THE EFFECT OF MORPHINE ON DOPAMINE NEURON ACTIVATION IN MALES AND CYCLING FEMALES

by

MICAH EIMERBRINK

Presented to the Faculty of the Graduate School of

The University of Texas at Arlington in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE IN PSYCHOLOGY

THE UNIVERSITY OF TEXAS AT ARLINGTON

December 2011

ACKNOWLEDGEMENTS

The events that resulted in the completion of this thesis were unexpected, and there are numerous individuals who influenced the progression of these events in an exemplary fashion. This is to say that I could never have achieved completion of this experiment, document, or degree without the support of key individuals.

It would be highly inappropriate to not first acknowledge the contribution of my parents. Any positive aspect of who I am is a result of their love and support. I am, and forever will be grateful for everything they have provided for me. To Dr. Yuan Bo Peng, who was willing to accept me as a graduate student, and provided a substantial portion of time and training to make this research possible. Fortunately, I was also able to rely on my lab mates, Christopher Haggins, and Ailing Li. I appreciate all their help, assistance, and patience as I slowly learned a small portion of the skills they possess. Again, I could not have completed this project without their assistance. Equally helpful were Samara Morris-Bobzean, and Torry Dennis who helped fill in holes in both knowledge and research skills required for this experiment. This project could not have been completed without them. I also appreciate the input and assistance of my other committee members, Dr. Linda Perrotti, and Dr. Perry Fuchs. The guidance of my full committee helped improve this experiment, and also helped me understand the level of commitment and determination required for academic research.

Finally, I would like to thank Priya Iyer. Her personal support during this process was invaluable, and I know I am a better person, both as an academic and individual, because of her influence on my life.

November 21, 2011

ABSTRACT

THE EFFECT OF MORPHINE ON VTA DOPAMINE
NEURON ACTIVATION IN MALES
AND CYCLING FEMALES

Micah Eimerbrink, M. S.

The University of Texas at Arlington, 2011

Supervising Professor: Yuan Bo Peng

The aim of this study was to evaluate the influence of estrogen on the electrophysiological response of ventral tegmental area (VTA) dopamine (DA) neurons to morphine (10 mg/kg; IP) in experimentally naïve adult male and intact adult female cycling Sprague-Dawley rats. Five groups were studied: males, proestrus females, estrus females, metestrus females, and diestrus females. Continuous 40 minute recordings were collected for each animal (5 minutes baseline, 5 minutes after a saline (0.9% NaCl) injection, and 30 minutes after morphing injection (10 mg/kg)). Results showed significant increases in VTA DA activity in the Metestrus and Male groups, no significant differences in activity in the Diestrus and Proestrus groups, and a significant decline in activity in the Estrus group after a morphine injection. Between group differences show that the Metestrus and Male group's DA activity significantly increased relative to the other groups at the corresponding time points. This study did not support the hypothesis that estrogen enhances VTA DA neuron activation following morphine administration.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

CHAPTER 1

INTRODUCTION

1.1 Overview

Understanding the effects of estrogen on opioid reward processing is important due to the abundant use of opioids as an analgesic, and because of the high addiction potential of chronic opioid use. Morphine is one opioid commonly prescribed for pain management, but is also known as a drug of abuse. These two uses of morphine are distinctly different, but are united by the rewarding perception of opioid use. This perception begins with morphine's influence on the ventral tegmental area (VTA) of the brain (Gysling & Wang, 1983). In the VTA, morphine produces an increase in dopaminergic (DA) neuron firing (Bozarth & Wise, 1984; Johnson & North, 1992; D. Zhang, Zhang, Jin, Zhang, & Zhen, 2008) that results in a rewarding experience (Bozarth & Wise, 1984). Morphine facilitates DA neuron activation by stifling the tonic inhibitory signals from gamma-aminobutyric acid (GABA) neurons (Johnson & North, 1992), allowing for an increased sensitivity to afferent signals. These signals are primarily glutamatergic (Kalivas, 2000), and can be influenced by estrogen (Foy et al., 1999; Kelly, Loose, & Ronnekleiv, 1992). Because estrogen levels fluctuate according to the female reproductive cycle, one can predict that the rewarding experience of morphine would also differ based on the phase of cycle. However, there is a lack of direct evidence of how fluctuation of estrogen will affect the dopaminergic neuronal activity in the VTA. The purpose of this study is to identify electrophysiological congruent fluctuations between estrogen levels and VTA DA neuronal activation following morphine administration.

1.2 Opioid Mechanism

Opioids have a long history as a potent analgesic. They utilize endogenous opioid receptors (δ, κ and μ receptors) which have seven transmembrane regions (S. Zhang et al., 1998) to activate G-proteins that can reduce neuronal activation by inhibiting adenylyl cyclase, increasing potassium ion channel permeability, and inhibiting calcium channel permeability (Childers, 1991). By inhibiting adenylyl cyclase the neuron's ability to produce cAMP is impaired. This decline in cAMP production prevents activation of second messenger systems, such as protein phosphorylation. Increases in potassium ion permeability inhibit the neuron by hyperpolarizing the membrane potential. Finally, inhibition of calcium channels at the presynaptic terminal prevents the influx of calcium, thereby inhibiting vesicular docking and subsequent neurotransmitter release into the synaptic cleft. These three processes decrease the functional ability of a neuron with an opiate receptor to propagate signals, including nociception. However, the same mechanism that can stifle nociceptive transmissions also activates the mesolimbic or reward pathway.

1.3 Mesolimbic Pathway

The mesolimbic pathway is also known as the reward pathway. It is comprised of dopaminergic (DA) neurons that originate in the ventral tegmental area (VTA) and project to the nucleus accumbens (NAcc) and prefrontal cortex (PFC). This pathway is reliably involved in the acute and chronic response to drugs and hedonic stimuli (Adinoff, 2004; Berke & Hyman, 2000; Clark & Overton, 1998), meaning that the acquisition of these stimuli involves altered neural activity in the VTA, NAcc, and PFC. Activation of these areas is significant because one of the functions of this pathway is to increase the salience of associated stimuli (Robinson & Berridge, 1993). Therefore, mesolimbic neural activation creates awareness between the subjective experience of reward, and the stimuli present during the elicited response. If a particular stimulus is perceived as rewarding to an individual or animal, one can predict activation of DA neurons in the VTA to stimulate the mesolimbic pathway and increase the association between

the stimulus and the response. This makes the mesolimbic pathway very important when attempting to understand drug reward.

1.4 Cellular Components of the VTA

The VTA is primarily comprised of DA neurons and GABA neurons (Oades & Halliday, 1987). Prior to arousal, the DA neurons in the VTA experience a tonic inhibition by local GABAergic interneurons (Johnson & North, 1992). This inhibition is due to the activity of GABA on a postsynaptic cell. When a GABA neuron releases GABA in the VTA, it binds to the $GABA_A$ receptor on the postsynaptic DA neuron. Once bound, a conformational change occurs that opens the receptor to increase the membrane permeability of the DA neuron to chloride ions (Kalivas, Duffy, & Eberhardt, 1990; Kalivas, Klitenick, Hagler, & Austin, 1991). This action is inhibitory because the influx of chloride ions hyperpolarizes the cell, and increases the amount of sodium influx required to reach the threshold potential to trigger an action potential. Within that model, one can increase the firing rate of DA neurons by either reducing the inhibitory inputs, or strengthening the excitatory inputs.

Various drugs of abuse exploit this pathway to produce reward through different mechanisms. Ethanol increases the firing rate of VTA DA neurons by reducing the K^+ flow out of the DA cell (Appel, Liu, McElvain, & Brodie, 2003), resulting hyperpolarization of neuronal membrane. Decreasing the flow of potassium reduces the hyperpolarization caused by the exodus of potassium from the cell, and increases the firing rate by heightening the cell's sensitivity to afferent signals. Cocaine stimulates an increase in DA neuron firing in the VTA at doses higher than 1.0 to 2.0 mg/kg by inhibiting voltage gated sodium channels on GABA neurons (Steffensen et al., 2008). Inhibiting the sodium channels prevents the influx of sodium that is required for an action potential and subsequent release of GABA. If the GABA neurons are unable to release GABA then the tonic inhibition of DA cells is removed, leading to facilitation of DA activity. This is similar to the mechanism used by morphine to create a rewarding experience.

Immunocytocheimcal labeling followed by election microscope detection has identified mu-opioid receptors (MOR) on GABA cells in the VTA (Garzon & Pickel, 2001). Additionally, VTA GABA cells possess the mRNA coding for the MOR (Mansour, Fox, Akil, & Watson, 1995). The presence of MOR is critical for the mechanism for morphine reward. When morphine binds to the MORs on VTA GABA neurons, the tonic inhibition of DA neurons is removed due to hyperpolarization of the GABA neurons (Johnson & North, 1992). The hyperpolarization of GABA neurons decreases the likelihood of activation by increasing the depolarization necessary to generate an action potential. Action potentials are required to release GABA from the presynaptic interneuron to the postsynaptic DA neuron, and a decline in firing rate reduces the inhibitory effect of GABA on DA neurons. The reduction of GABA inhibitory afferent signals decreases the amount of depolarization required for DA neuron activation.

The lower excitatory threshold of the DA neuron that results from the loss of GABA inhibition has interesting effects. Whole cell recordings from VTA DA neurons show a significant increase in the ratio of AMPA receptors to NMDA receptors within 24 hours of a single morphine exposure (Saal, Dong, Bonci, & Malenka, 2003). This effect was not unique to morphine. The same study found nicotine, ethanol, cocaine, and amphetamine all produced the significant ratio increases (Saal, et al., 2003), and a preceding study also found the effect in cocaine (Ungless, Whistler, Malenka, & Bonci, 2001). The commonality of AMPA to NMDA receptor ratio increase on VTA DA neurons across substance is significant because it suggests that these substances can enhance the strength of afferent signals into the VTA through a postsynaptic receptor mediated mechanism (Saal, et al., 2003; Ungless, et al., 2001). The addition of AMPA receptors into the DA neuron on the postsynaptic region of the synaptic cleft effectively primes the cell for activation by increasing the availability of potential binding sites for glutamate. This is highly relevant because the primary sources of afferent signals into the VTA are glutamatergic and emanate from the PFC (Carr & Sesack, 2000). Therefore, morphine

activates the mesolimbic pathway by binding to receptors on GABA cells, causing a disinhibition of DA neurons that increases sensitivity to the dominant source of afferent stimuli, glutamate.

1.5 Contribution of the VTA to Reward

Morphine administration results in a stimulation of VTA DA neurons that increases dopamine levels in the nucleus accumbens (Di Chiara & Imperato, 1988). However, this does little to explain what the stimulation of this pathway means behaviorally. Early research observed the connection between the firing rate of DA neurons and behavior unintentionally, noting that changes in environmental stimuli alter the burst firing pattern of DA neurons to produce an orientation to that stimuli (Freeman, Meltzer, & Bunney, 1985). This observation came post hoc as the researchers realized that DA neuron firing increased as the researcher's hand entered the cage during single cell recordings of freely moving animals. This is some of the first evidence that paired excitement of the VTA DA neurons with a specific influence on behavior - an orienting response. Following this observation it was discovered that a similar increase in burst firing exists in response to an auditory stimulus, manual stimulation of the whiskers, orientation behaviors toward a stimulus, and sniffing (Freeman & Bunney, 1987). The plasticity of this response indicates that various stimuli can produce activity in the VTA, and the nature of this stimulus appears to be arousing in nature. Moreover, the repeated orientation of attention to the stimulus indicates that activity in the VTA DA neurons can direct behavior.

When this idea was studied in morphine, behavioral patterns emerged to support the premise that VTA DA neuron activation can influence behavior. Some of the earliest research on the hypothesis involved conditioned place preference. This methodology evaluates an animal's preference for an environment after pairing that environment with a preferred stimulus. Rats demonstrate a conditioned place preference for environments associated with direct VTA morphine administration (Phillips & LePiane, 1980). The preference for associated environments is used as a measure to indicate that the administration of morphine into the VTA creates a positive experience for the animals.

Self administration studies provide tremendous support for a hypothesis predicting that a substance can elicit a rewarding experience. Animals can readily be trained to self administer morphine directly to the VTA via a lever press (Bozarth & Wise, 1981). Intentional behavior to maintain a morphine induced state demonstrates the reinforcing potential of morphine though observation of an increase in lever presses to administer morphine when compared to controls. By considering the self-administration data with conditioned place preference data, these two behavioral methodologies demonstrate that the effects of morphine can induce a preferential state. Also, animals will choose to perpetuate this state by intentionally administering morphine to maintain the sensation after proper training. Because morphine can activate VTA DA neurons (Gysling & Wang, 1983), a relationship between behavior and firing can be concluded such that activation of DA neurons in the mesolimbic pathway following morphine administration produces behavioral preferences for the elicited state.

Interestingly, while the VTA DA neurons respond with activity to arousing stimuli, it is important to note that they operate through a different mechanism than those that contribute to physical dependence on these substances. Animals trained to self administer morphine into the VTA fail to exhibit signs of physical dependence such as teeth chattering and “wet-dog” shakes (Bozarth & Wise, 1984). One explanation for this phenomenon is that VTA DA activation is involved in the reward processing of the event. Therefore, the VTA DA neurons respond to arousing stimuli with an orienting response, and the activation of these neurons is a preferable state of being (as seen by self administration studies); however, activation of these neurons is not sufficient for physical dependence. The lack of association with physical dependence indicates that VTA DA neurons potentiate the rewarding aspect of an experience.

1.6 The Role of the VTA in Motivation

The processing of reward is a critical feature of the mesolimbic pathway. However, there is another critical feature associated with activation-motivation. Limbic system activation precedes intentional behavior and is partially explained as the motivational component of action (Mogenson, Jones, & Yim, 1980). VTA DA neurons send signals to and receive signals from the limbic system via the amygdala (Maeda & Mogenson, 1981). These connections were discovered by stimulating one area while recoding the electrophysiological change in the other. Interestingly, these two studies can be paired with data showing a decline in motivational drive to acquire reward. Rats that receive lesions to the VTA show a significant motivational decline when they are forced to compete for water following an induced thirst (Papp & Bal, 1987). This means that the VTA is at least partially involved in synthesizing information and cues together to produce motivational influences on behavior. Moreover, a complete lesion to the VTA attenuates the motivation to acquire a reward.

The VTA signals that influence motivated behavior occur prior to an expected event to drive behaviors toward acquisition, rather than to maintain the behaviors during consumption (Blackburn, Phillips, Jakubovic, & Fibiger, 1989). Therefore, VTA motivated behavior results from alterations in the firing pattern of the DA neurons to produce a motivation drive toward acquisition. If changes in firing pattern of the VTA DA neurons are partially what transmit information about a particular stimulus, then a substance that alters these patterns would able to influence the subjective motivation.

Morphine is one substance capable of altering the firing pattern of VTA DA neurons (Gysling & Wang, 1983; Matthews & German, 1984). The changes in firing pattern are extremely important as alterations in the burst firing pattern influence the reinforcing properties downstream of the VTA (Bonci, Bernardi, Grillner, & Mercuri, 2003). Bonci, et al drew this conclusion after a review of available literature discussing the behavioral and physiological alterations caused by opioids. The acute influences of morphine induced rapid firing of VTA DA

neurons; however, this result wanes with chronic exposure until morphine is required to produce similar firing patterns seen with experimentally naïve animals (Georges, Le Moine, & Aston-Jones, 2006). This suggests that following chronic exposure to morphine, the mechanism regulating the burst firing patterns of VTA DA neurons is altered. This can also account for the subjective shift in motivation for morphine acquisition- what was once rewarding becomes required to normalize DA activity.

The physiological alteration of VTA DA neurons by morphine begins with the initial exposure (Vanderschuren, De Vries, Wardeh, Hogenboom, & Schoffelmeer, 2001; D. Zhang, Zhang, et al., 2008). Following the initial exposure to morphine there is a prolonged increase in burst and firing rate of DA neurons, as well as an increase in the slow oscillation of VTA DA neurons for up to at least 72 hours (D. Zhang, Zhang, et al., 2008). Zhang et al. (2008) observed that these changes were coupled with a decline in MOR G-protein activation demonstrating the ability of morphine to increase the sensitivity of the mesolimbic pathway by increasing the firing rate of DA neurons in the VTA. Moreover, it also shows that a single exposure to morphine can desensitize MOR as evidenced by a decline in G-protein activation.

Additionally, direct injections of morphine into the VTA eliminate the GABA long-term potentiation (LTP) (Nugent, Penick, & Kauer, 2007). When afferent glutamatergic signals induce LTP on postsynaptic GABA cells, there is a subsequent increase of presynaptic GABA release to the postsynaptic DA neurons in the VTA; morphine abolishes this effect. The mechanism of morphine to interrupt this effect is not fully understood, but it involves the interruption of nitric oxide (NO) as a retrograde messenger and its interaction with cGMP (Nugent & Kauer, 2008; Nugent, Niehaus, & Kauer, 2009). This is noted as the point of disruption because an increase in NO does not reestablish the effect, but an increase in cGMP (the target of NO) restores the LTP of GABA (Nugent & Kauer, 2008). The ability of morphine to disrupt afferent glutamatergic signals in GABA cells is an important feature that contributes to the disinhibition of DA neurons.

1.7 The Influence of Estrogen

The extent of influence sex has on opioid response is a current topic vast research interest. Studies of estrogen indicate that 17β-estradiol (E_2) exerts an influence on postsynaptic AMPA and NMDA receptors to perpetuate EPSPs in the hippocampus (Foy, et al., 1999). This activity is significant because the mechanism for LTP requires an extended EPSP from the afferent signal to provide the voltage necessary to expel Mg^{2+} from the NMDA receptor and extend the duration of the postsynaptic stimulation. The ability of estrogen to maintain LTP suggests a receptor mediated interaction that may not be limited to the hippocampus. Estrogen also has the ability to regulate the basal firing rate of DA neurons (D. Zhang, Yang, Yang, Jin, & Zhen, 2008). Estrogen replacement in OVX rats produced a significant increase in the basal firing rate of VTA DA neurons, however, a seemingly paradoxical relationship was found such that basal firing of OXV rats was higher when compared to intact rats in proestrus (D. Zhang, Yang, et al., 2008). While the exact relationship is unclear from this picture, the addition of estrogen in an OVX model did increase the basal firing rate of DA neurons suggesting that increases in estrogen result in increases in DA neuron activation (see Appendix A, Figure 1). This was precisely the premise the present study investigated.

