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Father's care uniquely influences male neurodevelopment

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Mammalian infants depend on parental care for survival, with numerous consequences for their behavioral development. We investigated the epigenetic and neurodevelopmental mechanisms mediating the impact of early biparental care on development of alloparenting behavior, or caring for offspring that are not one's own. We find that receiving high parental care early in life leads to slower epigenetic aging of both sexes and widespread male-specific differential expression of genes related to synaptic transmission and autism in the nucleus accumbens. Examination of parental care composition indicates that high-care fathers promote a male-specific increase in excitatory synapses and increases in pup retrieval behavior as juveniles. Interestingly, females raised by high-care fathers have the opposite behavioral response and display fewer pup retrievals. These results support the concept that neurodevelopmental trajectories are programmed by different features of early-life parental care and reveal that male neurodevelopmental processes are uniquely sensitive to care by fathers.

parental care | nucleus accumbens | prairie vole | social behavior | neurodevelopment

Significance

Early-life parental care is critical for mammalian infants and influences numerous neurological and behavioral outcomes. While many studies in rodents examine the impact of maternal care on such outcomes, fewer studies specifically examine how the composition of parental care from both mothers and fathers influence neurodevelopment and behaviors. Here, using the biparental prairie vole, we show that male offspring are uniquely sensitive to care by fathers: Males raised by high-care fathers have more synapses in reward circuitry and display high parental care toward infant pups later in life. This work provides evidence that neurodevelopmental processes are highly sensitive to care by fathers and provides insight into typical neurodevelopmental processes which may be disrupted in male-biased neurodevelopmental disorders.

Early-life experience, particularly the type of care received by parents, is a critical determinant of the developmental trajectory of neuropsychological outcomes. In humans, early-life stress from low care or unstable environments is associated with many adverse neuropsychological outcomes, including disruptions in emotion regulation (1), as well as changes to the structure and connectivity of limbic regions (2). More subtle variation in parental behavior also has long-lasting effects on neuropsychological outcomes in children. For example, maternal and paternal warmth is correlated with increased prosocial behavior (3), and maternal warmth early in childhood is associated with decreased activation in the medial prefrontal cortex and striatum during reward anticipation in early adulthood (4). While the effects of early-life experience in the form of parenting are well documented, the neurobiological mechanisms mediating the effects are not well defined.

Early-life experience can impact behavior later in life by changing the pace of development. In humans, early-life stress, particularly via low parental care, accelerates biological aging with implications for stress response and emotion regulation throughout the lifespan (5, 6). One biological indicator of aging is epigenetic age, which uses DNA methylation measurements to predict age (7). If one's predicted epigenetic age is higher than their chronological age, they would be age-accelerated—that is, they have aged faster than their chronological age suggests. We and others have shown that early-life social experiences impact epigenetic aging. Early-life experiences that increase epigenetic age include exposure to violence, trauma, low socioeconomic status, and poor peer relationships (8–12).

In rodent models, it is well established that variation in maternal care leads to individual differences in development and behavior (13–15). One relevant model for studying development of social behaviors is the prairie vole (*Microtus ochrogaster*), a species which displays human-like social behaviors including monogamous pair bonding as well as both maternal and paternal care of offspring (16, 17). Prairie voles display a naturally wide range of parental behaviors which are linked to offspring's variability in pair bond formation and alloparenting (parenting a pup that is not one's own) later in life (18–21). In voles, a single episode of handling on the first day of life transiently increases parental care behavior by the parents, leading to increased pair bond formation and parental care behavior in the offspring (22, 23).

In mice and rats, it is well established that low maternal care alters single gene and genome-wide gene expression throughout the lifespan in many brain regions (24–30). In voles, we have shown that higher amounts of pup-directed care early in life changes the epigenetic and transcriptional state of the offspring's brain. High early care leads to lower levels of DNA methylation and concomitant higher expression of the oxytocin receptor gene (*Oxtr*) in the nucleus accumbens (22, 31). This brain region is central to the reward pathway and a node of the social decision-making network critical for species-typical parental

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behaviors (32). Interestingly, genome-wide changes resulting from differences in early care have not been mapped in voles.

In addition to impacts on the epigenome and transcriptome, early-life care has consequences for neurodevelopmental outcomes including synapse development throughout the brain (33–36) and impacts the development of many neurotransmitter systems in the nucleus accumbens, including glutamatergic signaling (29), catecholamines (37), and opioids (38). These data suggest numerous circuitry differences in this region as a result of variation in early-life care. Importantly, much of this type of work is studied in species which typically only provide maternal care in the lab. Studies in the degu (*Octodon degus*), a biparental rodent, indicate that neurodevelopmental outcomes are not only sensitive to total parental care but may also be sensitive to the relative amounts of care by mothers and fathers (39, 40). Early-life care can also alter neuroimmune outcomes including the number and morphology of microglia (41, 42), the expression of cytokines (43, 44), and other immune regulators (45). In prairie voles, microglia proliferation is sensitive to social experience (46, 47). Altogether, these studies suggest that early-life care profoundly impacts the development and circuitry of the brain, including the nucleus accumbens, and suggest the need to study this in a biparental model.

Many studies of early-life experience use paradigms that are not naturalistic, including maternal separation and paternal deprivation, which may not recapitulate the typical childhood experience (48). Additionally, even more naturalistic early-life stress models such as the limited bedding and nesting model, which induces chronic stress to the mother, result in atypical parental behavior (49). Here, we examine the impact of biparental care on neurodevelopment using two types of parental care paradigms. The first is a handling model, where the parents of one group are directly handled (MAN1) and the control group is indirectly handled (MAN0). We have previously shown that this subtle manipulation increases biparental care during the first week of life (22). The second paradigm is a natural parenting observation where the breeding pairs are unmanipulated, and instead, the parental behaviors are directly quantified. Using these paradigms, we provide epigenetic and neuroanatomical evidence that high total biparental care slows maturation. We also find that gene expression in the nucleus accumbens is sensitive to early-life biparental care in male offspring only. Surprisingly, we found that care by fathers is associated with changes in excitatory synapses in the nucleus accumbens of male offspring only. Finally, we show that early-life care by fathers impacts alloparental care in a sex-specific manner: Males raised with more care by fathers display more pup retrievals while females raised with more care by fathers display fewer pup retrievals. These findings provide robust molecular evidence that male prairie voles are uniquely sensitive to differences in early-life care by fathers with consequences for neurodevelopment and behavior.

Results

High Early-Life Biparental Care Slows Epigenetic Aging. Prairie voles reared under conditions in which they are left relatively undisturbed, with no direct handling of either pups or parents for the first week of life (MAN0), display marked disruptions later in life in alloparenting, pair bonding, and parenting (23, 50). In contrast, offspring of parents that receive a brief direct handling manipulation (MAN1) on postnatal day (PND) 1, in which mothers and fathers are lifted by an investigator's gloved hand for 30 s while pups are attached to the mother's nipples by their milk teeth but never touched by the investigator, show later adult behaviors typical of this species. In rats, brief early handling leads to similar changes in adult offspring behavior (13, 51), likely because dams increase pup licking and grooming immediately after the handling episode (52, 53). Alternatively, rats receiving little to no early-life handling disturbance had increased anxiety-like behavior in adulthood (54). We have previously shown using this MAN0/MAN1 handling paradigm that the MAN1 condition leads to high-care parenting, which is consistent with handling experiments in rats (22).

One mechanism by which variation in early biparental care might alter behavioral outcomes later in life is by changing developmental trajectories. Previous work in prairie voles has examined how parental care and family structure impact developmental markers, though this literature has many conflicting results (19, 55–59). One study which examined developmental outcomes among either high-contact or low-contact parent dyads found that high-contact parenting slows offspring development: Offspring of high-contact parents opened their eyes later, ate solid food later, and left the nest later than those raised by lower-care parents (19). Studies which examine the role of parenting using paternal deprivation models where fathers are removed often find that pups raised with mothers only (and presumably lower care) have slower development (57, 58), though this manipulation is much more extreme and may be more akin to long-term maternal separation in rats, which does not have the same effects on offspring behaviors as brief maternal separations that lead to increased licking and grooming (53).

