MEASURING ACTIVITY IN THE AUDITORY SYSTEM OF WILDTYPE AND FRAGILE X MICE USING C-FOS PROTEIN EXPRESSION

By

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Abstract

Fragile X syndrome (FXS) is an autism spectrum disorder that is phenotypically characterized by social deficits and hypersensitivity to sensory input due to reduced or absent Fragile X mental retardation protein (FMRP) in the brain during development. There is ongoing investigation about the downstream changes in molecular and cellular systems of the brain, and the effects on the excitatory/inhibitory balance in sensory processing areas. In particular, it is important to study the responses to sound because individuals with FXS express auditory hypersensitivity and hyper-reactivity. The following proposed study will allow us to understand the activity levels of the brain at the cellular level in a circuit-like system. Wildtype (WT) and Fragile X model mice (*Fmr1* KO mice) of the C57 strain were exposed to 90 dB sound. 45 minutes following sound exposure, the animals were perfused, and brain tissues extracted and stained for c-Fos protein. The immediate early gene, c-Fos, is expressed in the brain as a result of neuronal activity making it difficult to capture with immunohistochemistry. In order to determine any signs of abnormal auditory activity, the presence of c-Fos was measured in brain structures involved in auditory information processing including the auditory cortex, the medical geniculate body of the thalamus and the inferior colliculus. The major aim of this experiment was to optimize the c-Fos staining protocol to clearly visualize the presence of c-Fos. The experiment manipulations suggest that depending on the manufacturer of the c-Fos primary antibodies, concentration changes and antigen retrieval may help increase the contrast between c-Fos signal and background fluorescence. These findings could help measure the amount of c-Fos staining in the brain of WT and *Fmr1* KO mice that were either exposed or not exposed to sound. The hypothesis of this experiment is that there is an increase in c-Fos density throughout the auditory cortex, thalamus, and inferior colliculus in the *Fmr1* KO mice exposed to sound than the WT mice.

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Introduction: Fragile X Syndrome (FXS) is a leading known genetic cause of autism and intellectual disabilities. FXS is caused by hypermethylation of the Fmr1 gene, which leads to the silencing of Fmr1 gene and reduced fragile X mental retardation protein (FMRP) production. This random mutation that arises in the human genome affects about 1 in 4000 males and 1 in 8000 females globally (Riley et al. 2017). Children with FXS show intellectual disability, anxiety, cognitive and social deficits and repetitive behaviors. There is also extreme sensory hypersensitivity including extreme reactions to sounds (Rotschafer and Razak, 2013; Chen and Toth, 2001). Sensory hypersensitivity is a consistent and highly debilitating symptom in children with FXS and may lead to increased anxiety, social and cognitive deficits. The *Fmr1* knockout (KO) mouse is a well-characterized mouse model of FXS. As in humans with FXS, sensory hypersensitivity is also present in the *Fmr1* KO mice. This phenotype has been characterized by electroencephalography (EEG), audiogenic seizure susceptibility, and single neuron recordings (Rotschafer and Razak, 2014). Thus, hypersensitivity is a translationally relevant phenotype in FXS. Given that circuits of sensory processing are likely more conserved across species than more complex cognitive and social behaviors, it will be feasible to address pathophysiology of brain circuits in FXS by studying sensory hypersensitivity. Moreover, there is a rich history of studies on sensory system development that could be utilized in understanding this neurodevelopmental disorder. Very little is known about development of cellular and circuit mechanisms in FXS or the role that FMRP plays in brain development.

