EFFECTS OF THE ESTROUS CYCLE ON NEURONAL ACTIVATION IN THE BED NUCLEUS OF THE STRIA TERMINALIS OF FEAR-CONDITIONED FEMALE RATS

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Effects of the estrous cycle on neuronal activation in the
bed nucleus of the stria terminalis of fear-conditioned female rats

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Women are much more likely than men to develop trauma- and anxiety-related disorders over their lifetime. One possible contributing factor is that unlike men, women experience large fluctuations in ovarian steroid hormones, like progesterone. Previous studies have suggested that high levels of progesterone reduce the expression of fear in female rats. The effect may be derived from allopregnanolone (ALLO), a metabolite of progesterone. ALLO, a positive allosteric modulator of ɣ-aminobutyric acid type A ($GABA_A$) receptors, may influence activity in the bed nucleus of the stria terminalis (BNST), a brain area that is important for the expression of conditioned fear. GABA is the major inhibitory neurotransmitter in the brain and contributes to the suppression of anxiety responses. The proposed study seeks to determine the effect of fluctuating progesterone levels on BNST cell activation during the expression of conditioned fear in female rats. We hypothesize that rats tested when progesterone levels are high will have lower BNST cell activation compared to rats tested when progesterone levels are low. To this end, animals will be trained and tested at estrous cycle stages with either high or low levels of progesterone under the Pavlovian fear conditioning paradigm. Progesterone levels will be measured using blood samples. BNST cell activation will be measured by quantifying the number of c-fos-expressing cells within this brain region. By exploring how progesterone

influences BNST cell activation during conditioned fear, a greater understanding of the susceptibility of women to trauma- and stress-related disorders, such as posttraumatic stress disorder (PTSD), may be achieved.

DEDICATION

I dedicate this research to my parents, Tracy Wang and Jay Haskin. Thank you both for your

endless enthusiasm and invaluable support to my lifelong education.

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I would like to thank Gillian Acca for being the finest graduate mentor a student could ask for. For three years at Texas A&M University, she has provided me with endless knowledge, instruction, and patience in my undergraduate research experience. Thank you for encouraging me every step of the way.

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Lastly, I would also like to extend thanks to the laboratory of Dr. Stephen Maren for generous scientific advice and technical support.

NOMENCLATURE

ALLO   allopregnanolone

$GABA_A$ γ-aminobutyric acid type A

BNST   bed nucleus of the stria terminalis

PTSD   posttraumatic stress disorder

Pro   animals in proestrus during training or testing

Di   animals in diestrus during training or testing

ITI   inter-trial interval

ELISA   enzyme-linked immunosorbent assay

PBS-Az   phosphate-buffered saline azide

NSB   nonspecific binding

B_o maximum binding

pNpp   p-nitrophenyl phosphate

DAB-nickel   nickel intensified diaminobenzidine

PBS   phosphate-buffered saline

PBST   phosphate-buffered saline with Tween-20

ABC   Avidin-Biotin Complex

ANOVA   analysis of variance

OV   oval

JU   juxtacapsular

ALD   anterolateral, dorsal portion

AMD   anteromedial, dorsal potion

ALV   anterolateral, ventral portion

AMV   anteromedial, ventral portion

FU   fusiform

AL   anterolateral

CHAPTER I

INTRODUCTION

A study accounting for the effects of the estrous cycle may shed further light upon the unique gender differences that exist in trauma- and stress-related disorders. For example, the prevalence of anxiety and depression is greater in women than in men (Leach et al., 2008). Furthermore, twice as many females are diagnosed with PTSD than males (Christiansen and Hansen, 2015). Possessing an in-depth understanding of how various hormones engage with and affect the brain is an imperative step towards developing future solutions to counter trauma- and stress-related disorders. Past studies have demonstrated significant sex differences in rats with respect to contextual fear (Maren et al., 1994). In particular, the stages of the estrous cycle have been shown to influence the expression of contextual fear (Markus and Zecevic, 1997). Additional reports have shown that the presence of allopregnanolone (ALLO), a metabolite of progesterone, is associated with reduced fear in female rats (Frye et al., 2000; Nagaya et al., 2015). Indeed, it has been generally indicated that the female sex bias existent in anxiety disorders is attributed to the complexity and fluctuations of female hormonal cycles (Donner and Lowry, 2013). To explore the effects of hormone fluctuations in females on conditioned contextual fear, the estrous cycle of female rodents serves as an excellent model. To understand how hormones affect the brain, one must understand the regions they target. In regards to the study of anxiety, particular interest has been brought to the bed nucleus of the stria terminalis (BNST). This brain region has been recognized for its involvement in the expression of fear (Jennings et al., 2013) and is hormonally regulated (Claro et al., 1995). Due to these findings, there is reason to believe that the fear-reducing effects of ALLO may be linked to reduced activation in the BNST. By

examining how progesterone affects neuronal activation in the BNST during the expression of conditioned fear, existing therapy for trauma- and stress-related disorders could be made more effective.

Objectives

Our objective is to evaluate how progesterone influences neuronal activation in the BNST during expression of conditioned fear in normal cycling female rats. We hypothesize that high levels of progesterone, which are representative of high levels of ALLO, will result in reduced activation of BNST neurons during the expression of conditioned contextual fear.

CHAPTER II

METHODS

Animals

In total, 82 female, Long-Evans rats (200-250 g; Harlan Laboratories, Indianapolis, Indiana) were used for this study. Each animal was individually housed in a clear plastic cage with unlimited access to food and water. The holding room followed a 14:10 h light: dark cycle and contained no other subjects for the duration of the experiment. The rats were handled for four days prior to smearing procedures to accustom them to the experimenter's touch.

Vaginal smears

For 2 complete cycles before behavioral procedures and continuing throughout behavioral procedures, vaginal smears were taken and characterized daily from the female rats between the hours of 11:00 am and 1:00 pm. This was done in order to predict the stages for each subject's estrous cycle. Smears were taken using a cotton swab moistened with distilled water. For each animal, the cotton swab penetrated the vagina approximately 1 cm to catch cell samples. These cell samples were smeared onto glass slides and observed for morphology using light microscopy. Figure 1 illustrates sample vaginal smears of all 4 estrous cycle stages. Nucleated cells indicated proestrus, cornified cells indicated estrus, and a mixture of both cell types complimented by the presence of leukocytes indicated metestrus and diestrus. Metestrus (also known as diestrus 1) exhibits a more even distribution of the 3 cell types whereas diestrus (also known as diestrus 2) predominantly exhibits leukocytes. As shown in Figure 2, a normal rat estrous cycle lasts for 4 to 5 days: 1 day of proestrus, 1-2 days of estrus, and 2 days of

metestrus/diestrus. The lowest levels of progesterone occur during diestrus, while the highest levels of progesterone occur in proestrus. Upon review of the smears after two weeks, only subjects with normal cycles were included in data analysis.