DNA methylation estimators of age can be used to determine whether an individual is developing at a faster or slower pace than predicted by their chronological age (7). Recently, a pan-mammalian methylation array and clock was developed to extend this tool to nonhuman species including prairie voles (60, 61). In order to determine how differences in the amount of early biparental care impact this biological marker of development, we manipulated parental care on PND 1 (MAN0 vs. MAN1), allowed parents to raise animals to weaning, and measured epigenetic age using DNA from the nucleus accumbens of juvenile offspring (Fig. 1*A*). We chose this region of the brain because it has been implicated in several species-typical social behaviors including pair bonding and alloparenting. We find that animals raised in the MAN1 condition, which receive higher total early-life biparental care (22), have slowed epigenetic aging ($F_{(1,11)}$ = 9.45, P = 0.011, Fig. 1*B*). Notably, the difference in the epigenetic ages of the two groups is over a week: The mean epigenetic age acceleration of the MAN0 group is +3.869 d, while the mean epigenetic age acceleration of the MAN1 (high-care) group is −4.928 d. There was no effect of sex ($F_{(1,10.48)}$ = 2.44, P = 0.148), nor an interaction between sex and handling condition ($F_{(1,10.48)}$ = 0.08, P = 0.777). These results provide evidence that higher biparental care leads to slower epigenetic aging, possibly through a mechanism that slows maturation generally.

High Early-Life Biparental Care Changes the Transcriptional Profile of the Male Brain. Changes in the epigenetic profile of the brain early in life may lead to distinct downstream transcriptional changes. To investigate how early biparental care impacts gene expression, we performed RNA sequencing on RNA derived from the nucleus accumbens of juvenile voles that were raised in the MAN0 or MAN1 environment (22). Differential expression analysis was performed separately for males (MAN0 vs. MAN1) and females (MAN0 vs. MAN1) because previous literature indicates sex-specific transcriptomic responses to early-life stress in other rodent species (28–30) and sex-specific behavioral responses to the handling manipulation in prairie voles (23). Analyses corrected at genome-wide significance identified 321 differentially expressed

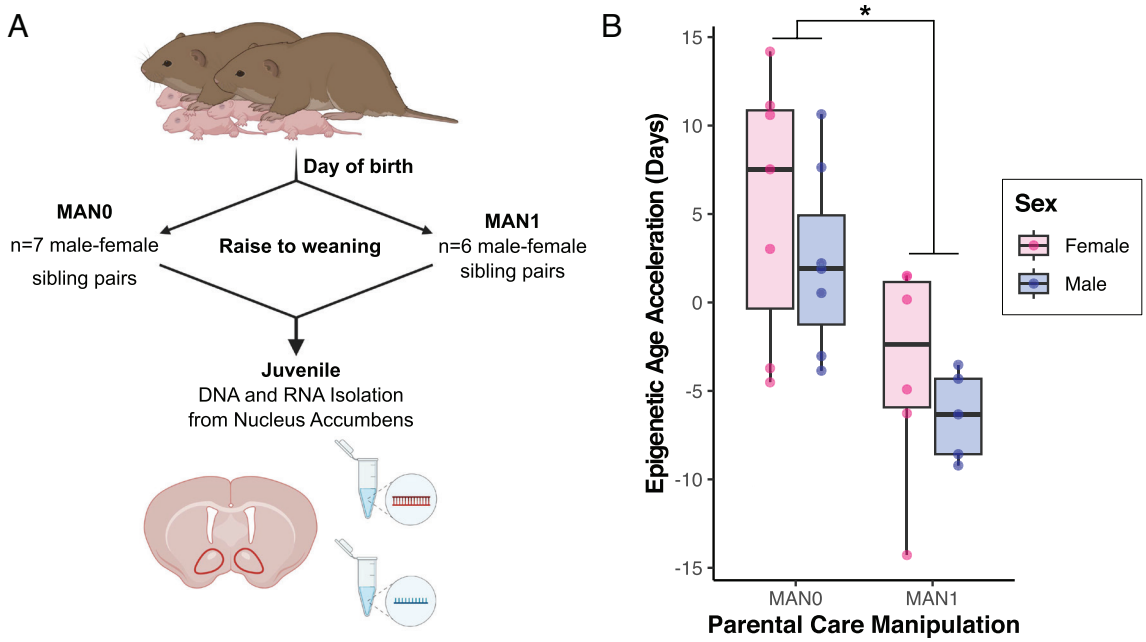

Fig. 1. High early life biparental care is associated with age deceleration in offspring. (*A*) Schematic showing experimental design. Litters were placed in either the indirect (MAN0) or direct (MAN1) handling condition and raised until weaning. DNA and RNA were then isolated from the nucleus accumbens. (*B*) Prairie voles raised in the MAN1 condition, which leads to an increase in parental care in the first week of life (22), show epigenetic age deceleration ($F_{(1,11)}$ = 9.45, P = 0.011). *P < 0.05.

genes (175 genes with higher expression in MAN1 and 146 genes with lower expression in MAN1) in male offspring (Fig. 2*A* and *SI Appendix*, Table S1). We note that we previously found that the MAN1 (high-care) condition leads to increased expression of the oxytocin receptor gene (*Oxtr*) (22), which we replicate here (*SI Appendix*, Table S1), though *Oxtr* does not receive an adjusted p-value because of its relatively low abundance in the nucleus accumbens. In females, three genes were differentially expressed (FDR < 0.1): ENSMOCG00000004642, *Ak8*, and ENSMOCG00000000123 (Fig. 2*A* and *SI Appendix*, Table S2). These results indicate a substantial sex difference in the genome-wide gene expression dynamics associated with early-life biparental care and suggest that females are resilient to changes in biparental care while males are highly impacted. To investigate biological processes that may be impacted by high early-life biparental care in the male brain, gene ontology analysis was performed separately for up-regulated and down-regulated genes. Up-regulated genes (genes with higher expression in MAN1 males) are involved in neuronal processes including neural development, synapse organization, and synaptic signaling (*SI Appendix*, Table S3). Further, up-regulated genes are localized to synaptic structures and are functionally involved in ion channel activity and cell–cell adhesion (*SI Appendix*, Table S3). Down-regulated genes (genes with lower expression in MAN1 males) are involved in metabolic processes and immune processes (*SI Appendix*, Table S4). A separate KEGG pathway analysis further supports these findings, with enriched KEGG pathways including neuroactive ligand–receptor interactions, axon guidance, and several synaptic and metabolic pathways (*SI Appendix*, Table S5).

High Early-Life Biparental Care Increases Expression of Genes Associated with Autism Spectrum Disorder. Given the male-specific effects of early-life experience on gene expression and social behavior (23), we tested whether the differentially expressed genes in males were enriched for autism risk genes. We found a significant enrichment of genes in the SFARI gene set (62) (Fig. 2 *B* and *C*), indicating that genes which are disrupted in autism spectrum disorder are also sensitive to early-life experience and are related to more subtle variability in social behaviors. Autism risk genes are typically loss-of-function. We find that of the 42 autism risk genes differentially expressed in males, 40 have higher expression in males that were raised in high early care environments (MAN1). To prevent misinterpretation of this data, it is critical to mention that while MAN0 male offspring have lower overall expression of autism risk genes, they still express functional products of these genes and display typical social behavior. Thus, parental care can modulate expression of autism risk genes but does not lead to an autism-like phenotype.

