

IS IT ALL IN THE FAMILY? THE EFFECTS OF EARLY SOCIAL STRUCTURE ON NEURAL–BEHAVIORAL SYSTEMS OF PRAIRIE VOLES (*MICROTUS OCHROGASTER*)

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Abstract—The transition to parenthood is generally associated with a reduction in anxiety or anxiety-like behavior across a wide range of species. In some species, juveniles provide supplementary parental care for younger siblings, a behavior known as alloparenting. Although the fitness consequences of alloparenting behavior have been a focus of evolutionary research, less is known about how alloparenting behavior impacts affective states. In the socially monogamous prairie vole (*Microtus ochrogaster*), most juveniles exhibit alloparenting behavior, making the species an ideal model for examining the effects of alloparenting on future behavioral outcomes. We randomly assigned juvenile voles to alloparenting (AL) or no alloparenting (NoAL) groups and behaviorally phenotyped them for anxiety-like and social behaviors using the elevated plus maze (EPM), open field test (OFT), startle box, social interaction test, juvenile affiliation test, and partner preference test. AL voles displayed more anxiety-like and less exploratory behaviors than NoAL voles, spending significantly less time in the open arms of the EPM and center of an open field. We dissected the CA1 region of the hippocampus and the bed nucleus of the stria terminalis (BNST) from brains of behaviorally phenotyped voles and nontested siblings as well. Decreased brain-derived neurotrophic factor (BDNF) expression in CA1 has generally been associated with increased anxiety-like behavior in other rodents, while an anxiogenic role for BDNF in BNST is less established. Western blot analyses showed that alloparenting experience increased expression of BDNF in the BNST but decreased BDNF expression in the CA1 region of hippocampus (CA1) of nontested voles. There were similar differences in BNST BDNF of behaviorally phenotyped voles, and BDNF levels within this region were negatively correlated with exploratory

behavior (i.e. time in center of OFT). Our results suggest that BDNF signaling in BNST and CA1 fluctuate with alloparenting experience, and they contribute to an increasingly complex “BDNF hypothesis” in which behavioral effects of this molecule are region-specific. © 2012 IBRO Published by Elsevier Ltd. All rights reserved.

Key words: alloparenting, family, anxiety, BDNF, Western blot.

INTRODUCTION

The developing brain is exceptionally plastic and sensitive to environmental experience (Fox, 1992; Horton and Hocking, 1997). In humans, early social experience is dominated by family encounters which can shape a person's social behavior as an adult (Lareau, 2003). These exchanges are typically regarded as positive experiences that can help individuals prepare for societal interactions and for interacting with their own offspring. In rodents, most data suggest that engaging in parental behaviors is anxiolytic, or conversely that lower levels of anxiety are necessary to engage in parental behavior. This reduction in anxiety has been hypothesized to facilitate foraging, care, and protective behaviors for newborns (Fleming and Luebke, 1981; Kinsley, 2011). In addition to behaviors that provide obvious advantages to offspring (e.g. heightened response toward pups and increased aggressiveness toward intruders), animals with parenting experience are, in general, less fearful than nulliparous animals during tasks designed to test emotional state (Fleming and Luebke, 1981; Hård and Hansen, 1985; Lonstein and Gammie, 2002). Although there is a growing body of literature focusing on the effects of parent–offspring interactions on adult behavior, much less is known about the effects of whole-family dynamics, including interactions with sibling peers.

Gene–environment interactions have proven to be important for understanding the development of psychiatric disorders into adulthood (Caspi et al., 2002). The combination of early-life adversity with genetic susceptibility greatly increases the risk for developing stress-related disorders later in life (Barr et al., 2004), and the consequences of stress exposure in animal models, particularly during the juvenile period, are robust on performance during tasks designed to quantify anxiety-like behavior in adulthood. Increases in anxiety-related behavior on these tasks following manipulations of

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Abbreviations: AL, alloparenting; BDNF, brain-derived neurotrophic factor; BNST, bed nucleus of stria terminalis; CA1, CA1 region of hippocampus; CRH, corticotropin-releasing hormone; EDTA, ethylenediaminetetraacetic acid; EPM, elevated plus maze; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NoAL, no alloparenting; OFT, open field test.

early-life experience are correlated with fluctuations in neural growth factors which are essential to axon guidance and growth during development (Cirulli et al., 2010). Brain-derived neurotrophic factor (BDNF), the mature form in particular (mBDNF), is an important mediator of cell survival and neuronal differentiation (Huang and Reichardt, 2001; Binder and Scharfman, 2004). In its unprocessed state, BDNF can induce cell death and dendritic atrophy due to its high affinity for the p75 receptor, but once the protein is cleaved into mBDNF it can bind to TrkB receptor tyrosine kinase, promoting neuronal plasticity and survival (Lu et al., 2005). These properties make BDNF an attractive candidate for long-lasting changes produced by early-life stressors, particularly within brain regions involved in stress response.

Neural modulations within the hippocampus may be particularly influential in the development of anxiety-like behavior. Direct administration of BDNF to the hippocampus produces antidepressant-like effects (Groves, 2007). Although rodent studies have found that the effects of stress on BDNF appear to be region-specific (Alleva and Francia, 2009), many have consistently reported that stressful events decrease hippocampal BDNF mRNA and protein (Kawahara et al., 1997; Duman and Monteggia, 2006; Tsankova et al., 2006; Martinowich et al., 2007). Natural environmental perturbations have been shown to influence BDNF gene expression as well, as rats that develop under maternal maltreatment have decreased BDNF gene expression (Roth et al., 2009). However, very little is known about how other early social encounters, including sibling interactions, affect BDNF gene expression.

