent offspring.

The purpose of the present study was to determine whether chronic morphine exposure during puberty affects the behavioral phenotype of adult offspring. The behaviors chosen were those that have previously been shown to be affected by variations in maternal care (Caldji et al. 1998; Kalinichev et al. 2003). Specifically, the present study examined the adult offspring of females exposed to a chronic morphine regimen during puberty on tasks that measure anxiety-like behaviors (elevated plus maze, novel environment) as well as on sensitization to the locomotor effects of morphine.

Material and methods

Pubertal morphine exposure in dams

Twenty-four 20-day-old female Sprague–Dawley rats [Crl:CD(SD)BR] were purchased from Charles River Breeding Laboratories (Kingston, MA, USA). All of the animals used in these experiments were maintained in accordance with the guidelines of the Committee for the Care and Use of Laboratory Animal Resources, National Research Council. All animals were housed two to four per cage in light- (on 0700–1900 hours) and temperature- (21–24°C) controlled rooms and provided food (Purina Rat Chow) and water ad libitum.

Beginning at 30 days of age, half of the subjects (n=12) began treatment with morphine sulfate (Butler Company, Columbus, OH, USA) using an increasing dose regimen for a total of 20 days. On day 1 of morphine treatment, rats received 2.5 mg/kg s.c. twice a day. Each subsequent day, the dose of morphine was increased by 2.5 mg/kg such that by the final day of treatment, subjects received two 50 mg/kg injections. The other 12 females served as age-matched controls receiving saline injections (s.c.) twice a day with volumes adjusted to match those of drug-treated females. Females were weighed and examined for vaginal opening at the time of each morning injection. Following the final drug treatment (50 days of age), females were observed for behavioral signs of withdrawal (i.e., wet dog shakes, burrowing, rearing). The observer was not blind to subject's condition. Subjects were observed hourly between 0800 and 1600 hours beginning the day after the final drug exposure. Observations continued the following day as well, again, between 0800 and 1600 hours. Behaviors were scored as either present or absent during a 5-min observation period. All subjects were then undisturbed until they reached 60 days of age.

Mating and postpartum assessment

At 60 days of age, all females were housed with males from our colony. Of the original 24 subjects, 11 morphine-treated and 12 saline-treated subjects became pregnant. On postpartum day 1 (parturition = postpartum day 0), all litters were weighed and culled to four males and four females. Pups were weighed on postpartum days 1, 5, 10, and 21. Following weaning on day 21, males and females were separated. Once subjects reached 60 days of age, they were assigned to either the elevated plus maze, novel environment task or the morphine sensitization paradigm. Only one male and one female per litter were used on any dependent measure to avoid potential litter effects.

Elevated plus maze in adult offspring

Testing was performed using an automated elevated plus maze (Hamilton-Kinder, San Diego, CA, USA), which consisted of two open arms without edges (10.8×50.2 cm), two closed arms (10.8×50.2×40.0 cm), and a center intersection (10.8×10.8 cm), which were elevated 85.1 cm off the floor. Testing was conducted in a quiet behavioral testing room, between 1100 and 1300 hours. All of the female subjects were tested during estrus. Subjects were brought into the testing environment 5 min prior to testing. Subjects were then placed on the center platform facing an open arm, at which point data collection commenced. Testing continued for 5 min. Measurements included overall activity (total number of beam breaks), distance traveled in the open and closed arms, and time spent on the open arm, closed arm or at the intersection. The measures used to assess anxiety were the distance traveled on the open arms as well as the percent of time spent on the open arms (Lister 1990; Pellow et al. 1985).

Response to a novel environment

To measure locomotor responses to a novel environment, subjects were placed in clear Plexiglas cages (45×25×20 cm) within an automated activity chamber (SmartFrame Activity Cage Rack System, Hamilton-Kinder). Activity was monitored for 30 min. Cages were changed between subjects to avoid any possible effects of prior odors.