The past and current literature in the field has played an important role in the development of the study that I am pursuing. The paper published in 1990 by Romand and Ehret describes the properties of neurons in the IC specifically the characteristic frequencies that arise

during development in order to better understand the role of the IC during auditory processing. Additionally, the paper published in 1979 by Shnerson and Willott, studies the response properties of developing C57 mice by looking more closely at the subtypes of neurons present in the IC. These properties are significant to my study which aims to determine the similarities and/or differences of WT and KO mice across strains by analyzing C57 mice and comparing them to FVB mice analyzed by Anna Nguyen in the lab. Next, the paper entitled, "Auditory processing in fragile X syndrome", by Rotschafer and Razak, demonstrate the abnormal response properties of neurons in the auditory information processing pathway using different types of encephalography that may contribute to the hypersensitivity to sound in FXS mice. Lastly, the paper published by Chen and Toth, shows that FXS mice are more susceptible to audiogenic seizures as well as the ability to use c-Fos immunohistochemistry as a marker for neuronal activity.

The proposed study will allow us to understand the activity levels of the brain at the cellular level in a circuit-like system by staining for the immediate early gene, c-Fos, which is expressed in the brain as a result of neuronal activity. The aim of this experiment is to measure the amount of c-Fos staining by comparing cell counts in the brain tissue of wildtype and fragile X mice both exposed and not exposed to sound. The hypothesis of this experiment is that there is an increase in c-Fos present throughout the auditory cortex, thalamus, and inferior colliculus in the *Fmr1* KO mice exposed to sound than the wildtype mice. During this study, there were issues that arose with the c-Fos protein staining. The original primary c-Fos antibody that was being used in the laboratory was no longer being manufactured. As a researcher, my goal was to perform the c-Fos staining with a well working protocol that could be properly collected and analyzed. This led to attempting to perform the c-Fos staining with another primary antibody that

did not turn out to be successful. Even with numerous changes made to the immunohistochemistry protocol to try and optimize the signal, the c-Fos staining was not subject to objective cell counting data analysis. Optimization attempts included adding specific reagents, reconfiguring the steps of the protocol, and adding an antigen retrieval process to the protocol. The aim of these experiments was to reduce the background signal and increase the brightness of staining. The c-Fos staining was more successful after attempting the protocol with a different primary antibody from the company, Synaptic Systems. With and without the antigen retrieval process included in the beginning of the protocol, I was able to learn how to use ImageJ, an image analyzing program, to objectively count the c-Fos stained cells.

Materials and Methods:

Mice: All mice of were on the C57bl/6 background. The ages of the mice ranged from P25 (postnatal day 25) -P29. The first round of sound exposure experiments and c-Fos staining was performed with mice that had a known genotype, either wildtype or *Fmr1* KO mice. In this first pass, there were 4 sets of mice with a total of 12 male mice; sets 1 and 4 were exposed to sound (n=6) whereas sets 2 and 3 were not (n=6). The second round of experiments was performed with mice from littermate control cages. Their tails were collected and sent for genotyping. The genotyping results were only able to determine the genotype of 3 mice. In this second pass, there were 2 sets of mice with a total of 8 male mice; set 2 was sound exposed (n=4) whereas set 1 was not sound exposed (n=4).

Sound Exposure: Groups of 2-4 mice were habituated in their cage within a sound attenuation booth (GK Inc., OR) for three hours. The control group of mice were not exposed to the sound whereas the experimental group was exposed to the sound. The sound stimulus that was presented in the experimental group of mice was a frequency modulated (FM) sweep from 5 kHz

to 50 kHz generated by the program, Sparkle (Portfors lab, Washington State Univ.). The repetition rate of the sound was 1 per second. The sound level of the stimulus was 90 decibel (dB) SPL which did not cause audiogenic seizures and was verified using a sound level meter. Five minutes before playing the sound, a video recording of all of the mice was started. Recording took place for both groups of mice to capture their movement during the experiment. The experimental group was then immediately exposed to the sound presentation for a duration of 15 minutes, after which the sound and video recording were turned off. All of the mice were left in the sound booth for an additional 45 minutes before perfusions to allow for the expression of the immediate early gene, c-Fos, in the brain.