Although rats and mice have been used as models for mother–offspring interactions, sibling–sibling care is less common in rodents (Lonstein and De Vries, 2000; Olazábal and Young, 2006). Prairie voles (*Microtus ochrogaster*), however, commonly reside in extended communal networks and perform a wide range of affiliation behaviors (Getz et al., 1993), some of which parallel family interactions in humans. In addition to being socially monogamous, prairie voles serve as “alloparents” from the juvenile period and into adulthood, helping their biological parents raise siblings (Getz et al., 1993; Bales et al., 2004). Rather than leaving the home nest, the majority of juvenile/young-adult voles in the wild (~60% of males and ~80% of females > 30 days of age) do not disperse but instead stay within colonies to care for future litters of pups (Getz et al., 1993). Various mammalian species that participate in cooperative breeding perform behaviors that range from feeding and allo-suckling to carrying pups (Russell, 2004). Other studies have previously observed alloparenting behaviors specifically in voles, such as huddling, pseudohuddling, time in/out of the nest, licking and sniffing, grooming, and retrieving pups (Stone et al., 2010; Keebaugh and Young, 2011). In addition to these behaviors toward siblings, carrying and side-by-side contact with unrelated pups have been observed in alloparenting tests (Roberts et al., 1996; Kirkpatrick and Kakoyannis, 2004). Studies examining spontaneous alloparenting behavior in prairie voles have

observed the majority (70%) of juvenile voles display these alloparental behaviors to novel pups (Roberts et al., 1998).

Many studies have tried to explain the cause of cooperative behaviors like alloparenting from various evolutionary perspectives, with a focus on the benefits of communal living (Bergmüller et al., 2007). How different forms of family living can influence future behavioral outcomes is not well known. In prairie voles, there are substantial individual differences in the intensity and types of alloparental behavior that voles perform, and this variability is correlated with parental behaviors with their own biological offspring (Ross et al., 2009; Stone et al., 2010). However, the benefits of alloparental behavior outside of reproduction (e.g. social, exploratory, locomotor) and, more importantly, its costs, have been understudied.

Here, we experimentally manipulated rearing conditions in prairie voles to test how early-life communal living experience influences anxiety-like behavior and the expression of BDNF in brain regions known to modulate this behavior (hippocampus and bed nucleus of the stria terminalis, BNST). We assigned voles to groups with standard weaning conditions and no alloparenting experience (NoAL) or to a communal living experience with alloparenting for two litters of siblings, which we have termed “alloparenting experience” (AL; see Stone et al., 2010). At sexual maturity, voles were behaviorally phenotyped using a series of standardized tests that examine anxiety-like behavior, with the hypothesis that alloparenting experience would have an anxiolytic effect. In order to further understand these effects at the molecular level, we quantified protein for mBDNF in the BNST and the CA1 region of hippocampus (CA1). We used an additional set of siblings that were not behaviorally-tested subjects to obtain tissue samples that could be tested for treatment effects on BDNF protein independent of extensive behavioral testing. Results from these experiments suggest robust effects following alloparenting experience, some of which are maintained after a series of behavioral tests.

EXPERIMENTAL PROCEDURES

Subjects

Weaning treatments. Laboratory-bred male and female prairie voles ($n = 9–12$ per sex per group, 40–50 g) were used from 14 separate breeder pairs. All voles were descendants of a wild stock initially trapped near Champaign, Illinois, and the stock was systemically outbred. Animals were kept on a 14 h light/10 h dark cycle with free access to food (High Fiber Rabbit Diet, Purina Mills) and water. Breeders and offspring were maintained on Sanichip bedding in twin polycarbonate cages (44 × 22 × 16 cm) connected with polycarbonate tubing (10.2 cm long × 7 cm diameter). All procedures in this study were consistent with the NIH Guide for the Care and Use of Laboratory Animals (N.I.H. Publications No. 80-23) and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Davis.

Subjects were randomly assigned into treatment groups as juveniles (20 days old) and were only selected from litters containing three or more pups. Pups were ear-clipped for individual identification. Treatment assignments were counterbalanced for breeder effects. NoAL subjects were weaned from their parents

at postnatal day 20, the standard age of weaning in our laboratory. At this time, NoAL voles were housed as pairs with same-sex/same-age siblings until the start of (and also during the duration of) behavioral testing (65–75 days old). Voles experience post-partum estrus, and their gestation length is 21 days (Hofmann and Getz, 1986; Wang et al., 1994); therefore, NoAL voles had no social exposure to younger siblings. The subject juveniles were removed from the parents, rather than removing the younger siblings, in order to eliminate potential exposure of juveniles to younger siblings (i.e. infants are often born overnight and NoAL subjects would then be exposed to younger siblings for an unknown duration).

Juveniles in the alloparenting group (AL) remained in the breeder cage with parents to care for two subsequent litters of younger siblings. Subjects that comprised this group derived from sets of four randomly selected juveniles (2 males and 2 females) that stayed with parents after they reached 20 days of age. Any remaining juveniles were removed at the time of selection, and the first litter of younger siblings was removed from the breeder cage at the normal time of weaning (20 days old) in order to minimize overcrowding. AL subjects were weaned from their biological parents by 63 days of age and also housed in same-sex/same-age sibling pairs for the duration of behavioral testing.

Once weaned as adults (AL voles) or juveniles (NoAL voles), the voles were group-housed with a same-sex sibling in polycarbonate cages (27 × 6 × 13 cm) for at least 1 week before beginning behavioral tasks. Sibling pairs were maintained in single-sex colony rooms so as not to induce estrus in female animals. To control for differing lengths of exposure to parents, we added soiled bedding from the parents' cage during weekly cage changes to NoAL voles once they were removed from their respective breeder cages (approximately 1/3 soiled bedding to 2/3 fresh bedding per cage), allowing for consistent exposure to parental scent and pheromones. However, it should be acknowledged that differing amounts of social stimulation from parents and siblings may also be an important factor, but it is likely that the number of social companions in the wild is variable and changes with dispersal and mortality. All animals were 65–75 days old at the start of behavioral testing. In addition to voles that underwent behavioral testing, a set of siblings to the tested voles underwent identical weaning conditions but did not undergo behavioral testing procedures. These nontested siblings were utilized to examine brain modifications due to weaning treatments that would be independent of exposure to behavioral testing.