Behavioral context

Behavioral training and testing were conducted in 7 identical observation chambers with the dimensions 30 x 24 x 21 cm (Med Associates, St. Albans, Vermont). Cabinet walls blocked off each chamber's view of other chambers. The chambers were composed of two aluminum sidewalls and a Plexiglas ceiling, back wall, and door. The floor was made up of rows of steel rods wired to a shock source and a steel tray underneath. The base of each chamber had a load cell platform, which measured the amount of immobility within the chamber. Before every behavioral procedure, the chamber interiors were wiped down with 1% acetic acid. During the behavioral procedures, speakers inside the chambers delivered the tone stimuli; the rods delivered the shock stimuli, the ventilation fans outside the chambers provided background noise, and the cage and room white lights were turned on.

Procedures

Each animal was assigned to 1 of 8 training-testing groups shown in Table 1. Rats assigned as proestrus (Pro) were predicted to have high levels of progesterone. Rats assigned as diestrus (Di) were predicted to have low levels of progesterone. The No-Shock group served as a control for the Shock group during fear conditioning. In behavioral training, the female rats were placed in the chambers and immediately observed through a live video feed. The whole training period included a 3-minute baseline period, 5 tone-shock pairings with a 1-minute inter-trial interval

(ITI) between each, and a final 1-minute wait period. Each tone was 10 seconds long at a frequency of 2 kHz and an intensity of 100 dB. Each shock was 2 seconds long at 1 mA. The control group was trained in the same conditioning conditions but excluding shock. Training was conducted in order to allow for the learned acquisition of fear for each animal. Behavioral testing began 1 normal cycle after training for each rat in the Pro, Pro and Di, Di groups; for rats in the Pro, Di group, training occurred after three estrous cycle stages whereas for rats in the Di, Pro group, training occurred after five estrous cycle stages. The context and handling procedures remained the same as that for training except with the absence of tones and shocks for all groups. The training and testing procedure is graphically illustrated in Figure 3. Behavioral testing was conducted in order to measure the level of fear retention from previous training.

Histology

Approximately 1 hour after behavioral testing, the subjects were euthanized with pentobarbital (100 mg/kg). Immediately upon death, blood was transcardially extracted from the animals and prepared for future enzyme-linked immunosorbent assay (ELISA) analysis. The rats were perfused with 0.1 M phosphate buffer solution followed by 0.1 M paraformaldehyde. The perfused brains were extracted and incubated in 4% formalin and then transferred to a 30% sucrose-formalin solution. After complete post-fixation, the brains were sliced in a cryostat and stored in 0.01% PBS/sodium azide (PBS-Az) before immunohistochemistry.

Enzyme-linked immunosorbent assay (ELISA)

A progesterone ELISA kit (Enzo Life Sciences, Inc.) was used to measure plasma progesterone levels. This laboratory technique allowed us to correlate circulating progesterone levels with freezing behavior and neuronal activity within the BNST. Collected blood was centrifuged at 1000 rpm for 10 minutes in order to collect the supernatant for the ELISA procedure. This supernatant was stored at -20 ˚C before use. Initially, 100 µl of Assay Buffer was pipetted into nonspecific binding (NSB) and maximum binding (B_o) wells. One hundred µl of 6 different standards and 100 µl of the supernatant from experimental samples were pipetted into the appropriate wells. All standards and samples were run in duplicate. Fifty µl of the Assay Buffer was pipetted into the NSB wells, followed by 50 µl of blue Conjugate for every well excluding the Blank and Total Activity wells. Fifty µl of yellow Antibody solution was added to each well except the Blank, Total Activity, and NSB wells. The wells were left to incubate at room temperature on a plate shaker at 500 rpm for 2 hours. Afterwards, the contents were removed and the wells were washed with 400 µl of wash solution 3 times. Once the wells dried after the washing, 5 µl of blue Conjugate was added to TA wells and 200 µl of p-nitrophenyl phosphate (pNpp) Substrate solution was added to every well. This solution was incubated for 45 minutes. Finally, 50 µl of Stop Solution was added to each well and analyzed by the PerkinElmer Victor X3 multimode plate reader at 405 nm.

Immunohistochemical methods

Brain sections were stained with antibodies and nickel intensified diaminobenzidine (DAB-nickel) for the black staining of c-fos. C-fos is an immediate early gene protein that reflects neuronal activation. By utilizing this immunohistochemical procedure, we were able to visualize

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the number and location of cells activated in the BNST during either the expression of conditioned contextual fear or as a result of re-exposure to a context that had been paired with tone presentations.

BNST sections of the brain were rinsed in a 12-well staining plate on a shaker with phosphate-buffered saline (PBS) for 10 seconds, followed by incubation in 0.3% H_2O_2 (10 µl of 30% H_2O_2 per mL PBS) for 15 minutes. Then, the sections were rinsed in PBS 3 times and incubated in the primary antibody, a polyclonal rabbit anti-c-Fos antibody (Millipore PC05; 1:10,000 in PBS-Tween; PBST), overnight. The next day the sections were rinsed in PBS 3 times and incubated in the secondary antibody, Alexa Fluor 488 biotinylated donkey anti-rabbit (1 µl per 1 mL PBST), for 1 hour. The sections were rinsed in PBS 3 times again, and incubated in Avidin-Biotin Complex (ABC; Vector Laboratories Vectastain) for 45 minutes, followed by a rinse in PBS 3 times. For the DAB-nickel reaction, 5 µl of DAB (5% DAB stock), 1000 µl of nickel (5% nickel ammonium sulfate stock), and 0.5 µl H_2O_2 (30% stock) for every ml of PBS was used to incubate the brain sections in for 10 minutes on a shaker. Following incubation, the sections were rinsed in PBS 3 times and stored in phosphate-buffered saline azide at 4 °C. Afterwards, the brain sections were mounted on glass slides with PBS and coverslipped.

