Paternal social enrichment effects on maternal behavior and offspring growth

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Paternal environmental experiences are significant predictors of developmental outcomes in offspring and can occur even in the absence of paternal care. Although there has been a recent focus on the role of environmentally induced changes in the male germline in producing these effects, the potential mediating role of mothers has not been investigated. A role for mothers in the transmission of paternal effects has been well acknowledged in behavioral ecology, which predicts that females will dynamically adjust their reproductive investment in response to the qualities of their mate. In the present study, we show that a lifetime of socially enriched compared with impoverished housing conditions shifts anxiety-like behavior and gene expression of male mice. Females that mate with enriched-reared males exhibit increased levels of pup nursing and licking toward their offspring, which are associated with changes in gene expression within the maternal hypothalamus. Significantly, these changes in maternal behavior are correlated with the general levels of anxiety exhibited by their male mates. Further, we show that paternal environmental enrichment results in increased growth of their offspring. These results suggest that maternal–paternal interactions at mating may guide offspring development, with significant implications for the transgenerational transmission of paternal environmental experiences.

differential allocation | fitness | maternal care | parental effects

Environmental factors (e.g., drugs, diet, stress) experienced by parents can alter the development of future generations. The study of parental influences, in both epidemiological and laboratory contexts, suggests diverse pathways through which these developmental effects can be achieved. In the matriline, environmental effects that induce changes in offspring can occur as a result of factors in utero and/or via postnatal maternal care (1–3). In contrast, little is known about the biological pathways through which the experiences of fathers influence offspring phenotype. Studies of paternal effects have typically focused on species in which biparental or exclusively paternal care is observed (e.g., prairie voles, *Peromyscus californicus*). In these species, paternal care may induce changes in offspring development via similar mechanisms as postnatal maternal care. However, few mammalian species (3–5%) provide paternal care, and paternal effects can occur in the absence of paternal care (4). The underlying mechanisms of these paternal effects are perplexing in nonmonogamous mammalian species because the biological information transmitted during fertilization is thought to be limited to paternal DNA.

Despite the seemingly limited paternal contribution to offspring development, there is evidence in humans that paternal age, nutrition, drug use, and stress are all predictors for the development of psychopathology and health risks in offspring (5–7). Importantly, some of these effects seem to arise preconceptually. Evidence in support of paternal effects, even in the absence of paternal care, raises important questions regarding the mechanisms driving these effects. One possibility is that the experiences of fathers become epigenetically encoded or imprinted into the germline (e.g., via DNA methylation, chromatin modifications, or RNA changes in sperm), are maintained after fertilization, and are directly inherited by future generations. This phenomenon has been reported to occur after in utero endocrine disruptor exposure (8), postnatal stress (9), alcohol exposure (10), and dietary changes (11). Many of the reported changes occur at genes within molecular pathways that direct and maintain large-scale changes to the epigenome across tissues, with broad implications for development. These molecular changes may play an important role in conferring ancestral epigenetic imprints across generations, although it has been argued that such strict inheritance of acquired epigenetic marks is rare and is limited to a small subset of genes (4).

An alternative explanation is that "paternal effects" could be indirectly mediated or facilitated by the mother. For example, perception of differences in the phenotypic quality of males (which may be induced by exposure to stress, drugs, or toxins) by female mates may lead to altered investment of maternal resources (during the pre- and/or postnatal period) toward offspring. Evolutionary biologists refer to this type of maternal adjustment in response to male mate quality as differential allocation (12). The differential allocation hypothesis (DAH) states that females mated with high-quality males should increase their investment in offspring—a phenomenon that has been shown to occur in several nonmammalian species (12–15). Thus, differential allocation may be an important phenomenon to consider when determining the pathways linking paternal experience/condition to offspring phenotype.

Although mechanistic studies exploring the origins of paternal effects are currently focused on the role of germline epigenetic variation (4), with limited focus on the mediating role of maternal influences, we suggest that the complex interactions between maternal and paternal effects make it difficult to separate the unique contributions of each parent. Integrating the theoretical and empirical perspectives provided by evolutionary biologists, here we investigate the influence of paternal experiences on maternal neurobiology and behavioral investment in offspring and the implications of these paternal effects for offspring fitness in inbred laboratory mice. We manipulated the social environment experienced by males during postnatal and postweaning development (social isolation vs. social enrichment) to shift male behavioral and neurobiological phenotypes to extremes. We then measured the extent to which paternal experience and/or behavioral variation influenced the subsequent

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maternal behavior of females toward offspring (in the absence of the male) during the postpartum period. On the basis of the predictions of DAH, we hypothesized that females would invest less in the care of offspring sired by socially isolated vs. socially enriched males, thus providing evidence for a maternal pathway through which paternal effects are achieved.

