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Cross-Generational Effect of Prenatal Morphine Exposure on Neurobehavioral Development of Rat Pups

R. ŠLAMBEROVÁ[1, 3], M.A. RILEY[2], I. VATHY[1, 2]

Departments of [1]Psychiatry and Behavioral Sciences, [2]Neuroscience, Albert Einstein College of Medicine, Bronx, NY, USA and [3]Department of Normal, Pathological and Clinical Physiology, Third Faculty of Medicine, Charles University, Prague, Czech Republic

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Summary

Prenatal exposure to opiates can have devastating effects on the development of human fetuses and may induce long-term physical and neurobehavioral changes during postnatal maturation. The present study was aimed at identifying cross-generational effects of prenatal morphine exposure in Sprague-Dawley rats. Pregnant rats were injected subcutaneously with either saline or morphine (10 mg/kg) twice daily during gestational days 11-18. Litter size, percentage of males and females, anogenital distances (AGDs), righting reflex, and body weight were assessed in prenatally morphine-exposed pups (first generation) and their offspring (second generation). Both prenatally morphine-exposed pups and offspring of prenatally morphine-exposed dams exhibited an increased latency to right. Additionally, second generation pups were slower in righting than first generation pups. During the early postnatal period the second generation pups weighed less than the first generation regardless of drug exposure. The AGDs of second generation male pups were decreased relative to the first generation. Our data provide important novel information about the trans-generational effects of maternal opiate abuse that may be useful for understanding/evaluating the teratogenic effects of prenatal opiate exposure.

Key words

Prenatal morphine exposure • Generational difference • Neurobiological development

Introduction

Clinical studies have demonstrated that prenatal exposure to opiates can have devastating effects on the development of human fetuses and as such may induce long-term physical and neurobehavioral changes during postnatal maturation (Zagon 1985). Experimental studies have also provided evidence on long-term effects of prenatal opiate exposure (Vathy *et al.* 1985, Vathy and Kátay 1992, Niesink *et al.* 1999, Šlamberová *et al.* 2001). Prenatal morphine exposure alters adult female reproductive physiology and behavior (Vathy *et al.* 1985, Vathy and Kátay 1992, Siddiqui *et al.* 1997). Thus, prenatally morphine-exposed female rats have irregular estrous cycles and reduced fertility, suggesting altered ovarian steroid-regulated release of circulating plasma luteinizing hormone (Siddiqui *et al.* 1997).

Friedler (1978) demonstrated that there is a transgenerational effect of morphine on mouse body weight and motor coordination when administered to

Fax +420 241 062 164
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

female mice prior to the gestation period. Additionally, Becker and Randall (1987) showed that female mice prenatally exposed to alcohol were more likely to have offspring with lower fetal weight. Similarly, more recent work by Lam *et al.* (2000) showed a lower weight gain and increased latency to right in pups from prenatally alcohol-exposed mice relative to the controls. Thus, there is evidence that alcohol and drug exposure may affect even second generation of animals. There are, however, no studies examining the effect of morphine administered to pregnant female rats on second generation of their progeny.

To determine whether prenatal morphine exposure on gestational days 11-18 has trans-generational effects, anogenital distances (AGDs), righting reflex, and body weight were investigated in prenatally morphine- and saline-exposed rats (first generation) and in the offspring of females exposed prenatally to morphine or saline (second generation). These parameters were compared in both males and females to ascertain whether there are sex-dependent alterations in morphine-exposed animals.

Methods

Animals and drug treatment

Thirty-six timed pregnant Sprague-Dawley female rats were purchased from Taconic Farms (Germantown, NY). Upon arrival at 8 days post-conception, pregnant dams were individually housed in maternity cages with food and water available *ad libitum*. Animals were maintained in a temperature-controlled (22-24 °C) colony room on a reversed 14-h (light): 10-h (dark) cycle with lights off at 11:00 h. The procedures for animal experimentation utilized in this report were reviewed and approved by the Institutional Animal Care and Use Committee of AECOM, which is in agreement with the Principles of laboratory animal care (NIH publication No. 85-23, revised 1985).

Pregnant rats were randomly assigned to receive either saline (control) or morphine injections. Beginning on post-conception day 11 and continuing through day 18, each pregnant female was injected subcutaneously (s.c.) twice daily at 08:00 h and at 20:00 h, with either 0.9 % physiological saline or morphine sulfate dissolved in saline. To decrease the possibility of resorption of pups or fetal death (Koren 2001), pregnant dams were gradually introduced to the higher dose of morphine. The dose of the first three morphine injections was 5 mg/kg, and the subsequent injections were 10 mg/kg (Vathy *et al.* 1985).

