DEFENSIVE BEHAVIOR AND HIPPOCAMPAL CELL PROLIFERATION:
DIFFERENTIAL MODULATION BY NALTREXONE DURING STRESS

by

MELISSA MARIE HOLMES

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Department of _Psychology_

The University of British Columbia
Vancouver, Canada

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Abstract

The present study was designed to investigate the role of endogenous opioids in both the expression of defensive behaviors and the suppression of cell proliferation that are induced by exposure to predator odor, trimethyl thiazoline (TMT). Adult male rats were injected with either naltrexone (5mg/kg) or saline 30 minutes prior to being transferred to a testing chamber where they were exposed to either TMT (250 µl) or control odor (distilled water 250 µl). Rats were videotaped and the expression of defensive and non-defensive behaviors was scored over the first fifteen minutes of exposure. Bromodeoxyuridine (BrdU, 200mg/kg), a thymidine analogue, was injected i.p. 15 minutes after exposure to the odors and the rats were perfused 1 hour after BrdU administration. As previously reported, the expression of defensive behaviors was increased after exposure to TMT. Pre-treatment with naltrexone attenuated the expression of defensive behaviors (defensive burying and directed stretch approach), independent of any drug effects on ability to perform the required motor patterns. TMT exposure rapidly suppressed the number of proliferating (BrdU-ir positive) cells in the dentate gyrus. In addition, naltrexone administration alone suppressed cell proliferation in the dentate gyrus. Thus, consistent with other reports, endogenous opioids mediate the expression of defensive behaviors in response to predator odor exposure. Furthermore, endogenous opioids may play a regulatory role in the control of cell proliferation in the dentate gyrus of adult male rats.

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Abbreviations

ANOVA	analysis of variance
BrdU	bromodeoxyuridine
NAL	naltrexone treated
NAL+TMT	naltrexone-treated with TMT exposure
SAL	saline-treated
SAL+TMT	saline-treated with TMT exposure
TMT	trimethyl thiazoline

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INTRODUCTION

Stress can have marked neural and behavioral effects across mammalian species. Specifically, chronic exposure to stress can result in neural atrophy and associated behavioral deficits (e.g., learning and memory) in rodents (Galea et al., 1997; Luine et al., 1994; Watanabe et al., 1992; Woods & Shors, 1998) and humans (de Leon et al., 1998). Exposure to acute psychosocial stressors has been shown to suppress cell proliferation in the dentate gyrus of developing rats (Tanapat et al., 1998), adult tree shrews (Gould et al., 1997) and adult marmoset monkeys (Gould et al., 1998). Preliminary work has also demonstrated that brief exposure to the risk of predation (i.e., predator odor) can induce a rapid significant suppression in cell proliferation in the dentate gyrus of adult male rats (Falconer & Galea, 2000; Galea et al., 1996).

Exposure to the risk of predation has also been shown to have profound effects on behavior. In rodents, brief exposure to the scent or call of a predator can elicit the expression of a repertoire of defensive behaviors including ultrasonic vocalizations and avoidance behavior (Blanchard & Blanchard, 1989; Blanchard et al., 1990a; Blanchard et al., 1990b; Blanchard et al., 1991; Blanchard et al., 1998; Hendrie & Neill, 1991; Zangrossi & File, 1992). Defensive behaviors likely assist the animal to survive stressful episodes as well as enable the animal to learn about the threat stimuli (Blanchard et al, 1990a; Blanchard et al., 1990b). The odorant molecule trimethyl thiazoline (TMT), a primary component to fox feces (Vernet-Maury et al., 1984), has been used as a predator odor stimulus as the fox is a natural predator of the rat (Catarelli & Chanel, 1979). Exposure to TMT has been shown to elicit defensive behavioral responses (e.g. freezing; Perrot-Sinal et al., 2000; Wallace & Rosen, 2000), stress-induced analgesia (Hotsenpiller & Williams, 1997), the suppression of cell proliferation in the dentate gyrus (Galea et al., 1996), and the induction of fast wave bursts in the dentate gyrus (Heale et al., 1994) in adult rodents.

Substantial evidence suggests a role for endogenous opioids in many physiological and behavioral stress responses (for review see McCubbin, 1993). Opioids are often released in response to a stressor resulting in opioid-dependent stress-induced analgesia (Grau et al., 1981; Miczek et al., 1982). Several types of stressors can induce opioid-dependent analgesia including shock (DeVries et al., 1979), risk of predation (Hendrie, 1991; Lester & Fanselow, 1985), and even novelty (Siegfried et al., 1987). Opioids have also been shown to both inhibit (Tapp et al., 1981) and stimulate (Buckingham, 1982) stress-induced hypothalamic-pituitary-adrenal axis activity; interestingly, these discrepancies may be a result of central versus peripheral opioid activity (Odio & Brodish, 1990). Additionally, opioid receptors are distributed throughout the neural circuitry involved in stress responses (Hammer et al., 1990), including the dentate gyrus of the hippocampus (Takemori & Portoghese, 1992).

