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Early Experience Affects the Traits of Monogamy in a Sexually Dimorphic Manner

ABSTRACT: *The goal of this study was to examine the effects of early life experiences on the subsequent expression of traits characteristic of social monogamy in prairie voles (Microtus ochrogaster). During cage changes parents and their offspring were either transferred between cages in a cup (zero manipulation, MAN0) or with a gloved hand (one manipulation, MAN1). Following weaning the offspring were tested for alloparental behavior. In adulthood they were tested for the capacity to form partner preferences, behavior in an elevated plus-maze (EPM), and corticosterone levels. MAN0 males (but not females) showed lower levels of alloparental behavior than MAN1 males. MAN0 females (but not males) were less likely to form pair bonds than MAN1 females. MAN0 animals of both sexes were less exploratory in the EPM than MAN1 counterparts. These experiments support the hypothesis that behaviors used to characterize monogamy are vulnerable in a sex-specific manner to early experience. © 2007 Wiley Periodicals, Inc. Dev Psychobiol 49: 335–342, 2007.*

The effects of early experiences can result in life-long changes in behavior and neurobiology, as well as contributing to later psychopathologies in humans (Plotsky, Sanchez, & Levine, 2001; Sanchez, Ladd, & Plotsky, 2001). Major depression, as well as schizophrenia and posttraumatic stress disorder, have been linked to stressful early experience and/or trauma (Brown, Cohen, Johnson, & Smailes, 1999; Kaufman, Plotsky, Nemeroff, & Charney, 2000). Animal models of early experience or maternal separation (MS) paradigms have a long history in developmental psychobiology (Levine, 2001). In rats, daily brief separations (15 min a day during postnatal weeks 2–3) often produced decreased stress reactivity in offspring, as well as high levels of licking/grooming and

arched-back nursing (LG-ABN) in dams and their female offspring. In contrast, daily longer separations (usually 3 hr a day) produced more reactive female offspring with higher levels of corticotrophin-releasing factor (CRF) receptor binding. Following longer periods of MS females also exhibited lower levels of LG/ABN toward their own offspring (Francis, Diorio, Liu, & Meaney, 1999). Handling studies also have often included a group that received no treatment. These animals typically appeared superficially similar to animals that had experienced longer separations, displaying higher stress reactivity, and "fearfulness" [reviews in Levine, 2001, 2002].

Studies of the effects of early experience have almost exclusively concentrated on species in which females are the sole caretakers of offspring. Social "monogamy" is usually characterized by male–female pair bonds and biparental care of offspring, and alloparenting (Kleiman, 1977). Thus in monogamous or cooperatively breeding species, such as prairie voles (Carter, DeVries, & Getz, 1995; Carter & Roberts, 1997) and humans (Fuentes, 1999; Kleiman, 1977), there is not only the potential for

care from the father or older siblings to influence offspring development, but also the potential for early experience to affect subsequent parental behavior and pair bonding behavior in the offspring.

Prairie voles are socially monogamous rodents in which 80–100% of males typically display alloparental care upon first exposure to an unrelated infant (Roberts, Williams, Wang, & Carter, 1998). Adult males and females of this species also form heterosexual pair bonds, which can be demonstrated experimentally by a preference for a familiar partner over a stranger (Williams, Catania, & Carter, 1992).

The expression of both alloparental behavior and partner preferences is sensitive to neonatal exposure to either oxytocin (OT) or an oxytocin antagonist (OTA) (Bales & Carter, 2003; Bales, Pfeifer, & Carter, 2004), with OT facilitating pair bonding and OTA reducing alloparenting in males. In these studies, two kinds of controls were used: animals were either handled on the first day of life or given an injection of isotonic saline. In some cases these control groups differed (Bales & Carter, 2003), suggesting the hypothesis that early, ''stressful'' experiences, including handling of the parents or offspring, might have long-term effects on adult social behavior. Alternatively, as in studies of ''handled'' versus ''unhandled'' infant rats, it is possible that early experiences, even relatively minor ones, might—via increased parental stimulation—facilitate the expression of later social behavior. In the present study we tested the hypothesis that a relatively minor amount of manipulation in the postnatal period, that is, handling during weekly cage changing, might alter the subsequent expression of social behaviors in prairie voles. Two behaviors often used to define social monogamy (Kleiman, 1977) were measured: alloparenting in the immediate postweaning period, and the display of a partner preference in adulthood. In addition, possible changes in emotionality or sympathetic reactivity were indexed by behavior in the elevated plus-maze (EPM) and by corticosterone levels.