Behavioral testing

Elevated plus maze testing. The elevated plus maze (EPM) can be used to interpret anxiety-like behavior, with individuals exploring the open arms of the apparatus considered to be showing lower levels of anxiety-like behavior (Pellow et al., 1985).

Early experience has been shown to affect performance in the EPM in voles (Bales et al., 2007). The EPM was administered on day 1 of testing, as behavior on this test is particularly sensitive to testing history (Bailey et al., 2007). Voles were acclimated to the testing room for 1 h prior to the start of EPM testing. The EPM was constructed with two open and two closed arms of identical length and width (67 cm × 5.5 cm). All subjects started testing after being placed in the middle area of the EPM (i.e. the "neutral" zone). Real-time behavioral observations on anxiety-like behaviors were recorded using behavioral software (Behavior Tracker 1.5, www.behaviortracker.com). We observed 41 males and females (12 NoAL females, 10 AL females, 9 NoAL males, 10 AL males), and we recorded duration for each zone, zone entries, and autogrooming behavior. The ratio of time spent in the open arms (calculated by dividing time spent in the open arms of the plus maze by the total time spent in either plus maze arms) was analyzed. Percentage of entries into the open arms was also calculated and analyzed. Following the EPM test, the voles were returned to their home cages.

Open field and social interaction testing. On day 2 of testing, voles underwent open field (OFT) and social interaction testing. The OFT can also be used to assess anxiety-related behavior in rodents by quantifying the amount of time the animal spends exploring an exposed center zone versus the sides of the arena (Crawley, 1999) (Fig. 2A). Animals were allowed to explore a large open field (89 × 63 × 60 cm) for 3 min. Durations within 12 cm of the sides and within a center zone located 16 cm from the sides (50 × 35.5 cm) were recorded using the Any-Maze video tracking system (Stoelting, Wood Dale, IL). Next, a small wire cage (14 × 17 × 14.5 cm) was introduced into the arena, and we recorded the amount of time the vole spent within 8 cm of the empty cage for an additional 3-min period (acclimation phase). Finally, a novel, same-sex virgin stimulus vole was placed into the wire cage for 3 min (social interaction phase). We recorded the amount of time the experimental vole spent interacting with the wire cage and the duration spent in the two corners opposite the wire cage (8 × 8 cm²). The arena was cleaned with 70% ethanol and dried before the start of each testing. Total distance traveled was also measured as an estimate of total activity.

Juvenile affiliation test. On day 3 of testing, animals were habituated to a 2-chamber test apparatus connected by a tube for 45 min. A nonrelated juvenile, same-sex stimulus vole was placed in the cage opposite of the experimental animal, giving the test animal a choice to approach or avoid a non-threatening social stimulus for a 10-min period. Each test was recorded on video, and affiliation-type behaviors were scored by an observer blind to treatment groups using Behavior Tracker 1.5 software. Behaviors scored for their total durations during the 10-min test included the following: autogrooming (self), licking and grooming juvenile, rearing, sniffing juvenile, and withdrawing from juvenile.

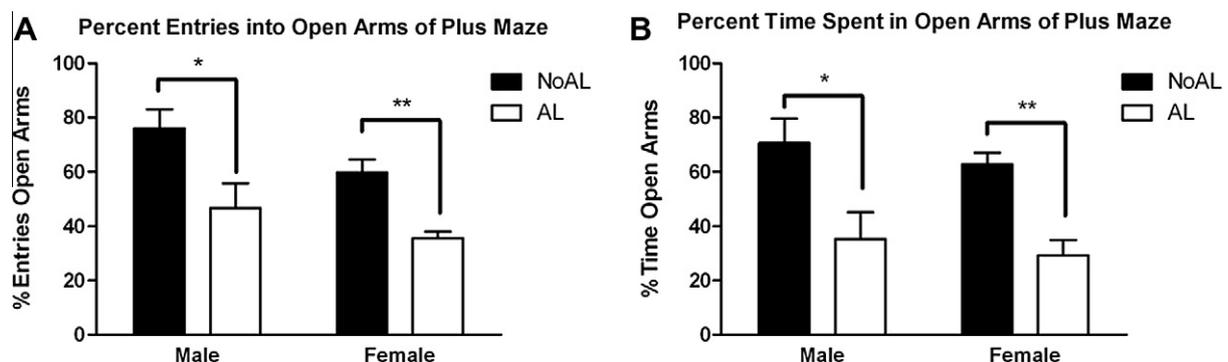


Fig. 1. Alloparenting voles displayed increased anxiety-like behavior on the elevated plus maze. Percentage of (A) entries and (B) time spent in open arms of EPM. Data are shown as mean + SE, with * $p < 0.05$ and ** $p < 0.001$ indicating significant differences.

