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Fatherhood reduces the survival of adult-generated cells and affects various types of behaviors in the prairie vole (*Microtus ochrogaster*)

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Abstract

Motherhood has profound effects on physiology, neuronal plasticity, and behavior. We conducted a series of experiments to test the hypothesis that fatherhood, similarly to motherhood, affects brain plasticity (such as cell proliferation and survival) and various behaviors in the highly social prairie vole (*Microtus ochrogaster*). In Experiment 1, adult males were housed with their same-sex cage mate (control), single-housed (isolation), or housed with a receptive female to mate and produce offspring (father) for 6 weeks. Fatherhood significantly reduced cell survival (assessed by bromodeoxyuridine labeling), but not cell proliferation (assessed by Ki67 labeling), in the amygdala, dentate gyrus of the hippocampus, and ventromedial hypothalamus, suggesting that fatherhood affects brain plasticity. In Experiment 2, neither acute (20 min) nor chronic (20 min daily for 10 consecutive days) pup exposure altered cell proliferation or survival in the brain, but chronic pup exposure increased circulating corticosterone levels. These data suggest that reduced cell survival in the brain of prairie vole fathers was unlikely to be due to the level of pup exposure and display of paternal behavior, and may not be mediated by circulating corticosterone. The effects of fatherhood on various behaviors (including anxiety-like, depression-like, and social behaviors) were examined in Experiment 3. The data indicated that fatherhood increased anxiety- and depression-like behaviors as well as altered aggression and social recognition memory in male prairie voles. These results warrant further investigation of a possible link between brain plasticity and behavioral changes observed due to fatherhood.

Keywords

amygdala; bromodeoxyuridine; hippocampus; Ki67; paternal behavior

Introduction

Motherhood has profound effects on physiology and neural plasticity (Taya and Greenwald, 1982; Caba et al., 1996; Numan and Insel, 2003; Kinsley et al., 2006), including the down-regulation of adult neurogenesis (generation and functional integration of new neurons in the adult brain) in several mammalian species. Specifically, motherhood reduces cell proliferation and survival in the dentate gyrus (DG) in rats, cell survival in the DG in

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California mice (*Peromyscus californicus*), and cell proliferation in the DG and subventricular zone in sheep (Leuner et al., 2007; Brus et al., 2010; Glasper et al., 2011). This motherhood-induced reduction seems offspring-dependent, as pup removal prevents the suppression of adult neurogenesis (Leuner et al., 2007).

Motherhood also leads to dramatic behavioral changes, including the shift from indifference to young to caring for offspring (Numan and Insel, 2003; Numan and Stolzenberg, 2009). Furthermore, mothers show hypo-responsiveness to various stressors such as noise, restraint, and immune challenge (da Costa et al., 1996; Windle et al., 1997; Shanks et al., 1999). Motherhood also increases aggression (Lonstein and Gammie, 2002), depression-like behaviors (for review see Workman et al., 2011), and spatial memory (for review see Macbeth and Luine, 2010).

Even though fatherhood also plays an important role in the well-being of offspring, especially in biparental species including humans (Videon, 2005; Graziano et al., 2009; Ahern et al., 2010), little is known about the effects of fatherhood on physiology, neuroplasticity, and behaviors. Nevertheless, some studies have shown that fatherhood modulates various neurotransmitter (Gubernick et al., 1995; Kozorovitskiy et al., 2006) and hormonal (Gubernick and Nelson, 1989; Storey et al., 2000; Ziegler and Snowden, 2000) systems. Additionally, the exposure of virgin males to pups and the induction of paternal behavior in normally non-paternal males increase the number of adult-generated cells in the DG of prairie voles (*Microtus ochrogaster*) and male mice, respectively (Ruscio et al., 2008; Mak and Weiss, 2010). Interestingly, fatherhood reduces hippocampal cell survival in the biparental California mouse (*P. californicus*) (Glasper et al., 2011). Furthermore, fatherhood in paternal species, similarly to motherhood, causes a behavioral shift toward pups (Bamshad et al., 1994; Gubernick and Teferi, 2000); however, we know virtually nothing about the effects of fatherhood on anxiety-like, depression-like, and agonistic behaviors.

The prairie vole (*M. ochrogaster*) is a socially monogamous species that displays spontaneous biparental behavior toward its offspring (Lonstein and De Vries, 1999). In addition, virgin males display paternal behavior toward conspecific pups (Bamshad et al., 1994). In the present study using this animal model, we assessed the effects of fatherhood on cell proliferation and survival in various brain regions. We then compared the effects of acute and chronic pup exposure on cell proliferation and survival in the brain. Lastly, we investigated the effects of fatherhood on anxiety-like, depression-like, and social behaviors.

Materials and methods

Subjects

Male prairie voles (*M. ochrogaster*), offspring of our laboratory breeding colony, were weaned at 21 days of age and housed in same-sex pairs in plastic cages [13 × 29 × 16 (H) cm] in a temperature-controlled (21 ± 1 °C) and light-controlled (14 : 10 light : dark with lights on at 07:00 h) environment with *ad-libitum* access to food and water. Subjects were used in experiments when they reached 70-120 days of age. All experimental procedures were approved by the Animal Care and Use Committee of Florida State University and conformed to the guidelines set forth by the NIH.

