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Amygdala Mechanisms Involved in Chemosensory Communication

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Abstract:

The amygdala is an almond-shaped region of the brain present in many vertebrates such as human, hamsters and mice. The amygdala is composed of several parts, of which the following are most relevant to my research: the basolateral amygdala (BLA) and the posterior medial amygdala (MeP). The BLA functions in learning and shows increased activation after the mouse has learned a behavior. The lateral paracapsular nuclei of the intercalated nucleus (ICNlpcn) project inhibitory neurons into the BLA and mediate its activity. Dopamine has been shown to inhibit the lpcn group and therefore relieve inhibition of the BLA (disinhibition). Dopamine also acts directly in the BLA to increase activation. Therefore, if a mouse is conditioned to prefer a particular odor (in this case steer urine), dopamine injection will cause an increase in BLA activation after learning due to both disinhibition and direct excitatory response. The medial posterior amygdala in the mouse mostly responds to olfactory stimuli from the same species (conspecific stimuli). The MeP is also thought to be under control of another ICN group, the caudal ICN (ICNc). If the same inhibitory relationship is seen between ICNc and MeP, the dopamine injection may cause disinhibition in the MeP and therefore the conditioned steer urine stimulus may show increased activation in the MeP despite being a stimulus from another species (heterospecific stimulus). My experiment tests whether the ICNc has inhibitory control over the MeP, and whether dopamine will cause inhibition in the ICNc and therefore cause disinhibition in the MeP.

Key Words: medial posterior amygdala (MeP), caudal intercalated nucleus (ICNc), dopamine (DA).

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AMYGDALA MECHANISMS INVOLVED IN CHEMOSENSORY

COMMUNICATION

By

IOANA STROE

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Biological Science

Honors in the Major Project Thesis

"Amygdala Mechanisms Involved In Chemosensory Communication"

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Part I – Prior to Experimentation

Introduction:

The amygdala is a region of the brain constituted by a group of nuclei that form an almond-shape and that may function together for the same purposes. For the purposes of this research, the following regions are important: the basolateral amygdala (BLA), the anterior medial amygdala (MeA), the posterior medial amygdala (MeP), the central amygdala (CeA), and several groups of intercalated nucleus cells (the caudal medial, medial paracapsular, and lateral paracapsular nuclei). The caudal ICN cells (ICNc) are located next to MeP. The medial paracapsular cells are located medially to the BLA, and next to the central amygdala (CeA). The lateral paracapsular cells are located lateral to the BLA. These groups of ICN cells modify the function of their adjacent main nuclei. The amygdala is found in many vertebrates such as humans, hamsters, and mice. In humans, the amygdala functions to process emotional reactions and in memory modulation, as well as in social cognition. Recent research has shown that in humans, the amygdala is responsible for processing memory of emotional events (Ferry et al 1999). This links the amygdala directly to emotional learning. The same is also true for animals like mice, since under fear conditioning, the BLA is activated in storing the memory of the aversive event (Takahashi et al 2007). There is also evidence the amygdala may be able to have long-term potentiation in discriminating between aversive events, and data has already shown the central nuclei of the amygdala are involved in fear responses. In the absence of a functioning amygdala, the fear response is impaired (Amunts et al 2005). Experiments have also shown the amygdala is responsible for consolidating memories, since mice that are trained to learn certain tasks will show greater retention of the behavior when injected with amygdala-stimulating drugs (Ferry et al 1999). Furthermore, upon exhibiting a learned behavior (including olfactory learning), the mouse basolateral amygdala shows increased activity (Schoenfeld et al 1999). The amygdala may play a role in unlearned responses to stimuli as well.

The amygdala's role in social behavior is also very important and has been widely-researched. For humans, who communicate mostly by visual and auditory signals, the amygdala has been shown to function in evaluating facial expression. The evaluation of facial expressions is proposed to be an unlearned response, which again points to the amygdala's importance in processing unlearned stimuli. For patients who recognize neutral facial expressions as threatening and fearful, hyperactivity in the amygdala is observed. For patients with borderline personality disorders, a greater activity of the amygdala is observed as compared to controls (Donegan et al 2003).

The amygdala is very important in processing social signals and distinguishing between positive and negative signals and their meanings. In hamsters, which communicate mostly by olfactory stimuli, the amygdala's activity is increased when the male hamster encounters chemosignals that are attractive to mating behavior (such as hamster vaginal fluid, HVF) (Fernandez-Fewell and Meredith 1994). The same principle should be true in mice, since they also communicate mostly by chemosensory signals, and the amygdala is responsible for processing and sorting through those signals.

Previous experiments done on male hamsters have shown that the medial amygdala distinguishes categorically between biologically relevant and irrelevant chemosensory signals such as hamster vaginal fluid, steer urine, hamster flank gland secretion, female mouse urine, etc. (Meredith and Westberry 2004). The hamster anterior medial amygdala responds to all these chemosensory stimuli used by different species to communicate reproductive readiness, social status, etc. The posterior medial amygdala responds to conspecific stimuli (belonging to the same species) in both hamsters and mice, but responds rarely to heterospecific stimuli (belonging to a different species). Thus, for example, hamster vaginal fluid

(HVF) activates the male hamster posterior medial amygdala but female (or male) mouse urine does not. Similarly, HVF does not activate the mouse posterior medial amygdala (MeP), but female mouse urine (FMU) activates it. The fact that the anterior medial amygdala, in both species, responds to both conspecific and heterospecific stimuli shows that the selectivity or categorical response is not dependent on the sensitivity of peripheral chemoreceptors: it is due to selective chemoresponses in the brain. More recent experiments have suggested that if a stimulus is biologically relevant to the animal, the posterior medial amygdala will respond regardless of whether the stimulus is conspecific or heterospecific. For example, in experiments done with mice, an odor from a collar worn by a cat for two weeks stimulates activation in the posterior medial amygdala as well as in the anterior medial amygdala (Samuelsen and Meredith 2009). This response shows the cat odor (naturally perceived as possibly dangerous for the mouse) elicits a response in the posterior medial amygdala despite of being a heterospecific stimulus. This brain response correlates to risk-avoidance behavior. Studies have shown that hamsters, which do not avoid the cat collar odor, also do not have increased activation in the posterior medial amygdala. Neither the hamsters nor the mice in these experiments had any previous experiences with cat odor, nor with any other heterospecific chemosensory signal. These results suggest the amygdala may possess an innate mechanism to evaluate the meaning and context of each stimulus for the animal, as well as the ability to form new evaluations for stimuli with which the animal has had previous experience.

The amygdala is the under control of other regions of the brain, as well as of chemicals of the brain and these controls may offer the explanation behind the mechanism of categorical determination of a stimulus' meaning by the amygdala. The intercalated nuclei, or ICNc are regions of the amygdala proposed to have inhibitory control over specific nuclei, particularly the basolateral amygdala and the central amygdala (Marowsky et al 2005). Another group of ICN cells, the caudal medial ICNc is adjacent to MeP and may be able to inhibit MeP responses. For example, studies done with hamsters suggest that hamster vaginal fluid (HVF) activates the MeP but does not activate the ICN (and in fact may even suppress it) (Meredith et al 2008). Female mouse urine (FMU), on the other hand, activates ICN but not MeP. This shows a reverse relationship between activation of the medial amygdala and the intercalated nucleus. This experiment will investigate the ICN involvement in the reciprocal pattern of activation of ICN and MeP in mice, by both mouse and steer stimuli. Some regions of the amygdala, specifically the BLA and the central amygdala (CeA) respond to the neurotransmitter dopamine by a decrease in inhibition, since dopamine inhibits their local cluster of ICN cells, which then in turn disinhibits the adjacent main amygdala nuclei (Marowsky et al 2005, Pape et al 2005). High dopamine levels in the ICN increase the inhibition of the ICN, which in turn reduces the inhibition in the BLA. This is called dis-inhibition. The lateral and medial paracapsular ICN (lpcn) cells are proposed to mediate the dopaminergic disinhibitory responses in the BLA and a medial (mpcn) group acts on the central amygdala. Increased dopamine levels have been shown to disinhibit the BLA and the central amygdala via D1 receptors, which means the suppression of the paracapsular ICN by dopamine is a functioning mechanism for promoting increased response of the amygdala, which may result in changed behavioral response of animals such as to heightened fear states, or to recognizing one's own species for reproductive purposes. The mechanism described above is illustrated by the following circuit:
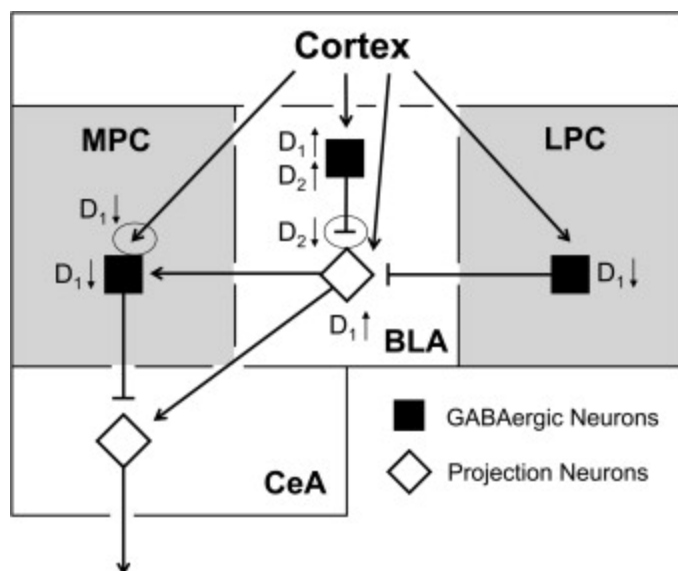
Figure from: Pape 2005: GABAergic neurons: gate masters of the amygdala, mastered by dopamine. Neuron. 2005 Dec 22;48(6):877-9. Figure shows the proposed circuit of ICN interaction with BLA and Ce as mediated by dopamine interactions.

Based on the research done, the following concepts will be tested in this experiment: 1) If BLA responds to conditioned odors and if the dopamine via D1 receptors causes disinhibition in the amygdala, and thus causes an increased response, then dopamine injected after training should cause an increase in activation of the BLA. 2) For the animals not receiving training, the BLA will not be active, and thus the dopamine may not have an effect in BLA activation. If there is no inhibition there to start with, there is nothing to disinhibit via the dopamine. 3) If the dopamine also inhibits the ICNc, and if the ICNc has the same relationship to MeP as the paracapsular ICN has to the BLA, then the MeP should be disinhibited. 4) For a conspecific stimulus (like the female mouse urine), the ICNc should not be active regardless of dopamine presence. Thus, if any effect is seen in ICNc, it is likely to be formed by a different mechanism. 5) For a heterospecific stimulus (like the steer urine), the ICN should be suppressed by the dopamine, and thus increased activation will be seen in the MeP.