CHAPTER 2

METHODS

2.1 Animals

This experiment used adult Sprague-Dawley rats bred at the University of Texas at Arlington with Institutional Animal Care and Use Committee approval. A total of 50 animals (12 male, 38 female) were used in this study, and formed a total of five groups. The male rats formed one group, but the females were assigned to one of four conditions based on the current phases of the estrous cycle.

2.2 Estrous Cycling Data

The four phases in the rat estrous cycle are proestrus, estrus, diestrus, and metestrus(Long & Evans, 1922; Mandl, 1951). These phases are distinguished from one another by comparing the proportions of three different cell types collected from a vaginal lavage and also by the variation in ovarian hormones (Marcondes, Bianchi, & Tanno, 2002). During proestrus the majority of the cells from a lavage sample are nucleated epithelial cells, estrogen levels reach their highest peak during this phase. Once the majority of these cells become denucleated and cornified the animal has transitioned into estrus. There is also a second smaller peak of estrogen that occurs during estrus. Equal proportions of leukocytes, cornified epithelial cells, and nucleated epithelial cells define metestrus. Finally, diestrus is characterized by a majority of leukocytes in a collected sample. During metestrus and diestrus the level of estrogen is similar and relatively stable until the animal begins to transition into proestrus where estrogen again increases. Please see Appendix B, Figure 2 then 3 respectively, for images of each phase with the corresponding cell types and a graphical representation of estrous hormone fluctuations.

Cell samples for phase identification were collected with a 10 µl normal saline vaginal lavage per protocol from Marcondes, Bianchi, & Tanno (2002). Cycling data was collected for at least 10 days prior to experimentation. Following collection, samples were immediately photographed under a microscope at 10x and/or 20x magnification for later assessment. On the day of experimentation (day 10 to 15 days after the collection began) a sample was collected after the animal was amnestied, and another sample was taken after experimentation was complete to verify phase.

Cycle phase assessment occurred in triplicate. The initial assessment was made at the time of collection, and confirmed by two independent reviewers. Reviewers received files containing the sequential photographs for each animal collected up to the completion of experiment. To help conceal the date of experimentation, ambiguous photographs were inserted after the final experimental photographs so that each set of photographs had at least 12 days of photographs to code. This helped ensure that the independent raters would be blind to the date of experimentation, and therefore be blind to the group placement (cycling phase) of the data collected.

2.3 Animal Preparation

Animals were anesthetized with chloral hydrate (400 mg/kg; ip), and were not considered anesthetized until they became unresponsive to tail and paw pinch, and a failure to exhibit a blink reflex. Once anesthetized, the animal's head was shaved to prepare the surgical area. The head was then isolated in a stereotaxic device prior to beginning surgery. An incision was made to expose the skull so that both bregma and lamda could be identified. A burr hole was drilled in the skull to permit access to the VTA (approximately 6mm from bregma). Anesthetization was maintained through booster doses of chloral hydrate (350 mg/kg; ip) as necessary throughout the procedure.

2.4 Electrophysiology

From bregma, the coordinates for insertion ranged from 5.6 to 6.2 mm caudal, .5 to 1 mm lateral, and 6.5 to 8 mm ventral (from the cortex) per previous research (Steffensen, Svingos, Pickel, & Henriksen, 1998). Tungsten microelectrodes (10-12 MΩ) were used to identify DA neurons by their spike shape and duration including biphasic (positive- negative) or triphasic (negative-positive-negative) action potentials (AP) with widths greater than 2 ms, and duration of at least 1.1 ms from the start of the AP to the lowest point in the negative trough (Grace & Bunney, 1983; Ungless, Magill, & Bolam, 2004). Spike2 software (CED, UK) was used to record approximately five minutes of baseline data, and another five minutes of data following a saline injection. Finally, a morphine injection (10 mg/kg, 10 mg/ml) was administered, and data was collected for 30 min following injection. Data was collected continuously for the entire 40 minutes. Once the data was collected, the recordings were analyzed and edited offline to ensure proper cell classification. An image of VTA DA projections is provided in Appendix C, Figure 4.

2.5 Histology

Electrode placement in the VTA was confirmed with histological verification. Animals were euthanized with a cardiac puncture after the recordings were complete, and the brains were extracted following euthansia. The tissue was preserved in formaldehyde and then immersed in sucrose for a minimum of 24 hours to remove all water. Following this immersion, the tissue was sectioned at 40 microns using a cryostat. Sections were slide mounted for a thionin staining, and the electrode tracks visible in the tissue confirmed placement in the VTA. The confirmation of electrode placement was also verified by an independent reviewer who was blind to the quality of data received from the animal to prevent bias.

2.6 Data Analysis

Data from Spike2 software was compared for significant differences in firing pattern between and within groups. Comparisons were made between mean baseline data (5 minutes

of recorded data), mean saline injection data (5 minutes of recorded data), and mean morphine injection data (30 total minutes separated into 6 time points of 5 minute increments). This design allowed for a consistent comparison of time increments while testing for alterations in firing frequency.

Two ANOVAs were run to accurately extrapolate information from the data (STATISTICA, StatSoft, OK). The first ANOVA was a 4 x 8 (group x time) that combined the diestrus and the metestrus group together due to the similar hormone levels at this time point, subsequently referred to as Model A. The second ANOVA, Model B, (5 x 8, group x time) separated the combined groups from Model A to provide information about each independent group.

CHAPTER 3

RESULTS

3.1 Animals

A total of 50 animals (12 males and 38 females) were used in this study, but data was only used from 35 animals (7 males and 28 females). All 7 males that were excluded died before the protocol was completed. Of the 10 females excluded, 2 were removed due to technological complications during the recording and the remaining 8 died before the protocol was completed. The following groups were formed from the 35 animals useable after data collection: Diestrus (n = 4), Metestrus (n = 6), Estrus (n = 8), Proestrus (n = 10), and Male (n = 7). The females used in this study ranged from 212 to 296 grams, and the males ranged from 361 to 464 grams.

3.2 Estrous Cycling Data

The phases of the estrous cycle progress in the following order over a period of four to five days: proestrus, estrus, metestrus, and then diestrus. During this study, cycling data progressed between stages in the correct pattern, but the duration of the diestrus phase was surprisingly short and lasted an average of less than one day (occasionally a matter of hours). This was accompanied by an increase in duration of proestrus and estrus that would each last an average of approximately two days.

The cycle phases were rated by independent reviewers with an accuracy of 83% agreement on phase at time of experiment. When the six discrepancies occurred an experienced consultant was contacted to resolve the discrepancy.

3.3 Histology

Electrode recordings for this experiment all took place between 5.75 and 6.25 mm caudal from bregma, .5 to .75 mm lateral, and 6.8 to 8.1mm ventral from the cortex. However,

72% of the recording occurred with placement at 6 mm caudal, .5 to .75 mm lateral and 7.1 to 7.6 mm ventral (from the cortex, see Appendix C, Figure 5, for diagram of electrode placement). These placements are consistent with previous research for VTA recordings (Steffensen, et al., 1998). Histology confirmed placement when electrode track was visible for the recordings, approximately 2/3 of all samples were verified. However, a lack of conformation for electrode placement was not considered grounds for elimination of data. The rational motivating this decision emerges from the consistency between the physical coordinates of electrode placement, the consistence between the placements (majority of recordings were collected from the same coordinates), the identification of the specific spike pattern for DA neurons, and the similar response of those cells to a morphine injection.

3.4 Hypotheses 1 and 2

The first and second hypotheses focused on the differences within groups after a morphine injection. The first hypothesis predicted that the burst firing rate of VTA DA neurons of each group would significantly increase following morphine injection when compared to baseline. The second hypothesis predicted that this increase would also be significant when compared to the saline time point. The results of these hypotheses are reported together for simplicity, because both seek to identify an increase in DA neuron activation from specific time points. The main effects of Model A and Model B are first presented to provide an introduction to the analyses. However, the hypotheses are primarily focused on the results of the simple effects. Therefore, after the main effects are provided the results are organized in the following sequence: proestrus, estrus, diestrus combined with metestrus (di/met), metestrus, diestrus, and male. When applicable, the simple effects for both models are presented.

Model A (4x8 ANOVA) failed to find a significant difference between the groups, $F(3,31) = 2.43$, $p>.05$. However, the model did find a significant effect for time, $F(6,186) = 2.16$, $p<.05$, but the interaction between group and time was also not significant, $F(18,186) = 1.05$, $p>.05$. Model B (5x8 ANOVA) found similar results. The model failed to find a significant difference

between groups, $F(3,31) = 2.43$, $p>.05$. There was a significant effect of time, $F(6,186) = 2.17$, $p<.05$, but the interaction between group and time again failed to find a significant result, $F(18, 186)= 1.05$, $p>.05$. The simple effects from these models with Fischer LSD post hoc were used to test the first and second hypotheses.

The proestrus group was the first group analyzed. It failed to support the first and second hypotheses. There was no significant change in DA neuron firing between baseline and any other time points for this group. This was also true for comparisons made between the saline time point and all other subsequent time increments. Additionally, the lack of significance was consistent across Model A and Model B (see Appendix D for all data figures for the first and second hypothesis, proestrus data presented in Figure 6). The estrus group also failed to support either of the hypotheses which predicted an increase in DA firing. However, the results show that there was a significant decline in DA neuron activation from baseline at time points: 5 min ($p<.05$), 10 min ($p<.05$), 15 min ($p<.05$), 20 min ($p<.05$), 25 min ($p<.001$), and 30 min ($p<.001$). This decline was also significant from the saline injection at time points: 15 min ($p<.05$), 25 min ($p<.05$), and 30 min ($p<.05$). The significant decline and p values were consistent between Model A and Model B. This decline is shown in Figure 7.

The next group evaluated was the met/diestrus group (the combination of metestrus and diestrus), and is specific to Model A. This group supported the first hypothesis at time point 20 min ($p<.05$) only; however, the second hypothesis was not supported (see Figure 8). Interestingly, when Model B split the combined groups and evaluated the metestrus and diestrus groups independently of each other, a different trend was discovered. Analysis of the metestrus data supported hypothesis 1 at time points: 10 min ($p<.05$), 15 min ($p<.05$), and 20 min ($p<.05$), but the data failed to support the second hypothesis. The diestrus group failed to support the first or second hypothesis, and the model failed to identify any significant change in DA firing from either baseline or following the saline injection. The metestrus data is shown in Figure 9, and the diestrus data in Figure 10.

The final group evaluated by both Model A and Model B was the group of males; this data supported both the first and second hypotheses. The first hypothesis was supported at time points: 5 min (p<.05), 10 min (p<.05), and 15 min (p<.05), and the second hypothesis was supported at time point 10 min (p<.05). This data is shown in Figure 11. When all groups are viewed side by side, the variation in the response to morphine becomes increasingly clear, see Figure 12.

3.5 Hypothesis 3

The third hypothesis predicted variation in the response of VTA DA neurons between groups based on cycle phase and thereby fluctuations in estrogen level. Specifically, this hypothesis predicted significant differences between groups at each time point based on the level of estrogen such that higher levels of estrogen would result in a significantly higher rate of VTA DA neuron firing for that respective group at that time point after a morphine injection. More specifically, this hypothesis predicted that the proestrus group would have the highest rate of DA neuron firing, followed by estrus, then metestrus/diestrus and then males. This hypothesis was also evaluated using Model A (4x8 ANOVA) and Model B (5x8 ANOVA). Before presentation of the effects identified by analyses it should be noted that none of the time points following morphine injection supported the hypothesis. The data presentation will begin with baseline comparisons and proceed in chronological order. Graphs in Appendix E will indicate significant relationships, but the p value is provided in text.

Each time point evaluated represents five minutes of recorded data. At baseline the only significant differences occurred in relation to the estrus phase. This difference was such that VTA DA firing was significantly higher at baseline when compared to the Met/Di group (p<.001), proestrus (p<.001), metestrus (p<.05), and diestrus (p<.05). Although this data appears to support the hypothesis, it is most likely not a valid effect because it was driven by an outlier (See Appendix F for data forming each group). Data for this time point is presented in Figure 13.

Following the saline injection additional differences were observed between groups that again failed to support the third hypothesis. The estrus group had an average DA firing frequency that was significantly higher than the diestrus (p<.05), metestrus (p<.05), and the met/diestrus (p<.05) groups. Again, this difference appears to support the hypothesis, but the effect was driven by an outlier. Additionally, the males were found to have a significantly higher average DA firing frequency following the saline injection than the diestrus (p<.05), proestrus (p<.05) and the met/diestrus (p<.05) groups. This data is presented in Figure 14.

The first set of data compiled after the morphine injection failed to support the third hypothesis, and showed that only the male group had a significantly higher rate of activation than all other groups- estrus (p<.001), proestrus, (p<.001), diestrus (p<.001), metestrus (p<.05), and met/di (p<.001). There were no other significant relationships at this time point (see Figure 15).

The next time point also failed to support the third hypothesis. Ten minutes after the morphine injection the average male firing rate of DA neurons was still significantly higher than the estrus (p<.001), proestrus (p<.001), diestrus (p<.001), and met/di (p<.001), but not the metestrus group. The met/di group's average DA firing frequency was also significantly higher when compared to the proestrus group (p<.05). Finally, the metestrus group had a significantly higher firing frequency than the estrus (p<.05), diestrus, (p<.05), and proestrus (p<.001) (see Figure 16).

This relationship between groups was carried over to the next time point, 15 minutes after the morphine injection. All results at this time point also failed to support the third hypothesis. The male group maintained a higher frequency of DA firing over the estrus (p<.001), proestrus (p<.001), diestrus (p<.001), and met/di (p<.05) groups. The met/di group was still significantly higher than the proestrus (p<.05) group. Additionally, the metestrus group was still significantly higher than the estrus (p<.05), proestrus (p<.05), and diestrus (p<.05) groups (Figure 17).

The next three time points (20, 25, and 30 minutes following morphine injection) also failed to support the third hypothesis. Two groups (males and metestrus) consistently provided significantly higher rates of DA neuron firing when compared to the other groups at the same time point. Twenty minutes after the morphine injection the males DA firing frequency was significantly higher than the estrus ($p<.05$), proestrus ($p<.05$), and diestrus ($p<.001$) groups, and the metestrus group was also significantly higher than the estrus ($p<.05$), proestrus ($p<.05$), and diestrus ($p<.05$) groups (Figure 18). After 25 minutes of morphine injection the males DA firing frequency was significantly higher than the estrus ($p<.05$), proestrus ($p<.05$), and diestrus ($p<.001$) groups, and the metestrus group was also significantly higher than the estrus ($p<.05$), proestrus ($p<.05$), and diestrus ($p<.05$) groups (Figure 19). At the final time point (30 minutes after morphine injection) the male group continued to show a significantly higher frequency of DA neuron firing than the estrus ($p<.001$), proestrus ($p<.05$), diestrus ($p<.05$), and the met/di ($p<.05$) group. The metestrus group was also significantly higher than the estrus ($p<.05$), proestrus ($p<.05$), and diestrus ($p<.05$). Data from this time point is shown in Figure 20

CHAPTER 4

DISSCUSSION

4.1 Interpretation of Results

The first two hypotheses analyzed the effect of morphine on DA neuron firing within each group of the study: proestrus, estrus, metestrus, diestrus, the combination of metestrus and diestrus, as well as a group of males. The first and second hypothesis predicted an increase in DA neuron activation following a morphine injection from baseline and after a saline injection respectively. The third hypothesis predicted this increase to be influenced by estrogen such that higher levels of estrogen would be associated with greater increases in DA neuron activity at corresponding time points. Because estrogen levels fluctuate throughout the estrous cycle, DA neuron activation was predicted to fluctuate in a respective pattern. The highest serum levels of estrogen occur during a spike in proestrus, and the second highest levels occur during a spike in estrus. Estrogen then falls to a relatively stable level during metestrus and diestrus before rising again during proestrus. If DA neuron activation rate is positively influenced by estrogen levels then the highest rate of activation would be identified during proestrus. Additionally, this activation rate would then correlate throughout the phases with the estrogen fluctuations. The male response was also expected to be less significant than the female response due to the deficit in ovarian hormone fluctuations, specifically estrogen. This was not the relationship identified by this experiment.

No significant change in DA neuron firing was identified in the proestrus group from baseline or from the saline injection following morphine administration, and thus did not support either the first or second hypotheses. The addition of morphine during the estrus phase

produced a significant decline in DA neuron activation from baseline at all time points following morphine administration, and a significant decline in DA neuron activation following the saline injection at the time points 15, 25, and 30. Viewed in isolation this data appears to support the idea that higher levels of estrogen attenuate a decline in DA activation because the proestrus data failed to produce a significant decline from either baseline or saline while the estrus phase resulted in a significant decline from both time points.

However, this notion is confounded by the significant increase of DA neuron activation with data collected during metestrus, when estrogen levels are lower relative to proestrus and estrus. Following morphine administration the metestrus group increased significantly from baseline at time points 10, 15, and 20, supporting the first hypothesis. However, there was no significant increase from the saline collection period. If estrogen is the hormone driving this response then the data collected during diestrus should mimic the metestrus data based on typical estrogen levels during this phase. Interestingly, when the diestrus data was analyzed independently of the metestrus data no significant increase or decrease was identified between any of the collection time points. Finally, when the male data was analyzed there was a significant increase following morphine injection from baseline at time points 5, 10, and 15, supporting the first hypothesis. There was also support for the second hypothesis with a significant increase at time point 10.

The data analyzed for the first and second hypotheses indicate that estrogen did not have the anticipated influence on DA neuron activation. Moreover, the data suggests that estrogen may attenuate DA neuron activation following morphine administration. This relationship can be inferred by the lack of a significant increase in DA neuron activation at any time point after a morphine injection during the two phases where estrogen is highest, proestrus and estrus. Also, the lack of congruence between the metestrus and diestrus phase may be attributed to the abnormally short length of the diestrus phase.

