Title: Prior sexual experience increases hippocampal cell proliferation and decreases risk assessment behavior in response to acute predator odor stress in the male rat

Authors: Mark D. Spritzer, Alex Weinberg, Victor Viau, Liisa A.M. Galea

PII: S0166-4328(09)00006-0
DOI: doi:10.1016/j.bbr.2009.01.003
Reference: BBR 5815

To appear in: *Behavioural Brain Research*

Received date: 5-9-2008
Revised date: 27-12-2008
Accepted date: 3-1-2009

Please cite this article as: Spritzer MD, Weinberg A, Viau V, Galea LAM, Prior sexual experience increases hippocampal cell proliferation and decreases risk assessment behavior in response to acute predator odor stress in the male rat, *Behavioural Brain Research* (2008), doi:10.1016/j.bbr.2009.01.003

Prior sexual experience increases hippocampal cell proliferation and decreases risk assessment behavior in response to acute predator odor stress in the male rat

Mark D. Spritzer[a,] , Alex Weinberg[a], Victor Viau[b], Liisa A.M.Galea[a*]

[a]Deptartment of Psychology and
[b]Department of Cellular and Physiological Sciences, The University of British Columbia, Vancouver, Canada.

*Corresponding author: Department of Psychology, The University of British Columbia, 2136 West Mall, Vancouver, B.C. V6T 1Z4; office: 604-822-3941; fax: 604-822-6536; E-mail: lgalea@psych.ubc.ca

Pages: 29
Figures: 3
Tables: 2

Abstract

Acute exposure to the predator odor trimethyl thiazoline (TMT) induces defensive behavior in the male rat, and this response is associated with a decrease in cell proliferation within the dentate gyrus of the hippocampus. Sexual experience appears to be protective, as it exerts anxiolytic-like effects and sustains gonadal function in the face of stress. To examine the influence of sexual experience on subsequent stress-induced defensive behavior and cell proliferation in the hippocampus we exposed adult male rats to TMT odor with or without prior exposure to sexually receptive female rats. A subset of rats were injected with the DNA-synthesis marker bromodeoxyuridine (BrdU; 200 mg/kg) during TMT exposure and perfused 24 h later to provide an index of cell proliferation within the dentate gyrus. In response to TMT, sexual experience reduced the duration of stretched attend postures, but had no significant effect on defensive burying. Furthermore, TMT induced a significant increase in cell proliferation in the dentate gyrus, but only in males with sexual experience. The results demonstrate an influence of socio-sexual experience on the magnitude of the behavioral and neural responses to predator odor stress.

Keywords: Sexual behavior; Risk assessment; Stress; Cell proliferation; Hippocampus; Dentate gyrus; Predator odor; TMT

1. Introduction

It is well established that exposure to stressors can inhibit reproductive physiology and behavior [39,48,49,53]. Clinical studies have shown that sexual dysfunction among men is comorbid with anxiety disorders [8,62] and depression [58,68]. Less understood are the potential effects of sexual experience upon subsequent physiological and behavioral responses to stressors. Many of the same brain regions that are activated by acute stressors are also activated by sexual interactions, suggesting potential links. For example, the medial preoptic area (mPOA), amygdala, and bed nucleus of the stria terminalis (BNST) are all activated by both predator-odor stress and by sexual experience among male rats [7,9,15,44]. Furthermore, sexual experience appears to exert anxiolytic effects. For example, anxiolytic effects can be induced among male rodents by pair-housing with a female [67], ejaculation [17,37,50], or exposure to female odors [30]. Following a sexual interaction, male rodents display increased circulating levels of testosterone [28,29], and testosterone exerts anxiolytic actions [12]. Stress-induced elevations in glucocorticoids typically inhibit the gonadal production of testosterone [22,48,65], but fail to reverse the gonadal hyperactivity induced by sexual stimulation [34,60]. Hence, sexual experience may afford some level of protection against the detrimental effects of stress.

Previous studies have used predator odor exposure as an ecologically relevant stimulus that reliably and effectively induces physiological and behavioral changes in rodents. Specifically, acute exposure of rats to 2,5-dihydro-2,4,5-trimethylthiazoline (TMT), an odorant extract of fox feces, induces significant increases in plasma corticosterone [9,40,59] as well as defensive burying [26], risk-assessment [14,25] and freezing behaviors [66]. Wild rat species avoid fox odors more than do shrews [11,23] and rats have a much lower olfactory detection threshold for TMT than do primates [33], suggesting that evolution has shaped the ability of rats

to detect and respond to TMT. Importantly, Perrot-Sinal et al.[43] determined that acute TMT exposure causes reduced exploratory behavior among reproductively active, but not reproductively inactive, male meadow voles, suggesting that changes in testosterone level may influence the behavioral response to predator stress.

The hippocampus is an important focal point for the effects of TMT. TMT exposure induces cellular activation of the dentate gyrus, CA1, and CA3 subregions of the hippocampus [9], elicits fast wave bursts in the dentate gyrus [24], and rapidly decreases cell proliferation in the dentate gyrus. The dentate gyrus possesses progenitor cells along the subgranular zone that retain the ability to divide and differentiate into neurons throughout adulthood [31]. Adult male rats given acute exposure to TMT show a decrease in cell proliferation within the dentate gyrus, at either 2 h [26,59] or 24 h after TMT exposure [14,27]. Curiously, the TMT-induced suppression in cell proliferation does not occur in female rats [14], suggesting that sex steroid hormones might contribute to the magnitude of the cellular response to TMT.

No previous studies have systematically examined the effects of sexual experience on neurogenesis in adult males, but there is evidence to suggest that social and sexual experiences and associated hormone changes can influence adult neurogenesis. For example, socially isolated male rats show reduced cell proliferation in the hippocampus relative to group-housed males [35,57]. Sexual experience can induce an increase in cell proliferation, at least in the subventricular zone. Specifically, female mice that engaged in mating display increased neurogenesis in the subventricular zone compared to non-mated females [55]. Testosterone levels increase among males following sexual interactions, and chronic injections of testosterone have been shown to increase hippocampal neurogenesis [56], suggesting that prolonged exposure

to elevated testosterone levels during sexual stimulation could have an impact on cell proliferation.

Taken together, previous studies suggest that sexual interactions protect against the detrimental effects of stress on behavior as well as on cell proliferation in the hippocampus. The current study examined the influence of sexual stimulation in adult male rats on the behavioral and neurological response to acute predator odor exposure. Due to anxiolytic effects, sexual interactions were expected to decrease defensive and risk assessment behaviors. Sexual interactions were also expected to influence the magnitude, and possibly direction, of the TMT-induced suppression of cell proliferation previously documented within the dentate gyrus.

2. Methods

2.1 Animals

Forty-eight adult male Long-Evans rats (approximately 60 days old, 250-275 g) were obtained from Charles River Canada as experimental subjects. In addition, sixteen adult female Sprague Dawley rats (220-270 g), obtained from the University of British Columbia Animal Care Facility, were used for sexual interactions. The difference in strain allowed easy distinction between the sexes during behavioral observations. Furthermore, males and females readily engaged in sexual interactions and different strains have been used previously to examine male sexual behavior [16,21]. Males and females were housed in separate colony rooms in opaque polyurethane bins with aspen-chip bedding. Males were pair-housed and females were housed individually. Rooms were temperature controlled (21 ± 1 ºC) with a 12:12 h light/dark cycle (lights on at 0700 h). Tap water and rodent chow (Lab Diet #5012; Jamieson) were provided *ad libitum* throughout the experiment. All animal procedures were approved by the animal care

committee at the University of British Columbia and were carried out in accordance with ethical guidelines set by the Canada Council for Animal Care.