Intracardial perfusions and brain extractions: The mice were perfused intracardially in a fume hood with 0.1 M Phosphate-Buffered Saline (PBS, pH = 7.4) and 2.5 % Paraformaldehyde (PFA, pH = 7.6). The mice were first anesthetized with isoflurane and then injected with a 1:10 dilution of sodium pentobarbital (Fatal Plus). Each mouse was perfused with 45 mL of 0.1 M PBS and 45 mL of 2.5% PFA. Brains were extracted and immediately stored in 2.5% PFA for 24 hours in the refrigerator then stored in 30% sucrose to allow for the brains to sink in the tube and be prepared for slicing.

Acquiring brain sections: Brains were sliced into sections of 40 micrometers thickness using a cryostat (Leica). The auditory cortex was identified by the appearance of the hippocampus and collected along with the medial geniculate body of the thalamus, and inferior colliculus which was identified by the appearance of the cerebellum. Upon slicing, the brain sections were stored in 0.1 M PBS in the refrigerator.

Materials for c-Fos staining: 0.1 M PBS (pH = 7.4), Normal Goat Serum (NGS), Triton X-100, Tween-20, secondary antibody (donkey anti rabbit 594); primary antibodies: Sigma-Aldrich c-Fos anti-rabbit and Synaptic Systems c-Fos anti-rabbit

c-Fos staining: Day 1: First, wash with 0.1 M PBS 3 times for 5 minutes each time. Second, block for 1 hour with 5% NGS in 0.1 M PBS. Third, wash with 0.1 M PBS for 10 minutes. Next, wash with 0.5% Triton X-100 for 10 min. Lastly, incubate in primary antibody on shaker in 4^0 C overnight: 1:200 c-Fos anti Rabbit, 1% NGS, 0.1% Tween-20 in 0.1 M PBS.

Day 2: First, wash with 0.1 M PBS 3 x 5 minutes. Second, incubate in secondary antibody for 2 hours: 1: 500 Donkey anti Rabbit 594, 1% NGS, 0.1% Tween-20 in 0.1 M PBS. Third, wash with PBS 3 times for 5 minutes each time. Lastly, mount sections with mounting medium and DAPI (Vectashield) onto slides, cover with glass, and use Cytoseal to seal edges.

Image acquirement and analysis: Images were obtained using a Confocal: Leica SP5 Inverted microscope and a Nikon H550L light microscope. Exposure to light was limited to prevent the fluorescent c-Fos staining to bleach out, becoming undetectable. Images were stitched together using the acquire large image tool and tile scan tool on the light microscope and confocal microscope, respectively. Analysis of the images involved measuring the number of cells marked by c-Fos staining to compare cell counts between the control and experimental groups for both genotypes. By using the ImageJ program, preparation of the images included summing up the z-stack images to obtain a single image which was then further modified. The single image was cropped to a width of 400 micrometers and the background signal was subtracted before applying the signal threshold to perform the cell count.

Staining Optimization: In order to verify that the analysis and statistical significance of the study were objectively determined, my goal was to improve the c-Fos immunohistochemistry protocol in order to improve the quality of the images and to perform the cell count. Optimization of the immunohistochemistry protocol included experiments that tested different concentrations of the primary antibody, a different order of certain steps and the use of novel reagents. Brain tissue from *Fmr1* KO mice that were exposed to sound were used in the c-Fos staining optimization experiments. I predicted to find the most c-Fos staining within these sections.

The first experiment was to test varying concentrations of the primary c-Fos anti-rabbit antibody including a concentration of 0 which served as a negative control, 1:50, 1:100, 1:150, 1:200 which was the concentration used in the original protocol, and 1:1000. The goal was to improve the primary antibody binding to the c-Fos antigen by testing concentrations that were higher than 1:200 (e.g., 1:50 and 1:100). All other steps in the protocol were kept the same.