METHODS

Early Manipulation

Subjects were laboratory-bred male and female prairie voles (*Microtus ochrogaster*), descendants of a wild stock originally caught near Champaign, Illinois. Stock was systematically outbred. Animals were maintained on a 14 hr light: 10 hr dark cycle and allowed food (high-fiber Purina rabbit chow) and water ad libitum. Breeding pairs were maintained in large polycarbonate cages ($44 \times 22 \times 16$ cm^3) and provided with cotton for nesting material. At 21 days of age offspring were removed and housed in same-sexed sibling pairs in smaller ($27 \times 16 \times 13$ cm^3) cages.

Ten pairs of prairie voles were initially used as breeders. The first treatment [zero manipulation (MAN0) or one manipulation (MAN1)] was assigned randomly for the pair's first litter. For the next litter, each pair received the opposite treatment. MAN1 litters received a normal colony cage changing within the first 2 days of life, which involved moving the parents between cages by lifting them by the scruff of the neck with a hand covered in a thick leather glove. Pups were attached to the mother by milk-teeth and therefore were not touched (if pups were not attached, researchers waited to perform the manipulation). MAN0 litters also received a cage change; however, all animals were transferred to the new cage in a clear plastic cup. Sitting animals were scooped into the cup. If the animal was moving, the cup was maneuvered in front of the animal as it walked into it. In this manipulation, infants would be supported by the cup while being moved, rather than dangling from the mother's nipples. This procedure was repeated a week later (~7–9 days of age). When pups were too large to be attached to the mother's nipples (14–16 days of age), we transferred them in the same manner as their parents (either scooped in the cup or handled by the scruff of the neck). These three episodes constituted the entire manipulation prior to weaning at 21 days of age.

For overall proportions of male alloparental care, results presented here represent three replications in two different laboratories, two replications at the University of Maryland and one at the University of Illinois, Chicago, with a total sample size of 67 males. The replications involving the measures in addition to male alloparenting were performed on multiparous pairs from the general breeding colonies that had not been previously used in this experiment. One male and one female from each litter of one of these replications were used for behavioral testing, giving group sizes of 8–10 for most behavioral and hormonal measures. Husbandry, housing, and manipulations were identical in both sites. All studies were approved by the Animal Care and Use Committees of the respective university and complied with National Institutes of Health ethical guidelines as set forth in the Guide for Lab Animal Care.

This methodology is somewhat different from the more traditional early handling (EH) or MS paradigms (Levine, 2002; Pryce, Mohammed, & Feldon, 2002) which involve removing infants from the mother's cage for either brief (EH: 3–15 min) or longer (MS: 3 hr or more) periods repeatedly during development. However, there is a long history of use of the ''no treatment'' group (Levine, 2002), which resembles the MS groups in its tendency toward hyper-responsiveness to stress.

The MAN1/MAN0 manipulations used here refer to how the parents are transferred during cage cleaning. We chose this manipulation based on preliminary data which indicated that even this apparently small difference in husbandry practices in the first weeks of life could influence behavior in this species. Previous studies have shown long-term effects of equally small manipulations (Denenberg & Whimbey, 1963). Preliminary data also revealed that the pups in MAN1 litters (vs. pups in MAN0 litters) received significantly more pup-directed behaviors from their parents (Tyler, Michel, Bales, & Carter, 2005).