Brain imaging

We conducted a regional analysis of the BNST sections of the brain by imaging them with the Axio Imager 2 Research Microscope by the Zeiss Company using the Zen Pro microscope software. All brain section images were taken at 10X magnification within a 24-tile area. Contrast was set at 1813 for black and 3625 for white. Upon image acquisition, the 24 tiles were

stitched together to create one comprehensive image of the brain slice. The left and right BNST were selected from this image at 2885 x 2885 units each and saved as TIF files for further cell counting analysis.

Cell counting analysis

All brain cell counts were conducted blindly, with the training-testing group of each rat unknown to the counter. The number of BNST cell nuclei expressing c-fos protein for each brain was quantified using the image processing software ImageJ64 and cross-checked with manual counting. The scale for all images was set to 1.550 pixels/μm. The multiple anterior and posterior regions within the BNST were partitioned according to their standardized pixel areas and template locations. The exact width and height of the BNST areas are specified in Table 2, and the BNST templates are illustrated in Figure 4. Each selected region of the BNST was copied onto a blank 1000 x 1000 pixel file. The black and white contrast level was set between 160-170 for the visualization of only black c-fos staining against a white background. This setting allowed the ImageJ64 program to count the number of black dots present, thereby counting the number of c-fos-positive cells present within that particular region of the BNST.

CHAPTER III

RESULTS

The freezing behavior in all training and testing groups was analyzed with analysis of variance (ANOVA). Levels of freezing in all training sessions were averaged across 1-minute ITls between shock-tone pairings (each training session had 5 ITIs). Levels of freezing in all testing sessions were averaged across 1-minute blocks (each testing session lasted 10 minutes). It was hypothesized that rats tested in proestrus would demonstrate lower levels of freezing, reflecting the anxiety-reducing properties of progesterone. However, our data reflected that female rats *trained* in proestrus demonstrated higher levels of fear in testing at a statistically significant margin.

Eighty-two female rats in total were trained and tested using either the Pavlovian fear conditioning paradigm or the modified control paradigm. Twenty-three rats were excluded due to irregular estrous cycles or blood levels that did not match their testing estrous cycles. These conditions left our experiment with a total of 59 rats for data analysis. The Shock group contained 31 rats and the No-Shock group contained 28 rats. The following group sizes were derived from the Shock group: n=7 in Di, Di, n=7 in Pro, Pro, n=10 in Di, Pro, and n=7 in Pro, Di. The group sizes for the No-Shock group included: n=8 in Di, Di, n=6 in Pro, Pro, n=7 in Di, Pro, and n=7 in Pro, Di.

Behavior

Fear acquisition during training did not differ between Pro and Di Shock groups. For the Pro and Di Shock groups in Figure 5A, levels of freezing in all groups were <10% before the initiation of tone-shock pairings. While the training session progressed with each trial, freezing increased similarly between the two Shock groups, with no statistically significant difference shown in percent freezing (p=0.62). The No-Shock groups remained at normal baseline for the entire duration of the training session (<10%; data not shown).

For all four Shock groups, as shown in Figure 5B, no interaction effect was established between the estrous cycle stage and fear expression during context testing (p=0.70). However, the training state exhibited a statistically significant effect on fear expression (p=0.03). The interaction bar plot ANOVA between estrous state and freezing percentage in Figure 5C further illustrates that rats trained in proestrus had statistically significant higher levels of freezing than rats trained in diestrus (p<0.0001). No-Shock animals did not exhibit freezing during the context test (data not shown).

Progesterone levels at testing

Assays of plasma progesterone levels after context testing indicated that animals in proestrus had significantly higher (p<0.0001) circulating progesterone (50.2 ± 2.3 ng/ml) compared to animals in diestrus (10.0 ± 1.0 ng/ml).

Cell counts

Activated cells indicated by c-fos staining were counted for 8 regions or subnuclei of the BNST: the oval nucleus (OV), juxtacapsular nucleus (JU), anterolateral nucleus (dorsal portion; ALD), anteromedial nucleus (dorsal portion; AMD), anterolateral nucleus (ventral portion; ALV), anteromedial nucleus (ventral portion; AMV), fusiform nucleus (FU), and anterolateral nucleus (AL). Figures 6 and 7 illustrate a series of bar plot ANOVAs for average quantity of c-fos-positive cells for all of the No-Shock and Shock groups. Of these 8 brain regions, the OV and JU regions (Figure 6A and B) were the only ones that demonstrated a main effect of tone-shock training ($p=0.003$ and $p=0.02$, respectively), showing overall increased activity as assessed by c-fos immunoreactivity. The OV nucleus was the only region to show a main effect of estrous cycle during context testing, as indicated by increased c-fos expression during diestrus ($p=0.03$). A significant interaction between training, training cycle stage, and testing cycle stage was found for the AMD and AMV regions (Figure 7A and B) such that c-fos expression was elevated in shocked animals that had been trained and tested in diestrus ($p=0.03$ and $p=0.048$, respectively).

CHAPTER IV

CONCLUSION

Our current data suggests that the estrous cycle plays a definitive role in the modulation of the acquisition of contextual fear in female rats. In contrast to our original hypothesis, the estrous cycle stage did not have a significant effect on fear expression during either training or context testing. However, our findings suggest that high levels of progesterone, and possibly high levels of its metabolite, ALLO, significantly affect fear acquisition during training to *later* generate greater fear expression in context testing. Furthermore, our c-fos expression analyses of the BNST illustrates an increased level of cell activation in the OV, JU, AMD, and AMV regions of the BNST as a result of training as well as both training and testing during diestrus, when progesterone levels are low. The brain cell analyses also demonstrate a high degree of variation in neuronal activation in different BNST areas as a result of contextual fear expression. Given the major role ALLO plays in the *acquisition* of fear in the training state, future studies will be conducted to further understand the influence of progesterone throughout the entire duration of the Pavlovian fear paradigm. Ultimately, our estrous cycle tracking analyses, behavioral results, and blood progesterone level assays suggest that high levels of progesterone may be in part responsible for the existent female sex bias in anxiety disorders such as PTSD. This finding, discovered within an experimental paradigm using the natural hormonal fluctuations of female animal subjects, consequently adds a new layer of complexity to a formerly straightforward understanding of the effects of ALLO in relation to anxiety.