The second experiment was to test whether the order of steps had an effect on the c-Fos staining. Particularly, the second and third steps of the protocol in day 1 were manipulated. Instead of introducing the brain sections to NGS before the detergent, I first washed the sections in a 0.5% solution of Triton X-100 then washed in 0.1 M PBS before incubating the sections to the NGS solution for one hour. NGS, normal goat serum, is used in immunohistochemistry as a blocking reagent, meaning it blocks the non-specific binding sites within the brain tissue. It prevents the c-Fos primary antibody from binding to those non-specific sites in order for the c-Fos primary antibody to bind to the specific c-Fos antigen. Triton X-100 is used in immunohistochemistry to wash the sections and to permeabilize the membranes of the cells within the tissue. It facilitates the process of the reagents such as the c-Fos primary antibody and fluorescently tagged secondary antibody entering the cells and binding to their respective sites.

The goal was to permeabilize the membranes of the cells before incubating in the blocking solution for the non-specific sites to be blocked more effectively. Then, ultimately, both the c-Fos primary antibody would bind to the c-Fos antigen and the fluorescently tagged secondary antibody would bind to the primary antibody more effectively as well. The expectation was that this manipulation would increase the brightness of the cell staining and increase the contrast of the background from the signal.

The third experiment was to test whether the addition of 0.1% Tween-20 to the blocking solution would improve the c-Fos staining. Tween-20 is another detergent used in immunohistochemistry to wash and permeabilize the membranes of the cells aside from Triton X-100. The goal was to improve the effectiveness of the blocking solution by adding the detergent which would allow the non-specific binding sites to be more accessible to the NGS. Therefore, decreasing the background fluorescence and increasing the contrast between the background and signal. Tween-20 was specifically chosen to be added to the blocking solution instead of Triton X-100 because comparatively, Tween-20 is a weaker detergent to Triton X-100 which is one of the reasons that it is used in solutions with other reagents such as the primary antibody solution.

The fourth experiment was to test whether the addition of 1% Bovine Serum Albumin (BSA) to the blocking solution would enhance the quality of the c-Fos staining and images. BSA is like NGS acts as a blocking reagent that blocks the non-specific binding sites of the tissue from the c-Fos primary and secondary antibodies. The goal was to decrease the background fluorescent staining and improve the quality of the images by incubating the sections in a solution made up of two blocking reagents.

The fifth experiment was to introduce a novel multistep process to the c-Fos immunohistochemistry protocol, commonly known as antigen retrieval. The antigen retrieval process was integrated to the beginning of the c-Fos immunohistochemistry protocol before washing with 0.1 M PBS, blocking with 5% NGS, and washing with 0.5% Triton X-100. The first step in the antigen retrieval process is to wash the sections with 0.1 M PBS three times for five minutes each time. Second, heat distilled water in a beaker to 80 degrees Celsius. Third, place 0.75 mL of citrate buffer solution into 1 mL sized tubes and place into hot distilled water bath to heat the citrate buffer solution. Fourth, place the brain sections into the 1 mL tubes with the citrate buffer solution and put back into the hot distilled water bath for 30 minutes. Lastly, take the tubes out of the hot water bath and let them cool to room temperature for about 20-30 minutes. The goal of antigen retrieval is to prepare the tissue for the overnight incubation with the c-Fos primary antibody.

Data:

Testing varying primary antibody concentrations (Sigma c-Fos primary antibody):

Brain tissue from the *Fmr1* KO mice that were exposed to sound from the first round of sound exposure experiments were used to test varying c-Fos primary antibody concentrations. The images acquired from this modified staining were compared to the acquired images from the original protocol. Figure 1 shows exemplary images of the data that indicated that there was improvement in the quality of c-Fos staining when the concentration of the primary antibody was increased from the original 1:200 concentration to 1:100. Sections from two different animals seemed to improve. Additionally, figure 2 shows that there was subtle improvement observed in the c-Fos signal when the concentration of the primary antibody was increased from the original 1:200 concentration to 1:150. The improvement with the 1:150 concentration was only visible in

one animal compared to the 1:100 concentration staining which was visible in both animals. In all, improvements were more remarkable with the 1:100 concentration of the primary antibody compared to the 1:150. There were no observed improvements in the quality of the images with the other tested concentrations: 0, 1:50, 1:200, and 1:1000.