Morphine sensitization

Morphine sensitization was performed in a separate group of adult male and female offspring. During the induction phase of the sensitization paradigm, subjects were removed from their home cage and placed in a clean cage within the automated activity chamber. Following a 30-min habituation period, subjects were injected subcutaneously with either 10 mg/kg of morphine sulfate (Butler Company) or saline. Subjects were then monitored for 90 min after which time they were returned to their home cage. This procedure was repeated daily for a total of 7 days. After the seventh day, subjects remained undisturbed in their home cage until the expression phase on day 14. To measure the expression of morphine sensitization, all subjects were again habituated to the activity chambers for 30 min

and then injected subcutaneously with a lower dose of morphine (5 mg/kg) and monitored for the next 90 min.

Statistical analysis

Body weight and vaginal opening data collected during the pubertal injection of the dams were analyzed using a *t* test. The presence or absence of withdrawal signs was analyzed using a Fisher's exact test. Pups' birth weights and postpartum body weight gains, as well as elevated plus maze behaviors, were also analyzed using a *t* test. Distance traveled in a novel environment was analyzed using a two-way repeated measures ANOVA with dam's pubertal treatment as the between-subjects factor and 5-min time interval as the repeated measure. The data examining the induction phase of morphine sensitization were analyzed using a three-way repeated measures ANOVA with dam's pubertal treatment and offspring's drug treatment (saline or morphine) as between-subjects factors and day as the within-subjects factor. The data examining the expression phase of morphine sensitization were analyzed using a two-way ANOVA with dam's pubertal treatment and the offsprings' treatment during the induction phase (morphine or saline) as factors. All adult male and female data were analyzed separately. Post hoc analyses were performed using the Tukey's test.

Results

Chronic morphine exposure during puberty resulted in a significant decrease in body weight gain in morphine-treated females (84.8±2.1 g) as compared to saline-treated controls (102.5±3.1 g; $t_{[22]}=-4.57$, $p<0.001$) during the 21-day exposure regimen (data not shown). However, no significant delay in mean day of vaginal opening was observed between morphine- and saline-treated females (33 vs 32 days of age, respectively). The most prevalent withdrawal sign was an increased occurrence of headshakes, which was most pronounced on the second day of observations, at which time, 75% of morphine-treated females displayed headshakes during at least one of the nine observation periods, as compared to vehicle-treated females in whom this behavior was never observed (Fishers' $p<0.01$).

On postpartum day 1, all litters were counted and weighed. No significant differences in pup birth weights nor in body weight gains during the postpartum period were observed between the two groups.