The typical duration of diestrus from previous experiments is approximately 28 hours (Mandl, 1951). The cycling data collected during this study revealed that diestrus lasted less than one day, and in most cases was undetected during the data collection. One reason for this reduction in cycling phase could be the housing proximity of females to males in the colony room. This suspicion is supported with data demonstrating that decreasing the proximity between males and females increases the time duration spent in proestrus, estrus, and metestrus relative to diestrus (Cooper, Purvis, & Haynes, 1972; Purvis, Cooper, & Haynes, 1971). These studies found alterations in cycling data similar to those observed in this study. The proximity measure in both studies allowed for several hours of cage sharing each day while prohibiting physical contact with a wire mesh barrier. This allowed for only visual, auditory, and olfactory cues to influence the cycling pattern. While these are not the exact conditions that were present in the colony room, housing females close to males on a permanent basis appears to be sufficient to replicate previously reported cycling discrepancies as demonstrated by the cycling data collected here. Therefore, it is reasonable to postulate that the similarity between the diestrus and proestrus data could be attributed to an early onset of ovarian hormones that regulate estrous cycle phase progression. Moreover, this onset would be more representative of naturally occurring phases where male/female interaction is not prohibited.

Also, while the significant decline from baseline and the saline time points during the estrus recordings appear to be different results from proestrus, these results must be approached with skepticism. Analysis of the differences between groups at each time point failed to find a significant difference between the estrus, proestrus, and diestrus groups at any of the measurement periods following morphine injection. This indicates that while there was a significant decline within the estrus data, this response is not significantly different from the response during proestrus or diestrus. However, this is not true with regard to the data collected from the metestrus group.

Data from the within and between groups analysis show that the response to morphine during metestrus resulted in a significant increase from baseline, but also in a sustained increase that was significantly higher than the other phases five minutes after administration. The metestrus response pattern also mimicked that of the male data. This is not surprising if the response is motivated by ovarian hormones, because these hormones are at their lowest levels during this phase. Based on the analyses preformed, a dichotomy within the data emerges which suggests that lower levels of ovarian hormones results in an increase in firing rate of DA neurons in the VTA following morphine injection.

Combining this electrophysiological evidence with behavioral observations increases the validity of this claim. In unpublished research, Morris-Bobzean, Chance, Dennis and Perrotti (2011) used a CPP paradigm with an OVX (ovariectomized) model to show that estrogen replacement reduces the preference for morphine associated environments compared to OVX animals who did not receive estrogen replacement. The combination of these two studies indicates that ovarian hormones can have a suppressive effect on the rewarding potential of morphine via a reduction in VTA neuron activation. However, an alternate explanation for these results may go beyond the role of just estrogen to another chemical, since estrogen was not measured directly.

4.2 Alternative Explanations

The ability of morphine to induce CPP has been established in both male and female rats (Karami & Zarrindast, 2008). Karami, et al. (2008) found that relative to males, intact female animals acquire CPP at lower doses of morphine, and that the females were more sensitive to the effects of morphine. Unfortunately, the researchers did not track the cycling status of the females. In a separate study, the acquisition of morphine CPP was effectively blocked in male rats through direct injections of a nitric oxide synthase (NOS) inhibitor into the VTA (Gholami, Zarrindast, Sahraei, & Haerri-Rohani, 2003). However, such an experiment has not been conducted that tests this effect in cycling females. The results from these two studies

indicate that the ability of morphine to induce CPP is related to a NO dependent pathway. Therefore, the ability of morphine to induce CPP and DA release in the VTA can be regulated by NO levels in addition to estrogen levels.

The estrous cycle of female rats is also subject to the influence of NO. NO deficiency can induce prolonged states of proestrus and/or estrus as long as the deficiency is maintained (Dunnam, Hill, Lawson, & Dunbar, 1999). Additional results from Dunnam, et al. (1999) study found that maintenance of these phases by NOS inhibitors in the drinking water also resulted in significantly higher basal serum levels of estradiol in the NO deficient rats compared to those who naturally cycled into proestrus. This means that inhibition of NO results in an increased duration of proestrus and estrus as long as the deficiency was maintained, and that the corresponding estrogen serum levels during this time are significantly elevated compared to experimentally naive animals. Moreover, the relationship between estrogen levels and NO was reportedly maintained in an OVX model, such that the addition of 17β-estradiol in OVX rats significantly attenuated NO increases after administration of NOS (Kauser, Sonnenberg, Tse, & Rubanyi, 1997). This indicates that even estrogen replacement OVX models are subject to the potential influence of NO on behavioral and physiological responses.

In regard to this study, it is possible that the influence attributed to estrogen may also be a consequence of alterations in NO levels throughout the study. NO levels are associated with phase progression such that a decline in NO results in an increase in serum estrogen accompanied by an indefinite maintenance of proestrus and/or estrus (Dunnam, et al., 1999). The extended duration of proestrus and estrus observed in this study induced by housing proximity to males (Cooper, et al., 1972; Purvis, et al., 1971) could also have decreased the NO levels in the animals. Likewise, the decline in NO could also account for the lack of predicted activity of VTA DA neurons because blocking NO results in the elimination of morphine induced CPP (Gholami, et al., 2003). This is also supported by the ability of NO to induce the release of DA in striatal slices in a dose dependent fashion (Zhu & Luo, 1992). Therefore, while the

results from this study could be the result from fluctuations in estrogen, it is equally likely that they are the result from the inverse fluctuations of NO or an interacting effect of estrogen and NO.

Reconciling this hypothesis is an avenue of research that has yet to be explored. One method of research would be to test the influence of estrogen in a NOS knockout model; this has yet to be experimentally documented. Also, data from this study should be replicated and expanded in order to make more conclusive interpretations in regard to the influence of estrogen on VTA DA neuron activation. This should include data from of an OVX model with estrogen replacement that includes a dose response element with respect to estrogen and morphine concentrations. Additionally, documentation of the various spike signatures located in the VTA should accompany this research to build a comprehensive understanding of the effect of morphine on cycling females. Such literature is currently absent from existing research.

However, this explanation does little to reconcile the evidence for receptor related influences of estrogen in the brain. Estrogens may diminish the effect of opioids, such as morphine, due to a sensitivity of the opioid receptor that specifically facilitates estrogen binding (Schwarz & Pohl, 1994). If this model is correct, estrogens could attenuate opioid sensitivity by blocking the binding site on the *μ, δ,* and κ opioid receptors. Also, progestins interact with $GABA_A$ receptors through an allosteric interaction to increase chloride ion membrane permeability (Barker, Harrison, Meyers, & Majewska, 1986; Majewska, Harrison, Schwartz, Barker, & Paul, 1986). This too could at least partially account for VTA DA neuron suppression with intact cycling females because progesterone is also present in these models, and VTA DA neurons contain $GABA_A$ receptors utilized by endogenous GABA from GABAergic interneurons (Kalivas, et al., 1990; Kalivas, et al., 1991). This interaction between progestins and the $GABA_A$ receptor could nullify the reduction in endogenous GABA transmission after morphine administration, and could also explain the results from this experiment.

4.3 Concluding Remarks

The contribution of this study extends only as far as the data permits. Experimentally naïve intact cycling female Sprague-Dawley rats show a significant increase in VTA dopamine neuron activation during metestrus. This response is similar to that of experimentally naïve male Sprague-Dawley rats. Female Sprague-Dawley rats in diestrus and proestrus experience no significant change in VTA DA neuron activation following morphine injection. Females in the estrus phase experience a significant decline in VTA DA neuron activation following a morphine injection. However, the difference in VTA DA neuron activation at each relative time point is such that only males and metestrus females experience a significant increase in activation compared to females in the diestrus, proestrus or estrus phase. In conclusion, females in the diestrus, proestrus, and estrus phase do not respond with significantly different VTA DA neuron activation following morphine injection.

APPENDIX A

PROPOSED MODEL OF MESOLIMBIC ACTIVATION

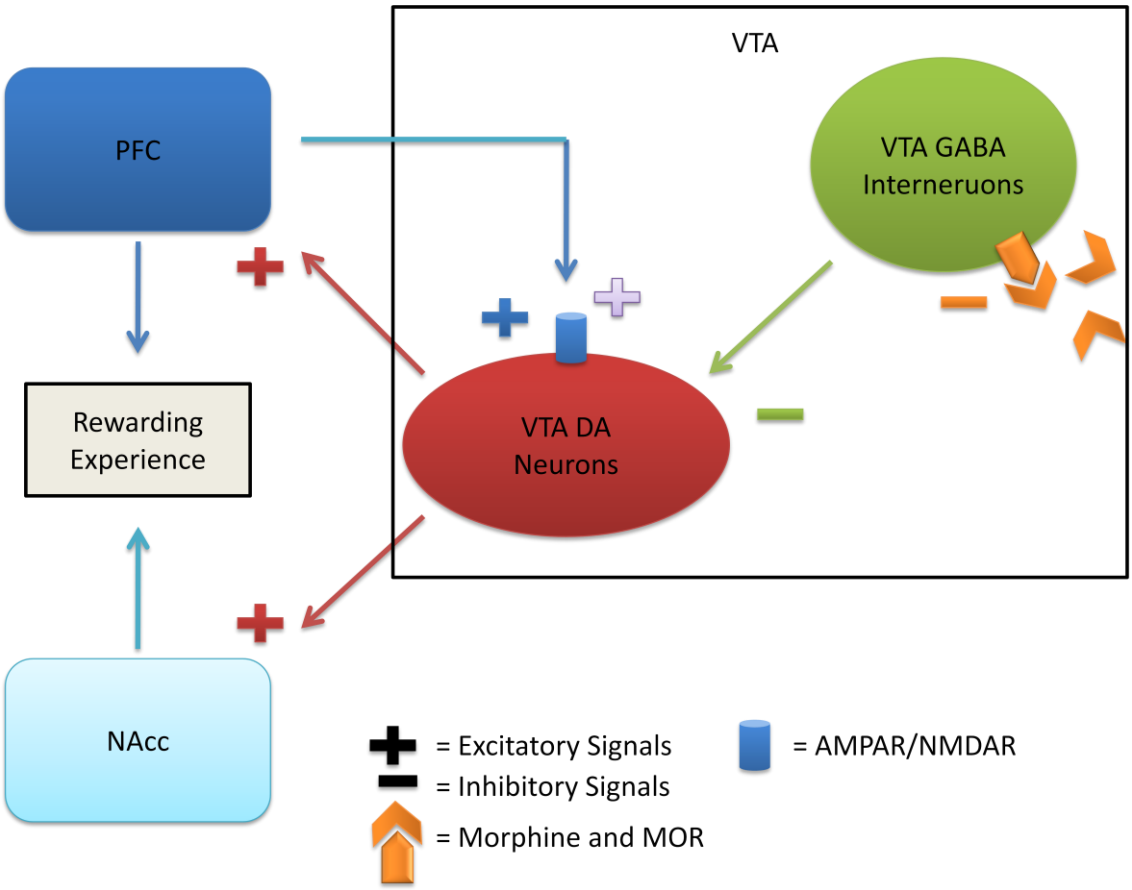

Figure 1: Simple Proposed Model of the Mesolimbic Pathway Activation. The black square identifies activity that occurs within the VTA. Arrows indicate projections and are color coded with the corresponding neuron they project from. Red is for DA, Green for GABA, Dark Blue is glutamate from PFC, Purple excitatory signal is representative of estrogen.

APPENDIX B

CYCYLING IMAGES

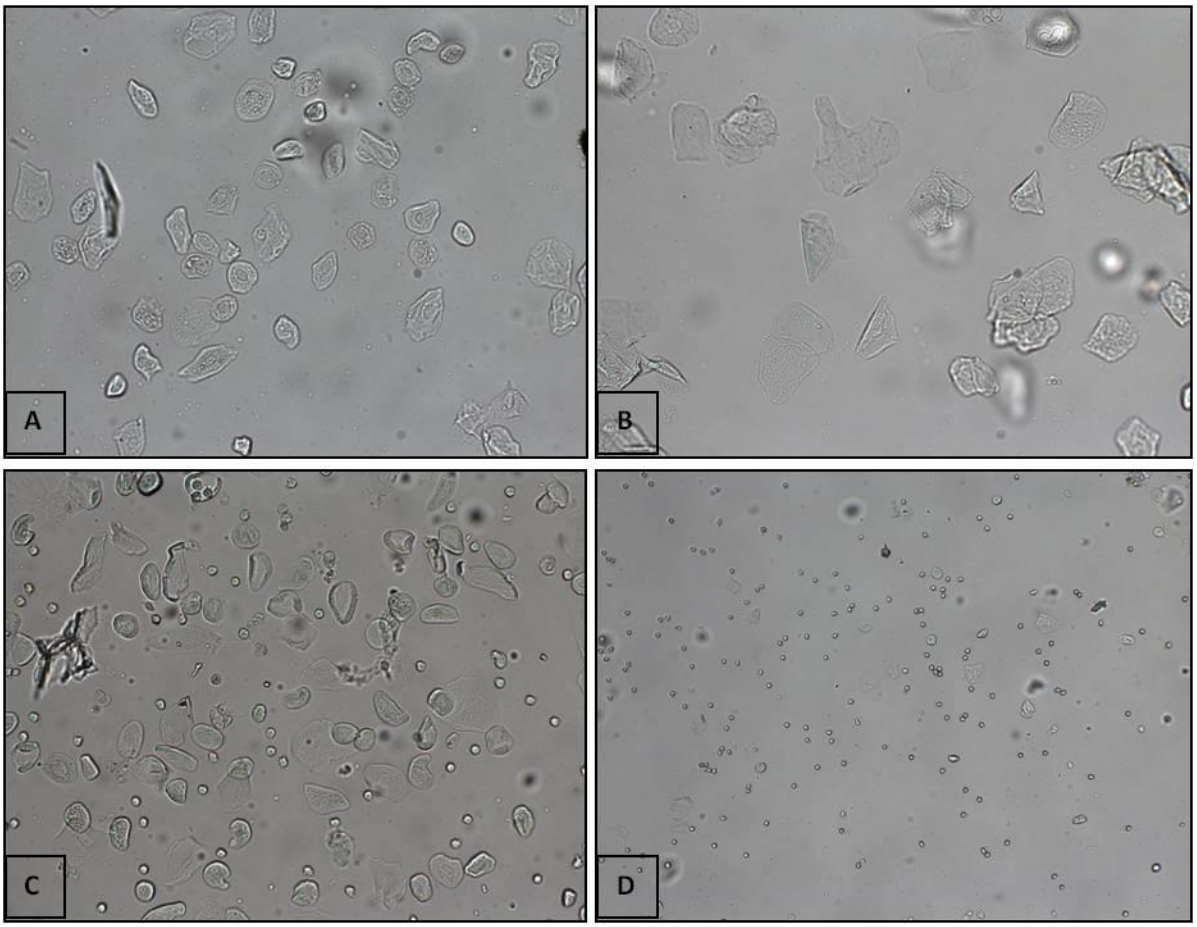

Figure 2: Photographs Collected from the Cycling Data. **A: Proestrus** (magnification 20x), characterized by nucleated epithelial cells; **B: Estrus** (magnification 20x), characterized by denucleated cornified cells; **C: Metestrus** (magnification 20x), characterized by approximately equal distributions of nucleated epithelial cells, cornified cells, and leukocytes; **D: Diestrus** (magnification 10x), characterized by a predominance of leukocytes.

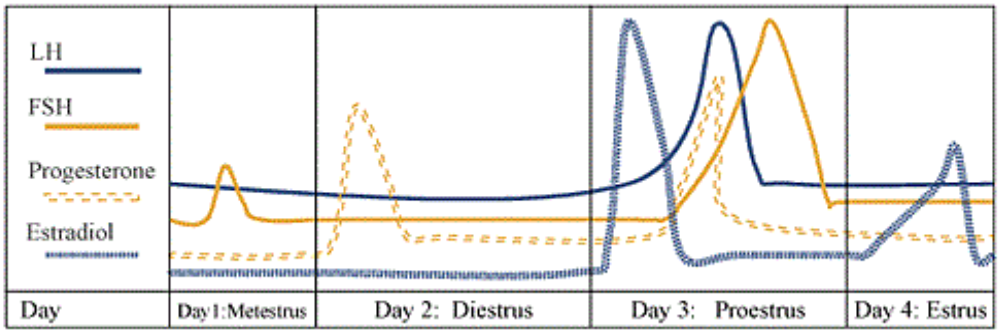

Figure 3: Phases of the Estrous Cycle with Hormones. The two peaks of estradiol occur during proestrus and estrus with relatively stable levels occurring during the metestrus and diestrus phase (Emanuele, Wezeman, & Emanuele, 2002).

APPENDIX C

DOPAMINE PROJECTIONS AND ELECTRODE PLACEMENT

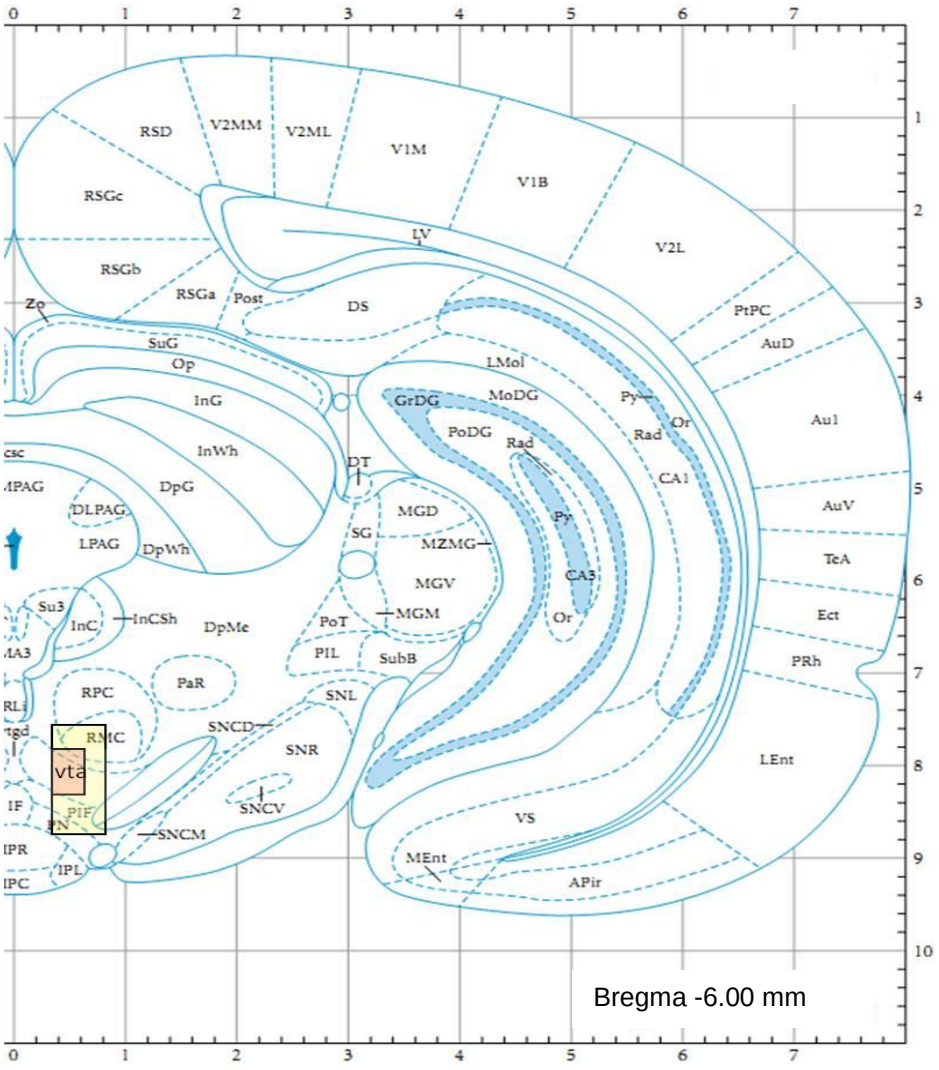

Figure 4: Electrode Placement. Atlas image displays the areas of the rat brain in stereotaxic coordinates. Yellow box = total range of recording coordinates in all distances from bregma (5.75 to 6.25 mm), red box = location of 72% of total electrode placements (G. Paxinos & Watson, 2005).