Higher Care by Fathers Is Associated with Increased Asymmetric Synapse Density in Male Offspring. RNA-sequencing results indicate in males only, high early-life biparental care leads to increased expression of genes related to neurodevelopment and synaptic transmission. A simple explanation for these results may be that male offspring raised by high-care parents have more synapses in the nucleus accumbens. We used electron microscopy to quantify the volumetric synapse density (Fig. 3*A*) and synapse type (asymmetric or symmetric, Fig. 3 *B* and *C*) in the nucleus accumbens core. The symmetric, Gray type II synapses (63) are formed by GABA immunoreactive, presumed inhibitory terminals (64). Asymmetric synapses, on the other hand, originate from typically excitatory neurons, including from the prefrontal cortex, amygdala, thalamus, and hippocampus (65, 66). The nucleus accumbens also receives inputs from diverse neurotransmitter systems including dopaminergic ventral tegmental area neurons, which can form symmetric and asymmetric synapses, and cholinergic interneurons (65, 67). Examining how early-life care impacts both excitatory and inhibitory synapses may indicate that specific circuitries are impacted by parental care while others are not.

We characterized 2,335 synapses (median 204 synapses per animal) from 433 electron micrographs (median 36 images per

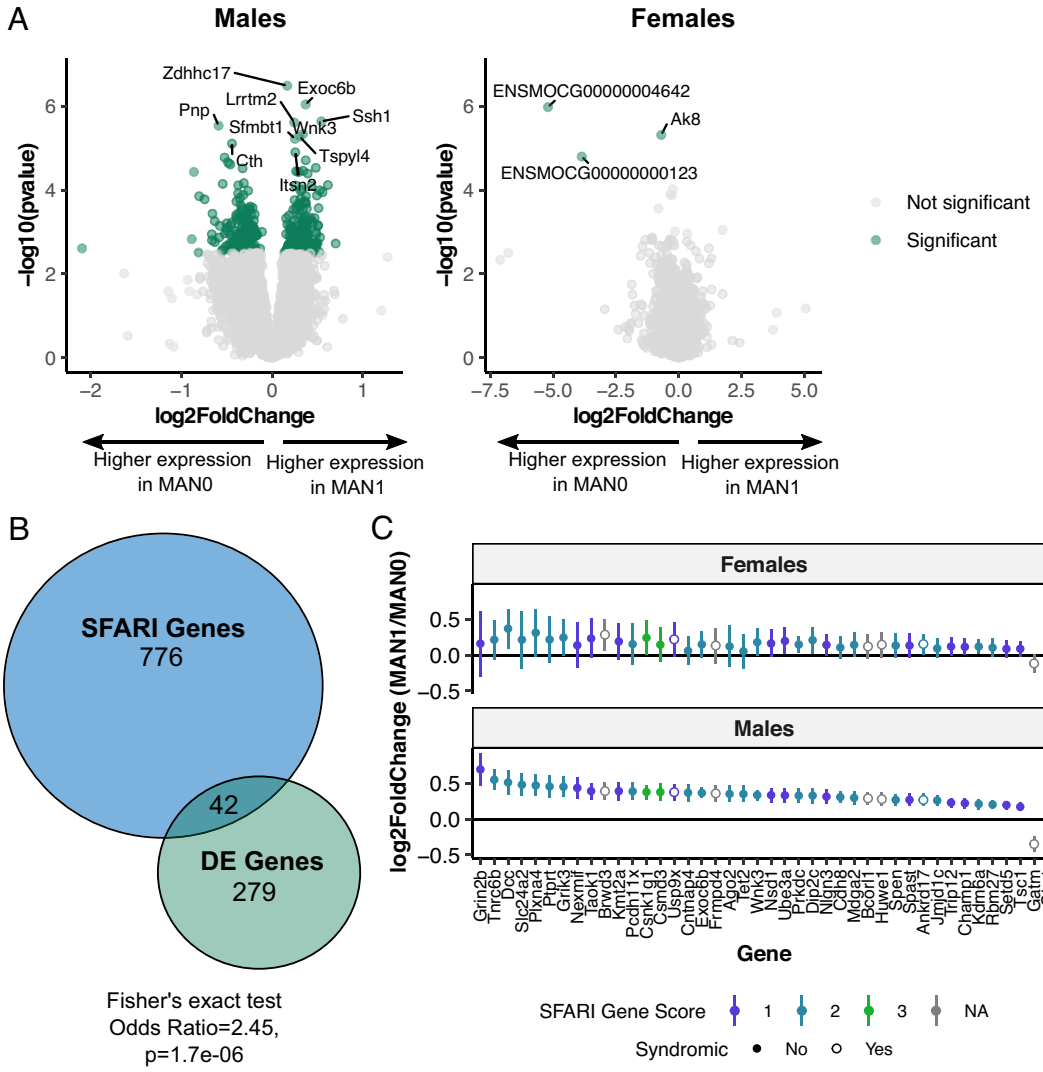

Fig. 2. High early-life biparental care specifically impacts the male nucleus accumbens transcriptome. (*A*) RNA-sequencing reveals 321 differentially expressed genes in males raised in MAN1 (high biparental care) and MAN0 (low biparental care) conditions, but only three differentially expressed genes in females. (*B*) Enrichment (n = 42) of SFARI autism risk genes in differentially expressed (DE) gene set in males. (*C*) Graph showing log2(Fold Change) of SFARI autism risk genes in both males and females. Points indicate log2(Fold Change), and bars indicate the SE of the estimate. Points are colored by SFARI risk category (1 = high confidence, 2 = strong candidate, 3 = suggestive evidence). Open points indicate genes responsible for syndromic forms of autism.

animal) in 11 animals (n = 6 sibling pairs, 6 female, and 5 males). The most common type of synapse observed was asymmetric onto spines (64.5%), followed by asymmetric onto dendrites (19.9%). Symmetric synapses were less common and occurred more frequently onto dendrites (10.9%) than spines (3.4%). Synapses onto cell bodies or axons were rare (<1%). Synapse density was calculated for each animal in three separate regions of interest in the nucleus accumbens core (68, 69). In each region of interest, we calculated total synapse density, asymmetric synapse density, and symmetric synapse density. Total parental care was quantified (*SI Appendix*, Fig. S1*A*), and we observed no association between litter size or sex ratio within the litter with total parental care (*SI Appendix*, Fig. S1 *B* and *C*), indicating that potential sex differences in results would not be due to males and females eliciting different amounts of behaviors from parents. Contrary to our hypothesis, there was no significant three-way interaction between sex, total parental care, and synapse type ($F_{(1,51)} = 0.496$, $P = 0.49$), but there was a significant interaction of total parental care and synapse symmetry ($F_{(1, 51)} = 13.3$, $P < 0.001$) such that increased total parental care was moderately associated with an increase specifically in asymmetric synapse density in both sexes (Fig. 3*D*).