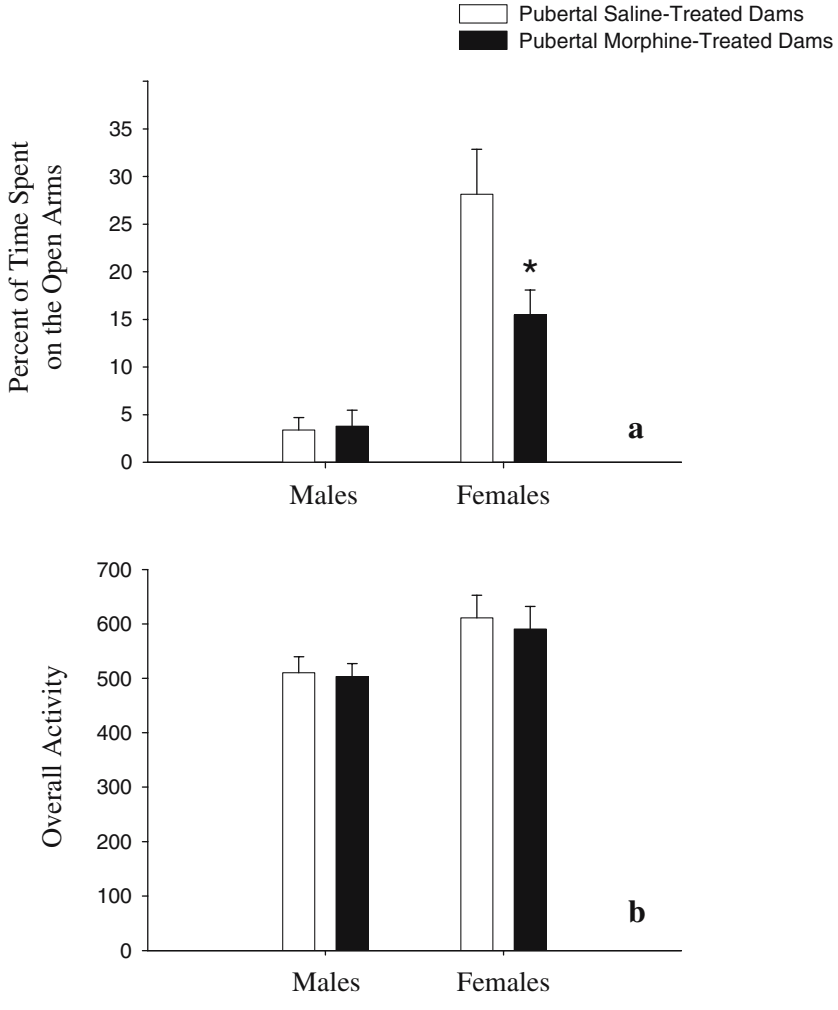

Fig. 1 Elevated plus maze behavior displayed by adult offspring of dams exposed to either morphine or saline during puberty. **a** The percent of time spent on the open arms. *$p<0.05$ as compared to female offspring of dams exposed to saline during puberty. **b** The overall activity (number of photobeam breaks) during the 5-min test. $N=10$ per group

Elevated plus maze in adult male and female offspring

As illustrated in Fig. 1, the pubertal treatment of the dam had a significant effect on the percent of time their female offspring spent on the open arms of the elevated plus maze ($t_{[18]}$=−2.348, p<0.03), with the offspring of morphine-exposed dams spending significantly less time on the open arm. No such effect was observed in male offspring. This effect was not due to a change in overall activity level, as no differences on this measure were observed in either gender as a function of their dam's pubertal treatment.

Response to a novel environment in adult male and female offspring

Similar to the findings reported on plus maze behavior, significant effects of the dam's pubertal treatment on locomotor activity in a novel environment were only observed in female offspring. A repeated measures two-way ANOVA on female offspring found significant main effects of time interval ($F_{[5,131]}$=18.71, p<0.001) and dam's pubertal treatment ($F_{[1,131]}$=5.22, p<0.04) with no interaction. As shown in Fig. 2, while both groups habituated to the novel environment during the 30-min test, the offspring of pubertal morphine dams had decreased levels of locomotor activity across all time points. No significant effects of dam's pubertal treatment were observed in males (data not shown).

Morphine sensitization

Adult male offspring induction

Significant main effects of dam's pubertal treatment ($F_{[1,26]}$=5.86, p<0.03), drug treatment ($F_{[1,26]}$=23.62, p<0.001) as well as day ($F_{[6,156]}$=5.65, p<0.001), were observed during the induction phase. In addition, there were significant interactions observed between dam's pubertal treatment×day ($F_{[6,156]}$=2.26, p<0.05) as well as drug treatment×day ($F_{[6,156]}$=13.83, p<0.001). These data are shown in Fig. 3 (top panel).

Post hoc analyses revealed significantly reduced locomotor activity following morphine treatment on days 1–3 of the induction phase, regardless of dam's pubertal treatment (all ps<0.05). Beginning on day 4, however, the male offspring of pubertal morphine-treated dams no longer demonstrated a reduction in locomotor activity following morphine, while the male offspring of pubertal saline-treated dams continued to demonstrate reduced locomotor activity (all ps<0.05). Finally, on the seventh day of treatment, while there were no significant effects of morphine treatment in either group, the offspring of pubertal morphine-treated dams, regardless of drug treatment, demonstrated higher levels of locomotor activity (p<0.05).