The concepts explained will be tested by the experimental protocol described below.

Experimental Protocol:

The following groups of animals will be tested:

Group 1: Without Training

- **Treatment:** *Saline Control Injection*
  Stimuli:
    - Control (no stimulus)
    - SU
    - FMU
- **Treatment:** *Dopamine Agonist (D1 – SKF38393) Injection*
  Stimuli:

- Control (no stimulus)
- SU
- FMU

Group 2: With Training

- **Treatment:** *Saline Control Injection*
  Stimuli:
  - Control (no stimulus)
  - SU
  - FMU
- **Treatment:** *Dopamine Agonist (D1) Injection*
  Stimuli:
  - Control (no stimulus)
  - SU
  - FMU

Each of the 12 categories will have 6 animals, for a total of 72 animals in the experiment.
The areas of the brain in which the number of activated cells will be counted are the following:

1. MeA
2. MeP
3. BLA
4. ICNc (caudalmedial ICN adjacent to MeP: may inhibit MeP)
5. ICNpcm (medial paracapsular ICN, medial to BLA and also adjacent to CeA: may inhibit CeA)
6. ICNpcl (lateral paracapsular ICN lateral to BLA: can inhibit BLA).

Methods:

This experiment is performed with adult (2-3 month old) male mice, strain C57 BL/6J. The animals are single-housed in clear plastic cages with clean bedding, and water *ad libitum*. Food will be restricted (see the Behavioral Control section for details).The animals are kept on a reverse light cycle of 12 hours dark/12 hours light photoperiod, with the lights off at 9 am and on at 9 pm. All procedures have been approved by the Florida State University Institutional Animal Care and Use Committee.

Stimulus Collection:

Before the experiment can be done, the stimuli must be collected.
-FMU is in stock and does not need to be collected for this experiment. The FMU in stock has been collected from three female mice housed in a metabolic cage for five days (therefore, it includes some contributions from all days of the 4-5 day estrous cycle). FMU is diluted 1:10 with distilled water, centrifuged, and the supernatant is saved.
-HVF is collected in the following manner: a labeled plastic vial is prepared, weighed, and its weight is written on the label. Three female hamsters are chosen that are in estrous (females that undergo

lordoses when momentarily exposed to a male), a small spatula is used to collect HVF from each hamster and place it in pre-weighed vial. After stimulus collection, the vial is weighed again to determine how much HVF was collected. Ten times the collected weight in water is added to the HVF sample; it is shaken well so the HVF dissolves in the water. The contents of the vial are placed in a centrifuge tube and are centrifuged at medium speed for 30 minutes. The supernatant is the stimulus. The stimulus is stored at -20 degrees Celsius until needed.

-SU is purchased, diluted 1:10 with distilled water, centrifuged, and the supernatant is saved.

Behavioral Conditioning:

The mice are to be single-housed for 3 days before beginning conditioning and their food intake is to be restricted to about 2.5 g of food per day until they weigh 85% of their body weight. When the desired body weight has been reached, the animals are fed the proper amount of food such that the desired 85% body weight is maintained. While they are being food deprived, the small bowl from the testing apparatus is inside the cage, so that each mouse becomes used to the new bowl as a source of food. Then the following protocol is done for each non-control mouse during training:
- give mouse the bowl with its regular food in its own cage (so that it becomes accustomed to eating its own food from the new bowl)
- give mouse the bowl with its regular food in the testing apparatus (so that it becomes accustomed to eating while inside the apparatus)
- give mouse the bowl with only a Cheerio in the apparatus (so that it becomes accustomed to eating the Cheerio from the bowl)
- give mouse two bowls with a Cheerio scented with a stimulus (so that it becomes accustomed to eating the scented Cheerio and to having two bowls to choose from)
- give mouse the two bowls inside the apparatus, one with a Cheerio above the stainless steel grid, and the other with a Cheerio below the stainless steel grid (so that it learns to choose the bowl with the accessible Cheerio)

For the non-control animals, after this protocol has been performed for a number of days until the mouse learns the behavior, one Cheerio is to be scented with SU and placed in the bowl above the gird and a non-scented Cheerio should be placed in the other bowl below the gird. The grid is made of a mesh stainless steel wire that has the same diameter as the bowl. The grid serves as a physical barrier for the Cheerio, since the mouse is unable to lift the grid and get to the Cheerio placed below it. The mouse is placed in the testing apparatus and is allowed to choose which bowl to go to and which Cheerio to try and eat. Since the unscented Cheerio is inaccessible below the grid, the only way to get a treat is to eat the SU-scented Cheerio and thus gain preference for it. Thus, allowing the mouse to eat the Cheerio with the SU is the positive conditioning, since the mouse is rewarded by accessing and eating the Cheerio with the SU stimulus on it. There is also the possibility of negative conditioning, in which the FMU-scented Cheerio is placed under the grid in one bowl, and an unscented Cheerio is placed above the grid in the other bowl. Inaccessibility may not be enough of a factor in negative conditioning for FMU, thus positive conditioning for SU will be done, with further consideration given to the negative conditioning later on depending on the results. SU is initially a relatively unimportant (neutral) stimulus for a mouse that does not activate MeP. Training will give it more importance to the mouse, since it is associated with the positive reward of a Cheerio.

For the control animals, the same behavioral conditioning process is done as described above, except the Cheerios are not scented with a stimulus. The control animals do not learn to prefer one Cheerio over another, since the Cheerios are unscented. During the conditioning process for all animals, regular food is to be given after the daily training has been performed, to avoid lack of participation of the animal due to satiation. Amounts of food should be adjusted to maintain 85% of free-feeding body weight.

After about 4 days of the conditioning process, the behavioral testing is performed 24 hours after the last conditioning day. Mice should run rapidly to the scented Cheerio by the end of conditioning.

Chemosensory Stimulation Experiment:

The behavioral experiment room is prepared before the animal is brought in. A clean cage with clean bedding is brought. The plastic cage lid is washed with soap and water. The lid of the cage is washed before and after each animal. Using gloves, the cotton applicators are broken off so they are only about an inch and a half long. All five of the cotton applicators used per mouse are to be coated in the same stimulus, or they are all to be clean if testing a control animal. Half an hour before the behavioral conditioning is done, the animal is brought into the room under dark conditions and is given an intraperitoneal injection with either D1 agonist or saline, depending on which group it belongs to. After 30 minutes have elapsed the mouse is ready to undergo the chemosensory stimulation.
After the mouse is put into the clean cage, it is allowed 2 minutes to acclimate. After the 2 minutes, the swab (either clean for control, or with FMU or HVF) is presented to the mouse. Each swab must be changed every 3 minutes for 15 minutes, for a total of 5 swabs per animal. The mouse's behavior is recorded on the keypad during the 15 minutes. The recordable behavior is the following: number of times the animal rears back on its feet, total rearing time throughout the experiment, number of contacts the animal makes with the swab, total contact time with the swab throughout the experiment, investigation of other parts of the swab, number and duration of grooming bouts, and number of "stretch-attend" movements toward the stimulus. The "stretch-attend" movements are considering to be risk-assessment behaviors. After the 15 minutes have elapsed, the mouse is returned to its cage and the timer is set for 30 minutes until the animal is ready to be perfused.

Perfusion:

After the animal is brought to the perfusion room, it is injected with 0.2 mL of Nembutal. After a minute, the animal's condition must be checked by pinching its paw and noting any reaction. If the mouse does not react, it is ready to be perfused. The perfusion is done placing its animal on its back, pinching and incising the skin on its abdomen, and cutting down the sides from the incision and then up to the front paws. The diaphragm is gently cut to expose the heart, and the right atrium is cut. The blunted perfusing needle is placed in the left ventricle of the heart and cold PBS solution is pumped though until the liver clears and blood flow has ceased. Following this, cold 4% paraformaldehyde fixative is pumped through until the muscles completely contract and 60 mL of the PFA have been used. After the needle is removed, the head is cut off at the neck and the body is placed in a body bag. The skin of the head is peeled off and the brain is carefully removed, protecting the ventral (amygdala) region especially. The brain is then placed in 4% PFA solution overnight at 4˚C. The next day, the brain is placed in 30% sucrose solution

until it sinks to the bottom of the container. The sucrose prevents the formation of ice crystals in the brain tissues during the freezing process and allows it to maintain a smooth consistency for proper sectioning.

Once the brains have sunk to the bottom, indicating complete saturation by the sucrose solution, they are sectioned to 40 micron sections on the microtome. The brain is placed on the freezing microtome stage and is covered with a weighing boat and dry ice is added over the weighing boat for a minute for faster freezing. The weighing boat protects the freezing stage, while allowing the brain to be covered and frozen by the dry ice. The sucrose prevents ice crystals from forming at this point in the sectioning process. The freezing is necessary so that the microtome blade glides smoothly through the tissue, without creating uneven cuts. Sections of brain start to be taken when the optic chiasm becomes visible to the eye, to ensure there are brain sections before the amygdala is reached so as not to miss any important sections. Every other section is taken for the primary plate and the rest are taken for the back-up plate. A section is taken, and placed in the 12-well plate in well #1. The following section is placed in the backup well plate. The following section is then place in well #2 of the primary plate and so on. One brain fits in 12 wells, so each well has 2-3 sections in it at the end of the sectioning process. After the brains are sectioned, they undergo the immunohystochemistry process.