Alloparental Care Testing

Animals were weaned at 21 days of age and tested for response toward an unfamiliar, unrelated pup. All animals were weighed at weaning, and treatment groups did not differ by weight. Animals were always weaned before the birth of the next litter in their home cage, thus ensuring that previous exposure to neonates had not occurred. Test animals were introduced into an apparatus, which consisted of two cages connected by a 5 cm clear tube, and given 45 min to acclimate. Two pups (1–3 days old) were then introduced into one cage. The test animal was exposed to the pups for 10 min (methods based on Roberts et al., 1998). If the test animal showed any pup-directed aggression, the test was stopped immediately and the pups removed and treated as necessary. Aggression displayed by 21-day olds rarely results in significant injury to the infant. Measures taken in our first replications were categorized as either parental or nonparental, with those animals performing huddling and retrieving behaviors classified as parental (Bales, Pfeifer, & Carter, 2004; Bales, Kim, Lewis-Reese, & Carter, 2004). A subsequent replication (sample size = 8–10 animals per group) was further scored from videotape by an observer blind to experimental treatment on behavioral software (Behavior Tracker, www.behaviortracker.com) for huddling, nonhuddling contact (any contact with pups not covered by another category), retrievals, licking/grooming, and aggression. Data analysis was carried out by an exact χ^2-test (Sokal & Rohlf, 1981) for data expressed as proportions and by Mann–Whitney U nonparametric tests (Sokal & Rohlf, 1981) for continuous variables, due to nonnormality of the data. All significance levels were set at $p < .05$ and all tests were two-tailed unless otherwise noted.

Elevated Plus-maze

At approximately 60 days of age (as adults), the same animals underwent an EPM test. This test examines responses to nonsocial stimuli associated with a novel environment, and also has been used as a form of mild stressor (Insel, Preston, & Winslow, 1995; Ramos & Mormede, 1998). Time spent in the closed arm of the EPM is considered a measure of anxiety or fear response, due to the fact that presumably most rodents find open spaces aversive. Behavior in the EPM is responsive to both anxiolytic and anxiogenic drugs, and fear responses in the EPM have been found to be fairly resistant to environmental conditions (Ramos & Mormede, 1998). Prairie voles may find open areas less aversive than do other rodents such as meadow voles, a closely related polygynous species (Stowe, Liu, Curtis, Freeman, & Wang, 2005), but EPM behavior in prairie voles has been shown to be responsive to manipulations such as injection of vasopressin (Dharmadhikari, Lee, Roberts, & Carter, 1997).

The EPM consisted of two open and two closed, opaque arms, each 67 cm long and 5.5 cm wide (Insel et al., 1995), elevated 1 m above the floor. Each vole was placed in the neutral area in the center of the EPM and its behavior scored for 5 min using Behavior Tracker. Plus-maze activity was indexed as time spent in the open arm/(time spent in the open arm + time spent in the closed arm). Data were analyzed by mixed model ANOVAs (Littell, Milliken, Stroup, & Wolfinger, 1996) in SAS 8.0 (SAS Institute, Cary, NC). The animal's litter and parents were included as random factors in the first run, and were dropped from the analysis if nonsignificant. All significance levels were set at $p < .05$ and all tests were two-tailed unless otherwise noted.

Partner Preference Testing

One week after the EPM test, animals underwent a partner preference test as an index of pair bonding. The experimental animal was exposed to an unrelated and previously unfamiliar, randomly selected animal of the opposite sex for a 6-hr period. Cohabitation began between 9 and 10 a.m. in the morning near the onset of the light cycle. Randomly assigned male and female partners were paired in a neutral cage during this period.

Immediately following the 6 hr period of cohabitation, experimental animals were placed in a testing apparatus where they were given a choice, over a 3-hr test period, of spending time with (1) the now familiar "partner"; (2) an unfamiliar opposite-sexed animal (otherwise comparable to the partner; here called the "stranger"); or (3) in an empty, neutral cage (Williams et al., 1992). Testing was conducted in an apparatus consisting of three identical polycarbonate cages ($27 \times 16 \times 13$ cm^3) attached by Plexiglas tubes (7.5×16 cm^2). The experimental animal was free to move throughout the apparatus while the two stimulus animals were loosely tethered within their separate chambers.