Adult male offspring expression

On the challenge day, a significant main effect of drug pretreatment ($F_{[1,26]}$=33.09, p<0.001) as well as a drug pretreatment×dam's pubertal treatment interaction ($F_{[1,26]}$= 33.09, p<0.02) was observed. As shown in Fig. 3 (bottom panel), morphine (5 mg/kg) increased activity in subjects treated with morphine during the induction phase when compared to saline controls within the same maternal condition (p<0.05). Thus, both groups demonstrated morphine sensitization. The offspring of pubertal morphine-exposed dams, however, exhibited a more robust sensitized response when compared to similarly treated offspring of pubertal saline-exposed dams (p<0.01).

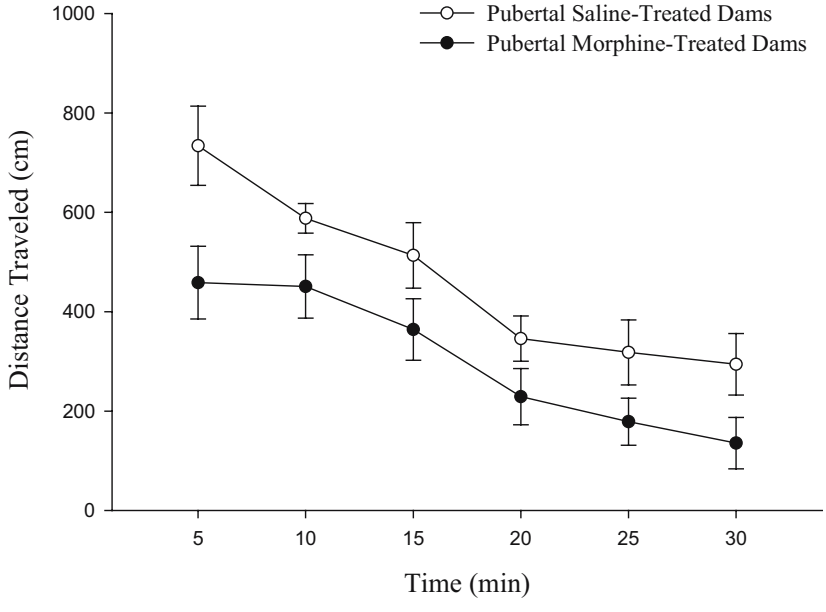

Fig. 2 Distance traveled in a novel environment displayed by adult female offspring of dams exposed to either morphine or saline during puberty. N=11 per group

