Biology Department Senior Thesis Proposal

c-fos Immediate Early Gene Expression After Olfactory Circuit Disruption in Mice

Introduction:

The world is plagued by various types of neurodegenerative and neuronal deficit disorders, such as Parkinson's disease and stroke, which directly affect over 50 million Americans each year (Brown et al. 2005). There are many medical treatments designed to prevent permanent damage from neural disorders. However, once the damage has occurred, there are very few treatments available to repair it. Neuroplasticity refers to changes in neuronal pathways and synapses resulting from a variety of stimuli, including environmental, psychological, and physiological changes (Demarin and Morović, 2014). By studying the natural mechanisms that induce plasticity in the brain, we may better understand the mechanisms that could be used to treat brain and spinal cord injuries and diseases.

The olfactory circuit is known for its ability to regenerate neurons and drive new neural connections (Huart et al, 2013; Mandairon and Linster, 2009). Unlike the cells found in most neural circuits, olfactory sensory neurons can be completely destroyed, yet are still able to regenerate and make connections through the proliferation and differentiation of basal stem cells (Graziadei & Monti-Graziadei, 1979; Calof & Chikaraishi, 1989; Schwob, 2002). This feature, along with the rapid turnover rate of cells throughout the olfactory bulb (OB), allows us to use the olfactory system as a model for studying neuroplasticity.

Odor detection and discrimination begins in the olfactory epithelium, where odor molecules bind to receptors on the olfactory sensory neurons (OSNs). These OSNs then relay odorant information to interneurons and output neurons in the glomeruli of the OB through

synapses. The main output neurons that synapse in the glomeruli are the mitral/tufted cells, found deeper in the bulb. These output neurons then send odorant information to other regions of the brain for further processing (Huart et al, 2013). A simplified version of this pathway and the surrounding structures is shown in Figure 1 (Blumenfeld 2010).

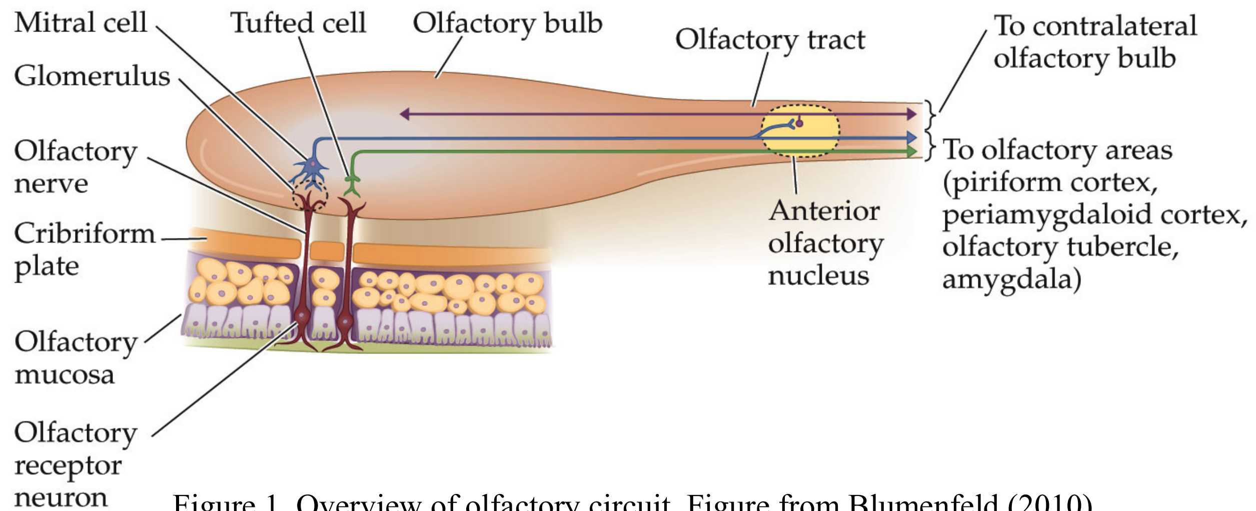

Figure 1. Overview of olfactory circuit. Figure from Blumenfeld (2010).