Results

Lifetime Social Enrichment Decreases Male Anxiety-Like Behavior. To shift the phenotype of isogenic males we reared BALB/c mice within two distinct physical and social environments. In the enriched condition (ENR), mice were communally reared during postnatal development and subsequently housed after weaning in a complex, enriched environment. In the isolated condition (ISO), mice were reared by a single dam and subsequently socially isolated. In adulthood, males that had experienced a lifetime of social and environmental enrichment showed reduced anxiety-like behavior compared with ISO males (Fig. 1). ENR males exhibited a reduced latency to enter the anxiogenic center area of an open-field apparatus [$t(44) = 4.30$, $P < 0.001$; Fig. 1*B*], made more entries into the center [$t(44) = -4.79$, $P < 0.001$], and spent more time in the center [$t(44) = -3.11$, $P < 0.01$] during a 10-min testing session. ENR males also produced fewer fecal boli during open-field testing [$t(44) = 4.90$, $P < 0.001$; Fig. 1*C*]. During testing in a light/dark activity box, ENR males exhibited a reduced latency to exit the dark chamber [$t(44) = 3.66$, $P < 0.01$; Fig. 1*D*] and spent less overall time in the dark chamber [$t(44) = 4.51$, $P < 0.001$; Fig. 1*E*]. ENR but not ISO males were found to have reduced anxiety-like behavior in comparison with a group of standard laboratory colony-reared BALB/c mice (i.e., raised by a single mother and then group-housed in a standard laboratory cage) in all measures of anxiety except for the number of fecal boli produced in the open field (Table S1), suggesting that the phenotypic differences observed were accounted for by the reductions in anxiety-like behavior induced through enrichment, rather than increases in anxiety-like behavior induced through social isolation.

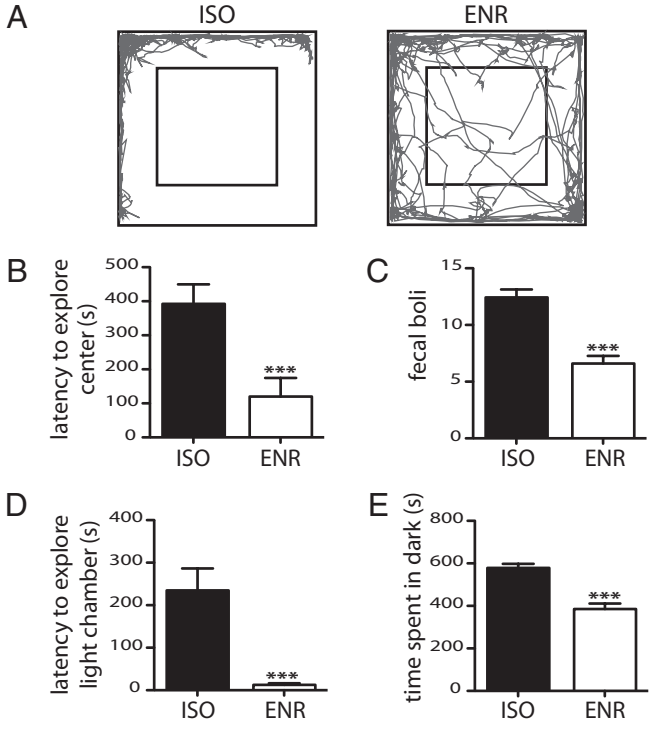

Fig. 1. Anxiety-like behavior of ISO- vs. ENR-reared males. (*A*) Representative traces of open-field activity indicating hypoactivity/reduced exploration by ISO compared with ENR males. (*B*) ENR males show a reduced latency to explore the center area of a novel open field. (*C*) ENR males produce fewer fecal boli than ISO males. (*D*) ENR males show a reduced latency to explore the light chamber of a light/dark box and (*E*) spend less time in the dark area during testing compared with ISO males. ***$P < 0.001$.

Social Enrichment Induces Increases in Hippocampal Gene Expression. Comparison of mRNA levels in the hippocampus of ENR vs. ISO males at 90 d of age indicated that ENR males had elevated hippocampal corticotropin-releasing hormone [*CRH*; $t(10) = -2.60$, $P < 0.05$] and *BDNF* [$t(10) = -2.44$, $P < 0.05$] mRNA (Table 1). ENR effects were specific to these gene targets, and no group differences were observed in hippocampal glucocorticoid receptor (*NR3C1*), methyl-CpG binding protein 2 (*MeCP2*), or *N*-methyl D-aspartate receptor subtype 2B mRNA levels—genes that have previously been shown to be affected by experiential factors (16).