Experiment 1: the effects of fatherhood on cell proliferation and survival

Subjects were injected intraperitoneally with a cell division marker, bromodeoxyuridine (BrdU) (100 mg/kg body weight; Sigma, St Louis, MO, USA), once daily (between 08:00 and 10:00 h) for 14 consecutive days. This injection paradigm has previously been used to label a large cohort of newly proliferated cells without causing neurotoxicity (Cameron and

McKay, 2001). Following the last BrdU injection, subjects were randomly assigned into one of three treatment groups: same-sex pair-housed control group (CTRL, subjects remained with their familiar same-sex cage mate; $n = 10$), single-housed isolation group (ISO, subjects were separated from their cage mate; $n = 10$), or placed with an intact, unrelated adult female (FATHER; $n = 7$). Subjects in all three groups were placed into new cages [$13 \times 29 \times 16$ (H) cm] at the beginning of the social environment treatment. Twenty-one days later, the FATHER group was checked daily for the presence of pups. To allow full paternal experience, the pups remained with their parents. On postnatal day 1 of the second litter (approximately 6 weeks after pairing with the female), subjects in the FATHER group and those that were time-matched for the CTRL and ISO groups were deeply anesthetized and perfused through the ascending aorta with 0.9% saline followed by 4% ice-cold paraformaldehyde solution.

Experiment 2: the effects of acute and chronic pup exposure on hypothalamic–pituitary–adrenal axis activation and cell proliferation and survival

As the FATHER group differed significantly from the other two treatment groups in the number of BrdU-labeled cells in selected brain areas, we examined whether such differences were due to pup exposure and the display of paternal behavior, independent of fatherhood. Adult males were paired with a receptive ovariectomized female that was estrogen-primed (one daily subcutaneous injection of estradiol benzoate at $1 \mu\text{g}/100 \mu\text{L}$ in sesame oil for three consecutive days) to ensure the female's sexual receptivity (Insel et al., 1995). At 24 h after pairing, males were injected with BrdU four times per day (6-h injection interval) for three consecutive days (50 mg/kg body weight). The dose and injection paradigm have previously been used to label a large cohort of proliferating cells that were of similar age at the time of treatment (Taupin, 2007). Further, mating and cohabitation significantly increases alloparental behavior and decreases infanticidal behavior in male prairie voles (Bamshad et al., 1994; Terleph et al., 2004). Following the 2-week cohabitation period during which the adult-generated cells reached an age characterized by enhanced sensitivity to stimulus-specific modulation (Tashiro et al., 2007), males were used in the following experiments.

Experiment 2a examined the effects of acute pup exposure on hypothalamic–pituitary–adrenal (HPA) axis activation and cell plasticity. Subjects were randomly assigned to one of three acute treatment groups: object ($n = 11$), pup ($n = 11$), or handle ($n = 10$) exposure. All subjects were placed individually into a large testing cage [$25 \times 45 \times 20$ (H) cm] with *ad-libitum* food and water at around 08:00 h and allowed to habituate for 3 h. Subsequently, subjects in the acute object and acute pup groups were exposed to a pup-sized plastic piece or to a 1–3-day-old unrelated, conspecific pup, respectively. The plastic piece or pup was placed at the opposite side of the cage, furthest away from the subject, and the subject's behavior was video recorded for 20 min. Subjects in the acute handle group remained undisturbed in the testing cage. Each pup was only used once. Immediately following the 20-min exposure test, each male was returned to its home cage (containing its female partner). Twenty-four hours later, subjects were deeply anesthetized, their cardiac blood was collected via right atrium incision, and subjects were perfused with 0.9% saline followed by 4% ice-cold paraformaldehyde solution.

Experiment 2b examined the effects of chronic pup exposure on HPA axis activation and cell plasticity. Subjects were randomly assigned to one of three chronic treatment groups: object ($n = 10$), pup ($n = 7$), or handle ($n = 10$) exposure. The chronic treatment exposure was identical to the acute treatment exposure as described above, except that testing occurred once daily for 10 consecutive days. Each subject in the chronic pup group was exposed to a pup from the same litter for the 10-day pup exposure. Immediately following

the 20-min exposure test, each subject was returned to its home cage (containing its female partner). At 24 h following the last exposure test, subjects were deeply anesthetized, their cardiac blood was collected and they were perfused as described above.

Experiment 3: the effects of fatherhood on anxiety-like, depression-like, and social behavior

Adult-generated cells have been implicated in a variety of behavioral functions (e.g. Shors et al., 2002; Jessberger and Kempermann, 2003; Ramirez-Amaya et al., 2006; Belnoue et al., 2011). As FATHER males differed from the CTRL and ISO males in the number of BrdU-labeled cells in a brain region-specific manner, we examined several types of behaviors in males with different reproductive experiences. Sexually naïve males were randomly assigned to one of three treatment groups: (i) remained in the same-sex pair-housing condition to serve as baseline controls (CTRL; $n = 13$), (ii) were placed with an ovariectomized, estrogen-primed female for mating and pair-bond experience (MATED; $n = 15$), or (iii) were placed with an intact female and remained with the female and subsequently their pups to obtain fatherhood experience (FATHER; $n = 10$). As pilot data indicated that fatherhood leads to changes in neurochemical systems at around postnatal day 6 (e.g. corticotrophin-releasing factor receptors; data not shown), our behavioral testing started at postnatal day 6 and was time-matched for subjects in the CTRL and MATED groups. All subjects were tested on a series of behavioral tests, one test per day in the following order: open field (OF), elevated plus maze (EPM), social discrimination (SD), resident intruder test, and forced swim test (FST). All testing started at 08:00 h.

Behavioral tests

The 10-min OF test was conducted, as previously described, to evaluate exploratory and anxiety-like behaviors (Pan et al., 2009; Lieberwirth et al., 2012). The plastic OF apparatus measured $56 \times 56 \times 20$ (H) cm and had a visual line grid that divided the apparatus into 16 squares, each measuring 14×14 cm. Each subject was placed into the center of the OF, and anxiety-like behaviors (i.e. frequency of center entries and duration spent in the center or corners) and an index of locomotion (frequency of line crosses) were quantified.