Odors induce a response in OB neurons, which causes the expression of immediate early genes (IEGs) (Bepari et al, 2012). IEGs activate transcription, either up-regulating or down-regulating gene expression (Perez-Cadahia et al. 2011). These transcription-level responses play a role in many cellular processes, including growth and differentiation (Perez-Cadahia et al. 2011). It is thought that long-term changes in neuronal connections and synaptic function may be related to IEGs, such as c-fos (Leslie and Nedivi, 2011). Additionally, because c-fos is stimulated rapidly after neuron activation, which is induced by odor exposure in the OB, the presence of fos protein can be used as an indication of recent neuronal activity (Guthrie et al. 1993; Hoffman et al. 1993). We are able to study the effects of experimental treatments on the OB by measuring and comparing fos positive cell levels throughout the bulb.

One such experimental treatment is sensory deprivation. Sensory deprivation, in this case, refers to a loss of sensory input through the OSNs into the OB. Since OB processing relies mostly on input from the environment, disrupting this input can have dramatic effects on the OB. Sensory deprivation can be induced using a weak detergent (Triton X-100) solution. Inducing sensory deprivation in this manner is cost effective and requires very little time, compared to surgical methods. The detergent solution degrades the nasal epithelium, and the OSNs in the olfactory epithelium, for a short period of time. The duration of degradation is dependent upon the concentration of detergent being used (Iqbal and Jacobs, 2010; Cummings et al. 2000). During this period of sensory deprivation, no sensory stimuli will enter the OBs through the OSNs, and the animal will be unable to smell (anosmic) (Fig 2A). The environment within the OB will change dramatically due to a lack of stimuli. Two known environmental changes in the OB include a decrease in dopamine synthesis and a decrease in tyrosine hydroxylase (TH) (Jin et al. 1996; Mast and Fadool, 2012), the rate-limiting enzyme of dopamine synthesis (Molinoff and Axelrod, 1971; Daubner et al. 2011). Since dopamine inhibits neuron activation in the OB (Hisa et al, 1999; Davila et al. 2003; Escanilla et al. 2009), we expect that this decrease in dopamine level will allow the OB to be more easily excitable (Hsia et al. 1999) once the OSNs are recovered. Approximately 72 hours after the last triton application, the nasal and olfactory epithelia are recovered (Fig 2C) and the animal exhibits normal investigative behavior (Iqbal and Jacobs, 2010). However, the OB circuits take longer to recover, as shown by the lower levels of dopamine and TH within the bulb, and therefore will still be disrupted (Baker et al. 1983). The recovery time required for the triton treated OBs to show no differences relative to PBS OBs is still unknown. We are able to study the changes within the OB as early as 24 hours after the last triton application (Fig 2B). At that time, the olfactory epithelium has recovered enough to allow

stimuli to enter the OB via OSNs, that is the animal can smell, but the OB circuit has not fully recovered (Fig 2C). Because the OB circuits are not recovered, the animal will not process odors in the same manner as a control animal. Behavior analysis from our lab has shown that triton treated animals are able to detect novel odors, but are less able to distinguish between odors. This is thought to be caused by fewer short axon cells, cells found in the glomerular layer that aid in glomerular communication, synapsing between the glomeruli of the OB. Loss of the short axon cells is caused by sensory deprivation (Jin et al. 1996). Since the presence of fos indicates recent neuronal activity, we will be able to identify any circuit changes in the OB caused by sensory deprivation, based on the location and amount of fos positive cells present. The goal of this study is to better understand the effect of sensory deprivation on OB activation by measuring c-fos expression.