2.2 Screening for Sexual Competence

All male subjects were handled daily for at least 5 days prior to the first day of screening for sexual competence. For both sex screening and experimental trials, behavioral estrus was induced among ovarectomized females by s.c. injections of 17β-estradiol benzoate (10 μg) 48 h prior to testing and progesterone (500 μg) 4 h prior to testing. All sexual testing was conducted in bi-level Plexiglas chambers (15 × 50 × 70 cm) with a platform (40 cm in length) centered 28 cm above the floor and ramps between levels [44,45]. Chambers were cleaned with 70% ethanol after each trial.

Each male received 4 sex-screening trials separated by 3-day intervals, and no male was exposed to the same female more than once. For each trial, a receptive female was placed into a bi-level chamber 5 minutes prior to adding a single male, and the pair was allowed to interact for 30 min. The third and fourth screening trial for each male was video recorded and scored for frequency of mounts, intromissions, and ejaculations [45]. Twelve males were screened per day, and four batches of 12 males were screened in all. The four males showing the least sexual behavior in each batch were eliminated from further testing. BestCollection software (ver. 7.0, Educational Consulting Inc.) was used to score the frequency of mounts, intromissions, and ejaculations [52] for each day of screening. Of the males that were eliminated, none ejaculated and 2 males exhibited intromission during the third and fourth screening trials only. All males retained for experimental testing engaged in frequent mounts and intromissions during screening and most (20 of 32) ejaculated at least once during the third and fourth screening trials.

2.3 Experimental Protocol

Experimental testing started five days after the completion of male screening. A 2×2 experimental design was used in which males were divided into four treatment groups of 8 males per group: no-sex/water, no-sex/TMT, sex/water, and sex/TMT. Males in the sex treatment groups were exposed to receptive females in bi-level chambers for 30 min over five consecutive days. No male was exposed to the same female more than once. In a separate room, males in the no-sex treatment groups were placed in an empty, clean bi-level chamber for 30 min over five consecutive days. Two males were tested at a time in each of the two rooms (i.e., sex room and no-sex room) using bi-level chambers that were visually isolated from each other. All trials were video recorded for behavioral analysis.

For TMT exposure, we followed a protocol that was previously shown to induce defensive behaviors and a decrease in cell proliferation within the dentate gyrus of adult male rats [14,26,27]. Immediately after exposure to the female or empty bi-level chamber, each male was placed in a Plexiglas box (29 × 30 × 46 cm) inside a fume hood for 20 min on the first four days of testing to habituate to the testing procedures. The testing boxes were filled with 5cm of corn cob bedding and a vial with a dry Kimwipe was placed in the corner. Two separate fume hoods were used, one for the TMT group and one for the control group, and two visually isolated chambers were used under each fume hood. On the fifth day, vials under one fume hood were filled with 150µl TMT and vials under the other fume hood were filled with 150µl of water. The fume hood prevented diffusion of odor throughout the room. The first 15 min was video recorded to determine the behavioral response to TMT or water. Immediately after video recording, all rats were given an i.p. injection of the thymidine analog BrdU (5-Bromo-2'deoxyuridine; 200

mg/kg body mass) to label dividing cells. Rats were then returned to the testing chambers for an additional 45 min of exposure (1 h total). The strong smell of TMT within the chambers did not dissipate noticeably during testing, indicating that rats were exposed to TMT for the full hour. Twenty-four hours later, rats were anaesthetized with a lethal dose of sodium pentobarbital (Euthanyl; Bimeda-MTC, Cambridge, ON, Canada) and perfused transcardially with 0.9% saline (60 ml) followed by 4% paraformaldehyde (120 ml). The cell cycle in adult male rats has been shown to be 24.7 h [6], and therefore the 24 h time period was used to measure cell proliferation (i.e., cells that have undergone one division) within the dentate gyrus. Brains were extracted and postfixed with 4% paraformaldehyde (4 °C) for 24 h. Brains were then cryoprotected with 30% sucrose in 0.1 M TBS (0.08 M Tris-HCl, 0.02 M Tris-base, 0.9 % saline, pH 7.4) and stored at 4 °C until slicing. Half the brains (N = 16) were mistakenly stored at -20 °C and were not viable for immunohistochemistry.

BestCollection software was used to score sexual behaviors and the behavioral response to TMT or water. The frequency of mounts, intromissions, and ejaculations was scored for all five days of sexual interactions. The frequency and duration of the following behaviors were scored during the first 15 min of exposure to TMT or water: defensive burying, stretched attend, contact with vial, rearing, and grooming. Defensive burying involved the rat using its forepaws to push and fling bedding toward the vial [47]. Stretched attend is a risk assessment behavior which was characterized by the rat extending the front half of its body toward the vial while its hind paws remained stationary [46]. Rearing involved rats raising their forepaws off the ground and stretching vertically. Grooming included licking any part of the body or vigorously rubbing licked forepaws on any part of the body.

2.4 Immunohistochemistry

Brains were sliced into 40 μm coronal sections through the extent of dentate gyrus in a bath of TBS using a vibratome (Leica VT1000S). 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.

Peroxidase immunohistochemistry was performed on free-floating tissue sections at 400 μm intervals (i.e., every 10^{th} section) through the rostrocaudal extent of the hippocampus to visualize BrdU-labeled cells. The sections were rinsed three times for 10 min between steps with 0.1 M TBS (pH 7.4) unless otherwise stated. Tissue was initially incubated for 30 min in 0.6% H_2O_2 to eliminate endogenous peroxidase activity. DNA was denatured by applying 2 N HCl for 30 min at 37°C, and this step was immediately followed by a 10 min incubate in 0.1 M borate buffer (pH 8.5) to neutralize the acid. Sections were blocked for 30 min with 3.0% normal horse serum (NHS; Chemicon, Temecula, CA, USA) in 0.1 M TBS and then incubated 48 h at 4 °C in mouse monoclonal antibody against BrdU (Roche Diagnostics, Laval, Quebec, Canada; 1:200 in 0.1 M TBS, 3% NHS and 0.1% Triton-X). Sections were next incubated for 4 h at room temperature in horse anti-mouse secondary antibodies (1:100 in 0.1 M TBS; Vector Laboratories, Burlington, ON, Canada). Sections were incubated for 2 h in avidin-biotin horseradish peroxidase complex (ABC Elite Kit; 1:50; Vector Laboratories). Sections were reacted for approximately 5 min in a solution of 0.02% diaminobenzidine (DAB; Sigma-Aldrich) and 0.003% H_2O_2 in 0.1 M TBS. The sections were mounted onto slides and dried overnight. Finally, sections were counterstained with cresyl violet acetate, dehydrated with ethanol, cleared with xylene, and coverslipped with Permount (Fisher Scientific, Nepeon, ON, Canada).