Recent studies indicate that in prairie voles, there is a specific effect of care by fathers on the development of social behaviors and neuropeptide systems in male offspring only (56, 70, 71). We tested the impact of parental care composition (calculated as the duration of care by fathers divided by total parental care duration), rather than total parental care, on synapse density. Parental care composition is operationalized as percent of total parental care provided by fathers (hereon referred to as percent care by fathers) because in prairie vole dyads, fathers are more likely to contribute to variation in care in part because a minimum amount of care is required by the mother to keep the pups alive (18). Using behavioral data from our breeding colony, we replicate results by Finton and Ophir (18) by examining care by mothers and fathers in prairie vole breeder pairs that have been categorized as high-, medium-, or low-care pairs. We find that care by moms does not differ among the different categories of dyads, but care by fathers does (*SI Appendix*, Fig. S2). Put another way, care by fathers drives population-level variation in biparental care. Though care by mothers and care by fathers are inversely related (*SI Appendix*, Fig. S1*D*), there is no correlation between total parental care and the percent care by fathers (*SI Appendix*, Fig. S1*E*), indicating that these are independent features of biparental care. Additionally, percent care by fathers is not impacted by either litter size or sex ratio (*SI Appendix*, Fig. S1 *F* and *G*). We found a significant interaction between sex, percent care by fathers, and synapse symmetry while accounting for total parental care ($F_{(1,50)} = 6.222$, $P = 0.016$) such that higher levels of care by fathers are associated with increased asymmetric synapse density in male offspring only (Fig. 3*E*). These results suggest that increased care by fathers

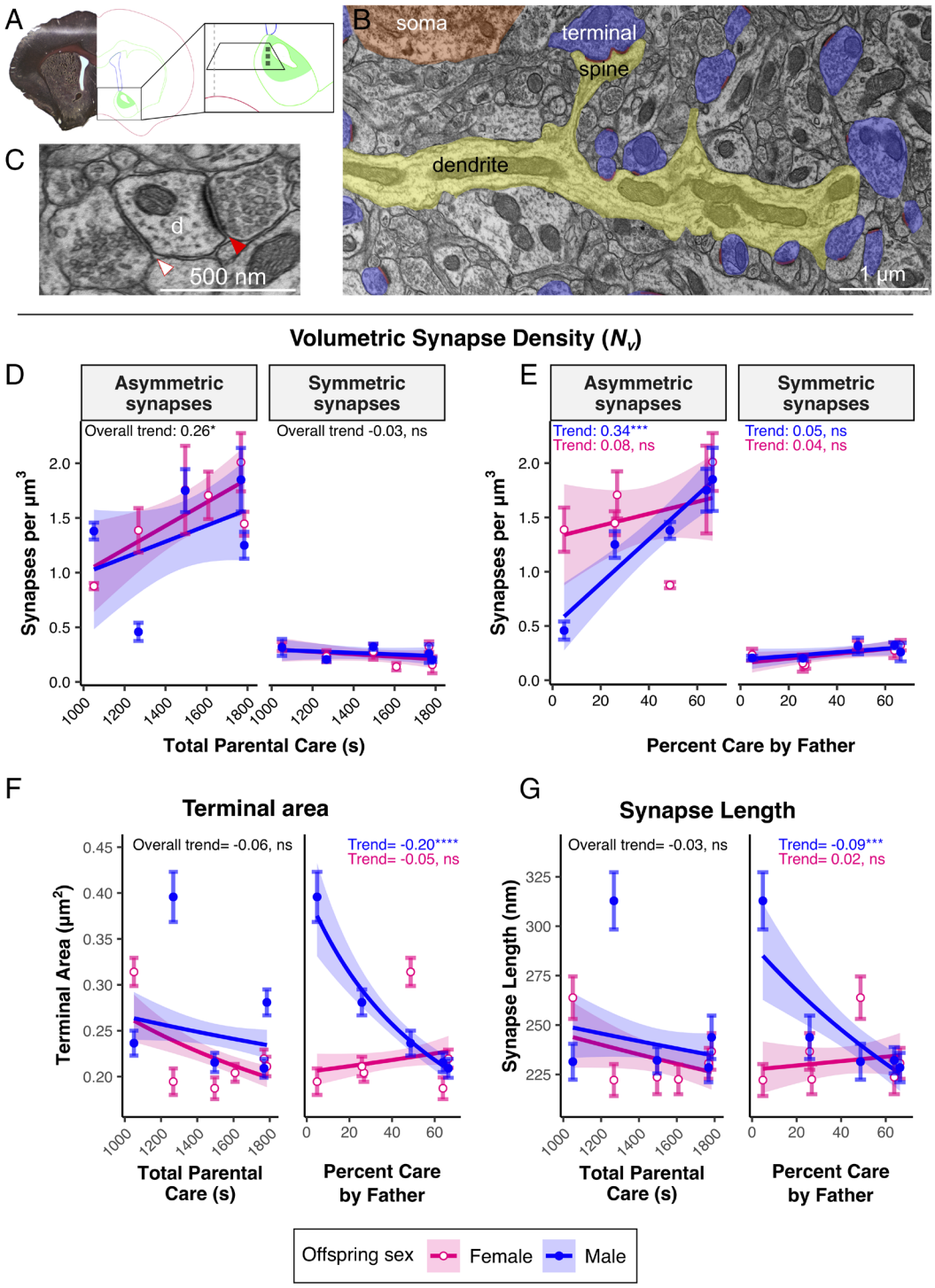

Fig. 3. High percent care by fathers is associated with an increase in excitatory synapse density and decrease in synapse size in male offspring only. (*A*) Example myelin stain and atlas of the brain section containing the nucleus accumbens (with the core filled in green). The zoomed inset contains the trapezoid sectioned for electron microscopy, and the shaded black boxes show where pictures were taken from. (*B*) Example electron micrograph at 12,000× magnification. A dendrite is pseudocolored in yellow. Terminals are blue and synapses are red. A cell soma is pseudocolored orange. (*C*) Cross-section of a dendrite (labeled "d") receiving two synapses, indicated by red arrowheads. The filled arrowhead shows an asymmetric synapse, where the postsynaptic density is dark and thick. The unfilled arrowhead shows a symmetric synapse, where the postsynaptic density is not darker or thicker than the presynaptic density. (*D*) There is a moderate effect of total parental care on asymmetric synapse density, but no effect on symmetric synapse density (total parental care × synapse symmetry interaction, $F_{1,51} = 13.3$, $P < 0.001$). (*E*) There is a significant three-way sex × percent care by father × synapse symmetry interaction ($F_{1,50} = 6.222$, $P = 0.016$) such that male offspring raised with more care by fathers have higher excitatory synapse density. Data points represent the average synapse density of three regions of interest in an animal, and bars represent the SE. (*F*) Male offspring raised with more care by fathers have more small-area synapses, while female offspring show no relationship (sex × percent care by father interaction, $\chi^2_{(1)} = 9.83$, $P = 0.002$). (*G*) Male offspring raised with more care by fathers have shorter synaptic zones (sex × percent care by father interaction, $\chi^2_{(1)} = 6.27$, $P = 0.012$). *$P < 0.05$, ***$P < 0.001$ ****$P < 0.0001$, ns $P > 0.05$

specifically alters the development of glutamatergic synapses in the nucleus accumbens in male offspring only. Notably, males raised with low care by fathers have much lower synapse density than their sisters, while no such sex difference exists in siblings raised with more care by fathers, suggesting that high care by fathers reduces sexual dimorphism in the nucleus accumbens.

The increased synapse density associated with increased care by fathers might occur because of a decrease in nucleus accumbens volume or a true increase in the synapse number. Nucleus accumbens volume was measured using serial myelin sections. There was no correlation between nucleus accumbens volume and total parental care ($\rho(11) = -0.03$, $P = 0.93$), nor was there a correlation between nucleus accumbens volume and parental care composition ($\rho(11) = -0.22$, $P = 0.51$). These results indicate that nucleus accumbens volume does not change with parental care, nor do changes in synapse density result from changes in nucleus accumbens volume.

