

DEVELOPMENTAL NEUROSCIENCE

Paternal deprivation impairs social behavior putatively via epigenetic modification to lateral septum vasopressin receptor

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Although it is well appreciated that the early-life social environment asserts subsequent long-term consequences on offspring brain and behavior, the specific mechanisms that account for this relationship remain poorly understood. Using a novel assay that forced biparental pairs or single mothers to prioritize caring for offspring or themselves, we investigated the impact of parental variation on adult expression of nonapeptide-modulated behaviors in prairie voles. We demonstrated that single mothers compensate for the lack of a co-parent. Moreover, mothers choose to invest in offspring over themselves when faced with a tradeoff, whereas fathers choose to invest in themselves. Furthermore, our study suggests a pathway whereby variation in parental behavior (specifically paternal care) may lead to alterations in DNA methylation within the vasopressin receptor 1a gene and gene expression in the lateral septum. These differences are concomitant with changes in social approach, a behavior closely associated with septal vasopressin receptor function.

INTRODUCTION

The quality and quantity of parental care can have profound impacts on the development of offspring. Several studies have demonstrated that high-quality parental care can substantially boost cognitive performance and healthiness of adult relationships, whereas parental neglect can have devastating consequences on the social, emotional, and even criminal behavior of adults later in life (1). These patterns are also well supported in nonhuman models of parental neglect (2, 3). For example, maternal separation in rats and paternal deprivation in mandarin voles reduces locomotor activity and increases anxiety-like behavior (4, 5), low parental contact during development increases aggressive behavior in prairie voles and California mice (6, 7), and prairie voles raised in the absence of a father show delayed species-typical partner preference formation (8). Despite this well-established relationship between early-life experiences and adult social behavior, there is a paucity of understanding for the mechanisms by which experience asserts these effects on the neural machinery that govern social behavior.

Epigenetic modification of DNA to alter gene expression is perhaps the most promising mechanism that could enable experiences in the natal environment to shape sociability and cognition in adulthood. Epigenetic modification can shift the genetic regulation of neural function (9) or alter the availability of the proteins constituting neuromodulatory systems that shape behavior (10, 11) by affecting translation and transcription. Although this field is still relatively nascent, much progress has been made recently, and a deeper understanding of how the genome can be modified by external forces is beginning to emerge. Multiple forms of epigenetic modification have the potential to produce functional gene modification at the epigenomic level (12). For example, environmental forces can cause changes in chromatin structure that increases (via histone acetylation) or decreases (via histone methylation) the access of large portions of DNA to the natural subcellular machinery that governs

transcription and translation. Whereas epigenetic modification of histones affects a number of gene loci, epigenetic modification of DNA via methylation of cytosine nucleic acids that are followed by guanine in the DNA sequence (i.e., CpG sites) is more specific and targets single genes. Unlike histone modification, DNA methylation is a stable signature of the epigenomic status of the regulatory sequence (12) and is therefore most suitable for explaining long-term processes underlying the maintenance of behavioral phenotypes that have been shaped by the early environment, especially when a focus on single genes (and their products) are justified. Epigenetic changes represent a molecular pathway through which long-term programming effects can be achieved (13). As knowledge of how gene expression can be modified grows, we continue to lack substantial understanding for the specific pathways connecting experience, gene expression, and behavior. Furthermore, there remains a lack of studies examining the mechanisms by which early-life experiences can alter gene expression and behavior. Most of what is known about the relationship between early-life experience and the neural and genetic mechanisms regulating adult social behavior comes from uniparental species, often in relatively restrictive laboratory settings (3, 14). However, few studies have considered the impact of paternal influence on offspring development or attempted to test parental behavior under realistic or ethologically relevant conditions. Nevertheless, studies using biparental rodents have shown that the presence of the father during development influences adult aggressive behavior (6) and is important for adult pair bonding (8), social recognition, and spatial memory (15).

The nonapeptides [e.g., vasopressin (VP) and oxytocin (OT)] are extensively involved in nearly every aspect of social behavior across vertebrates, including affiliation, parental behavior, social recognition, communication, and aggression (16). VP has three identified receptors (V1aR, V1bR, and V2R), and OT has just one (OTR). Most of the research on nonapeptide receptor impacts on social behavior has focused on V1aR and OTR, due, in part, to their wide distribution across several areas of the brain that regulate social behavior and their extensive implication in several areas of social behavior, perhaps most notably pair bonding (16, 17). Although the role of

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V1bR is beginning to emerge as an important modulator of social memory and aggression (18), its distribution appears to be relatively restricted to the hippocampus (HPC) and pituitary, its influence on behavior is comparably nuanced, and the developmental trajectories and sensitivity to early-life experiences of V1bR have not been investigated. Furthermore, V2R is primarily associated with peripheral functions of VP. In contrast, numerous factors experienced during development are known to substantially alter nonapeptide production and the gene expression of VP, OT, V1aR, and OTR. These early-life experiences include maternal separation (19), father removal (8, 15), communal rearing (2), and neonatal handling (20). The quantity of VP, OT, V1aR, and OTR throughout the forebrain shows profound plasticity over the course of development (21, 22), indicating the raw potential upon which social or environmental interventions could modify neural phenotype. Moreover, social and/or spatial environmental complexity also has the capacity to alter the density of V1aR and OTR in the brain (21). Notably, this study demonstrated that V1aR, specifically in males, is highly sensitive to environmental perturbations. It also highlighted a central need for experiments to improve the ecological relevance of experimental design, which, in turn, has the capacity to reveal more natural outcomes in brain function and behavioral phenotype.

Ecologically relevant designs allow for expression of behaviors more similar to those that occur in wild populations. For instance, parents must forage for food in nature, whereas laboratory animals in standard rodent cages have access to low-risk, high-energy food ad libitum, presumably alleviating energetic demands and natural tradeoffs associated with basic parental duties and survival experienced in the real world. Variation in parental care is surely attributable, in part, to how parents respond to their immediate context. Recapitulating elements of real-world tradeoffs will reveal behavioral variation that typical laboratory housing would presumably mask. Furthermore, considering the impact that parental care has on the development of offspring, it is striking that the mechanisms by which parental care, particularly paternal care, influences behavior largely remain a mystery.

To this end, we use a biparental and socially monogamous rodent, the prairie vole (*Microtus ochrogaster*), to examine how variation in parental care influences the development of offspring behavior and neural systems that modulate social behavior. Prairie voles are particularly well suited for asking questions about the role that the early-life family environment plays in developmental plasticity. Field studies show that prairie voles live in three types of social groups [communal group, male-female pair, and single female (23)], indicating that offspring naturally experience different types and amounts of parental care depending on the type of social group to which they are born. In addition, seminatural laboratory experiments that house prairie vole families in large pens demonstrate that mothers and fathers occasionally leave postnatal day (PND) 1 to 10 pups alone at the nest to forage (24).