The 5-min EPM test was also conducted to assess anxiety-like behaviors (Grippio et al., 2008; Lieberwirth et al., 2012). The testing apparatus was elevated 45 cm off the ground and consisted of two open (35×6.5 cm) and two closed [$35 \times 6.5 \times 15$ (H) cm] arms that crossed in the middle. Each subject was placed onto the center of the EPM facing an open arm. Anxiety-like behaviors (i.e. latency to enter the open arm and duration in the open and closed arms) and an index of locomotion (total arm entries) were quantified.

The SD test was conducted to assess social memory and discrimination. The three-chambered apparatus consisted of a center cage that was attached by hollow tubes (16 L $\times$ 7.5 radius cm) to two identical chambers [$13 \times 29 \times 16$ (H) cm], each containing a stimulus juvenile (30–40 days of age) inside a wire-mesh box (7.5 cm³). The SD protocol previously described for mice (Winslow, 2003) and rats (Landgraf et al., 2003) was modified to be suitable for prairie voles. Briefly, the subject was placed into the neutral center cage and was allowed to explore the two juveniles (J1 and J2) for 10 min during the familiarization trial (T1). The intertrial interval lasted 2 h during which the subject was placed into a holding cage with *ad-libitum* access to food and water. During the subsequent 10-min recognition trial (T2), the subject was exposed to one familiar juvenile (J1 or J2) from the familiarization trial and one unfamiliar stimulus juvenile (New). T1 and T2 were video recorded and the time that the subject spent investigating each juvenile during T1 and T2 was quantified.

The 10-min resident intruder test was conducted to assess agonistic behavior toward a stimulus male, as described previously (Gobrogge et al., 2007). Each subject was placed into a large cage [25 × 45 × 20 (H) cm] with approximately 20 g of home cage bedding (and *ad-libitum* access to food and water) for 3 h to establish residency. A weight-matched stimulus male (intruder) was then introduced into the cage and behavioral interactions were video recorded. The frequency and duration of the subject's aggressive behaviors toward the intruder (i.e. aggressive approaches, attacks, and chases) and non-aggressive olfactory investigation of the intruder (i.e. naso-genital and naso-nasal investigation) were quantified.

The 5-min FST was conducted to assess depression-like behaviors (Grippe et al., 2009; Lieberwirth et al., 2012). A clear tank [25 × 45 × 20 (H) cm] was filled with tap water (32 ± 1°C) to a depth of 13 cm. Each subject was placed into the middle of the tank and allowed to move around freely during the test. Depression-like behaviors (i.e. latency to immobility, frequency of immobility bouts, and immobility duration) and an index of locomotion (swim duration) were quantified. At 24 h after the FST, subjects were deeply anesthetized and atrial blood was collected.

Tissue preparation for immunohistochemistry

After postfixing in paraformaldehyde, the perfused brains were transferred into 30% sucrose in 0.1 M phosphate buffer for storage. The brains were sectioned coronally at 40 µm using a sliding microtome. To assess cell survival, one set of brain sections at 240-µm intervals was processed for BrdU immunohistochemistry using a rat anti-BrdU antibody (1 : 1000; Accurate, Westbury, NY, USA) and diaminobenzidine to visualize the staining. BrdU labeling was conducted using a previously established method (Lieberwirth et al., 2012). A second set of brain sections was processed for Ki67 immunohistochemistry to assess cell proliferation. The nuclear protein, Ki67, is only expressed in dividing cells and can be used as an endogenous proliferation marker (Scholzen and Gerdes, 2000). The sections were incubated in rabbit anti-Ki67 antibody (1 : 5000; Vector, Burlingame, CA, USA) and the staining was visualized using diaminobenzidine, following an established protocol (Lieberwirth et al., 2012). Both stains resulted in clearly labeled nuclei and visible structural landmarks (such as fiber bundles), as observed in our previous studies (Fowler et al., 2002; Lieberwirth et al., 2012), allowing for the identification of select brain regions.

Cell quantification and analysis

All microscope slides were coded to disguise the treatment condition until data quantification was completed. BrdU-immunoreactive (ir) and Ki67-ir cells were quantified in the granular cell layer, hilus, and molecular cell layer of the DG; the basolateral, central, cortical, and medial nuclei of the amygdala (AMY); and the ventromedial hypothalamus (VMH) (Experiments 1 and 2). BrdU-ir cells were also quantified in the external plexiform layer and the granular cell layer of the MOB (Experiment 1). BrdU-ir cells were quantified using a Zeiss Axioskop II microscope with the optical fractionator workflow (for stereological sampling parameters see Supplementary Table S1) of the Stereo Investigator software (MBF Bioscience, Chicago, IL, USA). Ki67-ir cells were quantified using a modified version of the optical fractionator method (West et al., 1991). Briefly, Ki67-ir cells were counted bilaterally on every sixth section and the sum of the counted cells across all sections for a given brain area was multiplied by six to obtain an estimate of the total number of Ki67-ir cells within the corresponding brain region (for detailed methods, see Leuner et al., 2009).