APPENDIX D

FIGURES FOR HYPOTHESIS 1 AND 2

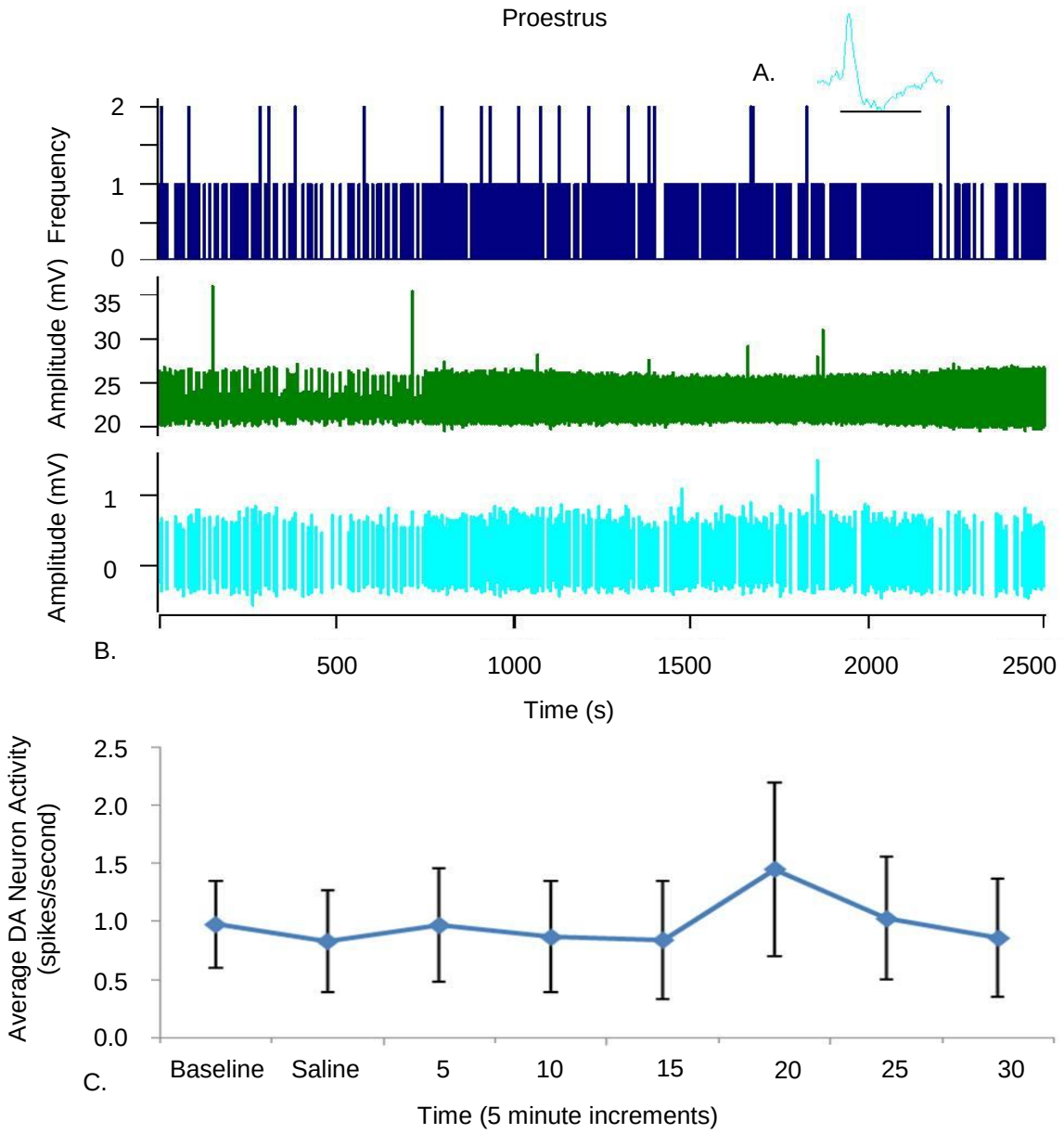

Figure 5: Proestrus Electrophysiological Data. **Image A** (top) is an isolated DA action potential from recordings. The solid line below the action potential represents a duration of 2ms. **Image B** (middle) is divided into three sections. The top is a histogram of DA action potential events during the recording. The middle portion is raw data recorded from the electrode. The bottom portion shows action potentials isolated from the raw data according to the template (Image A). Approximate injection times are 300s for saline, and 600s for morphine. **Image C** is the group average DA neuron activation at the corresponding time points, * = significant difference from baseline, + = significant difference from saline.

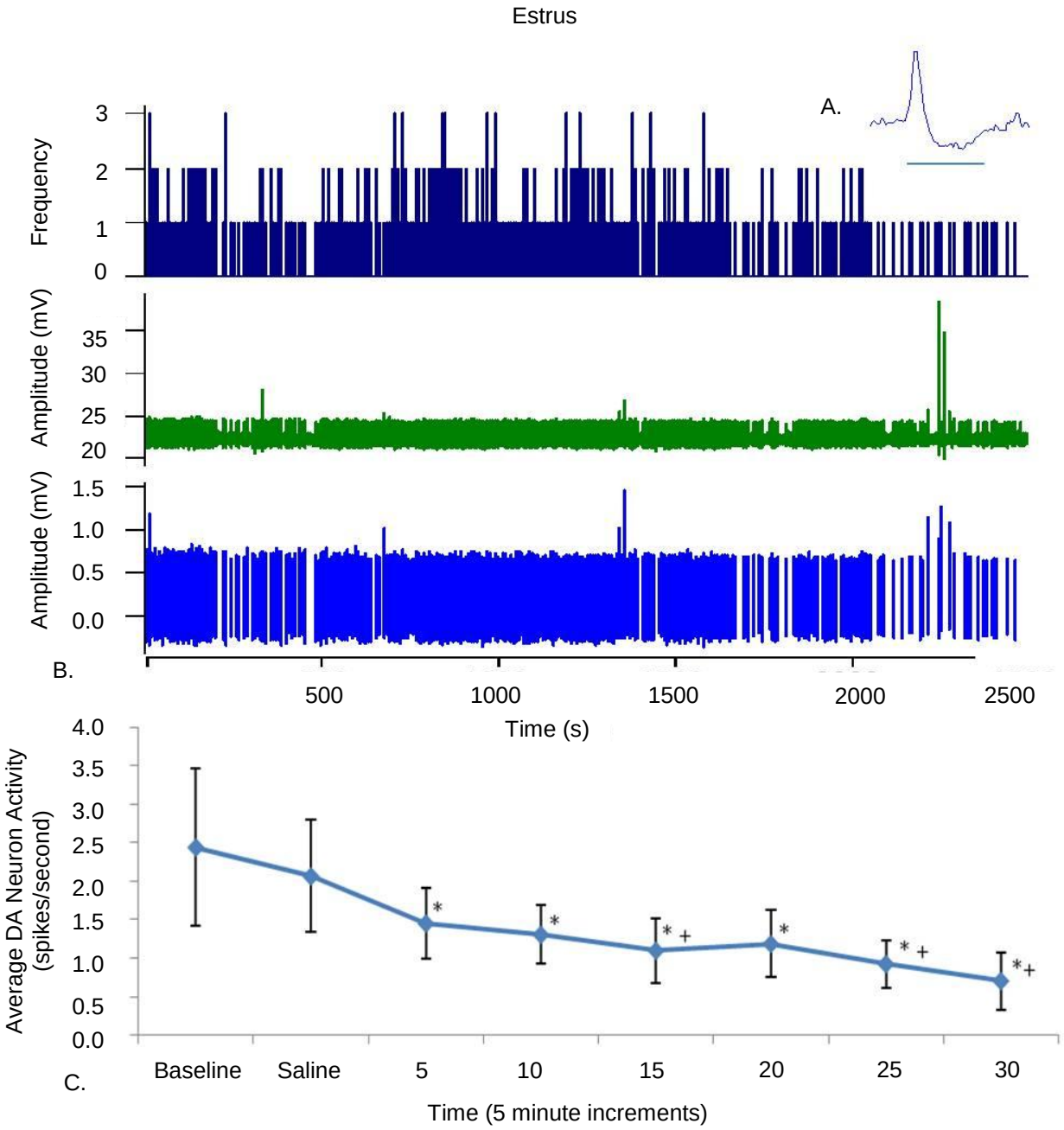

Figure 6: Estrus Electrophysiological Data. **Image A** (top) is an isolated DA action potential from recordings. The solid line below the action potential represents a duration of 2ms. **Image B** (middle) is divided into three sections. The top is a histogram of DA action potential events during the recording. The middle portion is raw data recorded from the electrode. The bottom portion shows action potentials isolated from the raw data according to the template (Image A). Approximate injection times are 300s for saline, and 600s for morphine. **Image C** is the group average DA neuron activation at the corresponding time points, * = significant difference from baseline, + = significant difference from saline.

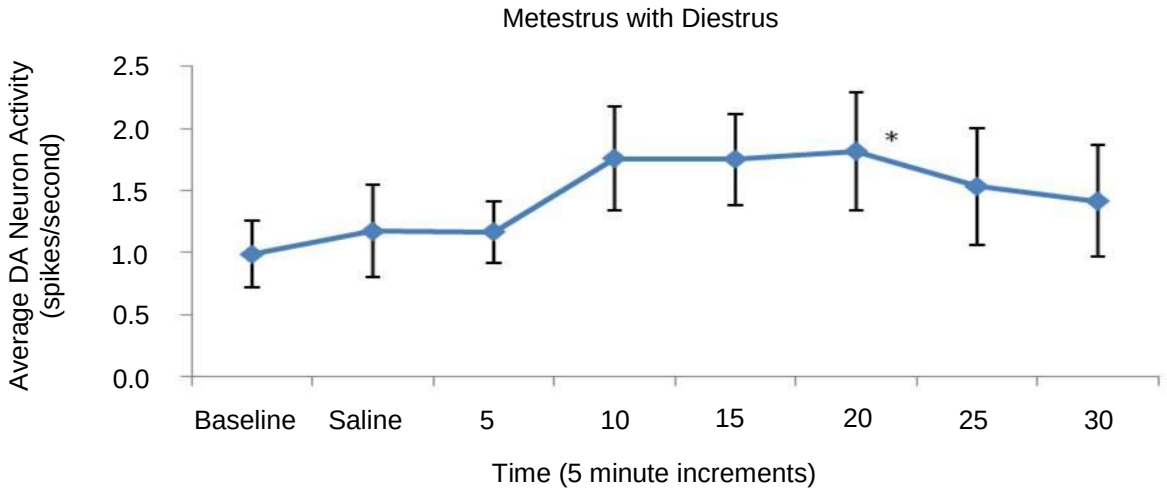

Figure 7: Metestrus with Diestrus. Presentation of the response of DA neurons to a saline and morphine injection; * = significant difference from baseline, + = significant difference from saline. Note: no significant difference from saline was found. No other data is presented in the figure because the data represents a composite of two groups.

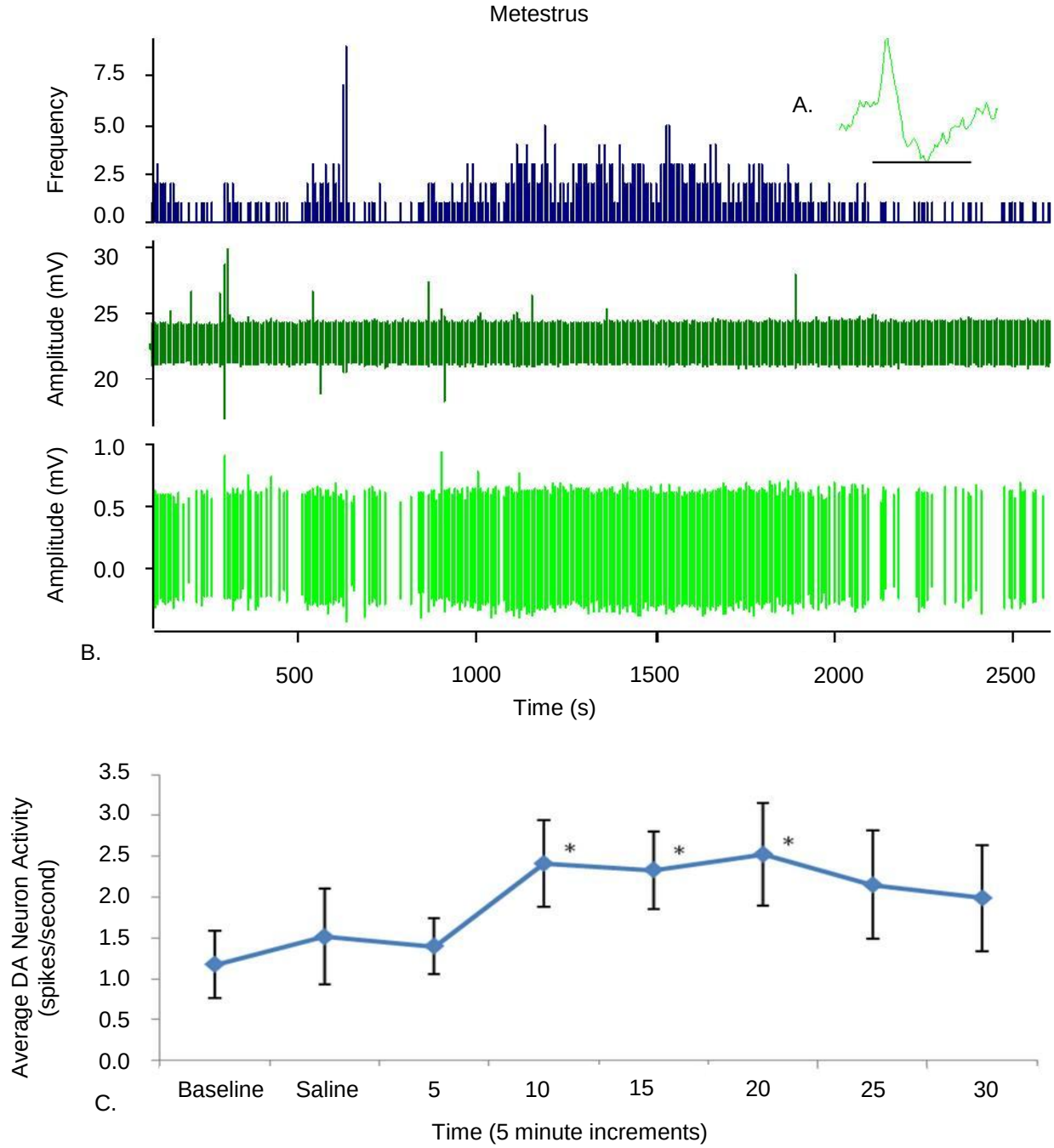

Figure 8: Metestrus Electrophysiological Data. **Image A** (top) is an isolated DA action potential from recordings. The solid line below the action potential represents a duration of 2ms**. Image B** (middle) is divided into three sections. The top is a histogram of DA action potential events during the recording. The middle portion is raw data recorded from the electrode. The bottom portion shows action potentials isolated from the raw data according to the template (Image A). Approximate injection times are 300s for saline, and 600s for morphine. **Image C** is the group average DA neuron activation at the corresponding time points, * = significant difference from baseline, + = significant difference from saline.