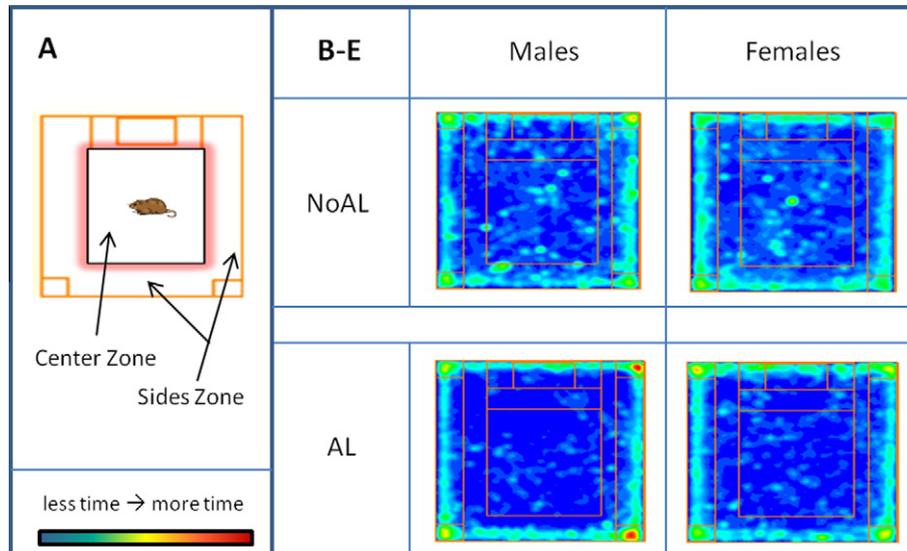


Fig. 2. Alloparenting experience decreased the amount of time subjects spent in the center of the open-field test. (A) The illustration describes the location of the “center zone” (highlighted in red) within an open-field arena. (B–E, clockwise from top left) Occupancy plots showing the average of the animals’ center position by group for (B) NoAL males, (C) NoAL females, (D) AL females, and (E) AL males. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Startle testing. On days 4 and 5 of testing, voles were placed in startle boxes for acoustic startle and tactile startle tests, respectively. Two SR-LAB Startle Reflex Systems were used simultaneously (San Diego Instruments, San Diego, CA). The startle chamber consisted of plexiglas cylinders (3.8 cm internal diameter × 12.8 cm length) mounted on a Plexiglas platform within a sound-attenuated apparatus outfitted with a loudspeaker and light above the cylinder. A decibel reader (RadioShack, Fort Worth, TX) was placed inside the startle chamber prior to testing to ensure that the acoustic stimulus was properly calibrated. A piece of copper tubing was inserted into the chamber prior to tactile startle testing so the tactile stimulus (air-puff) could be applied directly to the subject through the containing cylinder. A calibration procedure provided by the software accompanying the system (San Diego Instruments) was used to ensure consistency in startle amplitudes recorded by motion sensors across the two devices. Each test session was 20 min total. Animals were acclimated to the startle chamber for 5 min before being presented with acoustic (120 dB, 40 ms duration) or tactile (12-psi, 40 ms duration) startle stimuli that were presented alone or 100 ms following acoustic prepulses (20 ms) of 74, 78, 82, 86, and 90 dB. The trials were presented in seven consecutive blocks in pseudo-random order (49 trials total). The blocks also consisted of no-stimulus (background white noise of 70 dB) trials as baseline measurements of animal movement. Startle responses were recorded 65 ms following onset of the startle stimulus in arbitrary units of voltage change, with maximum startle amplitude (V_{max}) used as the dependent variable.

Partner preference testing. On days 5 and 6 of testing, voles were tested for the development of a partner preference using a standardized protocol for testing pair-bonding in voles. Females were exposed to a novel male stimulus vole for a 30-min cohabitation period. Males were exposed to an unfamiliar female vole for a 1-h period. At the time of separation for cohabs, nontested siblings were euthanized as detailed below. Testing began at (09:00), 3 h after lights-on. Voles were placed in a 3-chamber testing apparatus for a 3-h period immediately following the cohabitation period. The apparatus was made from three identical smaller polycarbonate cages that were described previously (Williams et al., 1992), which were attached by Plexiglas tubes (7.5 × 16.0 cm). Subjects were placed in the center chamber of

the apparatus. A familiar partner was tethered to one side chamber, and an unfamiliar opposite-sex vole was tethered in the other side chamber. Cohabitation periods and testing were monitored to ensure that no copulations took place. Tests were recorded in time-lapse video and scored later by an observer blind to experimental treatments. Time spent in physical side-to-side contact with the partner versus the stranger, duration in each cage, and entries to each cage were analyzed.

Following partner-preference testing, subjects were returned to their original cohabitation chambers with respective partners for an additional 24 h of cohabitation. A second partner-preference test was conducted using the same protocol as described above. Immediately following the second partner-preference test, voles were anesthetized with isoflurane gas and euthanized by cervical dislocation.

Tissue collection

To minimize the effects of isolation on the final day of testing, non-tested siblings were euthanized for tissue collection at the time of separation from their tested cagemates. Collection was performed as previously outlined (Trainor et al., 2003). Briefly, voles were anesthetized with isoflurane gas, euthanized, and decapitated. Brains were rapidly extracted, and 1 mm-diameter bilateral punch samples were taken from 2 mm-thick coronal sections kept frozen on a cold plate. Dissections were made from CA1 region of hippocampus and the bed nucleus of the stria terminalis (BNST). Punches were snap-frozen on dry ice and later homogenized in homogenization buffer over ice (7.4 pH, 20% glycerol, 0.4 mM NaCl, 20 mM HEPES, 5 mM MgCl₂, 0.5 mM EDTA in H₂O) with protease inhibitor (1% PMSF in EtOH). Following the final day of behavioral testing, subjects were euthanized as described above.

Western blotting

The levels of mBDNF protein were measured from the regions mentioned above by Western blot method. Western blot quantifications were normalized to the NoAL group voles (i.e. the

Table 1. Alloparenting experience had no effect on social behavior with novel peers or juvenile stimulus animals on social behavior tasks

Social behavior task	Male NoAL	Male AL	Female NoAL	Female AL
<i>Social interaction test</i>				
Time in interaction zone (empty)	127.5 ± 11.2	113.4 ± 18.4	97.4 ± 14.4	114.7 ± 11.0
Time in interaction zone (target)	146.1 ± 9.6	113.9 ± 20.1	134.2 ± 15.6	132.9 ± 8.1
Time in corners zone (empty)	4.3 ± 1.1	27.5 ± 15.4	6.1 ± 2.5	12.1 ± 4.1
Time in corners zone (target)	5.9 ± 4.0	33.5 ± 18.7	0.7 ± 0.4	3.9 ± 1.1
Total distance (empty)	14.8 ± 3.7	13.7 ± 2.2	15.1 ± 2.6	16.8 ± 2.5
Total distance (target)	9.3 ± 2.6	16.8 ± 7.6	10.8 ± 1.6	12.4 ± 1.8
<i>Juvenile affiliation test</i>				
Time spent autogrooming	36.9 ± 15.8	54.0 ± 18.0	31.7 ± 11.9	33.1 ± 7.1
Time spent licking and grooming juvenile	1.0 ± 1.0	2.5 ± 2.5	6.8 ± 4.5	1.5 ± 1.1
Time spent rearing	23.0 ± 11.1	15.1 ± 3.2	13.4 ± 7.1	24.5 ± 7.5
Time spent sniffing juvenile	99.9 ± 20.1	75.2 ± 13.1	108.4 ± 31.3	89.3 ± 18.1
Time spent withdrawing from a juvenile	2.2 ± 1.3	0.8 ± 0.5	0.5 ± 0.5	0.7 ± 0.4

All times in s, all distances in m.

standard weaning treatment). Levels of protein for AL vole groups are displayed as a percentage of the levels quantified from NoAL groups.