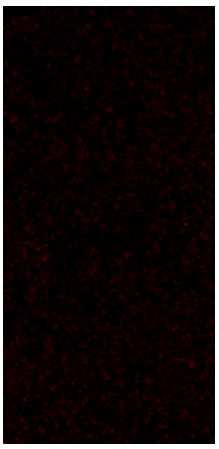
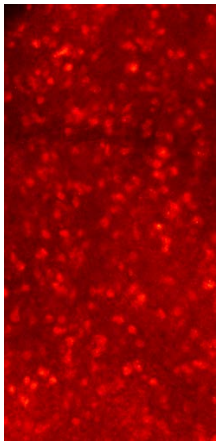
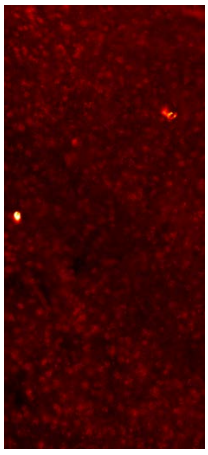
1a.  1b.  1c.  1d. 

Figure 1: Increased brightness in c-Fos signal and visualization of cells with a 1:100 primary antibody concentration (*1b* and *1d*) compared to a 1:200 concentration (*1a* and *1c*). Images *1a* and *1b* were obtained from the red labeled mouse in set 1 (DOB: 1/23/18). Images *1c* and *1d* were obtained from the none labeled mouse in set 1 (DOB:1/23/18). Set 1 of mice were *Fmr1* KO mice that were exposed to sound. The above images are of the IC.

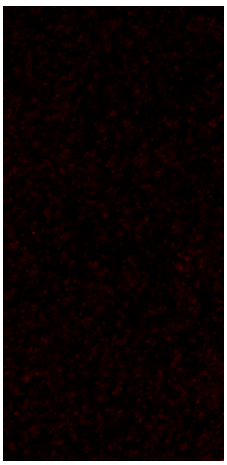
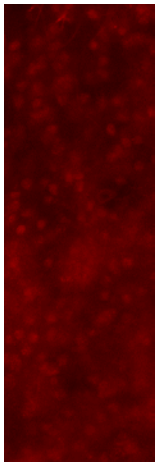
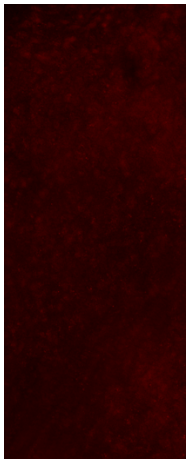
2a.  2b.  2c.  2d. 

Figure 2: Increased brightness in c-Fos signal and visualization of cells with a 1:150 primary antibody concentration (*2b*) compared to a 1:200 concentration (*2a*) in tissue from one animal. Images *2a* and *2b* were obtained from the red labeled mouse in set 1 (DOB: 1/23/18). Images *2c* (1:200) and *2d* (1:150) were obtained from the none labeled mouse in set 1 (DOB:1/23/18). Set 1 of mice were *Fmr1* KO mice that were exposed to sound. The above images are of the IC.

Manipulating the order of steps in the c-Fos protocol (Sigma primary antibody):

The next manipulation that was made to the c-Fos staining in an effort to further optimize the protocol was to change the order of the blocking and permeabilization steps. Instead of incubating the sections in the blocking solution first, the sections were first washed with a detergent solution to make the membrane more permeable. The primary antibody concentration of 1:100 was used in this experiment because it seemed to improve the overall quality of the c-Fos staining. Figure 3 shows the difference between the original staining (*3a*), the 1:100 primary antibody concentration staining (*3b*), and the combined 1:100 primary antibody concentration with the order of steps manipulation (*3c*). These staining experiments illustrate that permeabilizing the membranes of the cells before blocking the non-specific sites of the cells did not drastically improve the c-Fos staining. Further optimization was tested in order to reduce the background signal and to be able to perform cell counts