2.5 Cell Counting

All BrdU-labeled cells in the granule cell layer (including the subgranular zone) and hilus were counted by an experimenter blind to treatment group assignment. Every 10th section was counted through the entire rostrocaudal extent of the dentate gyrus (10-12 sections per brain), except at the uppermost focal plane, at 1000× magnification (oil immersion) on a light microscope (Nikon Eclipse 600). Cells observed within 20 μm of the inner edge of the granule cell layer were considered the subgranular zone (SGZ), and these cell counts were combined with the granule cell layer (GCL) counts (GCL+SGZ). Cells were considered BrdU-labeled if they were medium-sized (approximately 10-20 μm) and exhibited a dark brown stain (Fig. 1) [42]. BrdU-labelled cells were counted in the hilus and compared to counts in the granule cell region for a number of reasons: 1) to determine whether any experimentally induced effects on cell proliferation are due to generalized effects on blood brain permeability, 2) progeny from progenitor cells in the hilus give rise to a different population of cells that are mainly glial cells compared to progeny from progenitor cells in the subgranular zone which give rise to cells that are mainly neurons [5], and 3) new neurons in the hilus are considered ectopic [38,54]. No BrdU-labeled cells were found for one rat in the sex/TMT group following two attempts at immunohistochemical staining, and it was therefore removed from further analyses. Total number of BrdU-labeled cells per brain was estimated by multiplying the number of cells counted by ten[19,26] . Digital images were made of all sections at 20× magnification using ACT-1 (ver. 2.20, Nikon Corporation), and dentate gyrus areas were measured using Simple PCI (ver. 5.1, Comix Inc. Imaging Systems). Volumes of the dentate gyrus and hilus were estimated using Cavalieri's principle [20].

2.6 Testosterone Assay

Blood was collected from the chest cavity at the time of perfusion and samples were stored overnight at 4 °C. These blood samples were used to assay circulating testosterone levels for all subjects. Samples were centrifuged at 10,000 rpm for 15 min, and serum was decanted and stored at -20 °C. Serum testosterone was assayed using an ImmuChem Coated Tube RIA Kit (MP Biomedicals Inc., Costa Mesa, CA, USA). The lower limit of detection for testosterone was 0.2 ng/ml. The testosterone antibody had some cross-reactivity with DHT (7.8%), 5α-androsterone-3β, 17β-diol (2.2%), and 11-oxytestosterone (2.0%), but had no cross-reactivity with progesterone, estrogens, or glucocorticoids (all < 0.01%). The intra-assay coefficient of variation was 12.5%.

2.7 Data Analyses

Behaviors and serum testosterone levels were analyzed using two-way analysis of variance (ANOVA) with *stress* (water, TMT) and *sex* (sex, no-sex) as the between-subjects factors. Linear regression was used to determine the relationship between serum testosterone levels and each of the frequency and duration of behaviors recorded during TMT or water exposure. Total cell counts were analyzed using repeated-measures ANOVA with *cell layer* (i.e., GCL+SGZ and hilus) as a within subjects effect and *odor* and *sex* as between-subject factors. Post-hoc tests were conducted using Newman-Keul's procedure unless otherwise specified. A priori comparisons were subjected to Bonferroni corrections. Statistica 6.1 (Statsoft, Inc., Tulsa, OK) was used for all analyses, and the significance level was set at $\alpha = 0.05$.

3. Results

3.1 Sexual behavior

Males in the sex/TMT and the sex/water treatment groups experienced similar amounts of sexual interactions during the five days of testing. Specifically, there were no significant differences between the two groups in total number of mounts (p=0.97), intromissions (p=0.78), or ejaculations (p=0.68). Therefore, any differences between the sex/TMT and sex/water groups on other measures were probably not due to differences in sexual experience. Males mounted 12.1 ± 1.9 times, intromitted 7.8 ± 1.1 times, and ejaculated 0.49 ± 0.09 times on average during the 30 min trials.

3.2 Sexual experience modifies risk assessment behavior but not other behaviors

Prior sexual experience eliminated an increase in risk assessment behavior (stretched attends) induced by exposure to TMT (Fig. 2A, B). Specifically, a significant sex×stress interaction effect was found for both frequency and duration of stretched attends ($F_{1,28}$=6.92, p=0.014 and $F_{1,28}$=7.94, p=0.009, respectively). For frequency of stretched attends (Fig. 2A), the only significant difference among groups was that the no-sex/TMT group had a higher frequency than did the no-sex/water group (p=0.023). For duration of stretched attends (Fig. 2B), the no-sex/TMT group had a significantly longer duration than did any of the other groups (p<0.030), whereas all other pairwise comparisons were not significant (p>0.45). None of the main effects were significant (all p>0.10).

TMT exposure increased the frequency and duration of defensive burying ($F_{1,28}$=8.90, p=0.006; Fig 2C, $F_{1,28}$=8.88, p=0.006; Fig 2D, respectively), but there was no main effect of sex

or sex×odor interaction for defensive burying ($0.46<p<0.84$). TMT decreased the duration of vial contact irrespective of sexual stimulation prior to exposure to TMT ($F_{1,26}=4.25$, $p=0.049$; Table 1; data from 2 rats were not included as they were outliers) and had a tendency to decrease the frequency of vial contact ($F_{1,28}=3.16$, $p=0.086$; Table 1), indicating that rats avoided TMT. TMT exposure and sexual experience had no significant main or interaction effects on grooming and rearing behavior (Table 1). For frequency of grooming, the sex×stress interaction effect was nearly significant ($p=0.08$), with the sex/water group grooming more often than did any other group. No significant effects were found for duration of grooming (all $p>0.15$) or for the frequency and duration of rearing (all $p>0.09$).

3.3 Sexual experience increased cell proliferation only when males were also exposed to TMT

No significant differences were observed in the volume of the GCL or hilus among the groups (all $p>0.25$; Table 2), and therefore all further analyses were conducted using total cell count (BrdU+) estimates. The sex/TMT group exhibited more cell proliferation within the GCL+SGZ than did any of the other groups (Fig. 3). Specifically, planned comparisons revealed that the sex/TMT group had significantly more BrdU-labeled cells in the GCL+SGZ than did the no-sex/TMT group ($p=0.03$) or the sex/water group ($p=0.01$), but no significant differences were found between the sex/water group and the no-sex/water group or between the no-sex/TMT group and the no-sex/water group (both $p>0.40$). Overall, exposure to TMT resulted in a significant increase in cell proliferation in the GCL+SGZ relative to water control groups (main effect of stress: $F_{1,11}=7.31$, $p=0.02$), however this main effect is due to the higher counts of BrdU-labeled cells in the GCL+SGZ of the sex/TMT group (Fig. 3). There was also a strong trend for males engaging in sexual interactions to have a higher level of cell proliferation in the

GCL+SGZ than did males not engaging in sexual interactions (main effect of sex: $F_{1,11}$=3.89, p=0.07). As expected, there was significantly more cell proliferation in the granule cell layer than in the hilus (main effect of cell layer: $F_{1,11}$=279, p<0.001; Fig. 3), however none of the interaction effects with cell layer were significant (all p>0.15).

3.4 Testosterone levels

Serum was collected for testosterone assays 24 h after sexual interactions on the final day of testing. No significant differences between groups were observed in testosterone levels (interaction: p=0.15; main effects: p>0.25). It is noteworthy, however, that the sex/TMT group had the highest mean testosterone level (Table 3). Higher testosterone levels were predictive of increased grooming duration ($F_{1,30}$=5.42, r^2=0.15, p=0.027) and reduced rearing frequency ($F_{1,30}$=13.8, r^2=0.32, p=0.001) and duration ($F_{1,30}$=7.02, r^2=0.19, p=0.013). No other significant correlations were observed between testosterone levels and the behavioral response to TMT, sexual behaviors, or cell proliferation (p>0.10).

4. Discussion

This experiment tested the effects of prior sexual experience on risk assessment, defensive behavior and hippocampal cell proliferation of male rats exposed to acute predator odor. Previous studies demonstrated that male rats exhibit increased risk assessment behavior (stretched attend) and decreased hippocampal cell proliferation upon exposure to a predator odor [14,25,26,27,59]. In contrast, we found a strikingly different pattern in males that had engaged in sexual interactions for five consecutive days prior to exposure to TMT. Specifically, males that engaged in prior sexual interactions did not display the typical increase in risk assessment (i.e.,

stretched attends) in response to TMT and they exhibited an increase in hippocampal cell proliferation in response to TMT.