Two times Laemmli buffer was added to the homogenate at a 1:1 dilution and samples were placed on a shaker at 4 °C for 1 h. Proteins were denatured following heating on a heat block at 98 °C for 5 min and subsequently cooled on ice before running through SDS gel electrophoresis (15% bis-acrylamide resolving gel). A 0.2 µg sample of BDNF recombinant peptide (Genway, San Diego, CA) was run on each blot. After being separated through electrophoresis, protein was transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA) in transfer buffer with 20% methanol. Following transfer, membranes were rinsed in water and blocked with 5% skim milk in 1% Triton-X TBS (TBS-T) solution for 2 h at room temperature. After blocking, membranes were incubated overnight in primary rabbit anti-BDNF (sc-546, Santa Cruz Antibodies, 1:900) at 4 °C with gentle agitation. After washing the following day, membranes were incubated at room temperature for 1 h in peroxidase-conjugated anti-rabbit secondary antibody (Vector) diluted 1:2000 in TBS-T. Following final TBS-T washes, developing solution was applied (Bio-Rad) and the blot was imaged on a Bio-Rad ChemiDoc. Bands that were identified as the mature form of the BDNF protein (mBDNF) at approximately 14 kDa were quantified for relative intensity to the BDNF recombinant peptide using the Bio-Rad ImageLab software. The peptide also served as a positive control, confirming antibody specificity. After imaging, blots were stripped (10% SDS, beta-mercaptoethanol, Tris-HCl 6.7 pH solution) at 50 °C for 45 min and reprobed for β-actin as a loading control (Cell Signaling, Danvers, MA, 1:2000), and BDNF protein bands were normalized to their respective actin controls.

Statistical analyses

We tested whether pups from different breeder pairs showed significant differences in behavior or BDNF expression using a nested ANOVA design to test for breeder effects. This analysis was performed using the GLM procedure within SAS. Variation among the 14 different breeders was not significant for any of these variables. We then tested for effects of alloparenting experience and sex using two-way ANOVA. Weaning treatment effects on males and females were also analyzed separately using planned comparisons following a significant sex * treatment interaction or significant overall effect of weaning treatment. Analyses of Q-Q plots demonstrated that data from behavioral tests were normally distributed and variances were homogenous across treatment groups. We also used Pearson correlations to examine relationships between BDNF protein expression across brain areas with anxiety-like behavior on the EPM (i.e. entries

and percent time in open arms) and the OFT (i.e. time in center zone). All tests were two-tailed and significance levels were set at $p < 0.05$.

RESULTS

AL voles display anxiety-like behavior in the open field test and the elevated-plus maze

Voies that had alloparenting experience displayed increased anxiety-related behavior on the EPM. AL voles entered the open arms approximately 40% less than NoAL voles did (Fig. 1A) ($F_{(1,37)} = 15.500$, $p < 0.001$), and the difference was observed for both males and females ($p < 0.05$). AL voles also spent a reduced percentage of time in the open arms following entry (Fig. 1B, $F_{(1,37)} = 22.463$, $p < 0.001$), and there were significant differences in both males and females ($p < 0.05$). In addition, males made a significantly greater percentage of entries into the open arms when compared to females ($p < 0.05$). There was no significant sex * treatment interaction for entries or percentage of time spent in the open arms of the EPM.

Similar results were observed from the open field test. There was an overall effect of alloparenting experience on time spent in the center zone of the open-field arena, with voles that had alloparenting experience spending significantly less time in the center compared to NoAL voles (Fig. 2A, $F_{(1,35)} = 12.948$, $p = 0.001$). The effect of alloparenting experience was observed in both males (Fig. 2B, E, $p < 0.05$) and females (Fig. 2C, D, $p < 0.05$). NoAL voles also traveled over twice the distance AL voles traveled during OFT ($F_{(1,35)} = 10.001$, $p = 0.003$). There was no significant sex difference or significant sex * treatment interaction for time spent in the center zone or total distance traveled during the OFT.

Alloparenting experience had no effect on future social interactions with same-sex novel peer or juvenile voles

We did not find any differences in social behavior on the social interaction or juvenile affiliation tests ($p > 0.05$, Table 1).

Alloparenting experience has region- and sex-specific effects on levels of the mature isoform of BDNF protein in non-tested voles

Western blot analysis detected a signal specific to the molecular weight of mature BDNF at approximately 14 kDa. In animals that were not tested in behavior tests, we found that voles with alloparenting experience had approximately 50% less BDNF protein in CA1 hippocampus (Fig. 3A, $F_{(1,20)} = 12.883$, $p = 0.002$) compared to NoAL voles. There was no sex \times treatment interaction, as the effect of alloparenting experience was similar in males and females. Analyses of BNST BDNF protein in males and females showed an overall effect of alloparenting ($F_{(1,20)} = 27.032$, $p < 0.001$) and a sex \times treatment interaction: (Fig. 3B, $F_{(1,20)} = 5.429$, $p = 0.030$). Alloparenting experience had a dramatic effect on BNST BDNF in males, resulting in a 200% increase in BDNF levels over NoAL counterparts ($p < 0.001$). AL females had an 80% increase in BDNF compared to NoAL females (planned comparisons, $p < 0.02$).

