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Gonadal hormone-induced changes in adult male and female rats exposed to early postnatal handling are not altered by prenatal morphine exposure

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Abstract

The purpose of the present study was to investigate the long-term effects of early postnatal handling in several gonadal hormone conditions in adult male and female rats exposed prenatally to morphine or saline. An open-field apparatus was used to test locomotor activities such as line crossing, rearing, grooming, and anxiety-like behaviors such as visiting squares alongside the walls of the open field and boli dropping. Postnatal handling increased locomotor activities in gonadally intact males and in all groups of hormone-manipulated females, but did not change them in gonadectomized (GNX) males. Additionally, there was a decrease in anxiety-like behavior in ovariectomized (OVX) females after estradiol benzoate (EB) or EB and progesterone (P) replacement due to handling. Handling did not affect anxiety-like behaviors in OVX females or in GNX or gonadally intact males. Prenatal morphine exposure did not alter any open-field measures in handled or nonhandled animals when compared to saline controls. Thus, the present study demonstrates that early postnatal handling induces long-lasting changes in locomotor and anxiety-like behaviors of adult male and female rats regardless of their prenatal exposure to morphine. These changes are gonadal hormone specific.

Keywords: Prenatal morphine; Early postnatal handling; Open field; Gonadal hormones

1. Introduction

Early postnatal handling during lactation greatly reduces emotional reactivity and the responses of hypothalamic–pituitary–adrenal (HPA) axis to stress (Fernandez-Teruel et al., 1992; Meaney et al., 1991; Nunez et al., 1996; Plotsky and Meaney, 1993; Vallee et al., 1997, 1999). In response to different stressors, handled animals secrete less corticosterone and ACTH and more corticotropin-releasing factor than nonhandled animals (Ader and Grota, 1973; Hess et al., 1969; Levine, 1957, 1962; Meaney and Aitken, 1985; Meaney et al., 1989, 1991; Zarrow et al., 1972). In handled rats, the stress-induced increase of these hormones return faster to basal levels following the termination of the stressor. The basal levels, however, do not differ between handled and nonhandled animals (Meaney and Aitken, 1985; Meaney et al., 1989, 1991; Zarrow et al., 1972). Additionally, neonatally handled animals show less hippocampal neuronal death in contrast to that induced in nonhandled animals by higher concentrations of circulating glucocorticoids in adulthood (Meaney et al., 1988, 1991; Nunez et al., 1996; Plotsky and Meaney, 1993; Vallee et al., 1997, 1999; Weizman et al., 1999). Cognitive performance is also better in animals handled during first 3 weeks of life (Meaney et al., 1991; Vallee et al., 1999). Early handling increases accuracy and decreases the time to complete a task in learning tests (Meaney et al., 1991; Vallee et al., 1999).

In the open field, early handled rats show an increase in locomotor activity and a decrease in anxiety-like behavior, such as spending less time in the corners of an open field and dropping less boli than nonhandled rats (Fernandez-Teruel et al., 1990; Meerlo et al., 1999; Nunez et al., 1996; Vallee et al., 1997; Wakshlak and Weinstock, 1990; Weizman et al., 1999). Daily postnatal handling during the first

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21 days of life reverses the effect of prenatal stress in adult rats tested in the open field (Wakshlak and Weinstock, 1990). Additionally, handled animals spend more time on the open arms of the elevated plus-maze compared to nonhandled rats, which also demonstrates reduction in the anxiety-like behavior (Fernandez-Teruel et al., 1990; Meerlo et al., 1999; Vallee et al., 1997). Emotional reactivity is decreased after early postnatal handling; thus, postnatally handled animals show less reaction to being captured and handled during the emotional reactivity test conducted in adulthood (Ader, 1968; Fernandez-Teruel et al., 1992; Meerlo et al., 1999; Nunez et al., 1996; Vallee et al., 1997).