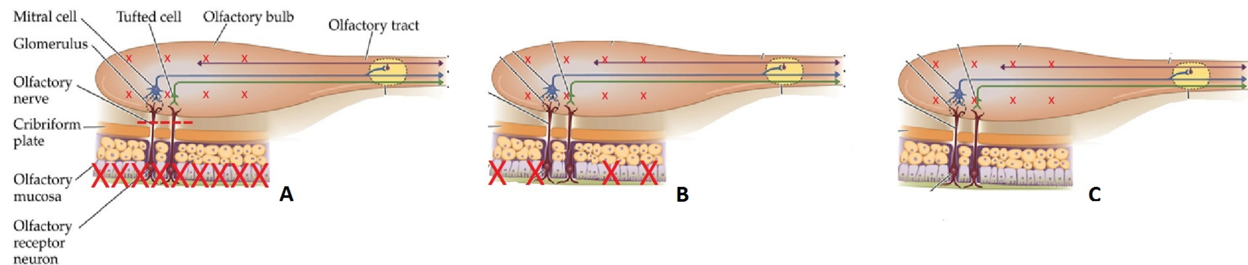

Figure 2. Effects of Triton X-100 treatment on olfactory epithelium and OB. The letter "X" symbolizes a disruption of the circuit where labeled. (A) Soon after Triton X-100 is introduced, the olfactory epithelium (mucosa) and OSNs are completely degraded. No sensory information will pass from the OSNs to the OB, causing drastic changes in OB connections. (B) Approximately 24 hours after Triton X-100 treatment, olfactory epithelium and OSNs show recovery and signals begin to reach the OB. OB environment is still disrupted. (C) Approximately 72 hours after Triton X-100 treatment, olfactory epithelium and OSNs are recovered and show full function. However, the OB environment is still disrupted. Figure has been edited from Blumendfeld (2010).

Hypothesis:

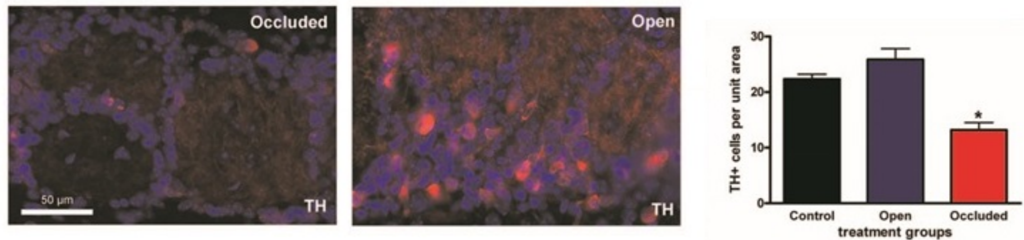

Figure 3. Tyrosine Hydroxylase (TH) immunolabeling (red) in the glomeruli layer of the OBs of an occluded and open naris. DAPI labeling of nuclei (blue) is also shown. Figure from Mast and Fadool (2012).

Based on a study by Mast and Fadool (2012), we can see that naris occlusion, an irreversible physical blocking of airflow into the naris, significantly lowers the amount of tyrosine hydroxylase in the olfactory bulb (Fig. 3). Rather than occluding the naris, we will be degrading the nasal and olfactory epithelia with triton. This lack of stimuli will affect the olfactory bulb circuits in a similar fashion to naris occlusion, but will be recoverable. Preliminary data show that triton treatment decreases the amount of dopamine in the OB. If this is the case, then we expect to observe increased levels of fos in the OBs of recovered triton treated animals, due to low OB dopamine levels, relative to the PBS treated animals. In this study, we aim to identify the link between dopamine presence in the OB and its relation to olfactory neuron activity, based on the presence of fos. We hypothesize that decreased OB dopamine will prevent neural inhibition, causing greater activation, marked by an increase in fos. By mapping fos expression, we will better understand the physiology and connections between the OSNs and the mitral/tufted cells of the olfactory circuit that send information to higher brain regions.

Experimental Plan:

We aim to manipulate the amount of dopamine in mouse OBs by intranasal irrigation with Triton X-100. The triton will be diluted with PBS to a final concentration of 0.1% (v/v).

