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SEXUAL INTERACTIONS WITH UNFAMILIAR FEMALES REDUCE HIPPOCAMPAL NEUROGENESIS AMONG ADULT MALE RATS

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Abstract

Recent experiments have shown that sexual interactions prior to cell proliferation cause an increase in neurogenesis in adult male rats. Because adult neurogenesis is critical for some forms of memory, we hypothesized that sexually induced changes in neurogenesis may be involved in mate recognition. Sexually naive adult male rats were either exposed repeatedly to the same sexual partner (familiar group) or to a series of novel sexual partners (unfamiliar group), while control males never engaged in sexual interactions. Ovariectomized female rats were induced into estrus every four days. Males were given two injections of BrdU (200 mg/kg) to label proliferating cells, and the first sexual interactions occurred three days later. Males in the familiar and unfamiliar groups engaged in four, 30 min sexual interactions at four-day intervals, and brain tissue was collected the day after the last sexual interaction. Immunohistochemistry followed by microscopy was used to quantify BrdU-labeled cells. Sexual interactions with unfamiliar females caused a significant reduction in neurogenesis in the dentate gyrus compared to males that interacted with familiar females and compared to the control group. The familiar group showed no difference in neurogenesis compared to the control group. There were no differences in the amount of sexual behavior (mounts, intromissions, ejaculations, or contact time) that the familiar and unfamiliar groups engaged in, indicating that the differences in neurogenesis were not due to the relative amounts of sexual activity. In a second experiment, we tested whether this effect was unique to sexual interactions by replicating the entire procedure using anestrous females. We found that interactions with unfamiliar anestrous females reduced neurogenesis relative to the other groups, but this effect was not statistically significant. In combination, these results indicate that interactions with unfamiliar females reduce adult neurogenesis and the effect is stronger for sexual interactions than for social interactions.

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Keywords

adult neurogenesis; sexual behavior; social behavior; familiarity; hippocampus; dentate gyrus

INTRODUCTION

The study of adult neurogenesis has provided exciting new insights regarding the neural mechanisms by which new memories are formed (Abrous et al., 2005; Kempermann, 2006). Among mammals, neurogenesis occurs throughout adulthood along the subgranular zone (SGZ) of the dentate gyrus within the hippocampal formation. Newly proliferated neurons from the SGZ migrate a short distance into the granule cell layer (GCL) of the dentate gyrus, where they extend functional axons into the CA3 region of the hippocampus (van Praag et al., 2002; Jessberger and Kempermann, 2003; Zhao et al., 2006). Young hippocampal neurons exhibit enhanced excitability, increased Ca^{2+} conductance, and a lower threshold for inducing long-term potentiation than do mature granule cells (Schmidt-Hieber et al., 2004; Ambrogini et al., 2010). These characteristics make young hippocampal neurons a particularly good substrate for memory formation. Considerable evidence indicates that enhanced hippocampal neurogenesis leads to improved memory (Shors et al., 2001; Snyder et al., 2005; Winocur et al., 2006; Dupret et al., 2007; Dupret et al., 2008), and reduced adult neurogenesis has been associated with a variety of neurodegenerative diseases, including age-related dementia (Klempin and Kempermann, 2007; Drapeau and Abrous, 2008). However, there are also numerous contradictory reports indicating that learning actually causes a decrease in adult neurogenesis (Ambrogini et al., 2004; Mohapel et al., 2006; Aztiria et al., 2007), and there is some evidence that elevated neurogenesis leads to increased forgetting in the adult brain (Akers et al., 2014). Novel learning paradigms are needed to clarify why certain types of learning involve increased neurogenesis while others are associated with decreased neurogenesis.

A wide variety of environmental factors induce changes in cell proliferation and/or the survival of new cells in the dentate gyrus, which in turn can cause changes in adult neurogenesis. In general, acute and chronic stress cause a decrease in neurogenesis (Mirescu and Gould, 2006). For example, acute exposure to aggressive resident males causes decreased neurogenesis among male rats (Thomas et al., 2007), and colony-housed subordinate male rats have reduced neurogenesis compared to dominant males (Kozorovitskiy and Gould, 2004). Social isolation can also be stressful for rodents, and socially isolated male rats have reduced hippocampal neurogenesis relative to group-housed or pair-housed males (Lu et al., 2003; Stranahan et al., 2006; Spritzer et al., 2011).

Contrasting the effects of social isolation, some recent studies have shown that certain social interactions can enhance adult neurogenesis (Gheusi et al., 2009; Lieberwirth and Wang, 2012). For example, male and female prairie voles exposed to pups for 20 min showed increased hippocampal neurogenesis relative to voles that were not exposed to pups (Ruscio et al., 2008). A similar effect was observed among male mice interacting with pups for two days (Mak and Weiss, 2010), but three weeks of paternal behavior reduced neurogenesis among monogamous California mice (Glasper et al., 2011). Sexual interactions have also

been shown to influence adult neurogenesis within the dentate gyrus. Among female mice, both sexual activity and exposure to male pheromones were shown to enhance neurogenesis (Shingo et al., 2003; Mak et al., 2007; Larsen et al., 2008). Among male rats, a single sexual interaction was sufficient to enhance hippocampal cell proliferation, and 14 consecutive days of 30 min sexual interactions caused a significant increase in neurogenesis (Leuner et al., 2010). Repeated sexual interactions (14–28 days) elevated neurogenesis levels among middle-aged male rats to that of young control rats (Glasper and Gould, 2013) and prevented a reduction in neurogenesis caused by chronic restraint stress among male mice (Kim et al., 2013). Thus, there is substantial evidence that the nature of a social interaction influences whether it will increase or decrease adult neurogenesis.