Immunohistochemistry:

Immunohistochemistry is the procedure done to localize specific proteins in the cells of a particular tissue (in this case, brain tissue). The procedure is based on the recognition of antibodies to antigens, hence the "immuno" in the title. The antibodies used in this experiment are polyclonal, which means they can bind to a variety of antigens. The antigens used in this experiment are the FOS and FRA (Fos-related antigen) proteins, which are indicative of a response by immediate-early genes (IEGs) in the brain. The IEG expression and the resulting FOS and FRA proteins indicate which areas of the brain have high levels of activity during the chemosensory stimulation experiments. The primary antibody is a rabbit-anti-FOS or FRA antibody that also recognizes FRA proteins. It is made by injecting a rabbit with FOS (or FRA) proteins, and purifying the antibodies the rabbit makes to the FOS , which include antibody components against FRA antigens. The secondary antibody is the goat-anti-rabbit biotinilated antibody. The secondary antibody is made by injecting goats with rabbit antigens, so that it makes antibodies that recognize any rabbit proteins. The primary antibody is made in rabbits, and the secondary antibody is made in goats. This accounts for the binding that occurs between primary and secondary antibodies, since the secondary is made to recognize and bind to the primary. The functioning principles of Immunohistochemistry are as follows: the primary antibody binds to the FOS or FRA protein in the nuclei of the brain cells, the biotinilated secondary antibody binds to the primary antibody. Then, a solution called Avidin-Biotin-Peroxidase Complex (ABC) binds to the secondary antibody, since its Avidin binds very tightly to the biotin of the secondary antibody. The Avidin has very many peroxidases that bind to it so that it provides for tremendous amplification of staining in the last step of the Immunohistochemistry procedure. Following the ABC binding to the secondary antibody, the tissues are placed in Diamino Benzidine solution (DAB). The peroxidases in the ABC react with the DAB and with H_2O_2 to produce the brown staining of the tissue sections where the primary antibody is bound to FOS or FRA. The brown staining is amplified because there are very many peroxidases bound to Avidin. The brown staining makes visualization of the FOS or FRA-containing nuclei in the tissues easily detectable under a light microscope. The following is the detailed procedure for immunohistochemistry:

Day 1:

A day after the brain is sectioned, the primary antibody procedure is done. The brain sections are washed twice in 0.1 M PBS solution for 5 minutes per wash, making sure to move sections to a new plate between washes by using a small brush. The sections are then incubated in a solution made up of 8.33 mL of 30% H_2O_2 in PBS for a final volume of 250 mL, for 15 minutes at room temperature. The final concentration of H_2O_2 is 1%. The H_2O_2 breaks up endogenous peroxidases to prevent staining of extraneous cells. Following the H_2O_2 incubation, the sections are washed again twice in 0.1 M PBS for 5 minutes each time, making sure to move sections to a new plate between washes. The sections are now incubated in 0.1% Triton x -100/5% normal goat serum/ 0.1 M PBS for 30 minutes. The goat serum binds to the mouse brain section anywhere that goat protein could bind. It therefore prevents the secondary goat-anti-rabbit antibody from binding. This prevents misleading staining. The Triton compound serves as a detergent, creating holes in the lipid bilayer of the brain cells, and thus allowing the primary (and secondary) antibody to more easily enter the cells. The sections are incubated in the primary antibody solution overnight (or about 20 hours) on a shaker at 4˚C. The solution of primary antibody is made at a ratio of 1:10,000 by adding 1 μL of primary FRA antibody (Santa Cruz SC-253) to 10 mL 0.1% Triton x-100/1% normal goat serum/0.1 M PBS.

Day 2:

The brain sections are washed twice in PBS for 5 minutes each time, changing plates between washes. A secondary biotinilated antibody solution in a ratio of 1:400 is made by adding 75 μL of goat-anti-rabbit secondary antibody to 30 mL of PBS. The secondary antibody binds to the primary antibody. The sections are incubated in solution for 2 hours. Half an hour before use, an ABC solution (Vector Laboratories) is prepared by mixing one drop each of A&B for every 5 mL PBS (use 30 mL PBS and 6 drops). The sections are incubated in ABC for one hour. The ABC (Avidin – Biotin – Peroxidase Complex) interacts with the secondary antibody and later with DAB to allow for protein staining. The brain sections are then washed in 0.1 M PB twice for 5 minutes each time, changing plates between washes. A solution of DAB (Diaminobenzidine) is then prepared by mixing 4 drops of buffer (vortex), 8 drops of DAB (vortex), and 4 drops of H_2O_2 (vortex) to every 10 mL of double distilled water. The ABC amplifies the protein staining since it provides more enzyme to react with the DAB and H_2O_2. The DAB stains the sections by interacting with the peroxidases that are bound to the Avidin, which is bound to the Biotin, which is bound to the secondary antibody, which is bound to the primary antibody, which is then bound to a FOS or FRA protein. The sections are left in the wells with the DAB until dark brown (about 2-5 minutes, keeping the time consistent). Sections are then removed and washed in PB twice for 5 minutes each time, changing plates between washes. The sections are now ready to be mounted, or to be stored at 4˚C until mounting.

Mounting Sections:

The mouse brain atlas is used to determine the most rostral section in each well. The most rostral section is taken from the first well, then from the next well, etc., so the sections are mounted in the original rostro-caudal order within the brain. The slide should contain 4 sections per column, and a total of five columns. After the sections are mounted and they dry on the slide, the sections are then dehydrated through a series of alcohols and xylenes, and cover-slipped using the protocol from the hood room. Once they slides are cover-slipped, the sections are ready to be counted.

Expected Results:

1. **Results of Conditioning:**
    The following expectations of results are based on data from the Meredith lab and elsewhere, already supported by the literature:
    - SU is an indifferent stimulus to the mouse, so the Cheerio will be a positive reinforcement to selecting the SU and the SU will change "value," by becoming a signal for available food for the animal.
    - The animals will gain a preference for SU by selecting the SU-scented Cheerio.
    - Change in BLA upon learned odor, since BLA is activated by the conditioning process.

    The following expectations of results are based on hypotheses based on the above-mentioned information supported by data:

    - For SU without dopamine: no expression in MeP or activation in ICN.
    - No change in MeP before and after learned odor.
    - The null hypothesis is that the MeA and MeP will not change in activation after the conditioning process has been done. However, a change in the MeP may be observed for mice who learn to prefer SU if the preference is a considerable one.

2. **Results of D1 Treatment:**

    The following expectations of results are based on data already supported by the literature:
    - SU appears to suppress MeP and is expected to activate ICN (based on hamster experiments showing heterospecific stimuli activate ICN and not MeP).
    - FMU activates MeP but is not expected to activate ICN.
    - Conditioned odors activate BLA (Schoenfeld et al 1999). After training, SU is expected to increase FRA expression in BLA.

    The following expectations of results are based on hypothesis based on the above-mentioned information supported by data:
    - Dopamine influences BLA, so a change is expected in BLA upon dopamine exposure in trained animals. No change expected in BLA upon dopamine exposure in untrained animals.
    - D1 agonist should inhibit the ICN cell group lateral to the BLA and decrease effects of inhibitory neurons in this ICN, so reduced inhibition is also expected in BLA (disinhibition in BLA) for trained animals.
    - MeP may show increased FRA expression since it could also be disinhibited like the BLA.
    - For FMU: ICN should not be active, and there should be no significant difference with the D1 injection.
    - For SU with dopamine: ICN activation may be suppressed; MeP may show activation even though SU is a heterospecific stimulus that normally doesn't activate MeP.

- For MeA, there are no clear expectations; it remains to be determined.

Meaning of Expected Results:

If the mouse gains a preference for the SU-scented Cheerio, the stimulus may then be perceived as relevant by the mouse, and its FRA expression in the MeP will be seen in response to SU. This would also suggest the MeP may be responsible for learned responses as well as unlearned. If a clear correlation is seen by the activation in ICNc and the inactivation in MeP (and vice-versa), then the data would suggest that ICNc controls the MeP via inhibitory projections into the MeP. If there is an increase in BLA activation after the dopamine treatment, that would suggest that dopamine influences BLA as well as the ICN. If a change in MeP is observed after conditioning, that would suggest that MeP is not only dealing with unlearned responses, but also with the mechanisms behind learned responses. The findings for MeA may shed some insight into its involvement with the chemosensory mechanisms of the amygdala under dopamine control as well.

<div align="center">Part II – Post Experimentation</div>

Relevant Changes to Predicted Protocol:

Stimulus

The originally chosen heterospecific stimulus, hamster vaginal fluid (HVF), was used in six trial animals. I concluded the HVF stimulation was not sufficiently up to previously established standards. It failed to give the expected levels of FRA expression in the MeA and was therefore discontinued from usage in subsequent animals. The heterospecific stimulus was changed to steer urine (SU). The steer urine was centrifuged; the supernatant was removed and diluted 1:10 with distilled water. Steer urine was considered a suitable replacement to HVF because not only is it also a heterospecific stimulus, but it is also a biological stimulus that mice may have encountered in the environment. Mice may naturally live in proximity to bovines. It appears to be the amygdala's role to distinguish between positive, negative, and neutral signals and assign them meanings. Previous studies have shown that steer urine is categorically distinguished by the medial amygdala, apparently as a neutral stimulus which activates MeA, but does not activate MeP. It is, therefore, a suitable replacement for HVF.

Number of Animals per Trial

The number of animals per trial was adjusted to compensate for time constrictions of the experiment. Three animals for each of the twelve groups were processed instead of the originally planned six per group. Due to issues with handling of the tissues either during sectioning, staining or mounting, I was left with two animals per group for most groups.

Areas of the Brain Counted

The medial paracapsular and lateral paracapsular intercalated nuclei (ICNmpcn and ICNlpcn) were not counted as originally planned in order to accommodate for allotted time to perform the experiment. The cells of these nuclei are widely dispersed and would be very time-consuming to count accurately.

Typical Conditioning Experience:

The experiment was performed typical to what is described in the "Methods" section above. It is important to note that perfume was not worn on the days of animal conditioning and testing procedures. The perfume could have potentially conditioned the animals to other stimuli that could have interfered with the outcome of the experiment and therefore perfume was not worn.

A typical conditioning experience for the animals following the preparations outlined above was as follows: the mouse was given three trials during each conditioning day. For the trained group, the three trials were with three SU-scented cheerios, and for the untrained group, the three trials were with three un-scented cheerios. The animals each spent anywhere between three to ten minutes in the testing apparatus, depending on the time it took for the animal to select the cheerio and eat it in each of the three trials. The conditioning process occurred daily for a total of seven days after the animal learned the testing protocol without the stimulus (or the plain cheerio in the case of the untrained animals). This differed by three days from the originally planned four days to ensure proper conditioning. A general observation made was that on average the animals receiving the SU-scented cheerios found their cheerios faster than the animals receiving un-scented cheerios during the conditioning process.

Results:

Behavioral Data:

Data on the behavior of the animals during their 15 minute exposure to either stimulus or control swabs is shown in the graphs in the appendix. These data are mainly important for monitoring the intensity of the investigation (as measured by swab-investigation time and number of contacts), which may affect the amount of FRA expression and is also an indicator of interest in the stimulus. The occurrence of unusual behavior such as an increase or suppression of grooming or rearing, or the appearance of special behaviors is also shown. Stretch-Attend behavior, considered a stereotyped risk-assessment behavior in mice was looked for but not seen in these experiments.