4.1 Sexual experience modified the risk assessment response to TMT

Our behavioral results support numerous previous studies showing an increase in risk assessment behavior (i.e., stretched attend) in response to acute exposure to TMT [14,26,27]. We also demonstrated that prior sexual experience eliminates this increased risk assessment in response to TMT. Stretched attend behavior is generally considered to be indicative of anxiety because this behavior involves investigation of a potentially harmful stimulus and is attenuated by anxiolytic drugs [3,4]. This suggests that sexual interactions may have been anxiolytic, causing a reduction in stretched attend behavior. Although not significant, we also found that sexual interactions prior to TMT exposure increased grooming and decreased rearing, further suggesting that sexual stimulation reduced anxiety in the face of stress [10,32]. In support of this interpretation, Edinger and Frye [13] found that four days of 10 min sexual interactions resulted in reduced anxiety-like behaviors in the open field and elevated plus-maze tests. Similarly, Westenbroek et al. [67] found that male rats exposed to chronic foot-shock and housed with a cycling female displayed less anxiety in an open field test than did males that were socially isolated.

Unlike stretched attend behavior, sexual experience had no effect on increased defensive burying induced by TMT. In contrast, Rodríguez-Manzo et al. [50] found that a single ejaculation resulted in reduced burying behavior toward a shock prod. However, the same study found that a decrease in burying did not occur among males allowed to copulate with a female for 4 h [50], suggesting that prolonged exposure to females, like the 5 days of sexual interactions

used in our study, may not induce changes in burying behavior. Defensive burying has been variously used as an index of anxiety and/or fearfulness and has been shown to be attenuated by many of the same anxiolytic drugs that attenuate stretched attend behavior [10]. However, some anxiolytic drugs have been shown to reduce the expression of freezing behavior without affecting defensive burying [10,32], suggesting that defensive burying represents an active avoidance strategy that is dissociable from more passive avoidance behaviors. Thus, defensive burying may be an index of active avoidance independent of initial risk assessment behavior. Therefore, we argue that while prior sexual experience is capable of modifying the evaluation of TMT as an aversive stimulus source (stretched attend), it does not appear to impact the active avoidance component of the animal's defensive repertoire (defensive burying).

The observed anxiolytic effect of sexual experience may be due, at least in part, to hormonal changes in the males. A sexual interaction with a female induces a rise in circulating testosterone among male rats [28,29], and elevated androgen levels lead to reduced anxiety behavior in rats [2,12,30]. Furthermore, metabolites of testosterone act upon the hippocampus to induce anxiolytic effects [12,18]. In the present study, we did not observe significant differences in serum testosterone levels among the treatment groups, but this may not be surprising as serum was collected 24 h after sexual interactions and exposure to TMT. Another possible hormonal mechanism involves prolactin, which also increases following sexual interactions [28,29] and has been shown to have anxiolytic effects [63,64]. The role of prolactin in the axiolytic effects of sexual interactions is an area that warrants further study.

4.2 Sexual experience resulted in increased cell proliferation in response to TMT

In the present study we found that sexual interactions for 5 days prior to exposure to TMT increased cell proliferation in the dentate gyrus compared to all other groups. This is in stark contrast to the suppression in cell proliferation seen in sexually naïve male rats [14,26,27,59]. As described above, sexual interactions seemed to reduce anxiety and/or fearfulness in response to TMT. This change in the perceived aversiveness of the stressor may have induced physiological changes that led to enhanced cell proliferation within the hippocampus. Intriguingly, other studies have shown that various social interactions can reduce anxiety and increase neurogenesis in response to stress. Pair-housing results in reduced anxiety-like behavior in male rats relative to singly-housed males [67]. Furthermore, Stranahan et al. [57] observed an increase in cell proliferation within the dentate gyrus among group-housed adult male rats exposed to cold water stress relative to singly-housed rats [57]. Lu et al. [35] also observed reduced cell proliferation in individually housed male rats relative to group-housed males. These findings, coupled with the present data, suggest that sexual or social interactions may reverse the stress-induced suppression of cell proliferation.

Interestingly, we also did not observe a significant stress-induced suppression of cell proliferation in males that did not engage in sexual interactions. This result may also be indicative of the neuroprotective effects of social interactions in general. Specifically, all of the males used in our study were screened for sexual competence 10 days prior to BrdU injection. This screening may have induced lasting physiological changes that prevented a TMT-induced suppression of cell proliferation, although the effects were not strong enough to induce the enhanced cell proliferation seen in males that had more recently experienced sexual interactions. Another key difference between the current study and past studies using TMT is that our animals

were pair housed throughout the experiment, whereas previous studies used individual housing. Among rats, social isolation is a stressor that has been shown to cause a decrease in cell proliferation within the dentate gyrus [35]. Therefore, TMT may suppress cell proliferation only among socially isolated rats. In support of this idea, Thomas et al. [61] found that acute TMT exposure had no significant effect on hippocampal cell proliferation among group-housed male rats. Finally, the effects of TMT may vary with the strain of rat used [51]. All previous studies testing the effects of TMT on cell proliferation have used Sprague-Dawley rats, whereas the current study used Long-Evans rats; therefore, acute exposure to TMT may reduce cell proliferation among Sprague-Dawley but not Long-Evans rats.

The functional significance of the observed changes in hippocampal cell proliferation remains unclear. Some evidence indicates that hippocampal neurogenesis is involved in the formation of olfactory memories [36,41]. The enhanced cell proliferation observed in the current study might be involved in the formation of memories related to sexual experience and/or TMT exposure. However, we did not observe enhanced cell proliferation in response to sexual interactions alone but only in combination with TMT exposure, suggesting that enhanced cell proliferation was not simply involved in learning mate identity. Fortin et al. (2002) demonstrated that the hippocampus is not required for rats to distinguish between familiar and novel odors, whereas a functional hippocampus is necessary to remember the sequence in which multiple odors are presented. Current theory suggests that encoding the timing of events may be one of the primary functions of adult neurogenesis [1]. Hence, the elevated cell proliferation observed in the current study may be involved in learning the temporal relationship between olfactory cues associated with mating and TMT. It is important to note, however, that cell proliferation does not

necessarily lead to adult neurogenesis, and further study is needed to determine whether the enhanced cell proliferation that we observed leads to an increase in functional neurons.

In summary, the current study demonstrates that sexual experience can influence an individual's response to acute stress. Males engaging in sexual interactions prior to predator odor exhibited reduced risk assessment behavior and increased hippocampal cell proliferation. These results suggest that sexual interactions cause decreased anxiety and/or fearfulness, which may in turn influence hippocampal cell proliferation. It remains to be determined whether the enhanced cell proliferation observed in the current study leads to changes in neurogenesis, or learning, and memory. An intriguing possibility is that the combined effects of sexual experience and acute stress influence memory formation in a way that is distinct from either of these events individually.

Acknowledgements

We thank Cindy Barha, Stephanie Lieblich, Pralle Kriengwatana, Alice Chan and Anne Cheng for their important contributions to this study. This work was funded by CIHR and NSERC operating grants to LAMG and VV. MDS was the recipient of a CIHR training grant. LAMG is a Michael Smith Senior Scholar.

Rferences

[1] Aimone JB, Wiles J, Gage FH. Potential role for adult neurogenesis in the encoding of time in new memories. Nat Neurosci 2006;9: 723-7.

[2] Bitran D, Kellogg CK, Hilvers RJ. Treatment of anabolic-androgenic steroid affects anxiety-related behavior and alters the sensitivity of cortical $GABA_A$ receptors in the rat. Horm Behav 1993;27: 568-83.