Many studies demonstrate that the opioid system is closely related to the responsiveness to different stressors (Akil et al., 1976; Amir and Amit, 1978; Appelbaum and Holtzman, 1984; Bardo et al., 1981; Bodnar, 1990; Olson et al., 1998; Pierce and Raper, 1995; Schnur et al., 1988; Stewart and Eikelboom, 1981; Stohr et al., 1999). Stress induces the release of endogenous opioids (Akil et al., 1976), produces opioid-like effects such as analgesia (Amir and Amit, 1978; Bodnar, 1990; Olson et al., 1998), and enhances the action of exogenous opiates on body temperature changes and locomotor and pain-related behaviors (Appelbaum and Holtzman, 1984; Schnur et al., 1988; Stewart and Eikelboom, 1981). However, there are only a few studies showing an interaction between the opioid system and early postnatal handling. Fernandez et al. (1999) demonstrate that postnatal handling significantly reduces the effect of morphine, which decreases the sensitivity to thermal pain in male and female rats. D'Amore et al. (1993) reports that postnatal handling reduces the antinociceptive effect of β-endorphin in male mice. Additionally, Attila (1989) shows a modifying effect of handling on noradrenaline turnover and metabolism in the hypothalamus, prefrontal cortex, and the lower brain stem after acute morphine administration.

Thus, the above studies show that early postnatal handling has long-term effects on anxiety-like behaviors, response to stress, and learning and memory. Further, stress response and early postnatal handling can be altered by the opioid system. However, there are no studies on whether handling-induced long-term effects are affected by prenatal drug exposure. Our previous work demonstrates that prenatal morphine exposure, during Gestation Days 11–18, has long-term effects on adult learning and memory (Šlamberová et al., 2001a) and response to stress (Šlamberová et al., in review). Spatial memory is impaired by prenatal exposure to morphine in adult male and female rats (Šlamberová et al., 2001a). Cold water stressor annuls differences between prenatally saline- and morphine-exposed animals, e.g. less floating of morphine- than saline-exposed males was observed when tested in the Porsolt swim test (Šlamberová et al., in review). We have also unpublished data showing higher corticosterone increase in morphine-exposed males after cold water stressor, while there was no difference in the basal level of corticosterone when compared to saline controls.

The effects of prenatal morphine exposure on spatial memory and response to stress are also affected by the presence or absence of female gonadal hormones (Šlamberová et al., 2001a, in review). Administration of both estradiol benzoate (EB) and progesterone (P) to ovariectomized (OVX) females annuls the impairment of prenatal morphine exposure on learning and memory (Šlamberová et al., 2001a). As a response to cold water stressor, an EB administration in OVX females increases floating observed in a modified Porsolt swim test (Šlamberová et al., in review). Thus, we demonstrated that prenatal morphine exposure alters spatial memory and response to stress, both of which can possibly be altered by early postnatal handling, in gonadal hormone-specific manner.

The present study is therefore designed to test the hypothesis that early postnatal handling induces changes in behaviors tested in the open field and that these are altered by prenatal morphine exposure and gonadal hormones in adult male and female rats.

2. Methods

Twenty time-mated pregnant Sprague–Dawley rats were purchased from Taconic Farms (Germantown, NY) on Day 8 after conception. Animals were weighed, housed individually in maternity cages, and maintained in a temperature-controlled colony room with free access to food and water on a reversed 14 h (light)/10 h (dark) cycle with lights off at 11:00 h. Pregnant rats were randomly assigned either to a saline- or a morphine-treated group. Morphine, dissolved in 0.9% physiological saline, or physiological saline injections were administered subcutaneously (sc) twice a day (08:00 and 20:00 h) during Gestational Days 11–18 (Vathy et al., 1985). The dose of the first three morphine injections was 5 mg/kg, and the dose of the remaining injections was 10 mg/kg. The day of birth was counted as Postnatal Day (PND) 0.

