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Date **May 08/02**
Abstract

Possible sex differences in response to predator odour exposure were examined in adult rats. The first study measured behaviour, cell proliferation, and survival in the dentate gyrus of adult males and females in response to acute and repeated trimethyl thiazoline (TMT, the main component of fox feces) or control odour exposure. Rats were injected with one of the cell synthesis markers bromodeoxyuridine (BrdU) or $[^{3}H]$ thymidine and perfused 24 hours (cell proliferation) after an acute TMT exposure or after 5 days (cell survival) of repeated TMT exposure. After acute exposure, cell proliferation and death were suppressed in TMT-exposed males compared to control males, but not in females. Repeated TMT exposure in males increased cell death and increased the survival of new cells born on Day 1 of exposure. Males initially expressed more defensive behaviours in response to TMT but this expression habituated after repeated TMT exposure. This habituation was concurrent with enhanced new cell survival, possibly indicating that learning enhanced new cell survival. In a second study, we assessed whether ovarian hormones altered the response to acute TMT exposure in females. Ovariectomized (OVX) females were given either a high dose of estradiol (EB) or a low dose of estradiol and progesterone followed by a high dose of estradiol (EB-P). TMT exposure did not affect cell proliferation in any group. However, hormone treatment affected the behavioural, hormonal, and cell death response to TMT. EB increased cell proliferation and decreased defensive behaviour whereas EB-P decreased cell proliferation and increased defensive behaviour. Thus, we demonstrated that pre-treatment with a low dose of estradiol and progesterone profoundly affected the behavioural and cellular response to later administration of estradiol. Taken together, acute TMT exposure suppressed both cell proliferation and death while repeated TMT exposure enhanced new cell survival and cell death in males. However, female rats did not show a change in cell proliferation, regardless of hormone condition, but OVX female rats exhibited increased cell death in response to acute TMT exposure. This is the first demonstration of a sex difference in cell proliferation and cell death in the adult dentate gyrus in response to stress.
# TABLE OF CONTENTS

Abstract ................................................................. ii.

Table of Contents ...................................................... iii.

List of Tables .......................................................... v.

List of Figures .......................................................... vi.

Preface and Acknowledgements ....................................... vii.

## CHAPTER 1 Introduction ............................................. 1.

1.1 Background ......................................................... 1.

1.2 Measuring Cell Proliferation, Cell Survival, and Cell Death ..... 3.

1.3 Measuring Behaviour ............................................... 4.

## CHAPTER 2 Sex Differences in Behaviour, Cell Proliferation, Cell Survival and Cell Death in the Dentate Gyrus due to Acute and Repeated Predator Odour Exposure in Adult Rats ......................................................... 6.

2.1 Materials and Methods ............................................. 9.

2.2 Results ............................................................... 16.

2.3 Discussion ........................................................... 27.

2.4 Functional Implications ........................................... 34.

2.5 Conclusions ........................................................ 34.

## CHAPTER 3 Estrogen modulates Hormonal, Behavioural and Cell Death but not the Cell Proliferation Response to Acute Predator Odour Exposure in Adult Females ......................................................... 36.

3.1 Materials and Methods ............................................. 39.

3.2 Results ............................................................... 45.

3.3 Discussion ........................................................... 61.

3.4 Implications ........................................................ 69.
CHAPTER 4 General Conclusions and Recommendations for Further Work

Bibliography
List of Tables

Table 1. Group mean (±SEM) percentage of BrdU-immunoreactive cells coexpressing a mature neuronal marker (NeuN) or a glial marker (GFAP). Page 18.

Table 2. Mean (±SEM) frequency or duration of nondefensive behaviours on Day 1 and Day 5. Page 26.

Table 3. Mean (±SEM) % cells immunoreactive for BrdU and NSE, BrdU and GFAP, or BrdU only in estradiol-treated females. Page 48.

Table 4. Mean (±SEM) plasma hormone levels in EB and VEH-treated females. Page 49.

Table 5. Mean (±SEM) frequency and duration of nondefensive behaviours in EB and VEH-treated females. Page 52.

Table 6. Mean (±SEM) plasma hormone levels in EP and VEH-treated females. Page 56.

Table 7. Mean (±SEM) frequency and duration of nondefensive behaviours in EP and VEH-treated females. Page 60.
List of Figures

Figure 1. Photomicrographs of representative images of A) a BrdU-labeled cell, B) a heavily-labeled and a lightly-labeled $^3$H-thymidine-labeled cell, C) a pyknotic cell, and D, E, F, and G are, respectively, images of a new neuron (BrdU and NeuN colabelled), a new glial cell (BrdU and GFAP colabelled), a new cell (BrdU only), and a new mature neuron (labelled with NSE and BrdU). Page 17.

Figure 2. A) Group means of the density of BrdU-labeled cells in the granule cell layer across sex and condition. B) Group means of the density of pyknotic cells in the granule cell layer on Day 1 across sex and condition. Page 19.

Figure 3. A) Group mean density of $^3$H-Thymidine labeled cells in the granule cell layer across sex and condition. B) Group mean density of pyknotic cells on Day 6 in the granule cell layer across sex and condition. Page 21.

Figure 4. Group means of the percentage of first, second, third, and fourth generation cells in males due to condition. Page 22.

Figure 5. Group means for A) frequency and B) duration of defensive burying and C) frequency and D) duration of stretched approach due to sex and condition across Day 1 and 5. Page 24.

Figure 6. A) Group means of the density of BrdU-labeled cells in the granule cell layer in estradiol (EB) or vehicle (VEH) treated females exposed to control odour or TMT. B) Group means of the density of pyknotic cells in the granule cell layer in EB and VEH treated females exposed to control odour or TMT. Page 47.

Figure 7. Group means for A) frequency and B) duration of defensive burying and C) frequency and D) duration of stretched approach due to EB or VEH treatment in females exposed to control odour or TMT. Page 50.

Figure 8. Group means for A) frequency and B) duration of freezing and C) the frequency of wet-dog shakes in EB or VEH treated females exposed to control odour or TMT. Page 51.

Figure 9. A) Group means of the density of BrdU-labeled cells in estrogen and progesterone (EB-P) or vehicle (VEH) treated females exposed to control odour or TMT. B) Group means of the density of pyknotic cells in the granule cell layer of EB-P and VEH treated females. Page 55.

Figure 10. Group means for A) frequency and B) duration of defensive burying and C) frequency and D) duration of stretched approach in EB-P and VEH-treated females exposed to control odour or TMT. Page 58.

Figure 11. Group means for A) frequency and B) duration of freezing and C) frequency of wet dog shakes in EB-P and VEH treated females exposed to control odour or TMT. Page 59.
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CHAPTER 1: Introduction

1.1 Background

Although it was once thought that new neurons were produced during a discrete period of development, it is now known that new neurons continue to be produced in the adult mammalian hippocampus (for review see Kempermann & Gage, 1999; Fuchs & Gould, 2000; Ormerod & Galea, 2000). New cells are produced from dividing progenitors in the subgranular zone of the dentate gyrus. The daughter cells migrate into the granule cell layer, extend axons into the CA3 region (Hastings & Gould, 1999; Markakis & Gage, 1999; Stanfield & Trice, 1988), and 2-3 weeks after birth express markers for mature neuronal proteins (see Kempermann & Gage, 1999). Many factors affect the birth of new cells in the hippocampus. Particularly relevant to this thesis: stress has been shown to suppress cell proliferation in males (Galea, Tanapat, & Gould, 1996; Gould, McEwen, Tanapat, Galea, & Fuchs, 1997; Gould, Tanapat, McEwen, Flugge, & Fuchs, 1998; Tanapat, Hastings, Rydel, Galea, & Gould, 2001), estrogen has been shown to increase cell proliferation (Ormerod & Galea, 2001; Tanapat, Hastings, Reeves, & Gould, 1999), and there are sex differences in cell proliferation depending on the stage of the estrous cycle in females (Tanapat et al., 1999).

Acute stress suppresses cell proliferation in the dentate gyrus of both developing (Tanapat et al., 1998) and adult male mammals (Galea et al., 1996; Gould, et al., 1997, 1998; Tanapat et al, 2001). This suppression of cell birth has been shown to be dependent on the presence of adrenal steroids in male rats (Tanapat et al., 2001). The acute stress-induced suppression in the number of new cells is transient, persisting for at least one week but disappearing by 3 weeks after exposure to the stressor (Tanapat et al., 2001).

There are sex differences in both stress responses and cell proliferation (Tanapat et al., 1999). Cell proliferation is increased transiently in female rats during a phase of the estrous
cycle in which estrogen is high (proestrus). Females in proestrus have more new cells than males or females in other phases of the cycle. However, there is not a sex difference in cell proliferation when comparing males and females in low estrogen phases of the estrous cycle. Estrogen treatment has also been shown to increase cell proliferation in ovariectomized females (Ormerod & Galea, 2001; Tanapat et al., 1999), suggesting that estrogen may play a role in the increase in cell proliferation during proestrus in intact female rats. Female rats show higher basal and stress responses of corticosterone (main glucocorticoid released due to stress in rats) than males (Critchlow, Liebelt, Bar-Sela, Mountcastle, & Lipscomb, 1963; Le Mevel, Abitbol, Beraud, & Maniey, 1979) and, depending on when in their estrous cycle they are tested, females differ from males in their behavioural stress responses (Kennett, Chaouloff, Marcou, & Curzon, 1986). No studies to date have investigated whether there is a sex difference or an effect of estrogen on the cell proliferation response to stress despite these observations that there are sex differences and effects of estrogen on the hormonal and behavioural responsivity to stress. Thus this thesis was designed to investigate possible sex differences (Chapter 2) and the effect of estrogen (Chapter 3) on cell proliferation in response to stress.

With chronic stress or high levels of cortisol (main glucocorticoid released due to stress in primates) there is a reduction in the volume of the human hippocampus (Sheline, Wang, Gado, Csernansky, & Vannier, 1996; Starkman, Gobarski, Berent, & Schteingart, 1992; Starkman, Giordani, Gobarski, Berent, Schork, & Schteingart, 1999). It is possible that this volume reduction is due to a suppression of neurogenesis and/or an increase in cell death. Therefore, this thesis examined the effects of predator odour stress on neurogenesis (cell proliferation and cell survival) and cell death in the dentate gyrus of the hippocampus.

It is possible that concurrent changes in behaviour in response to stress may be related to changes in cell proliferation and/or cell death in the hippocampus. The hippocampus is necessary for the expression of freezing behaviour (Blanchard & Blanchard, 1972; Takahashi,
1995, 1996; Takahashi & Goh, 1996; Takahashi & Kim, 1995), a behaviour which is increased following exposure to a stressor (Morrow, Redmond, Roth, & Elsworth, 2000; Morrow, Roth, & Elsworth, 2000; Wallace & Rosen, 2000). The hippocampus may also play a role in other behaviours that are elicited or suppressed due to stress. Therefore, the studies outlined in this thesis examine the relationship between cell death, cell proliferation, and behaviours expressed as a result of exposure to either acute or repeated stress.

1.2 Measuring Cell proliferation, Cell survival, and Cell death

New cells can be labelled with markers of DNA synthesis such as bromodeoxyuridine (BrdU) or $^3$H-thymidine. Both $^3$H-thymidine and BrdU are markers of DNA synthesis and label cells in the synthesis phase of mitosis. These markers are active for approximately two hours but, once incorporated into DNA, can be visualized for many years (Cameron & McKay, 2001). New cells can then be visualized by immunohistochemistry (for BrdU) or autoradiography (for $^3$H-thymidine). Previous studies have shown that BrdU and $^3$H-thymidine have equivalent labeling efficacies (Cameron & Gould, 1994; Miller & Nowakowski, 1988; Rietze, Poulin, & Weiss, 2000; Tanapat et al., 2001).

Progenitor cells divide asymmetrically and can give rise to a cell that differentiates into either a new glia or neuron and another progenitor cell that can divide further, giving rise to further generations of labelled cells (Nowakowski & Hayes, 2001). Therefore, there are more labeled cells 1 week after labeling than after 1 day reflecting progenitor cells and their progeny (Cameron Woolley, McEwen, & Gould, 1993). $^3$H-thymidine can be used as a label for investigating the number of times that a labelled cell divides. The dilution of the $^3$H-thymidine label is used to measure the number of first and subsequent generations of labelled cells (Angevine, 1965; Caviness, 1982; Luskin & Shatz, 1985a; Luskin & Shatz, 1985b; Miller, 1985; Polleux, Dehay, & Kennedy, 1997a, 1998; Polleux, Dehay, Moraillon, & Kennedy, 1997b;
Rakic, 1973). Therefore, \(^3\text{H}\)-thymidine is an appropriate label for measuring new cell survival. Cell death can be measured by examining the number of cells with pyknotic morphology. Pyknotic cells lack a nuclear membrane, and have a pale or absent cytoplasm and darkly stained spherical chromatin (Gould et al, 1991).

Measuring cell proliferation can be distinguished from measuring cell survival and depends on the amount of time that has elapsed between label injection and tissue perfusion (Ormerod & Galea, 2001b). Cell synthesis markers such as bromodeoxyuridine (BrdU) or \(^3\text{H}\)-thymidine actively label cells entering S-phase for two hours (Cameron & McKay, 2001). Newly labelled cells then complete one full mitotic division within approximately 24 hours (Cameron & McKay, 2001). Thus, factors that affect cell proliferation affect the amount of cells entering S-phase or the amount of cells that complete mitosis during this (approximately) 24 hour time period. Cell survival is measured by examining the number of these newly labelled cells that are still present at longer survival times (i.e. 1 week). Also, certain factors may affect the survival of newly labelled cells while others may affect the proportion of older cells in the granule cell layer that are dying (pyknotic cells).

New cells can differentiate into neurons or glia (but see: Seri, Garcia-Verdugo, McEwen, & Alvarez-Buylla, 2001). We can measure the phenotypes of newly-labeled cells by injecting BrdU, perfusing tissue 3 weeks later, and processing the tissue for immunohistochemistry using fluorescent probes conjugated with antibodies for BrdU and specific neuronal or glial proteins.