The rich knowledge of natural history for prairie voles highlights the strength that this species offers toward developing a powerful and ecologically relevant model for translational social neuroscience (17). In an attempt to loosely approximate a real-world tradeoff that parents experience, we raised prairie vole pups (i) in the presence or absence of a father and (ii) with parents that did or did not experience a tradeoff in caring for themselves or caring for their offspring. To force this tradeoff, we constructed home cage environments that required parents to leave the nest to obtain food (Fig. 1A). Food

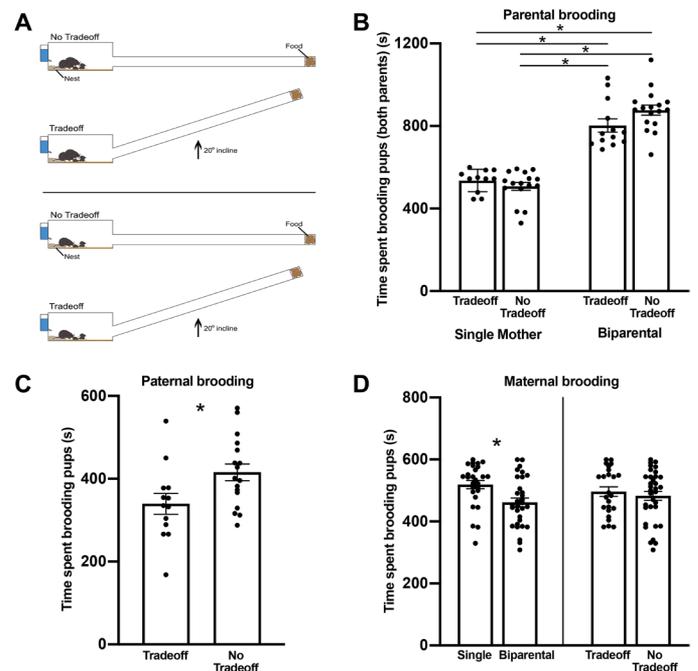


Fig. 1. The absence of a male co-parent increases maternal care and experiencing increased effort to feed impairs paternal care. (A) A diagram illustrating the Parental Tradeoff and No-Tradeoff conditions of the experimental design. All home cages were provided with water ad libitum and nesting material. Food for all families was placed in a wire hopper at the end of a 1.53-m plexiglass tube attached to the home cage. The plexiglass tube for families in the No-Tradeoff condition had no incline, whereas the plexiglass tube in the Tradeoff condition had a 20° incline. (B) Parental care. Mean (\pm SEM) total average brooding time (in seconds) from mothers and fathers that offspring received in the four rearing conditions from PND 0 to 21 (from an average of daily 10-min samplings). (C) Paternal care. Mean (\pm SEM) time that fathers in the Tradeoff and No Tradeoff conditions spent brooding offspring from PND 0 to 21 (from an average of daily 10-min samplings). (D) Maternal care. Mean (\pm SEM) time that mothers in Single Mother and Biparental families and Tradeoff and No Tradeoff families spent brooding offspring from PND 0 to 21 (from an average of daily 10-min samplings). Dots represent individual data. $*P \leq 0.05$.

was placed in a wire hopper at the end of a 1.53-m plexiglass tube attached to the home cage. The plexiglass tube for parents in the Tradeoff condition was set at a 20° incline. This design imposed a moderate energetic cost on parents; to feed, they were forced to climb a modestly steep incline at a distance not encountered under normal laboratory housing. Furthermore, suckling prairie vole pups latch to mothers' nipples with milk teeth and must be forcibly removed by parents with much resistance from the pups (25). The incline of the tubes was sufficiently steep such that mothers could not climb the tube with suckling offspring. Thus, mothers had to detach their suckling pups and leave them at the nest to feed themselves, and both parents had to exert effort to feed themselves. Parents that did not undergo the tradeoff manipulation (No Tradeoff) lived in cages with a plexiglass tube that was at the same level as the home cage (i.e., no incline), thus allowing mothers with suckling pups to take offspring with her to feed and for parents to exert nearly normal levels of effort to feed. As discussed below (see Results and Fig. 1; see the "Design" section in Supplementary Materials and Methods), our design significantly affected parental behavior and ultimately the quantity and quality of parental care that pups received.

RESULTS

Our experiment aimed to understand how parental variation in early life influences the development of behavior and social neural systems via epigenetic modifications. To this end, using a 2×2 design, we raised offspring in (i) Single Mother or Biparental and (ii) Tradeoff or No Tradeoff families (Fig. 1A) and tested them for a variety of social and cognitive behaviors once they reached adulthood. These behaviors included social approach, dominance behavior, partner preference, exploratory behavior, and spatial learning and memory. In addition, we examined nonapeptide receptor DNA methylation status of the OTR gene (*otr*) and V1aR gene (*avpr1a*), and gene expression for *avpr1a* only in brain regions important for social and cognitive behaviors (26). These brain regions included the (i) lateral septum (LS), which modulates reproductive and nonreproductive affiliation and plays a key role in modulating context-specific motivational states (27, 28); (ii) nucleus accumbens (NAcc), which is crucial for pair bonding (29); (iii) HPC, which is important for social recognition and spatial and contextual learning and memory (30); and (iv) retrosplenial cortex (RSC), which is involved in contextual space use and mating tactics (11). We examined data using statistical models that included Parental Partner Status (Single Mother or Biparental), Parental Tradeoff Status (Tradeoff or No Tradeoff), and Sex as fixed factors to (i) account for potential sex differences that might emerge as a result of these developmental manipulations and (ii) allow us to explore not only the interactions of Parental Partner Status and Parental Tradeoff Status (inherent in the 2×2 design) but also the main effects that these different aspects of our experimental manipulation may have had on the development of the brain and behavior of offspring.

Effects of experimental design on parental behavior

Brooding

We first sought to determine the effects of our experimental design on parental behavior to understand how variation in parental care influences the development of offspring. Home cages were video-recorded for 10 min daily from birth (PND 0) through weaning (PND 21), and the average daily amount of time spent brooding offspring from PND 0 to 21 was quantified for mothers and fathers. We used general linear models (GLMs) to examine parental behavior that pups in each rearing condition received. Because not all conditions contained both mothers and fathers and male and female parental care differs in function (31), we also used GLMs to assess how Partner Status (Single Mother versus Biparental) and Tradeoff Status (Tradeoff versus No Tradeoff) influenced parental behavior. All post hoc pairwise comparisons were adjusted using the Bonferroni correction.

When totaling the average amount of time mothers and fathers brooded pups under each rearing condition, GLM analysis revealed a main effect of condition [$F_{(3,53)} = 62.69$; $P < 0.01$; Fig. 1B]. Pups reared in Single Mother Tradeoff families received significantly less parental brooding compared to pups reared in Biparental Tradeoff families ($P < 0.01$; mean diff = -266.47) and Biparental No Tradeoff families ($P < 0.01$; mean diff = -341.10). Similarly, pups reared in Single Mother No Tradeoff families received significantly less parental brooding compared to pups reared in Biparental Tradeoff families ($P < 0.01$; mean diff = -294.79) and Biparental No Tradeoff families ($P < 0.01$; mean diff = -364.42). To understand how our manipulations within each rearing condition influenced parental care, we also analyzed the total average amount of parental brooding time that

pups received with Partner Status and Tradeoff Status as fixed factors. The statistical model revealed a main effect of Partner Status, with offspring raised in Single Mother families receiving significantly less brooding from parents compared to offspring raised in Biparental families [$F_{(1,53)} = 169.42$; $P < 0.01$]. Thus, even if single mothers compensate for a lack of a partner and/or if fathers down-regulate their parental effort, biparentally reared offspring receive more care than singly reared offspring overall.

We did not observe an effect of Tradeoff Status [$F_{(1,53)} = 0.35$; $P = 0.90$]. However, we did find a significant interaction between Partner Status and Tradeoff Status [$F_{(1,53)} = 4.44$; $P = 0.04$] on the total average amount of parental brooding time, with Biparental No Tradeoff parents brooding offspring more than Biparental Tradeoff families ($P = 0.03$; mean diff = 74.63). This difference suggests that the Tradeoff manipulation may have differentially affected mothers and father, which is explored below. Last, reflecting the main effect of Partner Status, the significant interaction between Partner Status and Tradeoff Status also showed that Biparental No Tradeoff families exhibited more brooding than Single Mother No Tradeoff families ($P < 0.01$; mean diff = 369.42) and that Biparental Tradeoff families exhibited more brooding than Single Mother Tradeoff families ($P < 0.01$; mean diff = 266.47), supporting the conclusion that the offspring receive different amounts of total care when they have two parents, independent of what the individual parents are doing.

