Postnatal Environment Affects Behavior of Adult Transgenic Mice

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Behavioral phenotypes of knockout mice are often interpreted as the effects of the absence of the gene product on adult behavior, yet behavioral differences among genotypes may be exaggerated or blurred by the postnatal environment. For example, mice develop in litters of varying sex ratios and genotypes, and it is possible that some of these behavioral differences may result from the composition of the litter. To determine whether these factors might play a role in the development of the behavioral characteristics that have become diagnostic of the knockout, offspring of parents heterozygous for a null mutation of estrogen receptor α (ERKO) were sexed and genotyped within 2 days of birth. Litters were then reconstituted, forming same-sex litters of equal numbers of ERKO and wild-type (WT) individuals that were tested in a standard resident-intruder paradigm. In this manner the effect of genotype would be evident without the potential confound of the presence of the opposite sex in the litter. Behavioral differences between the genotypes were more sharply defined than reported previously. ERKO females displayed only aggressive behavior whereas their WT littermates displayed only mounting behavior; both aggression and mounting behavior were greatly reduced in ERKO males. These data suggest that the postnatal environment such as litter composition may influence the development of sociosexual behaviors in ERKO mice. Exp Biol Med 229:935–939, 2004

Key words: maternal care; sex differences; genotype differences; mounting; aggression

Introduction

Mice with targeted deletions of the genome often exhibit behavioral deficits or exaggerations (see 1–4 for reviews). For example, both wild-type (WT) and estrogen receptor α knockout (ERKO) female mice exhibit male-typical mounting behaviors toward female intruders, but ERKO females are much more aggressive toward female intruders than their WT counterparts (5–8). This increased aggression is correlated with differences in androgen receptor (AR) in the brain and in circulating concentrations of androgens, with ERKO females having higher testosterone concentrations (7, 9) compared with WT and heterozygote (HTZ) females. Estrogen receptor α knockout male mice also exhibit significantly higher circulating levels of testosterone relative to WT males (7, 10, 11) and, relative to WT males, have comparable abundance of AR (12) and will also mount intruders, yet they do not display aggressive behavior toward male opponents (13, 14).

Although it is recognized that such alterations in behavior in knockout organisms may result from developmental effects of the mutation (1, 15), the possibility that other factors may also modulate subsequent expression of behavior in knockout mice has received little attention (16, 17). It is well known that in rodents both the prenatal (intrauterine) environment (18) and the postnatal (the composition of the litter and the behavior of the mother) environment (19, 20) influence adult sociosexual behavior. Previous studies of litter effects in mice have tended to focus on how litter composition affects the behavior of the mother (e.g., Ref. 21), documenting differences in behavior of individuals reared in isolation versus single-sex groups (e.g., Ref. 22), or demonstrating how the timing of sexual maturation in females (e.g., Ref. 23) or the incidence of social versus solitary play (e.g., Ref. 24) is influenced by raising individuals in litters of varying sex ratios. Other studies have demonstrated how stress reactivity and sociosexual behavior in the adult and their associated neuroendocrine mechanisms are influenced by the maternal behavior directed toward the infant (e.g., Refs. 19, 25, 26).

Because litters vary in their sex ratio and, in genetically manipulated litters created by heterozygote (HTZ) crossing, also in the representation of genotypes, both factors must be considered as potentially important contributors to an individual's behavioral development. In the present experi-

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ment we sought to define litter composition by creating litters of the same sex but with equal numbers of wild-type and knockout individuals. Hence, litters were reconstituted shortly after birth so that they consisted of a single gender but with a balanced genotype ratio. This enabled assessment of each factor without the confound of the other in shaping aggressive and mounting behavior in adulthood.

Materials and Methods

Mice. Mice were produced by mating male and nulliparous female mice, each heterozygous for a functional estrogen receptor α (ERα) gene obtained from the breeding colony maintained at the Rockefeller University. Two or three females were housed with a male until visibly pregnant (3–5 days before the day of delivery based on *post hoc* analyses), when they were singly housed. Original breeding pairs (mixed background of C57BL/6J and 129) were obtained from the National Institute of Environmental Health Sciences (27). Animal rooms were maintained on a 12:12-hr light:dark cycle at constant temperature (22°C). Food (PicoLab Rodent Diet 20, Oakville, Ontario) and water were available *ad libitum*. Experimental protocols adhered to institutional guidelines and the National Institutes of Health guidelines for the use of animals in research (IACUC approval number 01075). The litters used in the study were randomly selected. Maternal behavior did not affect the selection of the litters.