### 1.3 Measuring behaviour

During exposure to a stressful stimulus or environment, rats engage in very specific behaviours such as freezing, defensive burying, stretched approach, and avoidance of the aversive stimulus or context (Blanchard & Blanchard, 1969; Pinel & Treit, 1978). These behaviours do not require prior learning, and are expressed during the first exposure to the
stressful context or stimulus. These behaviours are considered to be defensive and are used to model animal ‘anxiety’ (Blanchard, Griebel, Rodgers, & Blanchard, 1998; Blanchard, Shepherd, De Padua Carobrez, & Blanchard, 1991; File, 1995). Defensive burying involves the rat using its forepaws to fling bedding towards a test object (Pinel & Treit, 1978), and may function to mask the aversive stimulus or scare off predators (see Pinel & Treit, 1978). Stretched approach is considered to serve an investigative function in assessing the threat of the aversive stimulus or context (Blanchard & Blanchard, 1969). Stretched approach is characterized by the rat extending its body towards the test object while its hind paws remain stationary (Pinel, Symons, Christensen, & Tees, 1989). Freezing is the term used to describe a complete cessation of movement (Fanselow & Bolles, 1979). Exposure to stress has also been shown to suppress certain other behaviours. These behaviours have been termed non-defensive (rearing, grooming, and direct contact with the stressful stimulus or context) (Blanchard et al., 1990; Perrot-Sinal et al., 2000; Shepherd et al., 1992; Wallace & Rosen, 2000, 2001). The amount that these defensive and non-defensive behaviours are expressed depends on the type of stressor and the environment in which the stressor is presented (Blanchard et al., 1998).

In the first study presented in this thesis (Chapter 2), we examined the effects of an acute and repeated exposure to a predator odour stressor on behaviour, cell proliferation, cell survival and cell death in both adult males and females. In the second study presented (Chapter 3), we investigated the effects of ovarian hormones on the adult female response to acute predator odour stress.
Exposure to stress can have profound effects on the morphology of the hippocampus. In humans, chronically elevated levels of the stress hormone cortisol are associated with smaller hippocampus volume in patients with both depression and post-traumatic stress disorder relative to controls (Sheline, Wang, Gado, Csernansky, & Vannier, 1996; Starkman, Gebarski, Berent, & Schteingart, 1992; Starkman et al., 1999). In elderly patients, higher fluctuations in cortisol are associated with both a reduction in cognition and hippocampus volume (Lupien et al., 1998; Lupien et al., 1999). In rodents, 21 days of restraint stress leads to dendritic atrophy in the apical dendrites of CA3 pyramidal neurons (Watanabe, Gould, & McEwen, 1992; Magarinos & McEwen, 1995) while in primates, 2-3 months of prolonged psychosocial stress leads to cell loss in the CA3 and CA1 regions of the hippocampus (Uno et al., 1994; Sapolsky, Uno, Rebert, & Finch, 1990; Uno, Tarara, Else, Suleman, & Sapolsky, 1989).

The dentate gyrus of the hippocampus is one of the few brain areas that has been confirmed to produce new neurons in adulthood (Altman and Das, 1965; Cameron, Woolley, McEwen, & Gould, 1993; Eriksson et al., 1998; Kornack & Rakic, 1999; Kuhn, Dickinson-Anson, & Gage, 1996; Seki & Arai, 1993). Acute stress has been shown to suppress cell proliferation in the dentate gyrus of adult male rodents and marmoset monkeys (Galea, Tanapat, & Gould, 1996; Gould, McEwen, Tanapat, Galea, & Fuchs, 1997; Gould, Tanapat, McEwen, Flugge, & Fuchs, 1998; Tanapat, Hastings, Rydel, Galea, & Gould, 2001) and a recent study has shown that cell proliferation is also reduced in the dentate gyrus after repeated psychosocial stress in adult tree shrews (Czeh et al., 2001). However, no work to date has studied the effects of repeated stress on the survival of new cells in the dentate gyrus.
No work has studied potential sex differences in cell proliferation and new cell survival in the dentate gyrus following acute and repeated stress despite evidence for sex differences in both cell proliferation (Galea & McEwen, 1999; Tanapat, Hastings, Reeves, & Gould, 1999) and behavioural and neural responses to stress (Blanchard, Shepherd, De Padua Carobrez, & Blanchard, 1991; Blanchard, Agullana, McGee, Weiss, & Blanchard, 1992; Blanchard, Blanchard, Rodgers, & Weiss, 1990; Galea & McEwen, 1999; Galea et al., 1997; Haleem, Kennett, & Curzon, 1988; Kennett, Chaouloff, Marcou, & Curzon, 1986; Klein, Lambert, Durr, Schaefer, & Waring, 1994; Perrot-Sinal, Ossenkopp, & Kavaliers, 2000; Shepherd, Flores, Rodgers, Blanchard, & Blanchard, 1992). Although females are more defensive than males when exposed to predator stress (D.C. Blanchard et al., 1991; R.J. Blanchard et al., 1990, 1992; Kennett et al., 1986; Klein et al., 1994; Shepherd et al., 1992) they are less susceptible to stress-induced hippocampal cell loss (Mizoguchi, Kunishita, Chui, & Tabira, 1992). Females also show a different profile of dendritic atrophy than males in response to stress, exhibiting atrophy of basal dendrites while males show atrophy of apical dendrites in the CA3 region (Galea et al., 1997).

In adult male rats, exposure to predator odour is considered a stressor as predator odour elicits hormonal (Holmes & Galea, 2002; Morrow, Redmond, Roth, & Elsworth, 2000a; Perrot-Sinal, Ossenkopp, & Kavaliers, 1999; Tanapat et al., 2001) and behavioural (D.C. Blanchard et al., 1991; R.J. Blanchard et al., 1990; File, Zangrossi, Sanders, & Mabbutt, 1993; Klein et al., 1994; Vernet-Maury, Polak, & Demael, 1984; Wallace & Rosen, 2000, 2001) stress responses. In particular trimethyl thiazoline (TMT or fox odour), the major component of fox feces (the fox is a natural predator of the rat (Funk & Amir, 2000), has been used as a predator odour. TMT has been shown to elicit freezing behaviour, reduce exploration (Vernet-Maury et al., 1984; Wallace & Rosen, 2000, 2001), and increase corticosterone levels (Holmes & Galea, 2002; Morrow et al., 2000a; Tanapat et al., 2001; Vernet-Maury et al., 1984).
Predator odour exposure also affects the morphology and electrophysiological response of the hippocampus. Exposure to TMT rapidly suppresses the proliferation of progenitor cells in the dentate gyrus of both adult (Galea et al., 1996; Holmes & Galea, 2002; Tanapat et al., 2001) and developing male rats (Tanapat, Galea, & Gould, 1998). The acute stress-induced suppression in the number of new cells persists for at least one week, but disappears 3 weeks after exposure (Tanapat et al., 2001). Also, exposure to TMT induces an electrophysiological fast-wave burst (approximately 20 Hz) in the dentate gyrus and olfactory bulb (Heale & Vanderwolf, 1994, 1999; Heale, Vanderwolf, & Kavaliers, 1994), which appears to be dependent on the entorhinal cortex and cholinergic interneurons in the dentate gyrus (Heale & Vanderwolf, 1999).

It is possible that the rapid dentate gyrus response to TMT may be related to changes in hippocampus-mediated behaviour. The hippocampus mediates the expression of certain defensive behaviour (Blanchard & Blanchard, 1972; Blanchard, Blanchard, & Fial, 1970; Takahashi, 1995, 1996; Takahashi & Goh, 1996), and has been implicated in contextual fear conditioning (Anagnostaras, Maren, & Fanselow, 1999; Antoniadis & McDonald, 2000; Bannerman et al., 2001; Chen, Kim, Thompson, & Tonegawa, 1996; Gisquet-Verrier, Dutrieux, Richer, & Doyere, 1999) and olfactory learning (Eichenbaum, Fagan, Mathews, & Cohen, 1988; Miller, Nonneman, Kelly, Neisewander, & Isaac, 1986; Staubli, Ivy, & Lynch, 1984; Staubli, Le, & Lynch, 1995). In the present study we examined the expression of defensive behaviours during a single exposure and after repeated fox odour exposures in order to determine whether there was a correlation between neurogenesis and defensive behaviour. Repeated exposure to fox odour gives the rats an opportunity to learn that the context in which the fox odour is administered is not associated with actual threat. This constitutes both contextual and olfactory learning that may involve the hippocampus. Engaging in hippocampus-dependent learning
increases new cell survival (Gould, Beylin, Tanapat, Reeves, & Shors, 1999). Thus, we investigated whether the survival of new cells would be affected by repeated TMT exposure.

It is important to distinguish between cell proliferation and cell survival when studying factors that affect neurogenesis (Ormerod & Galea, 2001b). Cell synthesis markers such as bromodeoxyuridine (BrdU) or \(^3\)H-thymidine actively label cells entering S-phase for two hours (Cameron & McKay, 2001). Newly labelled cells then complete one full mitotic division within approximately 24 hours (Cameron & McKay, 2001). Thus, factors that affect cell proliferation affect the number of cells entering S-phase or the number of cells that complete mitosis during this (approximately) 24 hour time period. We can investigate new cell survival by measuring the number of these newly labelled cells that are still present at longer survival times (i.e. 1 week). Certain factors may affect the survival of newly labelled cells while others may affect the proportion of older cells in the granule cell layer that are dying. Cell death can be measured by examining the number of cells with pyknotic morphology (Gould, Woolley, & McEwen, 1991).

In the present study we investigated the effects of acute and repeated TMT exposure on cell proliferation, new cell survival, cell death and behaviour in both male and female rats. We were also interested in whether changes in behaviour were related to changes in neurogenesis in response to predator odour stress.

2.1 Materials and Methods

Subjects

Subjects were male and female Sprague-Dawley rats (approx. 250-300 grams). Rats were obtained from the UBC Animal Care Center, housed singly in wire-mesh cages on a 12:12 hour light-dark cycle (lights on at 7:30 am). Housing temperature was maintained at 21±1°C. Animals were given free access to food (PMI Nutrition- Rat Diet) and tap water. All animal research was conducted in accordance with the guidelines of the Canadian Council on Animal
Care and the policies of the University of British Columbia. Every effort was made to minimize the number of animals used per group.

**Apparatus**

The test chambers consisted of 29 cm X 30 cm X 46 cm plexiglass boxes, lined with 5 cm of corn cob bedding. Each transparent test chamber was placed in a fume hood with 2-3 other test chambers, and the chambers were then visually isolated with opaque paper. A plastic vial filled with two Kimwipes was placed in the same corner of each testing chamber.

**Design and Procedures**

**Experiment 1.** To investigate the effects of a single exposure to fox odour on cell proliferation and cell death in male and female rats, rats were exposed to TMT (the major component of fox feces, Phero Tech Inc., Delta BC, CANADA) or a control odour (distilled water) for one hour. Rats were initially placed into the test chambers for 20 minutes a day for 5 days, in order to habituate them to the testing apparatus, fume hood, and empty odour vials. Testing began 24 hours after habituation day 5. TMT-exposed rats (n=10, 5 males and 5 females) were placed in test bins with a vial that contained a Kimwipe permeated with 150 ml of TMT. Control rats (n=10, 5 males and 5 females) were exposed to a vial containing a Kimwipe permeated with 150 μl of distilled water.

Fifteen minutes after the initial presentation of the odour, rats were given an intraperitoneal (i.p.) bromodeoxyuridine (BrdU) injection (100 mg/kg). Twenty-four hours after receiving BrdU, rats were given an overdose of sodium pentobarbital (2 ml/kg Somnitol, MTC Pharmaceuticals), perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer, and the brains extracted. Brains were processed immunohistochemically for BrdU.

These studies were designed to investigate whether or not there is a sex difference in response to TMT, therefore freely cycling females were used. Based on previously published data (Galea, Ormerod, Sampath, Kostaras, Wilkie, & Phelps, 2000), approximately 80-90% of
the females were predicted to be in diestrous. We performed statistical analysis (see results) to confirm that there was not significant variability in the females.

A separate group of male rats (n=6, 3 per group) underwent the same procedure as above (1 hour of odour exposure with BrdU injected 15 minutes into the odour exposure) but were perfused 3 weeks later. These brains were separately processed for combined immunofluorescence for BrdU and neuronal nuclei ((NeuN) a marker for mature neurons (Kempermann & Gage, 1999; Tanapat et al., 1999), and combined immunofluorescence for BrdU and glial fibrillary acidic protein (GFAP; an astroglial marker (Cameron et al., 1993)).

Experiment 2. To investigate whether repeated TMT exposure affects the survival of new cells, rats were injected with \(^3\)H-thymidine on the first day of TMT exposure and repeatedly exposed to TMT for five consecutive days (1 hr/day; 5 days was chosen to match the duration of exposure used by File et al. (1993)). Twenty-four hours after the fifth day of exposure rats were perfused.

Both \(^3\)H-thymidine and BrdU are markers of DNA synthesis, labeling cells in the synthesis phase of mitosis. Both markers are active for approximately two hours (Cameron & McKay, 2001). Previous studies have shown that BrdU and \(^3\)H-thymidine have equivalent labeling efficacies (Cameron & Gould, 1994; Miller & Nowakowski, 1988; Rietze, Poulin, & Weiss, 2000; Tanapat et al., 2001). We chose to use \(^3\)H-thymidine as a label for investigating cell survival as \(^3\)H-thymidine can account for the potential dilution of label after repeated cell divisions and thus makes it possible to estimate the timing of the final mitotic division (Angevine, 1965; Caviness, 1982; Luskin & Shatz, 1985a; Luskin & Shatz, 1985b; Miller, 1985; Polleux, Dehay, & Kennedy, 1997a, 1998; Polleux, Dehay, Moraillon, & Kennedy, 1997b; Rakic, 1973). After a pulse injection of \(^3\)H-thymidine, the intensity of signal can be used to approximate the population of labeled cells that are first generation (those cells that have quit the cell cycle after the first mitotic division) and those cells that are a result of subsequent divisions.
of the first generation cell. Measuring heavily and lightly labeled cells has been shown to effectively distinguish first generation cells from those of subsequent generations (Polleux et al., 1997b).

As in Experiment 1, rats (n=32, 8 males and 8 females in each of either TMT or control odour conditions) were habituated to the test chambers and then tested with a vial containing TMT or control odour (distilled water; approximately 150 µl of each). Rats were placed in the test bin 1 hour each day for 5 days. All rats were videotaped and scored for behaviour occurring during the first 15 minutes of TMT exposure on Day 1 and on Day 5. A subset of rats (n=5 per group) was given a single i.p. injection of $^3$H- thymidine (5.0 mCi/g body weight, New England Nuclear) fifteen minutes after testing began on Day 1. Twenty-four hours after Day 5 rats were given an overdose of sodium pentobarbitol (2 ml/kg Somnitol, MTC Pharmaceuticals), perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer, and their brains extracted. Brains were processed autoradiographically for $^3$H-thymidine.