Figure 3:

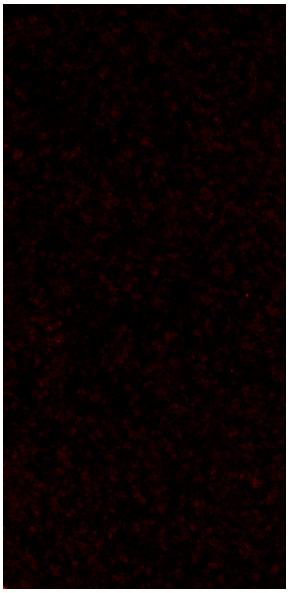
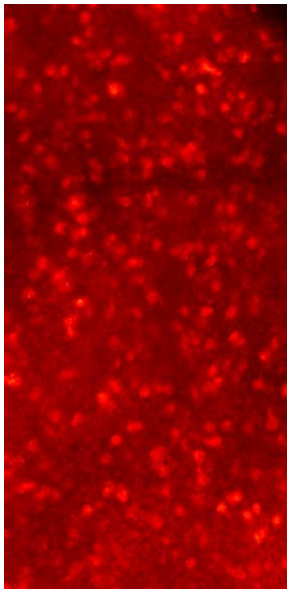
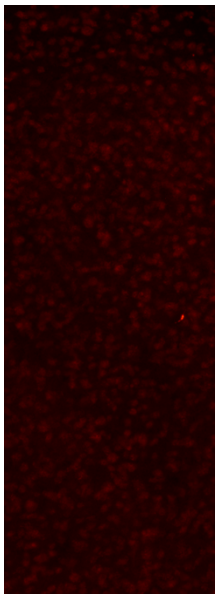
3a.  3b.  3c. 

Figure 3: *3a* shows the original 1:200 c-Fos staining in the IC. *3b* shows the 1:100 c-Fos staining in the IC. *3c* shows the manipulation of the steps combined with the 1:100 c-Fos staining in the IC. Images *3a*, *3b*, and *3c* were obtained from the red labeled mouse in set 1 (*Fmr1* KO, exposed to sound, DOB: 1/23/18).

Addition of 0.1% Tween-20 and BSA to the blocking solution (Sigma primary c-Fos antibody):

In an effort to permeabilize the membranes of the cells in the brain tissue and improve the effect of blocking the non-specific binding sites in the cells, 0.1% Tween-20 was added as a supplement to the blocking solution. The comparison between images *4b* and *4c* show that adding 0.1% Tween-20 to the blocking solution did not further improve the c-Fos staining. Similarly, BSA, another type of reagent that blocks the non-specific binding sites of the c-Fos primary antibody, was added to the blocking solution in the effort to reduce the background signal from the section images. Introducing BSA to the c-Fos staining did not lead to remarkable improvements in the quality of the images as depicted in images *4b* and *4d*.

Figure 4:

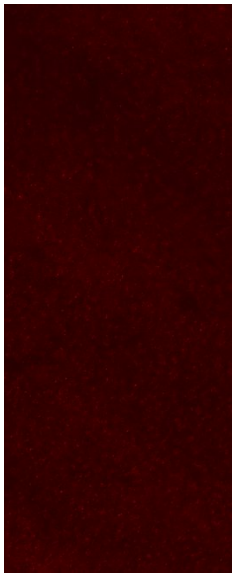
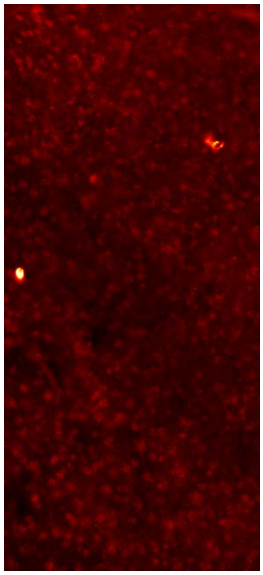
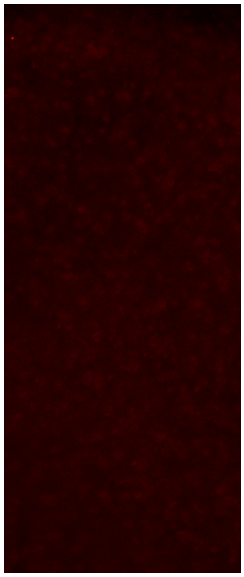
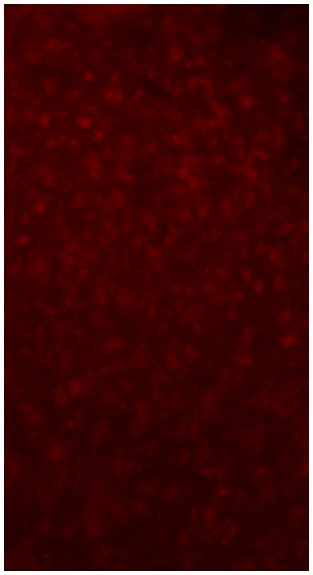
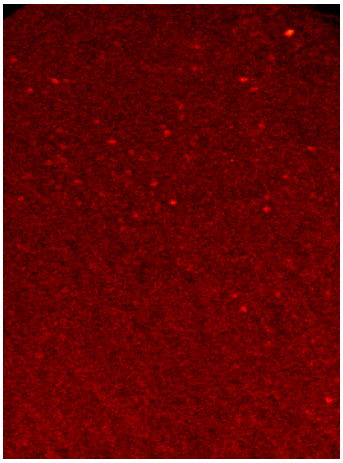
4a.  *4b.*  *4c.*  *4d.* 

Figure 4: Images *4a-4d* are all sections that belong to the none labeled mouse from set 1 (*Fmr1* KO, sound exposed, DOB:1/23/18). *4a* shows the 1:200 c-Fos staining of the IC. *4b* shows the 1:100 c-Fos staining of the IC. *4c* shows the addition of 0.1% Tween-20 combined with the 1:100 primary antibody concentration staining of the IC. *4d* shows the addition of 1% BSA combined with the 1:100 primary antibody concentration staining of the IC. *4c* and *4d* do not show improvements in the contrast of background to c-Fos signal compared to *4b*.

Addition of antigen retrieval to the c-Fos staining protocol (Synaptic Systems primary antibody):

The second pass of experiments performed in 3 sets of mice were stained with a different primary antibody than the one previously tested for optimization of the protocol. The sample c-Fos primary antibody from Synaptic Systems was tested with the original protocol and the addition of the antigen retrieval process to collect quantifiable images. The resulting images suggest that antigen retrieval seemed to improve the c-Fos staining within the tissue of sound exposed animals which is more apparent with the comparison of images *5a* and *5b*. Further experiments may be done to manipulate the antigen retrieval protocol in an effort to make the added process more efficient and effective.

5a.  5b. 

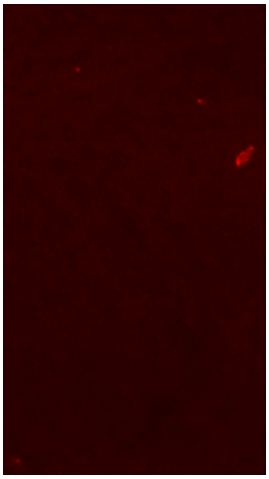
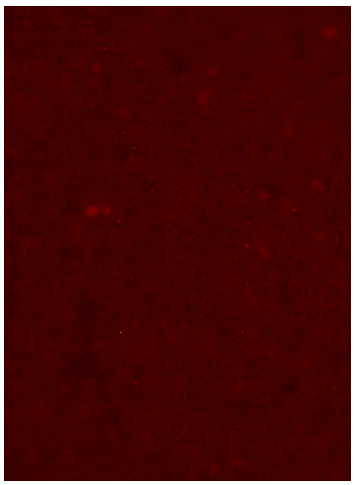
5c.  5d. 