Most past research testing the function of adult neurogenesis has involved spatial memory tasks due to the known role of the hippocampus in spatial cognition. The neurogenesis-enhancing effects of sexual experience are puzzling, in that the hippocampus is not directly involved in regulating sexual behavior (Hull and Dominguez, 2007). A study with hamsters showed that sexual experience had no effect on neurogenesis within regions of the brain specifically known to be involved with mating behavior (i.e., posterior medial amygdala and medial preoptic area) (Antzoulatos et al., 2008). However, chemically blocking neurogenesis throughout the brain was shown to impair sexual behaviors in male rats (Lau et al., 2011). Additionally, the hippocampus is involved in the formation of some types of social memories. For example, lesions of the hippocampus or blocking *c-fos* expression within the hippocampus impaired social transmission of food preferences (STFP) among rats (Clark et al., 2002; Countryman et al., 2005). In addition, training in the STFP task increased hippocampal neurogenesis (Olariu et al., 2005), indicating that hippocampal neurogenesis may play a role in the formation of some social memories. There is also some evidence that the hippocampus plays a role in social recognition. Lesions to the hippocampus disrupted long-term (24 h) and short-term (30 min) social recognition among mice, and control mice were shown to retain social recognition of other individuals for at least 7 days (Kogan et al., 2000). Exposure of hamsters to familiar social partners 24 h after an interaction caused an up-regulation of immediate early gene products in the hippocampus (Lai et al., 2005). Among rats, transection of the fimbria disrupts social memory, suggesting that the hippocampus is involved in forming social memories (Maaswinkel et al., 1996). Thus, it is plausible that hippocampal neurogenesis plays a role in social recognition.

Testing the role of neurogenesis during social interactions provides a new model for determining the function of adult neurogenesis. A functional hippocampus is important for learning a sequence of events (Fortin et al., 2002), and current theory suggests that one of the primary functions of adult neurogenesis may be to facilitate learning spatial or temporal relationships (Aimone et al., 2006). Neurogenesis may, therefore, play a role in learning to distinguish among individuals during future interactions. In support of this hypothesis, exposing female mice to the pheromones of a socially dominant male resulted in increased neurogenesis within the dentate gyrus, and chemically blocking neurogenesis in females eliminated their preference to mate with dominant rather than subordinate males (Mak et al., 2007). We specifically tested whether familiarity with a sexual partner influenced adult neurogenesis by comparing neurogenesis levels in male rats that had been exposed four

times to the same estrus female to those that had interacted with four different estrus females. A second experiment involved males interacting with anestrus females to determine if the effects of familiarity on neurogenesis were specific to sexual interactions.

The effects of environmental factors on adult neurogenesis depend upon the age of the newly proliferated cells. For example, hippocampal-dependent learning on the Morris water maze enhanced neurogenesis among cells that were 6–10 days old at the time of training, but not among cells that were 1–5 or 11–15 days old (Epp et al., 2007). This result is supported by a number of other studies with rats showing that training on hippocampus-dependent tasks specifically enhances neurogenesis among relatively young cells (3–11 days old) but not among older or younger cells (Dupret et al., 2007; Gould et al., 1999; Ambrogini et al., 2000; Sisti et al., 2007). Training sessions for social transmission of food preferences also enhanced cell survival when cells were 8 days old during training but not when cells were 16 days old (Olariu et al., 2005). Taken together, these past results indicate that a critical period may exist for environmental factors to influence adult neurogenesis. For rats, this critical period seems to be when cells are approximately 4–10 days old, which corresponds with the final stages of cell migration from the subgranular zone into the granule cell layer (McDonald and Wojtowicz, 2005; Brown et al., 2003), when axons are rapidly extending and integrating into the existing network (Hastings and Gould, 1999). Based on this information, we used injections of 5-bromo-2'-deoxyuridine (BrdU) to label actively dividing cells 3 days prior to the first interaction with a female, which was also 7 days prior to the second interaction with either a familiar or unfamiliar female.

Based on previous results (Leuner et al., 2010; Glasper and Gould, 2013), we predicted that sexual interactions would increase neurogenesis relative to control males that did not interact with females. We further predicted that if neurogenesis within the dentate gyrus is involved in learning the identity of sexual partners, then familiarity of partners should impact neurogenesis levels. We were less certain regarding the direction of any effects of familiarity upon neurogenesis. Exposure to a novel olfactory environment caused increased neurogenesis in the olfactory bulbs relative to mice that were exposed to the same environment (Veyrac et al., 2009), suggesting that exposure to a novel sexual partner might increase neurogenesis. Alternatively, exposure to a familiar female may enhance neuronal survival above that observed in males that are exposed to unfamiliar females. There is some evidence that neuronal survival is regulated by cell-specific input (Tashiro et al., 2006). In support of this idea, Olariu et al. (2005) demonstrated reduced hippocampal neurogenesis among rats that were trained to distinguish between two separate odor pairs, compared to rats that were trained to distinguish between only one odor pair. Thus, our experiment tested alternative hypotheses for the impact of social familiarity on adult neurogenesis.

EXPERIMENTAL PROCEDURES

Subjects

Adult male and female Long-Evans rats (approximately 55 days old) were obtained from Charles River Laboratory (Saint-Constant, Quebec, Canada). All rats were housed in clear polysulphone cages (21 × 42 × 21 cm) with Tek-Fresh Bedding (Harlan Laboratories, Indianapolis, IN) and free access to water and a soy-protein free rodent diet (Harlan Teklad

Diet 2020X). The males were pair housed, while the females were individually housed to minimize the transfer of olfactory cues among the females. Males and females were housed in separate rooms that were each temperature controlled (21 ± 1 °C) with a reversed 12:12 h light/dark cycle (lights off at 0700 h). All animal procedures were approved by the Middlebury College Institutional Animal Care and Use Committee and were carried out in accordance with ethical guidelines set by the National Institutes of Health.

All the female rats ($N=36$) were ovariectomized 7 days after they arrived at the facility. Aseptic, standard operating procedures were used and the animals were anesthetized with isoflurane (3.5–4.0% in oxygen during induction, 2.0–2.5% in oxygen during maintenance). The analgesic ketoprofen was administered just prior to starting surgeries (5 mg/kg body mass, s.c.). Ovaries were extracted through two small incisions made through the abdominal muscles. Absorbable chromic gut sutures (Ethicon, Somerville, NJ, USA) were used to ligate the ovary and close the muscle layer. The skin layer was closed using either nylon suture material (Ethicon) or surgical staples (Teleflex Medical, Research Triangle Park, NC, USA). Immediately after surgeries, topical antibiotic (vetropolycin) was applied to the incision sites and each female was injected with lactated Ringer's solution (1 ml/100 g body mass). Females were given 14–16 days to recover from surgeries prior to starting behavioral testing.