Because repeated morphine treatment attenuates maternal behavior (Šlamberová et al., 2001b), saline- and morphine-exposed pups were sexed, tattooed for identification, and crossfostered on PND 1. That is, each morphine-treated dam was paired with a saline-treated dam, and, thus, each mother received and raised some of her own and some of the adopted pups from the other litter receiving a different treatment (Vathy et al., 1985). Whenever possible, equal number of male and female rats were raised by each mother. The litters were adjusted to 10 pups. Because our previous work demonstrates no differences in weight, behavior, or neurochemistry related to whether the pups were raised by morphine- or saline-treated mothers (Vathy and Kátay, 1992; Vathy et al., 1994), all data reported are combined from morphine- and saline-exposed pups raised by both morphine- and saline-treated mothers. To ascertain that long-term effects of maternal care did not alter the open-field results, control statistics showing no differences between animals raised by

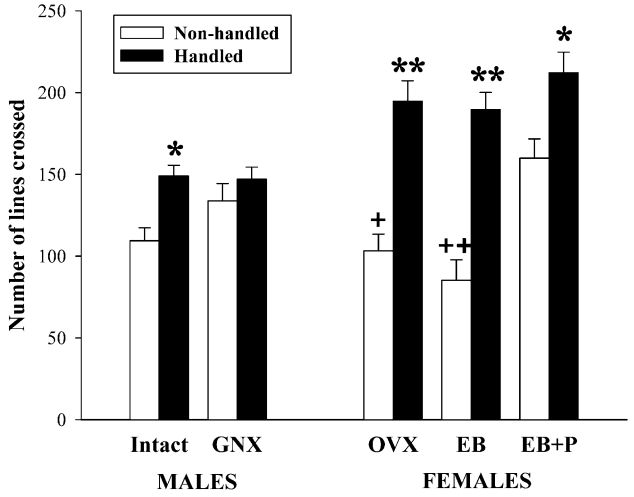

Fig. 1. Effect of postnatal handling on line crossing in the open-field of adult male and female rats. Values are averages from saline- and morphine-exposed animals in each group ($n=16$). Student–Newman–Keuls post hoc test shows * $P<.01$ and ** $P<.0001$ vs. nonhandled animals of the same hormonal background in both saline- and morphine-exposed rats. $^{+}P<.001$ and $^{++}P<.0001$ vs. nonhandled, OVX, EB- and P-treated females in both saline- and morphine-exposed rats.

saline- or morphine-treated mothers in all examined parameters within each testing group was performed.

After delivery, saline- and morphine-treated mothers with their pups were randomly assigned to either handled or nonhandled groups. Handling began on PND 1 and was conducted twice daily until weaning on PND 25. Morning handling begun at 08:00 h and the afternoon handling at 15:00 h. Handling consisted of removing the pups from the maternal cage and putting them for 15 min into another cage onto a heating pad to prevent hypothermia. This short separation is not considered to be maternal deprivation (Jans and Woodside, 1990; Leon et al., 1978; Meaney et al., 1991). The nonhandled pups were left with their mothers until weaning. Regular daily animal care was conducted the same way for all handled and nonhandled animals. On PND 25, all pups were weaned and housed individually.

Altogether, 64 adult male and 96 female rats were tested in the open field. Half of the males were left gonadally intact and half were gonadectomized (GNX). All females were OVX. Surgeries were performed at PND 65 under Metofane (Pitman-Moore, Atlanta, GA) anesthesia. Surgery and post-surgical care was approved by the Institutional Animal Care and Use Committee. GNX males were left undisturbed for 3 weeks and OVX females for 7–10 days. Some of the OVX females received EB and some of them EB and P. Hormones were administered subcutaneously; EB (3 μg/0.1ml/rat dissolved in peanut oil) 48 h and P (500 μg/0.1ml/rat dissolved in peanut oil) 3 h prior to open-field testing. There were 20 testing groups (8 animals/group) considering different prenatal drug exposure, postnatal handling, and gonadal hormone conditions.