Tests were recorded in time-lapse video and scored later by an observer blind to experimental condition. Tests were scored for the location of the experimental animal (alone or in the cage with the partner or the stranger), as well as the time spent in physical side-to-side contact with the partner and stranger. Total social contact was measured by the summed time spent in side-to-side contact with the partner and the stranger, while activity levels were measured by the number of entries into all cages. Data were analyzed by mixed model ANOVAs (see above). Dependent variables included time spent in side-to-side contact with the partner, time spent in side-to-side contact with the stranger, overall levels of social contact, and activity levels. In addition, data were analyzed as the proportion of time spent in side-to-side contact with the partner divided by total time spent in side-to-side contact with either the partner or the stranger. Data for most variables were transformed with the square or quad-root transformations due to nonnormality of the residuals. Variables that were nontransformable were analyzed by Mann–Whitney U test. Partner preference within groups was analyzed by paired t-tests (Sokal & Rohlf, 1981). Significance levels were set at $p < .05$ and all tests were two-tailed except where otherwise stated.

Corticosterone Levels

Approximately 1 week after the partner preference test, animals were anesthetized with a mixture of Ketamine and Xylazine and a blood sample obtained from the periorbital sinus. Blood was centrifuged to separate plasma, and diluted in assay buffer as necessary to give results reliably within the linear portion of the standard curve (1:2000). Samples were frozen at $-20°C$ until assay. Samples were run in duplicate with a commercial radioimmunoassay kit (MP Biomedical, Irvine, CA). This kit has previously been validated for use in the prairie vole

(Taymans et al., 1997). All samples were collected at the same time of day (between 10 a.m. and 12 p.m.) and within 5 min of disturbance. Cross reactivity with other steroids totals less than 1%. Intra-assay c.v.'s averaged 4.2%.

RESULTS

Alloparental Care

Male alloparental care was significantly affected by treatment, with lower proportions of MAN0 males displaying these behaviors (Fig. 1; exact χ^2, d$f = 1$, $\chi^2 = 29.16$, $p < .0001$). Several components of male alloparental care were lower in MAN0 males, including nonhuddling contact (Mann–Whitney test, $U = 52.5$, $p = .04$) and total time spent in pup-directed behavior including licking/grooming, sniffing, nonhuddling contact, and huddling (Mann–Whitney test, $U = 53.0$, $p = .046$) (Table 1). Female alloparental care, in contrast, was not affected either in overall proportions (50% parental, 50% nonparental, $n = 20$; exact χ^2, $\chi^2 = .00$, d$f = 1$, $p = 1.00$) or in any individual measures (Table 1).

Partner Preference

Males from neither MAN1 nor MAN0 groups showed a significant preference for the familiar partner. They also did not display any significant treatment differences in any of the measures examined, including side-to-side contact with the partner or stranger, total social contact, or the overall formation of a partner preference (Fig. 2). In contrast, although female MAN0 and MAN1 groups did not differ significantly in total time spent with the partner (Mann–Whitney test; $U = 95.5$; $p = .399$), stranger (Mann–Whitney test; $U = 71.0$; $p = .118$) or total contact (Mann–Whitney test; $U = 80.0$; $p = .658$), MAN0 and

MAN1 females did differ in the formation of a partner preference. While MAN1 females formed a significant preference for the familiar male (paired *t*-test; $t_8 = 3.77$; $p = .005$) MAN0 females did not (paired *t*-test; $t_7 = .5$; $p = .633$; Fig. 2) Female preferences were also analyzed as the proportion of time spent in contact with the partner, divided by total time spent in contact with either male. In this analysis, MAN1 females spent a significantly higher proportion of time in contact with their partner ($99 \pm .01\%$ of time spent with either male) than MAN0 females ($68 \pm 16.3\%$ of time spent with either male; Mann–Whitney test, $U = 55.0$, $p = .05$, one-tailed).