From the total amount of 36 litters, 16 (8 prenatally saline-exposed and 8 prenatally morphine-exposed females) were used to examine the effects of morphine on the first generation. Twenty litters were left to grow up and one female from each litter was used to examine the second generation.

First generation

The day of birth (gestation day 22) was designated as postnatal day (PND) 0. On PND 1, pups were sexed, and the AGDs were measured in all male and female pups. The number of pups and the percentage of males and females in each litter were noted. Black India ink was used to tattoo one footpad of pups for future identification. Pups from morphine- and saline-treated mothers were cross-fostered such that each mother raised a litter of which half the pups were her own and half were adopted from dams that received the opposite gestational exposure. The number of pups in each litter was adjusted to 10. On the day of weaning (PND 25) the experiment was discontinued.

Second generation

At PND 60 one female rat from each of the remaining 20 litters (10 saline- or 10 morphine-exposed) was randomly selected to reproduce and bear offspring. The rest of the animals were used in other studies. All 20 prenatally saline- and morphine-exposed female rats were smeared daily by vaginal lavage for a period of two weeks between PND 45-60 to assess estrous cyclicity. The smear was examined by light microscopy using 20 x magnification. To increase the probability of the successful impregnation, because the irregularity of estrous cycles, especially in prenatally morphine-exposed females, each female was housed for 4 days (duration of a regular cycle) with a sexually-experienced, adult male rat. The sexually mature, untreated males are kept in our colony room for mating only. After this mating period, females were individually housed in maternity cages to bear and raise their pups that were assigned as the second generation. Of the 20 first generation females mated, 13 were successfully impregnated (7/10 saline-exposed; 6/10 morphine-exposed), and used to bear and raise their offspring (second generation). The postnatal manipulation with litters was the same as in the first generation.

Data to be recorded

To assess trans-generational effects of morphine exposure, prenatally saline- and morphine-exposed pups (first generation) were compared with the offspring of prenatally saline- and morphine-exposed dams (second generation). On PND 1, the number of pups in the litter, number of dead pups, and percentage of males and females in each litter was recorded and compared between generations. AGD distances were measured on PND 1 for possible masculinizing or feminizing effects of morphine. Each pup was weighed in 5-day intervals between PND 1 and 25. The righting reflex, an index of neurobehavioral maturation and/or neurological impairment (Dodson and Miller 1985), was assessed in 4-day intervals between PND 1 and 12 (all pups righted by PND 12). Each pup was turned on its back, and the time it took for the pup to right with all four paws contacting the surface of the testing table was recorded as the latency to right, in seconds. A maximum of 60 s was given for each pup to right. If righting did not occur within 60 s, the test was terminated and a time of 60 s was assigned to that trial. All pups righted in less than one second by PND 12.

Statistical Analyses

The average of animals of the same sex and drug exposure in each litter was used as the unit of analysis. Because there were no differences between postnatal care of morphine- and saline-exposed mothers, the raising mother (biological vs. foster) was not taken as a factor for statistical analyses. Two-way ANOVA (drug exposure x generation) was conducted to analyze the number of pups in each litter. Three-way ANOVA (drug exposure x sex x generation) was used to analyze differences in AGD, weight, and righting reflex. For *post-hoc* comparisons, the Newman-Keuls test was conducted. Differences were considered significant if $p<0.05$.

Table 1. Effects of prenatal morphine exposure on first and second generation pups

Generation	Treatment	Number of Pups	Males (%)	Females (%)	Males AGD (mm)	Females AGD (mm)
First	*Saline*	13 ± 1	45 ± 7	55 ± 7	3.58 ± 0.04	1.77 ± 0.13 $^{+}$
	Morphine	12 ± 1	51 ± 4	49 ± 4	3.75 ± 0.11	1.51 ± 0.11 $^{+}$
Second	*Saline*	14 ± 1	55 ± 6	45 ± 6	2.93 ± 0.08 *	1.49 ± 0.04 $^{+}$
	Morphine	14 ± 1	53 ± 5	47 ± 5	2.38 ± 0.08 *	1.50 ± 0.05 $^{+}$

Values are the mean ± SEM. First generation: 8 saline litters; 8 morphine litters; Second generation: 7 saline litters; 6 morphine litters. AGD (anogenital distance): "n" = average of pups with the same sex and treatment in litter. First generation: 16 in each sex and prenatal exposure. Second generation: 13 in each sex and prenatal exposure. * $p<0.0001$ vs. first generation of both prenatal treatment. $^{+}$ $p<0.0001$ vs. males of the same generation and prenatal treatment