Analyses of BDNF from behaviorally tested males and females suggest that effects of alloparenting experience on BDNF expression are longer lasting in the BNST compared to the CA1. Overall, there was a significant main

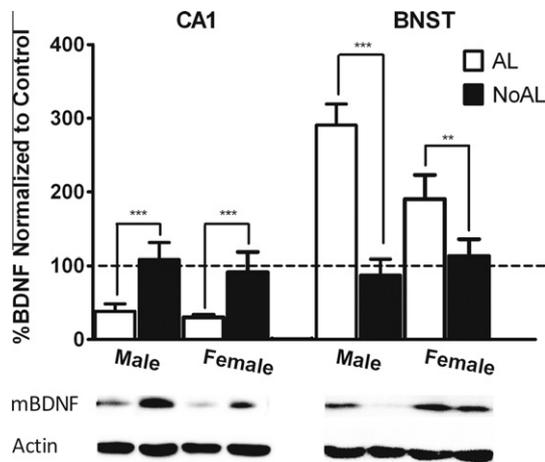


Fig. 3. Punches from hippocampus (CA1) and the BNST of nontested sibling voles showed that voles with alloparenting experience had (A) significantly reduced levels of mBDNF in male and female hippocampus. Conversely, AL males displayed (B) a nearly 300% increase in BNST mBDNF levels, and AL females also had significant increases in BNST BDNF when compared to NoAL voles. Data are shown as mean \pm SE, with ** $p < 0.02$ and *** $p < 0.01$ indicating significant differences from voles undergoing standard weaning treatments (NoA) using planned comparisons analysis.

effect of alloparenting experience on BDNF levels in the BNST, resulting in a 30% increase in protein in ALs versus NoALs (Table 2, $F_{(1,28)} = 6.969$, $p = 0.013$). Behaviorally tested AL males showed a nearly 40% increase in BNST BDNF over NoAL males (planned comparisons, $p < 0.01$). Tested females also showed a significant increase in BNST BDNF (planned comparisons, $p < 0.05$) that was similar to that observed in males, and there was no significant sex \times treatment interaction. Correlational analyses showed that BNST BDNF in tested voles was negatively correlated with time spent in the center zone of the OFT (Fig. 4, $r = -0.57$, $p < 0.001$). Although increases in BDNF were observed in CA1 for both tested NoAL males and females compared to same-sex counterparts in the AL group, these differences were not significant (Table 2, $p > 0.05$).

DISCUSSION

Early-life experiences are capable of inducing long-term effects on anxiety- and depressive-like behaviors (Coplan et al., 1996; Goel and Bale, 2009), but the impact of alloparenting experience on the brain and behavior has not been investigated. Using the extensive family networks of the prairie vole, we have demonstrated for the first time that alloparenting experience increases anxiety-like behaviors. The anxiogenic effects of alloparenting experience on behavior were limited to novel contexts, with no effects observed on measures of social behavior. Changes in behavior were associated with changes in BDNF expression in the BNST and CA1 hippocampus. Alloparenting experience decreased BDNF protein levels in CA1 but increased BDNF expression in BNST. The

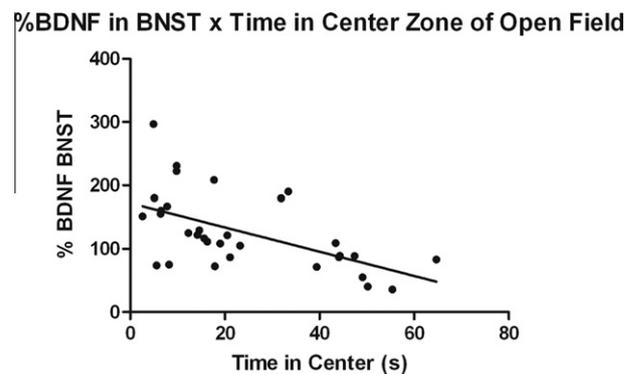


Fig. 4. BDNF protein expression in BNST (percent BDNF normalized to control) was negatively correlated with exploratory behavior (time in center zone) of the OFT.

Table 2. Behaviorally tested voles with alloparenting experience had significantly elevated BDNF in the BNST

Region	Male NoAL	Male AL	Female NoAL	Female AL
CA1	73.6 \pm 13.7	62.8 \pm 14.3	126.4 \pm 53.8	70.6 \pm 24.0
BNST	102.5 \pm 18.1**	159.4 \pm 24.0**	97.5 \pm 13.6*	145.5 \pm 20.3*

Values from Western blot are relative intensities normalized to voles undergoing standard weaning treatments (NoAL).

Data are shown as mean \pm SE.

* $p < 0.05$.

** $p < 0.01$, planned comparison NoAL versus AL.

changes in the BNST were particularly robust, as they were detected both in behaviorally phenotyped animals as well as nontested animals.

The effects of alloparenting experience on BDNF were region-specific. These findings are consistent with other studies that have reported region-specific associations of BDNF with behavior. In mice, social defeat stress increased BDNF protein in the nucleus accumbens (NAc) (Berton et al., 2006) but decreased BDNF mRNA in the hippocampus (Pizarro et al., 2004; Tsankova et al., 2006). Our findings are generally consistent with discoveries that have implicated BDNF action in the hippocampus as an anxiolytic mechanism. Less is known about the function of BDNF in the BNST, but evidence suggests that it may exert angiogenic effects. Exogenous BDNF infusions have increased transcription of corticotropin-releasing hormone (CRH) mRNA (Toriya et al., 2010) in the hypothalamus. The BNST contains populations of CRH neurons and receptors (Chalmers et al., 1995), and CRH infusions into the BNST increased anxiety-like behavior (Lee and Davis, 1997). Thus, while alloparenting experience induces anatomically specific changes in BDNF expression, they are all consistent with an increased anxiety phenotype.