EPM Test and Corticosterone Levels

There was a significant effect of treatment in the EPM test (ANOVA; $F_{1,32} = 4.84$; $p = .035$; Fig. 3), with MAN0 animals spending less time in the open arms (relative to total time in the open and closed arms). There was no sex difference in the time spent in open arms relative to total time in open and closed arms (ANOVA; $F_{1,32} = .10$; $p = .75$).

Females had higher baseline corticosterone levels than males (ANOVA; $F_{1,31} = 6.85$; $p = .014$). However, treatment was not a significant predictor of baseline corticosterone levels (ANOVA; $F_{1,31} = 1.5$; $p = .230$). Female MAN0 animals averaged 905.18 ± 159.27 ng/ml and female MAN1 animals averaged 606.00 ± 178.69 ng/ml, while male MAN0 animals averaged 404.48 ± 146.78 ng/ml and male MAN1 animals averaged 337.48 ± 102.55 ng/ml.

DISCUSSION

It has generally been assumed that the capacity to develop pair bonds and alloparental behavior are among the
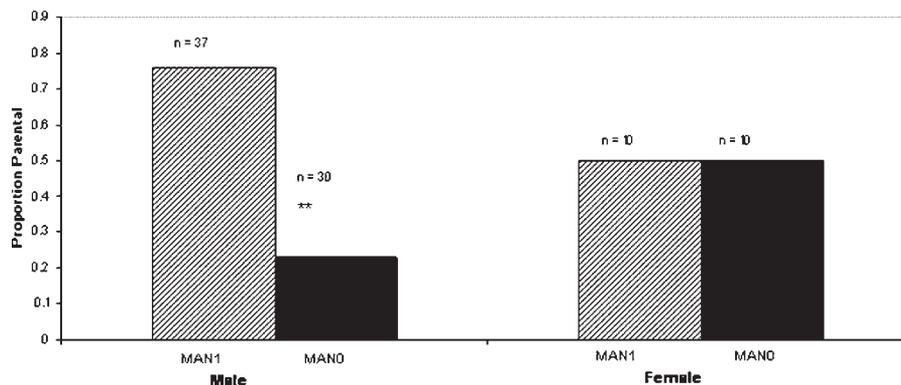

FIGURE 1   Proportion of animals that behaved parentally as a function of neonatal manipulation group ($n = 67$, $\chi^2 = 29.16$, $p < .0001$). Data for males include result of three replications in two different laboratories. Data for females are from a single experiment carried out at the University of Illinois, Chicago.

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Table 1. Means (+Standard Errors of the Mean) for Individual Behaviors in the Alloparental Care Test ($n = 10$ per Group).

Behavior	MAN0-Male	MAN1-Male	MAN0-Female	MAN1-Female
Total pup-directed behavior (s)	165.9 ± 74.8	$372.4 \pm 82.3*$	228.2 ± 74.8	209.9 ± 82.3
Huddling (s)	23.9 ± 23.9	82.6 ± 42.7	11.5 ± 11.2	23.2 ± 21.3
Nonhuddling contact (s)	49.0 ± 57.0	$171.7 \pm 48.3*$	131.4 ± 57.0	100.4 ± 48.3
Licking/grooming (s)	72.0 ± 20.1	80.1 ± 27.6	49.4 ± 20.1	66.4 ± 27.6
Retrievals (frequency)	3.7 ± 2.0	$1.6 \pm .7$	$.7 \pm .5$	$1.2 \pm .9$

*Significantly different from MAN0 male values at $p < .05$ when analyzed by Mann–Whitney U test.

defining features of species that are socially monogamous (Carter et al., 1995; Kleiman, 1977). However, data from the present study provide strong evidence that both alloparental care and the formation of partner preferences in later life can be affected by apparently minor changes in animal husbandry in the postnatal period.