**Behavioural Measures**

Using our TMT exposure paradigm, we preliminarily observed that TMT did not affect freezing behaviour in males (unpublished observations). Therefore in the present study we only measured the defensive behaviours stretched approach and defensive burying. These defensive behaviours are considered reliable measures of animal ‘anxiety’ (File, 1995) and have been shown to increase following exposure to aversive stimuli (Pinel & Treit, 1978; Blanchard, Blanchard, Rodgers, & Weiss, 1990). Defensive burying involves the rat using its forepaws to fling bedding towards a test object (Pinel & Treit, 1978), and in the present study, the test object is the vial containing TMT. Stretched approach is characterized by the rat extending its body towards the test object while its hind paws remain stationary (Pinel, Symons, Christensen, & Tees, 1989). Both the frequency and duration of 5 behaviours were collected: stretched approach, defensive burying, rearing, grooming and direct contact with the odour-containing
vial. These behaviours were chosen because we expected that exposure to TMT would increase the expression of defensive behaviours (stretch approach and defensive burying, respectively) and inhibit the expression of nondefensive behaviours (rearing, grooming, and direct contact with the source of the odour) (Blanchard et al., 1990; Perrot-Sinal et al., 2000; Shepherd et al., 1992; Wallace & Rosen, 2000, 2001).

**Histological Procedures**

All brains were stored overnight at 4°C in perfusate. The following day, brains were sectioned into 40 µm slices on an oscillating tissue slicer (OTS 3000, Electron Microscopy Sciences) at the level of the dentate gyrus (see below). In Experiment 1 and 2, the data from one male control was lost due to tissue damage.

**BrdU Labeling**

For peroxidase immunolabeling, sections were mounted on 2% 3-aminopropylsilane-treated slides, rinsed in phosphate buffer saline (PBS), incubated in H2O2, made permeable with Trypsin, and denatured with 2N HCl. The sections were then incubated with normal horse serum and anti-BrdU mouse monoclonal antibody (Boehringer Mannheim, 1:100 dilution) overnight at room temperature (20°C). Sections were rinsed in PBS and incubated in Biotinylated antibody anti-mouse IgG (1:29, Vector, Elite kit), rinsed in PBS, reacted using an ABC reagent (Vector, Elite kit) with 0.1 % diaminobenzidine (DAB), counterstained for Nissl substance with cresyl violet, and coverslipped with Permount.

**Fluorescence Immunohistochemistry**

Separate sets of slides were also double stained with immunofluorescent probes to assess either BrdU- and NeuN- or BrdU- and GFAP immunoreactivity. Slides were pretreated with 3% H2O2 in phosphate-buffered Saline (PBS) for 20 minutes, rinsed in tris-buffered saline (TBS), incubated for 2 h in deionised formamide solution (in 2XSSC) at 65°C, rinsed again, and DNA denatured in 2N HCl for 30 minutes. Sections were rinsed, incubated in 0.1 M borate buffer (10
min), blocked in 5.0% normal donkey serum (Jackson Immunoresearch) for 30 min, and then incubated for 60 hr in rat anti-BrdU (ascites 1:100; Serotec) and rabbit monoclonal anti-NeuN (1:2000; Polysciences), or rat anti-BrdU and mouse monoclonal anti-glial fibrillary acidic protein (GFAP 1:2000; Vector) at 4°C. Sections were then incubated in 5% normal donkey serum (Jackson Immunoresearch), rinsed, and incubated in a cocktail of donkey anti-rat fluorescein (FITC diluted 6µg/ml to visualize BrdU; Jackson Immunoresearch) or donkey anti-mouse Cy3 (diluted 6µg/ml to visualize GFAP or NeuN; Jackson Immunoresearch) for 4 hrs. Sections were rinsed and coverslipped with the anti-fading agent diazobicyclooctane (DABCO; 2.5% DABCO, 10% polyvinyl alcohol and 20% glycerol in TBS; Sigma).

\[^{3}H\]-Thymidine-labeling

Cells were considered labeled with \[^{3}H\]-thymidine when the number of silver grains on the cell body was greater than 20 times the background level and the cell exhibited granule cell morphology (see Fig 1B and Cameron et al., 1993; Ormerod & Galea, 2001a). For \[^{3}H\]-thymidine autoradiography, slides were dipped in autoradiographic emulsion (NTB2, Kodak) and stored at 4°C for 4 weeks. Then the slides were developed in Dektol (Kodak), fixed in Ektaflo (Kodak), counterstained with cresyl violet and coverslipped with Permount.

Slides were coded prior to the analysis in order to blind the experimenter to the treatment conditions. Six sections of the middle portion of the dentate gyrus (where the dentate gyrus is positioned horizontally beneath the corpus callosum and the suprapyramidal and infrapyramidal blades are joined at the crest; between A −3.3 and A −4.8 in rats). For each section, the total number of \[^{3}H\]-thymidine-labeled or BrdU-labeled cells were counted in the granule cell layer (including the subgranular zone) of the dentate gyrus under 1000x magnification on a Nikon Ellipse (E600) light microscope (see Figure 1A. and B.). Pyknotic cells were counted for all sections using the criterion set forth by Gould et al (1991). Briefly, pyknotic cells lacked a nuclear membrane, had pale or absent cytoplasm and darkly stained spherical chromatin (Figure
The cross-sectional area of the dentate gyrus was determined using AIS (Analytical Imaging System) software and the densities of new cells calculated (by dividing the number of new granule cells by total granule cell area). Density estimates are highly correlated with stereological estimates (Falconer & Galea, unpublished observations; Ormerod, Lee, & Galea, submitted).

As has been performed previously (Polleux et al., 1997b, 1998), we estimated the amount of first and subsequent generation \(^{3}\text{H}\)-thymidine-labeled cells in a subset of rats (we examined 4-6 sections per brain, N=12, 3 per group). To do this, we obtained grain counts for each labeled cell using a light microscope under 1000x magnification and AIS. We estimated the maximum number of silver grains per nucleus for each brain. Grain counts were corrected for nucleus diameter using the Appleton correction (Appleton, Pelc, & Tarbit, 1969) to account for different sizes of nuclei. The Appleton correction is defined as

\[
N_{\text{corr}} = \frac{N_{\text{obs}} 	imes D_{\text{obs}}}{D_{\text{Nmax}}},
\]

where \(N_{\text{corr}}\) is the corrected number of silver grains, \(N_{\text{obs}}\) is the observed number of silver grains, \(D_{\text{obs}}\) is the diameter of the observed nucleus, and \(D_{\text{Nmax}}\) is the diameter of the nucleus that has the maximum labeling intensity. We estimated that the number of silver grains per nucleus is halved at each subsequent division (based on Polleux et al., 1997b). Estimates of the numbers of first, second, third, and fourth generation \(^{3}\text{H}\) thymidine-labeled cells were obtained by counting heavily (indicated by labeling that is >50% of the maximum) and lightly (indicated by labeling that is <50%, <25%, and <12.5% of the maximum) labeled cells. We then expressed the number of first, second, third, and fourth generation cells each as a percentage of the total number of labeled cells.

\textit{BrdU-ir cell phenotyping}

In order to determine cell phenotype, tissue processed for combined immunofluorescence was examined using a confocal laser scanning microscope (BioRad 2000) on a 63X objective. BrdU-labelled cells from the middle portion of the dentate gyrus (four-six sections per brain)
were examined with Z-sections taken at 1 μm intervals. Optical stacks of 10 images were created with NIH Image for PC (http://www.scioncorp.com/pages/menu.htm) and imported into Adobe Photoshop for channel merging.

\textit{Data Analyses}

For Experiments 1 and 2, separate analyses of variance (ANOVAs) were calculated for the density of BrdU-ir cells and pyknotic cells, and the percentage of BrdU/NeuN-ir cells and BrdU/GFAP-ir cells with condition (TMT and control) and sex (male and female) as the between subjects factor. For Experiment 2, separate ANOVAs were calculated for the density of [\textsuperscript{3}H] thymidine-labeled cells and pyknotic cells with condition (TMT, control) and sex (male, female) as the between-subjects factor. A repeated measures ANOVA was also calculated for \textsuperscript{3}H thymidine-labeled cells in males with generation (first, second, third, and fourth) as the within-subjects factor and condition (TMT and control) as the between-subjects factor. For behavioural measures in Experiment 2, separate repeated-measures ANOVAs were used, with sex (male and female) and condition (TMT and control) as between-subjects factors and days (1 and 5) as the within-subjects factor. Unless otherwise indicated, Newman-Keuls was used for post-hoc testing (\(\alpha=0.05\)). While \(p\leq0.05\) will be referred to as statistically significant, it should be noted that these \(p\) values reflect the probability of the effect occurring solely by chance.

\textbf{2.2 Results}
Figure 1. Photomicrographs of representative images of A) a BrdU-labeled cell, B) a heavily-labeled (first generation) and a lightly-labeled $^3$H-thymidine-labeled cell, C) a pyknotic cell, and D, E, F, and G are, respectively, images of a new neuron (cell co-labeled with NeuN and BrdU), a new glial cell (co-labeled with GFAP and BrdU), a new cell that is not co-labeled with either GFAP or NeuN (BrdU labeling only), and a cell co-labeled with BrdU and NSE using confocal microscopy. GCL - granule cell layer, H - hilus. Scale bar represents 10 μm.
Experiment 1: Cell proliferation and cell death after a single odour exposure

Cell proliferation and cell death were suppressed in males but not females after a single TMT-exposure

Figure 2A shows the group mean density of BrdU-labeled cells in the dentate gyrus after a single odour exposure. TMT-exposed males (p≤0.04), but not TMT-exposed females, had fewer BrdU-labeled cells in the granule cell layer compared to their respective controls (interaction effect: F(1,15)=6.47, p≤0.02). We found no differences in the group variances (Bartlett χ²= 1.79, p≤0.62). At 3 weeks, there were no significant differences between groups in the percentage of BrdU-ir cells expressing NeuN (p≤0.63) or GFAP (p≤0.12) (Table 1).

Table 1. Group mean (±SEM) percentage of BrdU-immunoreactive cells coexpressing a mature neuronal marker (NeuN) or a glial marker (GFAP).

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>% BrdU and NeuN colabeled</th>
<th>% BrdU and GFAP colabeled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male control (n=3)</td>
<td>67.4 ± 14.5</td>
<td>23.9 ± 3.3</td>
</tr>
<tr>
<td>Male TMT (n=3)</td>
<td>55 ± 17.7</td>
<td>15.7 ± 2.6</td>
</tr>
</tbody>
</table>
Figure 2. A) Group means of the density of BrdU-labeled cells (measuring cell density on Day 1) in the granule cell layer across sex and condition. There was a significant suppression in cell proliferation in the male rats exposed to TMT but no evidence for a suppression in female rats exposed to TMT. B) Group means of the density of pyknotic cells in the granule cell layer on Day 1 across sex and condition. (n=5 for all groups). There was a significant suppression of pyknosis in response to TMT for the male rats only. Data is expressed in means ±SEM.
There was an interaction effect of sex by condition for the density of pyknotic cells (Figure 2B) in the granule cell layer \((F(1,15)=4.39, p<0.05)\). TMT-exposed males, but not TMT-exposed females, had a lower density of pyknotic cells than their respective controls \((p<0.05)\).

**Experiment 2: Behaviour, cell survival, and cell death after repeated odour exposure**

In both males and females, there was no difference in the density of new \((^3\text{H-thymidine-labeled)}\) cells after repeated TMT exposure.

Figure 3A shows the group mean density of \[^3\text{H}]-\text{thymidine labeled cells found in the granule cell layer after undergoing 5 days of repeated exposure to odour. These labeled cells represent both first generation cells surviving 6 days after they were labeled as well as cells generated by further mitotic divisions (subsequent generation cells). Female rats, regardless of group, showed a higher density of labeled cells in the granule cell layer (main effect of sex: \(F(1,16)=4.92, p<0.04\)) compared with males, regardless of condition. There were no other significant effects (interaction effect \(p<0.87\) or main effect of condition \(p<0.94\)).
Figure 3. A) Group mean density of $^3$H-Thymidine labeled cells (measuring cell density on Day 6) in the granule cell layer across sex and condition. There were no significant differences amongst groups. B) Group mean density of pyknotic cells on Day 6 in the granule cell layer across sex and condition. TMT-exposed animals, regardless of sex, exhibited more pyknosis than control-odour exposed animals (n=5 per group). Data is expressed in means ± SEM.

Figure 4 shows the percentage of first, second, third, and fourth generation cells in males after five days of exposure to odour. TMT- and control odour-exposed males did not differ in
the amount of first, second, third, and fourth generation cells. There was a significant main effect of cell generation (first, second, third, or fourth) \((F(3,12)=13.57, p<0.001)\), but there were no other significant main or interaction effects. Both TMT and control odour exposed males had more second generation than third or fourth generation cells \((p<0.01\) and \(p<0.001\) respectively).

**In both males and females, there was more cell death after repeated TMT exposure.**

Figure 3B shows the group mean density of pyknotic cells in the granule cell layer after repeated odour exposure. TMT-exposed rats, regardless of sex, tended to have more pyknotic cells in the granule cell layer than controls \((\text{main effect of condition}: F(1,16)=3.99, p<0.06)\).

There was neither a main effect of sex nor an interaction effect.

![Figure 4](image.png)

**Figure 4.** Group means of the percentage of first, second, third, and fourth generation cells in males due to condition. There were no differences between TMT- and control odour-exposed males in the percentages of each cell generation \((n=3\) per group). Data is expressed in means ± SEM.
TMT exposed animals engaged in more defensive behaviour than controls on Day 1 but not on Day 5.

**Defensive Burying**

Figure 5 shows the mean A) frequency and B) duration of defensive burying on Day 1 and Day 5 of testing. TMT-exposed rats, regardless of sex, buried more frequently (p ≤ 0.001) and longer (p ≤ 0.001) than controls on day 1 but not on day 5 (interaction effect for frequency and duration of defensive burying, F(1, 28)=27.33, p ≤ .0001 and F(1,28)=18.80, p ≤ .001, respectively), indicating that defensive burying behaviour habituated after 5 days of exposure to TMT.

**Stretched Approach Sequences**

Figure 5 shows the mean C) number and D) duration of stretched approach on Day 1 and 5. TMT-exposed rats engaged in stretched approach more frequently (p ≤ 0.01) and longer (p ≤ 0.02) on Day 1 but not on Day 5 (interaction effect for frequency (F(1,28)=27.33, p ≤ 0.0001) and duration (F(1,28)=10.69, p ≤ 0.003)), indicating that stretched approach behaviour habituated after 5 days of exposure to TMT.
Figure 5. Group means for A) frequency and B) duration of defensive burying and C) frequency and D) duration of stretched approach due to sex and condition across Day 1 and 5. TMT-exposed animals, regardless of sex, increased the expression of both defensive burying and stretched approach on Day 1. The expression of both defensive burying and stretched approached decreased (or habituated) by Day 5 of repeated exposure to TMT, in both male and female rats (n=8 per group). Data is expressed in means ±SEM.
TMT exposed animals engaged in less non-defensive behaviour than controls on Day 1 but not on Day 5.