Procedure

In two experiments, male subjects were assigned to three groups: 1) familiar, 3) unfamiliar, and 3) control. The females used for experiment 1 were induced into behavioral estrus via hormone injections. Specifically, females received s.c. injections of 17β -estradiol benzoate (10 μ g) 48 h prior to testing and progesterone (500 μ g) 4 h prior to testing. Females were given one week to recover from ovariectomy surgeries prior to starting hormone injections, and females had undergone two cycles of hormone injections prior to starting testing. In contrast, none of the females used in experiment 2 received hormone injections and were therefore all anestrus. Control males for both experiments remained housed in their home cages throughout the testing period. All subjects were pair housed throughout the experiment with another individual from the same treatment or control group. Twelve males were randomly assigned to the two treatment groups for both experiments. In experiment 1, we used only 8 control males, whereas for experiment 2 we increased this to 12 control subjects. Some subjects in experiment 1 were eliminated from the analyses for various reasons as indicated below (Statistical analysis section).

To habituate animals to the researchers, all subjects were handled 4–5 min per day for 4 consecutive days. The day after the last day of handling, all the male rats received two injections of BrdU (200 mg/kg body mass, i.p.) spaced 12 h apart (0800 h and 2000 h). BrdU (Sigma-Aldrich, St. Louis, MO, USA) was dissolved to 20 mg/ml in warm, sterile 0.9% saline containing 0.7% NaOH and filtered using a 0.22 μ m syringe filter. BrdU is a thymidine analog that is incorporated into dividing cells during the S-phase, thereby acting as a marker of cells that were actively proliferating at the time of BrdU injection. The dose and frequency of BrdU injections vary widely among neurogenesis studies, and we chose to use a double injection of a relatively high dose of BrdU in order to label a large population

of cells that were all dividing on the same day. BrdU is taken up by proliferating cells in the dentate gyrus of rats during the 8–9 h of S-phase, and the entire cell cycle is approximately 25 h (Cameron and McKay, 2001). Injections were spaced 12 h apart to avoid re-labeling the same cells while at the same time labeling the majority of cells that were actively dividing during a 24 h period. An identical injection protocol has been used in past studies of neurogenesis in rats (Snyder et al., 2005; Spritzer et al., 2011; McDonald and Wojtowicz, 2005), and doses as high as 300 mg/kg are non-toxic (Cameron and McKay, 2001). Injections were conducted 3 days prior to the first interactions with females (Fig. 1), which was also 7 days prior to the second interactions. Because cells actively divide for about 3 days after BrdU injections (Dayer et al., 2003), this protocol allowed us to test the effects of socio-sexual interactions on cell survival rather than cell proliferation.

Experimental males interacted with estrus or anestrus females in clear polycarbonate testing boxes (20×40×40 cm) with TekFresh bedding. The testing room had dim red lighting, and the experimenter left the room during testing. One female rat was placed in each testing box and allowed to acclimate for 5 min prior to interacting with a male for 30 min. Males were tested in groups of 4 at a time in adjacent boxes that were visually isolated from each other. Each female interacted with only one male each day. Between trials, the testing boxes were thoroughly cleaned with 70% ethanol and the bedding was replaced. Each male interacted with a female on four different days that were four days apart to correspond with the rat estrus cycle (Fig. 1). All trials were video recorded (Sony Handycam) for later quantification of behaviors. Event recording software (JWatcher ver.1.0) was used to score the frequency and duration of various behaviors for each male during all the 30 min trials. The number of attempted mounts, mounts, intromissions, and ejaculations were scored (Sachs and Barfield, 1976). During an attempted mount, the male assumes a copulatory position over the female by grasping her flanks, but unlike an actual mount, the male does not engage in pelvic thrusts. During experiment 2, males engaged in attempted mounts and mounts with anestrus females, but no intromissions or ejaculations were observed. The following non-sexual behaviors were also quantified in both experiments: wet dog shakes, aggression, rearing, and grooming. Wet dog shakes involve a short burst of side-to-side movement, and the frequency of wet dog shakes is negatively correlated with level of sexual activity (Watson and Gorzalka, 1990). Aggressive behavior included all instances of “boxing”, in which the male and female exchanged blows with the forepaws while standing on their hind legs, and all cases of one animal (male or female) forcefully pinning the other on its back (Blanchard et al., 1977). Finally, total amount of time that two rats were in contact was determined for each pair by summing the amount of time that the male: 1) followed the female within 1 cm, 2) actively sniffed any part of the female, 3) engaged in sexual interactions, and 4) engaged in aggressive interactions.

Sixteen days after the BrdU injections, rats were euthanized using a lethal dose of sodium pentobarbital (Fatal-plus, Virtech Pharmaceutical, Dearborn, MI) and perfused transcardially with 60 ml of 0.9% saline followed by 120 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) using a syringe pump. Brains were extracted and post-fixed with 4% paraformaldehyde at 4 °C overnight, followed by 48 h immersed in 30% sucrose in 0.1 M TBS (0.08 M Tris-HCL, 0.02 M Tris-base, 0.9% saline, pH 7.4) for cryoprotection. Brains

were then stored in 0.1 *M* TBS at 4 °C until sectioning. Using a vibrating blade microtome (Leica VT1000S, Wetzlar, Germany), each brain was sliced in a bath of 0.1 *M* TBS into 40 µm thick coronal sections through the entire hippocampus. Tissue was collected and stored in an antifreeze solution (0.05 *M* TBS, 30% ethylene glycol and 20% glycerol) at –20 °C until immunohistochemical processing.

Immunohistochemistry

Peroxidase immunohistochemistry was performed on free-floating tissue in a series of every 10th section (i.e., 400 µm intervals) through the rostro-caudal extent of the hippocampus to visualize BrdU-labeled cells. Sections were rinsed in 0.1 *M* TBS (pH 7.4) three times for 10 min between steps unless otherwise noted. Tissue was initially incubated for 30 min in 0.6% H₂O₂ to eliminate endogenous peroxidase activity. DNA was denatured by applying 2 *N* HCl for 30 min at 37 °C. This step was immediately followed by a 10 min incubation in 0.1 *M* borate buffer (pH 8.5) to neutralize the acid. Tissue was next blocked for 30 min in a solution of 0.1 *M* TBS, 0.1% Triton-X 100, and 3.0% normal horse serum (Vector Laboratories, Burlingame, CA, USA), followed by a 16 h incubation at 4 °C on a platform shaker in mouse monoclonal antibodies against BrdU (Roche Diagnostics, Indianapolis, IN, USA) at a concentration of 1:400 in blocking solution. Sections were then incubated in horse-anti-mouse secondary antibodies (1:100 in 0.1 *M* TBS; Vector Laboratories) for 4 h, followed by a 1.5 h incubation in avidin-biotin horseradish peroxidase solution (ABC Elite Kit; 1:50; Vector Laboratories). Sections were reacted for 3–5 min in a solution of 3,3'-diaminobenzidine (0.5 mg/ml; Sigma-Aldrich, Atlanta, GA) and 0.003% H₂O₂ in 0.1 *M* TBS. Next sections were mounted onto Superfrost/Plus microscope slides (Fisher Scientific, Suwanee, GA, USA) and dried overnight. Finally, slides were counterstained with cresyl violet acetate (0.2% in distilled water), dehydrated with ethanol, cleared with xylene, and coverslipped using Permount (Sigma-Aldrich).