[3] Blanchard RJ, Briebel G, Henrie JA, Blanchard DC. Differentiation of anxiolytic and panicolytic drugs by effects of rat and mouse defense test batteries. Neurosci Biobehav Rev 1997;21: 783-9.

[4] Blanchard DC, Blanchard RJ, Tom P, Rodgers RJ. Diazepam changes risk assessment in an anxiety/defense test battery. Psychopharmacology 1990;101: 511-8.

[5] Cameron HA, Woolley CA, McEwen BS, Gould E. Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. Neuroscience 1993;56: 337-44.

[6] Cameron HA, McKay RDG. Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. 2001;435: 406-17.

[7] Coolen LM, Peters HJPW, Veening JG. Anatomical interrelationships of the medial preoptic area and other brain regions activated following male sexual behavior: a combined Fos and tract-tracing study. J Comp Neurol 1998;397: 421-35.

[8] Corretti G, Pierucci S, De Scisciolo M, Nista C. Comorbidity between social phobia and premature ejaculation: study on 242 males affected by sexual disorders. Sex Marital Ther 2006;32: 183-7.

[9] Day HEW, Masini CV, Campeau S. The pattern of brain c-fos mRNA induced by a component of fox odor, 2,5-dihydro-2,4,5-trimethylthazoline (TMT), in rats suggest both systemic and processive stress characteristics. Brain Res 2004;1025: 139-51.

[10] DeBoer SF, Koohaas JM. Defensive burying in rodents: theology, neurobiology and psychopharmacology. Eur J Pharmacol 2003;463: 145-61.

[11] Dickman CR, Doncaster CP. Responses of small mammals to Red fox (*Vulpes vulpes*) odour. J Zool Lond 1984;204: 521-31.

[12] Edinger KL, Frye CA. Testosterone's analgesic, anxiolytic, and cognitive enhancing effects may be due in part to actions of its 5a-reduced metabolites in the hippocampus. Behav Neurosci 2004;118: 1352-64.

[13] Edinger KL, Frye CA. Sexual experience of male rats influences anxiety-like behavior and androgen levels. Physiol Behav 2007;92: 443-53.

[14] Falconer EM, Galea LAM. Sex differences in cell proliferation, cell death and defensive behavior following acute predator odor stress in adult rats. Brain Res 2003;975: 22-36.

[15] Fendt M, Endres T, Lowry CA, Apfelbach R, McGregor IS. TMT-induced autonomic and behavioral changes and the neural basis of its processing. Neurosci Biobehav Rev 2005;29: 1145-56.

[16] Fiorino DF, Coury A, Phillips AG. Dynamic changes in nucleus accumbens dopamine efflux during the Coolidge effect in male rats. J Nsurosci 1997;17: 4849-55.

[17] Freidin E, Kamenetzky G, Mustaca AE. Newly proliferated cells in the adult male amygdale are affected by gonadal steroid hormones. Learn Behav 2005;33: 277-86.

[18] Frye CA, Seliag AM. Testosterone increases analgesia, anxiolysis, and cognitive performance of male rats. Cogn Affect Behav Neurosci 2001;1: 371-81.

[19] Gould E, Beylin A, Tanapat P, Reeves A, Shors TJ. Learning enhances adult neurogenesis in the hippocampal formation. Nat Neurosci 1999;2: 260-5.

[20] Gundersen HJ, Bendtsen TF, Korbo L, Marcussen N, Moller A, Nielsen K, Nyengaard JR, Pakkenberg B, Sorensen FB, Besterby A. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. APMIS 1988;96: 379-94.

[21] Hamson DK, Watson NV. Regional brainstem expression of Fos associated with sexual behavior in male rats. Brain Res 2004;1006: 233-40.

[22] Hardy MP, Sottas CM, Ge R, McKittrick CR, Tamashiro KL, McEwen BS, Haider SG, Markham CM, Blanchard RJ, Blanchard DC, Saki RR. Trends of reproductive hormone of male rats during psychosocial stress: role of glucocorticoid metabolism on behavioral dominance. Biol Reprod 2002;67: 1750-5.

[23] Hayes RA, Nahrung HF, Wilson JC. The response of native Australian rodents to predator odours varies seasonally: a by-product of life history variation? Anim Behav 2006;71: 1307-14.

[24] Heale VR, Vanderwolf CH, Kavaliers M. Components of weasel and fox odors elicit fast wave bursts in the dentate gyrus of rats. Behav Brain Res 1994;63: 159-65.

[25] Hebb ALO, Zachoarko RM, Gauthier M, Trudel F, Laforest S, Drolet G. Brief exposure to predator odor and resultant anxiety enhances mesocorticolimbic activity and enkephalin expression in CD-1 mice. Eur J Neurosci 2004;20: 2415-29.

[26] Holmes MM, Galea LAM. Defensive behavior and hippocampal cell proliferation: differential modulation by naltrexone during stress. Behav Neurosci 2002;116: 160-8.

[27] Kambo JS, Galea LAM. Activational levels of androgens influence risk assessment behaviour but do not influence stress-induced suppression in hippocampal cell proliferation in adult male rats. Behav Brain Res 2006;175: 263-70.

[28] Kamel F, Frankel AI. Hormone release during mating in the male rat: time course, relation to sexual behavior, and interaction with handling procedures. Endocrinology 1978;103: 2172-9.

[29] Kamel F, Wright WW, Mock EJ, Frankel AI. The influence of mating and related stimuli on plasma levels of luteinizing hormone, follicle stimulating hormone, prolactin, and testosterone in the male rat. Endocrinology 1977;101: 421-9.

[30] Kavaliers M, Choleris E. Brief exposure to female odors “emboldens” male mice by reducing predator-induced behavioral and hormonal responses. Horm Behav 2001;40: 497-509.

[31] Kempermann G. Adult Neurogenesis: Stem Cells and Neuronal Development in the Adult Brain. USA: Oxford University Press, 2005, 448 pp.

[32] Koolhaas JM, Korte SM, DeBoer SF, Van Der Vegt, B.J., Van Reenen CG, Hopster H, De Jong IC, Ruis MAW, Blokhuis HJ. Coping styles in animals: current status in behavior and stress physiology. Neurosci Biobehav Rev 1999;23: 925-35.

[33] Laska M, Fendt M, Weiser M, Endres A, Hernandez Salazar LT, Apfelbach R. Detecting danger—or just another odorant? Olfactory sensitivity for the fox odor component 2,4,5-trimethylthiazoline in four species of mammals. Physiol Behav 2005;84: 211-5.

[34] Lemaire V, Taylor GT, Mormède P. Adrenal axis activation by chronic social stress fails to inhibit gonadal function in male rats. Psychoneuroendocrinology 1997;22: 563-73.

[35] Lu L, Bao G, Chen H, Xia P, Fan X, Zhang J, Pei G, Ma L. Modification of hippocampal neurogenesis and neuroplasticity by social environments. 2003;183: 600-9.

[36] Mak GK, Enwere EK, Gregg C, Pakarainen T, Poutanen M. Male pheromone-stimulated neurogenesis in the adult female brain: possible role in mating behavior. Nat Neurosci 2007;10: 1003-11.

[37] Martínez-Mota L, López-Rualcava A, Rodríguez-Mazo G. Ejaculation induces long-lasting behavioural changes in male rats in the forced swimming test: evidence for an increased sensitivity to the antidepressant desipramine. Brain Res Bull 2005;65: 323-9.