Percent Care by Fathers Impacts Synapse Morphology in Male Offspring Only. Circuitries are formed by convergence of different inputs originating from distinct brain regions. Terminals formed by each input tend to have characteristic morphological parameters which differ from other inputs. With this in mind, we next tested whether aspects of parental care were associated with cross-sectional terminal areas, which may be characteristic of specific inputs into the

nucleus accumbens. For example, in rats, terminals in the nucleus accumbens originating from the prefrontal cortex and amygdala tend to be smaller than terminals originating from the hippocampus or thalamus (72). There was no significant effect of total parental care ($\chi^2_{(1)} = 2.56$, $P = 0.109$), nor an interaction of total parental care and sex on terminal areas ($\chi^2_{(1)} = 1.40$, $P = 0.238$, Fig. 3*F*). There was, however, a significant interaction of sex and percent care by fathers ($\chi^2_{(1)} = 9.83$, $P = 0.002$, Fig. 3*F*) such that more care by fathers was associated with on average smaller terminals in male offspring only. Similarly, there was no effect of total parental care on synapse length ($\chi^2_{(1)} = 1.22$, $P = 0.270$), nor an interaction of total parental care and sex ($\chi^2_{(1)} = 0.24$, $P = 0.623$, Fig. 3*G*). There was a significant interaction of sex and percent care by fathers ($\chi^2_{(1)} = 6.27$, $P = 0.012$, Fig. 3*G*) such that more care by fathers was associated with on average shorter synapses in males only. Much like volumetric synapse density, there is a sex difference in both terminal area and synapse length in litters raised with less care from fathers, which is not present in the litters raised with more care from fathers. This provides further evidence that high care by fathers reduces sexual dimorphism in the nucleus accumbens. Additionally, these results indicate that males raised with high care from fathers have more characteristically smaller synapses. While we cannot make any claims regarding the specific circuitries that might be changing, it remains possible that high care by fathers leads to selective synaptogenesis of characteristically small excitatory inputs (prefrontal cortex or amygdala) or reduces pruning of characteristically large excitatory inputs (hippocampus or thalamus). Our findings cannot differentiate these possibilities. However, they do reveal a selective influence of percent care by fathers on the synaptic circuitries of the nucleus accumbens in male offspring.

Microglia Density but Not Morphology Is Impacted by Total Biparental Care in Both Sexes. Results indicate that male offspring raised with high biparental care have changes in expression of genes related to immune processes and synapse pruning by microglia. Specifically, we find that males raised with high biparental care have increased expression of *Cd47*, which prevents pruning by microglia, decreased expression of *C1qa,* which promotes pruning by microglia, and decreased expression of proinflammatory microglial gene *Skap2*, all of which we confirmed independently using RT-PCR (*SI Appendix*, Fig. S3) (73–75). Additionally, males with higher percent care by fathers have more synapses, which may be a result of reduced synapse pruning due to decreased microglia number or type (76). In the nucleus accumbens, microglial elimination of dopaminergic synapses during adolescence contributes to the development of social behaviors in rats in a sex-specific manner (77). Modulation of microglial synapse pruning activity by early-life experience may partly explain the differences we observed in gene expression and synapse density. To investigate this, we examined the number and morphology of microglia cells using Iba1 immunostaining. First, we examined the density of microglia. We found a moderate effect of total parental care on microglia density ($\chi^2_{(1)} = 4.43$, $P = 0.035$) where more care is associated with increased density, but no interaction with sex ($\chi^2_{(1)} = 0.94$, $P = 0.333$, Fig. 4 *A* and *B*). There was no effect of percent care by fathers ($\chi^2_{(1)} = 1.14$, $P = 0.286$), nor an interaction of percent care by fathers and sex ($\chi^2_{(1)} = 0.37$, $P = 0.541$, Fig. 4*C*). We next used Sholl analysis to examine the morphology of the microglia. We found no significant effects of sex, total parental care, or percent care by fathers on microglial morphology (all P values > 0.1). These results indicate that in both sexes, higher biparental care is associated with higher microglia cell density. This may be a result of a change in the pace of development. There is a well-defined time course of microglia cell density in the brain, which peaks during the early postnatal period and declines during the third postnatal week (78). This developmental trajectory is present in the prairie vole nucleus accumbens, where we find that microglia cell density decreases rapidly between postnatal days 14 and 24 (*SI Appendix*, Fig. S4). Because we show that higher biparental care is associated with increased microglia cell density, which is indicative of a less developed brain, these results further suggest that higher biparental care slows development.

Percent Care by Fathers Has Sex-Specific Effects on Pup Retrieval Behavior. Alloparenting behavior, or parental care toward offspring that is not one's own, is a commonly studied social behavior in prairie voles which is sexually dimorphic and sensitive to early-life experience (19, 23, 79). We suspected that aspects of parental care, including total parental care and percent care by fathers, may be related to social behavior during an alloparenting task (Fig. 5*A*). To test this, in a separate cohort of animals, we examined how early-life care impacted displays of alloparental behavior as a juvenile. As previously noted, while durations of care by mothers and fathers are inversely related, there is no association between total parental care and the percent care by fathers (see *SI Appendix*, Fig. S5 for descriptive analysis of parental behavior in this cohort; note that data from this cohort replicate data in *SI Appendix*, Fig. S1). We hypothesized that prairie voles raised with higher parental care would display more pup-directed behavior. Based on work in other monogamous rodents, we hypothesized that there would be a specific effect of care by fathers on pup retrieval behavior in male offspring (80, 81). In both sexes, total parental care was associated with less likelihood for a test animal to attack the stimulus pup ($\chi^2_{(1)} = 5.31$, $P = 0.021$) and, in nonattackers, a trend toward a higher duration of pup-directed care ($F_{(1,18.73)} = 4.08$, $P = 0.058$). There was no association of percent care by fathers with either of these behaviors (attack: $\chi^2_{(1)} = 0.01$, $P = 0.924$; pup-directed care: $F_{(1, 17.17)} = 0.13$, $P = 0.727$). We then examined the sex-specific impacts of total parental care and percent care by fathers on pup retrieval behavior in an alloparenting task. There was no effect of total parental care ($\chi^2_{(1)} = 0.03$, $P = 0.857$) nor an interaction of total parental care and sex ($\chi^2_{(1)} = 0.25$, $P = 0.618$) on total pup retrievals (Fig. 5*B*). There was, however, a significant interaction of percent care by fathers and sex ($\chi^2_{(1)} = 18.44$, $P < 0.001$) such that males raised with higher care by fathers show increased pup retrieval behavior, while females raised with higher care by fathers show a trend for a decrease in pup retrieval behavior (Fig. 5*C*).

Previous studies in prairie voles have associated genetic variation of *Oxtr* with affiliative behaviors (82, 83). We find no evidence that genetic variation of *Oxtr* at two SNPs, NT213739 and KLW2, are associated with alloparenting behaviors (*SI Appendix*, Fig. S6). Notably, this suggests nongenomic transmission of behavior, as it provides evidence that our results are not driven by fathers passing on specific genetic variants associated with parental behaviors onto their sons. However, without performing a cross-fostering experiment, we cannot rule out genomic transmission of parental behaviors. Still, these results indicate that receiving more paternal care is associated with higher pup retrieval behavior in male offspring and lower pup retrieval behavior in female offspring.

Discussion

The experiments in this study indicate that while both male and female prairie voles are sensitive to total biparental care, male prairie voles are additionally uniquely sensitive to the percent of that care which is provided by fathers. Specifically, we find that total biparental care is associated with slower development, evidenced by decreased epigenetic age of brain tissue, and higher