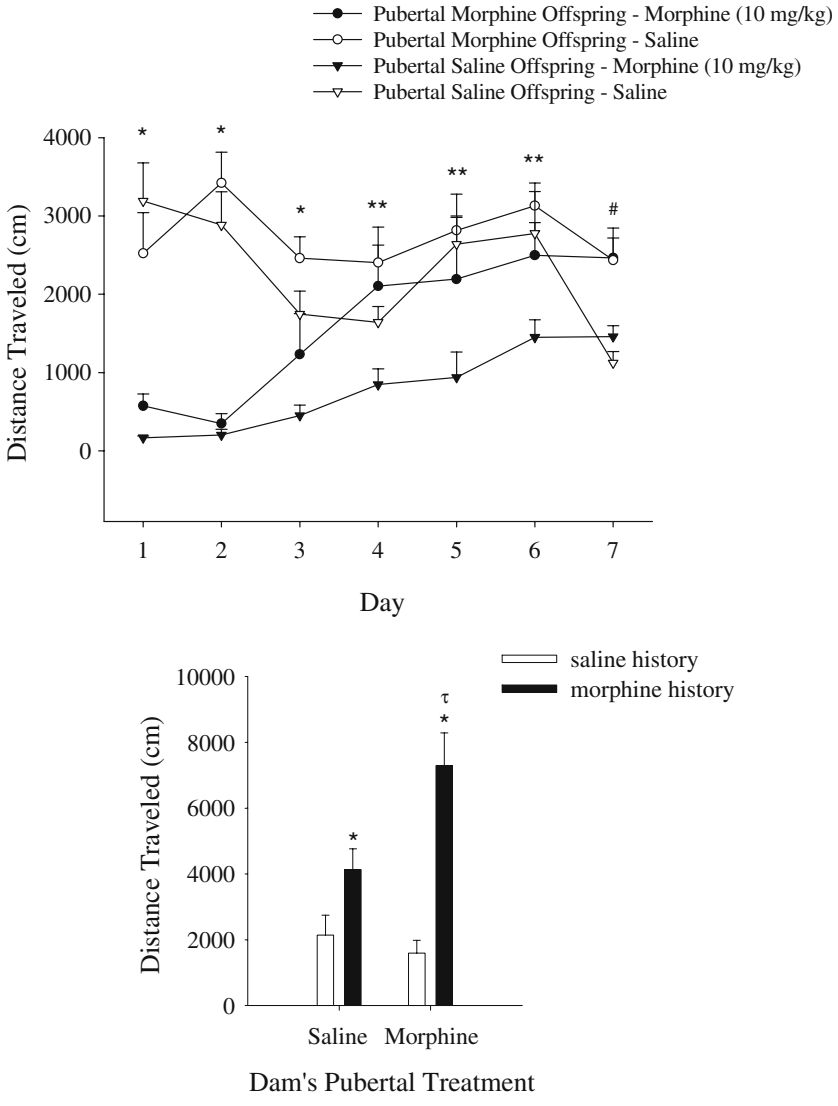

Fig. 3 *Top panel*, induction of locomotor sensitization to daily morphine injections (10 mg/kg). Distance traveled during the 90-min test session across the 7 days in adult male offspring of dams exposed to either morphine or saline during puberty. *$p<0.05$ as compared to saline-treated subjects within the same maternal condition. **$p<0.05$ morphine-treated subjects compared to saline-treated subjects in pubertal saline offspring. $^{\#}p<0.01$ pubertal morphine offspring as compared to pubertal saline offspring regardless of drug treatment. *Bottom panel*, expression of locomotor sensitization following a low-dose morphine (5 mg/kg) injection. Distance traveled during the 90-min session following 7 days of abstinence in the adult male offspring of dams exposed to either morphine or saline during puberty. *$p<0.05$ as compared to saline-pretreated subjects within the same maternal condition. $^{\tau}p<0.001$ as compared to morphine-pretreated male offspring of dams exposed to saline during puberty. N=8 per group

Adult female offspring induction

Significant main effects of drug treatment ($F_{[1,28]}$=12.88, $p<0.001$) and day ($F_{[6,168]}$=4.38, $p<0.001$) were observed during the induction phase. In addition, there were significant interactions observed between dam's pubertal treatment×day ($F_{[6,168]}$=2.87, $p<0.02$) as well as drug treatment×day ($F_{[6,168]}$=12.32, $p<0.001$). These data are shown in Fig. 4 (top panel).

Post hoc analyses reveal that both groups treated with morphine demonstrated a significant decrease in locomotor activity on the first 2 days of treatment ($p<0.01$), with effects that only approached significance ($p<0.08$) observed by the third day of treatment. By the fourth day of treatment, both groups demonstrated tolerance to the sedative effects of morphine. On the final day of treatment, however, the female offspring of pubertal morphine-exposed dams demonstrated significantly increased locomotor activity following morphine when compared both to saline-treated females within their maternal condition as well as when compared to morphine-treated offspring of dams exposed to saline during puberty ($p<0.02$)

Adult female offspring expression

On the challenge day, significant main effects of dam's pubertal treatment ($F_{[1,28]}$=4.85, $p<0.04$) and drug pretreatment ($F_{[1,28]}$=34.59, $p<0.001$) were observed. As shown in Fig. 4 (bottom panel), both groups of morphine-pretreated females displayed a sensitized locomotor response to the lower dose of morphine (5 mg/kg) on the challenge day, regardless of maternal condition (both ps<0.01). The significant main effect of dam's pubertal treatment was due to higher activity levels expressed in the offspring of pubertal morphine-exposed dams. This increased activity, however, was only significant in female offspring treated with saline during the induction phase ($p<0.05$).