Paternal Social Experience Influences Maternal Postnatal Investment in Offspring. Using a multilevel modeling approach, we assessed the frequency of postnatal maternal care of standard-reared BALB/c females mated with either ENR or ISO males. Importantly, males were removed after 2 wk of mating and were not present during the postpartum period. Females mated with ENR males (ENR-females) engaged in significantly higher levels of pup nursing across the first week postpartum [$t(22) = 2.75$, $P < 0.01$; Fig. 2*A*] and marginally higher levels of pup licking [$t(22) = 1.82$, $P = 0.08$; Fig. 2*C*]. These differences in maternal care were more pronounced during the early postpartum period. Compared with ISO-females, ENR-females nursed pups more frequently on the first two postpartum days [PPD1: $t(22) = 2.61$, $P < 0.05$; PPD2: $t(22) = 3.93$, $P < 0.001$; Fig. 2*B*] and licked more on the first day postpartum [$t(22) = 2.63$, $P < 0.05$; Fig. 2*D*]. For all other days, nursing and licking behaviors were not significantly different between groups, except on postpartum day 5, when both behaviors were significantly higher in ENR-females than in ISO-females [nursing: $t(22) = 2.26$, $P < 0.05$; licking $t(22) = 2.08$, $P < 0.05$]. Consistent with the social housing effects on male behavior, standard-reared females mated with standard-reared BALB/c mice were not found to differ in frequency of postpartum behavior compared with ISO-females, suggesting that paternally associated increases in maternal behavior were associated with the ENR treatment (Fig. S1).

Paternal Social Enrichment Affects Maternal Gene Expression. Although variation in early postpartum maternal behavior was not found to be associated with gene expression changes in the hypothalamus on PPD28 among ISO-females, a significant correlation was found among ENR-females. ENR-females that engaged in heightened levels of nursing during the first postpartum week had higher levels of *BDNF* mRNA [$t(8) = 3.43$, $P < 0.01$; Fig. 3*A*]. Analysis of PPD1 maternal behavior indicated that although no overall association between gene expression and behavior was detected for ISO-females, ENR-females that

Table 1. Hippocampal gene expression (mRNA) of ISO- and ENR-reared males

Gene	ISO	ENR
BDNF	1.01 ± 0.06	1.24 ± 0.07*
CRH	1.01 ± 0.05	1.28 ± 0.10*
NR3C1	1.01 ± 0.06	0.94 ± 0.10
MeCP2	1.01 ± 0.06	1.03 ± 0.15
NR2B	1.07 ± 0.16	1.14 ± 0.19

Relative fold expression of *BDNF*, *CRH*, glucocorticoid receptor (*NR3C1*), *MeCP2*, and *N*-methyl D-aspartate receptor subtype 2B (*NR2B*); *$P < 0.05$.

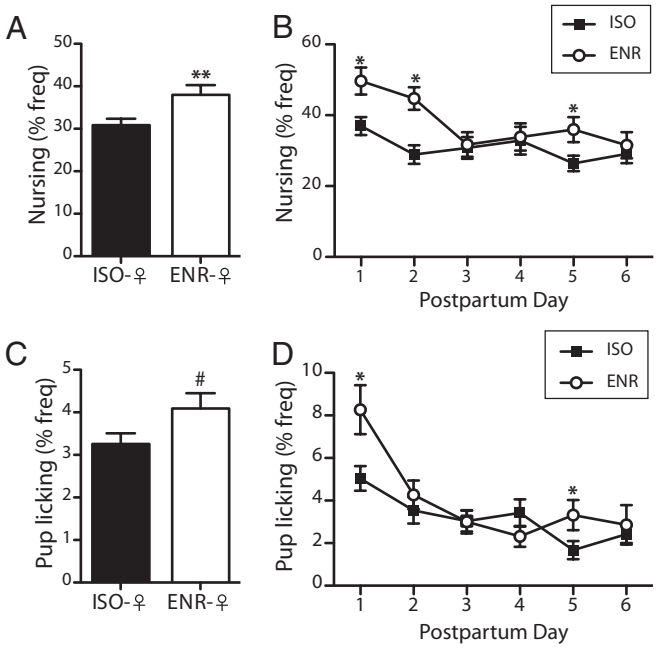

Fig. 2. Maternal behavior of females who mated with ISO-reared males (ISO-♀) or ENR-reared males (ENR-♀). (*A*) ENR-♀ exhibit elevated levels of nursing across the first postpartum week compared with ISO-♀. (*B*) Increased nursing levels of ENR-♀ were most pronounced during the first 2 postpartum days. (*C*) ENR-♀ exhibit marginally elevated levels of pup licking across the first postpartum week compared with ISO-♀. (*D*) Increased pup licking by ENR-♀ are most pronounced on postpartum day 1. $^{**}P < 0.01$; $^{*}P < 0.05$; $\#P < 0.10$.