It has been hypothesized that endogenous opioids may have a causal role in the expression of defensive behaviors that are seen following exposure to a stressor (Bolles & Fanselow, 1980). Specific support for this hypothesis comes from data demonstrating that acute administration of opioid *agonists* can induce a generalized increase in defensiveness including a significant increase in crouching behavior and time spent avoiding a predator stimulus (Blanchard et al., 1991). Furthermore, acute administration of opioid *antagonists* can increase latency to freezing (Coimbra et al., 2000). Additionally, endogenous opioids may also mediate the stress-induced suppression of cell proliferation in the dentate gyrus as opioid activity has been shown to influence cell proliferation and neurogenesis in the absence of stress (Eisch et al., 2000). These behavioral and neural stress responses may be mechanistically linked as the hippocampus is involved in the expression of certain defensive behaviors (e.g., avoidance; Blanchard & Blanchard, 1972) and dentate gyrus granule cells in particular have been shown to be essential for freezing behavior in developing rats (Takahashi, 1995). In the present experiment

we investigated the role of endogenous opioids in both the expression of defensive behaviors and the suppression of dentate gyrus cell proliferation in response to an acute ecologically-relevant stressor (predator odor).

MATERIALS AND METHODS

Subjects

Forty-eight male Sprague-Dawley rats (University of British Columbia Animal Care Centre, Vancouver BC) weighing approximately 400g were used in this experiment. Animals were single housed in opaque plastic bins with absorbent bedding in a temperature controlled colony room. They were maintained on a 12-hour LD cycle with food (Lab diet #5012, Jamieson) and water available ad libitum. All manipulations were performed according to the guidelines set out by the Canadian Council for Animal Care (CCAC) and the UBC Animal Care Committee. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

Apparatus

Testing was conducted in a clear Plexiglas chamber (29 cm x 30 cm x 46 cm) that was lined with approximately 6 cm of corncob bedding material. A small plastic vial containing one piece of Kimwipe tissue was placed in the same corner of the testing chamber for all animals. Either trimethyl thiazoline (TMT; Pherotech, Delta B.C.; approximately 250μl), a primary component of fox feces, or distilled water (control odor; approximately 250μl) was placed on the tissue in the vial immediately prior to testing. All testing took place in a fumehood to prevent diffusion of the odor throughout the room. A video camera was set up directly in front of the fumehood and testing chambers to record behavior for later analysis.

Procedures

Rats were handled and habituated to the testing apparatus and fumehood for 15 min a day for the 4 days prior to testing. Rats were assigned to one of four groups (n=12 per group): saline + control odor (SAL), naltrexone + control odor (NAL), saline + TMT odor (SAL+TMT), or naltrexone + TMT odor (NAL+TMT). On test day, all animals were injected with either naltrexone (5mg/kg i.p.) or saline 30 min prior to being placed in the testing chamber. Behavior was recorded on videotape for the first 15 min of exposure to either TMT or control odor. Immediately following behavioral assessment (i.e., 15 min after the presentation of the odor), a subset of animals (n=7 per group) was injected with bromodeoxyuridine (BrdU, 200mg/kg, i.p.), a marker of proliferating cells and their progeny. Rats remained in the chamber with the odor for one hour. Immediately after the hour, rats were given an injection of sodium pentobarbital (2ml/kg, i.p., Somnitol, MTC Pharmaceuticals) and transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer (PB). Brains were extracted, post-fixed overnight at 4°C and processed for BrdU immunohistochemistry (see below). Prior to perfusion, blood samples were collected from the right ventricle (N=12 per group) and were stored at 4° C. Serum was extracted following centrifution (4 g for 10 min) on the following day and then frozen at -70° C until radioimmunoassays were performed. Radioimmunoassays were performed to detect total corticosterone (CORT) as outlined by Weinberg and Bezio (1987). The intra-assay coefficient of variation was 1.55.

The following behaviors were scored for all animals with the observer blind to the experimental condition: defensive burying, stretch approach, rearing, contact with vial and resting/immobility. Defensive burying is characterized by the pushing of bedding toward the test object (i.e. vial with or without TMT odor; Pinel and Treit, 1978). Stretch approach is characterized by the hind paws remaining stationary while the animal extends and stretches the

front half of the body towards the test object (Pinel et al., 1989). Stretch approach sequences were divided into 'directed' stretch approach (when the sequence was performed directed to the vial) and 'undirected' stretch approach (when the behavior occurred anywhere else in the testing chamber). This distinction was made to assess the behavior towards the source of the odor as the odor permeates the testing chamber. Both frequency and duration of all behaviors were scored with the exception of resting/immobility (duration only).

Immunohistochemistry and Stereology

Brains were sliced into 40μm sections through the entire hippocampus using a vibratome (Leica) in a bath of 0.1M PB. Sections were treated with 0.2% H_2O_2 for 20 minutes, rinsed in PB, and mounted on to 3-aminopropyltriethoxysilane (3-AAS) treated slides. BrdU immunohistochemistry was performed as previously described (Ormerod & Galea, 2001). Briefly, cells were permeabilized with 0.05% Trypsin (Sigma Aldrich Chemicals) in Tris-HCl buffer (pH 7.5) containing 0.1% $CaCl_2$ DNA was denatured by applying 2N HCl for 30 minutes prior to application of 5.0% normal horse serum block for 30 minutes. Tissue was incubated overnight in mouse monoclonal antibody against BrdU (1:400; Boeringer Mannheim) at room temperature. Mouse secondary antibody (1:167; Vector Laboratories) was then applied and tissue was incubated for 2 hours prior to incubation in avidin-biotin horseradish peroxidase (1:50; Vector Laboratories) for 60 minutes. Finally, tissue was reacted for 10 minutes with 0.02% 3'3-diaminobenzidine (DAB; Sigma Aldrich Chemicals) and then counterstained with creysl violet, dehydrated, and coverslipped. Tissue was rinsed in 0.1M PB (pH 7.4) between all steps.