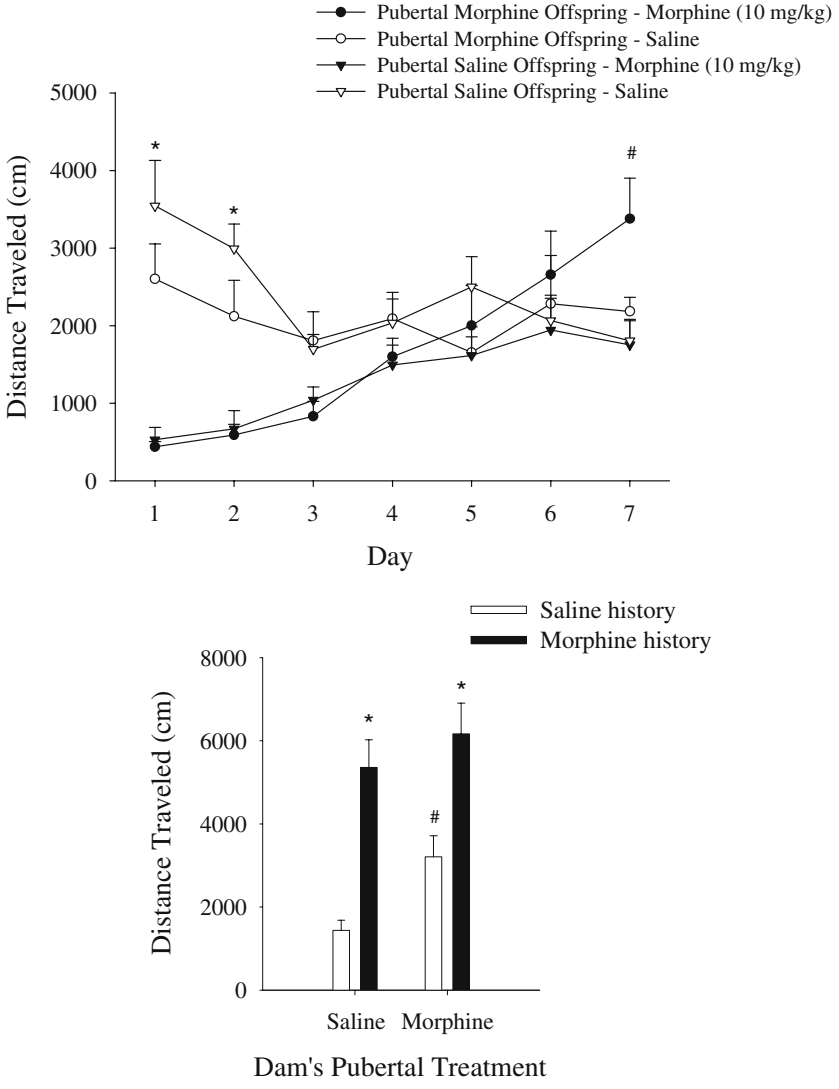

Fig. 4 *Top panel*, induction of locomotor sensitization to daily morphine injections (10 mg/kg). Distance traveled during the 90-min session was observed on days 1–7 in the adult female offspring of dams exposed to either morphine or saline during puberty. *p <0.05 as compared to saline-treated subjects within the same maternal condition. $^{\#}p$<0.01 as compared to morphine-exposed female offspring of dams exposed to saline during puberty. *Bottom panel*, expression of locomotor sensitization following a low-dose morphine (5 mg/kg) injection. Distance traveled during the 90-min session following 7 days of abstinence in the adult female offspring of dams exposed to either morphine or saline during puberty. *p<0.05 as compared to saline-pretreated subjects within the same maternal condition. $^{\#}p$<0.05 as compared to saline-pretreated female offspring of dams exposed to saline during puberty. N=8 per group