Immunofluorescent double-labeling was performed on free-floating tissue (*n*=3 brains/group) to determine the percentage of BrdU-labeled cells that were mature neurons (Sisti et al., 2007; Bruel-Jungerman et al., 2005; Barker and Galea, 2008). For each brain, one series of every 10th section was labeled for expression of BrdU and neuronal nuclei (NeuN), which is a maker of mature neurons (Mullen et al., 1992). Sections were rinsed in 0.1 *M* TBS (pH 7.4) three times for 10 min between steps unless otherwise noted. As for peroxidase labeling, DNA was denatured by incubating sections in 2 *N* HCl for 30 min at 37 °C, followed immediately by 10 min in 0.1 *M* borate buffer. Tissue was next blocked for 30 min in a solution of 0.1M TBS, 0.1% Triton-X 100, and 3.0% normal goat serum (Sigma-Aldrich), followed by a 16 h incubation at 4 °C on a platform shaker in primary antibodies: rat anti-BrdU monoclonal antibodies (1:200, AbD Serotec, Raleigh, NC, USA) and mouse anti-NeuN monoclonal antibodies (1:200, Millipore, Temecula, CA, USA) in blocking solution. Sections were blocked again for 30 min, followed by 16 h incubation at 4 °C on a platform shaker in secondary antibodies: Alexa Fluor488 goat anti-mouse IgG (1:200, Invitrogen, Eugene, OR) and Alexa Fluor568 goat anti-rat IgG (1:200, Invitrogen) in blocking solution. Sections were mounted on Superfrost/Plus slides, coverslipped with the anti-fading agent diazobicyclooctane (0.1 *M* TBS, 2.5% DABCO, 10% polyvinyl alcohol and 20% glycerol) and stored at –20 °C.

Microscopy

All BrdU-labeled cells in the dentate gyrus were counted by an experimenter blind to the subjects' group assignments. Every 10th section was counted through the entire rostro-caudal extent of the dentate gyrus (10–12 sections per brain) at 1000× magnification (100× oil immersion lens) using a light microscope (Zeiss Axio Imager D1). There were no significant differences in the number of sections scored between groups for either experiment (both $P>0.10$). Cells were considered labeled if they exhibited a brown punctate stain. For each section, all labeled cells in the GCL, SGZ and hilus were counted; however, labeled cells in the uppermost focal plane were excluded to avoid oversampling (i.e., the optical dissector method was used). Cells observed within 20 µm of the inner edge of the GCL were considered to be the SGZ, and these cell counts were combined with the GCL counts. Labeled cells were counted in the hilus and compared to counts in the GCL+SGZ to determine whether any experimentally induced effects influenced cell division in the brain more broadly rather than being specific to the neurogenic niche along the SGZ. Progeny from progenitor cells in the hilus give rise to a population of ectopic cells that are morphologically and physiologically distinct from the granule cells produced along the SGZ (McCloskey et al., 2006; Scharfman et al., 2007). Sections were also designated as being in the dorsal or ventral portion of the hippocampus (Banasr et al., 2006), with 3.70 mm from the interaural line as the division between dorsal and ventral (Paxinos and Watson, 1986). Some sections near this dividing line had portions of the granule cell layer that were discontinuous, and in these cases the dorsal and ventral portions were counted separately. Dorsal and ventral sections were discriminated because evidence suggests that they serve discrete cognitive functions, with memory functions confined to the dorsal hippocampus and emotional functions relegated to the ventral hippocampus (Fanselow and Dong, 2010). A stereological estimate of the total number of BrdU-labeled cells in the GCL+SGZ and the hilus was obtained for each rat by multiplying the number of cells counted by ten (i.e., inverse of the sampling ratio).

The percentage of BrdU-labeled cells that co-expressed NeuN was assessed for 50 randomly selected BrdU-labeled cells within the GCL+SGZ of each brain ($n=3$ /group). Five to six sections were sampled for each brain, such that cells were sampled evenly across dorsal and ventral sections. BrdU-labeled cells were visualized and photographed at 630× magnification using a confocal microscope (Zeiss, LSM 510 META) and associated software (Zeiss LSM Image Browser, ver. 4.2.0.121). Images were collected in sequential scanning mode to prevent cross-bleeding between detection channels. Confocal z-stacks were collected at 1 µm intervals, and cells were considered double-labeled if: 1) co-expression was observed throughout the z-stack, and 2) the NeuN label was intense relative to background staining intensity. Relative staining intensity was determined using the program ImageJ (ver. 1.42).

Statistical analysis

One subject from the familiar group in experiment 1 failed to perform any intromissions or ejaculations during the trials and was dropped from all analyses. Additionally, four subjects were removed from all analyses in experiment 1 because they had no visible BrdU-labeled cells (two rats from the familiar group and one each from the unfamiliar and control groups).

Thus, the final sample sizes for most analyses of experiment 1 were: $n=9$ (familiar), $n=11$ (unfamiliar), $n=7$ (control). For experiment 2, clear BrdU labeling was observed in all subjects and therefore the final sample size remained $n=12$ /group.

The estimated total number of BrdU-labeled cells was analyzed using repeated measures ANOVA with “treatment” (familiar, unfamiliar, or control) as the between-subjects factor and “region” (dorsal or ventral) and “cell layer” (GCL+SGZ or hilus) as separate within-subjects factors. Amount of sexual behavior and other behaviors observed during the 30 min trials were compared across the four testing days using repeated measures ANOVA, with “treatment” (familiar or unfamiliar) as the between-subjects factor and “day” (testing days 1–4) as the within-subjects factor. Whenever ANOVA revealed significant effects, Fisher’s LSD was used for post-hoc comparisons among groups (Field, 2009). Whether the amount of sexual activity influenced neurogenesis was tested by linear regressions of the total number of BrdU-labeled cells against the amount of sexual behavior summed across the four testing days for each rat. SPSS (ver. 20.0; Chicago, IL, USA) was used for all analyses, and the significance level was set at $\alpha = 0.05$ for all tests. All results are reported as mean $\pm$ SEM.