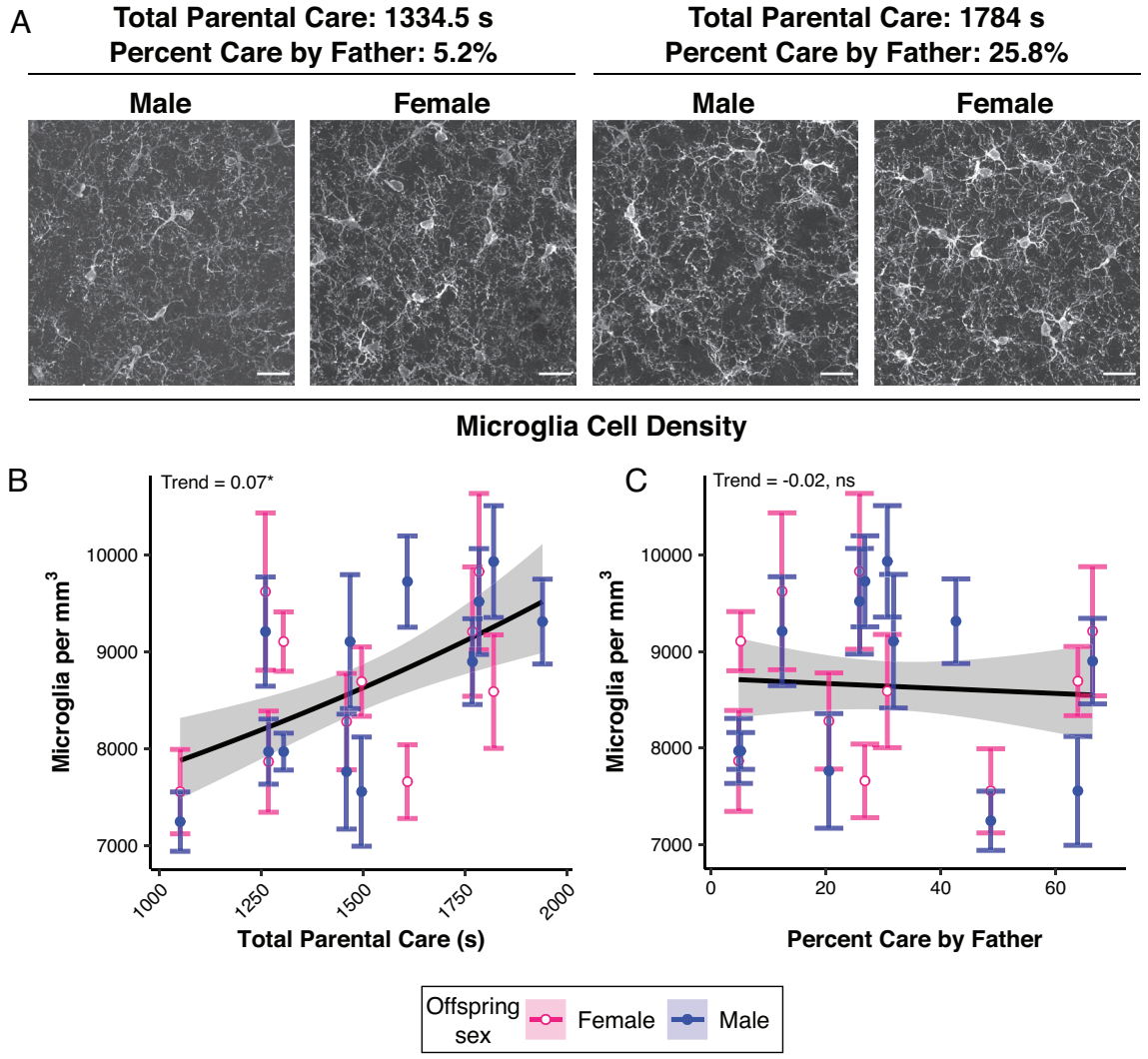

Fig. 4. Total biparental care is associated with microglia cell density in both sexes. (*A*) Example confocal microscopy images of Iba1+ cells in male and female offspring raised by low (from lower 25% of distribution) and high-care (from upper 25% of distribution) parents. (Scale bars, 20 μm.) (*B*) Total parental care is associated with higher microglia cell density ($\chi^2_{(1)} = 4.43$, $P = 0.035$). Data points represent the average microglia cell density of six regions of interest images in the nucleus accumbens core, and bars represent the SE. (*C*) No effect of percent care by father on microglia cell density ($\chi^2_{(1)} = 1.14$, $P = 0.286$). *$P < 0.05$, ns $P > 0.05$.

microglia cell density consistent with slowed aging. In male offspring only, we find that early care exerts differential effects on gene expression in the nucleus accumbens, especially of genes related to neurodevelopment and excitatory synaptic transmission. Using quantitative electron microscopy, we find that high care by fathers is associated with increased excitatory synapse density in the nucleus accumbens in male offspring only. These effects appear to be independent of microglia, as the density and morphology of microglia are not significantly related to early care in a sex-specific manner. Finally, we show that percent care by fathers has opposing effects on pup retrieval behavior in male and female offspring. Males raised with higher care by fathers are more likely to perform more pup retrievals than those raised with lower care by fathers and these behaviors are opposite in female siblings. These findings provide evidence that early-life experience fundamentally alters the development of male and female brains and alloparenting behavior in a sex-specific manner. This has widespread implications for studying how early-life experience relates to future mental health disorder, the underlying etiology of which may need to be understood to be different for men and women.

The RNA-sequencing results suggest that early biparental care sets up a male-specific transcriptional program that supports increased synaptic activity in the nucleus accumbens. Genes that are up-regulated by biparental care in males are involved in neurodevelopmental and synaptic processes, with enrichment for genes which produce proteins localized to asymmetric synapses. This network of genes malleable by early-life experience in males, which includes the oxytocin receptor, might support both the typical neurodevelopment of male prairie voles in particular and the development of species-typical social behaviors in both sexes. These results therefore might explain why reducing the function of one gene in this network, such as the oxytocin receptor, does not lead to widespread behavioral deficits (84, 85). Further, there is an enrichment of autism risk genes in the differentially expressed genes in males, with higher expression of genes typically disrupted in autism in high-care offspring. While variation in parental care does not alter expression of these genes to levels that might cause autism phenotypes (i.e., all voles in this study display species-typical social behaviors), it remains interesting that expression of these genes are highly variable and malleable by changes in parental care in male offspring only. Further studies of mechanisms by which autism risk genes are programmed by the environment may lead to insights into the male bias for autism and other neurodevelopmental disorders.

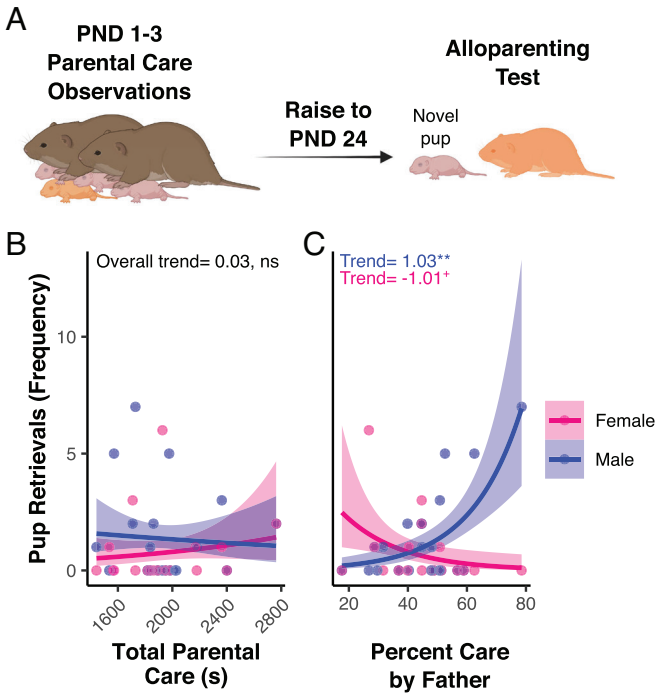

Fig. 5. Sex-specific impact of percent care by fathers on pup retrieval behavior in an alloparenting test. (*A*) Schematic showing experimental design. (*B*) There is no association between total parental care received and pup retrieval behavior during an alloparenting test ($\chi^2_{(1)} = 0.03$, $P = 0.857$). (*C*) There is a significant interaction of percent care by father and sex ($\chi^2_{(1)} = 18.44$, $P < 0.001$) such that males raised with more care by fathers display more pup retrievals as juveniles. Conversely, female siblings raised with more care by fathers display fewer pup retrievals as juveniles. $^{+}P = 0.059$, $^{**}P < 0.01$, ns $P > 0.06$

Using quantitative electron microscopy to examine the impact of early-life parental care on synapse density and morphology, we identify that excitatory synapse density is sensitive to total biparental care in both sexes, but the percent care by fathers is strongly associated with synapse density in male offspring only. These results are in line with work in other monogamous rodents that indicate male sensitivity to care by fathers. For example, paternal deprivation leads to synaptic changes throughout the brain in degus, including changes to both dopamine innervation and the number of parvalbumin-positive interneurons in the nucleus accumbens (37, 39, 86). We observe increased density of asymmetric synapses in males raised with more care by fathers and an increase in small-area terminals and shorter synaptic zones. These results may indicate upregulation of a specific excitatory input characterized by small terminals and short synapses (possibly prefrontal cortex or amygdala) or a generalized increase in synaptogenesis which would result in small, immature synapses. While increased care by fathers is associated with increases in excitatory synapse density in male offspring, there is no association between percent care by fathers and microglia cell density or morphology. It may be that the synapse density results are independent of microglial sculpting of accumbens circuitry or that differences in microglia cell density or morphology might be observed earlier in development.