[38] McCloskey DP, Hintz TM, Pierce JP, Scharfman HE. Stereological methods reveal the robust size and stability of ectopic hilar granule cells after pilocarpine-induced status epilecticus in the adult rat. Eur J Neurosci 2006;24: 2203-10.

[39] Menendez-Patterson A, Forez-Lozano JA, Fernandez S, Marin B. Stress and sexual behavior in male rats. Physiol Behav 1980;24: 403-6.

[40] Morrow BA, Redmond AJ, Roth RH, Elsworth JD. The predator odor, TMT, displays a unique, stress-like pattern of dopaminergic and endocrinological activation in the rat Brain Res 2000;864: 146-51.

[41] Olariu A, Cleaver KM, Shore LE, Brewer MD, Cameron HA. A natural form of learning can increase and decrease the survival of new neurons in the dentate gyrus. Hippocampus 2005;15: 750-62.

[42] Oremrod BK, Galea LAM. Reproductive status influences cell proliferation and cell survival in the dentate gyrus of adult female meadow voles: a possible regulatory role for estradiol. Neuroscience 2001;102: 369-79.

[43] Perrot-Sinal T, Ossenkopp K-, Kavaliers M. Influence of natural stressor (predator odor) on locomotor activity in the meadow vole (*Microtus pennsylvanicus*): modulation by sex, reproductive condition and gonadal hormones. Psychoneuroendocrinology 2000;25: 259-76.

[44] Pfaus JG. Neurobiology of sexual behavior. Curr Opin Neurobiol 1999;9: 751-8.

[45] Pfaus JG, Mendelson SD, Phillips AG. A correlational and factor analysis of anticipatory and consummatory measures of sexual behavior in the male rat. Psychoneuroendocrinology 1990;15: 329-40.

[46] Pinel JPJ, Mana MJ, Ward JA. Stretched-approach sequences directed at localized shock source by *Rattus norvegicus*. J Comp Psych 1989;103: 140-8.

[47] Pinel JPJ, Symons LA, Christensen BK, Tees RC. Development of defensive burying in *Rattus norvegicus*: experience and defensive responses. J Comp Psych 1989;103: 359-65.

[48] Retana-Márquez S, Bonilla-Jaime H, Vázquez-Palacios G, Martínez-García R, Velázquez-Moctezuma J. hanges in masculine sexual behavior, corticosterone and testosterone in response to acute and chronic stress in male rats. Horm Behav 2003;44: 327-37.

[49] Retana-Márquez S, Salazar ED, Velazquez-Moctezuma J. Effect of acute and chronic stress on masculine sexual behavior in the rat. Psychoneuroendocrinology 1996;21: 39-50.

[50] Rodríguez-Manzo G, López-Rubalcava C, Fernández-Guasti A. Anxiolytic-like effect of ejaculation under various sexual behaviour conditions in the male rat. Physiol Behav 1999;67: 651-7.

[51] Rosen JB, West EA, Donley MP. Not all rat strains are equal: differential unconditioned fear responses to the synthetic fox odor 2,4,5-trimethylthiazoline in three outbred rat strains. Behav Neurosci 2006;120: 290-7.

[52] Sachs BD, Barfield RJ. Functional analysis of masculine copulatory behavior in the rat. Adv Stud Behav 1976;7: 91-154.

[53] Sapolsky RM, romero LM, Munck AU. How do glucocorticoids influence stress responses? Integrating permissive suppressive, stimulatory, and preparative actions. Endo Rev 2000;21: 55-89.

[54] Scharfman H, Goodman J, McCloskey D. Ectopic granule cells of the rat dentate gyrus. Dev Neurosci 2007;29: 14-27.

[55] Shingo T, Gregg C, Enwere E, Fujikawa H, Hassam R, Geary C, Cross JC, Weiss S. Pregnancy-stimulated neurogenesis in the adult female forebrain mediated by prolactin Science 2003;299: 117-20.

[56] Spritzer MD, Galea LAM. Testosterone and dihydrotestosterone, but not estradiol, enhance survival of new hippocampal neurons in adult male rats. Dev Neurobiol 2007;67: 1321-1333.

[57] Stranahan AM, Khalil D, Gould E. Social isolation delays the positive effects of running on adult neurogenesis. Nat Neurosci 2006;9: 526-33.

[58] Sugimori H, Yoshida K, Tanaka T, Baba K, Nishida T, Nakazawa R, Iwamoto T. Relationships between erectiel dysfunction, depression, and anxiety in Japanese subjects. J Sex Med 2005;2: 390-6.

[59] Tanapat P, Hastings NB, Rydel TA, Galea LAM, Gould E. Exposure to fox odor inhibits cell proliferation in the hippocampus of adult rats via an adrenal hormone-dependent mechanism. J Comp Neurol 2001;437: 496-504.

[60] Taylor GT, Weiss J, Rupich R. Male rat behavior, endocrinology and reproductive physiology in a mixed-sex, socially stressful colony. Physiol Behav 1987;39: 429-33.

[61] Thomas RM, Urban JH, Peterson DA. Acute exposure to predator odor elicits a robust increase in corticosterone and a decrease in activity without altering proliferation in the adult rat hippocampus. Exp Neurol 2006;201: 308-15.

[62] Tignol J, Martin-Guehl C, Aouizerate B, Grabot D, Auriacombe M. Social phobia and premature ejaculatin: a case-control study. Depress Anxiety 2006;23: 153-7.

[63] Torner L, Maloumby R, Nava G, Aranda J, Clapp C, Neumann ID. *In vivo* release and gene upregulation of brain prolactin in response to physiological stimuli Eur J Neurosci 2004;19: 1601-8.

[64] Torner L, Toshci N, Pohlinger A, Landgraf R, Neumann ID. Anxiolytic and anti-stress effects of brain prolactin: improved efficacy of antisense targeting of the prolactin receptor by molecular modeling. J Neurosci 2001;21: 3207-14.

[65] Viau V. Functional cross-talk between the hypothalamic-pituitary-gonadal and -adrenal axes. J Neuroendocrinol 2002;14: 506-13.

[66] Wallace KJ, Rosen JB. Predator odor as an unconditioned fear stimulus in rats: elicitation of freezing by trimethylthazoline, a component of fox feces. Behav Neurosci 2000;114: 912-22.

[67] Westenbroek C, Snijders TAB, den Boer JA, Gerrits M, Fokkema DS, Ter Horst GJ. Pair-housing of male and female rats during chronic social stress exposure results in gender-specific behavioral responses. Horm Behav 2005;47: 620-8.

[68] Zajecka J, Dunner DL, Gelenberg AJ, Hirshfeld RM, Kornstein SG, Ninan PT, Rush AJ, Thase ME, Trivedi MH, Arnow BA, Borian FE, Manber R, Keller MB. Sexual function and satisfaction in the treatment of chronic major depression with nefazodone, psychotherapy, and their combination. J Clin Psychiatry 2002;63: 709-16.