In four replicates, experimental animals were sexed and genotyped within 2 days of birth. Records were kept of the sex ratio of the birth litter to determine the relative contribution each of the behavioral phenotypes had. During this period each pup received individual identification marks on the body by a Sharpie permanent marker, a procedure that was repeated each day. After genotyping, individuals were identified with individually specific toe clips for permanent identification. In all instances HTZ females that had contributed young to the study served as foster mothers; in only one instance was a pup fostered to its own dam. Littermates were separated in a systematic manner. Pups were reared in litters of six; all individuals survived to weaning. As in previous studies in this laboratory with these animals, individuals continued to be group-housed with littermates following weaning of the same sex but, unlike in previous studies, individuals were housed according to genotype. Two weeks before behavior testing, all animals were individually housed.

Reconstitution of Litters. The full set of offspring from 12 litters were reassigned to 12 unisexual litters with foster mothers (five all-male and seven all-female litters) each consisting of equal numbers of ERKO and WT individuals. As adults WT and ERKO individuals were chosen at random from each of the litters and tested.

Resident-Intruder Test. To determine whether litter composition influences sociosexual behavior in adulthood, 6 WT and 6 ERKO female mice and 8 WT and 11 ERKO male mice of the reconstituted litters were tested in a resident-intruder paradigm as in previous studies from this laboratory. All individuals were gonadally intact; the stage of estrous cycle at the time of testing was not determined for experimental females since this has been ruled out in previous studies in this laboratory to be an influential factor. Behavior tests lasted for 15 mins during the dark phase (4–8 hrs after lights off) under red light. Depending upon the sex of the resident animal, group-housed female or male Swiss-Webster mice were used as intruders to examine the levels of both within-sex aggression and sexual behavior. Females were tested with ovariectomized female intruders to avoid confounding effects of the endocrine status of the opponent. Males were tested with male intruders that were gonadally intact but had had their olfactory bulbs removed (OBX intruders). Expression of aggression in mice is mainly regulated by olfactory cues, and OBX intruders rarely show aggression. However, since their gonads were intact, OBX intruders can elicit aggressive behaviors from resident mice. By testing with OBX intruder mice, aggressive behaviors of resident animals, which were not influenced by any experience of defeat, were therefore measured.

Two categories of behaviors were quantified. An aggressive bout was defined as a continuous series of behavioral interactions, including at least one aggressive behavioral act (see below). Three seconds was the maximum amount of time that could elapse between aggressive behavioral acts to be considered part of the same aggressive bout. If more than 3 secs elapsed between two behavioral aggressive acts, the two behavioral acts were scored as two separate aggressive bouts. Aggressive behavior acts consisted of tail rattling, chasing, boxing, biting, lunge, offensive attack (often accompanied by biting), and wrestling. The number of animals that showed any aggressive behaviors, the latency to the first aggressive act (900 secs for nonresponders), and the number and cumulative duration of any aggressive behavior bouts was recorded in each test. In addition, male-typical mounting behavior and intromission patterns toward the intruder by resident male or female mice were also recorded, as well as the latency to first mount (900 secs for nonresponders) and the number and cumulative duration of male-typical mounting behavior. Females were tested at 28–30 weeks of age; males were tested at 26–29 weeks of age. Testing was terminated if wounding or bleeding occurred.

Statistics. Behavioral data were analyzed after they were log-transformed to normalize the distributions and remove nonhomogeneity. Differences in the number of individuals responding in the tests for aggressive behavior and sexual behavior were calculated using a chi-square test. Genotype differences in latency, frequency, and duration measures were analyzed by *t*-test (one-tailed because of predictions) in each sex.

Results

Aggressive and Mounting Behavior in Females. All of the ERKO females were aggressive toward the female intruder, while none of the WT females displayed aggressive behavior (likelihood ratio chi-square test, $P < 0.001$). None of the ERKO (0/6) females exhibited male-typical mounting behavior toward female intruders, whereas the WT (3/6) females mounted the female intruder (likelihood ratio chi-square test, $P < 0.05$).