Discussion

The present findings demonstrate that in female rats, repeated exposure to morphine during adolescence can significantly alter the behavioral phenotype of their adult offspring. With regard to measures of anxiety-like behavior, effects of the dams' prior morphine exposure were only observed in female offspring. Specifically, female offspring of dams exposed to morphine during puberty displayed increased anxiety-like behavior on both the elevated plus maze as well as in a novel environment. As significant variations in anxiety-like behavior in females have been associated with alterations in hormonal status (Marcondes et al. 2001; Mora et al. 1996), one possibility for the observed differences may be a shift in the endocrine system in the offspring of morphine-exposed dams. Indeed, we have previously observed a significant delay in vaginal opening in the female offspring of morphine-exposed dams (unpublished data), suggesting some modification in the neuroendocrine control of estrous cyclicity in these females.

The induction and expression of locomotor sensitization in response to morphine in adult offspring were also significantly affected by the pubertal experience of the dam. In males, during the induction phase, offspring of pubertal morphine-exposed dams became tolerant to the sedative effects of morphine more quickly than offspring of saline-exposed dams. In addition, repeated injections of either saline or morphine resulted in an overall increase in the activity of male offspring of morphine-exposed dams compared to offspring of saline-exposed dams. This effect does not appear to be due to differences in the acute stress response to the injection. When data were analyzed in 30-min time intervals (data not shown), the differences between the two groups treated with saline on induction day 7 were observed at the 30-min time period (p<0.05) but were even more robust at the 90-min time period (p<0.01). Thus, the increased activity observed in the male offspring of morphine-exposed dams during the induction phase suggests that they fail to habituate to the test environment. Perhaps future studies using a sensitization

paradigm in which the drug treatment is unpaired with the testing environment could help dissociate potential alterations in conditioned locomotor responses from alterations in morphine sensitivity.

During the expression phase, male offspring of pubertal morphine-exposed dams exhibited significantly enhanced sensitization. As discussed above, it is not clear whether this alteration in sensitization is the result of increased sensitivity to morphine, a change in the conditioned locomotor response, or some combination of these effects. Behavioral sensitization, however, has been shown to increase drug self-administration in laboratory animals (Piazza et al. 1989; Vezina et al. 2002). Based on this association, it has been suggested that sensitization may be a model of escalating drug use and craving in humans (Robinson and Berridge 1993). Thus, this augmentation of morphine sensitization may indicate that the dam's pubertal opiate exposure has the potential to significantly alter the drug use profile of adult male offspring.

In females, offspring of dams exposed to morphine during puberty demonstrated increased locomotor activity in response to morphine by the seventh day of exposure. Thus, these females develop morphine sensitization more rapidly than offspring of pubertal saline-exposed dams. During the expression phase, similar levels of locomotor sensitization were observed in both groups of females following the challenge dose of morphine. There were, however, differences in females that had been treated with saline during the induction phase. In these females, the offspring of pubertal morphine-exposed dams appeared to be less sensitive to the sedative effects of this lower dose (5 mg/kg) of morphine. It is difficult to discern whether this effect represents a true decrease in sensitivity to lower doses of morphine or whether it reflects a behavioral sensitization to repeated saline injections. Regardless, these findings suggest that morphine exposure during puberty can have transgenerational effects, which alter behavioral responses of adult offspring.

In the present study, morphine administration ended 10 days prior to mating; thus, direct effects of the drug on the fetus were not possible. Pubertal morphine exposure had no effect on birth weights, nor were there any effects on body weight gain of offspring during the neonatal period. Thus, the impact of the dam's prior opiate exposure does not appear to be due to gross alterations in physical development.