**Direct Vial Contact**

Table 2 shows the mean number and the mean duration of direct vial contact for each group on test Day 1 and Day 5. On both Day 1 and Day 5, TMT exposed rats contacted the odour-containing vial less frequently (main effect of condition: $F(1,28)=25.80, p<0.001$) and for a shorter duration (main effect of condition: $F(1,28)=39.47, p<0.0001$) than controls. Females tended to directly contact the vial for a shorter duration (main effect of sex: $F(1,28)=3.68, p<0.07$) than males, regardless of condition. These results suggest that avoidance of the vial containing TMT did not habituate in either male or female rats.

**Rearing and Grooming**

Table 2 also illustrates the group means for the frequency and duration of rearing and grooming on Days 1 and 5. TMT-exposed rats reared less frequently (main effect of condition: $F(1,28)=4.52, p<0.04$), and for a shorter duration (main effect of condition: $F(1,28)=4.96, p<0.03$) than controls. Rats reared more on Day 1 than on Day 5 (significant main effect of day: $F(1,28)=7.45, p<0.01$), indicating that rearing behaviour habituated after repeated odour exposure. Females reared longer than males on Day 1 (significant main effect of sex: $F(1,28)=7.38, p<0.01$) but not on Day 5. There were no significant interaction effects for frequency or duration of rearing. There were no significant interaction or main effects for frequency or duration of grooming.
Table 2. Mean (±SEM) frequency or duration of nondefensive behaviours on Day 1 and Day 5.

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Frequency of Direct Contact</th>
<th>Duration of Direct Contact (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 5</td>
</tr>
<tr>
<td>Male TMT (n=8)</td>
<td>4.12±2.58</td>
<td>0.62±0.49</td>
</tr>
<tr>
<td>Male Control (n=8)</td>
<td>11.00±3.47</td>
<td>8.12±1.26</td>
</tr>
<tr>
<td>Female TMT (n=8)</td>
<td>1.18±0.63</td>
<td>0.25±0.25</td>
</tr>
<tr>
<td>Female Control (n=8)</td>
<td>6.75±1.53</td>
<td>4.06±1.34</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Frequency of Rearing</th>
<th>Duration of Rearing (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 5</td>
</tr>
<tr>
<td>Male TMT (n=8)</td>
<td>21.18±4.0</td>
<td>14.06±4.5</td>
</tr>
<tr>
<td>Male Control (n=8)</td>
<td>34.50±5.9</td>
<td>22.50±3.4</td>
</tr>
<tr>
<td>Female TMT (n=8)</td>
<td>29.93±6.1</td>
<td>20.75±3.3</td>
</tr>
<tr>
<td>Female Control (n=8)</td>
<td>35.56±8.7</td>
<td>29.37±4.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Frequency of Grooming</th>
<th>Duration of Grooming (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 5</td>
</tr>
<tr>
<td>Male TMT (n=8)</td>
<td>4.25±1.8</td>
<td>4.00±1.1</td>
</tr>
<tr>
<td>Male Control (n=8)</td>
<td>5.88±1.4</td>
<td>4.50±1.1</td>
</tr>
<tr>
<td>Female TMT (n=8)</td>
<td>10.19±7.1</td>
<td>2.00±0.5</td>
</tr>
<tr>
<td>Female Control (n=8)</td>
<td>5.94±1.6</td>
<td>3.31±0.7</td>
</tr>
</tbody>
</table>
Behaviour did not correlate with cell proliferation

For experiment 2, Pearson product-moment correlations were performed for each group for the density of labeled cells in the granule cell layer and each behaviour exhibited on Day 1. There were no significant correlations between the density of labeled cells and any of the measured behaviours in either males or females (Experiment 2).

2.3 Discussion

Our results show a clear sex difference in the effects of predator odour on cell proliferation. Our findings support previous observations that exposure to fox odour (TMT) suppresses cell proliferation in the dentate gyrus of adult male rats (Galea et al., 1996; Holmes & Galea, 2002; Tanapat et al., 2001) and are consistent with a stress-induced suppression in cell proliferation in adult male tree shrews and primates following exposure to other stressors (Gould et al., 1997, 1998). We also found that the TMT-induced suppression of cell proliferation in adult males was accompanied by an increase in the expression of defensive behaviours (defensive burying, avoidance, and stretched approach), partially consistent with previous studies (Wallace & Rosen, 2000, 2001). Unlike our observations in male rats, TMT exposure did not suppress cell proliferation in female rats even though females responded to TMT with a similar increase in defensive behaviour. In both males and females, repeated exposure to TMT resulted in a reduction in the expression of defensive behaviour (defensive burying and stretch approach) but not in avoidance of the vial containing TMT. This indicates that, although some defensive behaviours habituated, the rats were still capable of responding to the presentation of TMT.

We have also demonstrated that repeated TMT exposure increases the survival of new cells in male rats. Tanapat et al (2001) have shown that males exposed to a single episode of TMT have fewer new cells 2 hours after labelling and this deficit in the number of new cells is still evident 1 week later. Unlike Tanapat et al.'s (2001) findings using a single TMT exposure, we found that after males are repeatedly TMT-exposed there is not a decrease in the number of
new cells approximately 1 week after labelling. This suggests that repeated TMT exposure enhances either the survival or the subsequent proliferation of new (labelled) cells. Our data suggests that TMT exposure is actually enhancing the survival of new cells rather than up-regulating cell proliferation as we found no difference between TMT and control odour-exposed males in the number labelled cells that were first and subsequent generation. Also, TMT-exposed males showed both suppressed cell birth and suppressed cell death after a single TMT exposure, suggesting that cell turnover was slowed. In contrast, after repeated TMT exposure there was an increase in cell death in both males and females. Thus, even though repeated TMT exposure may have enhanced the survival of new cells, repeated TMT exposure also increased the total proportion of granule cells that were dying. Thus, we have shown that the effects of repeated TMT on new cell survival and on cell death are dissociated.

**TMT exposure suppressed cell proliferation in males but not females**

A single exposure to TMT resulted in a significant suppression in cell proliferation in male rats consistent with previous studies using male rodents or primates (Galea et al., 1996; Gould et al., 1997). We did not see suppressed cell proliferation in TMT exposed females. This finding is intriguing considering that previous studies suggest that males may be more vulnerable to the neural effects of stress than females, as males exhibit neurodegeneration and apical dendritic atrophy in the hippocampus after chronic stress to a greater extent than females (Gould et al., 1997; Mizoguchi et al., 1992; Uno et al., 1989).

Estradiol levels are known to affect both cell proliferation (Tanapat et al., 1999) and stress responsivity (Viau & Meaney, 1991), and thus it is possible that females in proestrus (high levels of estradiol) may react differently to TMT than females in other stages of the estrous cycle. We did not control for estrous cycle variations in our females, however, our data suggests that the females were not likely in proestrus given that: 1) only proestrus females have a higher rate of cell proliferation relative to males (Tanapat et al., 1999) and the females in our study in
either condition did not have a higher rate of cell proliferation than their respective males and 2) we have evidence that neither ovariectomized females nor ovariectomized females treated with a high dose of estradiol show a TMT-induced suppression of cell proliferation (Chapter 3). Importantly, we have observed a sex difference in cell proliferation in response to TMT and our data suggests that this is not likely due to the females being in a high estrogen phase of the estrous cycle.

In males, repeated TMT exposure likely enhanced the survival of new cells.

Recent findings show that a single TMT exposure suppresses cell proliferation in males and this suppression is evident at both a 2-hour and a 1-week survival time after labelling (Tanapat et al., 2001). In the present study we replicated this suppression in the number of new cells in males after a single TMT exposure and a 24-hour survival time. However, we found no evidence of this suppression at a 1-week survival time after males were repeatedly exposed to TMT. We observed fewer labelled cells in males on the first day of TMT exposure, but if we allowed new cells labelled on the first day of TMT exposure to survive for five more days (while the rats underwent repeated TMT exposure), we no longer observed fewer labelled cells. This suggests that repeated TMT exposure resulted in either an increase in the survival of new cells or an enhancement in subsequent cell proliferation.

Consistent with previous studies, in all groups there was a greater number of labeled cells approximately 1 week after labeling compared with 1 day after labeling, which is likely due to further cell divisions over time (Cameron et al., 1993). Progenitors give rise to daughter cells that either exit the cell cycle or go on to divide further, giving rise to further generations of labelled cells (Nowakowski & Hayes, 2001). Specifically, males repeatedly exposed to TMT had 5 times the density of labeled cells (approx.) 1 week after labelling compared to 1 day after labelling while males repeatedly exposed to control odour had 1.76 times the density of labeled cells (approx.) 1 week after labelling relative to 1 day after labeling. Taken together, this
suggests that males repeatedly exposed to TMT have either an enhancement in the number of labelled cells that continue to proliferate or an enhancement in the number of labelled cells that survive. We analyzed differences in dilution of the $^3$H-thymidine label, as the signal intensity (heavily vs. lightly labeled cells) can effectively be used to approximate the population of labeled cells that are first generation (those cells that have quit the cell cycle after the first mitotic division) and those cells that are a result of subsequent divisions of the first generation cell (Smart & Smart, 1982; Polleux, 1997b). We found no difference in the amount of first and subsequent generation cells between conditions (TMT and control) in males. This suggests that cell survival is enhanced in TMT exposed males rather than cell proliferation being up-regulated. It would be of interest for future studies to verify the extent of label dilution in the adult dentate gyrus.

The survival of new cells over repeated odour exposure may have been enhanced in the TMT-exposed males due to hippocampus-dependent learning, which has been shown to increase new cell survival (Gould et al., 1999). Repeated TMT exposure provides an opportunity for rats to learn that the context in which the TMT is administered is not associated with the actual appearance of a fox, representing an opportunity to engage in both contextual and olfactory learning. The hippocampus has been implicated in both contextual fear and olfactory conditioning paradigms (Anagnostaras et al., 1999; Antoniadis et al., 2000; Bannerman et al., 2001; Gisquet-Verrier et al., 1999; Gisquet-Verrier et al., 1999; Miller et al., 1986; Selden, Everitt, Jarrard, & Robbins, 1991; Staubli et al., 1984, 1995). Contextual fear conditioning has been demonstrated in cat odour exposure paradigms (Dielenberg, Arnold, & McGregor, 1999; McGregor & Dielenberg, 1999, McGregor, Schrama, Ambermoon, & Dielenberg; Zangrossi & File, 1994). Thus it is possible that the hippocampus is involved in learning about the consequences of the TMT in our repeated TMT exposure paradigm. In our paradigm, all rats repeatedly exposed to TMT showed habituation of their expression of defensive behaviours
(discussed below). It is possible that this habituation-based learning may have increased cell survival through a hippocampus-dependent mechanism. Future studies could lesion the hippocampus to verify the hippocampal dependence of this TMT exposure paradigm.

A single TMT exposure suppressed cell death in males, while repeated TMT exposure increased cell death.

A single exposure to TMT suppressed pyknosis in males, consistent with observations that adrenal steroids modulate cell death (Cameron & Gould, 1994). Thus a single exposure to TMT stabilized cell turnover in males (suppressed cell proliferation and cell death). Interestingly, females exposed to TMT showed neither suppressed cell birth nor cell death. However, repeated exposure to TMT resulted in increased cell death in both sexes. Previous studies have found that longer-term (2-3 months) exposure to stress increases cell death in the CA1 and CA3 region of males (Mizoguchi et al., 1992; Sapolsky, Krey, & McEwen, 1985; Uno et al., 1989).

The differential results of repeated TMT exposure increasing cell death while enhancing new cell survival may be the result of combining both stress and learning. Repeated TMT exposure resulted in learning about the TMT (indicated by habituated defensive behaviour) and this learning may have enhanced cell survival. However, repeated TMT exposure may have also been stressful enough to increase granule cell death. Future studies should be undertaken to examine the complex relationship between stress and learning in eliciting or suppressing cell death.

Both males and females responded to TMT with an increase in defensive behaviour

On the first day of TMT exposure, TMT-exposed males and females exhibited higher levels of stretched approach and defensive burying. Increased defensive burying and stretched approach behaviour was accompanied by avoidance of the TMT-containing vial, indicating that both sexes could locate and avoid contact with TMT. These findings are consistent with
previous studies using predator odour (Blanchard et al., 1990, 1991; File et al., 1993; Klein et al., 1994; Shepherd et al., 1992; Wallace & Rosen, 2000, Zangrossi & File, 1994). In the present study, defensive burying was mainly directed towards the TMT-containing vial suggesting that the source of the odour (TMT-containing vial) was easily localized (Pinel & Treit, 1978; Pinel et al., 1989). Similar to previous studies using cat odour (Blanchard, Blanchard, & Hori, 1989; Williams & Scott, 1989), but unlike previous studies using TMT, (Morrow et al., 2000a; Morrow, Roth, & Elsworth, 2000b; Wallace & Rosen, 2000, 2001) we did not observe an increase in freezing behaviour in response to TMT exposure. However, in all of the studies that observed freezing in response to TMT no bedding was used in the test chamber and the test chamber was small in size (Morrow et al., 2000a, 2000b; Wallace & Rosen, 2000, 2001). Our test chamber contained bedding and was similar in size to Wallace and Rosen’ s (2000) large test chamber in which they did not observe freezing. Also similar to Wallace and Rosen (2000), we observed a decrease in non-defensive behaviours (we examined rearing and grooming) in rats exposed to TMT. Taken together, our results are consistent with studies (Blanchard et al., 1991) showing that exposure to a predator odour results in both an increase in the expression of defensive behaviour and a decrease in the expression of non-defensive behaviour in male and female rats.

Although both females and males have similar pressure for adapting appropriately to predation, several studies found that females react more defensively than males during situations of predator threat (D.C. Blanchard et al., 1991; R.J. Blanchard et al., 1990; Klein et al., 1994; Shepherd et al., 1992). In the present study we found that TMT-exposed females did not engage in more defensive behaviour than TMT-exposed males. Also contrary to previous studies (Klein et al., 1994; Perrot-Sinal et al., 2000), there was no sex difference in the amount of non-defensive behaviour expressed during predator odour exposure. The lack of a sex difference in the present study may have been due to several factors: the presence of bedding, the size of the
test chamber and the use of a different predator odour, all of which may affect the expression of defensive behaviour (see above).

**Both males and females habituated to the TMT**

After repeated exposure to TMT, both males and females decreased their defensive behaviours (stretched approach and defensive burying), suggesting that defensive behaviours habituated to repeated TMT exposure. Previous observations suggest that habituation to TMT may occur as rapidly as the second TMT exposure (Morrow et al., 1998). However, in our study both males and females continued to avoid the TMT-containing vial on the fifth day of TMT exposure, suggesting that while defensive behaviours habituated to repeated presentations of TMT, the rats were still able to detect and respond to the source of the odour. This finding is consistent with response to repeated presentations of cat odour (File et al., 1993; Zangrossi et al., 1994) in which male rats continue their avoidance of the predator odour source in response to five days of repeated exposure to cat odour. Stretched approach behaviour completely habituated in the TMT-exposed males but not the TMT-exposed females. This is partially consistent with observations that, unlike males, female fail to adapt to repeated restraint stress both behaviourally (using exploration and defecation as measures: Kennett et al., 1986; Haleem et al., 1988) and hormonally (corticosterone response: Galea et al., 1997).