Upon examining maternal care, we found that single mothers spent significantly more time brooding pups compared to Biparental mothers [$F_{(1,54)} = 8.78$; $P < 0.01$; Fig. 1C]. Thus, under this modest ecologically relevant testing paradigm, either single mothers compensate for the lack of a co-parent or biparental mothers relax their maternal caregiving as a function of having a partner. Although the effort to obtain food was increased in the Tradeoff condition, we did not observe a difference in brooding between Tradeoff and No Tradeoff mothers [$F_{(1,54)} = 0.56$; $P = 0.46$]. We also found no interaction of Partner Status and Tradeoff Status on maternal brooding [$F_{(1,54)} = 0.42$; $P = 0.52$]. In contrast, analysis of paternal behavior revealed that fathers in the Tradeoff condition spent significantly less time brooding offspring compared to fathers in the No Tradeoff condition [$F_{(1,28)} = 5.59$; $P = 0.03$; Fig. 1D]. Therefore, offspring raised in Tradeoff families received relatively reduced paternal care overall. These results also suggest that fathers are less willing to trade off effort for self-investment for offspring investment compared to females.

Weights

To determine whether experimental design affected the weight of parents and offspring, we weighed mothers, fathers, and offspring on PND 0, 5, 9, 13, 17, and 21. We used an average pup weight for each litter because we were unable to identify individual pups. We observed a main effect of Partner Status on maternal weight; single mothers weighed significantly less than biparental mothers on PND 0 [$F_{(1,54)} = 5.07$; $P = 0.03$; table S1], perhaps reflecting the stress associated with partner loss on single mothers (male partners were removed 18 days after pairing; see the “Design” section in Supplementary Materials and Methods) (32). We found no other differences in weight at any other offspring age in relation to Partner Status, Tradeoff Status, or an interaction of the two (all $P > 0.06$; table S1). Furthermore, we observed no differences in the weight of fathers in Tradeoff Status at any age of offspring development (all $P > 0.06$; table S1). Last, we found no significant weight differences in offspring at any age (all $P > 0.09$; table S1).

Effects of parental variation on offspring behavior

Offspring were weaned at PND 21 and transferred into standard rodent housing cages with their siblings. At PND 40, all offspring were sexed, and a subset of animals were randomly assigned as subjects if the litter contained two same-sex animals. Thus, all animals used as subjects were housed with one same-sex sibling (see the “Rearing procedure” section in Supplementary Materials and Methods). Subjects underwent behavioral testing for 2 weeks beginning at full adulthood (PND 50 to 55) to determine how variation in parental care during early development affected adult behavioral phenotype (details on testing order can be found in the “Adult behavioral testing” section in Supplementary Materials and Methods). GLM analyses were conducted on all behavioral data, with Sex, Parental Partner Status, and Parental Tradeoff Status as fixed factors. All post hoc pairwise comparisons were adjusted using the Bonferroni correction.

Partner preference

Subjects were tested in a partner preference test to assess how variation in parental care during early development influenced pair bonding as an adult (see the “Behavior” section in Supplementary Materials and Methods and fig. S2A). GLM analyses of a difference score in the time spent huddling with the partner versus stranger and of the time spent huddling with the partner yielded no main effects or interactions of Sex, Parental Partner Status, or Parental Tradeoff Status (all $P > 0.29$; table S2). However, offspring raised in Single Mother families exhibited significantly more huddling time with the stranger compared to animals raised in Biparental families [$F_{(1,65)} = 4.25$; $P = 0.04$; fig. S2B].

Previous studies have shown that being raised by a single mother impairs pair bonding in a sex-specific manner in prairie voles (8), and there are sex differences in partner preference formation and maintenance in prairie voles (33). Therefore, in light of these studies and because we found a main effect of Parental Partner Status (uniparentally- or biparentally-reared) on the time offspring spent huddling with strangers, we conducted separate planned GLMs for males and females raised in Single Mother and Biparental families. These GLM analyses yielded no main effect of Parental Partner Status for the difference score in time spent huddling with the partner versus the stranger, the time spent huddling with the partner, or the time spent huddling with the stranger for females (all $P > 0.15$). However, we did observe a main effect of Parental Partner Status on the time spent huddling with the stranger for males [$F_{(1,30)} = 4.33$; $P > 0.05$; fig. S2C], with Single Mother-reared males spending significantly more time with the stranger than Biparental-reared males. Yet, despite the finding in previous studies that Single Mother-reared males exhibit impaired pair bonds, we found no main effects for the difference score in the time spent huddling with the partner versus the stranger or the time spent huddling with the partner for males (all $P > 0.27$). Thus, under the conditions that we tested animals, although both Single Mother- and Biparental-reared males exhibit a partner preference, the bond that Biparental-reared males demonstrated was relatively stronger than those of males raised by just mothers. These subtle differences in bond strength suggest that Single Mother-reared males may be more prone to engage in extra-pair copulations than Biparental-reared males, given the tendency to spend more time with an opposite sex conspecific outside of the pair bond. Together, these findings also reinforce previous work suggesting that the lack of a father in early development influences male pair bonds more strongly than female pair bonds as adults (8).

Dominance

Subjects were tested in a dominance tube test (see the “Behavior” section in Supplementary Materials and Methods) to determine whether parental variation in the early-life family environment influenced adult dominance phenotype. We did not observe any main effects or interactions (all $P > 0.25$; table S2), suggesting that variation in the type and amount of parental care received did not influence offspring dominance assessed by this task.

Exploration of an open field

Subjects were tested in an open-field test to determine how parental variation in early-life influenced adult exploratory behavior (see the “Behavior” section in Supplementary Materials and Methods and fig. S2D). Animals raised in Single Mother families explored the center and inner zones of the open arena less than animals raised biparentally [Parental Partner Status main effects on the time spent in the inner zone, $F_{(1,67)} = 11.48$; $P < 0.01$; fig. S2E; center zone, $F_{(1,67)} = 8.89$; $P < 0.01$; fig. S2F]. Analyses did not reveal any main effects or interactions involving Sex and Parental Tradeoff Status on the time spent in the center and inner zones (all $P > 0.18$; table S2). We observed no differences in time spent in the outer zone or wall zone based on Sex, Parental Partner Status, or Parental Tradeoff Status (all $P > 0.29$; table S2). Furthermore, we did not find any main effects or interactions on locomotor activity (distance moved; all $P > 0.24$; table S2). Together, Single Mother-rearing appears to reduce anxiety or increase exploration in adult life.