The open-field testing was conducted during the dark phase (between 14:00 and 16:00 h) of the reverse light/dark cycle for 8 consecutive days. One animal from each group was tested on each day to avoid daily fluctuations in performance. The floor (90×90 cm) and the walls of the open field were painted black. White-lined grids on the floor delineated 36 squares (15×15 cm). Each animal was placed into the center of the open field in a testing room illuminated with red light and observed for 5 min. At the end of each test, an animal was removed from the open field and returned to its home cage, and the open field was cleaned. The number of lines crossed, the number of rearing, the number of grooming, and the number of boli released were recorded. The number of visited squares alongside the walls of the open field was counted as a percentage of the total number of squares visited.

2.1. Statistical analysis

A three-way ANOVA (Prenatal Drug Exposure× Handling×Sex/Hormones) and Student–Newman–Keuls post hoc test was used for data analysis. Differences were considered significant if $P<.05$. Because there were no differences between saline- and morphine-exposed animals in any analyzed measures, the data were collapsed across prenatal treatment groups in the table and figure presentations for better clarity.

3. Results

Generally, there was an increase in line crossing [$F(1,140)=82.86$, $P<.0001$] and rearing [$F(1,140)=27.05$, $P<.0001$] and a decrease in the number of squares visited alongside the walls of the open field [$F(1,140)=$

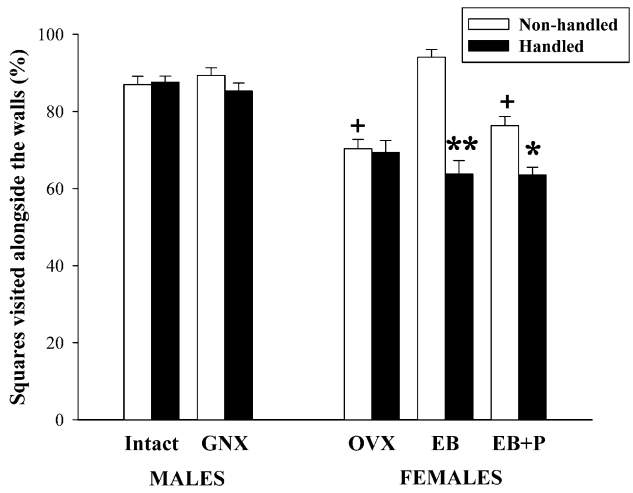

Fig. 2. Effect of postnatal handling on squares visited alongside the walls in the open-field of adult male and female rats. Values are averages from saline- and morphine-exposed animals in each group ($n=16$). Student–Newman–Keuls post hoc test shows * $P<.001$ and ** $P<.0001$ vs. nonhandled animals of the same hormonal background in both saline- and morphine-exposed rats. $^{+}P<.0001$ vs. nonhandled, OVX, EB-treated females in both saline- and morphine-exposed rats.

Table 1
Effect of postnatal handling on the number of boli released during the open field in adult male and female rats

Sex	Hormonal background	Nonhandled	Handled
Males	Intact	0.69 ± 0.3	0.6 ± 0.41
	GNX	2.56 ± 0.86^{++}	1.13 ± 0.4*
Females	OVX	0.25 ± 0.14^{+}	0.0 ± 0.0
	EB	1.69 ± 0.43	0.0 ± 0.0*
	EB + P	0.0 ± 0.0^{+}	0.0 ± 0.0

Values are averages from saline- and morphine-exposed animals in each group ($n = 16$).

* Student–Newman–Keuls post hoc test shows $P < .01$ vs. nonhandled animals of the same hormonal background.

$^{+}$ $P < .01$ vs. nonhandled, OVX, EB-treated females.

$^{++}$ $P < .001$ vs. intact males.