**There was no correlation between behaviour and cell proliferation**

In the present study, males but not females showed suppressed granule cell proliferation, however both males and females exhibited a similar increase in defensive behaviour after a single TMT exposure. Further, none of the behaviours that we observed correlated with the amount of cell proliferation in either males or females. Thus, changes in the expression of these defensive behaviours do not appear to be immediately or directly related to changes in cell proliferation. This is consistent with observations that changes in the amount of defensive behaviour do not directly correlate with changes in hormonal indices of stress (Holmes & Galea,
However, we did find that repeated exposure to TMT habituated defensive behaviours and likely enhanced the survival of new cells. This suggests that while cell proliferation does not relate to defensive behaviours per se, enhanced cell survival is related to learning about the stressful environment (as shown by the habituation of defensive behaviours).

2.4 Functional implications

New cells in the dentate gyrus have been shown to extend axons into the mossy fiber pathway (Hastings & Gould, 1999; Markakis & Gage, 1999; Stanfield & Trice, 1988) and to have enhanced electrophysiological properties (Snyder, Kee, & Wojtowicz, 2001). In the present study, the majority of cells produced in both TMT and control odour-exposed males became neurons three weeks after birth. However, the TMT-induced suppression in the number of new cells is no longer evident at 3 weeks (Tanapat et al., 2001) and therefore may not have a long-term functional impact.

If TMT exposure affects subsequent learning and memory, the suppression in cell proliferation may not necessarily be detrimental. Indeed, it is interesting that an acutely stressful event facilitates learning in male rats but not intact female rats (Wood & Shors, 1998). It is possible that this sex difference in learning in response to stress is related to the sex difference in the stress-induced suppression of cell proliferation that we have observed in the present study. In other words, the stress-induced suppression in cell proliferation may in fact be essential for the animal to learn about the episode. In this regard, it is interesting to note that TMT-exposed females did not habituate their defensive behaviours in response to repeated TMT exposure as much as TMT-exposed males (see discussion above).

2.5 Conclusions

The present study has demonstrated that there is a sex difference in the cell proliferation response to TMT exposure. Further, we have shown that changes in cell proliferation are not
related to changes in defensive and non-defensive behaviour in response to TMT exposure. We have found that, with repeated TMT exposure, defensive behaviours habituate in both males and females, suggesting that the rats are learning about the consequences of the predator odour exposure. Repeated TMT exposure led to an enhancement in the survival of new cells in males, which may have been due to habituation-based learning. We have also demonstrated a sex difference in the effect of TMT on cell death. Males but not females show suppressed pyknosis with a single TMT exposure. Repeated TMT exposure led to enhanced pyknosis in both males and females. Taken together, these data show that TMT differentially affects cell proliferation, new cell survival, and cell death in male and female rats. Studies should be undertaken to further elucidate the effects of hormonal status on the sex difference in cell proliferation and cell death in response to TMT exposure. Time course analysis on the extent of cell proliferation, cell survival, and cell death during repeated TMT exposures would also be of interest.
Stress can contribute to the onset of depression and anxiety, as the majority of both depressed patients and those with preliminary symptoms of anxiety have had an acute stressful precipitating event (Jacobs, Praag, & Gage, 2000; Morgan et al., 2001; Young, 1998). Furthermore, depressed patients have impaired negative feedback in hypothalamic-pituitary-adrenal (HPA) axis activity that is correlated with symptomology (Modell, Yassouridis, Huber, & Holsboer, 1997; Young, Haskett, Murphy-Weinberg, Watson, & Akil, 1991). Studies have reported that women are more likely to suffer from both anxiety and major depressive disorder than men (Kessler, McGonagle, Swartz, Blazer, & Nelson, 1993; Weissman & Merikangas, 1986; Williams et al., 1995). Consistent with these findings, there are also sex differences in animal models of anxiety and depression (File, 1995; Zimmerberg & Farley, 1993; Shepherd, Flores, Rodgers, Blanchard, & Blanchard, 1992; Blanchard, Shepherd, De Padua Carobrez, & Blanchard, 1991; Blanchard et al., 1998). It is possible that ovarian hormones mediate the sex differences in anxiety and depression (Young, 1998), as depressed patients have altered levels of ovarian hormones (Young, Midgley, Carlson, & Brown, 2000; Young & Korszun, 1998) and female rats show changes in both behavioural and HPA stress response across the estrous cycle (Viau & Meaney, 1991; Galeeva & Tuohimaa, 2001; Diaz-Veliz, Soto, Dussaubat, & Mora, 1989; Diaz-Veliz, Butron, Benavides, Dussaubat, & Mora, 2000; Diaz-Veliz, Baeza, Benavente, Dussaubat, & Mora, 1994).

Estradiol may dose-dependently affect both the behavioural and HPA response to stress. In ovariectomized females supra-physiological levels of estradiol increase (Handa, Burgess, Kerr, & O'Keefe, 1994; Handa & Burgess, 1992; Carey, Deterd, de Koning, Helmerhorst, & de Kloet, 1995; Viau & Meaney, 1991), while high physiological levels of estradiol decrease
(Young, Altemus, Parkison, & Shastry, 2001; Redei, Lifang, Halasz, & Aird, 1994) the HPA response to stress. Intact females in a stage of the estrous cycle in which levels of estradiol are high (proestrus) have an increased HPA response to stress relative to those in stages in which levels of estradiol are low (diestrus; Viau & Meaney, 1991). Studies have also shown that behavioural indices of “anxiety” are sensitive to stage of estrous cycle (Mora, Dussaubat, & Diaz-Veliz, 1996; Diaz-Veliz et al., 1989; Diaz-Veliz et al., 2000), with low estrogen females (diestrus) showing greater “anxiety” than high estrogen females (proestrus). Increased circulating estradiol has also been associated with a decrease in defensive behaviour (Diaz-Veliz, Urresta, Dussaubat, & Mora, 1991; Fernandez-Guasti & Picazo, 1992; Fernandez-Guasti, Martinez-Mota, Estrada-Camarena, Contreras, & Lopez-Rubalcava, 1999).

Progesterone can change the effects of estradiol on behavioural stress responses (Diaz-Veliz, Urresta, Dussaubat, & Mora, 1994; Frye, 2001; Galeeva et al., 2001; Picazo & Fernandez-Guasti, 1995). Progesterone antagonizes the anxiogenic effects of estradiol (Diaz-Veliz et al., 1994) and decreases “anxiety” as measured by the number of open arm entries in the elevated plus maze (Frye, 2001). Progesterone or its metabolite (3 α 5 α-THP) can also reduce immobility in the forced swim test (Martinez-Mota, Estrada-Camarena, Lopez-Rubalcava, Contreras, & Fernandez-Guasti, 2000), decrease “anxiety” in the elevated plus maze (Bitran & Dowd, 1996; Frye, 2001) and decrease time spent defensively burying a shock probe (Bitran, Dugan, Renda, Ellis, & Foley, 1999). Thus, progesterone and estrogen may interact to change stress responsivity in females.

The hippocampus may mediate the effects of estrogen and progesterone on stress responsivity. The hippocampus contains both estradiol and progesterone receptors (Guerra-Araiza et al, 2001; Milner et al., 2001; Shughrue, Lane, & Merchenthaler, 1997) and a high density of glucocorticoid receptors (Van Eekelan & De Kloet, 1992). Estrogen and progesterone treatment has been shown to both up- and down-regulate glucocorticoid receptor (GCR) mRNA
and protein (Burgess & Handa, 1992; Ferrini & De Nicola, 1991, Ferrini, Lane, & De Nicola, 1995; Turner, 1990; Patchev and Almeida, 1996) in the hippocampus. Both estrogen and progesterone can induce significant changes in hippocampal morphology. Estrogen and progesterone alter synaptic plasticity and dendritic branching in CA1 (Woolley & McEwen, 1993) and protect against hippocampal cell death in vitro (Nilsen & Brinton, 2002). Estrogen also protects against hippocampal cell death in vivo after kainic acid treatment ((Azcoitia, Sierra, & Garcia-Segura, 1998) and adrenalectomy (Frye, 2001). The effects of combined estrogen and progesterone treatment or progesterone treatment alone on adult neurogenesis in the dentate gyrus have not been studied, but estrogen treatment alone has been shown to significantly affect adult neurogenesis in the dentate gyrus in a time-dependent manner (Tanapat, Hastings, Reeves, & Gould, 1999; Ormerod & Galea, 2001).

Estrogen's ability to modulate the level of neurogenesis in the dentate gyrus may be important for its effects on stress, anxiety, and depression. Adult neurogenesis has been implicated in depression (Duman, Malberg, & Thome, 1999; Malberg, Eisch, Nestler, & Duman, 2000; Czeh et al., 2001) due to observations that chronic administration of antidepressants increase neurogenesis in the dentate gyrus of adult male mammals and that the timing of the increase in neurogenesis is temporally coupled with the time when drug treatment begins to reduce depressive symptoms. Furthermore, exposure to acute psychosocial and predator odour stress rapidly suppresses neurogenesis and cell death in male rodents and primates (Falconer and Galea, submitted; Gould, McEwen, Tanapat, Galea, & Fuchs, 1997; Gould, Tanapat, McEwen, Flugge, & Fuchs, 1998; Holmes and Galea, 2002; Tanapat, Hastings, Rydel, Galea, & Gould, 2001). Interestingly, intact females do not show suppressed neurogenesis or cell death when acutely exposed to a predator odour stressor (Chapter 2; Falconer and Galea, submitted). It is possible that ovarian hormones are important mediators of this sex difference in the neurogenesis response to stress because ovarian steroids modulate both hormonal responses to stress (Viau et
al., 1991) and neurogenesis (Tanapat et al., 1999). It is also possible that the effects of ovarian hormones on hormonal and behavioural stress responses are related to their effects on hippocampal neurogenesis. Therefore, in the present study we investigated the effects of estrogen replacement alone or in combination with progesterone on acute predator odour stress-induced changes in neurogenesis, cell death and behaviour.

3.1 Materials and Methods

Subjects

Subjects were female Sprague-Dawley rats (approx. 250-300 grams in weight). Rats were obtained from the UBC Animal Care Center and group-housed in metal hanging cages on a 12:12 hour light-dark cycle (lights on at 7:30 am). One week later, rats were ovariectomized and housed singly in bedding-lined (Care Fresh; Absorption Corporation) polyurethane cages during recovery. Animals were given free access to food (PMI Nutrition- Rat Diet) and tap water. Housing temperature was maintained at 21±1°C. All animals were treated in accordance with the guidelines of the Canadian Council on Animal Care and the policies on animal care established by The University of British Columbia. Every effort was made to minimize the number of animals used per group.

Apparatus

All testing was performed in test chambers placed in fumehoods (separate fumehoods were used for each odour). Test chambers consisted of 29 cm X 30 cm X 46 cm plexiglass boxes, lined with 5 cm of corncob bedding. Each transparent test chamber was placed in a fume hood with 2-3 other test chambers, and the chambers were then visually isolated with opaque paper. A plastic vial filled with two Kimwipes was placed in the same corner of each testing chamber.

Design and Procedures
Experiment 1  To investigate the possibility that estradiol regulates the number of BrdU-positive cells produced in female rats exposed to predator odour stress, female rats (n=40, 10 per group) were anesthetized with 2-Bromo-2-Chloro-1,1,1-Trifluoroethane (Halothane, flow rate 4%, MTC Pharmaceuticals, Cambridge, Ontario) and ovariectomized. Post surgery, rats were allowed to recover for one week before habituation. Rats were habituated to the test chambers 15 minutes/day for 5 consecutive days. The following day, rats were injected with either estradiol benzoate (10µg of estradiol benzoate (EB) in 0.1 ml sesame oil, sc) or vehicle (0.1ml sesame oil). Four hours later, both estradiol-treated rats (EB) and vehicle-treated rats (VEH) were exposed to either TMT (trimethylthiazoline, the major component of fox feces, Phero Tech Inc., Delta BC, CANADA) or a control odour (distilled water) for one hour. TMT-exposed rats (n=20, 10 EB, 10 VEH) were placed in test bins with a scintillation vial that contained a Kimwipe permeated with 150 µl of TMT. Control rats (n=20, 10 EB, 10 VEH) were exposed to a vial containing a Kimwipe permeated with 150 µl of distilled water. Rats remained in the odour-containing test chamber for a total of 1 hour, but were given an intraperitoneal (i.p.) bromodeoxyuridine (BrdU) injection (100mg/kg) after the first 15 minutes. Rats were videotaped and later scored for behaviour occurring during the first 15 minutes of TMT exposure.

Experiment 2  To investigate the effects of both estrogen and progesterone on the female response to predator odour female rats (n= 36, 9 per group) were bilaterally ovariectomized and implanted with capsules containing estradiol and progesterone (n=18) or capsules containing vehicle (n=18). Rats were implanted with silastic capsules (Dow Corning, Midland, MI, 0.062 ID, 0.125 OD) containing 17β-estradiol 3- Benzoate (Sigma, St Louis) dissolved in sesame oil (30 µg/ml, 10 mm/100 g BW) or vehicle (sesame oil) and capsules (0.132 ID, 0.183 OD) containing crystalline progesterone (Sigma, St. Louis; 20 mm/rat) or vehicle (empty capsules). These estrogen and progesterone capsules have been previously shown to deliver estradiol and
progesterone levels similar to those observed during diestrus (Brandi, 1990; Viau and Meaney, 1991).

As in experiment 1, rats were allowed to recover from surgery for one week and then habituated to test chambers 15 min/day for 5 days. The following day, rats that had received estrogen and progesterone implants were injected with estradiol benzoate (10μg of estradiol benzoate (EB) in 0.1 ml sesame oil, sc) and rats that had received vehicle implants were injected with vehicle (0.1ml sesame oil, sc). Four hours later, both estradiol and progesterone-treated rats (EB-P) and vehicle-treated rats (VEH) were exposed to either TMT or control odour (distilled water) for one hour. Rats were given an intraperitoneal (i.p.) bromodeoxyuridine (BrdU) injection (100mg/kg) after the first 15 minutes of odour exposure. Rats were videotaped and later scored for behaviour occurring during the first 15 minutes of odour exposure.

**Histological procedures**

For both Experiments 1 and 2, twenty-four hours after receiving BrdU rats were given an overdose of sodium pentobarbital (2 ml/kg Somnitol, MTC Pharmaceuticals), perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer, and the brains extracted. Prior to perfusion, blood samples were taken transcardially to verify serum estradiol and/or progesterone and corticosterone by radioimmunoassay. In Experiments 1 and 2, a separate group of rats (n=3 per group) was perfused 1 hour after odour exposure and blood collected in order to assay for estrogen and/or progesterone and corticosterone. All brains were stored overnight at 4°C in perfusate. The following day, brains were sectioned into 40 μm slices on an oscillating tissue slicer (OTS 3000, Electron Microscopy Sciences) at the level of the dentate gyrus. A subset of brains (5 per group) were then processed immunohistochemically for BrdU.