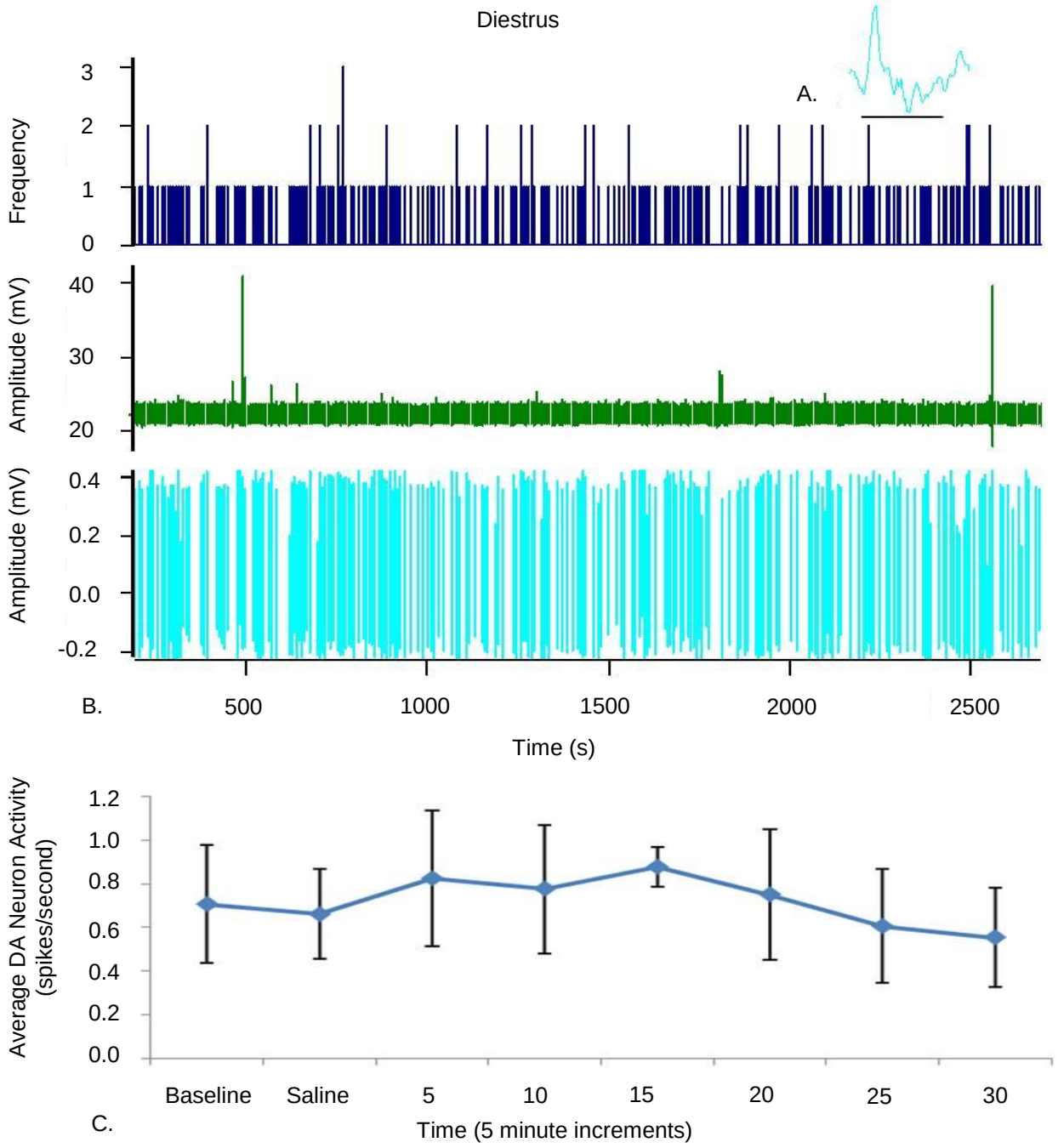

Figure 9: Diestrus Electrophysiological Data. **Image A** (top) is an isolated DA action potential from recordings. The solid line below the action potential represents a duration of 2ms. **Image B** (middle) is divided into three sections. The top is a histogram of DA action potential events during the recording. The middle portion is raw data recorded from the electrode. The bottom portion shows action potentials isolated from the raw data according to the template (Image A). Approximate injection times are 300s for saline, and 600s for morphine. **Image C** is the group average DA neuron activation at the corresponding time points, * = significant difference from baseline, + = significant difference from saline.

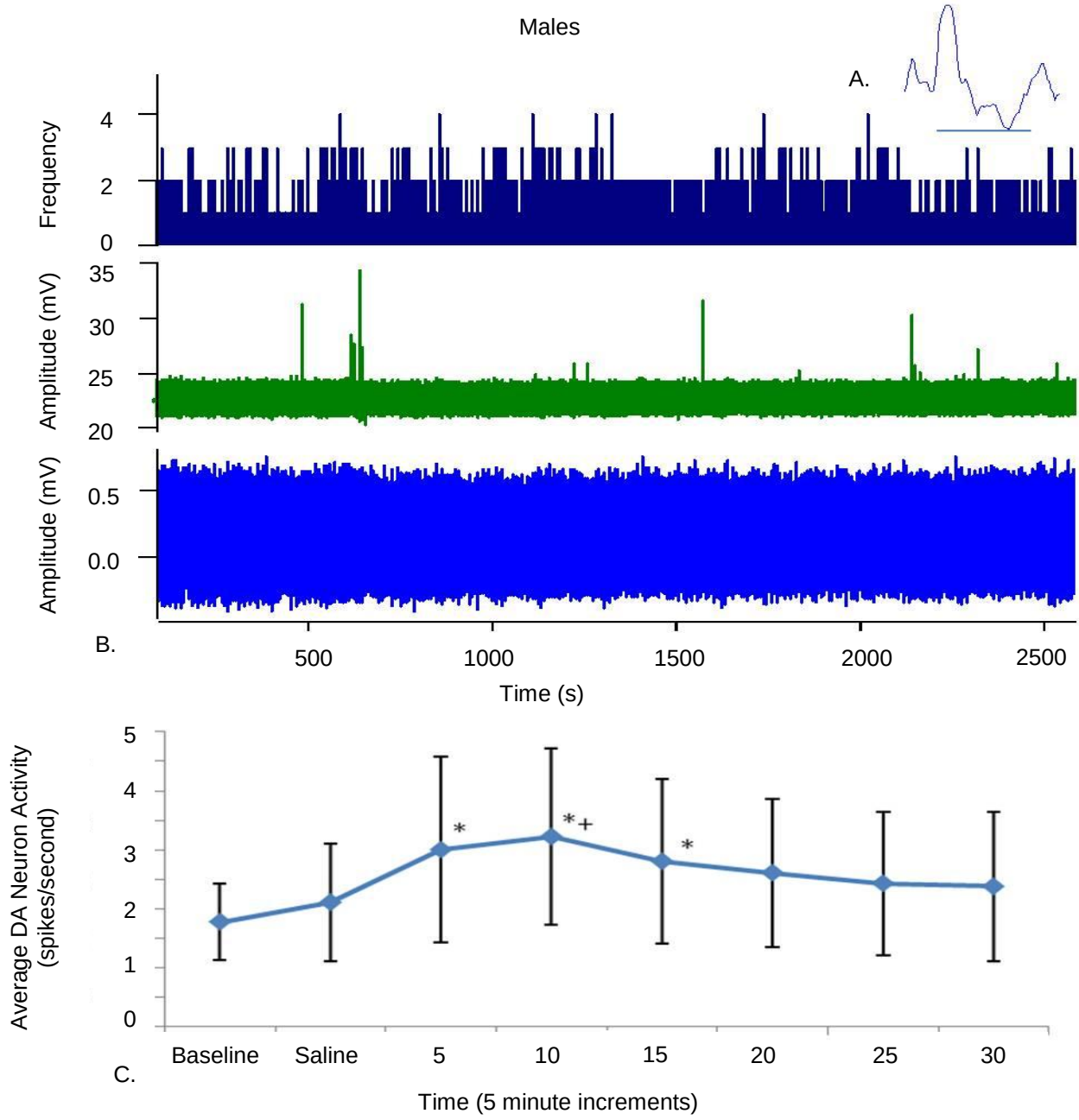

Figure 10: Male Electrophysiological Data. **Image A** (top) is an isolated DA action potential from recordings. The solid line below the action potential represents a duration of 2ms. **Image B** (middle) is divided into three sections. The top is a histogram of DA action potential events during the recording. The middle portion is raw data recorded from the electrode. The bottom portion shows action potentials isolated from the raw data according to the template (Image A). Approximate injection times are 300s for saline, and 600s for morphine. **Image C** is the group average DA neuron activation at the corresponding time points, * = significant difference from baseline, + = significant difference from saline.

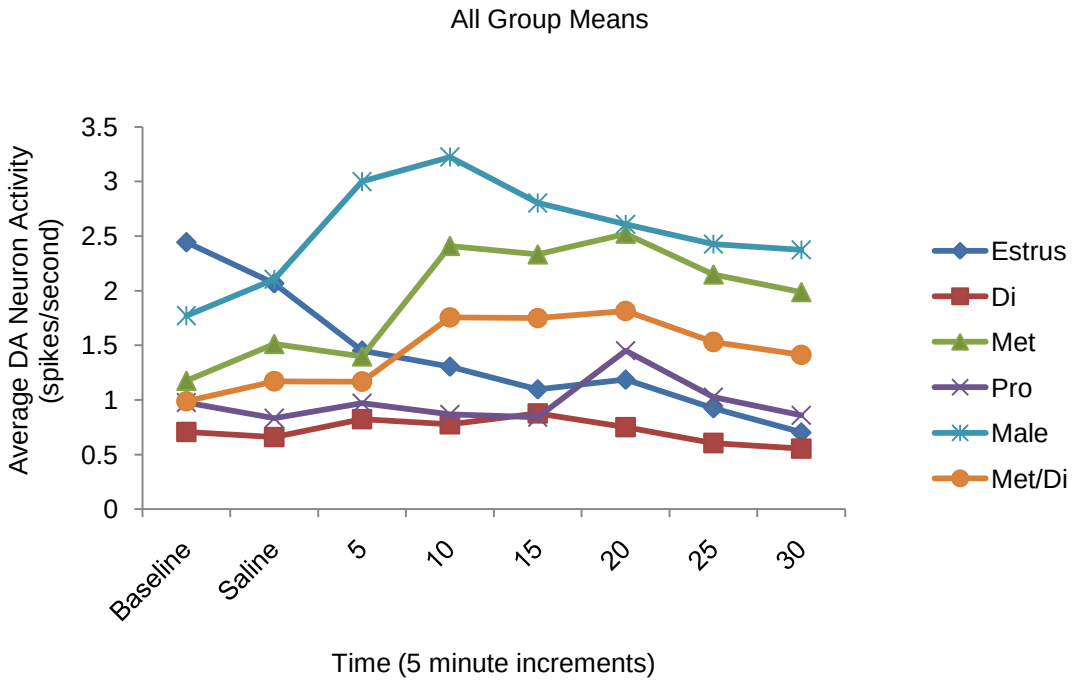

Figure 11: Comparison of All Group Means. Side by side comparison of means at each time point for each group. Error bars and significant differences omitted for graph clarity.

APPENDIX E

FIGURES FOR HYPOTHESIS 3

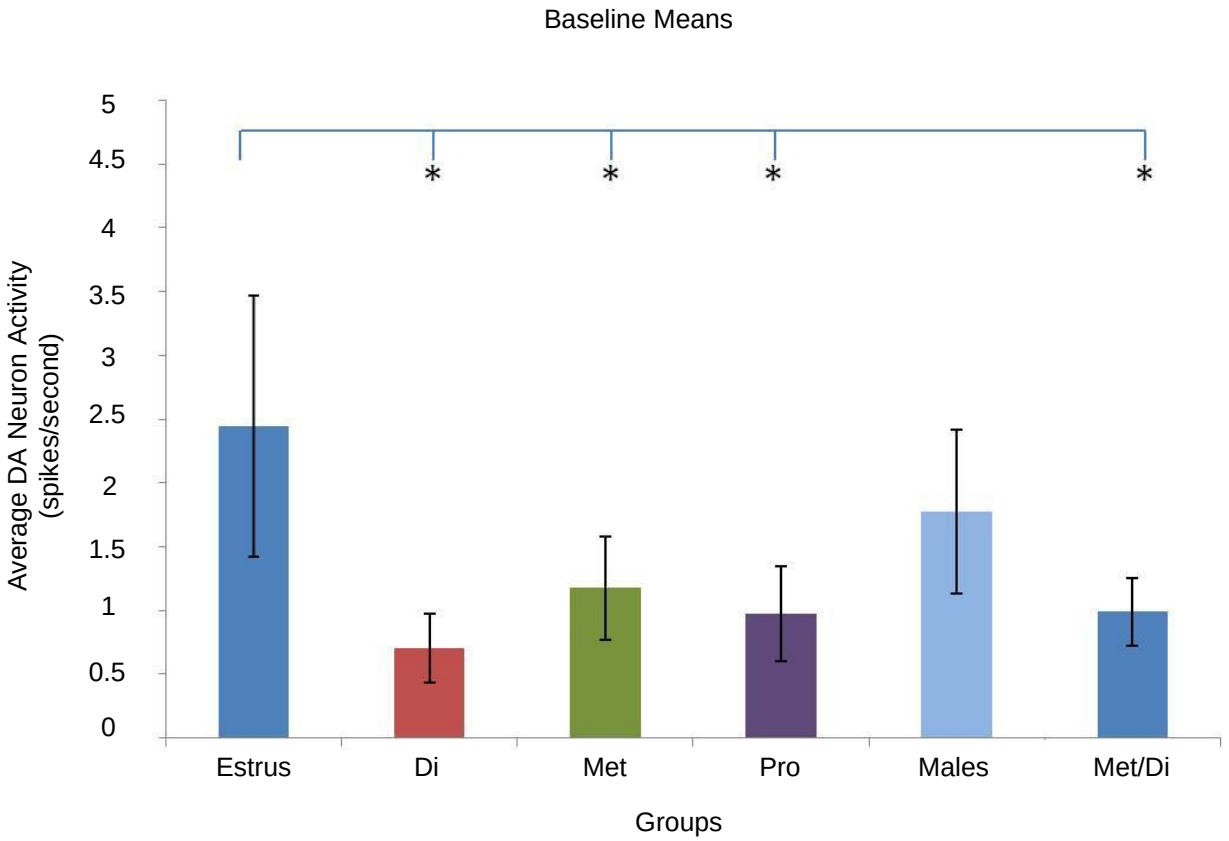

Figure 12: Baseline Means. Significant different rates of DA neuron firing between groups at baseline. Significance is not considered support of the third hypothesis because the baseline rate of DA neuron firing is inflated by an outlier. Significant differences between groups are indicated by *, see text for *p* value.

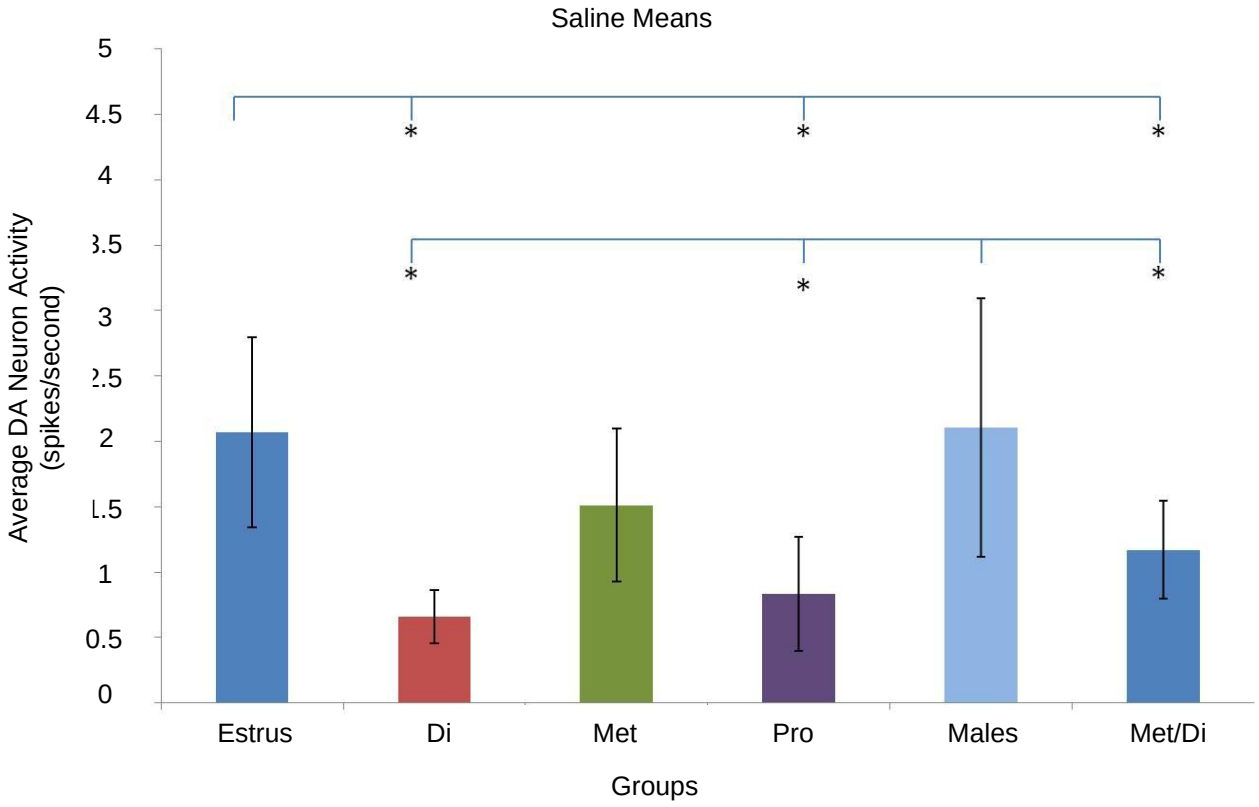

Figure 13: Saline Means. Significantly different rates of DA neuron firing between groups following a saline injection. Significantly higher rates of fire are not considered support of the third hypothesis because the baseline rate of DA neuron firing is inflated by an outlier. Significant differences between groups are indicated by *, see text for *p* value.