BrdU-positive cells in the granule cell layer (GCL) of the dentate gyrus were counted using a Nikon Eclipse ε600 light microscope (100x objective) with the experimenter blind to

experimental condition of the animal. BrdU-positive cells were counted if they were darkly stained and exhibited progenitor cell morphology (see Figure 1; Cameron et al., 1993). The majority of BrdU-labeled cells were located in the subgranular zone. Cells were counted in every twentieth section throughout the extent of the dentate gyrus region to determine stereological estimates. The granule cell layer (GCL) area was measured using Analytical Imaging Station software (AIS; Imaging Research Inc., Ontario, Canada). Estimations of total numbers of BrdU-labeled cells throughout the granule cell layer were made using a modified version of the optical fractionator method (West et al., 1991). Estimations of total granule cell layer volume were made using Cavalieri's principles (Gunderson et al., 1988).

Statistical Analysis

All behavioral, hormonal and histological variables (e.g. defensive burying) were individually analyzed using a two-way analysis of variance (ANOVA) with drug (saline, naltrexone) and odor (control, TMT) as the between-subjects factors. Behavioral data were analyzed from 45 rats, as data from three rats (2 SAL and 1 NAL) were lost due to equipment malfunction. Data from 25 of 28 rats were analyzed for histological measures as tissue from one SAL, one TMT, and one NAL+TMT, was damaged during processing. Unless otherwise stated, all post hoc analyses were performed using the Newman-Keuls procedure. Planned comparisons used a Bonferroni correction. All statistical procedures set $\alpha = 0.05$. While $p \leq 0.05$ will be referred to as statistically significant, these p values reflect the probability of the effect occurring solely by chance.

RESULTS

Exposure to TMT or pre-treatment with naltrexone attenuates the rate of cell proliferation in the dentate gyrus

TMT exposed groups had significantly fewer BrdU-labeled cells in the GCL than the control odor groups (main effect of odor; $F_{(1,21)}=12.613$, $p\leq0.002$; see Figure 2). Naltrexone-treated animals had fewer BrdU-labeled cells than saline-treated animals regardless of odor treatment (main effect of drug $F_{(1,21)}=6.479$, $p\leq0.019$; see Figure 2). There was no significant interaction effect for the number of BrdU-labeled cells in the GCL ($F_{(1,21)}=1.043$, $p\leq0.319$).

An ANOVA on GCL volume revealed that there were no significant main effects (drug: $F_{(1,21)}=0.155$, $p\leq0.698$; condition ($F_{(1,21)}=0.019$, $p\leq0.891$) or an interaction effect ($F_{(1,21)}=2.067$, $p\leq0.165$) which suggests that group differences in the numbers of BrdU-labeled cells were not due to group differences in GCL volume (see Table 1).

TMT exposure increases the serum concentration of corticosterone

TMT exposed animals, regardless of drug condition, had significantly higher levels of serum total corticosterone (CORT) (main effect of odor; ($F_{(1,46)}=9.224$, $p\leq0.004$); see Table 2) compared to animals exposed to control odor. There was no significant main effect of drug ($F_{(1,46)}=1.598$, $p\leq0.213$) nor an interaction effect ($F_{(1,46)}=0.217$, $p\leq0.644$).

TMT exposure increases the expression of defensive behaviors and pretreatment with naltrexone partially attenuates the expression of these defensive behaviors

Defensive Burying

There was a significant interaction effect between odor and drug for both frequency ($F_{(1,40)}=5.192$; $p\leq0.028$) and duration ($F_{(1,40)}=7.915$; $p\leq0.008$) of defensive burying (see Figure 3). There was also a significant main effect of odor on defensive burying for both frequency and duration ($F_{(1,40)}=5.50$, $p\leq0.024$; $F_{(1,40)}=20.80$, $p<0.001$, respectively) and drug

(F(1,40)=5.498, p≤0.024; F(1,40)=8.625, p≤0.005, respectively). Post-hoc analyses reveal that SAL+TMT animals displayed increased defensive burying compared to all other groups (frequency, p<0.002; duration, p<0.001, for all comparisons).

Stretch Approach

Directed Stretch Approach: There was a significant interaction effect between odor and drug for frequency (F(1,40)=4.34, p≤0.044; see Figure 4a) and a trend for an interaction effect for duration (F(1,40)=3.876, p≤0.056) of directed stretch approach (see Table 3). There was a main effect of odor for frequency (F(1,40)=14.28, p<0.001) and duration (F(1,40)=5.034, p≤0.03) of directed stretch approach. Similarly there was a trend for a main effect of drug for frequency (F(1,40)=4.34, p<0.051) but no effect on duration (F(1,40)=0.982, p≤0.328) of directed stretch approach. Post-hoc analyses revealed that SAL+TMT animals perform directed stretch approach more frequently than all other groups (p≤0.006 for all comparisons).