A separate group of female rats (n=6, 3 per group) underwent the same procedure as described in Experiment 1 (injected with EB or VEH, exposed 4 hours later to TMT or control odour (1 hour), BrdU injected 15 minutes into the odour exposure) but were perfused 3 weeks
later. These brains were processed for combined immunofluorescence for BrdU, Neuron-Specific Enolase (NSE) a marker for mature neurons (Iwanaga, Takahashi, & Fujita, 1989), and glial fibrillary acidic protein (GFAP; an astroglial marker (Cameron, Woolley, McEwen, & Gould, 1993).

**Hormone Assays**

Plasma estradiol assays were performed as described previously (Ormerod and Galea, 2001). Briefly, blood samples were stored overnight at 4°C and then were centrifuged at 10g for 10 min. Plasma estradiol was assayed using a Coat-A-Count kit (Diagnostic Products Corporation, Los Angeles, CA) modified for low expected levels of estradiol. Plasma progesterone was assayed using a Coat-A-Count kit (Diagnostic Products Corporation, Los Angeles, CA).

For experiment 1, plasma corticosterone levels were analysed using a radioimmunoassay protocol described by Weinberg and Bezo (1987). Briefly, antiserum was obtained from Immunocorp (Montreal, Canada) and tracer was obtained from Mandel Scientific (Guelph, Canada). Dextran-coated charcoal was used to adsorb and precipitate free steroids after incubation. For experiment 2, plasma corticosterone was assayed using a Coat-A-Count kit (Diagnostic Products Corporation, Los Angeles, CA).

For all assays, the intra-assay coefficient of variation was less than 5%.

**BrdU peroxidase immunohistochemistry**

Tissue was processed immunohistochemically to visualize BrdU-labeled cells. For peroxidase immunolabeling, sections were mounted on 3-aminopropylsilane-treated slides, rinsed in phosphate buffer saline (PBS), incubated in H2O2, made permeable with Trypsin, and denatured with 2N HCl. The sections were then incubated with normal horse serum and anti-BrdU mouse monoclonal antibody (Boehringer Mannheim, 1:100 dilution) overnight at room temperature (20 ± 1°C). Sections were rinsed in PBS and incubated in Biotinylated antibody
anti-mouse IgG (Vector, Elite kit), rinsed in PBS, reacted using an ABC reagent (Vector, Elite kit) with diaminobenzidine (DAB), counterstained for Nissl substance with cresyl violet, and coverslipped with Permount.

**Fluorescence Immunohistochemistry**

Separate sets of slides were also triple stained with immunofluorescent probes to assess BrdU-, NSE- and GFAP- immunoreactivity. Slides were pretreated with 3% H$_2$O$_2$ in phosphate-buffered Saline (PBS) for 20 minutes, rinsed in tris-buffered saline (TBS), incubated for 2 h in deionised formamide solution (in 2XSSC) at 65°C, rinsed again, and DNA denatured in 2N HCl for 30 minutes. Sections were rinsed, incubated in 0.1 M borate buffer (10 min), blocked in 5.0% normal donkey serum (Jackson Immunoresearch) for 30 min, and then incubated for 60 hr in rat anti-BrdU (ascites 1:100; Serotec), rabbit monoclonal anti-NSE (1:1000 Polysciences), and mouse monoclonal anti-glial fibrillary acidic protein (GFAP 1:2000;Vector) at 4°C. Sections were then incubated in 5% normal donkey serum (Jackson Immunoresearch), rinsed, and incubated in a cocktail of donkey anti-rat flourescein (FITC; to visualize anti-BrdU) donkey anti-rabbit Cy5 (to visualize NSE) and donkey anti-mouse Cy3 (to visualize GFAP; all diluted 6μg/ml; Jackson Immunoresearch) for 4 hrs. Sections were rinsed and coverslipped with the anti-fading agent diazabicyclooctane (DABCO; 2.5% DABCO, 10% polyvinyl alcohol and 20% glycerol in TBS; Sigma).

**Cell Density Measurements**

Slides were coded before analyses, and the code was not broken until all data was collected. For each brain processed for peroxidase immunohistochemistry, labeled cells were counted on every 10$^{th}$ section throughout the dentate gyrus and stereological estimates were then obtained. BrdU-ir (see Fig 1A) and pyknotic cells were counted in the granule cell layer and the subgranular zone (defined as approximately 50 μm between the granule cell layer and the hilus; Palmer et al, 2000). Cells were considered pyknotic if they lacked a nuclear membrane, had pale
or absent cytoplasm and darkly stained spherical chromatin (see Fig 1B and Gould, Woolley, & McEwen, 1991; Ormerod et al., 2001). Cells were counted at 1000X magnification on a Nikon Elipse (E600) light microscope and the total number of cells was estimated using a modified version of the optical fractionator method (West et al, 1991). The area of the dentate gyrus was determined using Analytical Imaging Station (AIS, Version 4.0, Imaging Research Inc) and dentate gyrus volume estimates were made using Cavalieri’s principle (West, Slomianka, & Gundersen, 1988).

Cell phenotypes were analyzed on slides treated for immunofluorescence. BrdU-labelled cells in the dentate gyrus on 4-6 sections per brain were taken from the middle portion of the dentate gyrus (where the dentate gyrus lies just beneath the corpus callosum and the infrapyramidal and suprapyramidal blades are joined at the crest; between A −3.3 and A −4.8). Cells were analyzed using a confocal laser scanning microscope (BioRad 2000) on a 63X oil objective. Z-sections were taken at 4 μm intervals. Optical stacks of 10 images were created with NIH Image for PC (http://www.scioncorp.com/pages/menu.htm) and imported into Adobe Photoshop.

Behvaioural Measures

We examined both the frequency and the duration of both defensive (stretched approach, defensive burying, freezing, and wet-dog shakes) and non-defensive behaviours (direct vial contact, rearing, and grooming) in response to a single odour exposure. Stretched approach, defensive burying, and freezing have been suggested to be behavioural indicators of stress and anxiety ((File, 1995; Blanchard & Blanchard, 1969; Blanchard, Mast, & Blanchard, 1975; Blanchard & Blanchard, 1986; Blanchard, Flannelly, & Blanchard, 1986; Blanchard, Blanchard, & Weiss, 1990; Blanchard, Blanchard, Rodgers, & Weiss, 1990). Stretched approach consists of the rat extending its body towards the test object while its hind paws remain stationary (Pinel, Symons, Christensen, & Tees, 1989). Defensive burying (Treit, Pinel, & Fibiger, 1981) involves
the rat using its forepaws to fling bedding towards a test object (in the present study, the test object is the vial containing TMT). Freezing is the term used to describe a complete cessation of movement (Fanselow & Bolles, 1979). Wet-dog shakes are shuddering movements of the head, neck, and trunk (Bedard & Pycock, 1977). Due to previous observations in males and females (Holmes and Galea, 2002; Falconer and Galea, submitted), we expected that rats exposed to predator odour (TMT) would increase the expression of certain defensive behaviours (stretched approach and defensive burying) and decrease the expression of certain non-defensive behaviours (rearing, grooming, and direct contact with the vial (ie. source of the odour)).

Data Analyses

For Experiments 1 and 2, separate analyses of variances (ANOVAs) were calculated for all dependent variables (total BrdU-ir cells, total pyknotic cells, percentage of BrdU/GFAP-ir cells, and percentage of BrdU/NSE-ir, and all behavioural measures) with condition (TMT and control) and treatment (EB and VEH or EB-P and VEH) as the between-subjects factor. Unless otherwise indicated, Newman-Keuls was used for post-hoc testing (α= 0.05).

Pearson product-moment correlation tests were conducted between total BrdU-ir cells or pyknotic cells and serum levels of estradiol, progesterone, and corticosterone or behaviour scores (frequency and duration of each behaviour examined: stretched approach, defensive burying, freezing, wet dog shakes (frequency only), direct contact, rearing, or grooming). In all statistical procedures, significance was set at α = 0.05. While p≤0.05 will be referred to as statistically significant, these p values reflect the probability of the effect occurring solely by chance.

3.2 Results

Experiment 1
Estradiol treatment increased cell proliferation but did not affect the cell proliferation response to TMT

EB-treated females had more BrdU-labeled cells than VEH-treated females regardless of odour exposure condition (main effect of treatment: $F(1,16)=8.698$, $p \leq 0.009$, Figure 6A). TMT exposure did not affect the number of BrdU-labeled cells in either EB or VEH-treated females (no significant main effect of condition, $p \leq 0.92$, nor significant interaction effect, $p \leq 0.74$).

TMT increased the number of pyknotic cells in VEH-treated but not EB-treated females (significant interaction: $F(1,14)=4.37$, $p \leq 0.05$; see Figure 6B).
Figure 6. A) Group means of the density of BrdU-labeled cells in the granule cell layer in estradiol (EB) or vehicle (VEH) treated females exposed to control odour or TMT. There was a significant enhancement of cell proliferation in EB treated females compared to VEH. B) Group means of the density of pyknotic cells in the granule cell layer in EB and VEH treated females exposed to control odour or TMT. TMT increased pyknosis only in VEH treated females. (n=5 per group). Data is expressed in means ±SEM.
In the subset of females (n=3) that were allowed to survive for 3 weeks after TMT or control odour exposure and labeled for combined immunofluorescence, there were no significant differences between the percentages of GFAP or NSE co-labeled cells (image shown in Figure 1G) or BrdU singly-labeled cells (p≤.24 to p≤.96). The group mean percentages for these combined immunofluorescent-labeled cells are seen in Table 3.

Table 3. Mean (±SEM) % cells immunoreactive for BrdU and NSE, BrdU and GFAP, or BrdU only.

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>% BrdU + NSE-ir</th>
<th>% BrdU + GFAP-ir</th>
<th>% BrdU-ir only</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB TMT (n=3)</td>
<td>59.4 (±8.4)</td>
<td>21.8 (±8.4)</td>
<td>13.9 (±7.4)</td>
</tr>
<tr>
<td>EB Control (n=3)</td>
<td>51.3 (±3.8)</td>
<td>24.4 (±6.2)</td>
<td>19.9 (±5.8)</td>
</tr>
<tr>
<td>VEH TMT (n=3)</td>
<td>44.4 (±5.1)</td>
<td>37.3 (±4.3)</td>
<td>6.4 (±1.2)</td>
</tr>
<tr>
<td>VEH Control (n=3)</td>
<td>39.6 (±7.5)</td>
<td>36.9 (±7.2)</td>
<td>19.8 (±3.1)</td>
</tr>
</tbody>
</table>

As expected, serum estradiol was significantly elevated in EB rats (main effect of treatment: F(1, 8)=10.04, p≤.01). *A priori* we were interested in the effects of EB treatment on corticosterone level in response to TMT. TMT increased corticosterone levels only in the VEH-treated females exposed to TMT (planned comparisons: p≤.03 in VEH-treated females and p≤.34 in EB-treated females, relative to their control groups; see Table 4).
Table 4. Mean (±SEM) plasma hormone levels in EB and VEH-treated females.

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Plasma Corticosterone (µg/dl)</th>
<th>Plasma Estradiol (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB TMT (n=3)</td>
<td>66.2 (±2.6)</td>
<td>141.6 (±34.2)</td>
</tr>
<tr>
<td>EB Control</td>
<td>53.4 (±12.9)</td>
<td>129.3 (±71.2)</td>
</tr>
<tr>
<td>VEH TMT (n=3)</td>
<td>87.2 (±4.6)</td>
<td>14.5 (±7.4)</td>
</tr>
<tr>
<td>VEH Control</td>
<td>48.5 (±9.1)</td>
<td>5.1 (±0.5)</td>
</tr>
</tbody>
</table>

Estradiol treatment did not affect defensive behaviour in response to TMT

Figure 7 illustrates the duration and frequency of defensive behaviours in the EB- and VEH-treated females: A) duration and B) frequency of defensive burying, C) duration and D) frequency of stretched approach. Figure 8 illustrates the A) duration and B) frequency of freezing and C) frequency of wet-dog shakes. Table 5 describes the frequency and duration of direct contact, grooming and rearing.
Figure 7. Group means for A) frequency and B) duration of defensive burying and C) frequency and D) duration of stretched approach due to EB or VEH treatment in females exposed to control odour or TMT (n=9 per group). Data is expressed in means ±SEM.
Figure 8. Group means for A) frequency and B) duration of freezing and C) the frequency of wet-dog shakes in EB or VEH treated females exposed to control odour or TMT (n=9 per group). Data is expressed in means ±SEM.
Table 5. Mean (±SEM) frequency and duration of nondefensive behaviors in EB and VEH-treated females

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Frequency of Direct Contact</th>
<th>Duration of Direct Contact (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB TMT (n=10)</td>
<td>9.8 (±1.8)</td>
<td>14.7 (±2.6)</td>
</tr>
<tr>
<td>EB Control (n=10)</td>
<td>10.1 (±2.1)</td>
<td>31.3 (±7.7)</td>
</tr>
<tr>
<td>VEH TMT (n=10)</td>
<td>10.6 (±1.9)</td>
<td>17.7 (±3.8)</td>
</tr>
<tr>
<td>VEH Control (n=10)</td>
<td>8.8 (±1.2)</td>
<td>24.5 (±4.5)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Frequency of Rearing</th>
<th>Duration of Rearing (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB TMT (n=10)</td>
<td>22.8(±3.0)</td>
<td>70.9(±11.5)</td>
</tr>
<tr>
<td>EB Control (n=10)</td>
<td>27.6(±4.5)</td>
<td>140.2(±29.2)</td>
</tr>
<tr>
<td>VEH TMT (n=10)</td>
<td>28.5(±2.0)</td>
<td>137.2(±21.4)</td>
</tr>
<tr>
<td>VEH Control (n=10)</td>
<td>22.5(±3.1)</td>
<td>80.7(±18.8)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Frequency of Grooming</th>
<th>Duration of Grooming (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB TMT (n=10)</td>
<td>7.40(±1.6)</td>
<td>131.4(±29.4)</td>
</tr>
<tr>
<td>EB Control (n=10)</td>
<td>8.70(±1.2)</td>
<td>110.3(±20.5)</td>
</tr>
<tr>
<td>VEH TMT (n=10)</td>
<td>8.90(±1.8)</td>
<td>124.8(±21.4)</td>
</tr>
</tbody>
</table>
TMT increased the expression of both defensive burying and stretched approach (main effects for duration and frequency of defensive burying $F(1, 36)=8.54, p<.006$ and $F(1, 36)=12.62, p<.001$, respectively; main effects for duration and frequency of stretched approach $F(1,36)= 45.35, p<.0001$ and $F(1, 36)= 56.38, p<.0001$, respectively). TMT exposure decreased the duration ($F(1,36)=5.46, p<.025$) but not the frequency ($p<.672$) of direct vial contact. In addition, there was a significant interaction effect of condition by treatment ($F(1,36)=8.82, p<.005$) for the duration of rearing. Post hoc comparisons indicated a non-significant trend for TMT to increase the duration rearing only in VEH-treated rats ($p<.07$).