RESULTS

Familiarity influenced neurogenesis

For experiment 1, in which males interacted with estrus females, there was a significant treatment $\times$ layer interaction ($F_{2,24}=3.41$, $P=0.049$). The main effect of treatment was not quite significant ($P=0.062$). Post-hoc analyses showed that within the GCL+SGZ (Fig. 2A), males that interacted with unfamiliar females had significantly fewer BrdU-labeled cells than males that interacted with familiar females ($P=0.045$) or control males ($P=0.043$). In contrast, the number of BrdU-labeled cells within in the GCL+SGZ did not differ between the control group and males that interacted with familiar females ($P=0.87$). Within the hilus (Fig. 2B), post-hoc analyses indicated that there were significantly fewer BrdU-labeled cells among males that interacted with unfamiliar females than among the control males ($P=0.020$), but the other pairwise comparisons were not significant (both $P>0.080$). As expected, there were significantly more BrdU-labeled cells in the GCL+SGZ than in the hilus ($F_{1,24}=304.8$, $P<0.0005$) and more BrdU-labeled cells in dorsal sections than in ventral sections ($F_{1,24}=56.79$, $P<0.0005$). The difference in cell counts between dorsal and ventral sections was larger in the GCL+SGZ than in the hilus, resulting in a significant region $\times$ layer interaction ($F_{1,24}=118.78$, $P<0.0005$). Brain region (i.e., dorsal vs. ventral) showed no significant interaction effect with treatment (both $P=0.34$), so the regional distinction is not shown in Fig. 2A and B.

BrdU-labeling showed a similar pattern in experiment 2 to that observed for experiment 1 (Fig. 2C and D), but the main effect of treatment was not statistically significant ($P=0.26$) and none of the interaction effects involving treatment were significant (all $P>0.17$). There were significantly more BrdU-labeled cells in the GCL+SGZ than in the hilus ($F_{1,33}=712.5$, $P<0.0005$) and more BrdU-labeled cells in dorsal sections than in ventral sections ($F_{1,33}=25.1$, $P<0.0005$). As for experiment 1, there was a more extreme difference between ventral and dorsal regions in the GCL+SGZ than in the hilus, resulting in a significant

region $\times$ layer interaction ($F_{1,24}=28.7$, $P<0.0005$). Considering the two experiments in combination, sexual interactions with unfamiliar females caused a decrease in the number of BrdU-labeled cells in dentate gyrus, whereas interactions with anestrous females, regardless of familiarity, had little effect on BrdU labeling (Fig. 2).

For both experiments, a majority (72–88%) of BrdU-labeled cells were co-labeled with the neuronal marker NeuN (Table 1; Fig. 3). Treatment did not have a significant effect on percentage of double-labeled cells for experiment 1 ($P=0.68$; $\eta^2=0.12$) or experiment 2 ($P=0.66$; $\eta^2=0.16$), indicating that interactions with females had no effect on the percentage of cells that differentiated into neurons.

Most behaviors varied across days and some varied between groups

Averaging the familiar and unfamiliar treatment groups together, males in experiment 1 engaged in species-typical levels of sexual interactions per day during the 30 min trials: 24.73 ± 2.63 mounts, 30.63 ± 2.06 intromissions, 2.32 ± 0.13 ejaculations. There was no significant main effect of treatment for mounts ($P=0.84$) or intromission ($P=0.20$), but males that interacted with familiar females had significantly more ejaculations than did males that interacted with unfamiliar females (Fig. 4C; $F_{1,18}=6.41$, $P=0.021$). There was a significant day $\times$ treatment interaction for intromissions (Fig. 4B; $F_{3,54}=2.92$, $P=0.042$), with rats in the familiar group engaging in more intromissions than rats in the unfamiliar group on all days except the fourth day. Mounts and ejaculations did not have significant interaction effects with day (both $P>0.55$). The frequency of all of the sexual behaviors varied significantly across the four days of testing. Both mounts and intromissions showed an initial rise followed by a decline in frequency per trial over the four days of testing (Fig. 4A, B; mounts: $F_{3,54}=5.89$, $P=0.001$; intromissions: $F_{3,54}=9.14$, $P<0.0005$). There was also a significant increase in the number of ejaculations per trial following the first day of testing (Fig. 4C; $F_{3,54}=6.67$, $P=0.001$), suggesting that males improved their sexual efficiency.

Turning to the non-sexual behaviors, there were no significant day $\times$ treatment interactions (Fig. 4D–4H; all $P>0.62$) and no significant effects of treatment for most of the behaviors measured (all $P>0.08$). The one exception was that males that interacted with familiar females showed significantly less rearing than did males that interacted with unfamiliar females (Fig. 4H; $F_{1,18}=7.38$, $P=0.014$). Indicative of decreasing anxiety, both wet dog shakes and rearing showed significant decreases over the four days of testing (Fig. 4G, H; WDS: $F_{3,54}=5.35$, $P=0.003$; rears: $F_{3,54}=7.34$, $P<0.0005$). Somewhat surprisingly, contact time with the female also decreased over the four days of testing (Fig. 4D; $F_{3,54}=3.66$, $P=0.018$), but this may be due to increased efficiency in the males sexual behavior and the fact that males often rested following ejaculations (resting behavior was not quantified). Paralleling the changes in mounting and intromissions, grooming showed a curvilinear pattern over the four days of testing (Fig. 4E; $F_{3,54}=3.86$, $P=0.014$). This was likely because males commonly groomed themselves after each intromission.

For experiment 1, regression analyses were used to compare the average amount of each sexual behavior per day (mounts, intromissions, and ejaculations) to the total number of BrdU-labeled cells in the GCL+SGZ of all rats in the two treatment groups (i.e., familiar and unfamiliar groups combined). No significant relationships were found (all $P>0.20$).

Analyzing the dorsal and ventral GCL+SGZ separately also showed no relationships with sexual behaviors (all $P>0.10$). Total contact time was also unrelated to the total number of BrdU-labeled cells in the entire GCL+SGZ ($P=0.49$), dorsal portion ($P=0.70$), and ventral portion ($P=0.39$). Thus, there is no evidence that the number of BrdU-labeled cells present in the GCL+SGZ was dependent on the amount of sexual activity or social contact that the male rats experienced with estrus females.