Learning and memory

We tested subjects in a Morris water maze test to examine influences of the early-life family environment on adult spatial learning and memory (see the “Behavior” section in Supplementary Materials and Methods). We assessed spatial learning using a linear mixed model to compare the latency to reach a hidden platform over the course of the first nine learning trials (table S3). Sex, Parental Partner Status, Parental Tradeoff Status, and Trial were used as fixed factors, and the subject was used as a random factor to control for multiple responses. As anticipated (34), a main effect of Trial indicated that all subjects improved finding the hidden platform over the nine trials [$F_{(8,536)} = 24.03$; $P < 0.01$]. We also observed a significant Trial \times Parental Partner Status interaction [$F_{(8,536)} = 2.96$; $P < 0.01$; fig. S2G]. Post hoc analyses showed a significant difference between animals raised in Single Mother and Biparental families only in Trial 2 (mean diff = -22.24 ; $P = 0.03$) and Trial 3 (mean diff = 23.66 ; $P = 0.02$), with Single Mother-raised animals reaching the platform more quickly than Biparental-raised animals in Trial 2 and vice versa in Trial 3. This seemingly perplexing finding may be explained by the timing of learning the test across trials. To that end, post hoc analyses also revealed that Single Mother-raised animals first reached the platform significantly more quickly in Trial 2 than in Trial 1 (mean diff = -27.78 ; $P = 0.02$), whereas Biparental-raised animals first found the platform significantly more quickly in Trial 3 compared to Trial 2 (mean diff = -37.54 ; $P < 0.01$). Thus, most of the learning occurred in Trial 2 for Single Mother-raised animals and in Trial 3 for Biparental-raised animals, suggesting that Single Mother-raised animals might have learned the test faster than Biparental-raised animals. We observed no other main effects or significant interactions for spatial learning performance (all $P > 0.15$; table S3).

We examined performance in the memory test using a GLM to analyze the latency to enter the location of the removed platform (platform zone) and the time spent swimming in the platform zone. We observed no differences in either measure of spatial memory

based on Sex, Parental Partner Status, or Parental Tradeoff Status (all $P > 0.25$; table S3).

Social approach

General social attraction/aversion was tested using a social approach testing paradigm (see the “Behavior” section in Supplementary Materials and Methods; Fig. 2A). Our analyses revealed a significant interaction between Sex and Parental Tradeoff Status [$F_{(1,65)} = 10.75$; $P < 0.01$; Fig. 2B]; males raised in Tradeoff families exhibited a longer latency to approach the stimulus animal compared to females raised in Tradeoff families (mean diff = 103.83; $P < 0.01$) and males raised in No Tradeoff families (mean diff = 128.00; $P < 0.01$). We also examined the time spent engaging in social investigation during the social approach test. Consistent with the latency to approach results, we found a significant interaction between Sex and Parental Tradeoff Status [$F_{(1,65)} = 7.08$; $P = 0.01$; Fig. 2C]; males raised in Tradeoff families spent significantly less time investigating the stimulus animal compared to females raised in Tradeoff families (mean diff = -63.69; $P = 0.05$) and males raised in No Tradeoff families (mean diff = -96.48; $P < 0.01$). Whether offspring were raised by single mothers or both parents did not affect offspring social approach or social investigation behavior (all $P > 0.07$; table S2). Thus, males raised under tradeoff conditions were relatively more reluctant to approach and investigate unfamiliar conspecifics.

Effects of parental variation on offspring nonapeptide gene expression and methylation status

Our overall goals in this study were twofold: (i) to characterize the ways in which early-life social experience in the natal nest can affect adult behavior (as just discussed) and (ii) to identify putative mechanisms by which early experience might affect the expression of adult behavior. As detailed above, epigenetic regulation of the nonapeptide system offers great potential toward addressing the second aim. Previous studies have shown that nonapeptide and nonapeptide receptor gene expression is regulated via methylation of CpG sites in promoter regions (35). Methylation in promoter regions is most frequently associated with transcriptional silencing; however, methylation at CpG sites along the gene body is often associated with increases in gene expression (36–38). Assessment of DNA methylation status of nonapeptide and nonapeptide receptor genes has primarily examined CpG sites in promoter regions, and thus, the role of methylation at CpG sites along the gene body has largely been unexplored. To gain a comprehensive understanding of how early-life social experiences influence gene expression and behavior via epigenetic modifications to nonapeptide genes, we analyzed methylation at CpG sites across the *avpr1a* and *otr* genes, extending beyond the promoter region.

We investigated the impact of being raised in (i) Single Mother or Biparental and (ii) Tradeoff or No Tradeoff families on nonapeptide

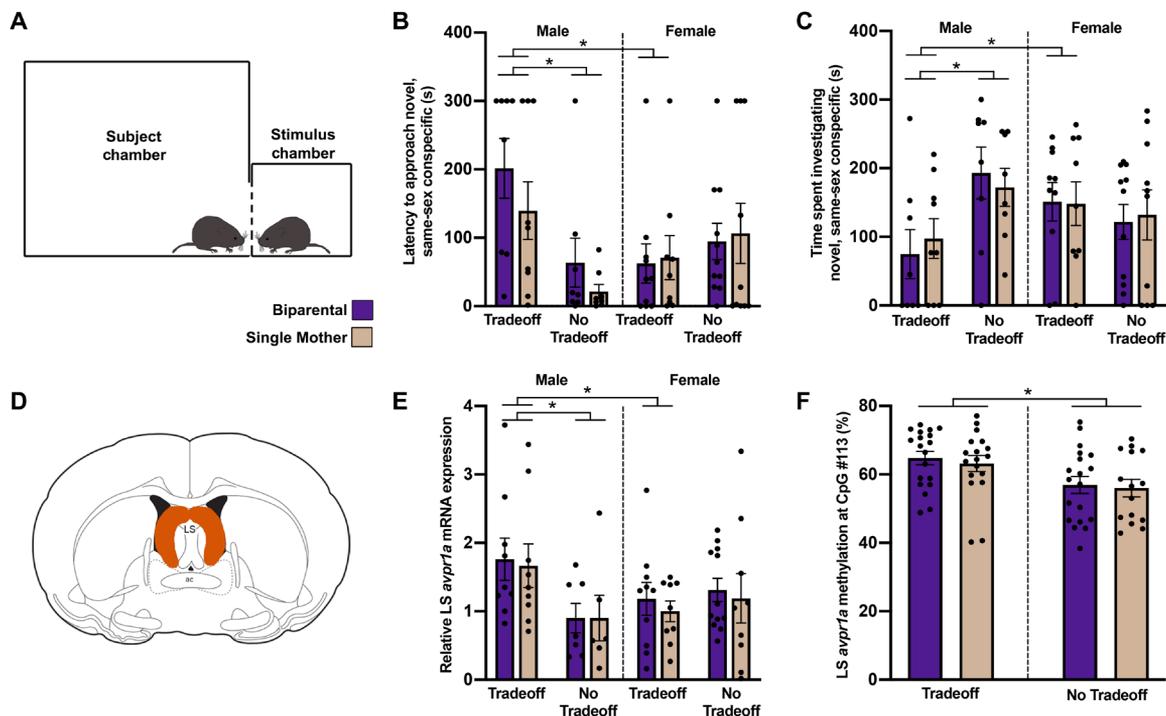


Fig. 2. Males raised in Tradeoff families exhibit retarded social approach behavior and increased *avpr1a* gene expression in the LS, and both sexes exhibit increased methylation of the *avpr1a* gene in the LS. (A) A schematic of the social approach test. Subjects were placed into a large chamber that contained a doorway, where a stimulus chamber was attached containing a novel, same-sex conspecific. (B) Mean (\pm SEM) time (in seconds) that male and female subjects raised in either Tradeoff or No Tradeoff and Biparental (purple) or Single Mother (beige) families took to approach a novel, same-sex conspecific and (C) spent socially investigating a novel, same-sex conspecific. (D) A schematic of a coronal section of a prairie vole brain illustrating the location of the LS in orange. AC, anterior commissure. (E) Relative vasopressin 1a receptor (*avpr1a*) gene expression in the LS. Mean (\pm SEM) relative LS *avpr1a* gene expression (mRNA) exhibited by male and female offspring raised in Tradeoff or No Tradeoff and Biparental or Single Mother families. (F) Mean (\pm SEM) LS *avpr1a* methylation at CpG #113 (%) exhibited by offspring raised in Tradeoff or No Tradeoff and Biparental or Single Mother families. Dots represent individual data. * $P \leq 0.05$.

receptor gene expression in four brain regions that are important for social and cognitive behaviors and express V1aR, OTR, or both [LS, NAcc, HPC, and RSC; see (26)]. The day after completion of behavioral testing, subjects were euthanized, and site-specific tissue punches were collected and processed via quantitative reverse transcription polymerase chain reaction (qRT-PCR) to determine *avpr1a* and *otr* mRNA levels (see Supplementary Materials and Methods). GLM analyses were conducted on all neural data, with Sex, Parental Partner Status, and Parental Tradeoff Status as fixed factors. All post hoc pairwise comparisons were adjusted using the Bonferroni correction.