Previous studies of sex differences in contextual fear have primarily focused on expression of fear in females in the Pavlovian fear paradigm without tracking of estrous cycle stage at both training and testing (Maren et al., 1994; Barker and Galea, 2010). Furthermore, those studies that have taken the estrous cycle into account have involved different training and testing methods (Markus and Zecevic, 1997). Previous studies regarding female contextual fear collectively suggest that female rats have lower levels of fear than male rats, and that female rats in proestrus demonstrate the lowest levels of freezing at testing. A great deal of this behavior has been attributed to hormone fluctuations, and ALLO has been suggested to be an important contributor to this gender bias (Nagaya et al., 2015). Our study contributes to the previous research done with analyzing female fear and the estrous cycle. We report that progesterone may act via its metabolite, ALLO, to significantly contribute to the acquisition of context fear such that its expression is enhanced. These data contrast with the results of previous studies and support the idea that ALLO may have a complex modulatory role in fear acquisition and expression. The increased level of c-fos staining observed in the OV and JU subnuclei suggests that training with shock selectively activates both anxiogenic (OV) and anxiolytic (JU) subregions of the BNST. The increased activation of neurons in the OV as well as those from the anxiolytic AMD and AMV regions in Di, Di animals suggests that decreased ALLO levels may result in greater cell activation at test although overall fear expression was low. Altogether, these results illustrate that the span of ALLO's effects on fear conditioning is greater than previously imagined.

Moreover, up until recently, many scientific studies utilizing female rats have rendered these animals sexless through ovariectomies in order to establish a baseline for hormone analysis (Gupta et al., 2001; Maeng et al., 2015). Ovariectomies have historically been preferred due to

the extensive degree of estrous stage monitoring required for studies involving cycling female rats. Indeed, the usage of intact females in fear conditioning has received little experimental attention. However, sudden removal of physiologically relevant processes in any animal can potentially change aspects of the studied phenomena itself. Because ovariectomies allow natural hormone synthesis and cycling to be bypassed within the body via artificial replacement of steroid hormones, it becomes extremely important to consider the validity of the neurological and behavioral results these artificial hormones generate. Thus, the results of our experiments provide unique and important insight to the way females process and express fear during natural hormonal cycle fluctuations.

Taken together, ALLO's potential influence on the acquisition of fear may play an important role in explaining the previous paradox between the apparent *immediate* anxiety-relieving effects of ALLO and the prominence of anxiety disorders in women (who generate naturally higher levels of ALLO than men do). ALLO's fluctuations within the female rat hormonal cycle may at least in part account for the anxiety gender bias in females. Other factors to consider include the effects other hormones of the estrous cycle have on conditioned fear. For example, deficiencies in estrogen have been related to the prevention of fear extinction (Zeidan et al., 2011). Our data support the recent hypothesis that hormone fluctuations contribute to anxiety disorders (Donner and Lowry, 2013). In relation to clinical applications, ganaxolone, a synthetic version of ALLO, is currently being tested for possible treatment for PTSD patients (Pinna, 2014). One must note that a significant difference between ganaxolone and progesterone is that while progesterone is metabolized relatively quickly by the body, ganaxolone takes far longer for the body to break down, subsequently preventing hormone fluctuations of ALLO that possibly lead to anxiety. In

light of this, our current findings suggest that new medications encouraging more stable levels of ALLO need to be monitored more closely in order to prevent drastic fluctuations of ALLO.

Ultimately, our work suggests that high levels of progesterone, and concomitantly high levels of its metabolite ALLO, reduce fear extinction after fear acquisition, allowing for greater fear expression in the future. The current relationship between neuronal activation and fear expression remains unclear given the high degree of variation among BNST regions. Further research will look at progesterone's neuronal effect in the BNST throughout the *entire* duration of the Pavlovian fear paradigm in order to determine the contributions of different BNST regions to conditioned fear.

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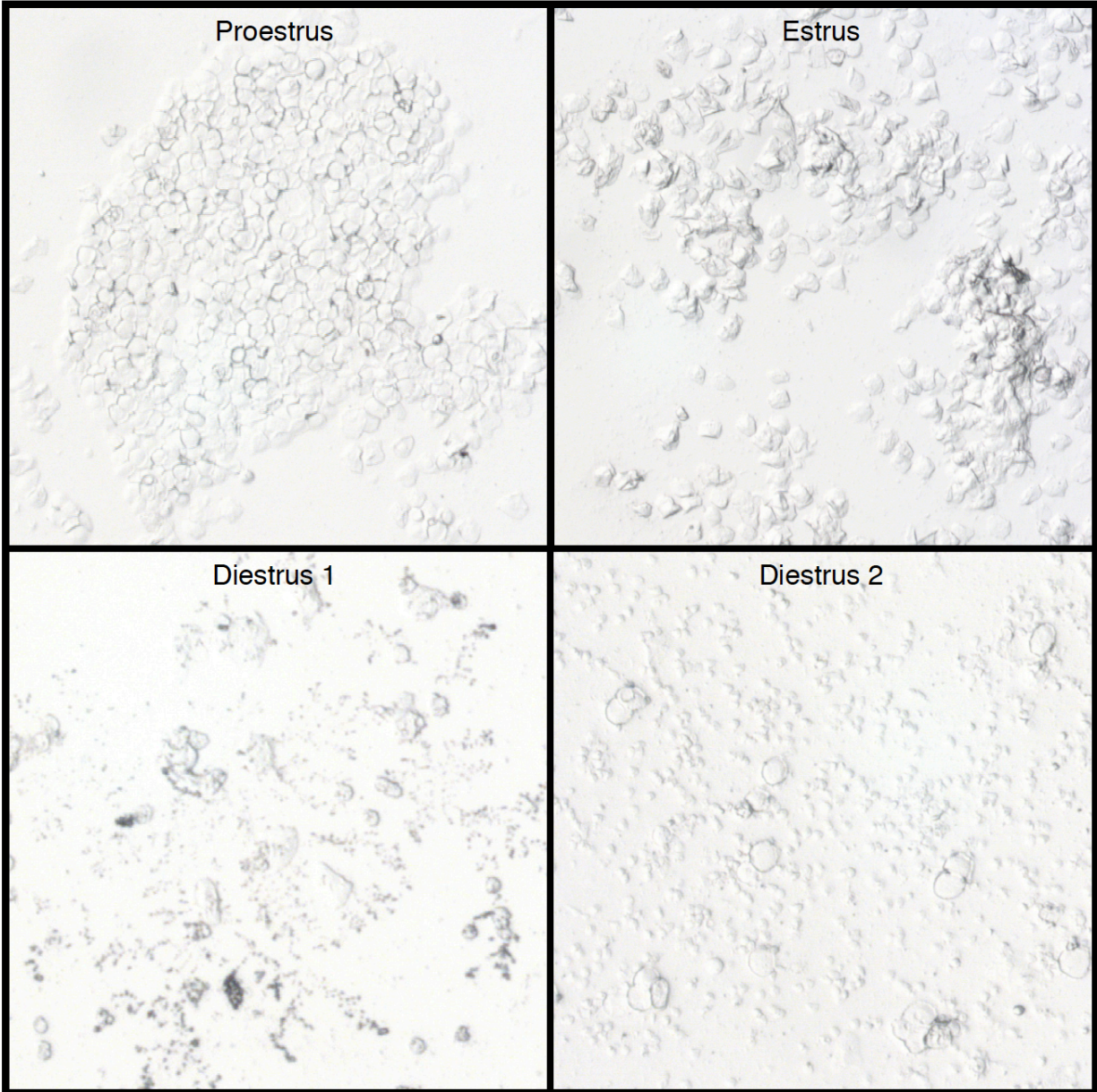
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APPENDIX A