40.34, $P < .0001$] and the number of boli dropped during the test [$F(1,140) = 8.66$, $P < .01$] in handled animals. Further, there was an interaction between handling and sex/hormonal condition in line crossing [$F(4,140) = 6.46$, $P < .0001$], in the number of squares visited alongside the walls of the open field [$F(4,140) = 14.58$, $P < .0001$], and in the number of boli dropped during the test [$F(4,140) = 2.45$, $P < .05$]. Postnatal handling increased the number of lines crossed in saline- and morphine-exposed, gonadally intact males and in all groups of females, but did not change it in GNX males (Fig. 1). Handled, OVX, EB-treated and OVX, EB- and P-treated females spent less time alongside the walls of the open field than nonhandled animals of the same hormonal treatment (Fig. 2). Further, handled GNX males and OVX, EB-treated females dropped less boli than nonhandled animals in the same gonadal hormone condition (Table 1).

There were no differences between prenatally saline- and morphine-exposed animals in any measures regardless of sex or gonadal hormone manipulation. When we compared sexes and adult hormonal background, there were significant main effects on line crossing [$F(4,140) = 9.04$, $P < .0001$], rearing [$F(4,140) = 17.22$, $P < .0001$], grooming [$F(4,140) = 7.68$, $P < .0001$], the number of squares visited alongside the walls [$F(4,140) = 27.17$, $P < .0001$], and the number of boli dropped during the open-field testing [$F(4,140) = 8.02$, $P < .0001$]. In general, males were less active, visited more squares alongside the walls, and dropped more boli than females, regardless of the handling, prenatal drug exposure, or the presence or absence of gonadal hormone. Intact males were considered less active than GNX males, because they crossed fewer lines (Fig. 1) and displayed less rearing (Table 2) regardless of postnatal handling.

Table 2
Effect of postnatal handling on the number of rearing during the open field in adult male and female rats

Sex	Hormonal background	Nonhandled	Handled*
Males	Intact	7.56± 0.78	12.87 ± 1.84
	GNX^{++}	18.69 ± 2.22	23.06 ± 2.18
Females	OVX	16.94 ± 1.67	24.06 ± 1.39
	EB	15.31 ± 1.18	21.88 ± 1.43
	EB+P^{+}	20.88 ± 1.76	25.38 ± 1.8

Values are averages from saline- and morphine-exposed animals in each group ($n = 16$).

* Student–Newman–Keuls post hoc test shows $P < .0001$ main effect of early postnatal handling.

$^{+}$ $P < .01$ vs. OVX, EB-treated females.

$^{++}$ $P < .0001$ vs. intact males.

Table 3
Effect of postnatal handling on the number of grooming during the open field in adult male and female rats

Sex	Hormonal background	Nonhandled	Handled
Males	Intact	0.88 ± 0.26	0.73 ± 0.27
	GNX	0.69 ± 0.27	0.5 ± 0.16
Females	OVX	1.0 ± 0.24	0.63 ± 0.2
	EB^{+}	2.44 ± 0.49	2.75 ± 0.8
	EB+P^{++}	3.56 ± 0.94	4.75 ± 2.01

Values are averages from saline- and morphine-exposed animals in each group ($n = 16$).

$^{+}$ Student–Newman–Keuls post hoc test shows $P < .05$.

$^{++}$ $P < .0001$ vs. OVX females.

In OVX females, there was a decrease in rearing (Table 2) when compared to OVX, EB- and P-treated females and in grooming (Table 3) when compared to OVX, EB- and OVX, EB- and P-treated females in both, the handled and nonhandled groups. Further, anxiety-like behaviors, such as the number of squares visited alongside the walls (Fig. 2) and the number of dropped boli (Table 1), were increased in nonhandled, OVX, EB-treated females when compared to OVX or OVX, EB- and P-treated, nonhandled females. Nonhandled OVX females after EB and P treatment crossed more lines during the open-field testing when compared to OVX or OVX, EB-treated, nonhandled females (Fig. 1).