For example, in the present study male MAN0 voles showed reduced levels of alloparental care in comparison to MAN1 males. Alloparental behavior in females did not differ as a function of these manipulations. MAN1, but not MAN0, females showed a partner preference. MAN0 males also did not exhibit partner preferences (however, neither did MAN1 males). The 6-hr period of cohabitation used in the present study was a compromise selected to allow comparisons between males and females. However, because males normally require longer to form significant partner preferences, the parameters used here did not differentiate between treatment groups in males. Whether partner preferences in males could be influenced by different periods of cohabitation remains to be determined.

The failure of females to show a partner preference is especially striking since the cohabitation period used here was six times longer than the duration normally required for pair bonding by females living under our current colony conditions. In this laboratory (as in many others) animals are routinely transferred between cages by a gloved hand, as in the MAN1 group here. Whether the apparent failure of MAN0 females to form a partner preference will continue to be seen after even longer periods of cohabitation also remains to be determined.

The long-term fitness consequences of alloparenting experience for the alloparents themselves have not been defined in prairie voles, although this experience is critical to normal parental behavior in many cooperatively breeding species (Tardif, 1997). Therefore, we might predict that this altered alloparenting experience could reduce the long-term prospects for normal parenting behavior in MAN0 males. It is also not known whether or not impaired pair bonding can affect fertility. However, preliminary data from this laboratory (Stone, Carter, and Bales, unpublished data) suggest that early MAN0 manipulations may significantly delay first reproduction in both males and females, perhaps through altered sexual behavior, partner preference formation, or both.
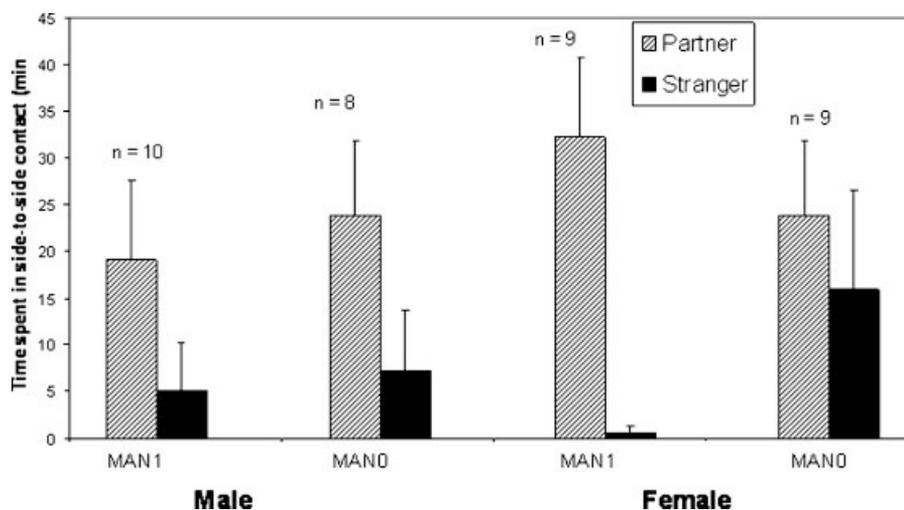

FIGURE 2   Time spent in side-to-side contact (min) by adult females and males as a function of neonatal manipulation group. MAN1 females showed a preference for the familiar male (paired t-test; $t_8 = 3.77$; $p = .005$), but MAN0 females did not (paired t-test; $t_7 = .5$; $p = .633$). Males did not differ by treatment group.
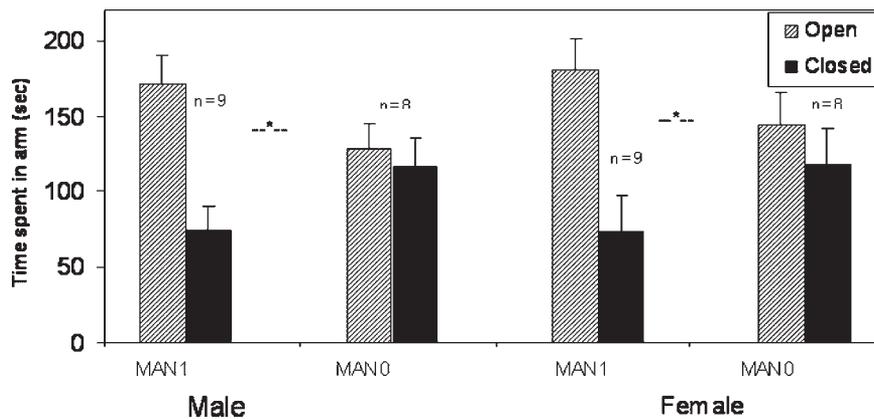
FIGURE 3 Time spent in the open or closed arms of the elevated plus-maze according to neonatal handling treatment and sex. Both MAN0 males and females spend proportionally higher amounts of time in the closed arms than in the open arms, therefore appearing more "anxious" ($F_{1,32} = 4.84$; $p = .035$).