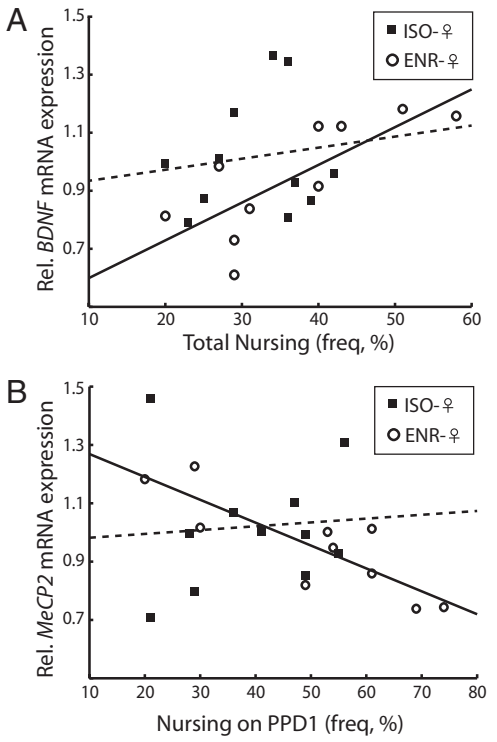

Fig. 3. Scatter plots (with best-fit regression lines) showing correlations between measures of hypothalamic gene expression and maternal behavior of females who mated with ENR males (ENR-♀) or males that were ISO-reared (ISO-♀). (*A*) Total postpartum nursing was positively correlated with *BDNF* mRNA expression in ENR-♀ (solid line) but not ISO-♀ (hatched line). (*B*) Nursing on PPD1 was negatively correlated with *MeCP2* mRNA expression in ENR-♀ (solid line) but not ISO-♀ (hatched line).

engaged in a high frequency of nursing behavior had lower levels of *MeCP2* mRNA [$t(8) = 4.48$, $P < 0.001$; Fig. 3*B*].

Environmentally Induced Changes in Paternal Anxiety-Like Behavior Are Associated with Maternal Behavior Toward Offspring. We examined whether anxiety-like behavior of males would predict subsequent frequency of postpartum nursing and licking by females. Although only marginal correlations were found for average maternal behavior across the postpartum period [i.e., latency to enter the center of the open field and nursing behavior: $t(22) = 1.81$, $P = 0.08$], male behavior did significantly predict maternal care on PPD1. A male's latency to enter the center of the open field was negatively related to PPD1 maternal nursing and pup licking [$t(22) = 1.95$, $P = 0.06$; $t(22) = 2.38$, $P < 0.05$, respectively; Fig. 4*A*]. Boli production during open-field testing was negatively related to nursing and frequency of pup licking [$t(22) = 2.02$, $P = 0.06$; $t(22) = 1.71$, $P = 0.10$, respectively; Fig. 4*B*]. The duration spent in the dark portion of the light/dark box was not associated with maternal licking behavior but was negatively related to PPD1 maternal nursing, such that females who mated with long-duration males nursed their pups significantly less than females who mated with short-duration males [$t(22) = 2.26$, $P < 0.01$; Fig. 4*C*]. Interestingly, although these analyses indicate that maternal behavior toward pups and paternal anxiety-like behavior were correlated, using a mediation analysis we found limited evidence that paternal anxiety-like behavior mediated the paternal effect on maternal behavior (all mediated effects <51%, all $P > 0.19$), suggesting that another phenotypic variable in combination with paternal anxiety-like behavior may have induced the observed differences in maternal care.

Paternal Enrichment Induces Variation in Offspring Outcomes. Litter weights at PN0 (postnatal day 0) and PN6, as well as individual pup weaning weights at PN28, were compared among ISO- and ENR-sired litters. Paternal condition did not affect sex ratio or PN0 litter size, nor did it affect survival to weaning (Table 2). Controlling for litter size, paternal condition did not affect litter weight at PN0 or PN6, but it did predict offspring weight at weaning (PN28). Offspring of ENR males weighed an average of 0.98 g more than offspring of ISO males [$t(21) = 2.84$, $P < 0.01$]. This growth effect was observed in both male and female offspring and was significant after controlling for maternal care. Offspring of standard-reared BALB/c mice were not found to differ in weight compared with offspring of ISO-males (ISO: 10.90 ± 0.23 g; standard-reared: 10.80 ± 0.26 g), suggesting that paternally associated increases in weight were associated with the ENR treatment.