Undirected Stretch Approach: TMT-exposed animals exhibited increased frequency (main effect of odor; F(1,40)=29.39, p≤0.001) but not duration (F(1,40)=2.379, p≤0.131) of undirected stretch approach compared to control odor-exposed animals (see Figure 4b and Table 3). There was no main effect for drug on either frequency (F(1,40)=1.219, p≤0.276) or duration (F(1,40)=0.858, p≤0.36) of undirected stretch approach. There was no interaction effect for either frequency (F(1,40)= 0.62, p≤0.44) or duration (F(1,40)=0.007, p≤0.933) of undirected stretch approach. The partial attenuation of naltrexone on directed but not undirected stretch approach may indicate that the animals are able to dissociate the source of the odor from general odor permeation in the chamber.

Direct Contact with the Vial

TMT-exposed animals regardless of drug treatment showed less frequency (main effect of odor; $F(1,40)=4.16$, $p\leq0.048$) but not duration ($F(1,40)=0.726$, $p\leq0.399$) of direct contact with the vial (see Table 4). Naltrexone-treated animals regardless of odor demonstrated less frequency (main effect of drug; $F(1,40)=4.33$, $p\leq0.044$) but not duration ($F(1,40)=1.88$, $p\leq0.178$) of direct contact with the vial compared to saline-treated animals. There was no interaction effect for frequency ($F(1,40)=1.536$, $p\leq0.132$) but a trend for an interaction for duration ($F(1,40)=3.903$, $p\leq0.055$) of contact with vial. Planned comparisons reveal that NAL+TMT animals contact the vial less frequently than SAL+TMT animals ($p\leq0.023$) and NAL animals ($p\leq0.024$), suggesting that naltrexone alters contact behavior *only* in the presence of TMT.

Rearing

There was no significant main effect of odor or interaction effects for either frequency (main effect of odor: $F(1,40)=0.207$; $p\leq0.652$; interaction effect: $F(1,40)=1.59$; $p\leq0.215$) or duration (main effect of odor: $F(1,40)=2.013$, $p\leq0.164$; interaction effect: $F(1,40)=1.008$, $p\leq0.321$) of rearing (see Table 4). There was a main effect of drug ($F(1,40)=5.522$, $p\leq0.024$) with naltrexone-treated animals rearing less frequently than saline-treated animals but no main effect of drug on duration ($F(1,40)=0.644$, $p\leq0.427$). Because *a priori* we wanted to determine whether pre-treatment with naltrexone altered rearing behavior, planned comparisons were conducted and revealed that there was no significant difference between SAL and NAL control groups but that NAL+TMT animals rear less frequently than SAL+TMT animals ($p\leq0.014$), suggesting that naltrexone alters rearing behavior *only* in the presence of TMT.

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Resting/Immobility

Naltrexone-treated animals rested significantly longer than saline-treated animals (main effect of drug; $F(1,40)=4.65$, $p\leq0.044$; see Table 4). There was no main effect of odor ($F(1,40)=0.003$, $p\leq0.96$) on duration of resting. There was no significant interaction effect for duration of resting ($F(1, 40)=2.37$, $p\leq0.132$). Because *a priori* we wanted to determine whether pre-treatment with naltrexone increased resting behavior, planned comparisons revealed that there was no significant difference in resting duration between SAL and NAL control groups ($p\leq0.67$), however, NAL+TMT rested longer than SAL+TMT ($p\leq0.012$), suggesting that naltrexone increases resting behavior *only* in the presence of TMT.

Correlations between CORT, BrdU-labeled cells and defensive behaviors

There were no significant correlations between stereological estimates of BrdU-labeled cells and either the frequency or duration of defensive burying or stretch approach (either directed or undirected). In addition, there were no significant correlations between CORT levels and either stereological estimates of BrdU-labeled cells or the expression of defensive behaviors.

DISCUSSION

The results of the present study show, consistent with previous studies (Falconer & Galea, 2000; Galea et al., 1996; Gould et al., 1997; Gould et al., 1998; Tanapat et al., 1998), that brief exposure to a stressor suppresses cell proliferation in the adult mammalian dentate gyrus. The present data also demonstrate that acute antagonism of endogenous opioid activity suppresses cell proliferation. To our knowledge this is the first demonstration of *antagonism* of opioid activity altering cell proliferation in the adult dentate gyrus. TMT-exposed rats exhibited

increased frequency and duration of defensive burying and stretch approach, consistent with previous reports using other stressors (Blanchard et al, 1991; Pinel & Treit, 1978; Pinel et al., 1989). Taken with the present data demonstrating a TMT-induced suppression in cell proliferation and a TMT-induced increase in serum CORT levels, it is clear that TMT is a robust perhaps ecologically relevant stress stimulus for inducing a variety of behavioral, neural, and physiological stress responses in adult male rats.

Rats that were pre-treated with naltrexone exhibited fewer defensive behaviors (both defensive burying and directed stretch approach) and altered rearing and resting behavior in response to TMT exposure than saline-treated animals, and this suppression of behaviors during stress was independent of any impairments in required motor patterns by naltrexone administration. This suggests that a possible stress-induced increase in endogenous opioids following exposure to TMT may mediate the expression of *certain* behaviors during exposure to stress but does not mediate the suppression in cell proliferation.