Blood collection and corticosterone measurement

Atrial blood (~400 µL) was collected into microcentrifuge vials containing 20 µL EDTA. The blood was centrifuged at 6000 rpm for 15 min at 4°C. After the aspiration of the plasma,

it was centrifuged again at 6000 rpm for 10 min at 4°C. The plasma was aliquoted into micro-centrifuge vials and stored at -80°C until processed for corticosterone (CORT) measurement using a CORT radioimmunoassay kit (Diagnostic Products Corp., Los Angeles, CA, USA) that had previously been validated in prairie voles (Stowe et al., 2005; Bosch et al., 2009; Lieberwirth et al., 2012). Plasma CORT (1 : 1000) was measured in 10-μL plasma samples in duplicates following the kit instructions. The detection limit of the CORT kit was 7.7 ng/mL. The intra- and interassay coefficients of variation were 2.85% and 2.11%, respectively.

Behavioral quantification

All behavioral videos were scored by a trained observer who was blind to the subject's treatment using the J-Watcher V1.0 software (Macquarie University and UCLA; <http://www.jwatcher.ucla.edu/>) to quantify test-specific behaviors. In Experiment 3, the subject's behaviors in each of the behavioral tests were quantified as described above. For the SD test, a1 and a2 represented the exploration times of J1 and J2, respectively, during T1, and a3 and b referred to the exploration times of the familiar and the new juvenile, respectively, during T2. In turn, the total exploration times for T1 and T2 were calculated as: $e1 = a1 + a2$ and $e2 = a3 + b$, respectively. In addition, the following measures of discrimination between the familiar and novel juvenile were calculated as: $d1 = b - a3$ and $d2 = d1/e2$ (see Supplementary Table S2; adapted from Akkerman et al., 2012a; Akkerman et al., 2012b; van Goethem et al., 2012). $d2$, a relative measure of discrimination, was corrected for exploration activity during the discrimination trial ($e2$) and can range from -1 (indicating complete preference for the familiar stimulus) to 1 (indicating complete preference for the unfamiliar stimulus). $e1$, $e2$, $d1$, and $d2$ were analyzed by one-way ANOVA to assess group differences in exploration and discrimination. Significant group differences for $d1$ and $d2$ were further analyzed. A paired-sample t -test comparing a3 and b was used to analyze the group difference for $d1$ and a one-sample t -test compared with 0 was used to further analyze group differences for $d2$.

Data analysis

Group differences in the numbers of BrdU-ir and Ki67-ir cells in each brain area, in the circulating CORT levels, and in the frequency and duration of the behaviors in each behavioral test were analyzed with a one-way ANOVA, followed by a Student Neuman–Keul's posthoc test. All statistical analyses were performed using PASW Statistic 18 software. Data were expressed as mean ± SEM, and $p < 0.05$ was considered statistically significant.

Results

Experiment 1: the effects of fatherhood on cell proliferation and survival

The social environment significantly affected cell survival in a brain region-specific manner, indicated by group differences in the number of BrdU-ir cells in the AMY ($F = 6.55$, $p < 0.01$) (Fig. 1A–C and G), DG ($F = 3.38$, $p < 0.05$) (Fig. 1D–F and G), and VMH ($F = 5.24$, $p < 0.05$) (Fig. 1G), but not the MOB ($F = 0.65$, $p = 0.53$; data not shown). Posthoc analyses for the AMY, DG, and VMH indicated that FATHER males showed a significant reduction in BrdU-labeling compared with the CTRL and ISO males. ISO males also showed a significant reduction in the number of BrdU-ir cells in the VMH, but not AMY or DG, compared with CTRL males (Fig 1G). Analyses of the amygdaloid subnuclei revealed that the social environment also affected cell survival in a subregion-specific manner (Table 1). Although no group differences were observed in the basolateral AMY ($F = 1.34$, $p = 0.28$), FATHER males had significantly fewer BrdU-ir cells in the central AMY than CTRL males ($F = 3.75$, $p < 0.05$). FATHER males also had fewer BrdU-ir cells in the cortical AMY than

males in both the ISO and CTRL groups ($F = 3.67, p < 0.05$). Furthermore, both FATHER and ISO males had significantly fewer BrdU-ir cells in the medial AMY compared with CTRL animals ($F = 7.60, p < 0.01$). Analyses of the subregions of the DG revealed that fatherhood, but not social isolation, significantly reduced the number of BrdU-ir cells in the hilus ($F = 3.91, p < 0.05$) and the molecular cell layer ($F = 5.50, p < 0.05$), whereas no group differences were found in the granular cell layer ($F = 2.18, p = 0.14$) (Table 1). In addition, no group differences were observed in the number of BrdU-ir cells in the subregions [i.e. external plexiform ($F = 0.28, p = 0.76$) and granular cell ($F = 0.85, p = 0.44$) layer] of the MOB (Table 1). The social environment did not affect cell proliferation, as assessed by the number of Ki67-ir cells, in the AMY ($F = 1.07, p = 0.37$), DG ($F = 1.15, p = 0.35$), and VMH ($F = 2.85, p = 0.10$) (Fig. 1H, Table 1).

Experiment 2a: the effect of acute pup exposure on basal corticosterone levels and cell proliferation and survival

Acute pup exposure did not affect basal CORT levels across treatment groups ($F = 0.68, p = 0.52$) (Fig. 2A). Furthermore, acute pup exposure did not affect Ki67-ir labeling in the AMY ($F = 0.91, p = 0.42$), DG ($F = 0.92, p = 0.42$), or VMH ($F = 0.25, p = 0.78$) (Fig. 2B). No group differences were found in the BrdU-ir labeling in the AMY ($F = 0.23, p = 0.80$), DG ($F = 0.80, p = 0.46$), or VMH ($F = 1.48, p = 0.25$) (Fig. 2C). In addition, no group differences were found in either Ki67-ir or BrdU-ir labeling in any of the subnuclei in the AMY and DG (Table 2).