For experiment 2, most of the behavioral variables measured showed no effect of treatment and no day $\times$ treatment interaction (Fig. 5; all $P>0.25$). However, males showed significantly more aggression toward familiar females than toward unfamiliar females (Fig. 5D; $F_{1,22}=7.66$, $P=0.011$). For grooming, there was a significant day $\times$ treatment interaction, with males in the familiar group engaging in more grooming than those in the unfamiliar group on days 3 and 4 of testing (Fig. 5C; $F_{3,66}=2.80$, $P=0.047$). However, post-hoc comparisons of grooming on each day showed only marginally significant differences between the groups on day 3 ($P=0.059$) and day 4 ($P=0.075$). Attempted mounts and aggression showed significant declines over the four days of testing, suggesting that the males learned to avoid the anestrus females (Fig. 5A and D; attempted mounts: $F_{3,66}=6.28$, $P=0.001$; aggression: $F_{3,66}=7.89$, $P<0.005$). Rearing also declined significantly over the testing days, suggesting that the rats were less exploratory as they became habituated to the testing chambers (Fig. 5F; $F_{3,66}=23.44$, $P<0.005$). Wet dog shakes increased significantly over the four days of testing (Fig. 5E; $F_{3,66}=10.25$, $P<0.005$), possibly indicative of increased anxiety during repeated interactions with anestrus females. Contact time with the female remained fairly stable, although there were significant fluctuations over the testing days (Fig. 5B; $F_{3,66}=8.69$, $P<0.005$). As for experiment 1, total contact time was unrelated to the total number of BrdU-labeled cells in the entire GCL+SGZ ($P=0.75$), dorsal portion alone ($P=0.34$), or ventral portion alone ($P=0.46$).

DISCUSSION

The goals of this study were to test the effects of sexual and social interactions on adult neurogenesis in the dentate gyrus and to specifically test the potential effects of social familiarity on neurogenesis. Intermittent sexual or social interactions with females did not increase neurogenesis among male rats. In fact, experiment 1 showed that sexual interactions with unfamiliar estrus females caused a significant decrease in hippocampal neurogenesis among males relative to that observed in control males and in males that interacted with familiar females. In contrast, interactions with anestrus females (familiar or unfamiliar) had no significant effects on neurogenesis (experiment 2). Sexual behaviors showed significant changes over the four days of testing in experiment 1, notably a significant increase in ejaculations. Although regression analyses showed no significant relationships between neurogenesis levels and amount of sexual behavior, males in the familiar group engaged in significantly more ejaculations than did males in the unfamiliar group. Over the course of the four interactions with anestrus females (experiment 2), there was a significant decrease in attempted mounts and aggression and a significant increase in wet-dog shakes. However, amount of contact with the anestrus females was not related to neurogenesis levels in the male subjects. Thus, our most interesting finding was that sexual

interactions with an unfamiliar female caused a decrease in adult neurogenesis among male rats, and there are multiple explanations for this effect.

The lower levels of sexual activity (ejaculations and intromissions) among males that interacted with unfamiliar estrus females compared to males that interacted with familiar estrus females corresponded with a significant decrease in neurogenesis. This suggests that sexual activity enhances neurogenesis, and this idea is supported by some past studies with rodents (Leuner et al., 2010; Glasper and Gould, 2013; Kim et al., 2013). It may be that females were more sexually proceptive or receptive toward familiar males than toward unfamiliar males (Pfaus et al., 1999). An alternative explanation is that familiarity with a female reduced anxiety in the males, resulting in increased sexual activity. Both oxytocin and testosterone are increased by sexual activity and both of these hormones cause an increase in neurogenesis (Spritzer and Galea, 2007; Leuner et al., 2012; Shulman and Spritzer, 2014). However, when the data from the two groups used in experiment 1 (familiar and unfamiliar) were combined, no significant relationships were found between any of the measures of sexual activity and number of BrdU-labeled cells. Additionally, there was no difference in neurogenesis between the rats in the familiar group and the control group. Clearly, the neurogenesis-enhancing effects of sexual activity cannot fully explain our results.

Sexual interactions with unfamiliar females may have induced a stronger stress response than interactions with familiar females, and elevated stress hormones could have caused a decrease in neurogenesis (Mirescu and Gould, 2006). Both acute and repeated aggressive interactions between pairs of male rats (social defeat stress) cause a significant decrease in neurogenesis (Thomas et al., 2007; Czeh et al., 2002; Van Bokhoven et al., 2011), and subordinate male rats living in colonies show decreased neurogenesis relative to dominant males (Kozorovitskiy and Gould, 2004). Therefore, it is plausible that social stress in a sexual context could also reduce neurogenesis. Acute and intermittent sexual interactions have been shown to elevate corticosterone levels in male rats (Bonilla-Jaime et al., 2003; Bonilla-Jaime et al., 2006), and corticosterone treatment causes a decrease in neurogenesis (Cameron and Gould, 1994; Brummelte and Galea, 2010; Brummelte and Galea, 2010). However, we observed the most sexual activity in the familiar group, so our results cannot be easily explained by differences in the level of the stress response caused by differences in sexual activity. Most previous studies involving repeated sexual interactions have involved males interacting with different females each day (Leuner et al., 2010; Glasper and Gould, 2013), and to our knowledge no studies have specifically compared whether sexual interactions with unfamiliar females induce a greater increase in glucocorticoids than interactions with a familiar female.

The “social buffering” hypothesis suggests that social interactions reduce the impact of stressors. In support of this idea, a number of studies have shown a reduced stress response (physiological and behavioral) among group-housed rats compared to socially isolated individuals (Ruis et al., 1999; Westenbroek et al., 2005). Of particular relevance, one study showed that young male rats introduced into a novel environment with a familiar same-sex partner had a reduced stress response compared to rats that were introduced into a novel environment with an unfamiliar same-sex partner (Terranova et al., 1999). This social

buffering seems to influence neurogenesis levels in the dentate gyrus. For example, social housing prevented a reduction in hippocampal neurogenesis caused by chronic foot shock among male rats (Westenbroek et al., 2004). Exercise was shown to enhance neurogenesis among socially housed male rats but not among socially isolated individuals (Stranahan et al., 2006; Leasure and Decker, 2009). Among male mice, three weeks of daily 90 min sexual interactions eliminated a reduction in neurogenesis caused by daily restraint stress (Kim et al., 2013). Thus, there is considerable evidence that social interactions can reduce the neurogenesis-impairing effects of stressors, and there is some evidence that familiarity influences the level of social buffering. Assuming that some aspect of our behavioral testing procedure was stressful (novel environment or the sexual interaction itself), perhaps interactions with a familiar sexual partner reduced the neurogenesis-impairing effect of this stress.