TMT exposure suppresses cell proliferation in the dentate gyrus independent of opioid activity

Consistent with preliminary reports (Falconer & Galea, 2000; Galea et al., 1996), we observed that brief exposure to TMT suppresses cell proliferation in the dentate gyrus of adult male rats. Our results are also consistent with other reports of stress effects on cell proliferation in the dentate gyrus whereby exposure to a psychosocial stressor has been shown to inhibit cell proliferation in the developing rat (Tanapat et al., 1998), adult tree shrew (Gould et al., 1997), and adult marmoset monkey (Gould et al., 1998). The present data also demonstrate that cell proliferation was suppressed in animals pre-treated with naltrexone *and* exposed to TMT, suggesting that a possible stress-induced release of endogenous opioids did not cause the TMT-induced suppression in cell proliferation.

Stress-induced opioid activity interacts with glucocorticoid release (Buckingham, 1982; Odio & Brodish, 1990; Tapp et al., 1981) and glucocorticoids suppress cell proliferation (Cameron & Gould, 1994; Cameron & Gould, 1996), thus it is possible that any effect of endogenous opioids on the stress-induced suppression of cell proliferation is mediated downstream via a glucocorticoid mechanism. Indeed we report a significant increase in serum CORT levels in TMT-exposed animals, suggesting that CORT may be a putative mechanism involved in the stress-induced cell proliferation. Although we do not report a significant correlation between CORT levels and cell proliferation, it is likely that this relationship is non-linear in nature as many other endocrine and transmitter systems may be involved. In particular, preliminary data demonstrate that the stress-induced suppression in cell proliferation may be partially mediated by cholinergic activity (Galea et al., 1996).

Pre-treatment with naltrexone suppresses cell proliferation in the dentate gyrus

In the present study, naltrexone administration alone suppressed cell proliferation in the dentate gyrus of adult male rats. It has been reported that chronic increases in opioid activity can suppress cell proliferation and neurogenesis in both the developing and adult dentate gyrus (Eisch et al., 2000; Hauser et al., 2000; Reznikov et al., 1999) as well as in a cultured cell line (Agarwal & Glasel, 1999). Specifically, while Eisch et al (2000) demonstrated that chronic administration of morphine for 5 days suppressed both cell proliferation and neurogenesis, acute morphine treatment did not affect cell proliferation. As the present results demonstrate that acute *antagonism* of opioid activity can suppress cell proliferation, it may be that there are differential optimal levels of opioid activity for the regulation of cell proliferation. Taken with the present data, it may be that opioids inhibit the proliferation of cells only following *chronic* administration of *exogenous* opiates (Eisch et al., 2000) and that an *acute* increase in *endogenous*

(present study) or *exogenous* (Eisch et al., 2000) opioids is not sufficient to alter cell proliferation. Additionally, there may be multiple opioid receptor systems involved in the regulation of adult cell proliferation. Multiple opioid receptor systems are active in the rat dentate gyrus including mu, delta, and dynorphin (Commons & Milner, 1996; Crain et al., 1986; Drake & Milner, 1999; Drake et al., 1994). While morphine and naltrexone are largely active at mu receptors, they do have partial activity at other receptor types (Dykstra et al, 1997; Takemori & Portoghese, 1992), thus it is possible that we have differentially manipulated opioid receptor activity due to differences in methodology (i.e. dose and/or time course of administration).

TMT-exposed rats are more defensive than control rats

TMT-exposed rats exhibited increased frequency and duration of defensive burying. Consistent with previous reports (Pinel & Treit, 1978), defensive burying was largely, although not exclusively, directed towards the source of the stimulus (data not shown). This result demonstrates that the animals were able to localize the source of the odor despite permeation of the odor throughout the testing chamber. TMT-exposed rats also exhibited increased frequency of stretch approach consistent with previous studies (Blanchard et al., 1991; Pinel et al., 1989).

The present results are consistent with numerous reports demonstrating that exposure to the risk of predation (i.e. predator odor or predator calls) results in an increase in the expression of defensiveness (avoidance) and stretch approach (Blanchard & Blanchard, 1989; Blanchard et al., 1990a; Blanchard et al., 1990b; Blanchard et al., 1991; Blanchard et al., 1998; Hendrie & Neill, 1991; Zangrossi & File, 1992). Furthermore, the present resting/immobility data is consistent with data demonstrating an increase in freezing in animals presented with a stress odor *only* when treated with naltrexone (Fanselow & Sigmundi, 1986). One study reports that TMT exposure produced a significant increase in duration of freezing (Wallace & Rosen, 2000),

inconsistent with the present results. However it has been postulated that expression of freezing behavior is contingent on subtle procedural parameters (Wallace & Rosen, 2000) and in the present study TMT was presented in a small vial while Wallace and Rosen (2000) presented TMT on a piece of Plexiglas. In the present study, presentation of TMT in a vial may have served to be a more localized stimulus which could alter the defensive strategy of the animal.