FRA Expression Data:

Due to the large number of groups and variables tested, the data were organized in various graphical representations for ease of viewing. The data were organized differently to show the effects of training or of dopamine treatment, or the differences between stimuli (control, female mouse urine, steer urine). Data are the means and standard errors for numbers of densely-stained FRA-positive nuclei within a region or subregion of the brain, with the counts for left and right sides averaged. Data are duplicated in graphs of the various subregions of the amygdala. Data were analyzed by Analysis of Variance (ANOVA) within each important anatomical region. Although there were three main experimental variables (training condition, dopamine/saline treatment and stimulus type), a three-way ANOVA was not used because of the small numbers of animals within each group. Instead, selected comparisons were made with two-way ANOVAs using the Sigma-Stat program. If the ANOVA had significant main effects or a significant interaction, posthoc pairwise comparisons were made within groupings, for example comparing the effects of saline vs. dopamine on FRA expression for the SU stimulus only within the stimulus-type groupings.

I will begin with a graphical representation of the various subregions together.
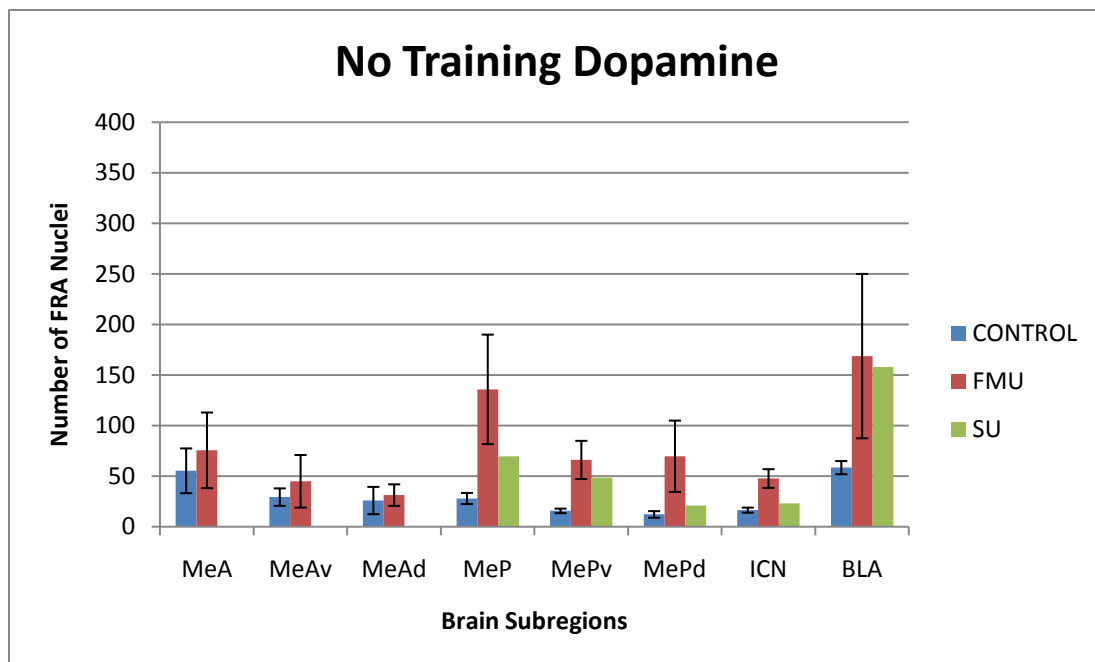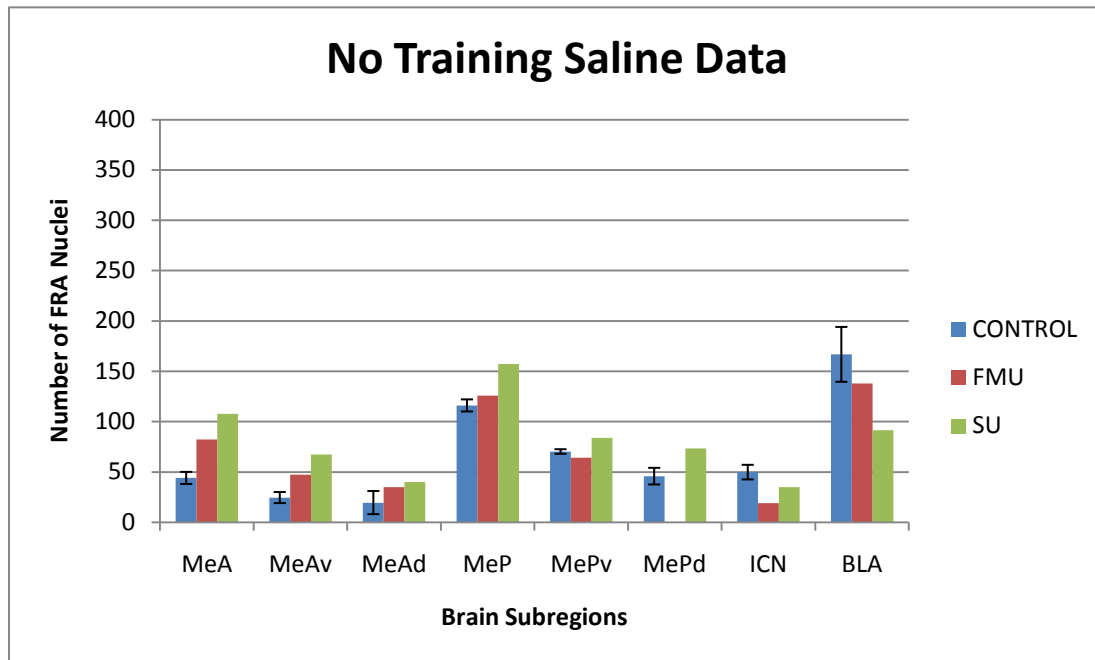
Combined Subregions Data:

Figure 1: Data for the no training groups for both the saline and dopamine conditions.
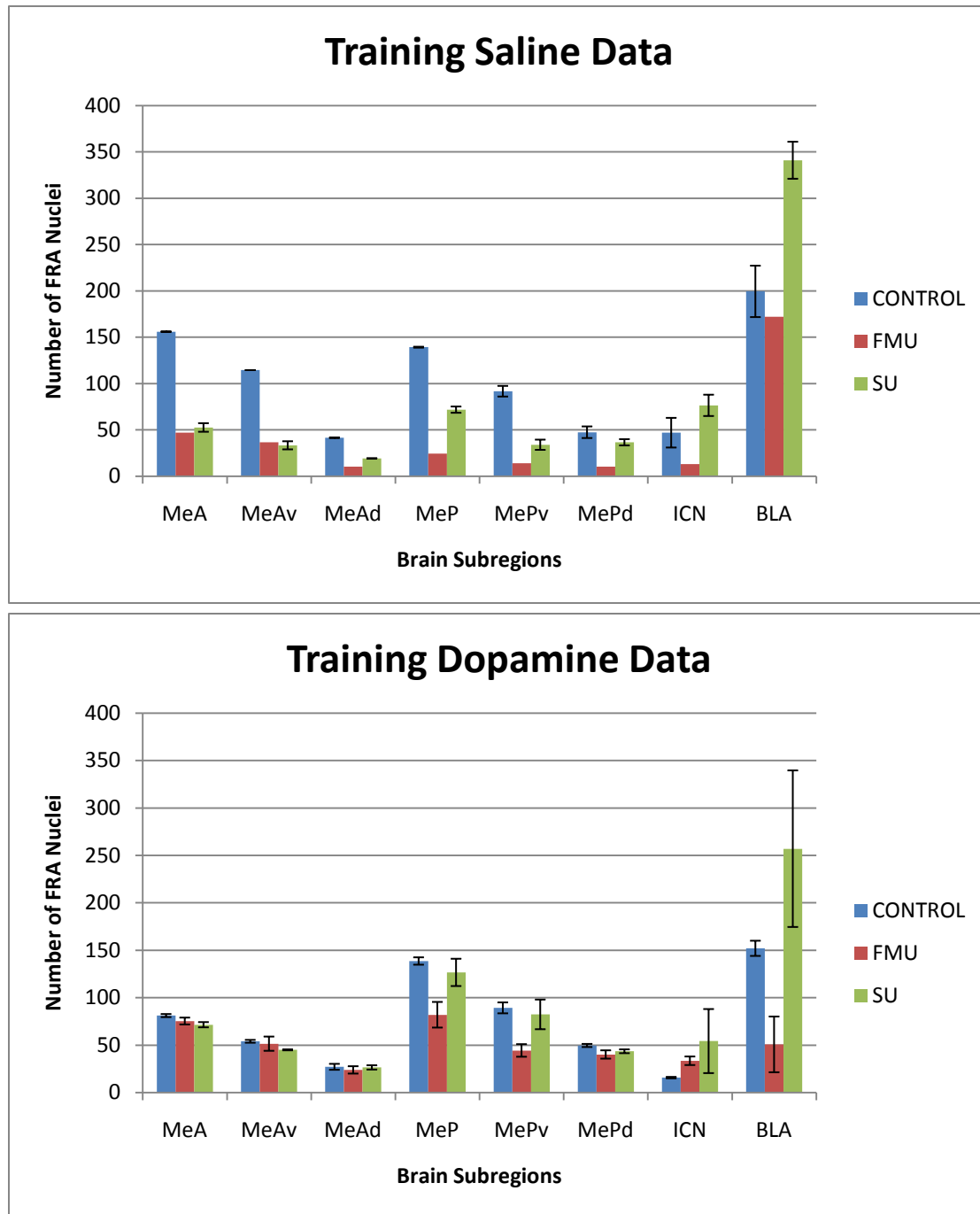
Figure 2: Data for the training groups for both saline and dopamine conditions.

Figures 1 and 2 were used mostly for ease of visual perception of all the data together. They will not be used in the discussion for the results. Separate graphs have been made for each important individual brain subregion as shown below.

Note: MeAd and MePd refer to the dorsal portion of the anterior and posterior medial amygdala, respectively. MeAv and MePv refer to the ventral portion of the anterior and posterior medial amygdala, respectively.

Discussion of Results:

Basolateral Amygdala (BLA) Data:

Effects of Training:

The BLA serves as a positive control for the effects of training and dopamine. The expected result for the effects of training in the BLA is that training should increase BLA activity for the learned odor, SU. From the visual observations based on Figure 3 from below, training increases activation in response to SU in both the saline and the DA condition. BLA does appear to register the increased salience of the odor. Several Two-Way ANOVAs were run using the Sigma-Stat program to determine the significance of these observations and the results are as follows:

1) When looking solely at the SU data, there is a significant main effect of training with an increase in activation in trained animals regardless of saline or DA treatment ($F=5.272$; $p<0.05$). This is the expected result, since SU is the trained odor and the BLA shows increased activation to learned odors.

SU data only:

Two-Way ANOVA; Factor 1: DA vs. Saline; Factor 2: Training vs. No Training.

2) When data are combined across the dopamine and saline treatments there is also a significant main effect of training ($F=6.674$; $p<0.05$). SU does show a significant increase in BLA activation with training ($p<0.05$); control and FMU stimulated groups do not ($p>0.05$). This is the expected result for the training group.