A previous study using the same pubertal morphine-exposure regimen found that dams receiving morphine during puberty had decreased levels of sucking-induced prolactin release (Byrnes 2005). This effect on prolactin secretion was only observed during early lactation (postpartum day 5) and had no effect on their offsprings' body weight gain. Maternal prolactin is the only source of prolactin available to the neonate in the early postpartum period and is critical for the development of the tuberoinfundibular dopamine system (Phelps et al. 2003). Thus, it is possible that a reduction in maternal prolactin could significantly affect the neural development of offspring. How such changes relate to the specific behaviors observed in the present study remains unclear. One possibility could be that alterations in hypothalamic development result in shifts in the endocrine profiles of these offspring. Several studies have shown that gonadal hormone levels can significantly affect behavior on the elevated plus maze and on novelty tasks (Frye and Walf 2004; Lund et al. 2005).

Alterations in maternal behavior latencies were not observed in our previous study, with all dams retrieving their pups into the nest and crouching over their litter to nurse (Byrnes 2005). More subtle variations in maternal care, however, may be one of the consequences of pubertal exposure to morphine. Studies have shown that alterations in maternal care, including changes in the frequency of licking and grooming behavior, can significantly affect the level of anxiety-like behaviors demonstrated by adult offspring (Caldji et al. 1998). In addition, brief neonatal handling as well as maternal separation has also been shown to alter locomotor responses in a novel environment as well as morphine sensitization (Kalinichev et al. 2002, 2003). These changes are believed to be due to an alteration in maternal behavior, which is induced by the separation. In fact, a recent study found that pups show few effects of maternal separation if their dam is given foster pups to care for during their absence (Huot et al. 2004).

Opioids are known to influence various components of maternal behavior, including placentophagia (Mayer et al. 1985), maternal motivation (Nelson and Panksepp 1998; Panksepp et al.1994), as well as the amount of time the dam spends nursing her pups (Byrnes et al. 2000). Indeed, one role of the endogenous opioid system appears to be inhibiting the more active components of maternal behavior (Mann et al. 1991). Exposure to opiates during puberty could alter the development of the opioid system, thereby altering the quality of maternal/pup interactions. Given the importance of maternal behavior on development, such a change could have long-lasting consequences for their offspring. Future studies using a cross-fostering paradigm will be necessary to determine the influence of postpartum dam-offspring interactions on the changes observed in the adult offspring of morphine-exposed dams.

The present study examined the potential impact of pubertal morphine exposure on future offspring. The results show that significant alterations in the adult behavioral phenotype as well as behavioral sensitization to morphine are observed in the offspring of pubertal morphine-exposed dams. While the present experiment observed changes following morphine exposure during puberty, it is not yet known whether similar changes in adult offspring might be observed when drug exposure occurs at other ages. Future studies are required to determine whether exposure occurring during the peripubertal period is critical for the changes observed in these adult offspring. Nonetheless, the present findings indicate that a dam's prior drug history can have long-lasting effects on her offspring, even when the exposure occurred prior to insemination. Moreover, the changes in morphine sensitization profiles observed in the offspring of pubertal morphine-exposed dams

suggest that a history of opiate exposure may increase the likelihood that future offspring will have a propensity toward drug abuse.

Given the trend toward increased opiate abuse in adolescent girls, studies examining the consequences that may be associated with drug use in this particular population could provide important insight into the potential problems that these girls, as well as their offspring, may face in the future. The fact that the transmission of these effects to the next generation occurred in the absence of any direct exposure of the fetus to opiates may also provide some insight into the nature of familial patterns of drug abuse. These results validate the use of this animal model to explore the long-term consequences of adolescent opiate abuse. Future experiments will attempt to determine the mechanisms that mediate the transmission of these effects to male and female offspring.

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