Estradiol treatment decreased both the duration (significant main effect: $F(1,36)=4.10, p<.05$) and frequency (significant main effect: $F(1,36)=5.10, p<.03$) of stretched approach behaviour. However, estradiol treatment tended to increase both the frequency and duration of freezing behaviour in both TMT and control odour-exposed females (main effects for duration and frequency of freezing: $F(1,25)=3.85, p<.06$ and $F(1,25)=3.78, p<.06$, respectively). A priori we were interested in how hormone treatment affected behaviour in response to TMT. Estradiol increased freezing only in rats exposed to TMT (planned comparisons $p<.02$).

Estradiol treatment did not affect the duration or frequency of any other behaviour ($p<.45$ to $p<.92$).

Both the frequency ($r=0.45, p<.10$) and duration ($r=0.40, p<.16$) of freezing tended to positively correlate with cell proliferation. Neither cell proliferation nor pyknotic cell counts were significantly correlated with any of the other behavioural measures. Serum levels of estradiol were not correlated with any of the behavioural measures.

**Experiment 2**

**Estrogen and progesterone treatment suppressed cell proliferation and cell death** but did not affect cell proliferation in response to TMT.
EB-P-treated females had fewer BrdU-labeled cells than VEH-treated females regardless of condition (main effect of treatment: \( F(1, 16)=4.88, p<0.04 \)). There was no significant main effect of condition or interaction (\( p<0.28 \) and \( p<0.47 \); see Figure 9A).

EB-P treatment tended to decrease the number of pyknotic cells in granule cell layer (main effect of condition: \( F(1, 16)=3.47, p<0.08 \); see Figure 9B) but there were no other significant main or interaction effects (\( p<0.38 \) and \( p<0.72 \)).
Figure 9. A) Group means of the density of BrdU-labeled cells in estrogen and progesterone (EB-P) or vehicle (VEH) treated females exposed to control odour or TMT. EB-P treatment significantly suppressed cell proliferation in females exposed to either EB-P or VEH. B) Group means of the density of pyknotic cells in the granule cell layer of EB-P and VEH treated females (n=5 for all groups). Data is expressed in means ±SEM.
As expected, serum estrogen and progesterone were significantly higher in EB-P-treated compared to the VEH-treated females (main effect of treatment for estrogen: $F(1, 32)=6.32$, $p \leq 0.017$, progesterone: $F(1, 32)=19.01$, $p \leq .0001$). Interestingly, plasma corticosterone levels did not differ between groups ($p \leq .66$ to $p \leq .90$; see Table 6).

Table 6. Mean (±SEM) plasma hormone levels in EP and VEH-treated females.

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Plasma Corticosterone (µg/dl)</th>
<th>Plasma Estradiol (pg/ml)</th>
<th>Plasma Progesterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP TMT (n=3)</td>
<td>60.47(±8.2)</td>
<td>17.95(±8.0)</td>
<td>11.10(±2.0)</td>
</tr>
<tr>
<td>EP Control (n=3)</td>
<td>69.20(±3.7)</td>
<td>37.83(±26.7)</td>
<td>12.08(±1.7)</td>
</tr>
<tr>
<td>VEH TMT (n=3)</td>
<td>55.09(±13.1)</td>
<td>6.91(±1.4)</td>
<td>4.72(±1.1)</td>
</tr>
<tr>
<td>VEH Control (n=3)</td>
<td>59.50(±11.6)</td>
<td>4.23(±0.8)</td>
<td>3.36(±0.8)</td>
</tr>
</tbody>
</table>
Estrogen and progesterone treatment increased defensive behaviour due to TMT exposure

Figure 10 illustrates the duration and frequency of defensive behaviours in the EB-P- and VEH-treated females: A) duration and B) frequency of defensive burying, and C) duration and D) frequency of stretched approach. Figure 11 illustrates the A) duration and B) frequency of freezing and C) frequency of wet-dog shakes. Table 7 describes the frequency and duration of direct contact, grooming and rearing.
Figure 10. Group means for A) frequency and B) duration of defensive burying and C) frequency and D) duration of stretched approach in EB-P and VEH-treated females exposed to control odour or TMT (n=9 per group). Data is expressed in means ±SEM.
Figure 11. Group means for A) frequency and B) duration of freezing and C) frequency of wet dog shakes in EB-P and VEH treated females exposed to control odour or TMT (n=9 per group). Data is expressed in means ± SEM.
Table 7. Mean (±SEM) frequency and duration of nondefensive behaviors in EP and VEH-treated females.

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Frequency of Direct Contact</th>
<th>Duration of Direct Contact (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP TMT (n=9)</td>
<td>0.44(±0.2)</td>
<td>1.50(±0.8)</td>
</tr>
<tr>
<td>EP Control (n=9)</td>
<td>3.0(±0.9)</td>
<td>9.67(±3.3)</td>
</tr>
<tr>
<td>VEH TMT (n=9)</td>
<td>0.11(±0.1)</td>
<td>0.11(±0.1)</td>
</tr>
<tr>
<td>VEH Control (n=9)</td>
<td>3.67(±0.4)</td>
<td>13.89(±1.9)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Frequency of Rearing</th>
<th>Duration of Rearing (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP TMT (n=9)</td>
<td>28.0(±6.9)</td>
<td>102.53 (±24.1)</td>
</tr>
<tr>
<td>EP Control (n=9)</td>
<td>19.56(±5.0)</td>
<td>68.67(±56.2)</td>
</tr>
<tr>
<td>VEH TMT (n=9)</td>
<td>30.78(±6.3)</td>
<td>164.45(±41.2)</td>
</tr>
<tr>
<td>VEH Control (n=9)</td>
<td>28.56(±3.0)</td>
<td>110.11(±13.5)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Frequency of Grooming</th>
<th>Duration of Grooming (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP TMT (n=9)</td>
<td>5.67(±1.1)</td>
<td>60.67(±12.3)</td>
</tr>
<tr>
<td>EP Control (n=9)</td>
<td>2.00(±0.6)</td>
<td>28.44(±11.9)</td>
</tr>
<tr>
<td>VEH TMT (n=9)</td>
<td>5.00(±0.6)</td>
<td>57.33(±12.3)</td>
</tr>
<tr>
<td>VEH Control (n=9)</td>
<td>4.22(±1.6)</td>
<td>43.11(±21.0)</td>
</tr>
</tbody>
</table>

TMT increased both the duration and frequency of stretched approach behaviours (significant main effects of duration: F(1, 32)=12.35, p≤.001 and frequency: F(1, 32)=11.63, p≤.002). TMT did not affect the duration (p≤.37) or frequency (p≤.61) of freezing behaviour. TMT
significantly increased the frequency of wet-dog shakes regardless of condition (main effect of
treatment: \( F(1, 32)=10.8, p \leq 0.003 \)). TMT exposure also increased the frequency of grooming
(\( F(1, 32)=4.238, p \leq 0.05 \)). As expected, TMT decreased the duration and frequency of direct vial
contact (significant main effects of duration: \( F(1, 32)=30.05, p \leq 0.0001 \) and frequency: \( F(1, 32)=38.91, p \leq 0.0001 \)). Neither TMT nor EB-P significantly affected the duration or frequency of
rearing.

*A priori* we were interested in the effect of EB-P treatment on defensive behaviour in
response to TMT. TMT significantly increased defensive burying in EB-P treated females
(duration: \( p \leq 0.009 \), and frequency: \( p \leq 0.005 \)) but not in VEH treated females (duration: \( p \leq 0.6 \) and
frequency: \( p \leq 0.5 \)). EB-P treated females exposed to TMT defensively buried more than VEH
treated females exposed to TMT (both duration and frequency \( p \leq 0.05 \)). EB-P treatment
suppressed the frequency (main effect of treatment: \( F(1, 32)=5.60, p \leq 0.02 \)) and tended to suppress
the duration (main effect of treatment: \( p \leq 0.08 \)) of freezing.

**Freezing behaviour positively correlated with cell proliferation**

Cell proliferation was significantly positively correlated with freezing behaviour
(duration: \( r=0.59, p \leq 0.008 \); frequency: \( r=0.65, p \leq 0.002 \)). Estradiol levels were positively correlated
with wet-dog shakes (\( r=0.47, p \leq 0.004 \)). There were no other significant correlations between
hormone levels or cell death and any of the behaviours that we examined.

### 3.3 Discussion

Our data suggests that the behavioural and neural response to estradiol treatment is
profoundly affected by pre-treatment with a low dose of estradiol and progesterone. Further, we
found that ovarian hormone treatment affected the behavioural, hormonal, and cell death
response but not the cell proliferation response to TMT. This is consistent with previous studies suggesting that females do not respond to TMT with changes in cell proliferation (Chapter 2) and suggests that the sex difference in cell proliferation in response to TMT exposure (Chapter 2) is not likely related to circulating levels of estradiol in adult females. Although female rats did not show altered cell proliferation in response to TMT, these data are the first to indicate that cell proliferation rates were positively correlated with freezing in female rats, indicating that a longer duration of freezing was associated with increased cell proliferation. An acute estradiol treatment (EB) enhanced cell proliferation whereas chronic treatment with combined estradiol and progesterone prior to estradiol treatment (EB-P) suppressed cell proliferation regardless of odour exposure condition. This is the first demonstration that pre-treatment with estradiol and progesterone suppresses the cell proliferation response to acute estradiol treatment. EB treatment alone also attenuated both the elevated levels of cell death and corticosterone in response to TMT. However, in striking comparison, EB-P treatment suppressed cell death in both odour exposure conditions without any significant effect on levels of corticosterone. EB and EB-P treatment also differentially affected behaviour. EB treatment did not significantly affect behaviour in response to TMT, whereas EB-P treatment increased defensive behaviour in response to TMT. Thus, consistent with the suggestions of others (Young et al., 2001), our data indicates that the duration of estrogen exposure and a potential interaction with progesterone are important to consider when examining the effects of estradiol on stress responsivity.

**Pre-treatment with estradiol and progesterone profoundly changed cell proliferation in response to estradiol treatment**

Consistent with previous studies, acute treatment with a high dose of estradiol increased the amount of new cells produced in the dentate gyrus (Ormerod & Galea, 2001; Tanapat et al., 1999). The majority of these newly produced cells became neurons after surviving for 3 weeks.
Remarkably however, we found that chronic treatment with a low dose of estradiol and progesterone before estradiol administration suppressed cell proliferation. In other words, estradiol treatment enhanced cell proliferation relative to vehicle whereas prior pretreatment with low level estradiol and progesterone (EB-P) eliminated the ability of the same dose of estradiol to enhance cell proliferation. Here we are the first to demonstrate that chronic treatment of estradiol and progesterone reverses the cell proliferation response to subsequent estradiol treatment. This outcome may reflect estradiol time-dependently suppressing neurogenesis, an effect previously shown in both rats and meadow voles (Ormerod and Galea, 2001). Ormerod and Galea (2001) have recently shown that estradiol initially enhances cell proliferation (4 hours post-injection of a 10 µg dose of EB (the same dose and administration time course used in the present study) but then subsequently suppresses cell proliferation (48 hours post-injection).

Although acute exposure to estradiol enhances cell proliferation via serotonin (Banasr, Hery, Brezun, & Daszuta, 2001), there are a number of other factors that may also contribute to estradiol’s effects on cell proliferation, such as N-Methyl-D-Aspartate receptors (NMDArs) (estradiol can negatively modulate NMDArs (Gazzaley, Weiland, McEwen, & Morrison, 1996; Weiland, 1992) which in turn increases cell proliferation (Cameron, Tanapat, & Gould, 1998), a variety of second messenger pathways or direct stimulation of gene transcription via estrogen receptors (Shughrue et al., 1992). Chronic estradiol exposure may suppress cell proliferation by increasing adrenal steroids (Ormerod, Lee and Galea, submitted). Although we did not see an increase in circulating corticosterone in EB-P treated females, this does not preclude chronic estradiol treatment having an effect on adrenal activity. Others have found increases in adrenal mass (an indicator of increased HPA activity) in response to chronic estradiol treatment but not an accompanying increase in circulating corticosterone (Ormerod, Lee and Galea, submitted; Young et al., 2001).
Chronic exposure to estradiol in EB-P treated females (1 week) in the present study may have changed the ability to respond to subsequent estradiol treatment. Chronic EB-P treatment could downregulate estrogen and progesterone receptors in ovariectomized females (Shughrue et al., 1992) and change the expression of glucocorticoid receptors (GR) (Ferrini & De Nicola, 1991, Ferrini et al., 1995; Burgess & Handa, 1992; Patchev, Hassan, Holsboer, & Almeida, 1996) and therefore affect the physiological response to both estrogen and corticosterone. Chronic hormone treatment likely acted to counteract ovariectomy-induced upregulation of estradiol (and progesterone) receptors (Shughrue et al., 1992). Thus, fewer estradiol receptors may have been available to respond to the high dose of estradiol in the group pretreated with estradiol and progesterone. This change in the number of estradiol receptors may have led to changes in the cell proliferation response to estradiol treatment. Chronic progesterone administration in EB-P treated females may also have influenced cell proliferation, potentially by affecting the cholinergic, GABAergic, or serotonergic systems or estrogen, all of which modulate cell proliferation (for review see Ormerod, Falconer, and Galea, in press).

**Estradiol did not affect cell proliferation in response to TMT**

We have previously shown that males but not females exhibit suppressed neurogenesis in response to TMT (Chapter 2). TMT suppresses cell proliferation in males but not in females (Chapter 2). In the present study, we found that estradiol in ovariectomized females did not affect the amount of cell proliferation in response to TMT. In males, adrenal steroids suppress cell proliferation (Cameron & Gould, 1994; Gould, Cameron, Daniels, Woolley and McEwen, 1992; Schlessinger, Cowan & Gottlieb, 1975) and mediate the suppression of cell proliferation in response to TMT (Tanapat et al., 2001). Our results also suggest that increases in adrenal steroids are not related to suppressed cell proliferation in females. As evidence, we found that corticosterone was significantly elevated in vehicle treated females in response to TMT exposure.
but the number of labelled cells did not differ from control odour-exposed females. The present study suggests that activational levels of ovarian hormones or adrenal steroids in adult females do not affect the cell proliferation response to TMT. Taken together, our results may suggest that gonadal hormones present during development may organize the sex difference in cellular response to TMT.