Voles that stay home during adolescence and early adulthood are more anxious

In general, human and rodent studies suggest a relationship between parental behaviors and lower levels of anxiety. Lactating rat dams displayed lower levels of anxiety-like behavior than virgin females (Neumann, 2001; Wartella et al., 2003; Lonstein, 2007; Kinsley and Lambert, 2008), and breast-feeding mothers have also reported lower levels of anxiety compared to mothers using bottles (Fleming et al., 1990). In female rats, decreases in anxiety-like behavior at parturition have been linked to upregulated oxytocin activity (Bosch, 2011), but these changes appear to be independent of increased oxytocin release during parturition or suckling (Lonstein, 2005). In addition, in the biparental California mouse, male parents have exhibited decreased anxiety-like behavior when compared to sexually inexperienced males (Bardi et al., 2011), and mating induced a 2-week elevation in male plasma oxytocin level (Gubernick et al., 1995). In a previous study, we found that male prairie voles with alloparenting experience performed more parental behaviors (e.g. licking and grooming) toward their own offspring (Stone et al., 2010).

Thus, we hypothesized that alloparenting experience and exposure to a communal living environment would increase exploration and social behavior in tested voles. Opposite to our predictions, voles that were separated from the colony as juveniles displayed less anxiety-like behavior and more exploratory behavior. These findings appear to be counterintuitive and in conflict with the literature. However, unlike the aforementioned parenting studies in rats and California mice, female alloparents in this study did not experience the hormones associated with pregnancy or parturition, and male alloparents were not exposed to a pregnant female mate. Our behavioral data suggest that the relationship between sibling interactions

is fundamentally different from parent–offspring interactions, at least in the context of anxiety-like behavior.

It may be that the early experience of alloparenting induces an angiogenic signal indicating a dangerous outside environment. Although communal groups have been observed in prairie vole populations across seasons, they are most prevalent during the late autumn and winter months (Getz et al., 1993). In fact, voles are more than twice as likely to reside within communal groups during these months compared to the months between March and mid-October and the size and stability of the group also increase during this period (Getz and Carter, 1996). However, mortality also increases as this period progresses and vegetation/food sources dwindle. Because the formation of these groups coincides with increased environmental stressors, being part of a communal group may serve as an indicator of winter and harsh conditions. Under these conditions, juveniles would likely find dispersal to an outside habitat unsuitable, and it is therefore an advantage to remain in the natal nest. A different form of communal living has been observed in house mice (*Mus musculus*), in which multiple dams sometimes share a common nest (Hayes, 2000). Consistent with our data, mice raised in communal nests had increased anxiety-like behavior on the EPM and OFT as adults (Branchi et al., 2006). While the effects of alloparenting experience on anxiety-like behavior resemble that of communal nesting, they appear to be mediated by different neurobiological mechanisms.

Hippocampal BDNF and anxiety-like behavior

Our results are consistent with previous observations that social deprivation stress intensified anxiety-like behavior and decreased hippocampal BDNF (Berry et al., 2012). In contrast, antidepressant treatments have increased hippocampal BDNF expression and decreased anxiety-like behaviors (Nibuya et al., 1995). Additionally, the majority of pharmacological experiments suggest that BDNF signaling in the hippocampus is anxiolytic. Infusions of BDNF into a rat hippocampus have induced increases in time spent in the open arms of the EPM (Cirulli et al., 2004). Loss-of-function studies have been more complicated, both because of evidence for region-specific effects of BDNF and also because BDNF knockout is lethal (Ernfors et al., 1994; Lyons et al., 1999). One intriguing example has come from studies of the Val-Met mutation in the BDNF gene (Egan et al., 2003). The mutation is a single-nucleotide polymorphism, resulting in methionine substitution for valine at codon 66, and is associated with reduced secretion of BDNF by neurons and reduced hippocampal size in both humans (Bueller et al., 2006) and mice. BDNF^{Met/Met} mice have also exhibited increased anxiety-like behavior (Chen et al., 2006). Decreased BDNF activity has been observed in mice with deletion of one copy of the BDNF gene (+/−) (Ernfors et al., 1994). The action of BDNF also mediates sensitivity to the environment. Wild-type mice assigned to environmental enrichment with increased social (i.e. > 10 cagemates) and sensory (i.e. toys, tunnels, and ladders) stimulation have amplified dendritic spine formation in CA1 hippocampus and increased exploratory behavior on the OFT compared to mice housed in standard cages

(i.e. 2–3 cagemates with no toys). These effects of enrichment are blunted in BDNF +/– mice (Zhu et al., 2009). Intriguingly, the effect of alloparenting experience on hippocampal BDNF expression resembles the effects of early-life stressors. For instance, exposing juvenile rats to predator scent stress has decreased BDNF mRNA in adult CA1 hippocampus compared to controls, and this change in BDNF is associated with increased anxiety-like behavior on the EPM (Kozlovsky et al., 2007; Bazak et al., 2009). Thus, our behavioral observations, in combination with molecular findings, support an anxiogenic effect of alloparenting experience.

Not all studies have reported an anxiolytic link for hippocampal BDNF function. For example communal nest rearing in mice has produced pups with increased anxiety-like behavior and increased hippocampal BDNF levels in adulthood when compared to mice that were raised in standard rearing conditions (Branchi et al., 2006). On the other hand, we observed *decreased* levels of BDNF in the hippocampus in animals with alloparenting experience, which is more consistent with an anxiety phenotype. There are major differences between the two studies which may contribute to the alternative outcomes. Voles assigned to alloparenting experience were exposed to infants during adolescence and were not weaned until adulthood (postnatal day 63), whereas mice raised in communal nests were not exposed to younger siblings and were weaned before puberty (postnatal day 25). Additionally, in the mouse study, mice were individually housed for 3 weeks prior to behavioral testing, whereas the voles were group-housed. Social isolation has been found to increase BDNF protein expression in rat hippocampus (Meng et al., 2011). Finally, in the studies of communal nesting, BDNF measurements were made from behaviorally-tested mice. In our study, the effects of alloparenting experience on hippocampal BDNF were weaker in behaviorally phenotyped voles, suggesting that behavioral testing influenced hippocampal BDNF expression. It is likely that some or all of these factors may have altered the relationship between BDNF and anxiety-like behavior across studies.