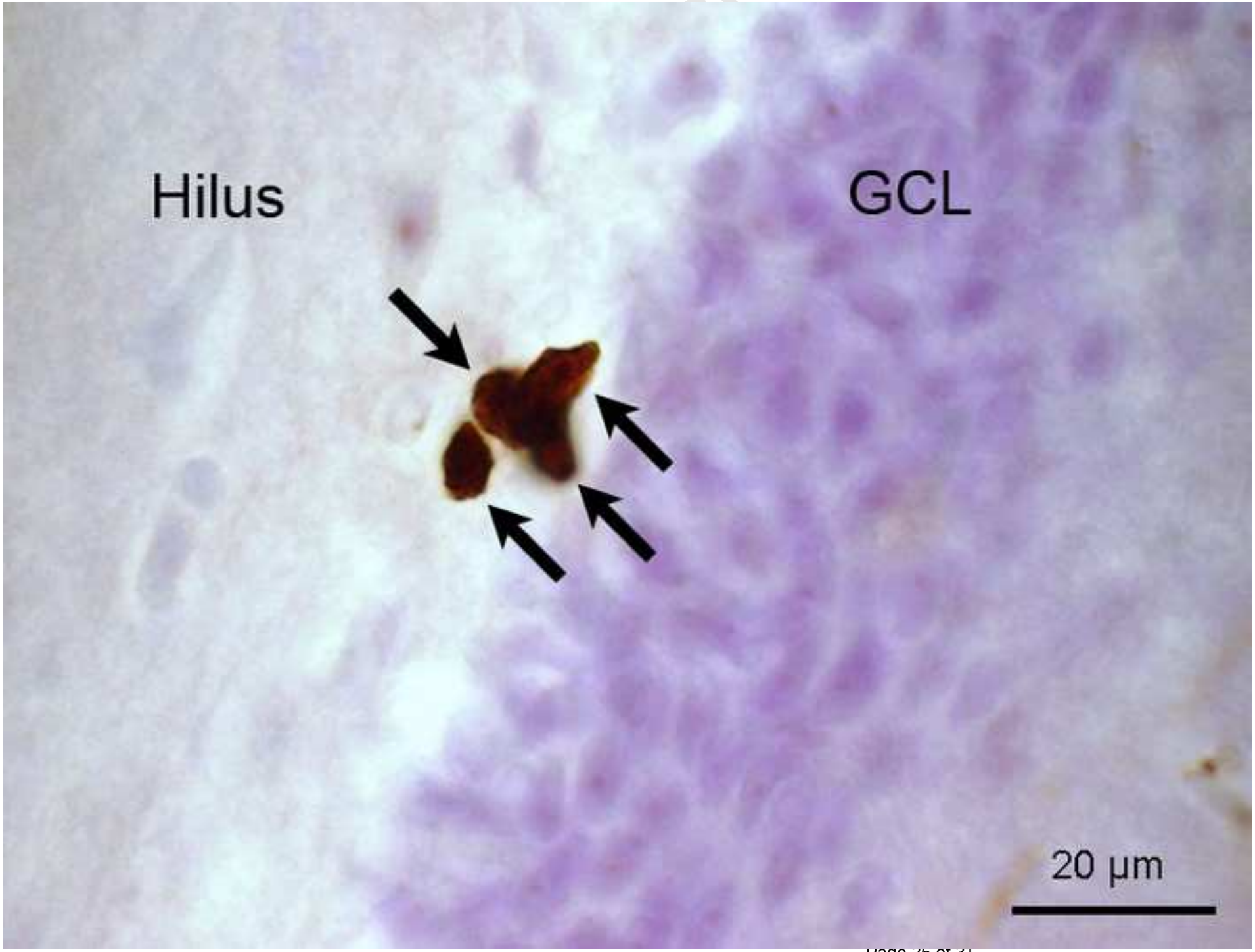
Figure Legends

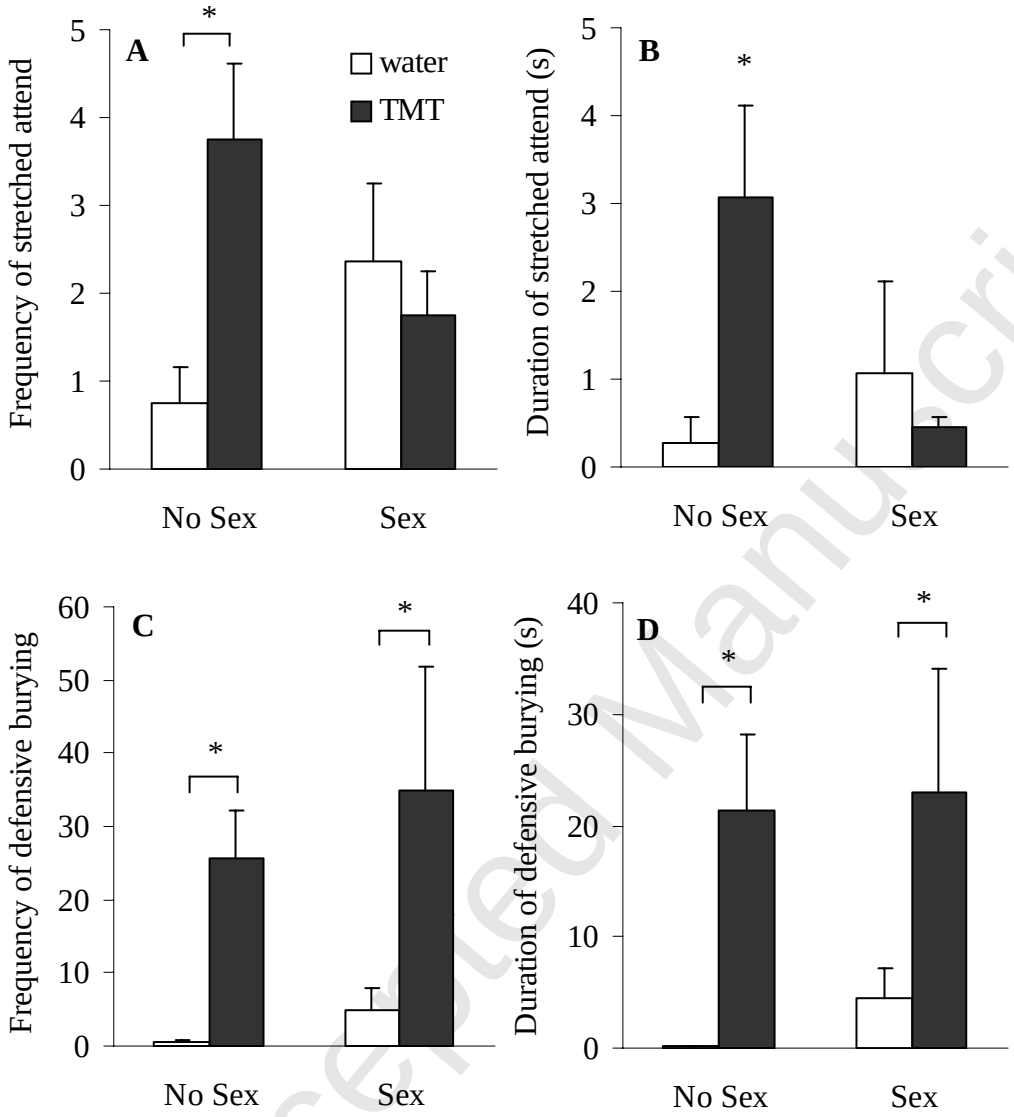
Fig. 1. Photomicrograph (× 1000 magnification) of clusters of BrdU-labeled cells along the edge of the granule cell layer (GCL) of the dentate gyrus of an adult male rat. Representative BrdU-labeled cells are indicated by arrows. The scale bar represents 10 μm.