Experiment 2b: the effect of chronic pup exposure on basal corticosterone levels and cell proliferation and survival

Chronic pup exposure caused a significant increase in the basal level of CORT in comparison to the chronic handle and chronic object groups ($F = 5.32, p < 0.05$; Fig. 2D). However, chronic pup exposure did not affect Ki67-ir labeling in the AMY ($F = 0.85, p = 0.44$), DG ($F = 0.64, p = 0.52$), or VMH ($F = 0.16, p = 0.86$) (Fig. 2E). No group differences were found in the BrdU-ir labeling in the AMY ($F = 0.50, p = 0.63$), DG ($F = 0.41, p = 0.67$), or VMH ($F = 0.19, p = 0.83$) (Fig. 2F). Further, no group differences were found in either Ki67-ir or BrdU-ir labeling in any of the subnuclei in the AMY and DG (Table 2).

Experiment 3: effects of fatherhood on anxiety-like, depression-like, and agonistic behavior as well as social discrimination

Anxiety-like behaviors in the OF and EPM tests showed behavior-specific differences between treatment groups, an effect that seemed to depend on the experience of fatherhood. In particular, there were no group differences in the number of center entries ($F = 0.97, p = 0.39$), the center duration ($F = 2.51, p = 0.10$), and the number of line crosses ($F = 0.57, p = 0.57$) in the OF test, whereas the FATHER group spent significantly more time in the corners of the OF than the CTRL and MATED groups ($F = 8.18, p < 0.005$) (Fig. 3A-C). In the EPM test, the latency to open arm entry ($F = 3.01, p = 0.09$) and the total number of arm entries ($F = 0.75, p = 0.49$) did not differ across treatment groups, whereas FATHER males tended to show a lower frequency of entering open arms ($F = 3.03, p = 0.07$) and had a significantly lower ratio of open to total arm duration compared with the CTRL and MATED groups ($F = 6.05, p < 0.01$) (Fig. 3D-F).

Group differences were also found in the depression-like behavior. Males with mating experience (MATED and FATHER groups) had a shorter latency to immobility ($F = 12.22, p < 0.005$), a higher number of immobility bouts ($F = 10.45, p < 0.005$), and a longer duration of immobility ($F = 5.68, p < 0.05$) than the CTRL group, whereas no group differences were observed for the swim duration ($F = 2.43, p = 0.11$) (Fig. 3H-K).

In the resident intruder test (Fig. 4A and B), the MATED group showed a higher frequency ($F = 3.41, p < 0.05$) and duration ($F = 5.76, p < 0.01$) of aggressive approaches compared with the CTRL and FATHER groups. The MATED group also exhibited a higher frequency of attack behavior than the other two groups ($F = 8.77, p < 0.005$), whereas both MATED and FATHER groups had a longer attack duration compared with the CTRL group ($F = 9.07, p < 0.005$). The MATED group also displayed a higher frequency of chase behavior compared with the FATHER group, which, in turn, showed a higher frequency than the CTRL group ($F = 15.63, p < 0.001$). The MATED group also displayed a longer chase duration as compared with the CTRL group ($F = 14.26, p < 0.001$). We also combined approach, attack, and chase behaviors to obtain a total aggression index. The MATED group showed a higher total aggression frequency ($F = 14.27, p < 0.001$) and duration ($F = 13.05, p < 0.001$) than the FATHER group, which, in turn, was higher in both behavioral measurements than the CTRL group. For the olfactory investigation in the resident intruder test, there were no group differences in the frequency ($F = 2.09, p = 0.14$) or duration ($F = 2.40, p = 0.11$) of naso-genital investigation (Fig. 4C and D). However, the MATED groups exhibited a lower frequency ($F = 5.51, p < 0.05$) and duration ($F = 5.57, p < 0.05$) of naso-nasal investigation than the CTRL group, and the same behavioral patterns were found in the frequency ($F = 5.94, p < 0.01$) and duration ($F = 4.28, p < 0.05$) of the total olfactory investigation index (combined naso-genital and naso-nasal investigations) (Fig. 4C and D).

In the SD test, the measures of exploration, namely e1 ($F = 0.27, p = 0.77$) and e2 ($F = 0.60, p = 0.56$), did not differ across treatment groups (Fig. 5A). However, the indices for discrimination, d1 ($F = 4.42, p < 0.05$) (Fig. 5B) and d2 ($F = 5.30, p < 0.05$) (Fig. 5C), were significantly different between treatment groups. Subsequent paired-sample *t*-tests for d1 indicated that the CTRL group spent significantly more time investigating the novel juvenile as compared with the familiar juvenile during the discrimination trial [$t_{(6)} = 3.79, p < 0.01$] (Fig. 5B). However, such differences in juvenile investigation were not observed in the MATED [$t_{(8)} = 1.35, p = 0.21$] or FATHER ($t = 0.47, p = 0.66$) groups. Lastly, one-sample *t*-test posthoc analysis showed that the d2 value of the CTRL group differed significantly from 0 ($t = 3.78, p < 0.01$), whereas the d2 values of the MATED ($t = 1.38, p = 0.21$) and FATHER ($t = 0.92, p = 0.39$) groups did not differ significantly from 0 (Fig. 5C).