BDNF in BNST and anxiety-like behavior

Our data also suggest a potentially novel anxiogenic role for BDNF signaling in the BNST. The effect of alloparenting experience on BDNF expression in the BNST was the opposite of that observed in the hippocampus. The BNST is part of the “extended amygdala,” with connections to the central and medial nuclei of the amygdala (Alheid et al., 1998). It is an important locus for unconditioned anxiety-like behavior in particular, as opposed to conditioned fear behaviors (Davis, 1998; Singewald et al., 2003; Davis et al., 2009). Also, chronic stress has been found to increase BDNF mRNA expression in the BNST (Hammack et al., 2009). To our knowledge, this is the only study that has examined BDNF protein expression in this nucleus, and our study is the first to report effects of the social environment on this protein. Increased BDNF could induce greater anxiety-like behavior through downstream neuropeptide signaling, such as corticotropin-releasing hormone (CRH). There is considerable evidence that intracerebral CRH is anxiogenic (Koob and Heinrichs,

1999), specifically via CRH receptor 1 signaling (Kehne and Cain, 2010), and infusions of BDNF into the lateral ventricle have increased CRH transcription in the paraventricular nucleus of the hypothalamus (Naert et al., 2011). The BNST contains both CRH cell bodies as well as CRH receptors that may be stimulated by hypothalamic CRH neurons. Increased BNST BDNF in AL voles could be a product of increased activation of these neurons.

Increased BDNF signaling could also regulate neuroplasticity within the BNST following stimulation from pituitary adenylate cyclase-activating polypeptide (PACAP) (Hammack et al., 2010), a peptide that has recently been associated with fear response and mood disorders in humans (Ressler et al., 2011). Chronic unpredictable stress increases dendritic length (Pêgo et al., 2008) and increases the number of branch points observed in the dendritic arborization of BNST neurons (Vyas and Mitra, 2002). BDNF signaling-induced plasticity could influence anxiety-like behavior by altering the conductance of GABA receptors. Application of BDNF to slice cultures reduces the frequency and amplitude of spontaneous inhibitory postsynaptic currents (IPSPs) (Ohbuchi et al., 2009). In general GABA inhibition of the BNST has anxiolytic effects. Chronic infusion of the GABA synthesis inhibitor L-allylglycine into the BNST increased anxiety-like behavior of rats on the EPM (Sajdyk et al., 2008), whereas muscimol (a GABA_A receptor agonist) infusions into the BNST inhibit predator odor-induced freezing behavior in rats (Fendt et al., 2003). BDNF-induced modulations of BNST neuronal activity and their relation to anxiety-like behavior should be further examined.

Interestingly, increases in BNST BDNF protein in AL voles were maintained following extensive behavioral testing in our tested voles, and alloparenting experience resulted in a 120% larger increase in BNST BDNF in males than females. Initial studies on CRH alluded to sex differences in functionality due to its presence in areas involved in sexual behavior (e.g. MPOA) (Aguilera et al., 1987). In prairie voles, males have significantly higher CRFR₂ binding in the BNST than females, and this sex difference is not found in promiscuous rats, mice, or nonhuman primates (Lim et al., 2005). Further evidence from prairie voles suggests that DA cells in BNST may have a larger effect on male behavioral responses to early social environment than females. Male prairie voles have greater expression of dopaminergic cells (TH-ir) in the BNST than females, and males also have greater activity in these cells (c-Fos-ir) following exposure to various housing conditions and social stimuli (i.e. housed within colony, alone, with sibling) (Northcutt and Lonstein, 2009). Additionally, male mice have a larger BNST with more cells than females (Chung et al., 2000). More recently, CRH and its distribution of receptors have been shown to produce sexually-dichotomous responses to stress. Female CRHR₂ knockout mice have displayed decreased climbing time compared to wild-type females during the forced swim test, but this difference is not observed in male mice (Bale and Vale, 2003). Our evidence suggests that changes in BNST BDNF associated with anxiety-like behavior are greater in male prairie voles compared to females.

CONCLUSIONS

Our results show that living in complex family groups exerts long-lasting effects on BDNF expression and anxiety-like behavior. It appears that prolonged contact with parents and siblings creates a fundamentally different behavioral and neurobiological phenotype compared to prolonged contact with parents only. The most robust molecular effects of alloparenting experience were observed in the BNST. There has been a growing interest in the effects of BNST activity on anxiety in humans (Somerville et al., 2010), and our data suggest that modulations of BNST signaling may contribute to anxiety-like behavior in rodents. Our data also suggest certain aspects of family structure may influence anxiety. Human studies have reported conflicting results, with one study reporting a positive correlation between adolescent anxiety symptoms and family size (Ozer et al., 2008), and a negative correlation in a second study (Bayer et al., 2008). Currently it is unclear whether certain aspects of sibling interactions are anxiogenic, thus increasing the development of anxiety disorders later in life (Merikangas, 2005).

There is evidence that aspects of family living can be stressful in humans. Domestic violence is the most frequent form of physical abuse, and other forms of psychosocial aggression (e.g. shouting, swearing, or threats) occur in approximately 90% of U.S. households (Straus and Gelles, 1988; Straus and Field, 2003). Clinical studies suggest that differences in family structure can influence vulnerability to stress-induced mood disorders, such as anxiety and depression, and there is substantial evidence that the early postnatal environment is a critical factor for the development of anxiety later in life (Tweed et al., 1989; Heim and Nemeroff, 2001; Gross et al., 2002). Further study of alloparenting behavior in voles should provide useful insights for interpreting and designing future studies of the impact of the family environment on anxiety.

Acknowledgments—This study was supported by the National Institutes of Health Grant R21 MH090392 to B.C.T., R01 MH073022, HD060117 to K.L.B. G.G. was supported by the National Institutes of Health Neuroscience Training Grant T32 MH 82174-3. The authors thank Cindy Clayton, for veterinarian assistance; Paul Hastings, Danica Peterson, and Dana Dooley for helpful discussions; and Joy Greenberg for comments on the manuscript. The authors also thank Luana Griffin, Gillian Meyers, Heather Voigt, Raymond Wong, Ellen Seeley and Jon Judkins for technical assistance.

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