Figure 1

Estrous Cycle Smear Samples

8x magnification

Figure 2

Rat Estrous Cycle

Circulating Progesterone Level

Estrus | Metestrus | Diestrus | Proestrus | Estrus
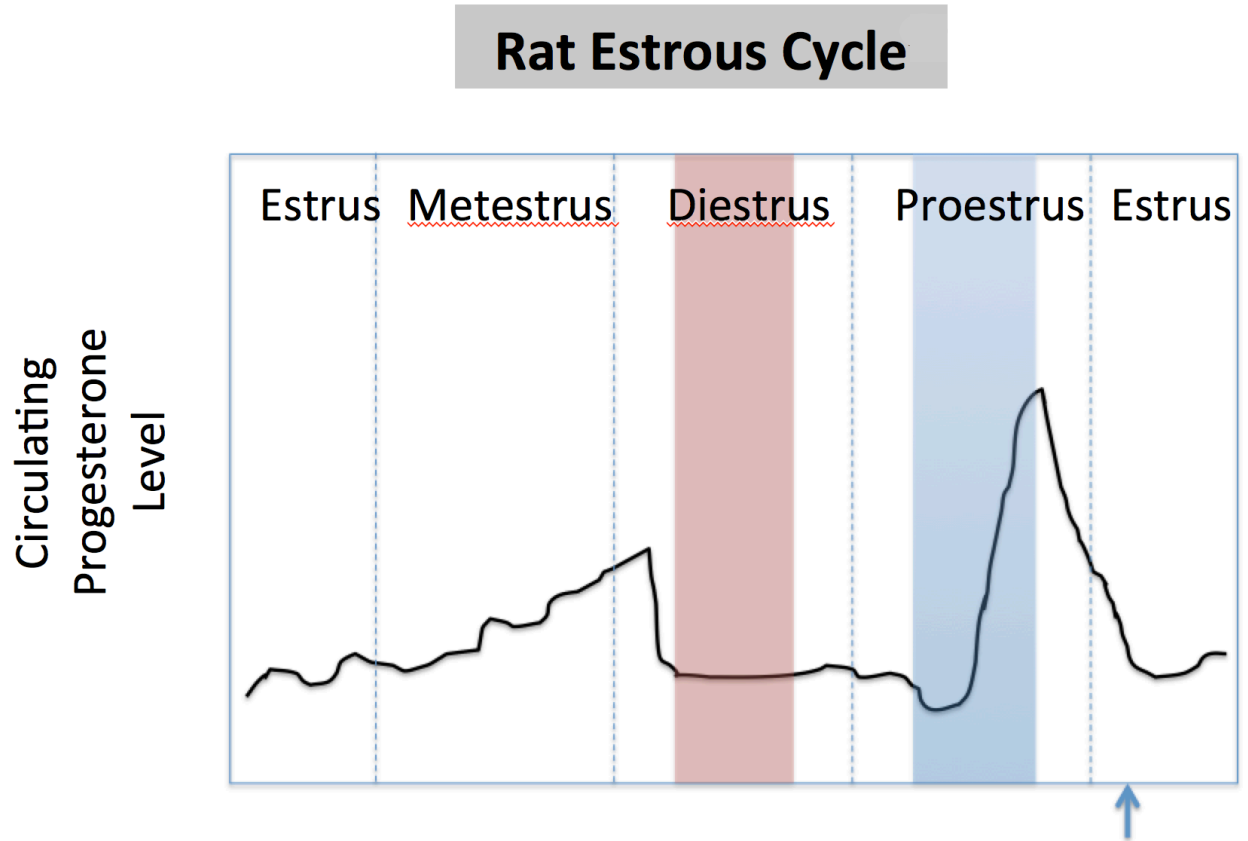
Table 1

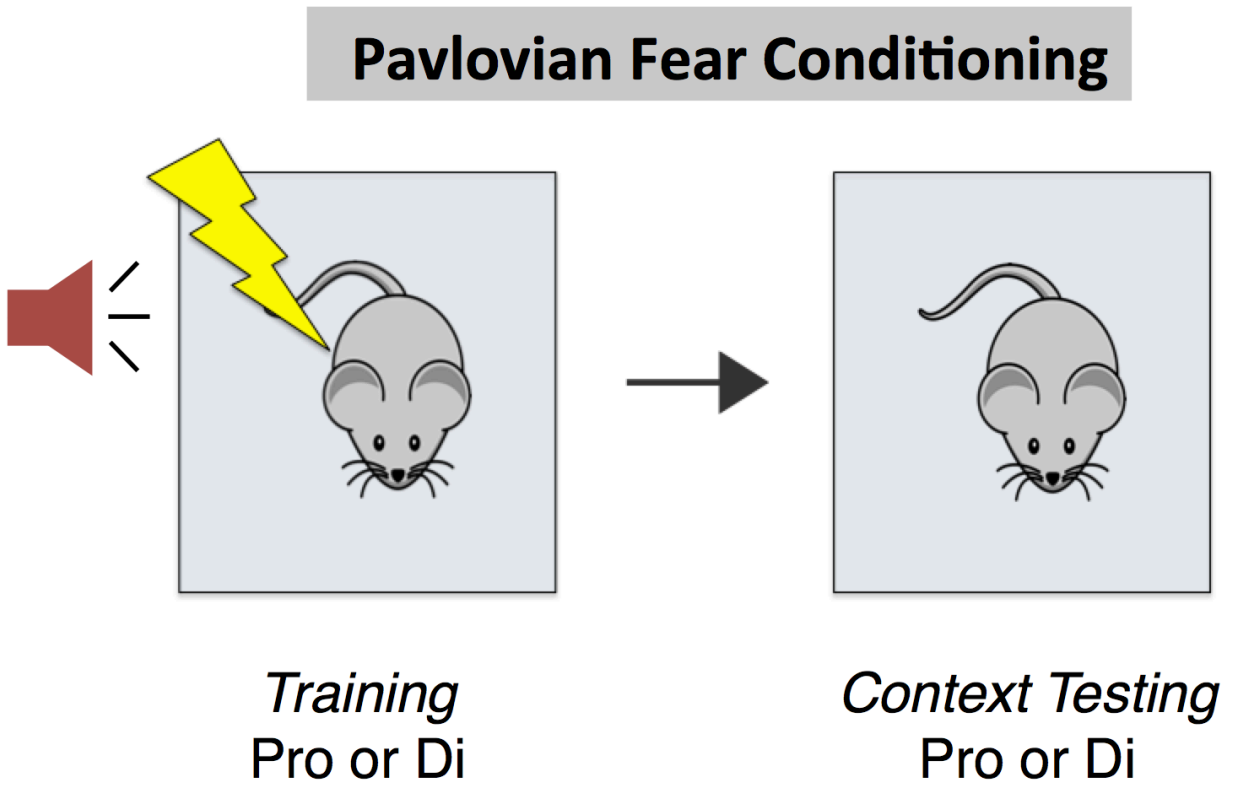| Training-Testing Groups	
Shock	**No-Shock**
Proestrus-Proestrus	Proestrus-Proestrus
Proestrus-Diestrus	Proestrus-Diestrus
Diestrus-Proestrus	Diestrus-Proestrus
Diestrus-Diestrus	Diestrus-Diestrus