There was a significant difference between ERKO and WT females in the latency [$t(10) = 2.93$, $P = 0.007$], frequency [$t(10) = 2.93$, $P = 0.007$], and duration [$t(10) = 2.98$, $P = 0.006$] of aggressive behavior to a female intruder (Fig. 1). There were also significant differences between ERKO and WT females in the display of male-typical mounting behaviors in latency [$t(10) = 2.08$, $P = 0.03$], frequency [$t(10) = 2.21$, $P = 0.03$], and duration [$t(10) = 2.23$, $P = 0.03$] measurements (Fig. 1).

Aggressive and Mounting Behavior in Males. - Most of the WT male mice (6/8) responded aggressively to a male intruder, while only 1 of 11 of the ERKO males responded aggressively to the male intruder (likelihood ratio chi-square test, $P < 0.005$). More WT males (6/8) displayed mounting behavior toward to the intruder compared with the ERKO males (3/11) (likelihood ratio chi-square test, $P < 0.05$).

There was a significant difference between ERKO and WT males in the latency [$t(17) = 3.18$, $P = 0.002$], frequency [$t(17) = 4.17$, $P = 0.0003$], and duration [$t(17) = 4.81$, $P = 0.0001$] of aggressive behavior toward a male intruder (Fig. 1). There were also significant differences between ERKO and WT males in the frequency [$t(17) = 2.03$, $P = 0.03$] and duration of mounting behaviors [$t(17) = 2.12$, $P = 0.02$], but not in the latency to first display (Fig. 1).

Discussion

In all previously reported studies using ERKO mice, individuals were raised in natural litters having a mixture of sexes and genotypes. In such instances, both ERKO and WT females exhibit mounting and aggressive behaviors toward female intruders. While the ERKO female is more aggressive than WT female littermates, both genotypes mount female intruders equally. However, the prenatal and postnatal environments could exaggerate or blur the behavioral differences that characterize knockout mice. We find that when males are removed from the litter and ERKO and WT females are raised together, the behavioral differences become absolute; ERKO females are only aggressive toward female intruders and fail to mount them, whereas WT females are completely nonaggressive and only mount intruders.

The individual's position in the uterine horn relative to the sex of neighboring fetuses has a significant effect on its morphology, physiology, and behavior when it reaches adulthood (18). This effect is due to the diffusion of sex steroid hormones from fetal neighbors across the fetal