Discussion

Previous studies have shown that motherhood reduces cell proliferation and survival in the DG in various mammalian species including the California mouse (*P. californicus*), Sprague Dawley rat, and sheep (*Ovis aries*) (Leuner et al., 2007; Brus et al., 2010; Glasper et al., 2011). Similarly, fatherhood suppresses hippocampal cell survival in the biparental California mouse (*P. californicus*) (Glasper et al., 2011). Consistent with and in extension of this finding, the present study using the biparental prairie vole showed that fathers, compared with sexually naive controls, had reduced cell survival in the DG. This observed reduction in cell survival primarily affected the hilus and molecular cell layer, layers of the DG containing mostly interneurons (van Strien et al., 2009). However, it did not significantly reduce the cell survival in the granular cell layer, the layer containing granular cells that project via the trisynaptic circuit to the CA3 region (Hastings and Gould, 1999; van Strien et al., 2009). Due to these anatomical differences, it is likely that these three layers contribute differently to the function of the DG.

Furthermore, the present study documented for the first time that the experience of fatherhood also reduced cell survival in the AMY and VMH, non-traditional neurogenic brain regions that play important roles in social behaviors. This fatherhood-induced reduction in cell survival seemed to be brain region-specific, as no such effect was observed in the MOB. In addition, this effect was selective to distinct stages of adult neurogenesis, as

it only affected cell survival but not cell proliferation. Is it possible that the observed reduction in cell survival in the FATHER group was due to mating, rather than the experience of fatherhood? Although the current study cannot rule out this possibility, previous research across various species (including rats, mice, and prairie voles) showed that mating usually results in either no change or the increase of cell proliferation and cell survival in several distinct brain regions (for review see Lieberwirth and Wang, 2012). It should be noted that fathers of biparental species, such as the prairie vole, engage in all aspects of parental behaviors, except nursing, displayed by mothers (Lonstein and De Vries, 1999). Therefore, the display of parental behaviors in biparental species may cause a similar parenting experience in both sexes. Overall, the data obtained in the current study and the study in the male California mouse (Glasper et al., 2011) mirror the findings in females (Leuner et al., 2007; Pawluski and Galea, 2007; Brus et al., 2010), demonstrating that the experience of parenthood impairs cell proliferation and/or survival in a brain region-specific manner. Future investigations are needed to assess the effect of fatherhood on neuronal differentiation and the integration of the new neurons in the existing neural circuitry.

Although the motherhood-induced suppression of cell proliferation/survival appears to be pup-dependent (Leuner et al., 2007; but see Pawluski and Galea, 2007), the mechanism by which fatherhood suppresses cell survival in biparental species is currently unknown. As father voles display extensive levels of paternal behavior (Lonstein and De Vries, 1999), we predicted that the display of paternal behavior may have contributed to the observed reduction in cell survival. However, our data indicated that neither acute nor chronic pup exposure in the absence of fatherhood altered cell proliferation or survival in any of the brain areas examined in the prairie vole. These data suggest that the level of pup exposure and the display of paternal behavior may not be the sole factors reducing cell survival in prairie vole fathers. It should be noted that, in a previous study in male prairie voles, acute pup exposure was found to increase the number of BrdU-ir cells in the DG at 48 h after a single BrdU injection (Ruscio et al., 2008). These observed differences across studies may be due in part to the different life history of the male voles, i.e. sexually-naïve male voles in the study by Ruscio et al. (2008) and paired male voles that cohabited with a female for 2 weeks to enhance the likelihood of spontaneous paternal behavior (Bamshad et al., 1994; Terleph et al., 2004) in the current study. In addition, several other methodological differences, such as the BrdU injection paradigm, the number of pups used during the pup exposure test, as well as the interval between the BrdU injection and the termination of the subject, might also have resulted in discrepancies between the two studies.

One interesting finding of the current study is that the chronic, but not the acute, pup exposure significantly increased the basal CORT levels in male prairie voles, suggesting that a high level of paternal investment, similarly to maternal investment, can activate the HPA axis (as assessed by measuring CORT levels) (Leuner et al., 2007). In rats, this increased HPA axis activity probably plays a role in the motherhood-induced suppression of hippocampal cell survival (Leuner et al., 2007). In male prairie voles, however, chronic pup exposure increased the levels of circulating CORT but did not alter BrdU or Ki67 labeling in any of the brain regions examined, suggesting that a CORT-mediated mechanism is unlikely to be responsible for altered cell survival in father voles. Interestingly, long-term social isolation did not alter basal CORT levels but decreased cell proliferation and survival in the DG and AMY in female prairie voles, suggesting that isolation-induced neural plasticity in female voles is also unlikely to be mediated via a mechanism involving CORT (Lieberwirth et al., 2012). As previous studies have indicated that fatherhood modulates various neurotransmitter systems (e.g. oxytocin and vasopressin) (Gubernick et al., 1995; Kozorovitskiy et al., 2006) and causes hormonal changes (including prolactin and estrogen) (Gubernick and Nelson, 1989; Storey et al., 2000), future studies should investigate whether such changes may play a role in the reduction of adult-generated cells due to fatherhood.

A previous study in the female prairie vole demonstrated that 6 weeks of social isolation significantly reduced cell survival in the AMY, DG, and VMH as well as cell proliferation in the DG and MPOA (Lieberwirth et al., 2012). In male voles in the present study, however, 6 weeks of social isolation only reduced cell survival in the medial amygdala and VMH. Such sex differences may be explained by the differential sensitivity of female and male prairie voles to social isolation (Grippe et al., 2007; Martin and Brown, 2010). In general, social isolation has been proposed as a model of depression in female rodents as they are more sensitive to social isolation than males (Anisman and Matheson, 2005). Female prairie voles show a reduction in sucrose intake (anhedonia), enhanced depression-like behaviors in the FST, and increased activation of the HPA axis in response to a stressor (Grippe et al., 2007; Lieberwirth et al., 2012). In humans, women are more likely to suffer from depression, display more severe depressive symptoms, and have a poorer clinical outcome than men (Palanza, 2001). It is of interest to note that social isolation in male prairie voles reduced cell survival in the medial amygdala and VMH, limbic brain regions that belong to the 'social behavior network' (Newman, 1999).