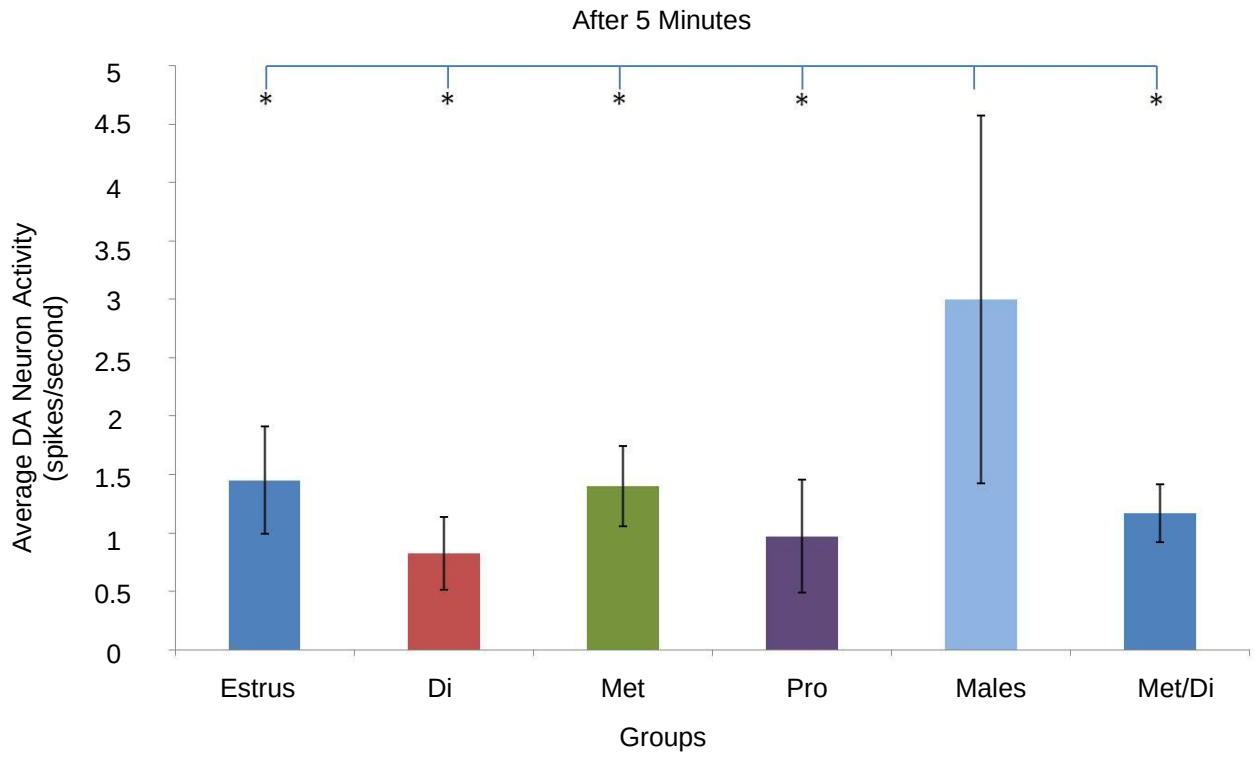

Figure 14: 5 Minutes Following Morphine Injection. Significantly different rates of DA neuron firing between groups for the first set of five minutes of data collected 5 minutes after morphine injection. Significant differences between groups are indicated by *, see text for p value.

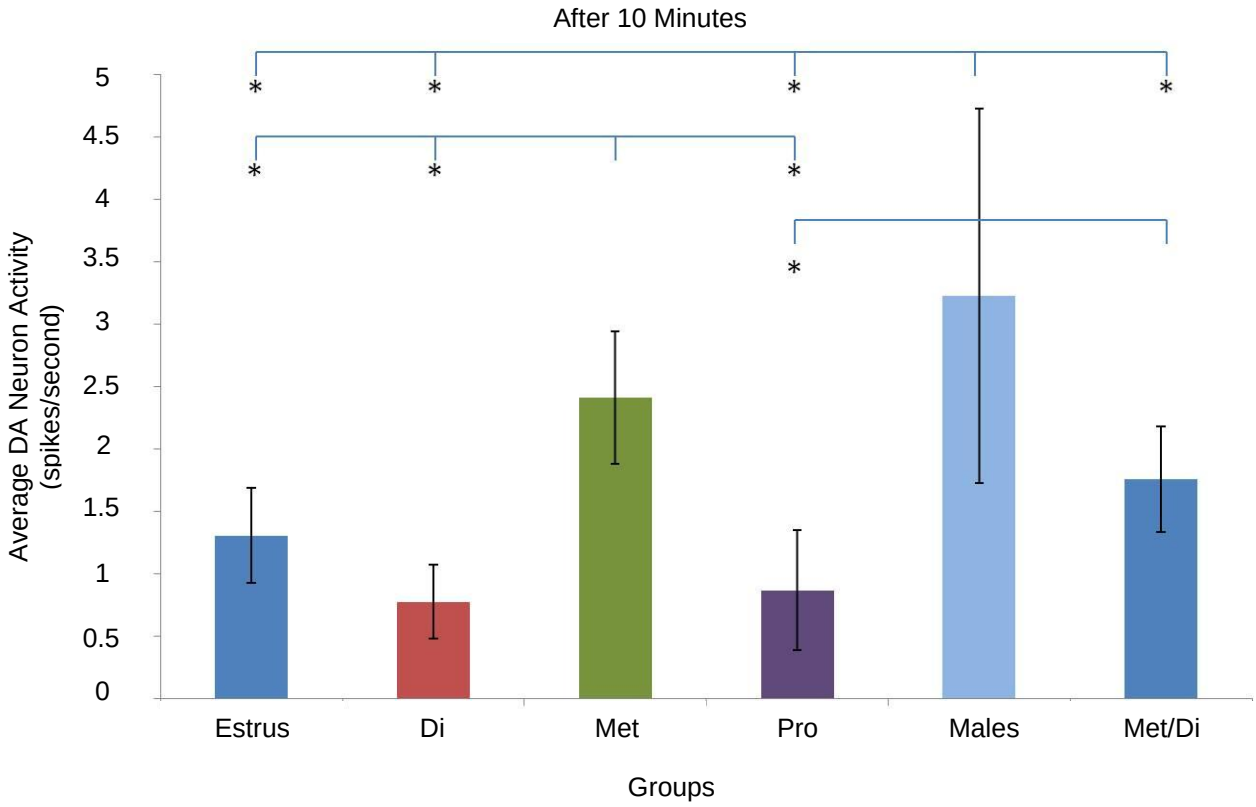

Figure 15: 10 Minutes Following Morphine Injection. Significantly different rates of DA neuron firing between groups for the second set of five minutes of data collected 10 minutes after morphine injection. Significant differences between groups are indicated by *, see text for *p* value.

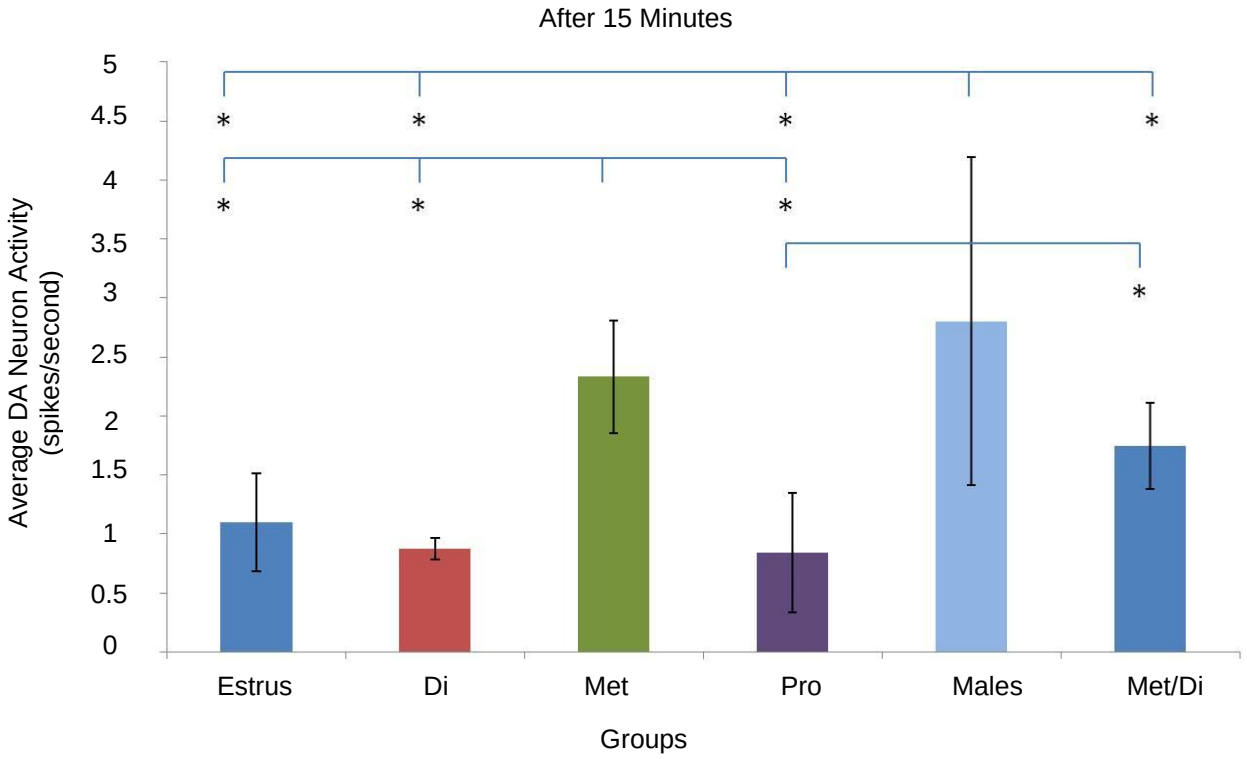

Figure 16: 15 Minutes Following Morphine Injection. Significantly different rates of DA neuron firing between groups for the third set of five minutes of data collected 15 minutes after morphine injection. Significant differences between groups are indicated by *, see text for *p* value.

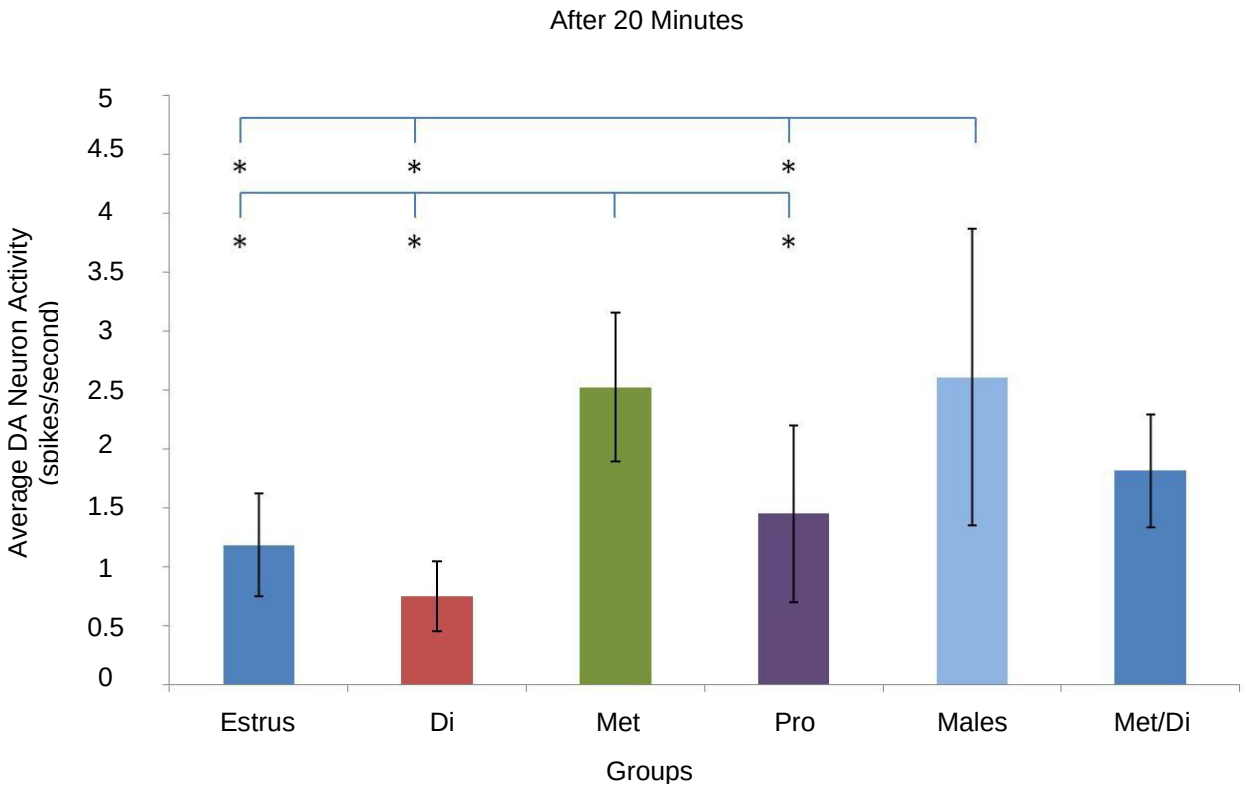

Figure 17: 20 Minutes Following Morphine Injection. Significantly different rates of DA neuron firing between groups for the fourth set of five minutes of data collected 20 minutes after morphine injection. Significant differences between groups are indicated by *, see text for *p* value.

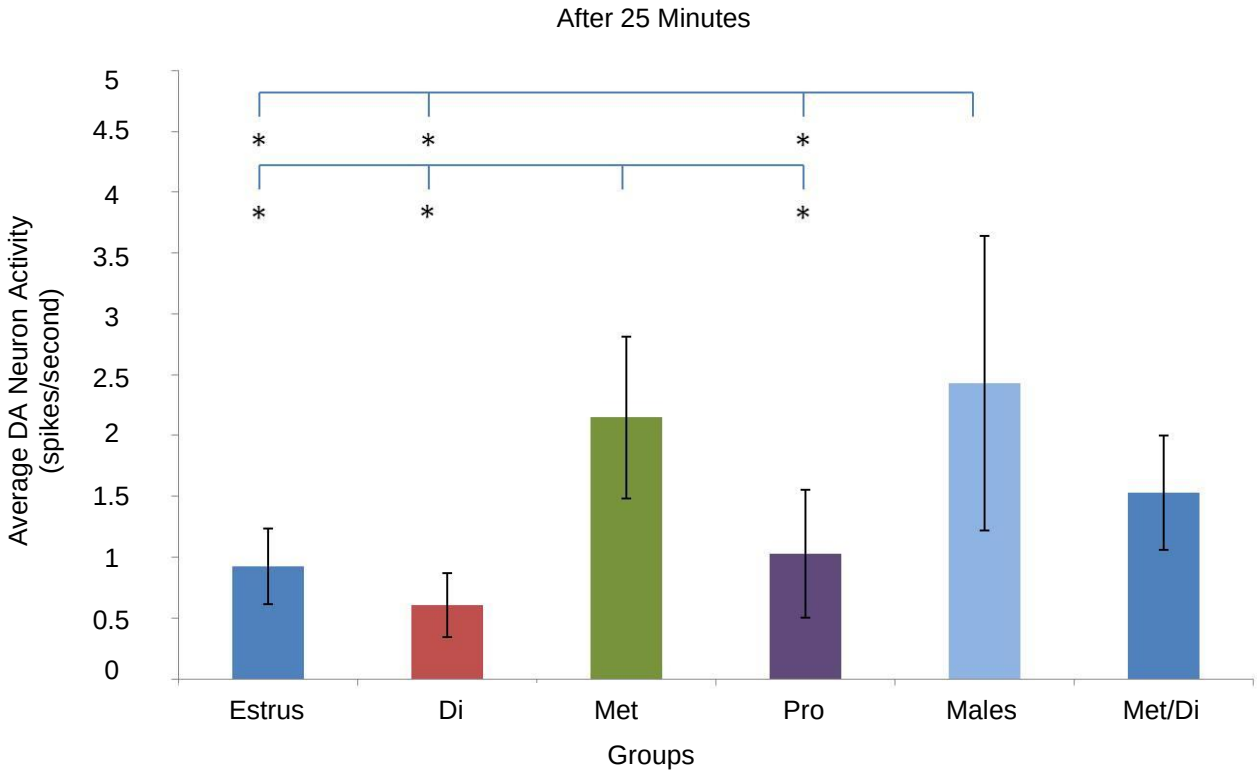

Figure 18: 25 Minutes Following Morphine Injection. Significantly different rates of DA neuron firing between groups for the fifth set of five minutes of data collected 25 minutes after morphine injection. Significant differences between groups are indicated by *, see text for *p* value.

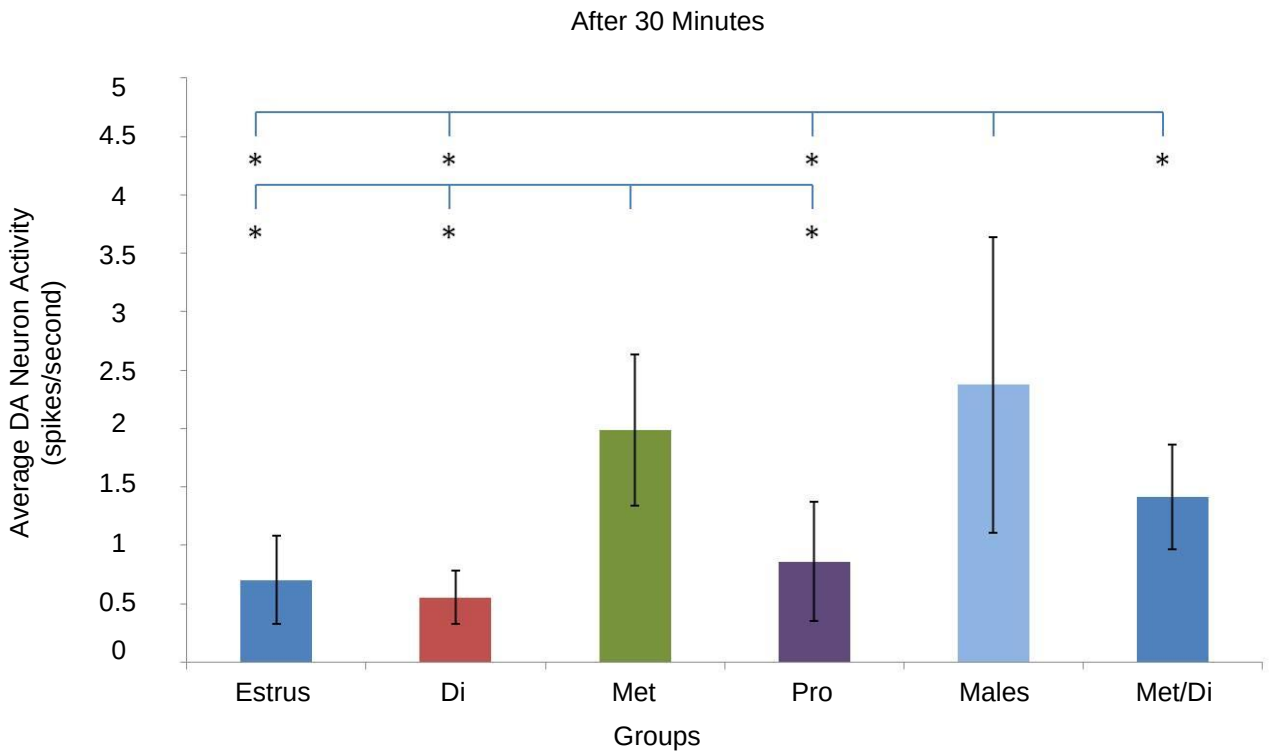

Figure 19: 30 Minutes Following Morphine Injection. Significantly different rates of DA neuron firing between groups for the sixth and final set of five minutes of data collected 30 minutes after morphine injection. Significant differences between groups are indicated by *, see text for p value.