Discussion

Environmental experiences have enduring effects on neurobiology and behavior, and there is increasing evidence for the impact of paternal experiences on developmental outcomes in offspring. In the present study, we show that social experiences lead to divergent phenotypes in males and that these phenotypes have implications for the level of postnatal reproductive investment of their female mates toward offspring, with consequences for offspring fitness. These data provide evidence for differential allocation of maternal resources dependent on the social experience of male mates, a finding that has significant implications for our understanding of the mechanistic pathways through which paternal effects are achieved.

Social experience across the lifespan has been shown to modulate stress responsivity and anxiety-like behavior across species (17–19). In BALB/c mice, heightened anxiety-like behavior in adulthood has been attributed to the reduced levels of postpartum maternal care received in infancy (20, 21). In the

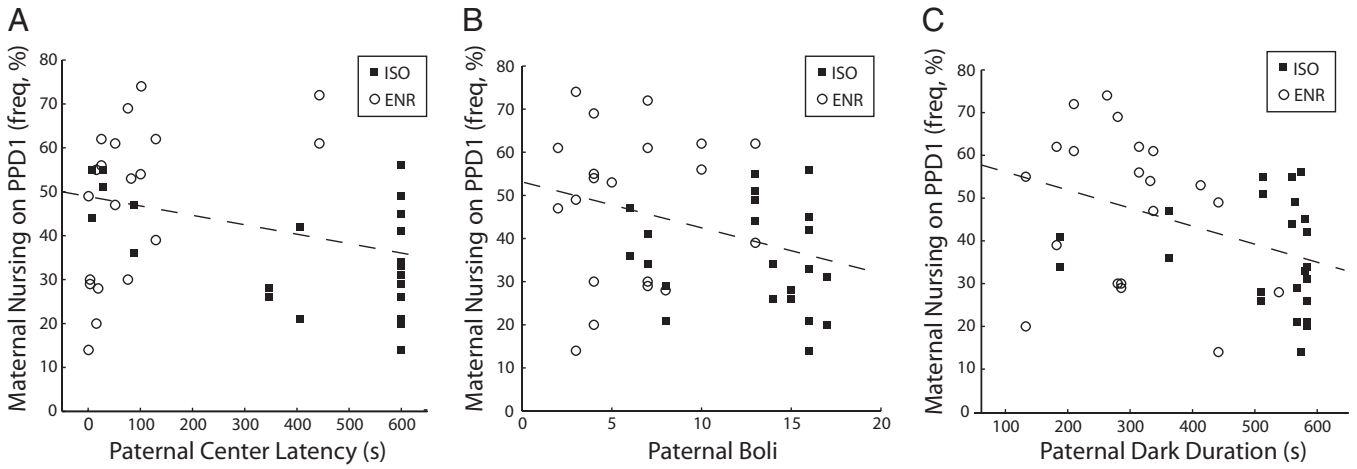

Fig. 4. Scatter plots (with best-fit regression lines) showing correlations between paternal anxiety-like behavior and subsequent maternal behavior of the mate. The (*A*) latency to enter the center of a novel open field, (*B*) number of boli defecations in a novel open field, and (*C*) time spent in the dark chamber of a light/dark box arena was negatively correlated with nursing on PPD1.

present study we illustrate the combined effect of postnatal communal rearing and postweaning enrichment (ENR males) on ameliorating this phenotype, an effect consistent with previous studies of complex housing and social stimulation (17, 18). In contrast to the effects of enrichment, we find that social isolation exerts little effect on anxiety-like behavior in BALB/c mice. The effect of social enrichment vs. social isolation is also observed on measures of hippocampal expression of *BDNF* and *CRH*, genes implicated in the expression of anxiety-like behavior. Previous studies have shown that genetic deletion of *BDNF* results in increased anxiety-like behavior, whereas the converse is evident after overexpression of *CRH* (22, 23). Increased levels of BDNF within the hippocampus are thought to reduce anxiety-like behavior by enhancing structural plasticity and providing trophic support to neurons (24). Enriching the postnatal and/or postweaning environment can increase levels of BDNF (25–27), whereas maternal neglect and postweaning social isolation lead to reduced levels of BDNF in the brain (28, 29). *CRH* expression is similarly sensitive to experiential factors (30). Although stress-induced levels of CRH potentiate anxiety-like responses, in the absence of stressors, basal levels of CRH in the hippocampus (and other extrahypothalamic regions) have been associated with hypervigilance and arousal (31). Thus, increases in hippocampal *CRH* observed in ENR males in the present study may underlie their increased exploratory behavior.