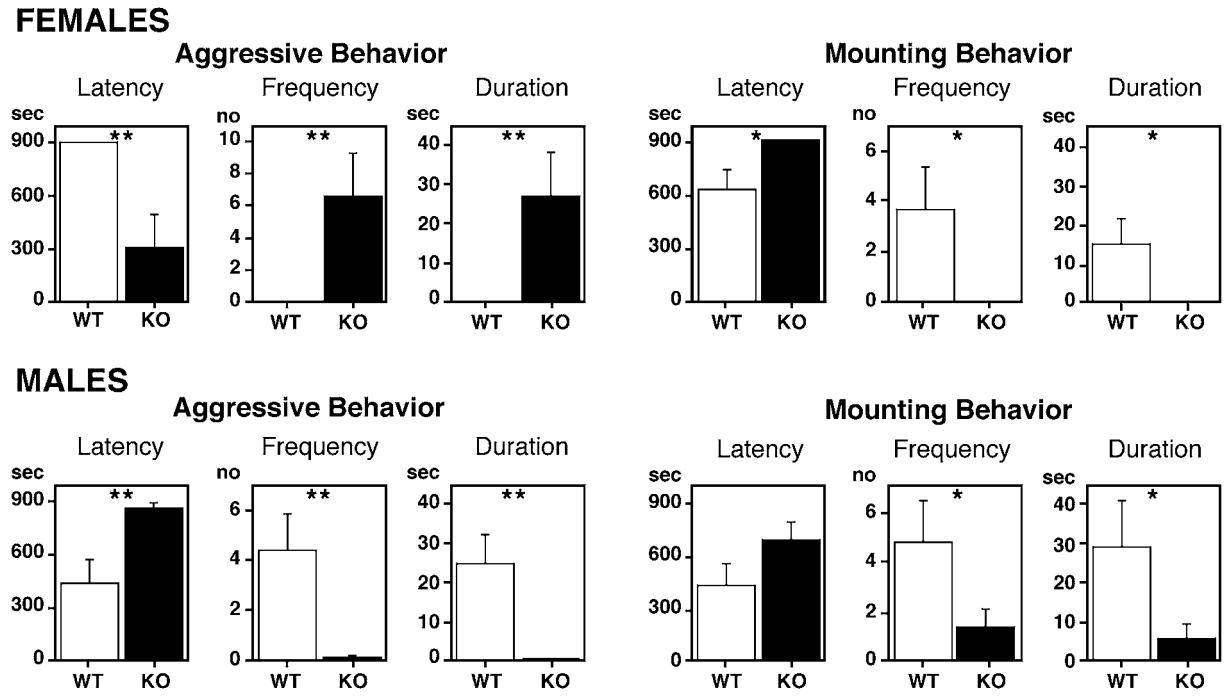

Figure 1. Frequency of aggressive and mounting behavior in genetically manipulated mice raised in single-sex groups with mixed genotypes. Tests with female mice involved ovariectomized female intruders (top panel), and tests with male mice involved olfactory-bulbectomized male intruders (bottom panel). Mice were genotyped within 2 days of birth and the litters reconstituted to contain equal numbers of wild-type (WT) or knockout (KO) male or female mice. Values are group mean and standard errors. Statistical analysis was computed on log-transformed data (* $P < 0.05$, ** $P < 0.01$).

amniotic and chorionic membranes. In the present study, potential prenatal effects were not controlled, but the sex ratio of natural litters, which reflects the number of males and hence the probability of any one fetus residing next to a male, was noted for each female. There was no evidence that sex ratio in the birth litter had a significant effect on the behaviors exhibited by the KO or WT animals in the resident-intruder tests.

Female-female mounting has been interpreted as an index of sexual arousal in some species, whereas in others it is related to social dominance (28, 29), and it is possible that the failure of ERKO females to display these behaviors indicates that they were not sexually motivated. Parallels also exist with the males. Previous experiments indicate that ERKO and WT males mount male intruders equally. However, when ERKO males are raised in all-male litters with WT males, most fail to display mounting behavior toward male intruders. Taken together, this suggests that in the absence of females (or males) in the litter, ERKO individuals fail to exhibit the normal sociosexual behavior repertoire when they reach adulthood.

While these studies do not tell us the means by which sex and genotype ratios of the litter might affect the development of adult sociosexual behaviors, they do present several intriguing possibilities. It is well known that the "society of the litter" can profoundly affect the development of adult sexuality and its underlying neural substrates in rodents. Building on a long history of research in developmental psychobiology, Meaney and colleagues have shown how the nature and amount of care a pup receives from the mother modulates the effects of the altered maternal behavior and hence the stress reactivity of the pup later in life (19). Moore and colleagues demonstrated that mother rats lick the anogenital region of male pups more than they do female pups and, further, that this difference accentuates the copulatory behavior of the pup when it reaches adulthood (25, 26). Both authors have also documented that such differences in maternal care can lead to differential development of the neural substrates that subserve behavior (see also 20, 30). It is known that circulating concentrations of androgens are elevated in ERKO females (8, 9) and, further, that the increased licking of the anogenital area of male pups is due to the presence of an androgen-dependent attractive molecule in the urine of male pups (25). While it is not known yet whether ERKO female pups also have elevated levels of androgens, it is possible that in the all-female/mixed-genotype litters the mother treats the ERKO female as if it were a male. Another possibility is that the presence of ERKO females suppresses the development of aggression in WT female littermates in all-female litters.

The majority of studies on the behavior of rodents do not control for the early social environment. While studies have demonstrated that housing male rodents in same-sex groups *following weaning* can ameliorate the effects of social isolation (31, 32), it is remarkable that there apparently has been only one study of the behavioral outcomes of animals *raised* in same-sex litters. Hard and Larsson (33) reduced natural litters of rats to a single male pup, all-male litters containing six pups, or to mixed-gender litters containing three male and three female pups; it is not clear how animals were housed after weaning. Males were then tested daily over a 25-day period as adults with sexually receptive females. One quarter of the isolated males never ejaculated, whereas all of the males raised with females did ejaculate; the cumulative percentage of males reared in all-male litters lagged that of the males raised in heterosexual litters, but by 1 week of testing all but one of the isosexually reared males were exhibiting complete sexual behavior patterns. Alteration of the sex ratio in biparental species also results in the alteration of behavior of the young in adulthood. The zebra finch is a small colonially breeding bird, in which both the male and female parent cares for the young. If males are removed from the breeding colony when nestlings are less than a week old, the female young fail to show a sexual preference for males when adult (34).