Pre-treatment with naltrexone partially attenuates the expression of defensive behaviors

Rats that were pre-treated with naltrexone demonstrated fewer defensive behaviors (both defensive burying and directed stretch approach) in response to TMT exposure than saline-treated animals. This suggests that, as hypothesized by Bolles and Fanselow (1980), the stress-induced increase in endogenous opioids may mediate the expression of behaviors during exposure to stress. Other reports investigating the role of opioids in defensive behavior have also suggested that opioid activity is related to defensive behaviors. It has been demonstrated that morphine administration combined with exposure to a cat can induce increased crouching behavior and avoidance of the stimuli (Blanchard et al., 1991; Vivian & Miczek, 1998). However, another defensive behavior, ultrasonic vocalizations, responds differently to opioid administration. Morphine administration elicits a *decrease* in antipredator ultrasonic vocalizations (Blanchard et al., 1991, Shepherd et al., 1992; Vivian & Miczek, 1998) while naloxone (an opioid antagonist) administration resulted in an increase in ultrasonic vocalizations to vibrissae stimulation (Blanchard et al., 1991). Taken together, these data imply a possible dissociable effect of opioid activity on defensive behaviors, with activation of opioid activity either increasing or decreasing the expression of different defensive behaviors (Blanchard et al., 1991; Vivian & Miczek, 1998). This in itself may not be so surprising as in the entire repertoire of defensive behaviors there are many antagonistic behaviors (i.e. freezing and fleeing are

defensive behaviors but are inversely related). Furthermore, different stressors may differentially affect opioid involvement in the stress response as naltrexone either enhances freezing during presentation of a stress odor (Fanselow & Sigmundi, 1986) or increases the threshold to freezing following brain stimulation (Coimbra et al., 2000).

Relationship between neural and behavioral effects

There were no significant correlations between cell proliferation and expression of defensive behavior. This is not surprising as the data demonstrate differential effects of opioid manipulations on these measures whereby opioids appear to be involved in the stress-induced expression of defensive behaviors but not the stress-induced suppression in cell proliferation. Thus, it is appears that these neural and behavioral effects are governed by different mechanisms and not directly linearly related with each other. This is perhaps surprising given that the hippocampus has been implicated in the modulation of the expression of defensive behaviors (primarily freezing) in the developing and adult animal (Blanchard & Blanchard, 1972; Takahashi, 1995). However, the periaqueductal grey (a site of extensive opioid activity) also appears to be involved in the neural control of other defensive behaviors (Coimbra et al., 1996; Sante et al., 2000; Siegfried et al., 1990).

Functional and evolutionary significance for a stress-induced suppression in cell proliferation

The induction of defensive behaviors by exposure to the risk of predation has obvious immediate and evolutionary benefits for the organism (i.e. survival of the episode). However, the TMT-induced suppression of cell proliferation in the dentate gyrus is more difficult to interpret. One possibility is that, assuming that cell proliferation is metabolically expensive, the organism prevents metabolic expenditure on cell proliferation in response to stimuli that are immediately compromising to survival. This is supported by preliminary data from our

laboratory (Falconer & Galea, 2000) demonstrating that the suppression in cell proliferation is no longer present following repeated exposure to TMT (i.e. that cell survival is not affected), this change in cell survival rate is accompanied by a reduction in the expression of defensive behaviors suggesting that the animal 'learns' that the stimulus is not directly threatening. An additional somewhat related possibility is that the stress-induced suppression in cell proliferation is important for learning about the stressful episode. There is evidence to suggest that neurogenesis in the hippocampus may have a role in hippocampus-dependent learning and memory (Gould et al., 1999; Shors et al., 2001). Interestingly, while chronic stress has been shown to impair spatial learning (Luine et al., 1994), acute stress has been shown to enhance learning in male rats (Woods & Shors, 1998). At present it is not clear whether an acute stress-induced decrease in cell proliferation is causally related to enhanced learning about the stressful episode. Future research investigating the relationships between acute and chronic stressors and their effects on cell proliferation and neurogenesis may allow us better understand the mechanisms that mediate these effects while potentially increasing present understanding of the functional importance of the stress-induced suppression of cell proliferation.

CONCLUSIONS

Brief exposure to TMT induces the rapid induction of defensive behaviors and suppression of cell proliferation in the dentate gyrus of adult male rats. Pre-treatment with naltrexone partially attenuates the expression of defensive behaviors (defensive burying and directed stretch approach) independent of drug effects on ability to perform motor patterns. Pre-treatment with naltrexone significantly suppresses cell proliferation in animals exposed to the control odor and does not attenuate the TMT-induced suppression of cell proliferation. These data suggest that a possible stress-induced release of endogenous opioids may be involved in the

mediation of defensive behaviors but not the stress-induced suppression of cell proliferation.

These data also suggest that endogenous opioids may play a regulatory role in the control of cell

proliferation in the dentate gyrus of adult male rats independent of other manipulations.

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Table 1. Mean (±SEM) volume (mm^3) of granule cell layer.

Group (n)	Volume (mm^3) of granule cell layer
SAL (n=6)	3.489 (±0.169)
NAL (n=7)	3.897 (±0.191)
SAL+TMT (n=6)	3.840 (±0.224)
NAL+TMT (n=6)	3.607 (±0.296)

* indicates a significant difference (p≤0.05) from SAL condition

Table 2. Mean (±SEM) serum total CORT
 levels (µg/dl).

Group (n)	Serum total CORT
SAL (n=12)	25.996 (±3.520)
NAL (n=12)	28.206 (±4.721)
SAL+TMT (n=12)	37.848 (±4.570)*
NAL+TMT (n=12)	45.820 (±5.406) [a]

* indicates a significant difference (p≤0.05)
from SAL condition. [a] indicates a difference
(p≤0.10) from SAL condition.