We hypothesized that environmentally induced variation in male phenotype could affect their mate's maternal investment. Indeed, we find that females mated with ENR males engage in higher frequencies of postpartum maternal care, particularly during the early postpartum period. Alterations in maternal investment arising from mate quality are a well-characterized phenomenon, occurring across a wide variety of species (13). In most maternal investment studies, however, it has been difficult to dissociate genetic signals of paternal attractiveness (i.e., "good genes") from nongenetic signals of paternal attractiveness, such as paternal experience. In the present study, males were genetically identical and only differed in the social environment experienced across their lifespan. Our findings strengthen the idea that nongenetic aspects of male quality are able to drive differential allocation in mammalian female mates.

Table 2. Characteristics of litters and offspring sired by ISO- and ENR-reared males

Measure	ISO	ENR
PN0 litter size	5.29 ± 0.34	5.23 ± 0.43
PN0 litter weight (g)	7.81 ± 0.45	7.77 ± 0.53
PN6 litter size	5.36 ± 0.34	5.00 ± 0.41
PN6 litter weight (g)	20.89 ± 1.19	20.61 ± 1.32
Percent male pups	42.45 ± 5.06	36.03 ± 4.98
Pup weight on PN28 (g)	10.97 ± 0.24	11.94 ± 0.25**

**$P < 0.01$.

Variation in behavioral characteristics in rodents is associated with levels of maternal care experienced during postnatal development (18, 25, 32, 33). These phenotypic effects may be achieved through maternal epigenetic programming of offspring gene expression, inducing individual differences that persist into adulthood (29, 30, 34). Thus, the paternally induced alterations in maternal behavior we observe could affect multiple developmental outcomes. Female experiences (e.g., stress exposure, social/isolation rearing) affect her postpartum maternal care (1, 2, 29). Our data provide evidence that a father's experience can induce similar effects, thus exposing a pathway through which paternal experiences can influence offspring development. The recent finding that the effects of chronic social defeat stress in isogenic male mice are not completely transmitted to offspring when sired using in vitro fertilization lends further support to this maternal mediation hypothesis (35).

From a mechanistic perspective, it is important to note that the environmentally induced anxiety level of males did not completely predict the degree of maternal investment, consistent with previous findings that postnatal maternal behavior was not altered after mating with males selected solely on the basis of anxiety-like behavior (36). In addition to changes in anxiety-like behavior, the differential rearing experience of ISO and ENR males is likely to have altered other behavioral characteristics (e.g., social and aggressive behaviors, mating style) that may induce the observed differential maternal investment. Any aspect of male phenotype that induces mate preference in females could be an important predictor of maternally driven paternal effects, a process that may be further elucidated through broader phenotypic assessment of males during mating and mate-preference testing. In birds, there is evidence that changes in investment are related to the perceived quality of the male rather than any intrinsic or genetic differences (14). Studies in house mice have shown that females mated with a preferred male gave birth to larger litters and that these offspring were found to be socially dominant and to have decreased mortality rates compared with

offspring of females mated with nonpreferred males (37). Although mate preference is often thought to occur on the basis of genetically driven phenotypic features, experiential factors (e.g., in utero food restriction and toxin exposure) of males can shift female preferences toward nonexposed males (38, 39). Male phenotype could also have direct effects on the reproductive physiology of females (i.e., shifting reproductive and stress hormone levels) during the mating period. For example, experiences (e.g., stress) can shift mating strategies and the sexual behavior of males (e.g., rates of intromission), which has significant consequences for successful pregnancy and parturition (40–42). Importantly, these effects could occur independent of female-driven selection of preferred mates.