Figure 3

Pavlovian Fear Conditioning

Training
Pro or Di

Context Testing
Pro or Di

Figure 4

BNST Regional Mapping
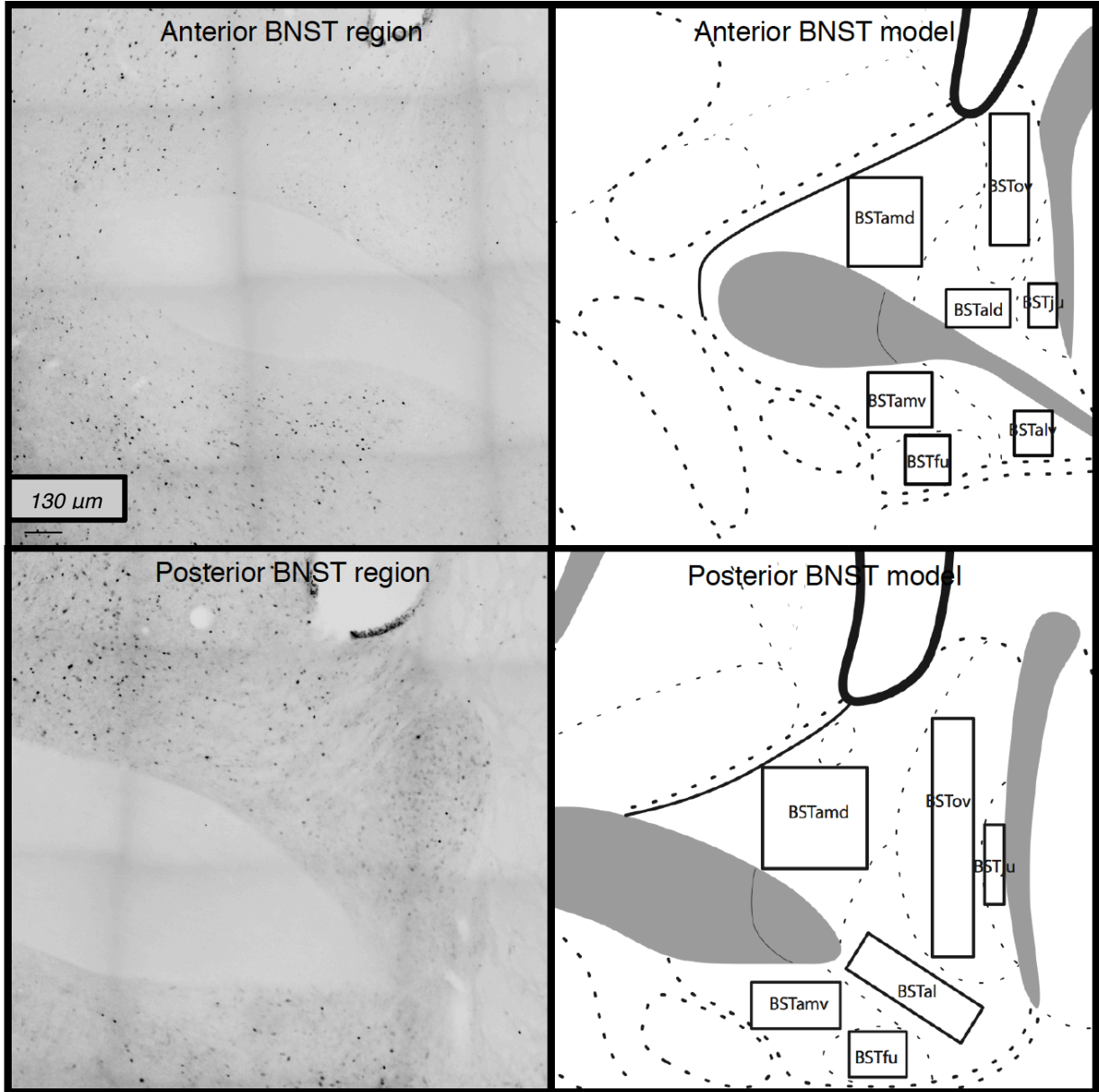
Table 2

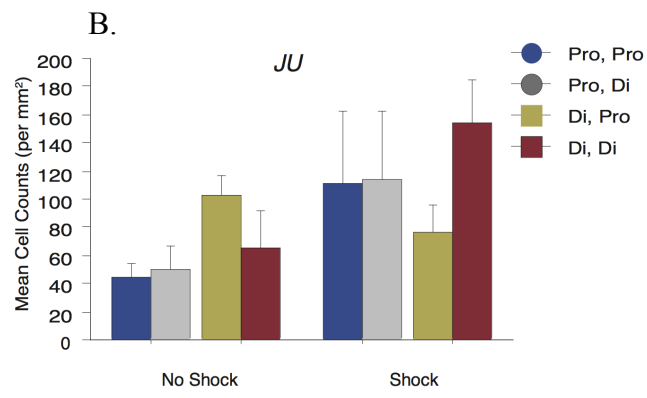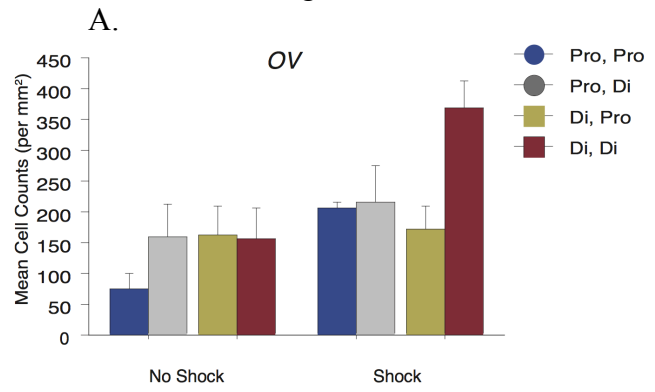| Standardized BNST Pixel Areas					
BNST Anterior	**Width**	**Height**	**BNST Posterior**	**Width**	**Height**
OV	201.24	522.45	OV	205.11	623.07
JU	89.01	131.58	JU	77.4	251.55
FU	263.16	143.19	FU	270.9	170.28
AMV	344.43	197.37	AMV	445.05	178.02
AMD	363.78	340.56	AMD	421.83	352.17
ALD	243.81	100.62	AL	445.05	174.15
ALV	135.45	154.8			