Results

As shown in Table 1, there were no generation or drug differences in the number of pups *per* litter. The percentage of male and female pups *per* litter was also comparable in all groups (Table 1). In AGDs (Table 1), there was a main effect of sex [$F(1, 108) = 719.4$; $p<0.0001$] and generation [$F(1,108) = 47.02$; $p<0.0001$] and a significant interaction between drug exposure, sex and generation [$F(1, 108) = 20.73$; $p<0.0001$]. Second generation males had shorter AGDs than the first generation males regardless of drug exposure. There were no generational or drug effects on ADGs in females. As expected, males had longer AGDs than females.

In the righting reflex, there was a main effect of generation [$F(1, 108) = 5.33$; $p<0.05$]; second generation pups were slower to right than first generation pups regardless of sex or drug exposure (Fig. 1A). Additionally, there was a main effect of drug exposure [$F(1, 108) = 8.81$; $p<0.01$]; morphine-exposed pups were slower to right than saline-exposed pups regardless of sex or generation (Fig. 1B). The increased latency to right was more pronounced between PND 1-4 than at later ages.

There was a main effect of generation on body weight during PND 1-5 [$F(1,108) = 6.44$; $p<0.05$]; PND 6-10 [$F(1,108) = 4.73$; $p<0.05$]; and PND 16-20 [$F(1,108) = 9.49$; $p<0.01$]. Thus, the second generation pups weighed less than first generation pups regardless of sex or prenatal drug exposure (data not shown). There

were, however, no morphine exposure-induced differences in the first or second generation.

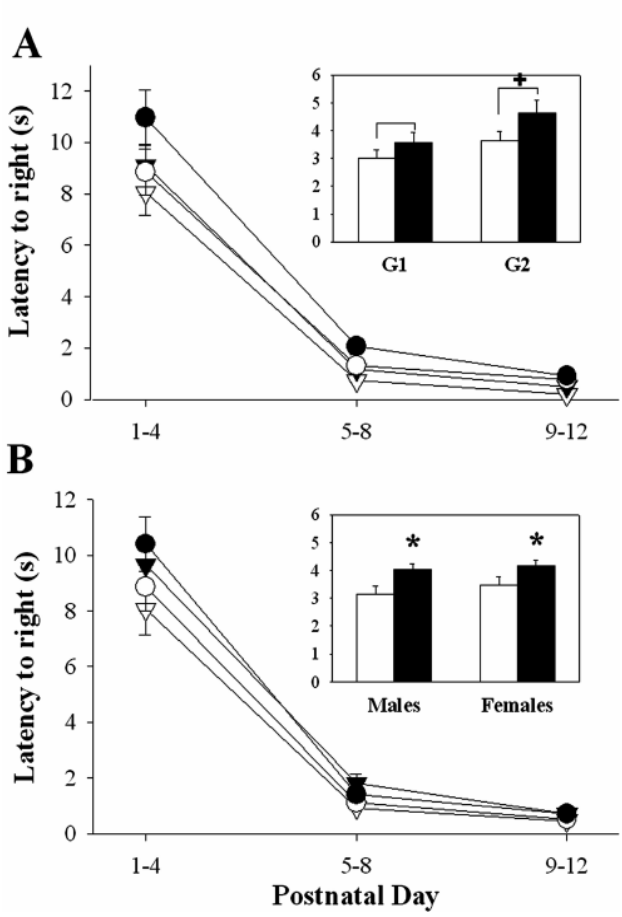

Fig. 1. Effect of prenatal morphine exposure on the righting reflex in two generations of rats. Values are mean ± SEM; "n" = average of pups with the same sex and treatment in litter. Insets are averages of the latencies to right between PND 1 and PND 12. Plot A, trans-generational effect: Triangles are generation 1, circles are generation 2 regardless of sex. Open symbols and columns are prenatally saline-exposed rats, filled symbols and columns are prenatally morphine-exposed rats. G1 = Generation 1, G2 = Generation 2; + $p<0.05$ (ANOVA). Plot B, main effect of prenatal morphine exposure: Triangles are males, circles are females regardless of generation. Open symbols and columns are prenatally saline-exposed rats, filled symbols and columns are prenatally morphine-exposed rats. * $p<0.01$ (ANOVA)