4. Discussion

Our data demonstrate that prenatal morphine exposure does not alter early postnatal handling-induced behavioral changes in the open field of adult male and female rats. Because there are no studies examining the effects of opiates on open-field activities after early postnatal handling, no comparisons can be made between the work of others and the results of the present study. However, the present work shows novel findings that the effect of early postnatal handling may be influenced by adult gonadal hormones in male and female rats. Because all previous behavioral studies tested the effect of postnatal handling only in intact males (Fernandez-Teruel et al., 1990; Meerlo et al., 1999; Vallee et al., 1997; Wakshlak and Weinstock, 1990; Weizman et al., 1999) or intact females without considering the stages of estrus cycles (Wakshlak and Weinstock, 1990; Weizman et al., 1999), our data stands alone regarding gonadectomy and/or hormone replacement.

The present data in adult, intact male rats are in agreement with others showing that early postnatal handling increases locomotor activity in the open field in males (Meerlo et al., 1999; Vallee et al., 1997; Weizman et al.,

1999). However, unlike others (Fernandez-Teruel et al., 1990; Meerlo et al., 1999; Vallee et al., 1997; Wakshlak and Weinstock, 1990; Weizman et al., 1999) demonstrating decrease in anxiety-like behaviors such as boli dropping and the amount of time spent in the corners of the open field, or on the closed arms of the elevated plus-maze, the present study shows no such effects in adult, intact male rats. One possible explanation for these differences may be that we evaluated anxiety-like behaviors as a percentage of squares visited alongside the walls and not as the time spent in the corners. Another possibility is that in the present study, the animals were repeatedly exposed to maternal injections of either saline or morphine during gestation. Prenatal exposure to saline injection may have also acted as a prenatal stressor, which could have induced long-lasting changes in behaviors (Peters, 1986; Šlamberová et al., in review).

In female rats, the present study shows that handling increases locomotion regardless of their hormonal background and decreases anxiety-like behavior in OVX females with gonadal hormone replacement. This agrees with data of Wakshlak and Weinstock (1990) and Weizman et al. (1999) showing an increase in locomotor and a decrease in anxiety-like behaviors in intact female rats. Thus, in the present study, OVX females with hormone replacements show similar behavioral effects like intact females in the work of others (Wakshlak and Weinstock, 1990; Weizman et al., 1999). Interestingly, early postnatal handling does not alter anxiety-like behaviors of OVX females in the present study. Moreover, there is a decrease in the anxiety-like behavior of OVX females when compared to OVX females with hormone replacements. Thus, it seems that the presence or the absence of female gonadal hormones elicits or eliminates differences in anxiety-like behaviors between handled and nonhandled female rats. Unfortunately, there are no other studies investigating changes in adult behaviors after early postnatal handling in OVX and OVX, gonadal hormone-replaced females.

However, we can compare the effects of female gonadal hormones in nonhandled females in the present study with others showing conflicting reports about the effect of female gonadal hormones on open-field behaviors. Consistent with the present study, Luine et al. (1998) demonstrated that administration of estrogen increases locomotor activity of rats in the open field. In contrast, Palermo-Neto and Dorce (1990) showed that repeated injection of estrogen alone or in combination with P decreases locomotor activities in the open field. Anxiety-like behavior in females also changes in response to gonadal hormone fluctuation or replacement. Mora et al. (1996) demonstrated that in intact female rats during proestrus and estrus, when estrogen levels are high (Turner and Bagnara, 1976), they visit more often the open arms of an elevated plus maze than during other phases of the estrous cycle. Additionally, P injection increases the number of entries into the open arms of the elevated plus maze (Mora et al., 1996). In the present study, nonhandled OVX, EB-treated females display more anxiety-like behavior (visited more squares alongside the walls and dropped more boli) than nonhandled, OVX or OVX, EB- and P-treated females. These differences as a function of gonadal hormones on female behavior may be due to different doses or routes of hormone administration in OVX females. It is, however, still evident that gonadal hormones affect locomotor and anxiety-like behaviors in adult female rats. Additionally, our data, like others (Alonso et al., 1991; Brotto et al., 2000; Fitch and Denenberg, 1998), demonstrate that females are generally more active and less anxious than males in the open field.