Related to the concept of paternal effects via maternal investment is the consequence of inherited paternal genetic/epigenetic variation by offspring that could lead to variations in the level of care that offspring receive. In rodents, rates of ultrasonic vocalizations, suckling ability, and locomotor activity are all influenced by paternal genes and can regulate the amount of care offspring receive from their mother during the pre- and postnatal periods (43, 44). Therefore, the experience of fathers could have indirect effects on maternal behavior by driving greater resource extraction (via prenatal priming and placental function) from the mother through their effects on the epigenetic variation of fetal tissues (43). Embryo transfer and cross-fostering manipulations may allow for further dissection of the biological and behavioral pathways through which these paternal effects on maternal investment are achieved, although it should be noted that these strategies have significant methodological limitations in this context due to (*i*) the epigenetic disruption associated with gamete extraction and in vitro fertilization (45), (*ii*) cross-fostering effects on postnatal maternal behavior (33, 46), and (*iii*) the complex interactions between maternal and pup characteristics in the prediction of postnatal maternal investment (47).

The consequence of paternal social experience can also be observed within the brain of females, particularly in variation of hypothalamic *BDNF* and *MeCP2* gene expression that persist beyond the mating period and are observed at the time that offspring are weaned. BNDF plays a critical role in neuronal and behavioral plasticity, and reduced nurturing behaviors in postpartum rats are associated with decreased *BDNF* mRNA levels (29). Thus, we would anticipate finding increased *BDNF* among ENR-females compared with ISO-females. However, we find that *BDNF* levels do not differ across the mating groups but emerge as an interaction between the levels of maternal behavior exhibited by the female and the social experiences of her mate. In other words, maternal behavior and *BDNF* mRNA levels are only correlated in ENR-females. A similar interaction is found when examining hypothalamic levels of *MeCP2*. As a methyl-binding protein, MeCP2 can bind to promoter regions of genes and modulate gene activity, and in the case of *BDNF* can reduce the expression of this gene (48). Thus, changes in *BDNF* may be part of a larger network of neural changes that occur in response to the experience of mating with ENR males. The role of MeCP2 in maternal behavior has not been previously explored, although its reduced expression levels may be an indicator of the general level of gene activity within hypothalamic circuits (49). Our findings suggest that reduced MeCP2 levels are associated with reduced levels of maternal behavior. This effect could be explored further through overexpression or inhibition of hypothalamic *MeCP*2 in females mated with ENR vs. ISO males and analysis of the time course of hypothalamic gene expression consequent to these manipulations. The lack of association between these neurobiological changes and the maternal behavior of ISO-females may suggest that alternative mechanisms regulating postpartum behavior are induced when females are mated with isolation-reared males.

Consistent with the accumulating evidence for environmentally induced paternal effects on phenotypic characteristics of offspring (5–9, 35, 36), in the present study we show that the offspring of ENR males were heavier at weaning compared with offspring of ISO males. Increased growth during the juvenile period has been shown to have fitness consequences in a wide variety of species (50), and in laboratory mice low weaning weight is a predictor of stressor susceptibility and the ability to overcome infection and dietary challenges (46, 51). Studies conducted in a number of species have identified a link between paternal condition and growth rates in offspring (36, 52–54), and these effects have been attributed to differential allocation of maternal resources that are based on mate quality/preference (14, 15). The weight differences we observed remained even after controlling for postpartum maternal behavior, indicating that prenatal metabolic programming or late postpartum maternal behavior (preweaning) may account for this particular fitness outcome. Quantifying prenatal maternal investment in mammals is difficult because important factors may include gestational food intake, hormonal release, and other physiological changes that depend on interactions between the mother, placenta, and fetus.

In humans there is compelling evidence that paternal age, nutrition, and drug use predict the development of psychopathology and health risks. Interestingly, the effects observed in human epidemiological studies are qualitatively similar to paternal effects observed in species of laboratory rodents that do not engage in postnatal paternal care (4). Although much of the evidence is, at this stage, preliminary, there is emerging support that environmental induction of paternal germline epigenetic changes can have consequences for multiple generations of offspring. The potential role of mothers in these paternal effects has typically not been explored. In the present study, we show that maternal investment is partially dependent on the social and environmental experience of her mate. Thus, the inheritance of paternal genetic/epigenetic variation can have both direct effects on offspring and/or indirect effects on offspring via maternal investment. The complex relationship between paternal, maternal, and offspring phenotypes, and the effect of the environment on this dynamic, represent challenging yet important factors in the study of the mechanisms driving paternal effects.