Table 3. Mean (±SEM) duration of directed and undirected stretch approach (seconds).

Group (n)	Duration of Directed Stretch Approach	Duration of Undirected Stretch Approach
SAL (n=10)	3.385(±0.638)	3.637 (±1.997)
NAL (n=12)	5.458(±1.776)	2.402 (±0.760)
SAL+TMT (n=11)	12.311(±3.41)	5.419 (±01.266)
NAL+TMT (n=11)	6.04(±1.462)	4.39 (±0.626)

* indicates a significant difference from SAL control group (p≤0.05)

Table 4. Mean score (±SEM) for frequency or duration of non-defensive behaviors.

Group (n)	Frequency of Contact with Vial	Duration of Contact with Vial (s)	Frequency of Rearing	Duration of Rearing (s)	Duration of Resting (s)
SAL (n=10)	6.20(±1.05)	18.26(±5.61)	16.1(±3.55)	58.36(±16.81)	238.11(±62.04)
NAL (n=11)	5.42(±0.67)	22.05(±5.72)	13.17(±2.47)	61.23(±18.83)	276.11(±58.78)
SAL+TMT (n=12)	5.46(±1.34)	25.31(±9.54)	18.27(±3.10)	52.48(±10.52)	139.81(±45.08)
NAL+TMT (n=12)	2.36(±0.47)[a]	4.31(±0.91)	b8.55(±1.30)[a]	26.82(±6.01)	367.36(±75.99)[a]

* indicates a significant difference ($p \leq 0.05$) from SAL condition

[a] indicates a significant difference ($p \leq 0.05$) from SAL+TMT condition

Figure Captions/Legends

Figure 1. Photomicrograph of a bromodeoxyuridine (BrdU)-labeled cell located in the subgranular zone between the granule cell layer (GCL) and the hilus. Scale bar = 10μm.

Figure 2. Number of BrdU-labeled cells in the granule cell layer of the dentate gyrus. * indicates a significant difference from SAL control group (p≤0.05).

Figure 3. Frequency (A) and duration (B) of defensive burying. * indicates a significant difference from SAL control group (p≤0.05).

Figure 4. Frequency of directed (A) and undirected (B) stretch approach. * indicates a significant difference from SAL control group (p≤0.05).