Data for saline treated and DA treated groups combined:

Two-Way ANOVA; Factor 1: Training vs. No Training; Factor 2: Stimuli (CON, FMU, SU).

3) Interestingly, when looking solely at the control (clean swab) data, there is a significant main effect of training ($F=10.121$; $p<0.05$), with an increase in BLA activation after training for the dopamine treated animals. This apparent increase in control is due to a decrease in FRA expression with DA treatment in the untrained animals – and is small when comparing it to the increase in SU with training.

CON data only:

Two-Way ANOVA; Factor 1: Training vs. No Training; Factor 2: Saline vs. DA.

Effects of Dopamine

The BLA also serves as a positive control for the effects of dopamine via D1 receptors. From previous publications I would expect BLA activity to increase with DA for all stimuli that activate the local ICN cell group, the lateral paracapsular nucleus (lpcn). However, I did not have enough time to count activated cells in the paracapsular nuclei. If there were some background level of activity in the lpcn ICN cells, DA would be expected to increase BLA activation. However, the dopamine does not act on BLA as expected for this scenario.
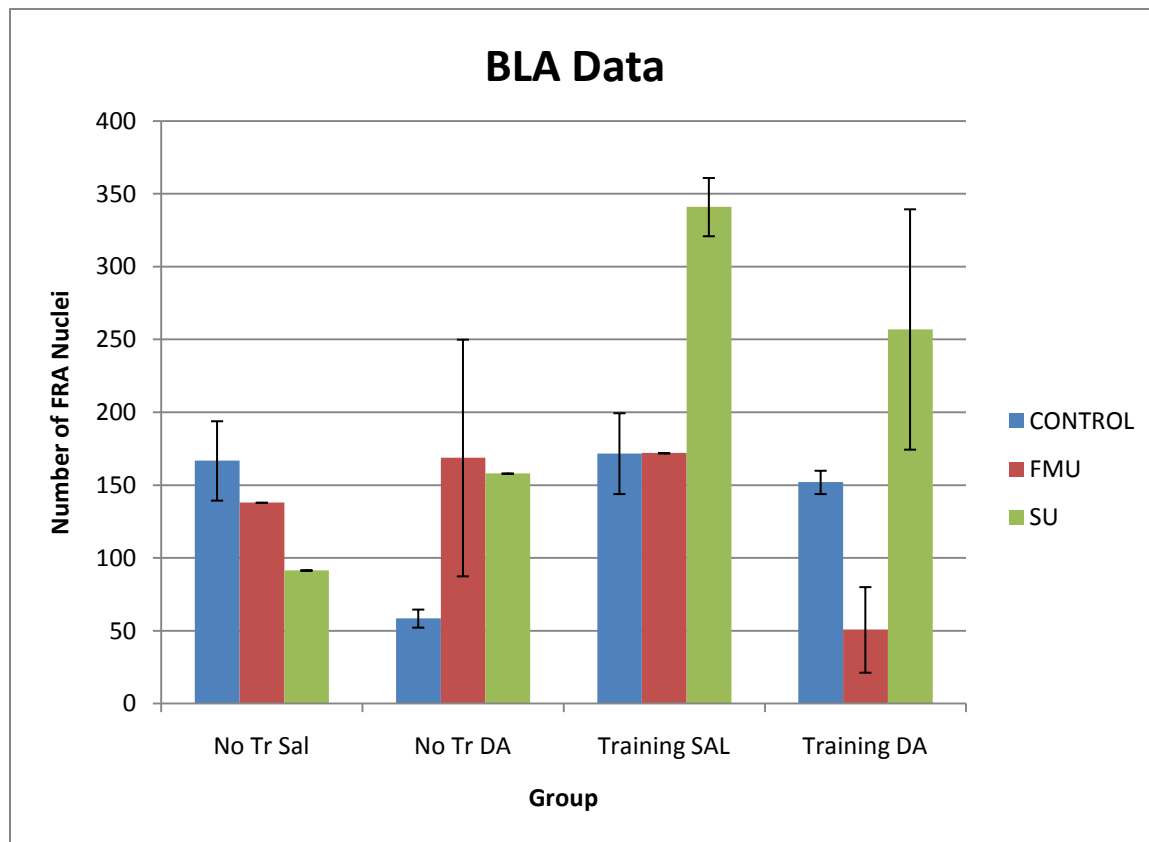
BLA Data:

Figure 3: Basolateral Amygdala (BLA) data for all stimuli and all groups.

From visual observations of Figure 3 above, dopamine is shown to decrease activity for control in the no training condition and for FMU in the trained animals. Figure 3 gives no evidence of increased BLA activation for any stimulus or condition, suggesting no background inhibition from lcpn. To test the significance of the data, several Two-Way ANOVAs were run in SigmaStat for the BLA data which give the following conclusions:

1) When combining data across training conditions (combining data from animals that did not receive training and that did), DA treatment does not show significant difference over saline treatment in BLA activation for control, FMU, or SU. According to expected results, DA should show increased activity in the BLA for all stimuli if there were some ongoing inhibition from lpcn. However, there is no significant main effect of treatment, suggesting there may not be background inhibition.

Data for Training and No Training groups combined:
Two-Way ANOVA; Factor 1: Training vs. No Training; Factor 2: Stimuli (CON, FMU, SU); No significant main effect or interaction.

2) When looking at the training data group only, there is a main effect of stimulus-type (F=6.633; p<0.05). Steer urine shows a significant increase in BLA activation over control, whether the animals were treated with saline or with dopamine but with no significant effect of DA for any stimulus group.

Data for trained animals only:
Two-Way ANOVA; Factor 1: Saline vs. DA; Factor 2: Stimuli (CON, FMU, SU).

3) The ANOVAs on SU data only and CON data only (1 and 3 in the "training" analysis above), show an effect of training (p<0.0), but not of DA (p>0.05).

Significance of Results in the BLA:

The BLA shows increased activation with training for SU, the learned odor. This is the expected result, and a change in brain response after training is consistent with the SU odor being successfully learned during conditioning. The expected results include that dopamine might increase activation for SU under training conditions, and that DA would not make a difference under the no training conditions, when activation of BLA (and lpcn) is not expected. The latter was also observed. The BLA does not show increased activation with SU after dopamine treatment for the no training group. The BLA response also shows some changes for control and FMU groups with dopamine and/or training, although the changes are very small when comparing them to the changes of activation by SU. The DA did not affect the BLA quite as expected. An increased activation might be expected with dopamine for all stimuli that activate BLA, if they also activate the potentially inhibitory ICN type cells of the lateral pcn. FMU does not activate BLA above control levels and neither it nor SU show a significant increase with DA. This may mean that there was no activation of lpcn by any stimulus or in any condition. It may be possible in the future to find the time to count the FRA-positive lpcn cells to test this prediction. However, the BLA was not significantly activated for the untrained conditions with or without dopamine, and this is also expected.

Medial Posterior Amygdala (MeP) Data:

Effects of Dopamine and Training:

The expected results for the MeP are that dopamine should increase MeP activity via disinhibition by the ICN – if there is any activation or background activity in the ICN. The FMU stimulus is particularly expected to increase MeP activity as shown by previous publication since it is a conspecific stimulus and an important signal for male mice (Meredith and Westberry 2004). For SU, MeA but not MeP is expected to show activation. MeP may show increased activation under the DA condition because ICN is expected to be activated by SU and suppress MeP response. There is no clear prediction for the effect of training. BLA is expected to be activated by the trained odor but whether the MeA/P would increase or decrease activation is not clear. From visual observations of Figure 4 (below) for the saline groups, FMU and SU do not give a significant response in MeP compared to control regardless of training or no training conditions. For the dopamine groups, however, FMU does show an increase in activation compared to control and SU does not, both of which are as expected for control (or saline) conditions. The dopamine group also shows a decreased MeP response to SU for the no training condition, which is not expected. However, for the training condition, DA does show increased activation for SU for dopamine-treated animals which is expected. To determine the significance of these results, several Two-Way ANOVAs were run that gave the following conclusions:

1) There is a significant interaction between the effect of training and the effect of dopamine treatment (F=16.134; p<0.05) with an increase in activation of MeP by SU, which was expected.

SU data only:

Two-Way ANOVA; Factor 1: DA vs. Saline; Factor 2: Training vs. No Training.

2) There is also a significant decrease (p<0.05) in SU activation of MeP for the saline treatment for the training groups compared to the no training groups. This is given by the posthoc pariwise comparison (Tukey Test) under the SU only ANOVA data.
SU data only:
Two-Way ANOVA; Factor 1: DA vs. Saline; Factor 2: Training vs. No Training.

3) There is also a significant interaction between the effects of training and dopamine treatment with a main effect of dopamine treatment (F=13.823; p<0.05) with a significant increase in MeP activity in the trained control animals.
CON data only:
Two-Way ANOVA; Factor 1: DA vs. Saline; Factor 2: Training vs. No Training.

4) The FMU data for either group does not show significant difference (p>0.05).