Finally, we show a sex-specific behavioral phenotype associated with increased care by fathers in prairie voles. Specifically, we show that care by fathers is related to pup retrieval behavior such that males raised with more paternal care display more pup retrievals during an alloparenting task, while female offspring tend to display fewer pup retrievals when raised with more care by fathers. Previous work in prairie voles indicates that males are uniquely sensitive to care by fathers, which relates to social behavior and neuropeptide receptor density throughout the lifespan (56, 58, 70). Our results add to this body of literature by providing further evidence in a natural parenting paradigm that the percent care provided by fathers impacts neuroanatomical and behavioral outcomes in male prairie voles only. These results are also consistent with studies in California mice which suggest that paternal pup retrievals are associated with pup retrievals by male offspring later in life (81). However, California mouse female offspring also display higher pup retrieval behavior when raised by fathers that display high pup retrieval (87). Our study is different from this previous work in California mice in three critical ways (other than species): First, the studies in the California mouse manipulate paternal pup retrieval behavior while we do not. Second, the age of the offspring differs: We use juveniles while the previous studies use adults. Third, and perhaps most importantly, our study uses an alloparenting test where the test subject is exposed to a pup that is not theirs, while the studies in the California mouse expose the test subject to their biological offspring. This may account for the different behaviors in females, as female prairie voles are generally less alloparental than males as juveniles (88). Altogether, our results suggest that male prairie voles are uniquely sensitive to care by fathers in terms of neurodevelopmental processes and alloparenting behavior. Further work should focus on specific circuitries that are sensitive to care by fathers and are related to alloparenting and other social behaviors.

One possible explanation for our results is that high care by fathers alters the developmental trajectory of male offspring such that there is increased synapse density, which ultimately increases social behavior throughout the lifespan. A second explanation is that high care by fathers alters the pace of development such that at the juvenile time point, there are differences in gene expression, nucleus accumbens ultrastructure, and social behavior which converge by adulthood. Further, these explanations are not necessarily mutually exclusive: Differences in pace of development may lead to dramatic differences during development and more subtle differences in adulthood. While direct examination of these features in adulthood is necessary to determine which explanation may be more likely, there is good evidence from this study and others supporting either explanation. First, effects of early-life experience and paternal care impact social behavior in prairie voles throughout the lifespan, suggesting that effects do not converge by adulthood. There is a strong impact of early-life experience on partner preference behavior in adulthood (56, 89). However, developmental timing is also impacted by early-life care, with evidence suggesting that prairie voles raised by low-care parents reaching developmental markers such as eye opening, eating solid food, and leaving the nest earlier than those raised by high-care parents, though not all studies support this finding (19, 55–58). Our microglia cell density results also support this explanation, as voles raised with low biparental care show decreased microglia cell density which is indicative of a more mature brain. Future work examining how early-life biparental care impacts brain development and aging throughout the lifespan is necessary to resolve these two explanations.

Our results indicate that parental care, and in particular, the percent of that care which is provided by fathers, is associated with gene expression programs and synapse density and morphology in male offspring only. We provide evidence that this is a true sex difference and not a result of differential biparental care of male and female offspring by showing that features of parental care including total care provided and the percent of care by the father are not impacted by the litter size or the sex ratio within the litter (*SI Appendix*, Figs. S1 *B*, *C*, *F*, and *G* and S5 *B*, *C*, *F*, and *G*) consistent with previous reports that male and female prairie voles do not receive different amounts of care (90). While it is possible that females are not as sensitive to early-life experience, a more

likely explanation is that effects of early-life biparental care in females may be found in other brain regions or at other developmental time points. For example, the handling manipulation used in this study has sexually dimorphic impacts on behavior: In males, MAN1 (high-care parents) increases alloparenting behaviors as juveniles, while in females, MAN1 increases pair bond formation as adults (23). Additionally, we do provide evidence that total parental care impacts aging and neurodevelopmental processes in females, but we may have examined a time point where differences in gene expression and neuroanatomical outcomes are more consistent in male offspring. Future studies should aim to examine the timing of developmental changes associated with early-life experience in female prairie voles.

One limitation of these experiments is that gene expression, neuroanatomy, and behavior were not measured in the same animal cohorts. These experiments are interested in baseline potential for social behavior, not response to social stimuli, so we did not perform gene expression or anatomy experiments on animals after an alloparenting test. Further work should be done to connect excitatory synapse density to social behavior directly. A second limitation of these experiments is that the natural parenting experiments presented cannot differentiate between effects of early experience and genetic effects. However, a previous cross-fostering experiment indicates that alloparental behaviors are largely driven by rearing parents, not genetic parents (90). A cross-fostering study to determine how development of synapse density in the nucleus accumbens is impacted by both genetic and environmental effects would build upon our results. Finally, we cannot rule out the possibility that the subtle differences in handling in the MAN0 and MAN1 paradigm directly impact the pups by inducing a differential stress response. The experience of the pups in the MAN1 condition includes a period of time where they are dangling from the mother, while in the MAN0 condition, pups are always supported by the bottom of the cup. We do not know whether this induces a differential stress response, which could impact neurodevelopmental outcomes in a sex-specific manner. However, we believe that the relevant mechanism in this handling paradigm is the differential biparental care that occurs as a result, in part because our results indicate that male offspring are sensitive to variation in biparental care, with differences that are consistent across the handling and natural parenting paradigms. Future work examining stress responses in pups resulting from early-life disturbances might clarify these results.

Overall, results from these experiments indicate that male prairie voles are uniquely sensitive to their early rearing environment, with care by fathers being very important for neurodevelopment and the development of alloparenting behaviors. Our results are relevant to understanding male-specific neurodevelopmental processes which may be associated with male bias for neurodevelopmental disorders, including autism. Prairie voles are an excellent model for these studies given their enriched rearing environment resulting from biparental care. Further, our results indicate that while many features of offspring development are related to total parental care in both sexes, including aggression toward a pup, excitatory synapse density, and gene expression, males are additionally sensitive to variation in the percent of this care which comes from fathers, which impacts specific neurodevelopmental and behavioral phenotypes.

Materials and Methods

Animal Model. Subjects were laboratory-bred prairie voles (*Microtus ochrogaster*) descended from a wild-caught stock captured near Champaign, Illinois, previously used in our study of *Oxtr* DNA methylation and expression (22). The procedures for the animal care, the MAN paradigm, tissue collection, and DNA and RNA isolation are detailed in *SI Appendix, Materials and Methods*. Data showing a transient increase in parental care by early handling (MAN1) were previously published (22). Thirteen male–female sibling pairs from the MAN paradigm experiment were used for RNA sequencing and epigenetic clock experiments. Additionally, 11 male–female sibling pairs were used for immunohistochemistry, a subset of which (6 sibling pairs) were used for electron microscopy. An additional 23 animals were used for a developmental time course of microglia cell density. For alloparenting testing, 27 male–female sibling pairs were used. The total number of animals used in these studies is 125.