Although many studies have indicated that motherhood leads to dramatic behavioral changes (Lonstein, 2005; Darnaudery et al., 2007; Lonstein, 2007; Agrati et al., 2008), the current study is the first to show that fatherhood experience affects various behaviors in male prairie voles. We observed a subtle increase in anxiety-like behaviors in father voles. In contrast, motherhood in rats reduced (Lonstein, 2005; Agrati et al., 2008) and fatherhood in the biparental California mouse (*P. californicus*) did not alter (Chauke et al. 2012) anxiety-like behaviors. These data, together, indicate that parenthood may have species-specific effects on anxiety-like behavior in rodents. Further, our data showed that sexual experience (MATED and FATHER males) increased depression-like behaviors as assessed in the FST. Although it is well-known that women have the highest risk of developing depression during the postpartum period (Brummelte and Galea, 2010; Hubner-Liebermann et al., 2012), studies to assess the effects of fatherhood on developing depression have been largely elusive (Spector, 2006). Additional studies using other behavioral tests (e.g. sucrose preference to measure anhedonia) are needed to further assess the role of fatherhood in paternal depression.

Fatherhood is also associated with changes in social behaviors in prairie voles. Mating significantly increased aggression in male prairie voles in previous studies (Getz et al., 1981; Winslow et al., 1993; Gobrogge et al., 2007) and the current study. Interestingly, father voles showed a level of aggression intermediate between sexually-naïve and mated males, indicating that fatherhood may reduce mating-induced aggression. These data are consistent with the data from studies in seasonally breeding birds showing reduced aggression following the onset of fatherhood as a potential trade-off between paternal behavior and aggression (Hegner and Wingfield, 1987; Ketterson and Nolan, 1992). Father voles also showed an intermediate level of olfactory investigation between sexually-naïve and mated males. As fatherhood improved social memory in mice (Mak and Weiss, 2010), it was expected that FATHER voles might show an improvement in social memory. Interestingly, our data demonstrated that the sexual and fatherhood experience led to a deficit in SD for juveniles. Although the physiological and neurochemical mechanisms underlying such changes in behavior need to be determined (Albers et al., 2006; Egashira et al., 2009; Leonard and Winsauer, 2011), these data suggest that sexual and fatherhood experience may alter memory function and its related behavioral expression in male prairie voles.

Adult neurogenesis has been shown to play an essential role in mediating distinct cognitive and behavioral functions (Imayoshi et al., 2009). For example, adult-generated MOB neurons play an important role in olfaction including odor discrimination and social recognition (Enwere et al., 2004; Mak and Weiss, 2010), whereas adult-generated

hippocampal neurons mediate spatial functions such as acquisition and long-term retention of learning and memory tasks (Shors et al., 2001; Clelland et al., 2009; Jessberger et al., 2009). Further, adult-generated SVZ cells may mediate maternal behavior (Furuta and Bridges, 2009). As the AMY, VMH, and DG have been implicated in a variety of behaviors, including anxiety- and depression-like behaviors, aggression, and social interactions (Kollack-Walker and Newman, 1995; Wang et al., 1997; Lonstein et al., 1998; Cushing et al., 2003; Gregus et al., 2005), it is possible that the fatherhood-induced reduction in cell survival in these brain areas may play a role in the observed behavioral changes in male prairie voles. This speculation and the causal relationship between altered cell survival and behaviors need to be examined in further studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AMY	amygdala
BrdU	bromodeoxyuridine
CORT	corticosterone
CTRL	control
DG	dentate gyrus
EPM	elevated plus maze
FST	forced swim test
HPA	hypothalamic–pituitary–adrenal
ir	immunoreactive
ISO	isolation
OF	open field
SD	social discrimination
VMH	ventromedial hypothalamus

Fig. 1. Social environment affected long-term survival (assessed by BrdU labeling), but not cell proliferation (assessed by Ki67 labeling), in the brains of male prairie voles. Compared with same-sex pair-housed males (CTRL), single-housed males (ISO) showed fewer BrdU-labeled cells in the VMH (G) and prairie vole fathers (FATHER) had fewer BrdU-labeled cells in the AMY (A, D and G), DG of the hippocampus (B, E and G), and VMH (G). * $p < 0.05$; ** $p < 0.01$. Error bars represent SEM + mean. Scale bar, 250 μ m.

Acute Pup Exposure

Chronic Pup Exposure

Fig. 2.

Acute pup exposure did not affect basal CORT levels (A) or cell proliferation (B) and survival (C) in the AMY, DG of the hippocampus, and VMH. Chronic pup exposure significantly increased basal CORT levels (D), without affecting cell proliferation (E) and survival (F) in the AMY, DG, and VMH.

Open Field **Elevated Plus Maze** **Forced Swim** **Fig. 3.**

Social experience alters anxiety- and depression-like behaviors in male prairie voles. In the OF test, the number of center entries (A) and the duration of time spent in the center (B) did not differ across groups. Fatherhood significantly increased the duration of time spent in the corners of the apparatus (B). Social experience did not affect locomotor activity (C). In the EPM, social experience did not alter the latency of open arm entry (D). Fatherhood reduced the ratio of open : total arm duration (E) and reduced the number of open arm entries (F), without affecting the total number of arm entries, an index of locomotion (G). In the FST, sexual experience (mating and fatherhood experience) reduced the latency of immobility (H). Sexual experience increased the number of immobility bouts (I) and duration of immobility (J), without affecting swim duration (K).