PBS lacking Triton X-100 will be delivered as a control. 10µl of 0.1% Triton or PBS will be delivered to each nostril with a micropipette. Treatments will be delivered on alternating days for five days, each group containing approximately six animals. On the sixth day (24 hours after the last application), behavioral techniques will be used to expose animals to an odor, in order to induce c-fos expression in the OB. This will be done using an olfactometer. This device is a behavioral test chamber that allows the researcher to control odorant exposure through a pneumatic system and to measure animal behavior. Once the mice have completed treatment and behavioral tasks, they will be euthanized and their olfactory bulbs will be harvested (Mast and Fadool, 2012). The OBs will be post-fixed in a 4% paraformaldehyde (PFA) solution for at least 24 hours. They will then be transferred to a 30% sucrose solution for at least three days. The OBs will be protected in an optimal cutting temperature (OCT) compound and sectioned with a cryostat at 16µm thickness (Biju et al. 2008). Once sectioned and mounted on gelatin coated slides, we will use immunohistochemical techniques (Biju et al. 2008) to detect fos. For fluorescence microscopy, the primary antibody will be anti-cfos from Santa Cruz Biochem (#SC-52; diluted 1:1000) and the secondary will be Alexa Fluor 594 donkey anti-rabbit from Jackson Immuno Research (#711-585-152; diluted 1:200). For bright field imaging, an Avidin-Biotin Complex (ABC) labeling method will be used with the same 1:1000 primary antibody and a biotinylated donkey anti-rabbit secondary from Jackson Immuno Research (#711-055-152, diluted 1:200). The biotinylated secondary antibody has an extremely high affinity for avidin (Edward and Meir, 1980). Once complexed, we can use a variety enzymatic labeling techniques to visualize the fos positive cells. A no primary control treatment will be used as a negative control. Fluorescence and bright field microscopy images will be taken with a Nikon Eclipse

E400 microscope. Fluorescence images will be taken with a Cool Snap MYO camera, while bright field images will be taken with a Nikon Sight camera.

Image data will be analyzed using ImageJ software to quantify the amount of detected fos. This will allow us to more accurately measure the amount of fos positive cells per unit area, compared to manually counting fos positive cells. Preliminary trials have allowed us to develop a macro in ImageJ for counting fos positive cells (Fig. 4). From the preliminary trials, the number of fos positive cells obtained by manual counting and automated counting varied by less than 8% (Fig. 5). The image analysis process begins with converting the color image to a grey scale image. The background, which is automatically determined by the software, is then subtracted, allowing the fos positive cells to appear brighter and any background to appear black (Fig. 6). Figure 6 shows the background subtraction applied to a negative control image, as well as a treatment image. A brightness threshold is then applied to the image. Thresholds are determined by first using the “measure” function in ImageJ, which allows us to identify the minimum, maximum, and average brightness found throughout each image. We then set the threshold above the average intensity, and adjust it within 10% of the average intensity to minimize any background/noise that may still be present. Cells that are at or above the set threshold are considered fos positive, and are labeled red, like in Figure 4B.

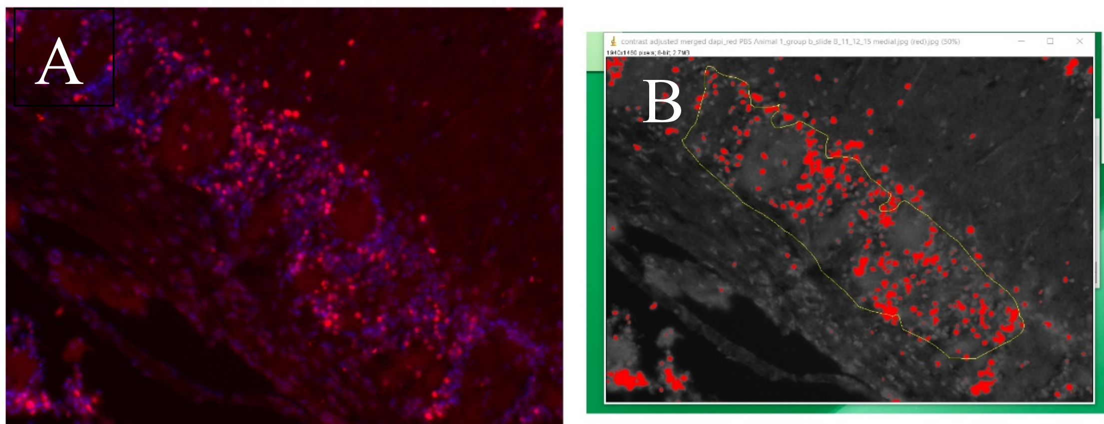

Figure 4. (A) fos positive labeling (red) and DAPI labeling (blue) in the glomerular region of a PBS treated animal. (B) ImageJ macro for identifying and automatically counting fos positive cells.