Discussion

The present study demonstrates that prenatal morphine exposure alters neurobehavioral development in two generations, as demonstrated by increased latencies to right. Pups from morphine-exposed dams of both generations had an increased latency to right relative to pups from saline-exposed dams. The finding that the righting reflex of drug-naive offsprings (second generation) of prenatally morphine-exposed dams is slower than offsprings of prenatally saline-exposed dams suggests that the effects induced by prenatal morphine exposure may be present during neuronal development of the second generation. Our results support the findings of Friedler (1978), who observed the lack of motor coordination in second generation of morphine-exposed mice between PND 14 and 17. Additionally, Lam *et al.* (2000) demonstrated an increased latency to right in offspring of prenatally ethanol-exposed mothers relative to rat pups from control mothers. The neuromotor deficits induced by prenatal alcohol exposure in second generation rats suggested a role for altered oocytic mitochondrial DNA (Lam *et al.* 2000). Because mitochondrial DNA is inherited exclusively from the mother and oocytic maturation begins in early fetal life (Lam *et al.* 2000), prenatal drug administration may induce mutagenic effects in oocytes of the developing, drug-exposed fetus. These effects may then lead to impaired neurodevelopment in the second generation.

Our results do not show a significant morphine-induced decrease in body weight. The finding that prenatal morphine exposure does not alter the body weight of first generation pups agrees with our previous observations (Vathy *et al.* 1985, 1993). However, it does not correlate with clinical reports showing decreased birth weights in infants prenatally exposed to illicit drugs such as heroin (van Baar *et al.* 1994). The contradictory findings between our results and those that show a opiate-induced decrease in body weight of exposed animals (van Baar *et al.* 1994) may be due to different doses and/or routes of the drug administration. Interestingly, examination of body weight in 5-day intervals revealed a significant main effect of generation during early postnatal period, i.e. the second generation pups weighed less than the first generation regardless of drug exposure. However, this decrease in body weight was limited to PND 1-10, with both generations achieving comparable body weight after PND 10. These findings of a transient decrease in body weight in the early postnatal period are novel and have not yet been published in clinical or basic science literature to the best of our knowledge.

The AGD is widely accepted in rodent studies as a measure of sexual dimorphism resulting from prenatal exposure to androgens, and is longer in males than females (Palanza *et al.* 2001). Our previous studies (Vathy *et al.* 1983, Vathy 1999) and the current observations in the first generation pups suggest that prenatal morphine exposure neither masculinizes the females nor feminizes the males. Novel findings of the

present study are the data showing that second generation male pups had shorter AGDs than first generation male pups regardless of drug exposure. Because the group of animals with shorter AGDs correlate with those of lower body weight, it is possible that an overall decrease in body size and weight of second generation pups at birth, may account for the observed decrease in AGD in both males and females. However, the decrease in AGDs are significantly lower only in male pups, while the decrease is not significant in females. Thus, it seems that male pups from second generation were somewhat feminized or demasculinized when compared to first generation male pups regardless of drug exposure. This finding is very interesting, but difficult to explain. It is possible that the different breeding conditions in the first and second generation caused the generational differences. The first generation dams were purchased pregnant at eight-day post-conception from Taconic farms, while the second generation was bred in our colony laboratory. However, why the males would be more affected by breeding conditions than females is not clear. Future studies may keep track of the feminizing effect in second generation males by monitoring other sexual markers and maturation of these animals, such as the time of testes descent and sexual behavior. Moreover, it would also be interesting to test male sexual behavior in first generation saline- and morphine-exposed males with stimulus-untreated females, as was conducted in the present report on females. It could show whether paternal drug-exposure induces differences in second generation pups in these conditions and whether saline- and morphine-exposure induced these changes separately. Such results should allow us to demonstrate whether maternal and/or paternal opiate exposures transmit long-term alterations in animals of the second generation.

In conclusion, to the best of our knowledge the present study is the first to demonstrate second generation effects of morphine in offspring of prenatally morphine-exposed rats. The delayed neurological maturation observed in our studies may support findings of neurobehavioral deficits seen in children of abusers of this substance (Johnson and Leff 1999, Kolar *et al.* 1994). Future studies may investigate underlying neurochemical changes that can contribute to neurological impairments.

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Reprint requests

I. Vathy, Department of Psychiatry and Behavioral Sciences, Albert Einstein College of Medicine, 1300 Morris Park Ave., Ull. 111, Bronx, NY 10461, USA. Fax (718) 430-8772. E-mail: vathy@aecom.yu.edu