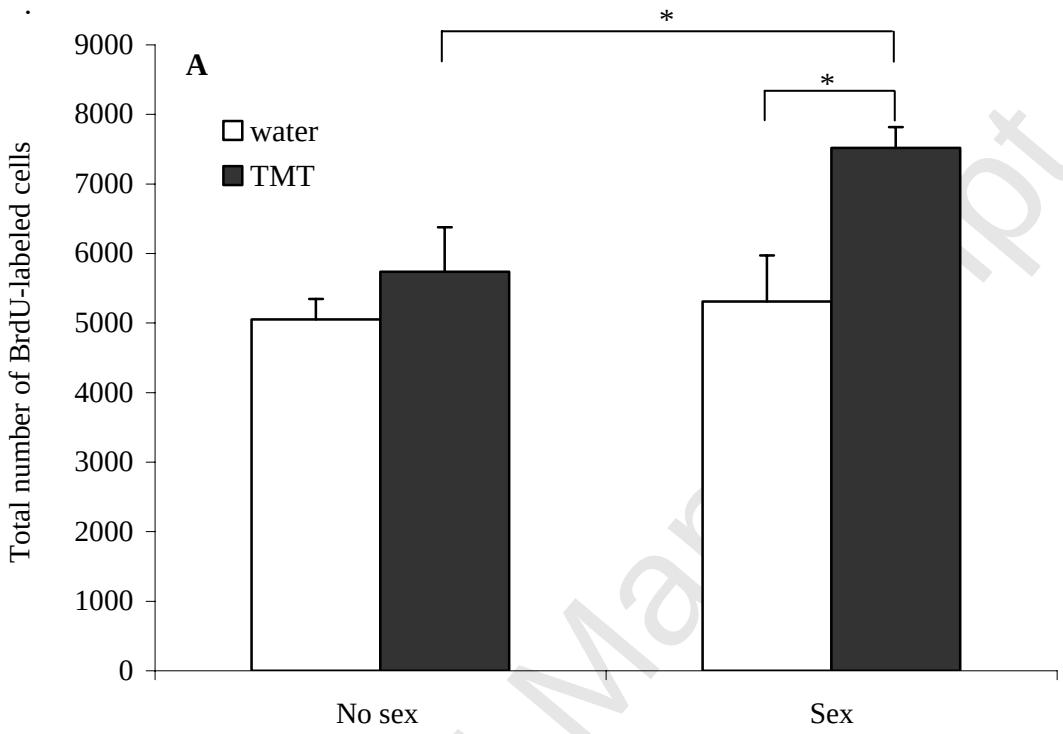
Fig. 2. Mean (±S.E.M.) frequencies and durations of defensive behaviors by rats during first 15 min of exposure to fox odor (TMT) or a control odor (water). Prior to exposure to odors, rats received either 5 consecutive days of sexual interactions or 5 days without sexual interactions (N=8 per group). TMT exposure caused a significant increase in stretched attend behavior only among males that had not experienced prior sexual interactions (A, B). TMT exposure caused a significant increase in defensive burying, but prior sexual interactions had no significant effect on defensive burying (C,D). Asterisks indicate significant differences using post-hoc tests (p<0.05)

Fig. 3. Total number of BrdU-labelled cells (mean ±S.E.M.) in the GCL+SGZ (A) and hilus (B) of the dentate gyrus among males that were exposed to 60 min of predator odor (TMT) or control odor (water) and had experienced either 5 days with or without sexual interactions prior to exposure to odors. Planned comparisons revealed that males in the sex/TMT group had significantly more new cells than did males in the no-sex/TMT and sex/water groups. No significant differences were observed among the groups within the hilus. Asterisks indicate significant differences using planned comparisons (p<0.05).

Fig. 1







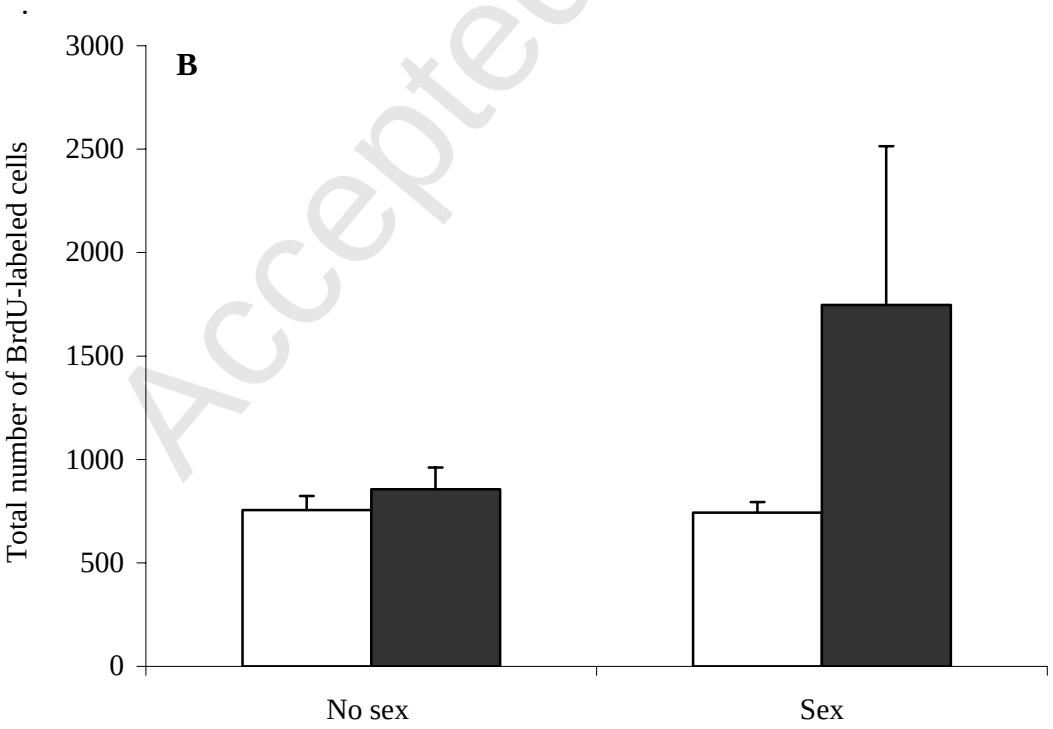


Table 1

Mean (±S.E.M.) duration and frequency of grooming, rearing, and vial contact during exposure to fox odor (TMT) or a control odor (water).

Treatment	No-sex/water	No-sex/TMT	Sex/water	Sex/TMT
Frequency of vial contact	10.3 ± 3.4	8.5 ± 1.6	12.4 ± 2.2	5.5 ± 2.2
Duration of vial contact (s)	23.9 ± 7.6[a]	17.2 ± 1.0[b]	58.1 ± 22.0[a]	11.1 ± 7.1[b]
Frequency of grooming	4.1 ± 1.8	5.0 ± 1.0	11.4 ± 3.0	5.1 ± 3.0
Duration of grooming (s)	35.4 ± 24.7	16.9 ± 3.6	70.0 ± 31.4	65.8 ± 30.5
Frequency of rearing	44.3 ± 5.8	44.3 ± 4.6	55.0 ± 7.7	34.5 ± 5.3
Duration of rearing (s)	185.3 ± 36.6	154.1 ± 26.9	190.4 ± 42.6	107.3 ± 20.5

[a, b]Significant differences between groups ($p<0.05$).

Table 2

Mean (±S.E.M.) volume (mm^3) of the granule cell layer (GCL) and hilus for the four treatment groups.

Treatment	No-sex/water	No-sex/TMT	Sex/water	Sex/TMT
GCL	2.06 ± 0.09	2.15 ± 0.11	2.16 ± 0.04	2.28 ± 0.23
hilus	4.74 ± 0.25	4.82 ± 0.23	4.94 ± 0.24	5.20 ± 0.31

Table 3

Mean (±S.E.M.) serum testosterone levels 24 h after behavioral testing for rats from the four treatment groups. No significant differences were observed among the groups.

Treatment	No-sex/water	No-sex/TMT	Sex/water	Sex/TMT
Testosterone (ng/ml)	2.25 ± 0.29	1.85 ± 0.35	2.12 ± 0.39	2.87 ± 0.47