Figure 1

Figure 2
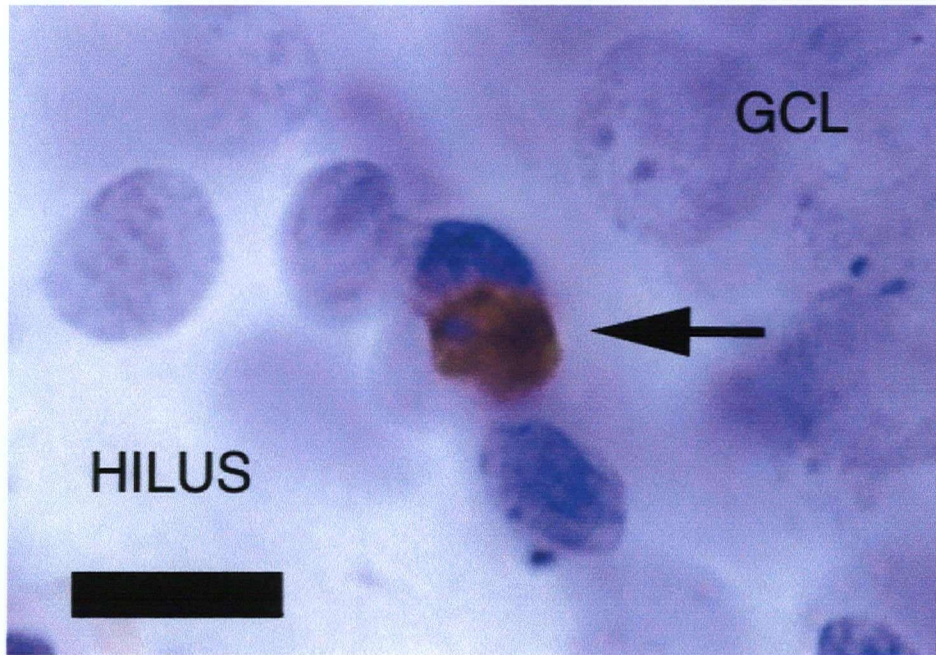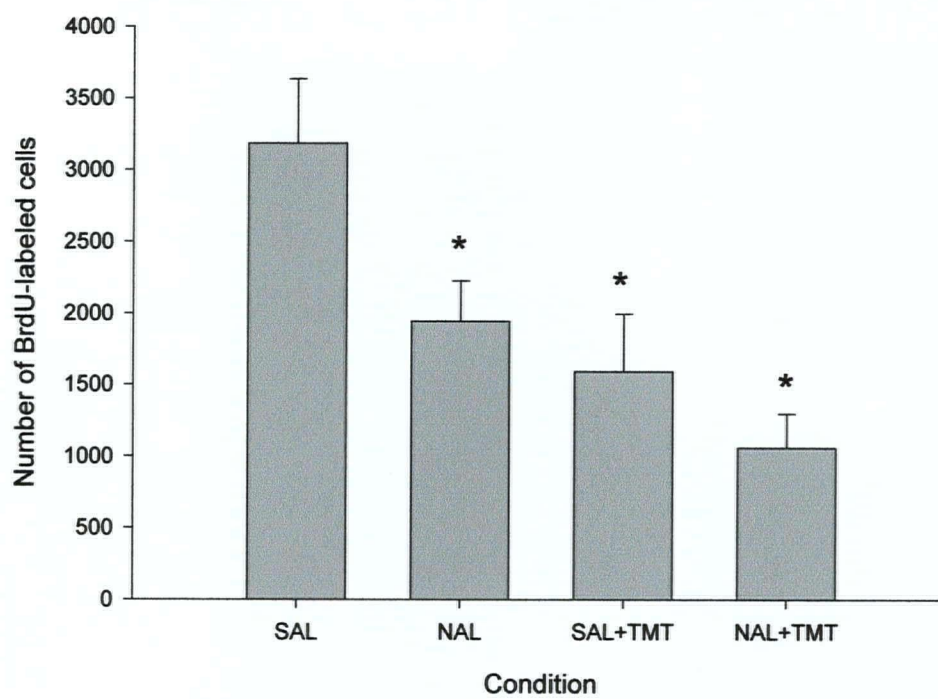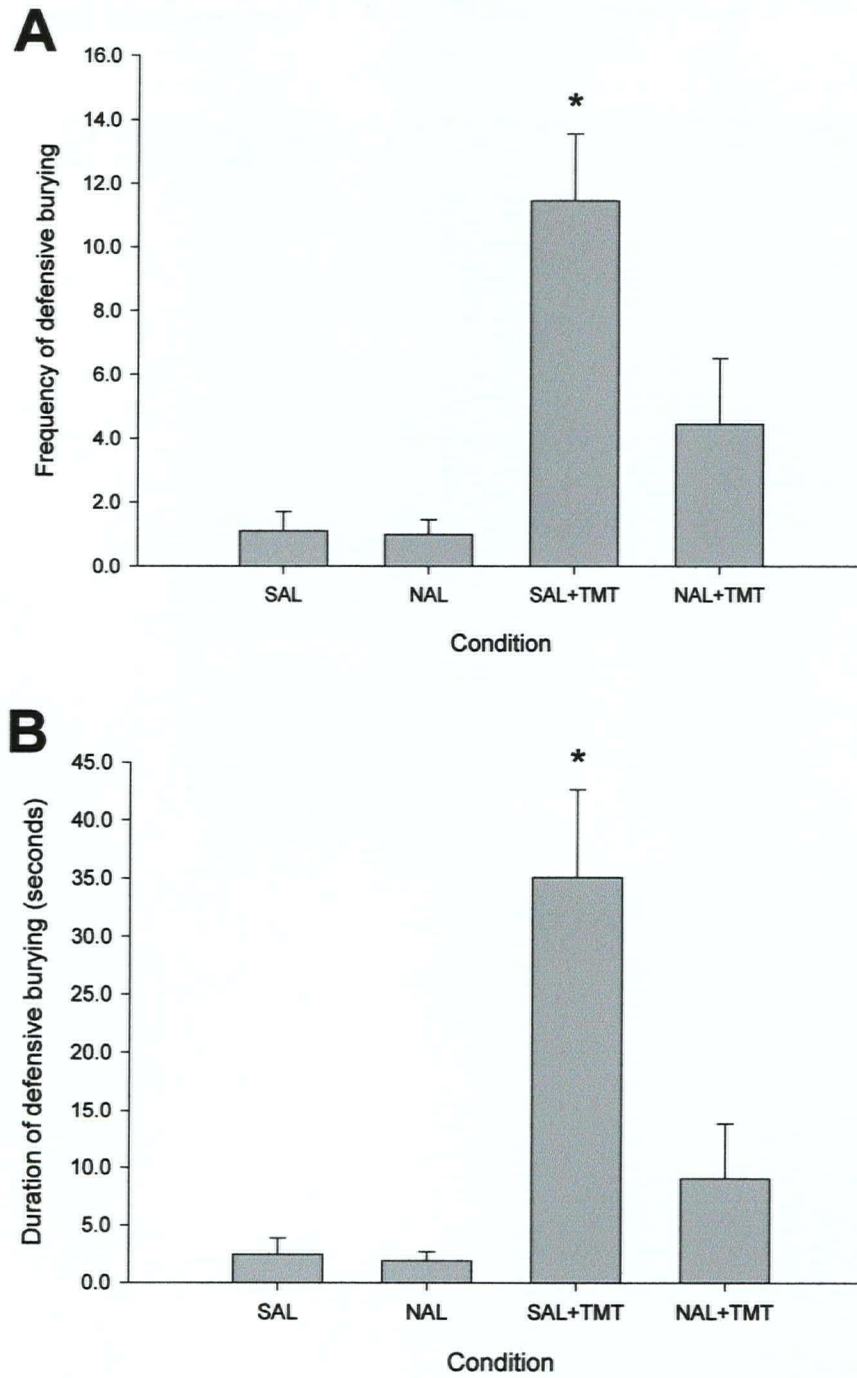
Figure 3

A

B

Figure 4