APPENDIX F

DATA FORMING EACH GROUP

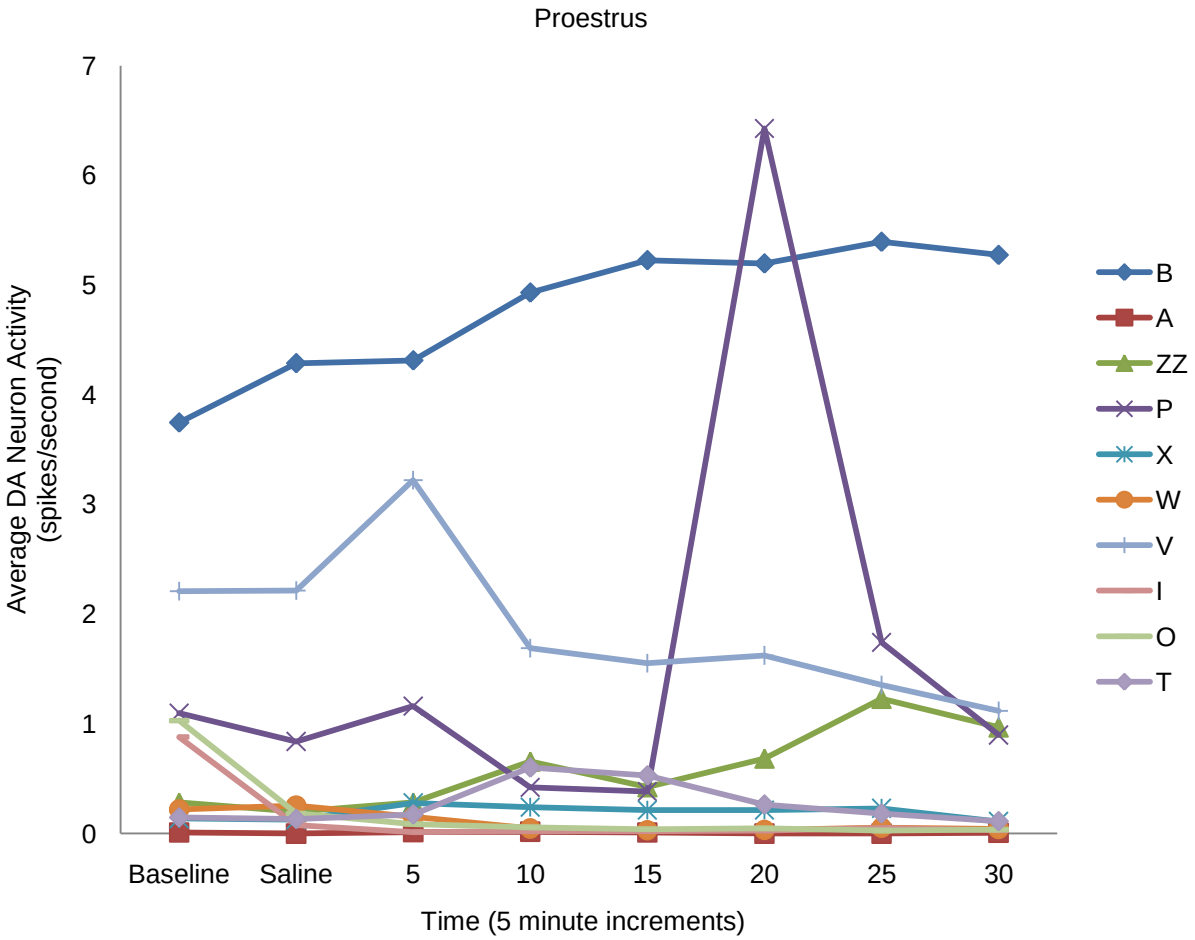

Figure 20: Proestrus Group Data. All data included in group mean for the Proestrus group.

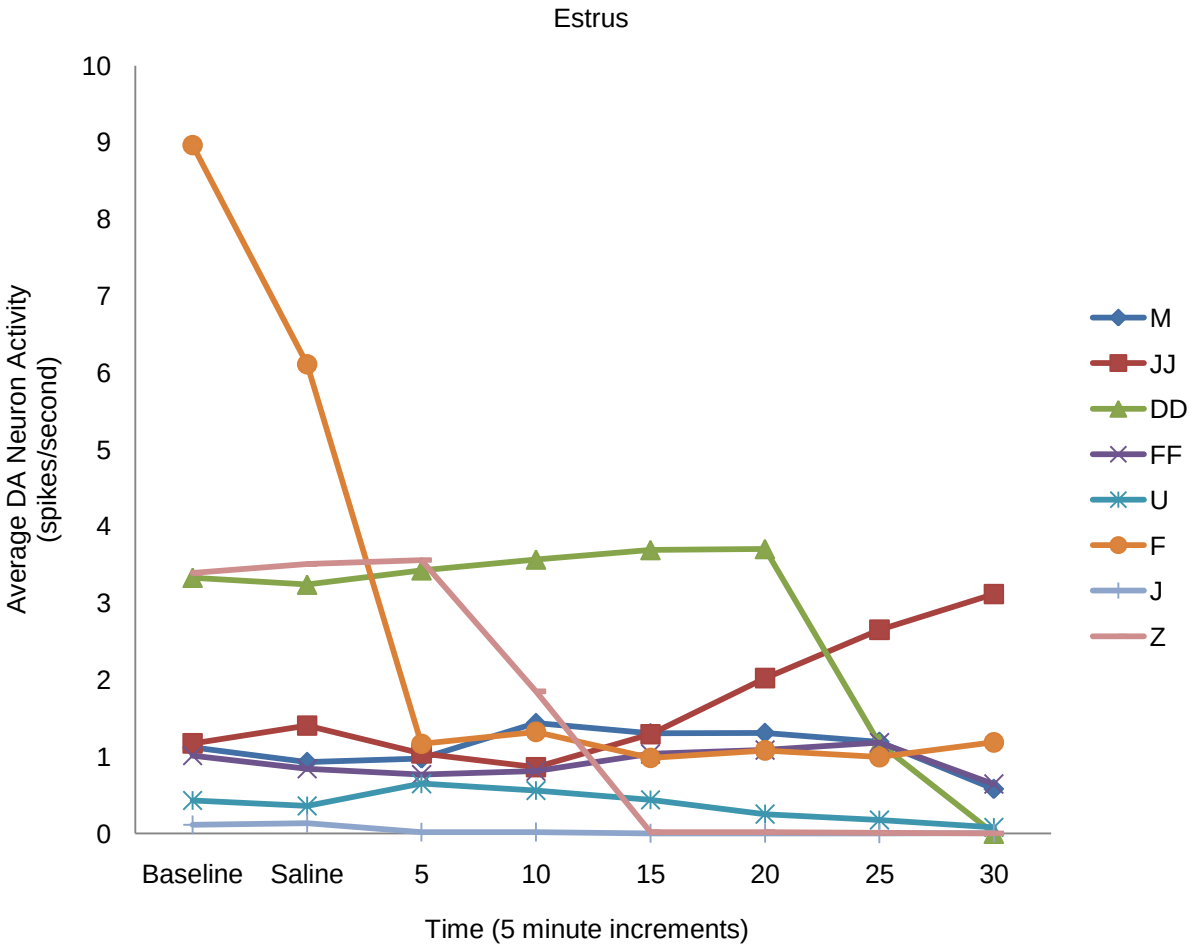

Figure 21: Estrus Group Data. All data included in group mean for the Estrus group.

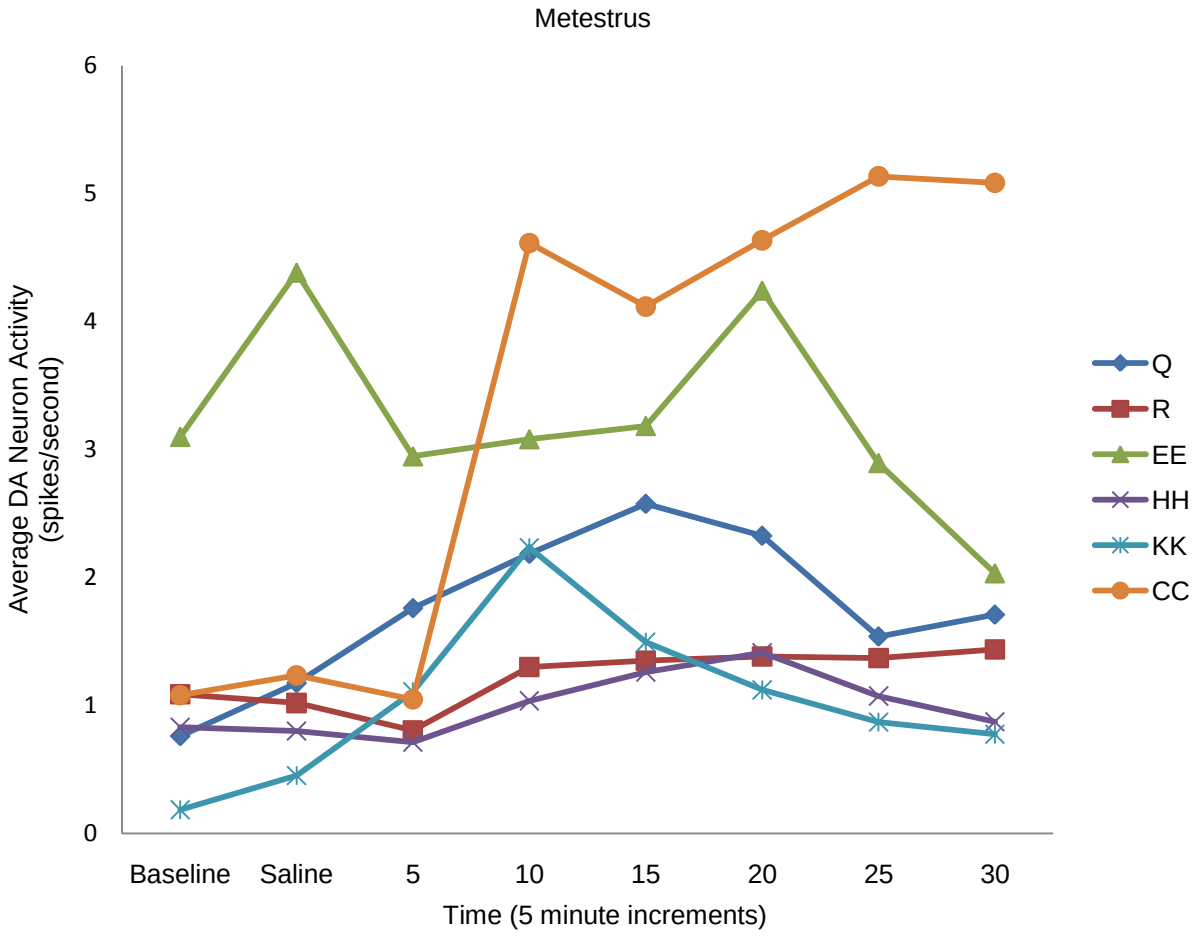

Figure 22: Metestrus Group Data. All data included in group mean for the Metestrus group.

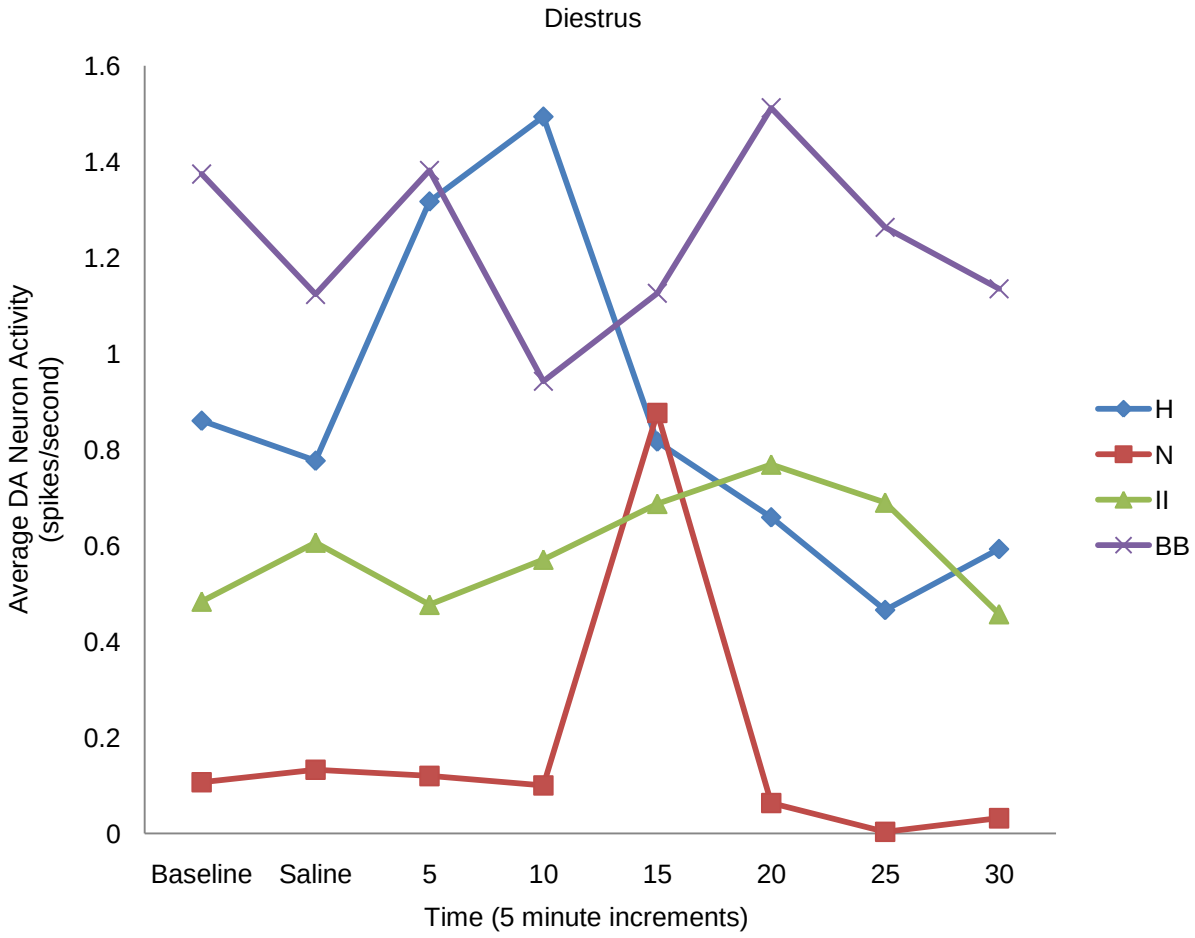

Figure 23: Diestrus Group Data. All data included in group mean for the Diestrus group.

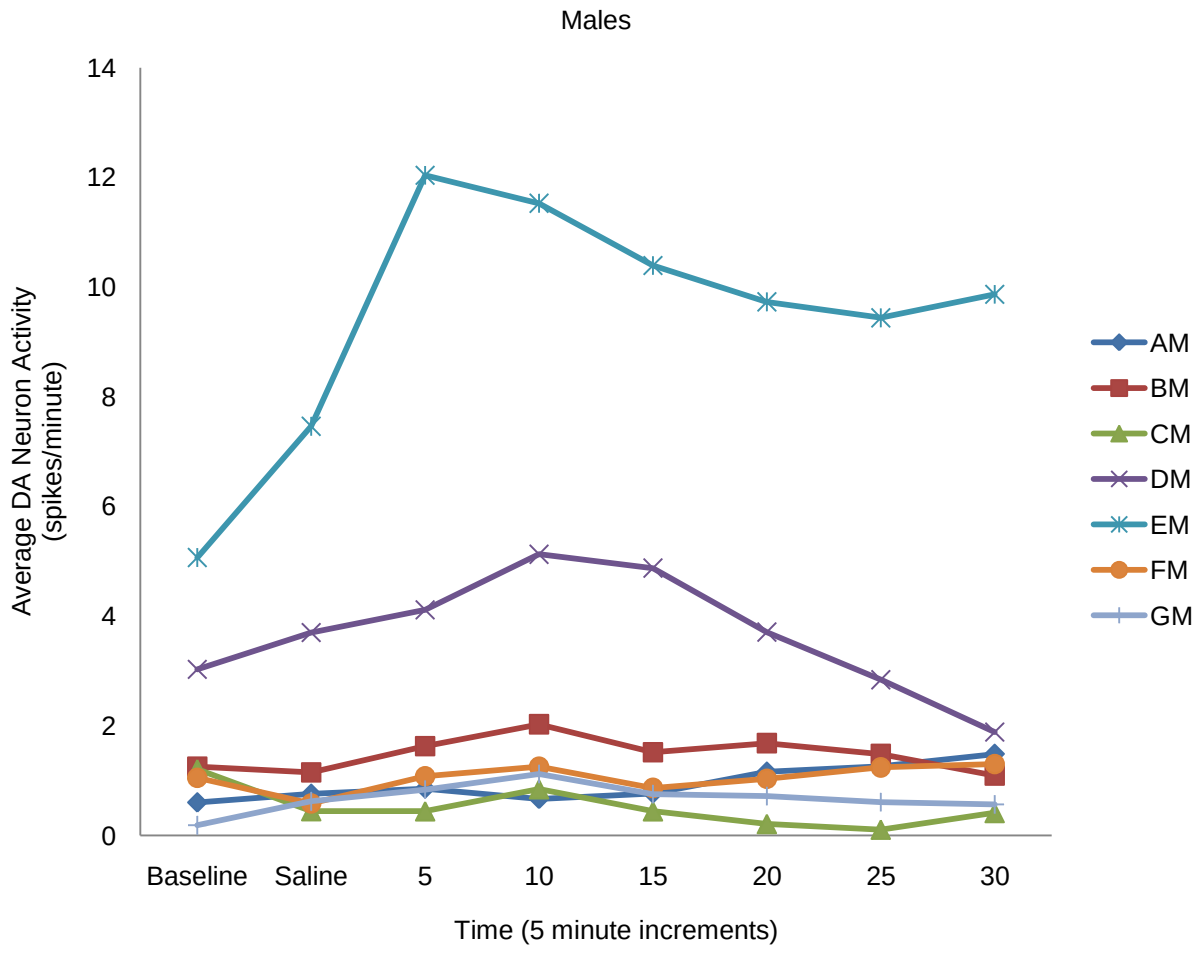

Figure 24: Male Group Data. All data included in group mean for the Male group. Rat EM is of particular interest due to the extreme response, and dramatic increase from baseline. See figure 25 for the Male after all outliers are removed.