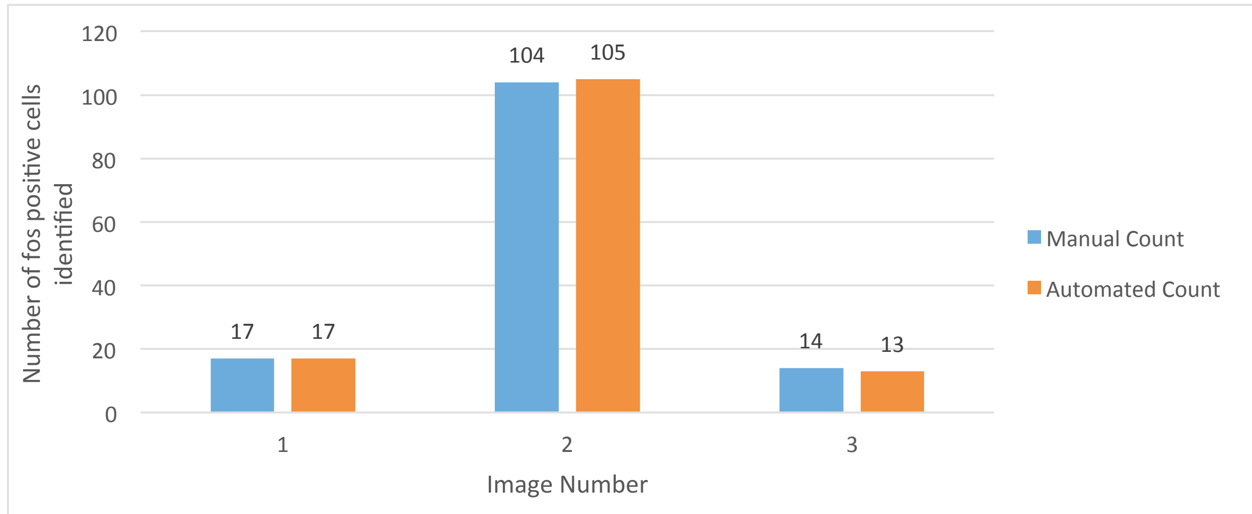

Figure 5. Comparison of manual counting and ImageJ automated counting. Counts were performed on three separate images to validate the accuracy of automated counting. Different regions of the bulb were analyzed for each image to test the validity of automated counting throughout the bulb. Image 1- dorsal glomerular region. Image 2- central granule region. Image 3- ventral glomerular region. Automated counts showed a percent difference of less than 8% compared to manual counts.

The number of fos positive cells per treatment will be measured and we will look for differences in labeling quantity using ImageJ, similar to Figure 5, as well as visually identify changes in activity patterns throughout the bulb. The data shown in Figure 5 was not caused by treatment effects, but is a display of automated counting throughout the whole OB. Statistical analysis, likely a T-Test, will be applied to the quantitative data to identify any significant differences between treatment groups. Our aim is to compare overall OB differences between treatment groups, as well as region-specific differences between treatment groups. The T-Test will give us insight into whether or not the treatment significantly affects OB activation. We believe our experiment will yield valid data to support the idea that the amount of fos present is inversely related to the amount of dopamine present in the olfactory bulb.