One alternative to the present interpretation is that the animals used in this study were several generations removed from earlier studies and subject to genetic drift. Another interpretation may relate to the fact that the after-weaning environment in the present study was controlled such that individuals were housed with same-genotype littermates, whereas in the earlier studies individuals were housed in mixed-genotype groups. Finally, this study is not meant to be definitive, since it does not tell us about how these effects are mediated; that is, if the mother behaves differently toward mutant individuals and, if so, whether these differential behaviors are correlated with the behavioral phenotypes exhibited. To answer this question it will be necessary to reconstitute litters having all genotypes and both sexes represented and then monitor the mother's behavior toward individual pups. For example, an expanded design would include a two-way analysis of variance with genotype composition (same vs. different) and sex ratio composition (all-female, all-male, and mixed) in a 2×3 design. Similarly, to control for genotype differences in circulating hormone levels, future studies must be conducted using gonadectomized animals given the same hormonal treatment. It will also be important in future studies to test all experimental animals with the same stimulus animals (e.g., males tested with both male and female stimulus animals). Finally, focal observations are essential to determine the nature and frequency of behaviors exhibited by the dam to pups of different genotypes.

Despite these caveats, the results obtained in this initial investigation remain of interest, namely, that genotype differences in sociosexual behavior were exaggerated when litter composition was controlled. This suggests that the litter environment may provide epigenetic influences that further shape the neural mechanisms controlling sociosexual behaviors in knockout mice in much the same way that they

influence adult behavior of wild-type individuals. Thus, in behavioral phenotyping studies of genetically manipulated animals it is important to understand and control for the potential role of the rearing environment in the development of the behavior of interest (35, 36). The present study indicates that when the behavior of interest is related to the social context, litter composition is an important consideration.