The amount of V1aR gene expression in the LS showed a significant interaction of Sex and Parental Tradeoff Status [LS relative normalized *avpr1a* mRNA expression: $F_{(1,63)} = 6.42$; $P = 0.01$; Fig. 2, D and E]. Specifically, males raised in Tradeoff families exhibited significantly higher levels of V1aR gene expression in the LS than No-Tradeoff males (mean diff = 0.81; $P < 0.01$). Females, however, did not differ on the basis of Parent Tradeoff Status (mean diff = 0.16; $P = 0.53$). In addition, males exhibited significantly higher levels of LS V1aR gene expression than females in Tradeoff families (mean diff = 0.62; $P = 0.02$), but males and females raised in No-Tradeoff families did not differ (mean diff = 0.35; $P = 0.22$). Furthermore, males exhibited more V1aR gene expression than females in the RSC [relative normalized *avpr1a* mRNA: $F_{(1,63)} = 6.51$; $P < 0.01$]. However, neither early-life experience manipulation (Parental Partner Status and Parental Tradeoff Status) differed, and no interactions therein resulted, for *avpr1a* mRNA in the RSC (all $P \geq 0.06$; table S4). Last, we observed no main effects or interactions for *otr* mRNA in the LS, NAcc, or HPC (all $P > 0.10$; table S4).

Because gene expression of V1aR in the LS was the only instance in which early-life social experiences corresponded with gene expression differences, we assessed DNA methylation of the *avpr1a* gene in tissue punches taken from the LS via pyrosequencing. We identified and investigated methylation at 19 CpG sites across the *avpr1a* gene [4 CpG sites in the promoter region, 13 CpG sites across exon 1, and 2 CpG sites in the 3' untranslated region (3'UTR); see the "Pyrosequencing" section in Supplementary Materials and Methods and fig. S2, E to G]. Across these sites, only methylation at CpG #113 in the 3'UTR region showed a significant relationship with any manipulation of early-life experience. Specifically, animals raised in Tradeoff families exhibited significantly greater DNA methylation than animals raised in No-Tradeoff families at this site in the 3'-UTR region of the *avpr1a* gene within the LS [$F_{(1,63)} = 11.01$; $P < 0.01$; Fig. 2F]. We did not find any main effects or interactions for other LS V1aR CpG sites (all $P > 0.06$; table S4). Notably, we also found that LS V1aR gene expression significantly predicts DNA methylation at CpG #113 [Pearson's $R = 0.24$; $F_{(1,69)} = 4.21$; $P = 0.04$; Fig. 3A], indicating that more methylation at CpG #113 related to higher *avpr1a* mRNA levels in the LS. Together, our results indicate that differences in LS V1aR gene expression appear to result from epigenetic modification of a downstream 3'UTR site of the *avpr1a* gene in the LS that is putatively attributable to early-life social experiences made possible in Parental Tradeoff families.

LS neural correlates of social approach behavior

Evidence that early-life experience shapes brain phenotype must be paired with clear links to concomitant changes in behaviors associated with those neural mechanisms before a truly compelling account of how developmental context putatively affects functional behavior. To this end, we sought to identify potential behaviors susceptible to

the epigenetically altered V1aR gene expression in the LS just reported. We revisited our results for which behavioral data also demonstrated a main effect and significant interactions involving Parental Tradeoff Status. Only the performance in social approach tests showed this pattern. For brain-behavior relationships of other behaviors, see Supplementary Results.

Recall that latency to approach an unfamiliar same-sex conspecific was greatest in males raised in Tradeoff families (see above). The same pattern of behavior was evident when including relative LS V1aR gene expression as a covariate with Sex, Parental Partner Status, and Parental Tradeoff Status as fixed factors [GLM: Sex \times Parental Tradeoff Status; $F_{(1,60)} = 9.23$; $P < 0.01$; Fig. 3B] and when including LS *avpr1a* methylation at CpG #113 as a covariate with those fixed factors [GLM: Sex \times Parental Tradeoff Status; $F_{(1,62)} = 10.37$; $P < 0.01$; Fig. 3B]. In addition, a GLM analysis including the same fixed factors on the time spent socially investigating the stimulus yielded significant interactions when relative LS V1aR gene expression was used as a covariate [Sex \times Parental Tradeoff Status; $F_{(1,60)} = 6.34$; $P = 0.01$; Fig. 3B] and when LS *avpr1a* methylation at CpG #113 was used as a covariate [Sex \times Parental Tradeoff Status; $F_{(1,60)} = 6.75$; $P < 0.01$; Fig. 3B]. Together, these results indicate that the relationship between social approach behavior and LS *avpr1a* gene expression and DNA methylation significantly differ on the basis of the sex of the animal and whether or not the subject was raised in a Tradeoff or No-Tradeoff family.

To more clearly understand the relationships between social approach behavior and LS V1aR gene expression and DNA methylation, we conducted separate linear regressions for males and females raised in Tradeoff and No-Tradeoff families. LS *avpr1a* DNA methylation at CpG #113 directly related to the latency to social approach but only in males raised in Tradeoff families [Pearson's $R = 0.49$; $F_{(1,15)} = 4.79$; $P = 0.04$; Fig. 3, C to F, and table S5]. Relative LS *avpr1a* gene expression showed no relationship with the latency to approach a novel, same-sex conspecific (all $P > 0.24$; table S5). Similarly, although relative LS *avpr1a* gene expression did not significantly predict the time spent investigating the stimulus animal for males or females reared in Tradeoff or No-Tradeoff families (all $P > 0.21$; table S5), a linear regression revealed that LS *avpr1a* DNA methylation at CpG #113 significantly predicts social investigation, specifically in males reared in Tradeoff families [Pearson's $R = 0.51$; $F_{(1,15)} = 5.38$; $P = 0.04$; Fig. 3, G to J, and table S5].

These data indicate a putative link between social approach, a behavior that represents the initial steps to all subsequent social behavior, and epigenetic modification of a gene that, as we have demonstrated, is influenced in a sex-specific manner by early-life social experience. Notably, no other behaviors were significantly predicted by LS *avpr1a* DNA methylation or gene expression (see Supplementary Results). Early-life experience also resulted in differential gene expression of V1aR in the LS, which significantly related to methylation of the *avpr1a* gene in that brain region. Together, these results highlight a promising system in which determining whether differential experiences with parental care in the natal nest directly leads to epigenetically modified gene expression that induces reductions in social behavior.