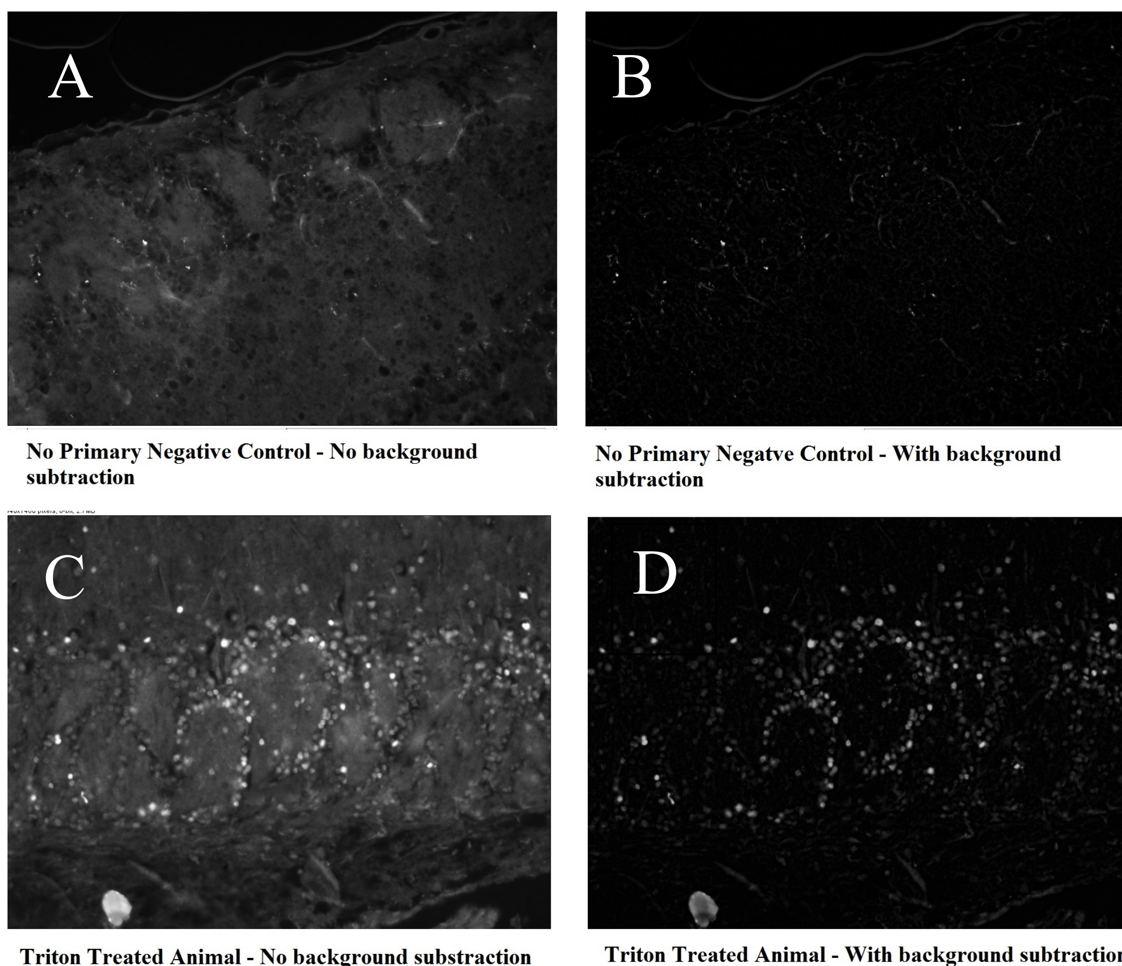

Figure 6. Display of background subtraction. All images were adjusted to grey scale then the background subtraction was applied. (A) No primary tissue before background subtraction. (B) No primary tissue after background subtraction. (C) Triton treated tissue before background subtraction. (D) Triton treated tissue after background subtraction.

These studies will be conducted in the Mark Jefferson Science Complex, in the areas including, but not limited to, the vivarium, the SEM laboratory, and the CMBB laboratory. Equipment and resources utilized will include, but are not limited to: basic laboratory supplies, microscopes, an olfactometer, approximately 12 mice, triton, antibodies, and odorants. The odorants may include, but are not limited to, amyl acetate, acetophenone, and 2-hydroxyacetophenone. These odorants have been previously shown to elicit fos expression in the

olfactory system (Glinka et al. 2012; Guthrie et al. 1993). The procedure has been approved by the EMU Institutional Animal Care and Use Committee (IACUC), protocol #2014-060.

Timeline:

The research will primarily be conducted in Winter 2016 and Fall 2016, as a continuation from the studies conducted during Fall 2015. The animals completed treatment and the olfactory bulbs were collected in February 2016. The bulbs have also been sectioned and immunohistochemistry was completed in March 2016. Tissue imaging should be completed by the end of the Winter 2016 semester. Preliminary data was presented at the Undergraduate Symposium in April 2016. During Fall 2016, images will be analyzed and data will be compiled. I will also begin writing the majority of my Honors Thesis during this time. The overall conclusions from my thesis project will also be presented at the Undergraduate Symposium in Winter 2017.

Conclusion:

The mechanisms behind olfactory bulb function are not well understood. By studying the pathways involved in plasticity, we could open new doors in medical research, and advance the treatment of neurological disorders. The present study will replicate/verify some known techniques, but also guide us in a direction which will help us better understand the physiology and circuitry that is found within the olfactory bulb.

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