Based on the larger literature in this field (Levine, 2001; Levine, 2002; Meaney, 2001), we hypothesize that the observed group differences are due at least in part to differential parental stimulation, although the present study did not directly test this hypothesis. It is equally possible that some aspect of the handling experience itself (such as dangling unsupported from the mother, as in MAN1, or being transferred while supported by a cup, as in MAN0) could result in long-term changes in behavior (Denenberg, 1999; Tang, 2001; Tang, Akers, Reeb, Romeo, & McEwen, 2006). The present study did not involve either separation from the parents or deliberate stimulation of pups, beyond that elicited by our differential cage transfer procedures. Differences such as those seen here might occur during routine animal care, and could inadvertently contribute to individual and sex-specific variations in later behavior or physiology.

Sex differences in reactivity to unfamiliar or stressful stimuli are a common finding in rodent research, even in socially monogamous species such as prairie voles in which behavior and anatomy show comparatively little sexual dimorphism (Carter, 2003; Carter et al., 1995; Kleiman, 1977; Carter et al., 1997). The data shown here are consistent with the more general notion that stimuli from mammalian infants are potentially fear-inducing and that timidity must be overcome to allow the expression of infant-directed caregiving (Fleming & Leubke, 1981). These findings are also consistent with the previous research in rats suggesting that "no treatment" or reduced stimulation in early life is associated later with increased anxiety and hyper-responsivity to challenges later in life (Levine, 2002). Corticosterone levels did not differ significantly between groups in a single measurement taken at the end of our study. However, behavioral responses in the EPM revealed that MAN0 animals of both

sexes were less exploratory and possibly more "anxious" than MAN1 animals. These results, taken in conjunction with our other behavioral findings, suggest that reactions to social versus nonsocial experiences may rely on different mechanisms.

The effects of neonatal handling manipulations on the HPA axis have been shown to be sexually dimorphic in some rat studies (Papaioannou, Gerozissis, Prokopiou, Bolaris, & Stylianopoulou, 2002; Park, Hoang, Belluzi, & Leslie, 2003). Corticosterone levels were not significantly different as a function of treatment in the present study. However, these samples were collected without any deliberate attempt to challenge these animals. In a preliminary experiment we found that corticosterone levels in MAN0 males were slower than those in MAN1 males to return to baseline after a swim stressor (Kramer, Bales, and Carter, unpublished data), but this phenomenon requires further study.

In summary, the neonatal period is characterized by behavioral and neuroendocrine plasticity, and experiences during this period may have epigenetic, although not necessarily permanent, consequences (Weaver, Diorio, Seckl, Szyf, & Meaney, 2004; Weaver, Meaney, & Szyf, 2006). The results of the present study reveal that even traits that have been used to define species-typical social systems, such as the presence of alloparenting and the capacity to form pair bonds, can be influenced by apparently minor changes in early experience. The effects of early experience also differed between males and females; however, these sex differences were not mediated by basal stress hormones or anxiety. These and related findings in other species may also offer insights into the mechanisms underlying individual or sex differences in the vulnerability to psychopathologies (Carter, 2006; Plotsky et al., 2001; Sanchez et al., 2001)

as well as differential responsiveness to therapeutic treatments (Nemeroff et al., 2003).

NOTES

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