DISCUSSION

We have demonstrated that ecologically relevant variation affects the behavior of parents, thereby altering the nature of caregiving that

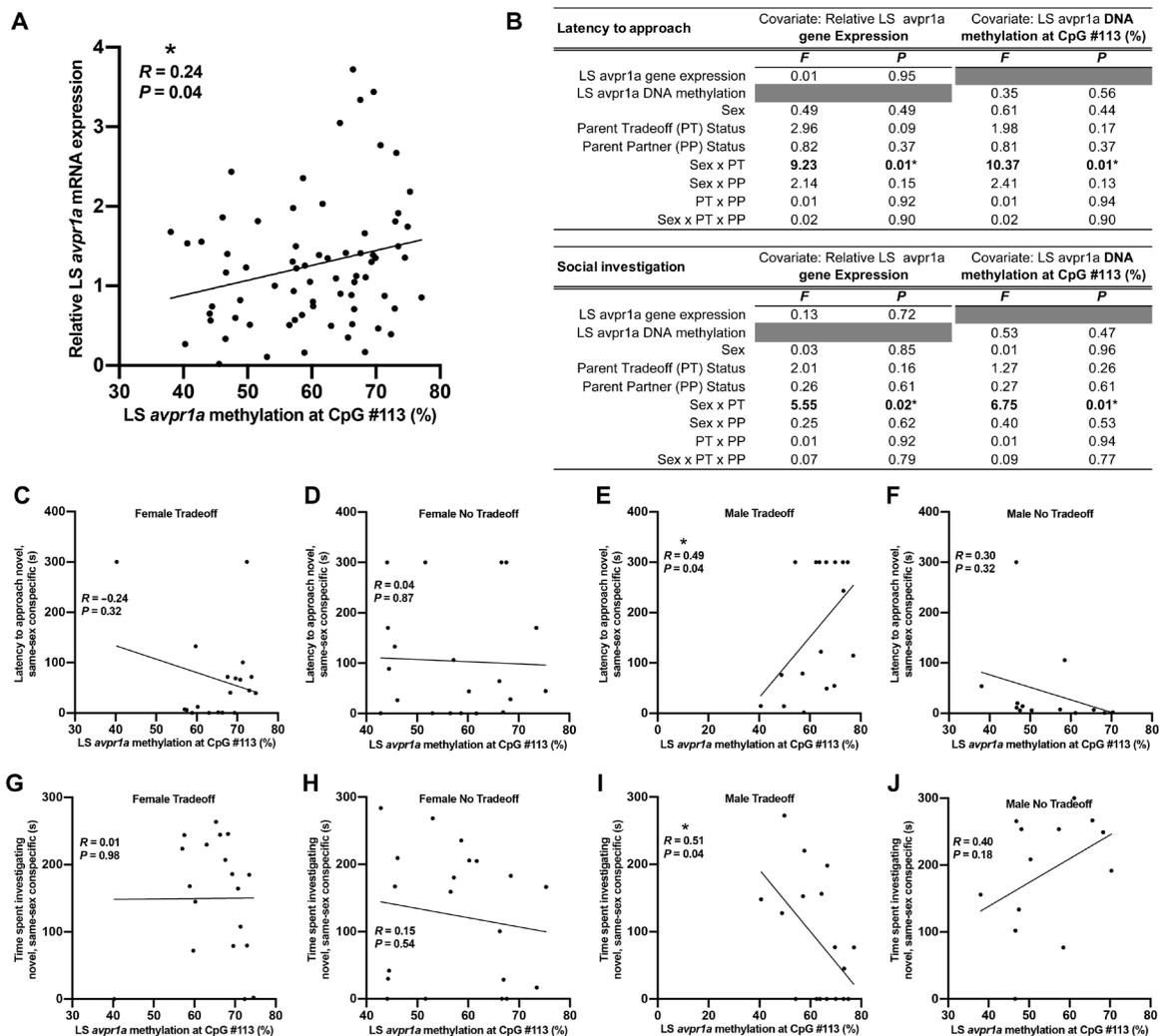


Fig. 3. *avpr1a* gene expression and methylation in the LS predict social approach behaviors in males reared in Tradeoff families. (A) Relative LS *avpr1a* gene expression (mRNA) positively correlates with LS *avpr1a* methylation at CpG #113 (%). (B) GLM results examining the latency to approach and time spent investigating a novel, same-sex conspecific from the social approach test with sex, Parental Partner (PP) Status, and Parental Tradeoff (PT) Status as fixed factors and either relative LS *avpr1a* gene expression or LS *avpr1a* methylation at CpG #113 as a covariate. All $F_{(1,60)}$. (C to F) Regression of methylation of the *avpr1a* gene in the LS at CpG #113 and the latency to approach a novel, same-sex conspecific (in seconds) from the social approach test for males and females raised in Tradeoff and No Tradeoff families. (G to J) Regression of methylation of the *avpr1a* gene in the LS at CpG #113 and the time spent socially investigating a novel, same-sex conspecific from the social approach test for males and females raised in Tradeoff and No Tradeoff families. Values in bold font and * indicate $P \leq 0.05$. Female No-Tradeoff/Biparental ($n = 11$), Female Tradeoff/Biparental ($n = 10$), Female No-Tradeoff/Single ($n = 9$), Female Tradeoff/Single ($n = 9$), Male No-Tradeoff/Biparental ($n = 7$), Male Tradeoff/Biparental ($n = 8$), Male No-Tradeoff/Single ($n = 6$), and Male Tradeoff/Single ($n = 9$).

offspring receive. In turn, the developmental trajectory of offspring behavior was directly affected by the variability in parental care that our manipulation produced. In the process, we highlight the importance of the father in the development of offspring social behavior and for co-parenting with mothers. For instance, we report that single mothers compensate for the lack of a co-parent, and mothers choose to invest in offspring over themselves when faced with a tradeoff. In contrast, fathers choose to invest in themselves over their offspring. Profound deficits in social approach was just one key consequence of paternal neglect observed in offspring, and this behavior related to epigenetic modification of the *avpr1a* gene and the magnitude of V1aR gene expression. These modifications were found in a nucleus of the brain for which V1aR has been intimately tied to

deficits in social behavior. We are unaware of any other study that provides evidence, direct or indirect, that draws a line from natal nest social experience to epigenetic modification and neural function within a nucleus known to modulate prosocial behavior to direct deficits in one of the most important elements of social behavior: the initiation of social behavior by way of the decision to approach a novel animal.

Postnatal social environment affects adult behavior: Mothers, fathers, and ecologically relevant paradigms

The influence of paternal care on offspring development is profoundly understudied. The few nonprimate mammalian studies that have examined paternal influences on development involve rearing rodent

offspring in the presence or absence of fathers (8). We extended this literature by using a novel assay that both manipulated father presence/absence and induced variation in parental care by forcing a tradeoff on all parents to choose between caring for themselves or caring for their offspring.

Although our prime focus was to investigate the consequences of social manipulations in the natal nest on offspring, it should not go unnoticed that manipulating the parenting context also produced differential experiences for the parents. For example, our results contradict previous studies that have shown that biparental rodent mothers do not compensate for absent fathers (25). We believe that our results are very likely due to our manipulation of a novel, ecologically relevant context that elevated the challenges parents face in the laboratory, thereby removing a laboratory-induced ceiling effect on the observable variability in parental care. In addition to single mothers increasing maternal care in the absence of a co-parent, they invested in their offspring over themselves in the tradeoff context. On the other hand, fathers were more inclined to invest in themselves over offspring. These discordant patterns of decision-making highlight interesting sex conflict differences based on parental investment theory that are common among nonmonogamous species but have been thought to be markedly reduced or absent among monogamous species (39). Our data suggest that among the socially monogamous prairie voles, classic sex differences in parental investment are extremely relevant and can potentially have transgenerational effects on social behavior (see below).