Significance of Results in the MeP:
The fact that the data on FMU is not statistically significant is rather troubling, since I expected FMU to show an increase in activation in the MeP, with perhaps an additional increase with dopamine treatment (if there was any background inhibition from ICN). From visual observations, the FMU does appear to activate the MeP compared to control, but since there are not very many animals in this group, the true significance of this observation is unknown. The fact that SU does significantly activate MeP under the dopamine and training conditions also is expected and suggests that dopamine serves to disinhibit the MeP and allow it to become activated by heterospecific stimuli where it normally does not. In trained animals, ICN is activated by SU and shows some reduction in activity with dopamine. This is the result expected for untrained animals but was not seen. The fact that the control also showed activation with dopamine and training compared to dopamine and no training is interesting. This may show the amygdala is indeed responsible for unlearned stimuli as well. Although control is not a particular olfactory stimulus, it may very well be a stimulus for the animals, since they have not had previous experience with the cotton swabs used as control.
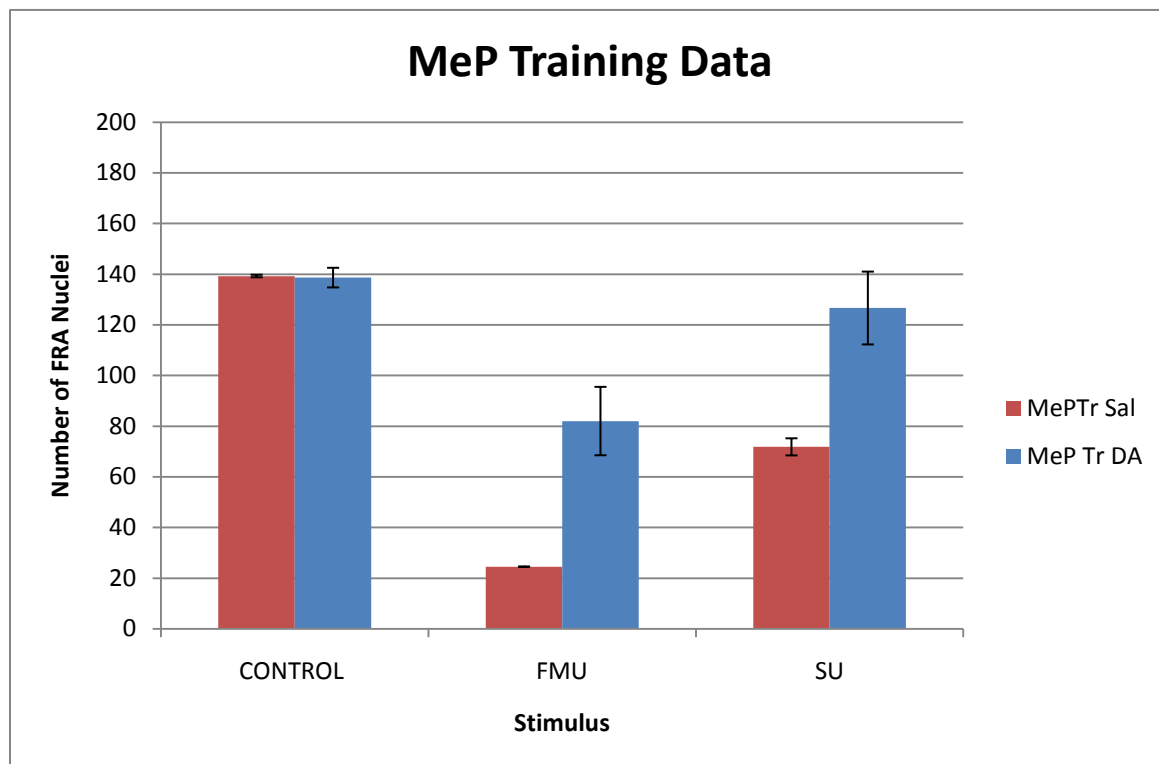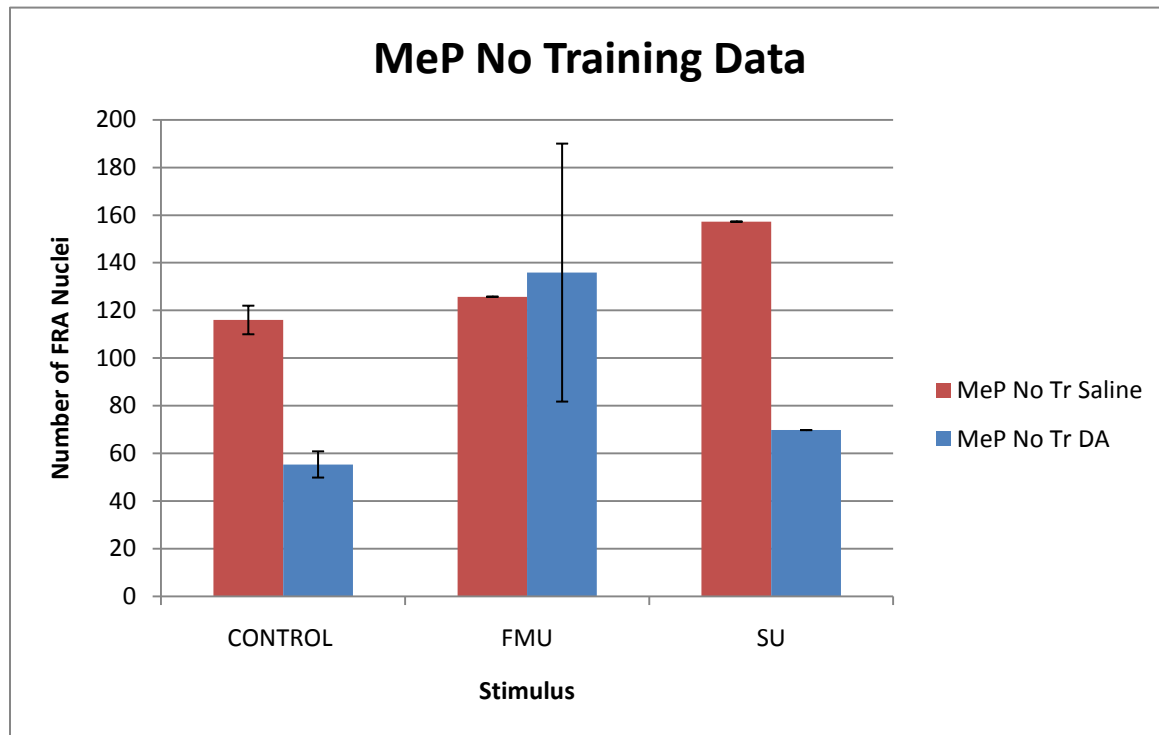
MeP Data:

Figure 4: MeP Data for both training and no training conditions, including saline and dopamine

Intercalated Nucleus (ICN) Data:

Effects of Dopamine and Training:

The expected result for dopamine in the ICN is inhibition of ICN. For the FMU stimulus, dopamine is not expected to make a difference in ICN expression, since ICN is expected to be inactive during FMU stimulation under saline condition. ICN is expected to show increased activity for SU under control (saline, no training) conditions, which was not observed. An increase in SU activation of ICN is seen in the trained animals. There is also a decrease with dopamine treatment, as expected. Whether these results correspond in any way to results without training in previous experiments is not clear. From visual observations in Figure 5 (below), ICN activity is decreased by dopamine for both SU and control for both training and no training groups. This is expected. For the no training group, FMU may suppress ICN, as the equivalent conspecific female stimulus HVF does in the hamster. For dopamine within both the training and the no training groups, FMU increases activation in ICN. Perhaps DA relieves FMU inhibition of ICN. To determine the statistical significance of these results, several Two-Way ANOVAs were run on the ICN data: Two-Way ANOVA combining across Training/No Training conditions (Factor 1: DA vs. Saline; Factor 2: Stimuli (CON, FMU, SU)) and Two-Way ANOVA combining data across DA/Saline treatments (Factor 1: Trained vs. Untrained; Factor 2: Stimuli (CONT, FMU, SU)). Both Two-Way ANOVAs showed that none of the results described above were significant ($p > 0.05$). This may be due to the small number of animals per group.

Significance of Results in the ICN:

Although many of the expected results seem to have occurred in the ICN from the interpretations given by Figure 5, the ANOVA results did not show any significant differences ($p > 0.05$) for the occurrences described above. This may be due to the small number of animals per group, or it may be simply because the results are not significant. Based on Figures 4 and 5, the reciprocal relationship expected between MeP and ICN occurs between saline and dopamine, but overall, the reciprocal relationship expected between the two is not evidenced. Because the ICN data is not significant, it is difficult to draw a conclusion whether the ICN inhibits the MeP and whether dopamine inhibits the ICN and therefore disinhibits MeP. Because some of the results for BLA and MeP are significant, the dopamine may act elsewhere in the brain to disinhibit the BLA and the MeP.
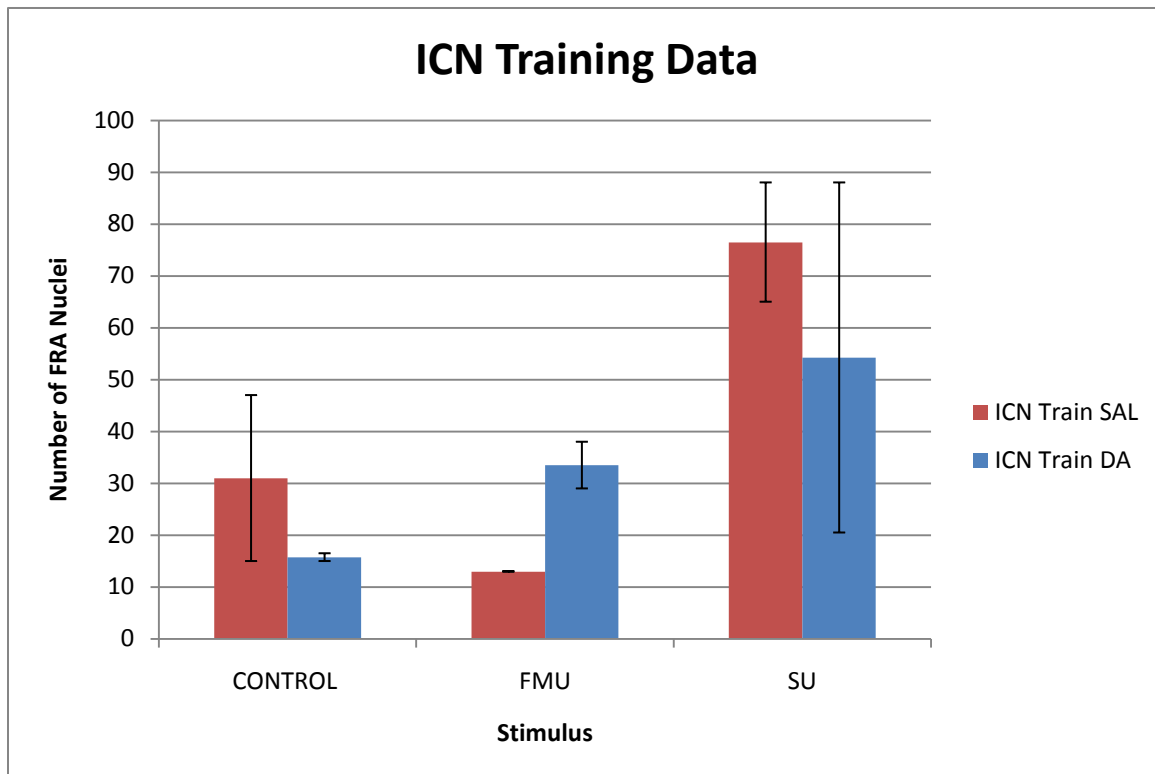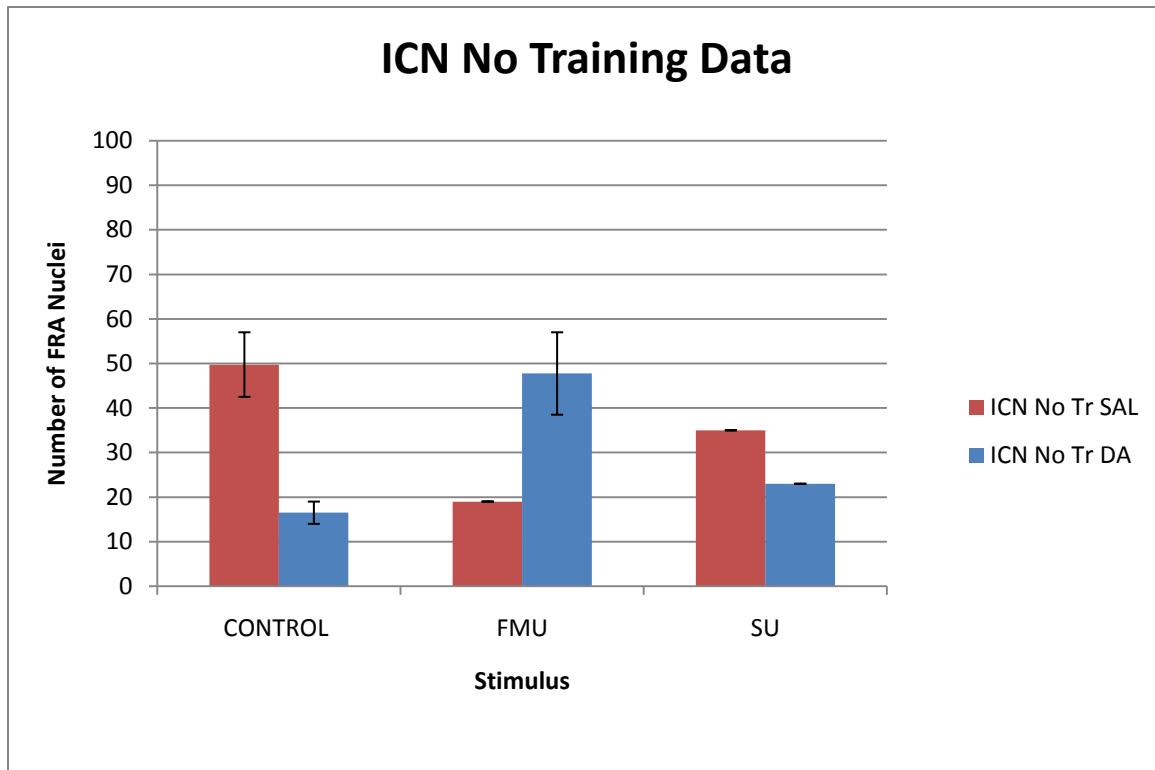
ICN Data:

Figure 5: ICN Data for both training and no training conditions, including all groups for saline and dopamine.

Results of Behavioral Data:

Figures 6-9 (see appendix) do not show any major differences between the behaviors of the animals in each group. In particular there were only a few cases where increased FRAS expression was associated with an increase in intensity of investigation; and in each case the changes were relatively small. Therefore, I can conclude that the results in the BLA, MeP and ICN are not a result of the animal's behavior during the testing process and rather a result of the exposure to the stimulus (or control).

Future Directions:

In future experimentation, the number of animals in each group must be increased to further verify the significance of the findings. It will also be helpful to determine if there are other areas of the brain that affect the BLA and MeP and whether those areas are affected by dopamine. Counting the FR-positive nuclei in the lpcn cells would also be useful. Another interesting experiment to think about is testing the hypothesized reciprocal relationship between MeP and ICN in another, larger experiment with and without dopamine.

Acknowledgements:

Thank you to my committee members: Dr. Michael Meredith, Dr. Thomas Houpt and Dr. Elaine Hull! Your guidance has made my project possible. Special thanks to Dr. Michael Meredith, Ariel Simonton, Lindsey Silz, and Dr. Chad Samuelsen for your support throughout the years I have been in the laboratory that have led me to completing this project. I appreciate all your help and support! I could not have completed this experiment without you all.

Appendix

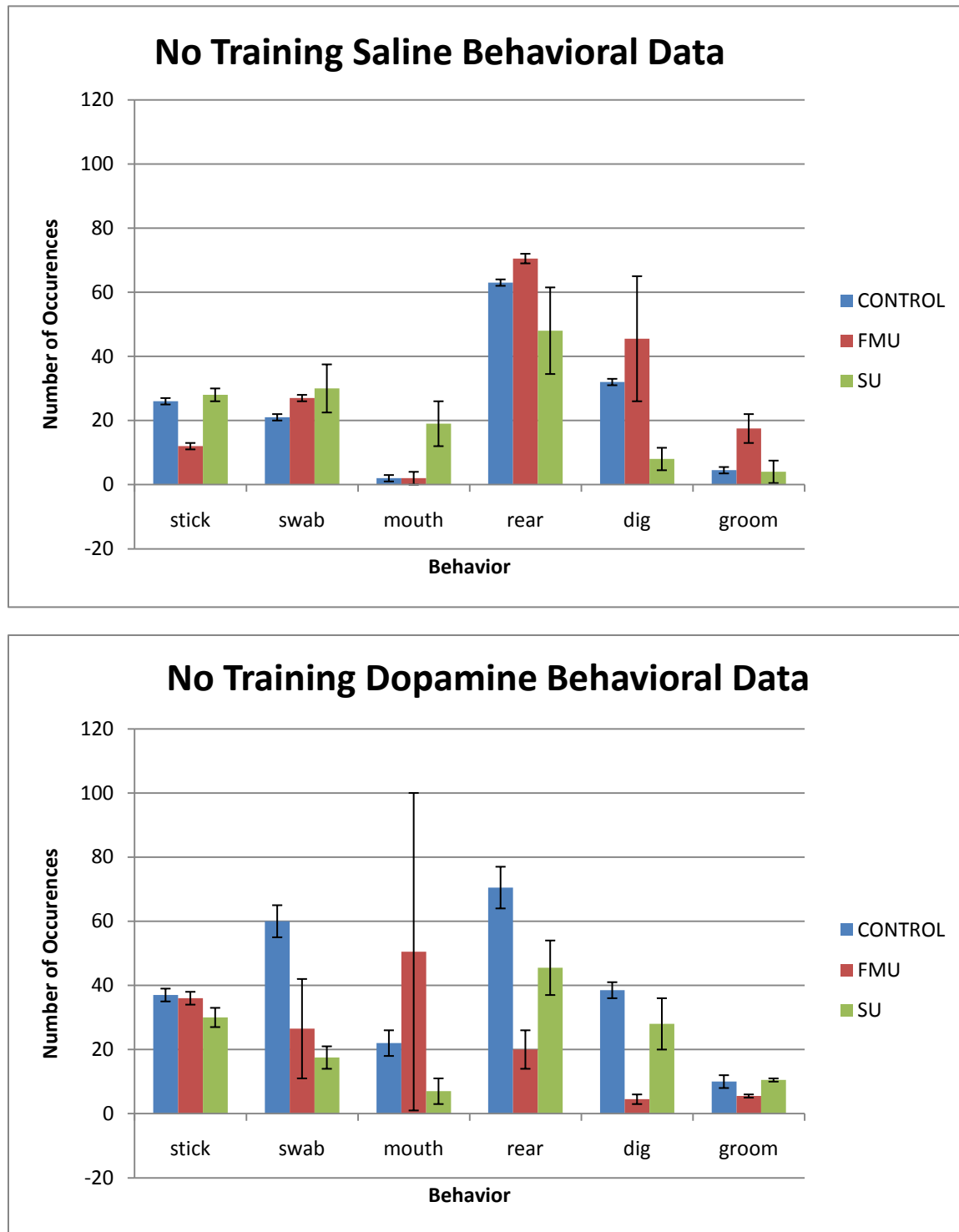**Behavioral Testing Data – Occurrences of Behavior**

Figure 6: Behavioral data for the no training condition. Number of occurrences of each behavior exhibited and recorded during testing is shown.
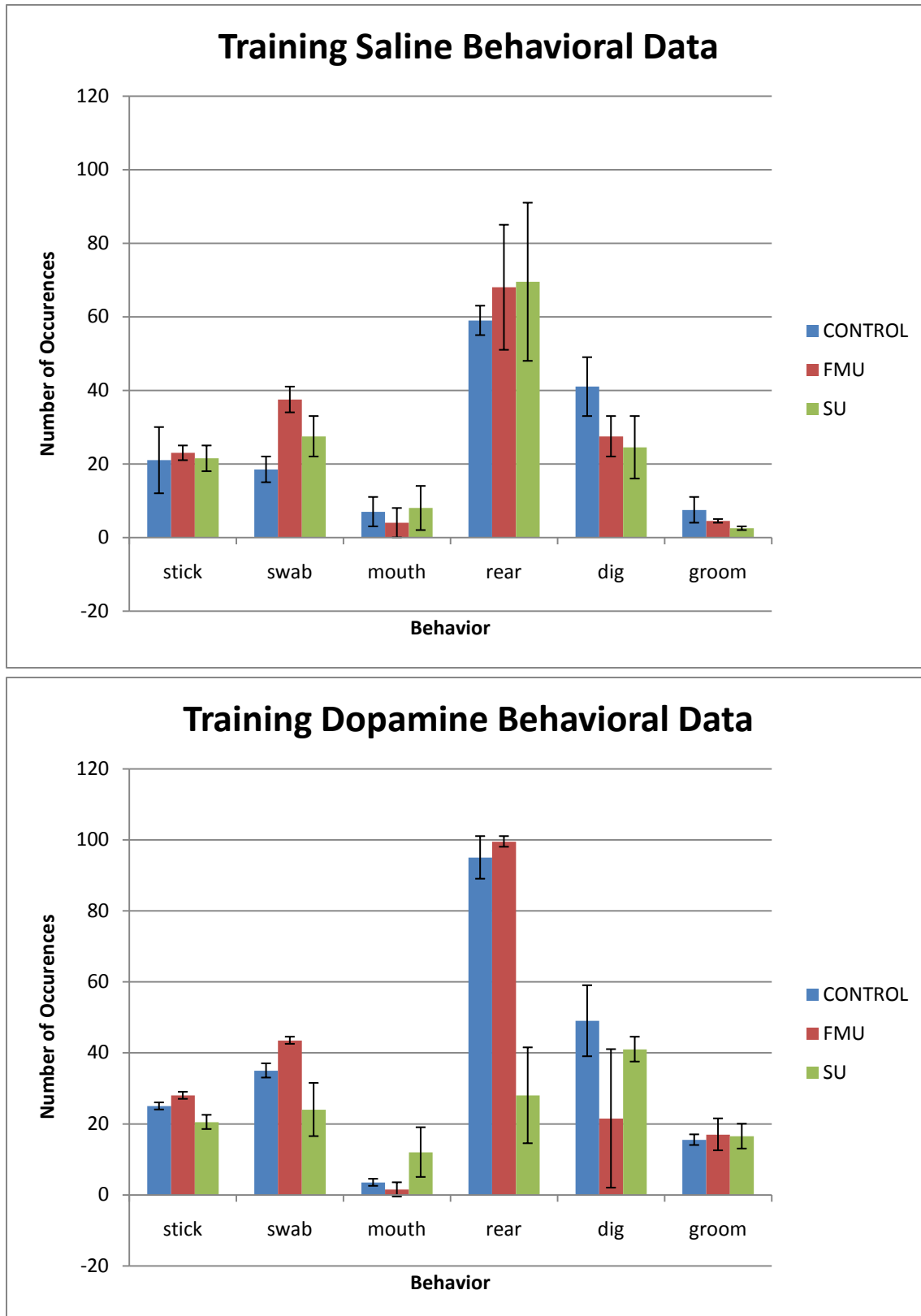
Figure 7: Behavioral data for the training condition. Number of occurrences of each behavior exhibited and recorded during testing is shown.