Figure 5

A.
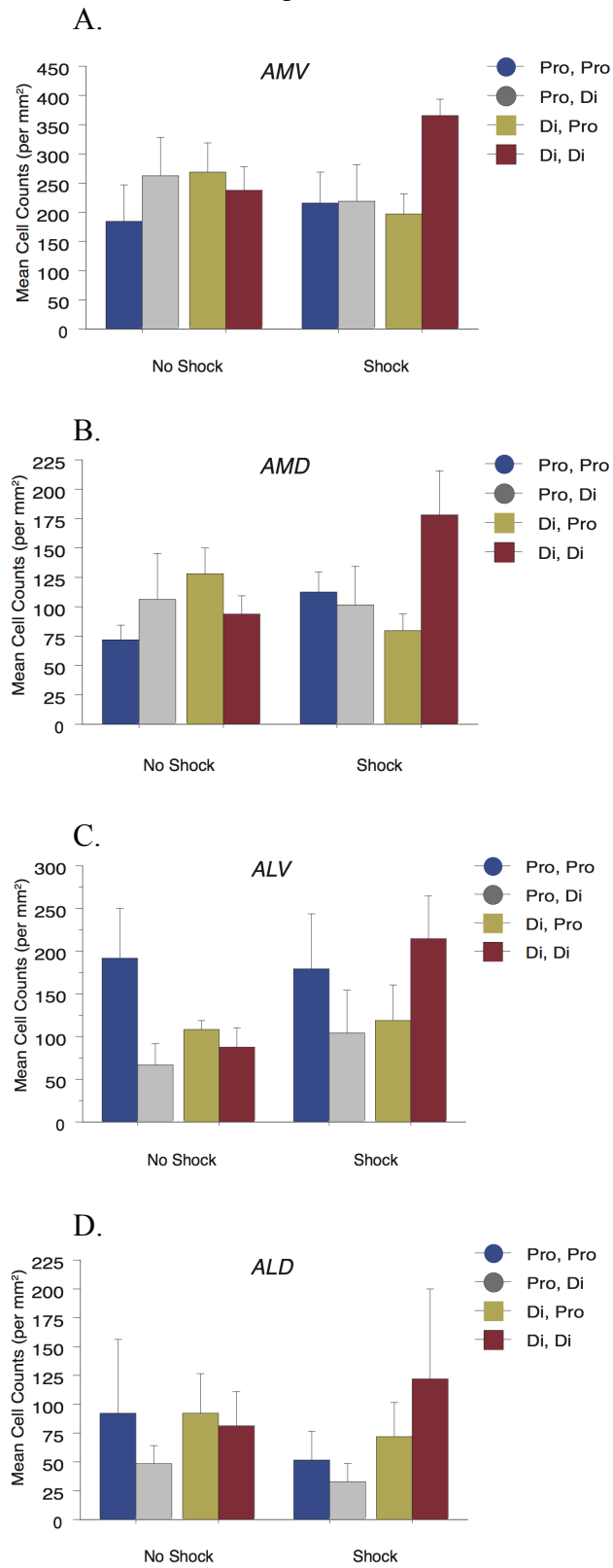
B.

C.
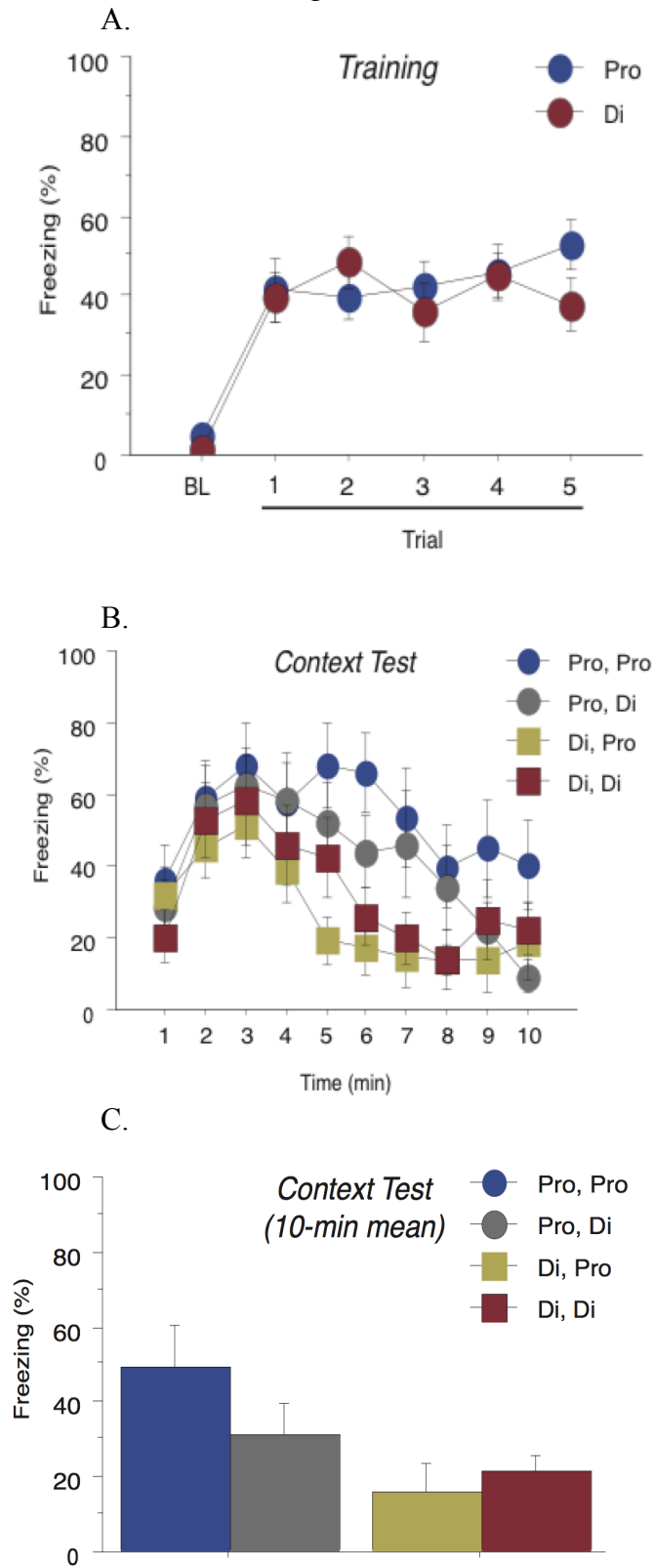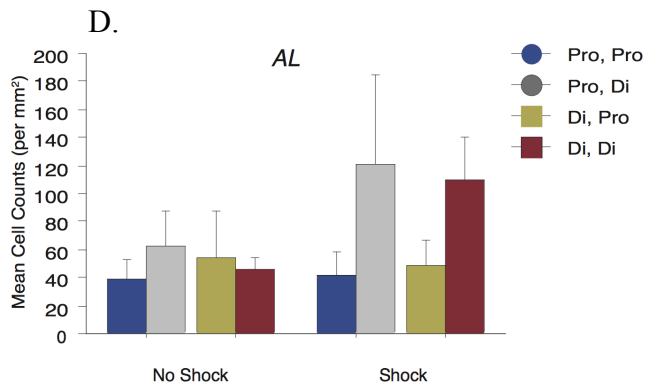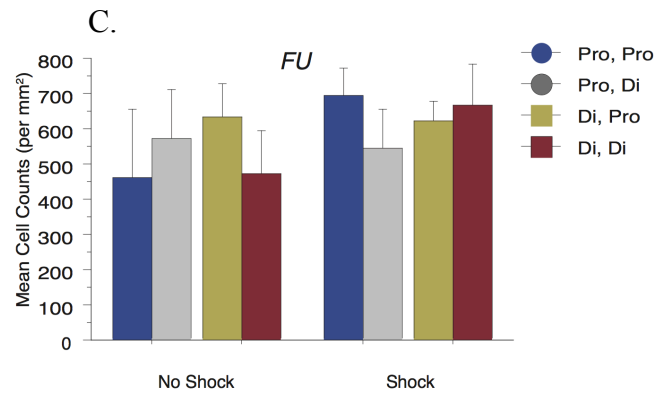
Figure 6

Figure 7

FIGURE LEGENDS

Figure 1

Representative images of wet, unstained vaginal smears taken through each of the 4 stages of the rat estrous cycle. Proestrus shows nucleated cells, estrus shows cornified cells, diestrus 1 (also called metestrus) shows a mixture of both cell types with the presence of leukocytes, and diestrus 2 (also called diestrus) predominantly shows leukocytes.

Figure 2

A schematic pattern of rat progesterone levels in the blood throughout the 4-day rodent estrous cycle based on previous studies (Butcher et al., 1974; Smith et al., 1975). Shaded columns at diestrus (pink) and proestrus (blue) indicate the lowest and highest levels of progesterone during the cycle, respectively. The blue arrow indicates the timing of ovulation.

Figure 3

A schematic diagram of the Pavlovian fear conditioning experimental design. On the left is shown a rat receiving tone-shock pairings in a specific context during training. On the right is shown a rat placed in the same context with no tones or shocks during context testing. The experimental groups consisted of animals trained and tested during either the same estrous cycle phase (Pro, Pro or Di, Di) or different estrous cycle phases (Pro, Di or Di, Pro).

Figure 4

Representative section of the anterior (top left) and posterior (bottom left) portions of the anterior BNST stained for c-fos protein. Analysis templates based on the Swanson brain atlas (2003) are shown next to their respective brain sections and labeled for BNST nuclei of interest: oval (OV), juxtacapsular (JU), fusiform (FU), anteromedial (ventral portion; AMV), anteromedial (dorsal portion, AMD), anterolateral (dorsal portion, ALD), anterolateral (ventral portion, ALV), and anterolateral (AL). Activated cells (c-fos positive) within each outlined area were counted as part of the indicated brain region.