It is possible that the differences in neurogenesis between the familiar and unfamiliar groups in experiment 1 have functional significance. Numerous past studies have shown that various types of social interactions can cause changes in neurogenesis among rodents (Lieberwirth et al., 2012), but only a few studies to date have tested the functional significance of these changes. Intra-ventricular injections of AraC (neurogenesis inhibitor) eliminated a social preference for dominant males over subordinate males among female mice (Mak et al., 2007). Blocking prolactin signaling caused a reduction in neurogenesis and impaired pup recognition among male mice (Mak and Weiss, 2010). These studies with mice provide some evidence that adult neurogenesis is necessary for social recognition, but it is unclear how a reduction in neurogenesis among the male rats exposed to unfamiliar estrus females could facilitate social memory formation.

There is some evidence that a combination of cell death and neurogenesis is necessary for new memory formation. In one study with mice, blocking neurogenesis caused an improvement on a spatial working memory task that required mice to forget previously learned information (Saxe et al., 2007). A negative correlation was found between the number of older cells (2 months old) and younger cells (4–7 days old) among mice living under environmental enrichment (Llorens-Martín et al., 2010), suggesting that older cells may interfere with the survival of younger cells or vice versa. Dupret et al. (2007) demonstrated that cell death was enhanced among cells in the dentate gyrus that were 3 days old during spatial learning, whereas cell survival was enhanced among cells that were 7 days old during learning (Dupret et al., 2007). We used this result as the basis of our experimental timeline (Fig. 1), with the first exposure 3 days after BrdU injection and the second exposure 7 days after BrdU injection. Males were either re-exposed to the familiar female or exposed to an unfamiliar female for the first time on day 7, and we assumed that this timing would allow any potential effects of social familiarity (or novelty) to have their strongest effect. However, the results of Dupret et al. (2007) might also suggest that exposing males to a sexual interaction for the first time 3 days after BrdU injection could have led to selective death of BrdU-labeled cells, assuming that some critical learning was occurring during that first exposure. This interpretation is unsatisfactory, however, because it does not explain why we observed a decrease in neurogenesis in only the unfamiliar group and not the familiar group. It has been proposed that new neurons in the dentate gyrus may be encouraged to survive through an input-dependent, cell-specific mechanism (Tashiro et al.,

2006; Deng et al., 2010). Based on this idea, re-exposure of males to the same female (familiar group) may have reinforced memories of that particular female, encouraging young neurons to survive, whereas exposing males to a different female may have encouraged the young neurons to undergo selective cell death. In support of this idea, re-exposing mice to a water maze caused selective reactivation of neurons in the dentate gyrus that were born during initial training on the water maze (Trouche et al., 2009). Furthermore, Olariu et al. (2005) found that a single training session on a STFP task resulted in an increase in neurogenesis in the dentate gyrus, whereas decreased neurogenesis occurred when rats were exposed to two separate training sessions (5 days apart) involving different odor pairs. Thus, reduced neurogenesis among the unfamiliar group in experiment 1 may either be an adaptive mechanism for eliminating un-needed cells or it may simply be a side-effect of elevated stress hormone levels.

We have some evidence that males were able to distinguish between familiar and unfamiliar females. For experiment 1, males ejaculated more often with familiar than unfamiliar females. Additionally, males in the unfamiliar group showed significantly more exploratory rearing than did males in the familiar group, likely because they were engaged in less sexual behavior. For experiment 2, males in the familiar group were more aggressive and displayed more grooming behavior than did males in the unfamiliar group. Because all of these effects were subtle, documenting changes in memory may be difficult with our methodology. Past studies have quantified social memory by showing that adult male rats will investigate an unfamiliar juvenile male more than a familiar juvenile male (Maaswinkel et al., 1996; Bannerman et al., 2001). We observed no differences in investigatory behavior in either of our experiments, which could mean either that males are consistently interested in females regardless of familiarity or that male social memory does not last four days. One past study found that males were able to distinguish between familiar and unfamiliar juveniles after 30 min of isolation, but not after 24 h of isolation (Squires et al., 2006). Given that we did observe some differences in behavior in males' response to familiar and unfamiliar females, our data suggest that social memory may last as long as four days in male rats. Pair-housing males with females could increase familiarity and allow better quantification of social memory in future studies.

We observed a statistically significant decrease in neurogenesis when males were exposed to unfamiliar estrus females (experiment 1) but not when they were exposed to anestrus females (experiment 2), which suggests that different hormonal changes were induced by these two social contexts. Bonilla-Jaime et al. (2006) found that interactions with estrus and non-estrus females produced a similar increase in corticosterone relative to control males, which suggests that the difference in the results of our two experiments cannot be fully explained by differences in corticosterone. However one older study noted that males that had 14 days of 30 min interactions with an anestrus female had significantly lower corticosterone levels compared to males that engaged in four 30 min sexual interactions separated by four days with the same partner (Szechtman et al., 1974). This suggests that the stress response may diminish over time when males are re-exposed to an anestrus female but not when they are re-exposed to an estrus female. Assuming that elevated corticosterone induces decreased neurogenesis (Cameron and Gould, 1994), differences in the stress response could explain why familiarity had a significant effect in experiment 1, but not in

experiment 2. Other hormonal changes in male rats that are induced by sexual interactions (e.g., elevated testosterone and prolactin) are comparable to hormonal changes observed when male rats are exposed to anestrus females (Kamel et al., 1975), making it unlikely that such changes could explain the differences that we observed between experiments 1 and 2.