**Estradiol protected against TMT-induced increases in corticosterone and cell death**

Estradiol treatment (EB) reduced both the increased corticosterone response and the increased cell death response to TMT exposure that was evident in vehicle-treated females. Prior pretreatment with estradiol and progesterone (EB-P) altered both the cell death and corticosterone response to TMT. There is a curvilinear relationship between corticosterone level and cell death in the dentate gyrus, as both very low levels (via adrenalectomy) and very high levels (via stress) of corticosterone increase cell death, while an optimal level of corticosterone suppresses cell death. (Cameron & Gould, 1996; Mizoguchi, Kunishita, Chui, & Tabira, 1992; Sapolsky, Uno, Rebert, & Finch, 1990; Reagan & McEwen, 1997). These data point to the possibility that the corticosterone response to TMT is related to the cell death response to TMT.

EB-P treatment tended to decrease cell death. These findings are consistent with observations that both estrogen and progesterone are neuroprotective in adulthood (Wise, Dubal, Wilson, Rau, & Liu, 2001). Estrogen and/or progesterone may decrease cell death by inducing the expression of anti-apoptotic proteins such as Bcl-2 (Nilsen et al., 2002) or by acting as an antioxidant (Culmsee, Vedder, Ravati, & Junker, 1999) and/or changing neurotrophic factor expression in the dentate gyrus (Gibbs, 1998, 1999).

Vehicle-treated females that received implants (Experiment 2) had higher levels of corticosterone than those that received injections (Experiment 1). This is consistent with previous findings that exposure to silastic implants significantly increases plasma corticosterone
levels relative to non-implanted controls (Fernandes, McKittrick, File, & McEwen, 1997; Kipp & Fox, 1989). A chronic stressor (Armario, Garcia-Marquez, & Jolin, 1987; Mizoguchi et al., 2001) can lead to habituation of the HPA response to stress. Chronic exposure to mild stress blunts the corticosterone response to a subsequent stressor (Armario et al., 1987). Thus, the chronic exposure to silastic implants might increase circulating levels of plasma corticosterone and blunt the corticosterone response to TMT. Indeed, these changes occurred in our vehicle-treated females: levels of circulating corticosterone were elevated in the implanted vehicle group relative to the non-implanted vehicle group and there was a significant corticosterone response to TMT in the non-implanted group only. Consistent with the idea that corticosterone levels are an important indicator of cell death in the dentate gyrus, vehicle-treated females that received implants and had higher corticosterone levels (Experiment 2) had more pyknotic cells than those that received injections (Experiment 1).

Pretreatment with estradiol and progesterone changed the effect of estradiol on the behavioural response to TMT

EB treatment did not specifically affect defensive behaviour in response to the predator odour stressor. Rather, we found that EB treatment decreased the expression of stretched approach behaviour in both control odour and TMT-exposed females. This is partially consistent with studies showing that increased circulating estradiol is associated with a non-significant decrease in defensive burying ((Diaz-Veliz et al., 1991; Fernandez-Guasti et al., 1992; Fernandez-Guasti et al., 1999). This suppression of defensive behaviour was not a result of changes in general motor activity, as rearing behaviour did not change in response to EB treatment. In contrast, EB-P treatment affected defensive behaviour specifically in response to TMT; EB-P treatment increased defensive burying only in TMT-exposed females. This increase in defensive behaviour in response to TMT may suggest that EB-P treated females are more “anxious” in response to predator odour (consistent with previous suggestions (Blanchard et al.,
Thus, pretreatment with estradiol and progesterone increased the defensive behavioural response to TMT in estradiol treated females. Contrary to our findings, many studies have found that low levels of estradiol are associated with greater expression of certain behavioural indices of “anxiety”. Females in diestrus (low levels of estradiol and progesterone) spend less time in the open arms of the elevated plus maze than females in proestrus and estrus (high levels of estradiol and progesterone) (Galeeva et al., 2001; Mora et al., 1997; Zimmerberg et al., 1993) and ovariectomized rats exhibit less exploration of open arms than intact rats in the elevated plus maze (under bright light) (Mora et al., 1997). However, lower levels of “anxiety” observed during high estrogen and progesterone stages of the estrous cycle may be due to high levels of progesterone rather than high levels of estrogen. High levels of circulating progesterone and infusions of progesterone into the hippocampus have been associated with less anxiety in the elevated plus maze and social interaction tests (Frye, 2001; Mora et al., 1997), and a decrease in defensive burying (Frye, 2001). Further, estrogen antagonizes the anti-anxiety effects of progesterone (Mora et al., 1997) and diazepam (Nomikos & Spyraki, 1988) in the elevated plus maze. We did not observe an anxiolytic effect of a low dose of progesterone in our EP treated females. This is partially consistent with observations that a low dose of progesterone does not change the effects of estradiol treatment on HPA activity in response to stress (Young et al., 2001). However, it may be important to distinguish hormonal indicators of stress from behavioural measures of “anxiety”, as discussed below.

The hormonal response to TMT was not related to the behavioural response to TMT

Even though EB treatment protected against the increase in corticosterone in response to TMT (indicating less of a hormonal response to stress), EB did not affect behaviour in response to TMT. Also, EB-P treatment increased behavioural measures of “anxiety” but did not affect
the hormonal stress response to TMT. This suggests that the hormonal stress response and the expression of certain defensive or "anxious" behaviour are not related. This is supported by findings that during diestrus (low levels of estradiol and progesterone) there is an increased expression of certain behavioural indices of "anxiety" (Galeeva et al., 2001; Zimmerberg et al., 1993) but there is a decreased HPA response to stress (Viau and Meaney, 1991) relative to proestrus (high levels of estradiol and progesterone).

Our finding that a high dose of EB decreased the corticosterone response to TMT is in apparent contrast with observations that treatment with a high dose of EB has been shown to increase the sensitivity of the hypothalamic pituitary adrenal axis (HPA) to stress (Burgess & Handa, 1992; Carey et al., 1995; Viau & Meaney, 1991) and that there is an enhanced HPA responsivity to stress during proestrus, when levels of both estrogen and progesterone are high (Viau & Meaney, 1991). However, it is possible that intact females in proestrus show a higher HPA stress response as a result of an increased level of progesterone or interactions between estrogen and progesterone rather than high levels of estrogen per se (Young et al., 2001). Interestingly, many studies in stressed and non-stressed intact females have shown enhanced HPA activity during late proestrus, when levels of both progesterone and estrogen are high, relative to other stages of the estrous cycle including early proestrus (when only estradiol levels are elevated) (Carey et al., 1995; Pollard, White, Bassett, & Cairncross, 1975, Ogle & Kitay, 1977, Buckingham, Dohler, & Wilson, 1978). Further, in at least one of these studies examining the effects of estradiol on HPA response to stress the hormone administration protocol has led to plasma hormone levels in the supra-physiological range (approximately 470-550 pg/ml of EB or approximately five times the levels that we obtained in our females (Viau and Meaney, 1991) or has chronically administered high levels of estradiol (75 pg/ml for 21 days) (Burgess and Handa, 1992). As Young and colleagues (2001) suggest, the dose of estradiol is likely also important in influencing the HPA response to stress. Indeed, our results are consistent with Young et al's
findings (2001) that a high physiological dose of estradiol suppresses the HPA response to stress.

**Freezing behaviour was related to cell proliferation**

We are the first to demonstrate that freezing behaviour is significantly correlated with cell proliferation in female rats exposed to predator odour. EB treated females showed both enhanced cell proliferation and freezing whereas EB-P treated females showed both suppressed cell proliferation and freezing relative to vehicle-treated females. This positive relationship between freezing behaviour and cell proliferation is interesting considering that we may expect a negative correlation in males. Males have been shown to freeze more (Morrow, Redmond, Roth, & Elsworth, 2000; Morrow, Roth, & Elsworth, 2000; Wallace & Rosen, 2000) and show suppressed cell proliferation (Tanapat et al., 2001, Chapter 2; Holmes and Galea, 2001) in response to TMT exposure. However, in previous studies that used the same behavioural paradigm as the present study (Chapter 2; Holmes & Galea, 2001) we did not see any change in freezing behaviour (potentially as a result of having bedding in our testing chambers (see Chapter 2; Falconer and Galea, submitted)) and found that cell proliferation was suppressed in response to TMT in males. Therefore, it is certainly plausible that cell proliferation is correlated with freezing behaviour in males, as no studies have systematically measured both freezing behaviour and cell proliferation in males using the same testing paradigm. The correlation between freezing behaviour and cell proliferation in the dentate gyrus is intriguing and appropriate considering that the development of freezing behaviour is dependent on an intact dentate gyrus (Takahashi, 1996; Takahashi, 1995; Takahashi & Goh, 1996; Takahashi & Kim, 1995) and that the expression of freezing in adulthood is dependent on the hippocampus (Blanchard & Blanchard, 1972).

3.4 Implications

Our data demonstrates that the effects of estradiol on the hormonal, neural, and
behavioural response to acute stress can be dissociated. Estradiol treatment can *decrease* the hormonal and cell death stress response while *increasing* the defensive behavioural response to acute stress. We are also the first to observe that freezing behaviour is related to cell proliferation in the dentate gyrus of adult females in response to TMT. We have demonstrated that chronically pre-exposing ovariectomized females to a low dose of estradiol and progesterone can change the subsequent hormonal, behavioural and neural stress response to estradiol treatment. Consistent with the suggestions of others (Young et al., 2001), our data indicate that the duration of estrogen exposure and a potential interaction with progesterone are important factors to consider when examining the effects of estradiol on stress responsivity. Future studies should further examine this dynamic ability of ovarian hormones to differentially modulate aspects of the stress response.
These papers illustrate that there are sex differences in both cell proliferation and cell death in the dentate gyrus of adult rats in response to predator odour stress. Neither changes in estradiol nor adrenal hormones in adult females appear to significantly affect cell proliferation in response to predator odour. This suggests that the sex difference in the cell proliferation response to predator odour is not likely due to changes in estradiol or adrenal levels in adult females. It is possible that the sex difference in the cell proliferation response to stress is a result of the influence of hormones in adult males or in males and/or females during development. Alternatively, progesterone levels may play a role in the cell proliferation response to stress in adult females. Our work raises the possibility that adult males and females possess very different neural mechanisms for responding to predator odour.

This thesis has also provided evidence that cell death and cell proliferation in response to predator odour stress are related in males but are dissociated in females. For example, in males both cell proliferation and cell death are suppressed whereas in ovariectomized females cell proliferation is unaffected while cell death is increased in response to predator odour exposure. This suggests that cell turnover is stabilized in males but not females when exposed to predator odour. In males, stabilizing cell turnover may be a homeostatic defense mechanism and therefore help the system adapt to acute stress. In other words, suppressing cell turnover during acute stress may be related to the operation of systems such as the HPA to preserve homeostasis and help the body adapt to challenges. This implies that the lack of suppressed cell proliferation with predator odour exposure in females might be maladaptive. Alternatively, the suppression of cell turnover in response to predator odour stress in males may be a response to changes in blood flow during an acutely stressful event. Interestingly, there are sex differences in circulation during stress (Eikelis & Van Den Buuse, 2000). Thus, the sex difference in the cellular response
to predator odour may be a result of this difference in blood flow. Palmer and colleagues (2000) offer support for this hypothesis, as they hypothesize that hematopoetic stem cells are precursor cells that give rise to new neurons in the hippocampus and that increases in neurogenesis in response to running wheel activity in rats (van Praag, Kempermann, & Gage, 1999) are a result of increases in blood flow.

The suppressed cell proliferation following acute predator odour stress in males is transient (is evident up to 1 week after cell birth but not 3 weeks after cell birth as shown by Tanapat et al., 2001). We have shown that the deficit in new cells following acute stress is affected by subsequent experience; the number of new cells in predator odour exposed males is restored to control levels with repeated predator odour exposure. Therefore, it is unlikely that the suppression of cell proliferation changes the number of cells incorporated into existing circuitry and therefore subsequent hippocampus-dependent learning.

Although the suppression of cell proliferation in response to acute predator odour exposure in males unlikely affects learning 3 weeks later, it is possible that the suppression affects memory in the short-term as acute stress has been shown to reversibly and transiently suppress the neural mechanisms involved in hippocampus dependent memory (Kirschbaum et al., 1996; McEwen & Sapolsky, 1995). Indeed, an acute exposure to TMT has been previously shown to disrupt short-term working memory in male rats (Morrow, Roth, & Elsworth, 2000). There are also sex differences in short-term memory following acute stress in humans (Wolf et al., 2001) and in rats (Wood & Shors, 1998). In humans, increases in cortisol are associated with poorer performance on a recall test in males but there is no relationship between cortisol and memory in females (Wolf et al., 2001). This sex difference in humans may be comparable to our observation that male rats show a negative relationship between the cellular and corticosterone response to TMT while females exposed to estradiol do not show a relationship between
corticosterone and cell proliferation. Thus, sex differences in the cellular response to acute predator odour stress may be related to sex differences in short-term memory. Further, in rats hippocampus-dependent learning is enhanced in males but not females 24 hours after an acute stressor (Wood & Shors, 1998). This suggests the possibility that the suppressed cell proliferation following acute stress in males may be advantageous for later learning (24 hours later).

Although we did not observe an effect of predator odour exposure on cell proliferation in females treated with ovarian hormones, a very intriguing finding was that the cell proliferation response to ovarian hormone treatment was sensitive to the hormone treatment protocol. Pretreatment with estrogen and progesterone completely reversed the direction of cell proliferation in response to subsequent estrogen treatment. This observation has critical implications for the study of hormonal effects on cell proliferation. This data suggests that it may be important to use intact females before attempting to model the effects of hormones in females because response to hormonal treatment is sensitive to both dose and length of hormone administration (as suggested by Viau, V., in press; Young et al., 2001).

We have shown that the sex differences in cell proliferation and cell death in response to acute predator odour stress are not related to differences in the behavioural response to predator odour. However, in our second study we found that changes in cell proliferation as a result of estrogen treatment were positively related to the expression of freezing behaviour. Although we did not see a relationship between freezing behaviour and cell proliferation in males, it is possible that our behavioural paradigm did not allow easy discrimination of changes in freezing behaviour (perhaps due to a floor effect and/or the presence of bedding (see Wallace & Rosen, 2000) making it difficult to observe a possible relationship between freezing and cell proliferation. Future studies could try to discern whether a similar positive relationship exists
between freezing and cell proliferation. Further, studies could examine whether the positive 
relationship between freezing behaviour and cell proliferation is preserved in intact females.

In summary, there are sex differences in the cellular response of the adult hippocampus to 
predator odour exposure. Changes in cell proliferation may be related to freezing behaviour 
and/or short-term hippocampus-mediated memory. Studies should directly examine this 
possibility, potentially by acutely administering an anti-mitotic agent to the hippocampus and 
testing the subsequent effects of this treatment on learning and freezing behaviour in males and 
females.
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