Figure 5

Conditioned freezing in female rats trained and tested at estrous stages that were either the same (Pro, Pro or Di, Di) or different (Pro, Di or Di, Pro). A, Mean percentage of freezing (±SEM) during the five-trial training session. Data are shown for a 3-min pre-trial period followed by 5 tone-shock pairings. Freezing was measured before the first conditioning trial (baseline, BL) and for the 1-min period after each conditioning trial. B, Mean percentage of freezing (±SEM) to context over 10 min after one complete estrous cycle (Pro, Pro or Di, Di), three estrous cycle stages (Pro-Di), or five estrous cycle stages (Di-Pro) after training. C, Mean percentage of freezing (±SEM) to context over the complete 10-min test session shown in B for the indicated training/testing conditions.

Figure 6

Mean counts (±SEM) per mm^2 of c-fos immunopositive cells in different regions of the anterior

BNST of female rats that were either untrained (No Shock) or trained (Shock) prior to context

testing. A, oval nucleus (OV). B, juxtacapsular nucleus (JU). C, fusiform nucleus. D,

anterolateral nucleus.

Figure 7

Mean counts (±SEM) per mm^2 of c-fos immunopositive cells in different regions of the anterior

BNST of female rats that were either untrained (No Shock) or trained (Shock) prior to context

testing. A, anteromedial nucleus, ventral portion (AMV). B, anteromedial nucleus, dorsal portion

(AMD). C, anterolateral nucleus, ventral portion (ALV). D, anterolateral nucleus, dorsal portion

(ALD).