Our results differ from two previous studies showing that sexual activity increased neurogenesis among male rats (Leuner et al., 2010; Glasper and Gould, 2013), but this discrepancy was likely due to a variety of methodological differences between studies. As in the previous studies, we used a 30 min interaction period between males and females, so in this regard all three experiments are identical. However, in our study rats engaged in only four sexual interactions separated by four days, whereas Leuner et al. (2010) employed 14 consecutive days of sexual interactions. Additionally, in the Leuner et al. (2010) study rats engaged in sexual interactions prior to BrdU injections, whereas in our study all the sexual interactions occurred three days or more after BrdU injections. By three days after BrdU injection, only about 25% of labeled cells are still actively proliferating (Dayer et al., 2003), so it is possible that sexual interactions increase neurogenesis exclusively through an increase in cell proliferation. In contrast, our results suggest that interactions with unfamiliar females after the period of cell proliferation lead to reduced survival of new neurons. In one of their experiments, Glasper and Gould (2013) exposed male rats to 28 days of sexual interactions and injected BrdU daily during the first 14 days of sexual interactions. This design resulted in sexual interactions during both the cell proliferation and cell survival stages of neural development, which in turn led to a significant increase in neurogenesis (Glasper and Gould, 2013). This suggests that a sexually-induced increase in cell proliferation may be able to override the reduction in neurogenesis that we observed. Alternatively, daily sexual interactions may affect neurogenesis differently than the intermittent interactions that we used. Because we were specifically interested in the effects of familiarity, this necessitated four-day gaps between sexual interactions to correspond with the female estrus period. In on-going experiments, we are testing the relative impact of daily sexual interactions on cell proliferation and cell survival.

CONCLUSION

Male rats that had sexual interactions with unfamiliar females showed reduced hippocampal neurogenesis compared to males that interacted with familiar females, and this effect of familiarity was less pronounced when males interacted with anestrus females. This may have been a side-effect of a heightened stress response among males that mated with unfamiliar females or it may be an adaptive mechanism for eliminating memories of a previous mate that could interfere with memories of the new mate. Sexual interactions seem to potentially reduce the survival of new neurons while also enhancing the proliferation of new cells in the dentate gyrus (Leuner et al., 2010). Disentangling these divergent effects of sexual activity upon neurogenesis will be important for determining the utility of male-female interactions as a method for quantifying social memory and for determining the underlying physiological changes responsible for socially induced changes in neurogenesis.

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Abbreviations

ANOVA	analysis of variance
BrdU	5-Bromo-2'deoxyuridine
GCL	granule cell layer
NeuN	neuronal nuclei
SEM	standard error of the mean
s.c.	sub-cutaneous
SGZ	sub-granular zone
STFP	social transmission of food preferences
SVZ	subventricular zone
TBS	Tris-buffered saline
WDS	wet dog shakes

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Research Highlights

- Sexual interactions with unfamiliar females reduced neurogenesis in male rats.
- Interactions with anestrus females had no effects on neurogenesis in male rats.
- Some behaviors differed when males interacted with familiar and unfamiliar females.
- Neurogenesis levels were not directly correlated with amount of sexual activity.

Fig. 1.

Timeline for experiment 1 (A) and experiment 2 (B). For both experiments, all male subjects were handled 4–5 min per day for four days prior to two injections of BrdU spaced 12 h apart. In experiment 1, males interacted with estrus females (E) on four days spaced four days apart. In experiment 2, males interacted with anestrus females (A) on four days spaced four days apart. For both experiments, all rats were perfused and brains extracted 16 days after BrdU injections.

Fig. 2.

Total number of BrdU-labeled cells (mean ± SEM) in the dentate gyrus of males that interacted with familiar, unfamiliar, or no females (control) during 30 min trials. Among males that interacted with estrus females, there were significantly fewer labeled cells within the GCL+SGZ of the unfamiliar group than among the other two groups (A). Additionally,

there were significantly fewer labeled cells within the hilus of the unfamiliar group compared to the control group (B). Letters indicate groups that differ significantly (Fisher's LSD: $P < 0.05$). In contrast, there were no significant differences in the number of labeled cells among males that interacted with anestrus females (C,D). Photomicrographs at 1000 $\times$ magnification show dark brown BrdU-labeled cells between the GCL and the hilus (scale bars = 20 μ m). Representative cells are shown for each of the two experiments: a male that interacted with unfamiliar estrus female (E) and from a male that interacted with a familiar anestrus female (F).

Fig. 3.
Confocal images (630× magnification) of a representative cell within the granule cell layer (GCL), with a BrdU-positive cell shown in red and NeuN-positive cells shown in green. The merged image at the right includes orthogonal planes.

Fig. 4.

Mean ($\pm$ SEM) frequency of behaviors for males repeatedly exposed to the same estrus female (familiar) or exposed to four different estrus females (unfamiliar). Four days of 30 min interactions were conducted with four days between interactions. The first three graphs (A–C) show frequency of sexual behaviors and the other graphs (E–G) show non-sexual behaviors. Most behaviors showed no significant day $\times$ treatment interactions for any of these behaviors (all $P > 0.10$), but there was a significant interaction for intromissions (B). Ejaculations (C) were significantly more common in the familiar group ($*P = 0.021$), and

rearing (H) was more common in the unfamiliar group than the familiar group (* $P=0.047$). Treatment had no significant effect on any of the other behaviors (all $P>0.08$). Most of the behaviors differed significantly over the four days of testing, and significant differences among days are indicated by letters (Fisher's LSD: $P<0.05$). Aggressive acts were infrequent and did not differ in frequency over the four days of testing (F).

Fig. 5.

Mean ($\pm$ SEM) frequency of behaviors for males repeatedly exposed to the same anestrus female (familiar) or exposed to four different anestrus females (unfamiliar). Four days of 30 min interactions were conducted with four days between interactions. For grooming (C), there was a significant day $\times$ treatment interaction ($P=0.047$). There were no other significant interactions (all $P>0.12$). Males in the familiar group had significantly more aggressive interactions with the females (D) than did those in the unfamiliar group ($P=0.011$). The treatment effect was not significant for any of the other behaviors (all

$P > 0.26$). Frequency of attempted mounts, rearing, contact time, wet dog shakes (WDS), and aggression differed significantly between days (all $P < 0.001$), and significant differences among days are indicated by letters (Fisher's LSD: $P < 0.05$).

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Table 1

Percentage (mean $\pm$ SEM) of BrdU-labeled cells in the GCL+SGZ that were co-labeled with NeuN for both experiments ($n=3$ /group).

Experiment	Group	Percent cells co-labeled
1	Control	77.2 ± 3.5
	Familiar	80.7 ± 0.7
	Unfamiliar	80.0 ± 3.1
2	Control	82.7 ± 1.8
	Familiar	83.3 ± 3.6
	Unfamiliar	86.0 ± 1.6