Sex conflicts can have major repercussions on the physiological condition of mothers. Energetic costs associated with mammalian maternal care are known to be high (39), so it is not terribly unexpected that mothers that lack the opportunity to share the costs of parental investment will pay a greater price. Individually shouldering the weight of offspring care might even have implications for survival; although unexpected deaths in our study were uncommon, most of the animals that did not survive were single mothers (see the “Rearing procedure” section in Supplementary Materials and Methods). These results highlight the importance of the dynamics between caregivers not only on the offspring but also on the co-parent. In the current case, the consequences resulting from mothers’ apparent prioritization of offspring above themselves appear to exacerbate the physiological toll that single mothers must endure. Results such as these raise a larger question about the extent to which social dynamics within a family unit can be attributable directly to the dyadic interactions between offspring and one (or each) parent or the second-order effects that result from interactions (or lack of interactions) between parents and how they then interact with offspring.

Our data demonstrated that although fathers spend less time brooding pups in the tradeoff condition, the overall care these pups received (from both fathers and mothers) did not differ. This discordance in the data raises the provocative point that the quality and quantity of parental care provided by mothers and fathers are not equivalent. Fathers provide different forms of paternal care ranging from direct to indirect care (40, 41), and the nature of care that fathers provide often differs from that of mothers (31, 42). Our results lean into and extend this notion, suggesting that even though the quantity of total care that pups received did not significantly differ, the quantity of paternal care did, and that shift in the proportion of maternal and paternal care produced a significantly different qualitative experience for the offspring. Although we were unable to deconstruct the nature of differences in maternal and paternal care

(and the total amounts of each), Lonstein and De Vries (31) reinforce this interpretation and document the types of behaviors that father and mother prairie voles provide. Thus, the altered access to the kinds of behaviors attributable to fathers that resulted from our manipulation could provide a mechanism by which experiences during development lead to behavioral and neural consequences in adulthood.

Variation in parental care in early life influenced the development of several behaviors important to aspects of prairie vole life history. For example, we found that being raised without a father significantly resulted in males and females spending less time exploring the inner and center zones of a novel open field, which can be interpreted as reflecting a more anxious or less exploratory phenotype. Many studies have demonstrated that a variety of experiences in early life, including reduced parental care, affect the development of cognitive function, increase anxiety-like behavior and stress responses, and reduce exploration [(3), but see (8)]. Our results suggest that offspring raised with both parents might be more likely to leave the nest. If so, then these same offspring might be more likely to establish their own breeding units rather than contributing to the communal breeding units established by their parents, demonstrating a putative link between parental care and the form of reproductive tactics that offspring ultimately adopt.

Exploring space effectively requires some degree of memory formation to create references (e.g., a “spatial map”). Like studies before ours (34), we found that prairie voles were capable of learning to locate the platform over the entire course of the water maze test and identify its location based on spatial cues. We found that animals raised without fathers learned the test more quickly than biparentally raised animals. Specifically, Single Mother–raised animals exhibited most of the learning between the first two trials, whereas Biparentally raised animals showed most of the learning later, between the second and third trials. Although this is a potentially subtle difference, it hints at the possibility that pups raised only by mothers are more readily prepared to face challenging environments requiring quick learning.

Prairie voles are a socially monogamous rodent best known for their ability to form pair bonds (23). It is interesting that both males and females raised without fathers spent more time huddling with the stranger than biparentally reared animals. Previous work has shown that only male prairie voles raised by single mothers exhibit impaired pair bonds (8). Because of this previous sex-specific finding, we also conducted planned separate analyses for the sexes. In a male-focused analysis, we found that Single Mother–reared males spent more time huddling with a stranger than Biparentally reared males. In contrast, the strength of the partner preference among females did not differ in a female-specific analysis. This suggests that the main effect in our overall model (which considered both male and female partner preferences) may largely be driven by male behavior. Together, these data indicate that the presence of a father is crucial to the formation of adult social bonds in males and appears to facilitate nonpromiscuous prosocial behavior in both sexes. It remains an open question whether the presence of a father in the natal nest explains the tendency for some bonded prairie voles to pursue and engage in extra-pair copulations, while others forgo such infidelity (43).

Dominance and aggression have a tremendous influence on access to resources necessary to acquire mates and successfully reproduce. We had intended to assess this using the dominance tube test, and our results demonstrated that the degree to which prairie

voles are dominant or subordinate is not sensitive to the forms of early-life social experience that we manipulated. Although dominance hierarchies have been well defined in other rodents, vole dominance has not been explored much. In contrast, prairie voles are well known to exhibit selective aggression against strangers after a bond has been established (27). A large qualitative difference exists between general dominance and proclivity for aggression, particularly selective aggression. Unfortunately, we did not assess selective aggression, and it is unclear whether early-life social experiences affect this form of fitness-enhancing agonistic behavior. Considering the impact that the presence and absence of fathers had on male pair bonding, we predict that (male) selective aggression would be sensitive to the presence of fathers in the natal nest, even though general dominance does not appear to be.

Together, our results indicate that variation in access to paternal care in early life affected the development of nonreproductive and reproductive behaviors important to prairie vole life history. These results indicate that the presence or absence of fathers has the potential to shape behaviors that are important in taking the first steps toward leading an independent, reproductively successful adult life and the particular form that they may take.

The transgenerational socio-(epi)genomics of approach behavior

The initiation of social behavior is contingent on one animal approaching another, perhaps making social approach the single most important component of social behavior. Without social approach, the ability to acquire mates, defend territories, establish social bonds and coalitions, and a number of other fitness-enhancing behaviors would be impossible. We showed that social approach is influenced by the Tradeoff context, demonstrating that males, but not females, raised in Tradeoff families exhibited social avoidance. Although it is difficult to directly identify what it was about the Tradeoff context that shaped male development, we believe that it is most likely attributable to the quality, quantity, and nature of paternal interactions; offspring had either no access to fathers or relatively less access to fathers when living in biparental contexts. Attributing retarded social approach and decreased social investigation in the Tradeoff families to paternal care is consistent with several studies that have shown that paternal care influences the expression of social investigation and contact, often in male but not female offspring (5). Our results appear to have resulted from increased disengagement by fathers, when they were present at all, rather than the simple father-present or father-absent scenario that these other studies used.

To understand how variation in parental care influences the development of the social brain, we examined gene expression for V1aR and OTR in four brain regions that are important for the modulation of social and cognitive behaviors. The only brain region for which we observed a significant difference based on manipulation of the early-life family environment was the LS (see Supplementary Discussion for brain regions that had nonsignificant findings). The LS is a brain structure rich in V1aR and OTR that integrates social, spatial, and stress-related processes and is believed to critically contribute to the modulation of context-specific motivational states and behavioral responses appropriate to specific environmental stimuli (28). The social approach test used in the present study used a same-sex stimulus animal. Veenema *et al.* (44) demonstrated that blockade of LS V1aR enhanced same-sex social play in males, suggesting that VP-V1aR binding may have antisocial functions in males. Consistent

with this hypothesis, we found that males raised in Tradeoff families (i.e., those with retarded social approach and decreased social investigation) exhibited significantly higher levels of LS *avpr1a* mRNA than males raised in No-Tradeoff families and females raised in Tradeoff families. We observed no effects or interactions for LS *otr* mRNA, suggesting that LS *avpr1a* mRNA may be particularly susceptible to the influence of parental care during postnatal development. Although previous work has shown that LS V1aR binding is sensitive to maternal separation in rats (45), our study is among the first to examine the influence of the father on the development of LS V1aR gene expression [but see (8)]. These studies both indicate that, like latencies to social approach, septal V1aR increases as a result of decreased interactions with parents (see above).