Methods

Animals, Husbandry, and Breeding. Adult male and female BALB/c mice (F0; ~3 mo of age) purchased from Charles River were used to generate offspring. Mice were housed in the Department of Psychology at Columbia University, with lights on at 2200 hours and off at 1000 hours, and provided with ad libitum access to food and water. All procedures were conducted in accordance with Columbia University Institutional Animal Care and Use Committee standards. For standard rearing, each male was mated with three females, and males were removed from the cage after approximately 2 wk. Females were singly housed starting on gestational day 16. Mice were allowed to give birth and left undisturbed (except for weekly cage cleaning) until weaning (PN28), at which time male pups were housed four per cage in a standard laboratory cage. The isolated condition (ISO) differed in that a single female was mated with a single male (to prevent the effects of prenatal social stimulation). At weaning, pups were housed alone in a standard laboratory cage for the remainder of the experiment. For enriched rearing (ENR), during the mating period a male was placed into an enriched environment with three females. The enriched environment consisted of four standard laboratory cages connected by Plexiglas tubes and contained toys and a running wheel. Three litters (all of which had given birth within the same 12-h period) were rehoused to a single enriched environment to create communal rearing conditions. At weaning, ENR pups were placed 12 per cage into same-sex groups into an enriched environment. These manipulations ensured that ENR pups received a lifetime of enrichment. A maximum of two male adult F1 pups were selected for behavioral testing from each litter. Males (n = 24 per group) selected for behavior testing were first tested at PN75 in the open field and then 1 wk later tested in the light/dark box. Two weeks after testing, a subset of mice (n = 6 per group) used

for anxiety testing were killed and brains extracted for hippocampal gene expression analysis. A subset of males (n = 12 per group) was used for mating. On PN90, all mice assigned to the mating condition were removed from their housing conditions, and a single male was placed in a mating group with two standard-reared females for 2 wk. On approximately gestational day 16, females were singly housed. On the day of birth (PN0), litters were weighed and counted. These litters were observed from PN1 to PN6 to determine postnatal levels of maternal care, and all pups were weighed and counted at PN6 and at weaning (PN28). A subset of dams (n = 12 per group) was killed and brains extracted for hypothalamic gene expression analysis.

Maternal Observations. The procedure for assessing maternal behavior in mice has been described previously (21). Each dam was observed for four 1-h periods per day by an observer blind to paternal condition from PN1 to PN6, resulting in a total of 480 observations of each litter. The frequency of the following behaviors were scored: mother in nursing posture over pups, mother in contact with pups but not in a nursing posture, mother licking and grooming any pup, nest building, self-grooming, eating, and drinking.

Open Field Test. The open field apparatus used was a 24 × 24 × 16-inch Plexiglas box with black walls and a white floor. On the day of testing, the mouse was removed from its home cage and placed directly into one corner of the open field. After a 10-min session, the mouse was returned to its home cage. All testing was conducted under red lighting conditions. Behaviors were video recorded. Behaviors scored using Ethovision (Noldus) included (*i*) center area exploration, defined as the time spent in the inner (12 × 12-inch) area, (*ii*) latency to enter the center, and (*iii*) distance traveled.

Light/Dark Test. The light/dark testing apparatus was a 11.5 × 19 × 11-inch (outer dimensions) Plexiglas box, consisting of a light compartment (11 × 11 inches) and a smaller dark compartment (11 × 7 inches) connected by a door (3 × 3 inches) in the center of the wall separating the two compartments. A 60-W bulb located 16 inches above the center of the light compartment provided bright illumination. Mice were placed in the center of the dark compartment facing the door and were allowed to explore the box for 10 min. Time (seconds) spent in the light and dark compartments and the latency to exit the dark chamber were scored using Ethovision (Noldus).

Quantitative Real-Time PCR Analysis. RNA was isolated from the whole hippocampus (ventral and dorsal) of males and whole hypothalamus of females using the AllPrep DNA/RNA Mini Kit (Qiagen) and reverse transcribed to cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR applications (Invitrogen). Quantitative RT-PCR was performed with 1 μL of cDNA using an ABI 7500 Fast Thermal Cycler and the Fast SYBR Green Master Mix reagent (Applied Biosystems). All primer probes (Sigma-Aldrich; Table S2) were designed to span exon boundaries ensuring amplification of only mRNA. For each gene, C_T values were normalized to cyclophillin A (endogenous control). Relative expression values were obtained by the $\Delta\Delta C_T$ method.

Statistical Analyses. Because of the hierarchical nature of our data (i.e., an individual male was mated with two females, which produced litters with multiple pups), we used a multilevel modeling approach. Random intercepts for male ID (and where appropriate, female ID) were estimated to correct for repeated sampling (55). In the "fixed" portion of the model (the level 1 effects), we tested for differences in offspring weight by paternal experimental condition. To test whether the males' anxiety-like behaviors mediated the paternal effect on maternal behavior, we conducted a mediation analysis using maternal behaviors averaged by male ID (56). All analyses were conducted with Stata v12.0.

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