Aggression **Olfactory Investigation** **Fig. 4.**

Sexual experience altered aggression and olfactory investigation behavior displayed in the resident intruder test. Sexual experience in the absence of fatherhood (MATED) significantly increased the number (A) and duration (B) of agonistic approaches, attacks, and chases, whereas fatherhood reduced this mating-induced increase. Furthermore, treatment did not alter the frequency (C) or duration (D) of naso-genital olfactory investigation, whereas sexual experience (MATED and FATHER groups) reduced the frequency and duration of naso-nasal olfactory investigation. Bars labeled with a different letter differ significantly from each other as determined by posthoc Student Neuman–Keul's test in which a significant main effect was detected in the ANOVA ($p < 0.05$). Error bars represent SEM.

Fig. 5.

Sexual experience impaired SD. (A) There were no group differences in the exploration duration during the familiarization (e1) or discrimination (e2) trial in the SD test. (B) CTRL males spent a longer time investigating the novel compared with the familiar juvenile stimulus; no such preference was observed after sexual experience (MATED and FATHER males). Bars labeled with an asterisk indicate a significant difference in paired t-test between investigation of the novel and familiar juvenile. (C) The relative discrimination (d_2) differed significantly across groups. Posthoc analysis revealed that CTRL males showed SD, whereas MATED and FATHER males did not. Bars labeled with an asterisk differ significantly from CTRL males. Error bars represent SEM.

Table 1
Number of cells labeled for proliferation markers in the subregions of the AMY, hippocampus, and main olfactory bulb

Brain area	BrdU labeling		Ki67 labeling			
	CTRL	ISO	FATHER	p	CTRL	ISO
AMY						
BIA	1620.4 ± 99.6	1512.5 ± 104.2	1338.9 ± 156.3	ns (0.28)	70.0 ± 13.9	49.3 ± 9.5
CeA	1492.9 ± 73.3	1332.0 ± 118.0	1035.8 ± 155.1*	< 0.05	50.0 ± 12.0	65.3 ± 16.2
CoA	2793.5 ± 174.5	2740.5 ± 298.5	1790.2 ± 351.6*	< 0.05	194.0 ± 32.9	122.7 ± 15.2
MeA	2287.4 ± 188.7	1765.6 ± 120.1*	1380.1 ± 151.8*	< 0.005	98.0 ± 25.0	52.7 ± 7.3
Hippocampus						
GCL	4797.7 ± 506.7	4443.2 ± 457.0	3172.0 ± 697.6	ns (0.14)	154.0 ± 46.4	69.3 ± 18.4
Hilus	1425.1 ± 152.8	1363.1 ± 123.9	902.4 ± 78.2*	< 0.05	370.7 ± 97.1	246.7 ± 50.1
MCL	2116.7 ± 129.7	1958.8 ± 204.5	1316.2 ± 124.8*	< 0.05	105.3 ± 25.7	66.7 ± 13.8
Main olfactory bulb						
EPL	10 118 ± 847.6	11 309 ± 2099	9740.7 ± 1063	ns (0.76)		
GCL	119 859 ± 7715	106 847 ± 8136	108 465 ± 7495	ns (0.44)		

Means with asterisks differ significantly from CTRL as determined by posthoc Student Neuman-Keul's test in which a significant main effect was detected in the ANOVA. Data are expressed as mean ± SEM. BIA, basolateral; CeA, central; CoA, cortical; MeA, medial amygdala; GCL, granular cell layer; MCL, molecular cell layer; EPL, external plexiform layer; GCL, granular cell layer.

Table 2
Number of cells labeled for proliferation markers in the subregions of the AMY and hippocampus

BrdU labeling			Ki67 labeling					
Brain area	aHAN	aOBJ	aPUP	p	aHAN	aOBJ	aPUP	p
AMY								
BIA	1592.1 ± 157.9	1535.1 ± 232.5	1571.0 ± 99.8	ns (0.98)	30.0 ± 9.2	25.2 ± 4.5	21.6 ± 5.2	ns (0.66)
CeA	928.1 ± 112.4	955.5 ± 97.5	1065.1 ± 179.3	ns (0.77)	16.7 ± 5.1	15.0 ± 3.3	16.2 ± 5.1	ns (0.96)
CoA	2228.8 ± 140.8	2217.1 ± 266.0	2363.1 ± 254.3	ns (0.89)	46.7 ± 11.2	39.6 ± 9.7	24.6 ± 5.6	ns (0.18)
MeA	1672.9 ± 118.4	1868.9 ± 211.3	2001.2 ± 230.6	ns (0.55)	18.7 ± 4.5	19.2 ± 3.6	16.2 ± 4.5	ns (0.86)
Hippocampus								
GCL	1350.4 ± 154.2	1392.0 ± 163.3	1421.9 ± 139.0	ns (0.95)	10.0 ± 3.9	18.0 ± 7.2	12.0 ± 3.0	ns (0.52)
Hilus	1109.1 ± 94.3	1261.0 ± 81.0	1311.7 ± 76.0	ns (0.24)	65.3 ± 24.8	48.0 ± 18.4	30.6 ± 8.6	ns (0.38)
MCL	983.5 ± 108.5	893.6 ± 93.0	1128.8 ± 125.4	ns (0.31)	8.7 ± 3.8	3.6 ± 1.6	3.0 ± 1.0	ns (0.38)

Data are expressed as mean ± SEM. BIA, basolateral; CeA, central; CoA, cortical; MeA, medial amygdala; GCL, granular cell layer; MCL, molecular cell layer; aHAN, acute handle; aOBJ, acute object; aPUP, acute pup.