Although it is well established that early social experiences can have long-term consequences on behavior, the mechanisms mediating these effects are poorly understood. DNA methylation is a gene-specific epigenetic mechanism that offers great promise toward understanding how developmental trajectories are established in early life. We investigated DNA methylation across the *avpr1a* gene from tissue punches of the LS and found that the only component of the V1aR gene that exhibited significant methylation was at CpG #113 in the 3'-UTR. DNA methylation of CpG #113 was significantly higher in both males and females raised in Tradeoff families, and this methylation significantly and positively correlated with LS *avpr1a* mRNA expression. Notably, although DNA methylation is most frequently associated with transcriptional silencing, this is caused by methylation in promoter regions, whereas methylation at CpG sites along the gene body is often associated with increased gene expression (36–38). The significant positive association between LS *avpr1a* DNA methylation and gene expression strongly suggests that this relationship was due to the significant modification located at this downstream 3'-UTR site. These findings underscore the importance of examining CpG sites outside of the promoter region.

Developmental plasticity in LS V1aR expression was observed only in males, and it is known that male V1aR (and OTR) density throughout the forebrain is profoundly more sensitive to socioenvironmental factors during development [e.g., in (21)]. The observation that both male and female offspring demonstrated similarly high levels of LS *avpr1a* methylation at CpG #113 suggests that the female brain should be comparably susceptible to DNA modification. However, the fact that Tradeoff-reared female V1aR gene expression in the LS did not increase the way it did in males suggests that females are more resilient to perturbations in family social interactions compared to males. Furthermore, methylation only significantly correlated with the behavior of Tradeoff-raised males and not females. Together, these results support the conclusion that altered interactions with parents, resulting from tradeoffs rooted in parental investment, appear to affect LS *avpr1a* DNA methylation in offspring, which might alter gene expression in males, but not females, and result in retarded social approach behavior. These findings suggest a putative mechanism through which variation in parent-offspring interactions could lead to long-term shifts in brain function and behavior.

CONCLUSION

Developmental plasticity is ubiquitous in nature and is crucial for phenotypic diversity and adaptation (46). Thus, it is important to understand the forces that can shape an organism and the mechanisms

upon which they can act. Behavioral, neural, genetic, and physiological traits are all shaped by environmental influences and are particularly malleable early in life (3, 47). Plastic adjustments in response to early-life experiences can have profound effects on the development of social behavior that persist into adulthood (47). However, the mechanisms by which the early-life environment can influence behavior are not clearly understood. Our study suggests that in an ecologically relevant laboratory context, prairie vole single mothers compensate for the lack of a co-parent and, when faced with a tradeoff, choose to invest in their offspring over themselves. However, when faced with the same tradeoff, fathers choose to invest in themselves over their offspring. The social instability in the postnatal nest that this produced was sufficient to bend the developmental trajectories of offspring (and for males in particular) in ways that altered numerous aspects of behavior that presumably contribute to behavior important to vole life history. Our investigation of nonapeptide receptor gene expression and DNA methylation identified a putative mechanism mediating the interaction between varying life experiences and neural and behavioral phenotypes. Although our findings indicate that parental care throughout early development may interact with genetic and epigenetic mechanisms to shape adult behavioral phenotype in a sex-specific manner, continued work will be necessary to determine a causal role of nonapeptide-mediated social approach behavior. These studies will contribute to the growing body of literature examining the direct role of the LS in mediating early-life stress-induced social impairments [e.g., see (48) for septal dopamine-mediated behavior]. Direct assessment of nonapeptide-mediated behavior is necessary to fully understand the mechanisms underlying environmentally susceptible social behavior, and our study identifies a clear target for neurogenetic manipulation experiments that seek to directly link epigenetically modified septal V1aR with social approach behavior in males.

MATERIALS AND METHODS

Detailed descriptions of the methodology are provided in Supplementary Materials and Methods. All experiments were approved by the Institutional Animal Care and Use Committee of Cornell University (2013-0102).

Animals

All prairie voles used in this study were obtained from our breeding colony, from pairs that were offspring of wild-caught animals captured in Champaign County, Illinois, USA. All animals were housed in standard polycarbonate rodent cages (29 cm by 18 cm by 13 cm) lined with Sani-Chips bedding and provided nesting material. A 1.53-m-long plexiglass tube with an internal diameter of 7.6 cm was attached to cages and contained a wire food hopper at the end. Rodent chow (Laboratory Rodent Diet 5001, LabDiet, St. Louis, MO, USA) and water were given ad libitum. Animals were kept on a 14-hour light:10-hour dark cycle. Ambient temperature was maintained at $20^{\circ} \pm 2^{\circ}\text{C}$.

Design

We used a two-factor design to induce parental variation in the early-life family environment. The first factor describes the presence/absence of the father. Here, families were assigned to one of two conditions: a Single Mother or Biparental family. In Single Mother families, fathers were removed from the home cage 18 days after pairing

with the female and before the birth of pups. The second factor was the Tradeoff Status of parents. Food for all families was placed in a wire hopper at the end of the 1.53-m plexiglass tube attached to the home cage. The plexiglass tube for parents in the Tradeoff condition had a 20° incline. This angle was sufficiently steep to require more effort by both fathers and mothers to reach food. For example, mothers could not climb the tube with suckling offspring attached to their nipples and were required to detach and leave behind pups to feed. Parents that did not undergo the tradeoff manipulation lived in cages with a plexiglass tube that was at the same level as the home cage (i.e., no incline), requiring minimal effort to feed and allowing mothers with attached suckling pups to take offspring to feed with them.

Families were moved to the testing apparatus home cages on the day pups were born. Family units remained there until PND 21, when offspring were weaned and moved into standard rodent housing cages. Once the offspring reached PND 50 to 55, all subjects underwent behavioral testing.

Behavioral testing

All subjects underwent a partner preference test to measure strength of pair bonds, a dominance tube test to determine dominance status, an open-field test to assess exploratory behavior, the Morris water maze test to evaluate learning and memory, and a social approach test to measure same-sex affiliation. For detailed descriptions and behavioral testing order, see the “Adult behavioral testing” section in Supplementary Materials and Methods.

Histology

Following the completion of behavioral tests, subjects were euthanized by CO_2 suffocation, and brains were immediately extracted. Brains were flash-frozen using powdered dry ice and stored at -80°C . Tissue punches were collected from target brain regions inside a -20°C cryostat and shipped on dry ice to the University of Arizona Genetics Core for RNA extraction and qRT-PCR and to EpigenDx for DNA extraction, multiplex PCR amplification, library preparation, and targeted NextGen bisulfite sequencing. For full details on tissue processing, see Supplementary Materials and Methods.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/6/36/eabb9116/DC1>

[View/request a protocol for this paper from Bio-protocol.](#)

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Acknowledgments: We thank M. L. Rice for assistance with maintenance of family home cages and scoring behavioral videos. A special thank you to Karen Ophir (1949–2020), who so often prioritized her family over herself. **Funding:** The authors acknowledge the support from the National Institutes of Health Eunice Kennedy Shriver National Institute of Child Health and Human Development (HD081959 to A.M.K. and HD079573 to A.G.O.). **Author contributions:** A.M.K. and A.G.O. designed the experiment. The experiments were carried out by A.M.K. with assistance in rearing animals and conducting behavioral tests from J.Y.O. and R.A.W. The manuscript was written by A.M.K. and A.G.O. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 26 March 2020
 Accepted 17 July 2020
 Published 2 September 2020
 10.1126/sciadv.abb9116

Citation: A. M. Kelly, J. Y. Ong, R. A. Witmer, A. G. Ophir, Paternal deprivation impairs social behavior putatively via epigenetic modification to lateral septum vasopressin receptor. *Sci. Adv.* **6**, eabb9116 (2020).

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Sci Adv 6 (36), eabb9116.
DOI: 10.1126/sciadv.abb9116

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