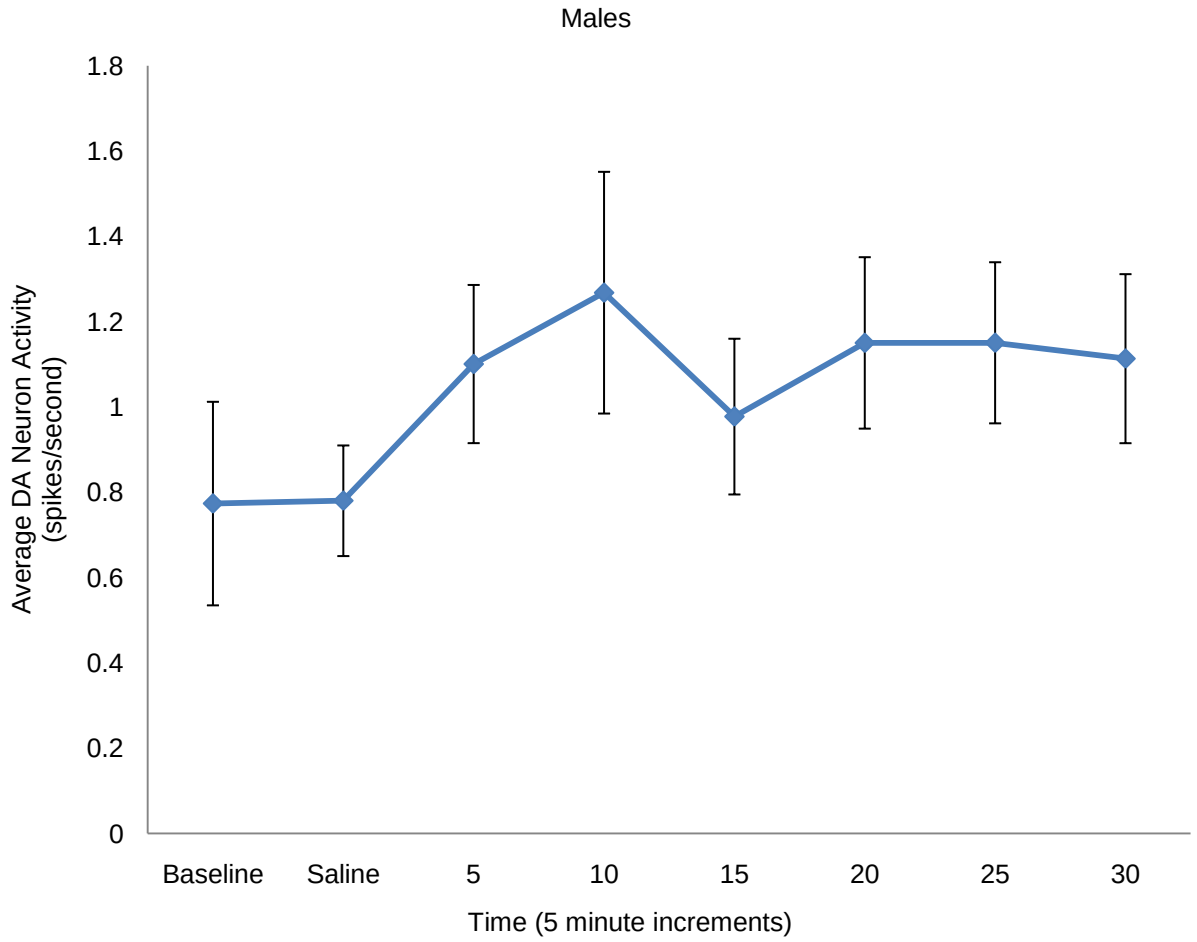

Figure 25: Male Electrophysiological Data with Outliers Removed. Outliers were determined with a box and whisker plot for each time point. All data from an animal was removed if they presented as an outlier on any of the time points. This left a sample size of n = 4, but the trend of the response is similar to that of the complete dataset shown on page 42.

REFERENCES

Adinoff, B. (2004). Neurobiologic processes in drug reward and addiction. Harv Rev Psychiatry, 12(6), 305-320. doi: K5U67XCPQMC2WT9U [pii]10.1080/10673220490910844

Appel, S. B., Liu, Z., McElvain, M. A., & Brodie, M. S. (2003). Ethanol excitation of dopaminergic ventral tegmental area neurons is blocked by quinidine. J Pharmacol Exp Ther, 306(2), 437-446. doi: 10.1124/jpet.103.050963jpet.103.050963 [pii]

Barker, J. L., Harrison, N. L., Meyers, D. E., & Majewska, M. D. (1986). Steroid modulation of GABAA receptor-coupled Cl- conductance. Clin Neuropharmacol, 9 Suppl 4, 392-394.

Berke, J. D., & Hyman, S. E. (2000). Addiction, dopamine, and the molecular mechanisms of memory. Neuron, 25(3), 515-532. doi: S0896-6273(00)81056-9 [pii]

Blackburn, J. R., Phillips, A. G., Jakubovic, A., & Fibiger, H. C. (1989). Dopamine and preparatory behavior: II. A neurochemical analysis. Behav Neurosci, 103(1), 15-23.

Bonci, A., Bernardi, G., Grillner, P., & Mercuri, N. B. (2003). The dopamine-containing neuron: maestro or simple musician in the orchestra of addiction? Trends Pharmacol Sci, 24(4), 172-177. doi: S0165614703000683 [pii]

Bozarth, M. A., & Wise, R. A. (1981). Intracranial self-administration of morphine into the ventral tegmental area in rats. Life Sciences, 28(5), 551-555. doi: 10.1016/0024-3205(81)90148-x

Bozarth, M. A., & Wise, R. A. (1984). Anatomically distinct opiate receptor fields mediate reward and physical dependence. Science, 224(4648), 516-517.

Carr, D. B., & Sesack, S. R. (2000). Projections from the rat prefrontal cortex to the ventral tegmental area: target specificity in the synaptic associations with mesoaccumbens and mesocortical neurons. J Neurosci, 20(10), 3864-3873. doi: 20/10/3864 [pii]

Clark, D., & Overton, P. G. (1998). Alterations in excitatory amino acid-mediated regulation of. [Article]. Addiction Biology, 3(2), 109 - 135.

Cooper, K. J., Purvis, K., & Haynes, N. B. (1972). Further observations on the ability of the male to influence the oestrous cycle of the underfed rat. J Reprod Fertil, 28(3), 473-475.

Di Chiara, G., & Imperato, A. (1988). Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proc Natl Acad Sci U S A, 85(14), 5274-5278.

Dunnam, R. C., Hill, M. J., Lawson, D. M., & Dunbar, J. C. (1999). Ovarian hormone secretory response to gonadotropins and nitric oxide following chronic nitric oxide deficiency in the rat. Biol Reprod, 60(4), 959-963.

Emanuele, M. A., Wezeman, F., & Emanuele, N. V. (2002). Alcohol's effects on female reproductive function. Alcohol Res Health, 26(4), 274-281.

Foy, M. R., Xu, J., Xie, X., Brinton, R. D., Thompson, R. F., & Berger, T. W. (1999). 17beta-estradiol enhances NMDA receptor-mediated EPSPs and long-term potentiation. J Neurophysiol, 81(2), 925-929.

Freeman, A. S., & Bunney, B. S. (1987). Activity of A9 and A10 dopaminergic neurons in unrestrained rats: further characterization and effects of apomorphine and cholecystokinin. Brain Res, 405(1), 46-55. doi: 0006-8993(87)90988-7 [pii]

Freeman, A. S., Meltzer, L. T., & Bunney, B. S. (1985). Firing properties of substantia nigra dopaminergic neurons in freely moving rats. Life Sci, 36(20), 1983-1994.

Garzon, M., & Pickel, V. M. (2001). Plasmalemmal mu-opioid receptor distribution mainly in nondopaminergic neurons in the rat ventral tegmental area. Synapse, 41(4), 311-328. doi: 10.1002/syn.1088 [pii]

Georges, F., Le Moine, C., & Aston-Jones, G. (2006). No effect of morphine on ventral tegmental dopamine neurons during withdrawal. J Neurosci, 26(21), 5720-5726. doi: 26/21/5720 [pii]10.1523/JNEUROSCI.5032-05.2006

Gholami, A., Zarrindast, M. R., Sahraei, H., & Haerri-Rohani, A. (2003). Nitric oxide within the ventral tegmental area is involved in mediating morphine reward. Eur J Pharmacol, 458(1-2), 119-128. doi: S0014299902026961 [pii]

Grace, A. A., & Bunney, B. S. (1983). Intracellular and extracellular electrophysiology of nigral dopaminergic neurons--1. Identification and characterization. Neuroscience, 10(2), 301-315.

Gysling, K., & Wang, R. Y. (1983). Morphine-induced activation of A10 dopamine neurons in the rat. Brain Research, 277(1), 119-127. doi: 10.1016/0006-8993(83)90913-7

Johnson, S. W., & North, R. A. (1992). Opioids excite dopamine neurons by hyperpolarization of local interneurons. J Neurosci, 12(2), 483-488.

Kalivas, P. W. (2000). A role for glutamate transmission in addiction to psychostimulants. Addict Biol, 5(3), 325-329. doi: ADB325 [pii]10.1111/j.1369-1600.2000.tb00199.x

Kalivas, P. W., Duffy, P., & Eberhardt, H. (1990). Modulation of A10 dopamine neurons by gamma-aminobutyric acid agonists. J Pharmacol Exp Ther, 253(2), 858-866.

Kalivas, P. W., Klitenick, M. A., Hagler, H., & Austin, M. C. (1991). GABAergic and enkephalinergic regulation of locomotion in the ventral pallidum: involvement of the mesolimbic dopamine system. Adv Exp Med Biol, 295, 315-326.

Karami, M., & Zarrindast, M. R. (2008). Morphine sex-dependently induced place conditioning in adult Wistar rats. Eur J Pharmacol, 582(1-3), 78-87. doi: S0014-2999(07)01330-1 [pii]10.1016/j.ejphar.2007.12.010

Kauser, K., Sonnenberg, D., Tse, J., & Rubanyi, G. M. (1997). 17 beta-Estradiol attenuates endotoxin-induced excessive nitric oxide production in ovariectomized rats in vivo. Am J Physiol, 273(1 Pt 2), H506-509.

Kelly, M. J., Loose, M. D., & Ronnekleiv, O. K. (1992). Estrogen suppresses mu-opioid- and GABAB-mediated hyperpolarization of hypothalamic arcuate neurons. J Neurosci, 12(7), 2745-2750.

Long, J. A., & Evans, H. M. (1922). The oestrous cycle in the rat and its associated phenomena. Berkeley,: Univ. of California Press.

Maeda, H., & Mogenson, G. J. (1981). Electrophysiological responses of neurons of the ventral tegmental area to electrical stimulation of amygdala and lateral septum. Neuroscience, 6(3), 367-376.

Majewska, M. D., Harrison, N. L., Schwartz, R. D., Barker, J. L., & Paul, S. M. (1986). Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. Science, 232(4753), 1004-1007.

Mandl, A. M. (1951). The phases of the oestrous cycle in the adult white rat. Journal of Experimental Biology, 28, 576-584.

Mansour, A., Fox, C. A., Akil, H., & Watson, S. J. (1995). Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. Trends Neurosci, 18(1), 22-29. doi: 016622369593946U [pii]

Marcondes, F. K., Bianchi, F. J., & Tanno, A. P. (2002). Determination of the estrous cycle phases of rats: some helpful considerations. Braz J Biol, 62(4A), 609-614. doi: S1519-69842002000400008 [pii]

Matthews, R. T., & German, D. C. (1984). Electrophysiological evidence for excitation of rat ventral tegmental area dopamine neurons by morphine. Neuroscience, 11(3), 617-625.

Mogenson, G. J., Jones, D. L., & Yim, C. Y. (1980). From motivation to action: Functional interface between the limbic system and the motor system. Progress in Neurobiology, 14(2-3), 69-97. doi: 10.1016/0301-0082(80)90018-0

Morris-Bobzean, S., Chance, D., Dennis, T., & Perrotti, L. (2011). Estrogen influences morphin reward and activation of hypothalamic orexin neurons. Neuronscience Meeting Planner. Abstract. Society for Neuroscience. Washington , DC.

Nugent, F. S., & Kauer, J. A. (2008). LTP of GABAergic synapses in the ventral tegmental area and beyond. J Physiol, 586(6), 1487-1493. doi: jphysiol.2007.148098 [pii] 10.1113/jphysiol.2007.148098

Nugent, F. S., Niehaus, J. L., & Kauer, J. A. (2009). PKG and PKA signaling in LTP at GABAergic synapses. Neuropsychopharmacology, 34(7), 1829-1842. doi: npp20095 [pii]10.1038/npp.2009.5

Nugent, F. S., Penick, E. C., & Kauer, J. A. (2007). Opioids block long-term potentiation of inhibitory synapses. Nature, 446(7139), 1086-1090. doi: nature05726 [pii]10.1038/nature05726

Oades, R. D., & Halliday, G. M. (1987). Ventral tegmental (A10) system: neurobiology. 1. Anatomy and connectivity. Brain Res, 434(2), 117-165.

Papp, M., & Bal, A. (1987). Separation of the motivational and motor consequences of 6-hydroxydopamine lesions of the mesolimbic or nigrostriatal system in rats. Behav Brain Res, 23(3), 221-229.

Paxinos, G. (1995). The rat nervous system (2nd ed.). San Diego: Academic Press.

Paxinos, G., & Watson, C. (2005). The rat brain in stereotaxic coordinates: Elsevier Academic Press.

Phillips, A. G., & LePiane, F. G. (1980). Reinforcing effects of morphine microinjection into the ventral tegmental area. Pharmacol Biochem Behav, 12(6), 965-968.

Purvis, K., Cooper, K. J., & Haynes, N. B. (1971). The influence of male proximity and dietary restriction on the oestrous cycle of the rat. J Reprod Fertil, 27(2), 167-176.

Robinson, T. E., & Berridge, K. C. (1993). The neural basis of drug craving: an incentive-sensitization theory of addiction. Brain Res Brain Res Rev, 18(3), 247-291.

Saal, D., Dong, Y., Bonci, A., & Malenka, R. C. (2003). Drugs of abuse and stress trigger a common synaptic adaptation in dopamine neurons. Neuron, 37(4), 577-582. doi: S0896627303000217 [pii]

Schwarz, S., & Pohl, P. (1994). Steroids and opioid receptors. J Steroid Biochem Mol Biol, 48(4), 391-402. doi: 0960-0760(94)90080-9 [pii]

Steffensen, S. C., Svingos, A. L., Pickel, V. M., & Henriksen, S. J. (1998). Electrophysiological characterization of GABAergic neurons in the ventral tegmental area. J Neurosci, 18(19), 8003-8015.

Steffensen, S. C., Taylor, S. R., Horton, M. L., Barber, E. N., Lyle, L. T., Stobbs, S. H., & Allison, D. W. (2008). Cocaine disinhibits dopamine neurons in the ventral tegmental area via use-dependent blockade of GABA neuron voltage-sensitive sodium channels. Eur J Neurosci, 28(10), 2028-2040. doi: EJN6479 [pii]10.1111/j.1460-9568.2008.06479.x

Ungless, M. A., Magill, P. J., & Bolam, J. P. (2004). Uniform inhibition of dopamine neurons in the ventral tegmental area by aversive stimuli. Science, 303(5666), 2040-2042. doi: 10.1126/science.1093360303/5666/2040 [pii]

Ungless, M. A., Whistler, J. L., Malenka, R. C., & Bonci, A. (2001). Single cocaine exposure in vivo induces long-term potentiation in dopamine neurons. Nature, 411(6837), 583-587. doi: 10.1038/3507907735079077 [pii]

Vanderschuren, L. J., De Vries, T. J., Wardeh, G., Hogenboom, F. A., & Schoffelmeer, A. N. (2001). A single exposure to morphine induces long-lasting behavioural and neurochemical sensitization in rats. Eur J Neurosci, 14(9), 1533-1538. doi: 1775 [pii]

Zhang, D., Yang, S., Yang, C., Jin, G., & Zhen, X. (2008). Estrogen regulates responses of dopamine neurons in the ventral tegmental area to cocaine. Psychopharmacology (Berl), 199(4), 625-635. doi: 10.1007/s00213-008-1188-6

Zhang, D., Zhang, H., Jin, G. Z., Zhang, K., & Zhen, X. (2008). Single dose of morphine produced a prolonged effect on dopamine neuron activities. Mol Pain, 4, 57. doi: 1744-8069-4-57 [pii]10.1186/1744-8069-4-57

Zhang, S., Tong, Y., Tian, M., Dehaven, R. N., Cortesburgos, L., Mansson, E., . . . Yu, L. (1998). Dynorphin A as a potential endogenous ligand for four members of the opioid receptor gene family. J Pharmacol Exp Ther, 286(1), 136-141.

Zhu, X. Z., & Luo, L. G. (1992). Effect of nitroprusside (nitric oxide) on endogenous dopamine release from rat striatal slices. J Neurochem, 59(3), 932-935.

BIOGRAPHICAL INFORMATION

Micah Eimerbrink earned his undergraduate degree in psychology from Texas State University in San Marcos, Texas. After graduation he returned to the DFW area to work for the Department of State Health Services (DSHS) where he worked as a Public Health Technician in the Tuberculosis Department before being promoted to Community Health Specialist (CHS). As a CHS, Micah worked with rural communities to form coalitions focused on addressing health concerns specific to the respective county. Micah's work with DSHS was concluded when he decided to return to academic study in pursuit of his PhD in Psychology.

He began his graduate career under the mentorship of Dr. Andrew Baum. His research focus under Dr. Baum's guidance was on the relationship between stress and cancer, and he specifically wanted to use tissue culture as a modality for research. After Dr. Baum's tragic death, Micah was accepted into the lab of Dr. Yuan Bo Peng. In Dr. Peng's lab Micah was trained in electrophysiology, and various surgical procedures. However, following receipt of his master's degree from UTA Micah will transfer to Texas Christian University (TCU) to pursue training in tissue culture.