DNA Methylation Array and RNA-Sequencing. DNA methylation was measured using the custom Illumina chip "HorvathMammalMethylChip40" which measures DNA methylation at CpG sites with wide conservation in mammals. Detailed methods regarding data analysis and epigenetic clock measurements are provided in *SI Appendix, Materials and Methods*. RNA-sequencing was completed at the University of Virginia Genome Analysis and Technology Core (RRID:SCR_018883). Detailed methods for RNA-sequencing quality control and analysis are provided in *SI Appendix, Materials and Methods*. Methods used for RT-PCR validation of RNA-sequencing results are provided in *SI Appendix, Materials and Methods*.

Natural Parenting Behavior Scoring. Parenting behavior was observed for litters used for neuroanatomy experiments (*SI Appendix*, Fig. S1), colony breeders (*SI Appendix*, Fig. S2), and alloparental behavior experiments (*SI Appendix*, Fig. S5). Scoring of natural parenting behavior was completed according to previously published methods which are detailed in *SI Appendix*, *Materials and Methods* (19).

Tissue Preparation for Neuroanatomy. Ten male–female sibling pairs were used for immunohistochemistry, and a subset of six sibling pairs were used for electron microscopy. Animals were deeply anesthetized using isoflurane gas and transcardially perfused with Tyrode's solution (137 mM NaCl, 5.5 mM dextrose, 1.2 mM $MgCl_2$, 2 mM KCl, 0.4 mM NaH_2PO_4, 0.9 mM $CaCl_2$, and 11.9 mM $NaHCO_3$) until fluids ran clear followed by a fixative solution containing 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer for 4 to 5 min. Brains were postfixed in the skull overnight in a 4% paraformaldehyde solution at 4 °C. Brains were then removed, blocked, and sectioned coronally at 60 μm using a vibratome (Leica VT1000S) and collected in four series. One of the series was mounted on subbed slides for myelin staining. All other sections were rinsed in 1% sodium borohydride and stored in 0.05% azide in 0.01 M PBS at 4 °C until immunohistochemistry and electron microscopy. An additional cohort of animals was used for a time course of microglia cell density. Male–female sibling pairs were collected from our breeding colony on postnatal days 14, 20, and 30. They were deeply anesthetized with an overdose of Euthasol and transcardially perfused with Tyrode's solution until fluids ran clear followed by 4% paraformaldehyde in 0.1 M phosphate buffer for 4 to 5 min and further treated as described above.

Myelin Staining and Light Microscopy. Sections mounted on subbed slides were rehydrated in 0.02 M PBS for 2 min and incubated in 0.2% $HAuCl_4$ for 12 to 15 min at 60 °C (91). Once fine myelinated fibers were differentiated, the slides were transferred to an intensification solution of 0.2% $KAuCl_4$ for 2 min. Finally, sections were incubated in 1% sodium thiosulfate for 3 min and rinsed three times in 0.02 M PBS. Slides were treated through a series of ethanol solutions for dehydration and through xylenes for clearing the lipids from the tissue. All slides were cover slipped using the DPX mounting media (Sigma Aldrich, St. Louis, MO). Sections were photographed using a Leica microscope (model LMDC 888011) and Leica MC170 digital camera.

Images of myelin stains were examined, and sections including the nucleus accumbens were opened in Image Pro Plus, version 5.1 (Media Cybernetics). The area of the nucleus accumbens was calculated by tracing the nucleus accumbens. Each area was multiplied by 240 to calculate a volume (each section represents four sequential 60-μm sections), and these volumes were summed to calculate total volume of the nucleus accumbens.

Iba1 Immunohistochemistry. One series of sections from light microscopy brains (n = 22) were stained for microglia markers. See *SI Appendix, Materials and Methods* for detailed description of immunohistochemistry procedures.

Coronal sections containing the nucleus accumbens were chosen for confocal microscopy based on the following anatomical markers: a) the corpus callosum is present at the midline; b) lateral ventricles extend ventrally into the nucleus accumbens; and c) hippocampal formation is not present. Chosen sections were then imaged using a Carl Zeiss LSM 800 microscope using a 40× objective in 3D Z stack mode (0.825 μm steps). For each section, three images were taken from the nucleus accumbens core from each hemisphere, totaling six images per section. Z stacks were imported into Fiji software (NIH) (92). Labeled cells were manually counted by a researcher blinded to parental care. Details regarding Sholl analysis of microglia and statistical analysis of microglia data are included in *SI Appendix, Materials and Methods*.

Electron Microscopy. One series of sections were resin-embedded for electron microscopy from 12 brains according to previous published procedures which are repeated in *SI Appendix, Materials and Methods* (69). Images were taken from three regions of interest in the nucleus accumbens core, just medial to the anterior commissure which was easily identified via the bundle of myelinated axons. From each region of interest, 12 to 15 nonoverlapping EM images were captured at 12,000× magnification. One male had poor ultrastructure and was not used for further analysis.

Electron Microscopy Image Analysis and Quantification. Each EM micrograph was examined using Image-Pro Plus software, version 5.1 (Media Cybernetics) by a researcher blinded to sex and parental care. In each micrograph, the number of synapses were counted, and the terminal profile areas, synapse lengths, and effective sampling area were calculated. Profiles were identified as synaptic terminals if they met the following criteria: 1) a vesicle docking at the membrane, 2) at least three vesicles within the same profile, and 3) parallel alignment of the postsynaptic plasma membrane with that of the terminal. The length of the synapse was measured along the length of the parallel-aligned plasma membranes. If the synapse was perforated, the synapse length included the perforation. Synapses were identified as either asymmetric if a postsynaptic density was present or symmetric if a postsynaptic density was absent. If a single terminal contacted two different postsynaptic profiles, each contact was considered its own synapse. The postsynaptic profile was classified as a spine, dendritic shaft, terminal, or cell body. A dendritic shaft was identified by the presence of microtubules organized in parallel and/or the presence of mitochondria. Cell bodies were identified by the presence of organelles such as endoplasmic reticulum, Golgi apparatus, or nucleus. Terminals were identified by the presence of vesicles. Spines were identified by the absence of mitochondria and microtubules. Additionally, the presence of a spine apparatus was used for classifying postsynaptic profiles as spines but was not necessary. The effective sampling area was calculated as the total area of the individual micrograph minus the area of cell bodies, blood vessels, and myelinated axons, as these elements are not expected to receive synaptic contact.

Volumetric synapse density was calculated as previously described ($N_v = N_A/d$, where N_A is the number of synapses per square micrometer, and d is the average synapse length in micrometers) (68, 69). This calculation was performed for each region of interest, resulting in three measurements of synapse density per animal. Statistical methods used to analyze volumetric synapse density and synapse morphology are described in detail in *SI Appendix*, Materials and Methods.

Alloparenting Behavior. On PND 24, one male and one female from each litter (n = 27 litters) were observed in an alloparenting test to examine social behavior toward a novel, unrelated pup (1 to 3 d old) according to previously published methods which are detailed in *SI Appendix, Materials and Methods* (19). Alloparenting behavior was analyzed for three features: attack, total duration of pup-directed behavior, and frequency of pup retrievals during the test. Statistical analysis of alloparenting behavior is described in detail in *SI Appendix, Materials and Methods*. Separately, we also tested for an effect of genotype at SNPs NT213739 and KLW2, which have previously been associated oxytocin receptor gene expression in this species (82). Methods for genotyping these SNPs are reported in *SI Appendix, Materials and Methods*.

Data, Materials, and Software Availability. RNA sequencing data have been deposited in Gene Expression Omnibus (GSE229256) (93). All study data are included in the article and/or supporting information.

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