In contrast, after postnatal handling there is no such hormone effect on open-field activities. Thus, it seems that handling annuls the effect of gonadal hormones on locomotor and anxiety-like behaviors in nonhandled animals. It was shown that early postnatal handling during lactation reduces emotional reactivity and the responses of HPA axis to stress (Fernandez-Teruel et al., 1992; Meaney et al., 1991; Nunez et al., 1996; Plotsky and Meaney, 1993; Vallee et al., 1997, 1999) and decreases corticosterone and ACTH plasma levels (Ader and Grota, 1973; Hess et al., 1969; Levine, 1957, 1962; Meaney and Aitken, 1985; Meaney et al., 1989, 1991; Zarrow et al., 1972). In addition, Viau and Meaney (1991) demonstrated that estrogen increases the level of ACTH and corticosterone in adult, OVX female rats. Moreover, the HPA and hypothalamic–pituitary–gonadal (HPG) axes are functionally connected (Rivier and Vale, 1985). It is then likely that in the present study, the effect of postnatal handling, which annuls the effect of gonadal hormones on locomotion and anxiety-like behavior, might be due to alterations in the functional relationship between the HPA and HPG axes.

Based on the results of the present and our previous studies, it is difficult to ascertain the underlying mechanism(s) by which the functional interaction of HPA and HPG axes is disturbed. As mentioned above, we demonstrated that prenatal saline, as well as morphine injections, may have acted as prenatal stressor and have the same long-term effects on stress responses during adulthood (Šlamberová et al., in review). Similarly, in the present study, there are no differences between saline- and morphine-exposed animals when the effect of postnatal handling, which is known to influence the HPA axis (Fernandez-Teruel et al., 1992; Meaney et al., 1991; Nunez et al., 1996; Plotsky and Meaney, 1993; Vallee et al., 1997, 1999), is investigated. However, our earlier work, which investigated adult male and female sexual behaviors (Vathy and Kátay, 1992; Vathy et al., 1983, 1985), demonstrated that prenatal morphine exposure sex-dependently alters such HPG regulated behaviors in male and female rats (Vathy and Kátay, 1992; Vathy et al., 1983, 1985). Both receptive and proceptive behaviors are impaired in morphine-exposed female rats while saline-exposed females display the full expression of both receptive and proceptive responses to the mounting attempts of stimulus males (Vathy et al., 1983, 1985). Interestingly, the binding characteristics and receptor dynamics of estrogen and P are

similar in both saline- and morphine-exposed females (Vathy et al., 1985). Thus, the behavioral alterations do not correlate with altered binding characteristics of female gonadal hormones in morphine-exposed females. Based on these studies, it seems that prenatal morphine exposure does not affect the HPA axis per se, but it affects the HPG axis. On the other hand, early postnatal handling seems to influence both HPA and HPG axes. Therefore, it is possible that any disturbance during the prenatal and/or postnatal development may have long-term effects on the HPA and HPG axes. Future studies are on the way to examine functional interaction between the HPA and HPG axes in prenatally saline- and morphine-exposed rats.

In conclusion, the present study demonstrates that early postnatal handling alters locomotor and anxiety-like behaviors. These handling-induced alterations are not influenced by prenatal exposure to morphine, but they are affected by male and female gonadal hormones. These interesting results may enhance our understanding about prenatal drug exposure and early postnatal handling and, subsequently, they could facilitate investigations in maternal drugs of abuse and nurturing of offspring. More studies are required to assess the mechanism(s) by which handling during early development influences the effects of gonadal hormones on adult male and female behaviors.

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