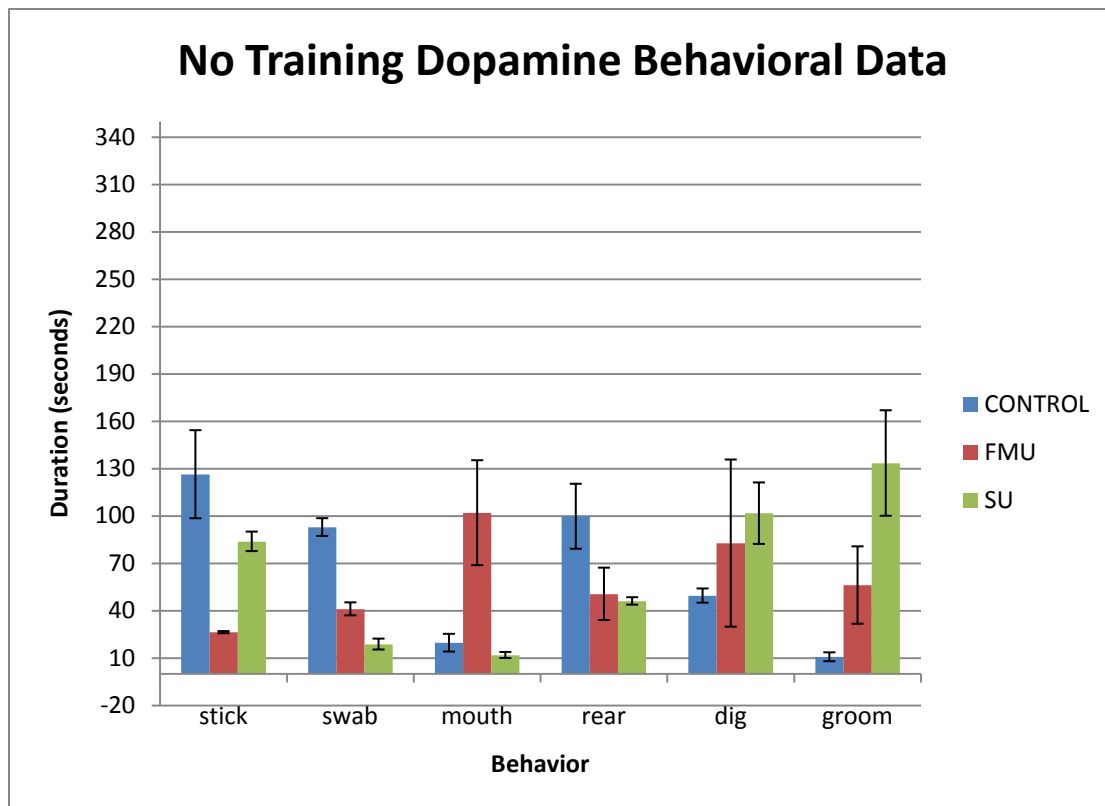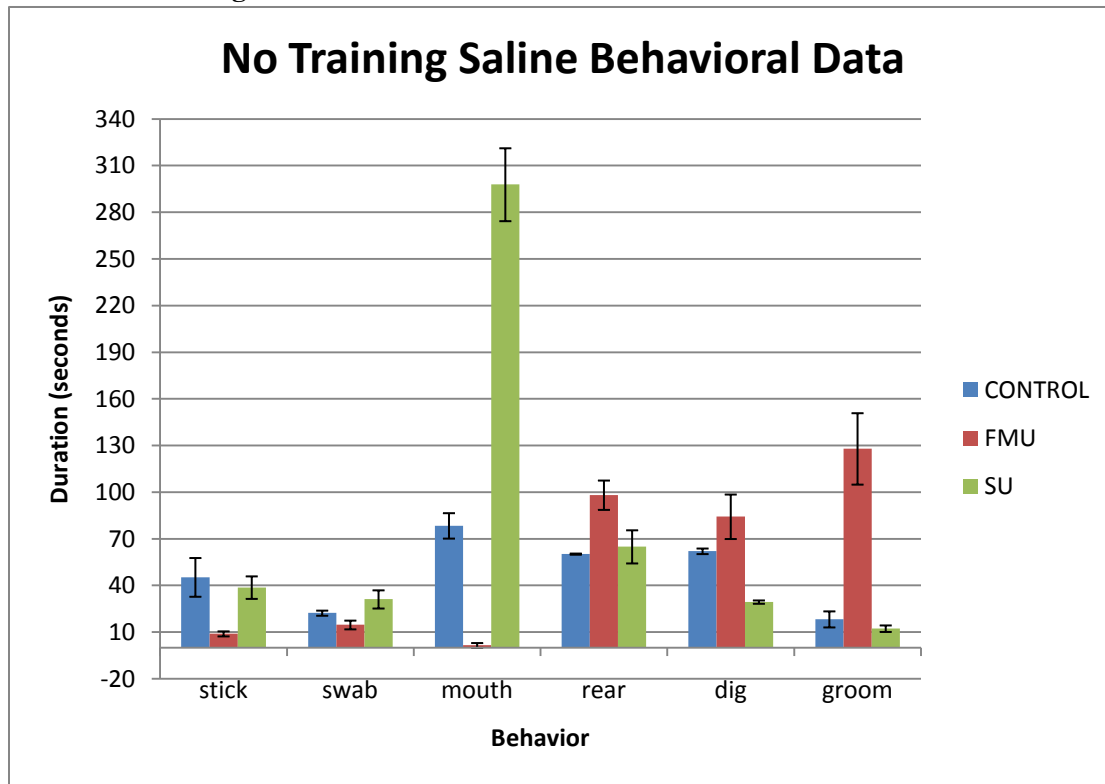**Behavioral Testing Data – Durations of Behavior**

Figure 8: Behavioral data for the no training condition. The durations of the various behaviors exhibited and recorded during testing are shown.

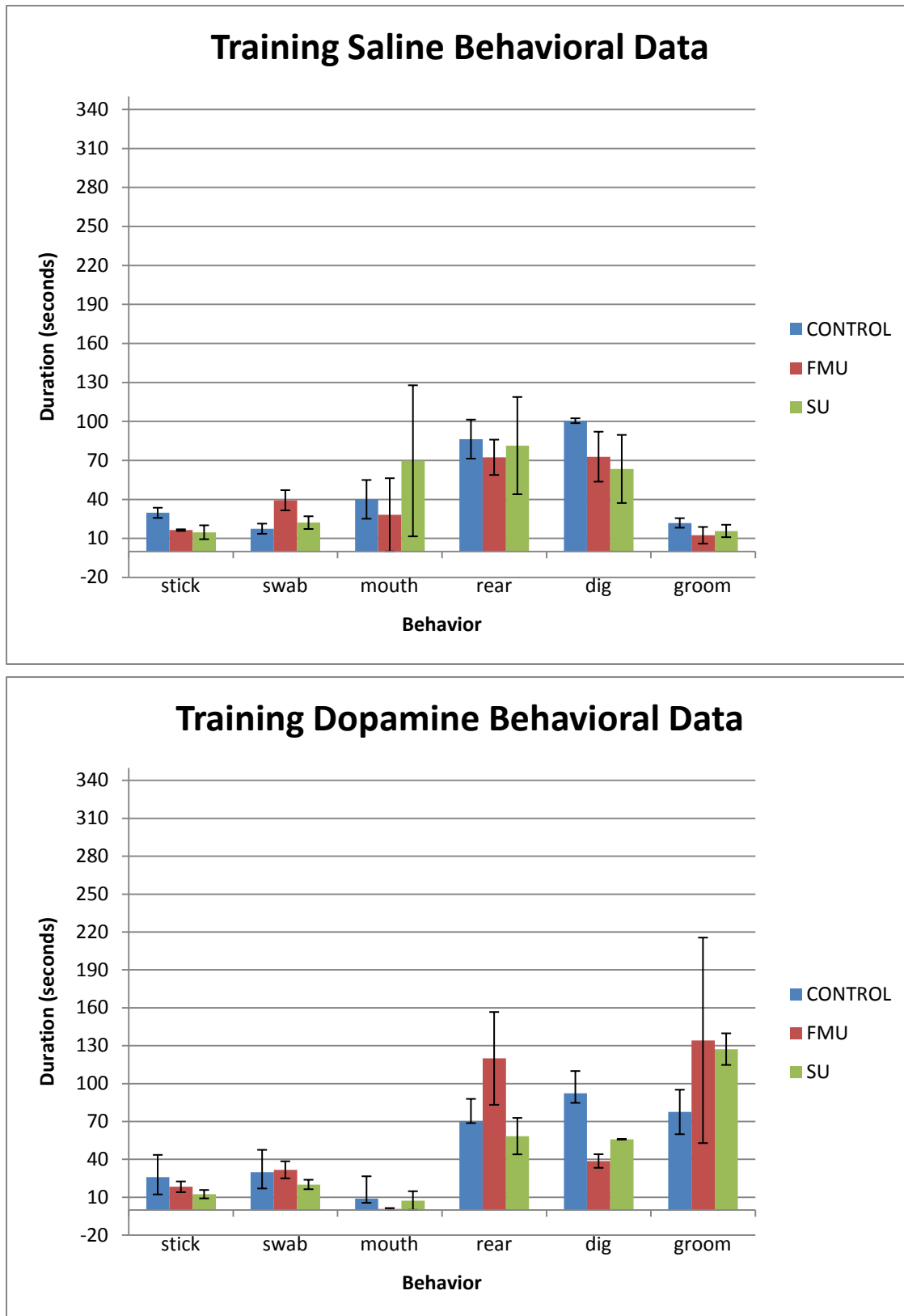Figure 9: Behavioral data for the training condition. The durations of the various behaviors exhibited and recorded during testing are shown.

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