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1. Nelson RJ, Chivavegatto S. Molecular basis of aggression. Trends Neurosci 24:713–719, 2001.
2. Nelson RJ, Young KA. Behavior in mice with target disruption of single genes. Neuroscience Biobehav Rev 22:453–462, 1998.
3. Ogawa S, Korach, KS, Pfaff DW. Differential roles of two types of estrogen receptors in reproductive behavior. Curr Opin Endocrin Diabetes 9:224–229, 2002.
4. Pfaff DW, Berrettini WH, Joh TH, Maxson SC. Genetic Influences on Neural and Behavioral Functions. Boca Raton: CRC Press, 1999.
5. Ogawa S, Lubahn DB, Korach KS, Pfaff DW. Reversal of sex roles in genetic female mice with disruption of estrogen receptor gene. Neuroendocrinology 64:467–470, 1996.
6. Ogawa S, Eng T, Taylor J, Lubahn DB, Korach KS, Paff DW. Roles of estrogen receptor-α gene expression in reproduction-related behaviors in female mice. Endocrinology 139:5070–5081, 1998.
7. Rissman EF, Wersinger SR, Taylor JA, Lubahn DB. Estrogen receptor function as revealed by knockout studies: neuroendocrine and behavioral aspects. Horm Behav 31:232–243, 1997.
8. Rissman EF, Wersinger SR, Fugger HN, Foster TC. Sex with knockout models: behavioral studies of estrogen receptor alpha. Brain Res 835:80–90, 1999.
9. Lindzey J, Korach KS. Developmental and physiological effects of estrogen receptor gene disruption in mice. Trends Endocrin Metab 8:137–145, 1997.
10. Couse JF, Korach KS. Estrogen receptor null mice: what have we learned and where will they lead us? Endocrine Rev 20:358–417, 1999.
11. Lindzey J, Wetsel WC, Couse JF, Storker T, Cooper R, Korach KS. Effects of castration on chronic steroid treatments on hypothalamic gonadotropin-releasing hormone content and pituitary gonadotropins and estrogen in male wild-type and estrogen receptor-α knockout mice. Endocrinology 139:4092–4101, 1998.
12. Wersinger SR, Sannen K, Villalba C, Lubahn DB, Rissman EF, DeVries GJ. Masculine sexual behavior is disrupted in male and female mice lacking a functional estrogen receptor alpha gene. Horm Behav 32:176–183, 1997.
13. Ogawa S, Lubahn DB, Korach KS, Pfaff DW. Behavioral effects of estrogen receptor gene disruption in male mice. Proc Natl Acad Sci U S A 94:1476–1481, 1997.
14. Ogawa S, Washburn TF, Taylor J, Lubahn DB, Korach KS, Pfaff DW. Modifications of testosterone-dependent behaviors by estrogen receptor-α gene disruption in male mice. Endocrinology 139:5058–5069, 1998.
15. Ogawa S, Pfaff DW. Genetic contributions to the sexual differentiation of behavior. In: Matsumoto A, Ed. Sexual Differentiation of the Brain. Boca Raton, FL: CRC Press, p11, 1999.
16. Gingrich JA, Hen R. The broken mouse: the role of development, plasticity and environment in the interpretation of phenotypic changes in knockout mice. Current Opin Neurobiol 10:146–152, 2000.
17. Miczek KA, Maxson SC, Fish EW, Faccidomo S. Aggressive behavioral phenotypes in mice. Behav Brain Res 125:167–181, 2001.
18. Ryan BC, Vandenbergh JG. Intrauterine position effects. Neurosci Biobehav Rev 26:665–678, 2002.
19. Meaney MJ. Maternal care, gene expression and the transmission of individual differences in stress reactivity across generations. Ann Rev Neurosci 24:161–192, 2001.
20. Fleming AS, Kraemer GW, Gonzalez A, Lovic V, Shah A, Rees S, Melo A. Mothering begets mothering. Physiol Pharm Behav 73:61–75, 2002.
21. Alleva E, Caprioli A, Laviola G. Litter gender composition affects maternal behavior of the primiparous mouse dam (*Mus musculus*). J Comp Psychol 103:83–87, 1989.
22. Namikas J, Wehmer F. Gender composition of the litter affects behavior of male mice. Behav Biol 23:219–224, 1978.
23. Drickamer LC. Effect of size and sex ratio of litter on the sexual maturation of female mice. J Reprod Fertil 46:369–374, 1976.
24. Laviola G, Alleva E. Sibling effects on the behavior of infant mouse litters (*Mus domesticus*). J Comp Psychol 109:68–75, 1995.
25. Moore CL. Maternal contributions to mammalian reproductive development and divergence of males and females. In: Slater PJP, Rosenblatt JS, Snowdon CT, Milinski M, Eds. Advances in the Study of Behavior. New York: Academic Press, pp47–118, 1995.
26. Moore CL, Wong L, Daum MC, Leclair OU. Mother-infant interactions in two strains of rats: implications for dissociating mechanism and function of a maternal pattern. Devel Psychobiol 30:301–312, 1997.
27. Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O. Alternation of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci U S A 90:11162–11166, 1993.
28. Beach FA. Sexual attractivity, proceptivity, and receptivity in female mammals. Horm Behav 7:105–138, 1976.
29. Dagg AI. Homosexual behavior and female-male mounting in mammals. Mamm Rev 14:155–185, 1984.
30. Fleming AS, O'Day D, Kraemer G. Neurobiology of mother-infant interactions: experience and central nervous system plasticity across development. Neurosci Biobehav Rev 23:673–685, 1999.
31. Bakker J, van Ophemert J, Slob AK. Postweaning housing conditions and partner preference and sexual behavior of neonatally ATD-treated male rats. Psychoneuroendocrinol 20:299–310, 1995.
32. Cooke BM, Chowandadisai W, Breedlove SM. Post-weaning social isolation of male rats reduces the volume of the medial amygdala and leads to deficits in adult sexual behavior. Behav Brain Res 117:107–113, 2000.
33. Hard E, Larsson K. Dependence of adult mating behavior in male rats on the presence of littermates in infancy. Brain, Behav Evol 1:405–419, 1968.
34. Adkins-Regan E. Development of sexual partner preference in the zebra finch: a socially monogamous, pair-bonding animal. Archives Sexual Behav 31:27–33, 2002.
35. Smotherman WP, Robinson SR. Caveats in the study of perinatal behavioral development: utility of fetal study. Neurosci Biobehav Rev 18:347–354, 1994.
36. Francis DD, Szegda K, Cambell G, Martin WD, Insell TR. Epigenetic sources of behavioral differences in mice. Nature Neurosci 6:445–446, 2003.