Figure 5:

Addition of antigen retrieval in the c-Fos staining protocol performed in sound exposed mice of C57 background. Images *5a* and *5b* are taken from sections that belong to the orange labeled mouse from set 2 (sound exposed, DOB: 1/21/19). *5a* shows the c-Fos staining of the IC without antigen retrieval. *5b* shows the c-Fos staining of the IC with antigen retrieval. *5c* and *5d* are taken from sections that belong to the blue labeled mouse from set 2 (WT, sound exposed, DOB: 1/21/19). *5c* shows the c-Fos staining of the IC without antigen retrieval. *5d* shows the c-Fos staining of the IC with antigen retrieval.

Discussion:

As an undergraduate researcher, performing the sound exposure experiments, collecting mice brain tissue, and optimizing the c-Fos immunohistochemistry staining has led to my understanding of the process to quantify cellular differences in the brain among animals of wildtype and *Fmr1* KO genotypes. Furthermore, I have been able to work through the challenges of c-Fos staining that became apparent during the collection of c-Fos staining in the auditory cortex, medial geniculate body of the thalamus, and inferior colliculus. The c-Fos gene product is synthesized upon immediate early gene transcription of the c-Fos gene making c-Fos protein expression time sensitive. This means that animal perfusions and brain extractions must be performed in an adequate amount of time after sound exposure in order to collect c-Fos in the brain tissue. Additionally, immunohistochemistry is a multi-step process consisting of many concentration dependent reagents and time sensitive steps which are essential to prepare the brain tissue for antibody binding and to clearly visualize the c-Fos stained cells. As an undergraduate researcher, I performed an array of experiments manipulating the c-Fos staining protocol in order to record and evaluate the effectiveness of each of its steps. Throughout the staining process, it became clear that reagents such as the primary antibody that binds to the c-Fos antigen and the fluorescently tagged secondary antibody that binds to the primary antibody are sensitive to changes in their concentration. Also, depending on the antibody manufacturer, different concentrations may be more useful than others. In my studies using brain tissue from C57 mice, the c-Fos anti rabbit primary antibody from Sigma worked more effectively at a concentration of 1:100 (1%) in 0.1 M PBS whereas the c-Fos anti rabbit primary antibody from Synaptic Systems worked at a concentration of 1:200 (0.5%) in 0.1 M PBS. I found that it is critical to test concentrations of the primary antibody to ensure that the c-Fos antigens are

captured and visualized. The effectiveness of the blocking reagent is also important to consider during immunohistochemistry due to the possibility of non-specific antibody binding to occur within the cells. Throughout my studies, I found that the combination of Bovine Serum Albumin and Normal Goat Serum, two different types of blocking reagents, did not drastically improve the quality of the c-Fos staining. In an effort to decrease the amount of non-specific background signal, future experiments may be performed to test the effectiveness of Normal Donkey Serum compared to other blocking reagents with the use of the donkey anti rabbit secondary antibody in this protocol. The incorporation of antigen retrieval seemed to increase the effectiveness of using antibodies to detect and visualize c-Fos protein expression as a marker for neuronal activation. Future c-Fos staining experiments may include the antigen retrieval process into the protocol depending on the effectiveness and efficiency of the multi-step procedure. During the second pass of experiments, I determined that the Synaptic Systems primary antibody worked more effectively at a 1:200 concentration with and without antigen retrieval which is the protocol that I followed to collect preliminary cell counts. Although I was able to quantify c-Fos for some images, the process of cell counting was more valuable for my understanding of the data collection process rather than conclusive results. Nonetheless, it is apparent that there are many vital components to the success of immunohistochemistry like the preparation of primary antibody, permeabilization of the cell membrane, and blockage of non-specific binding sites within the tissue which were illustrated in my c-Fos staining experiments.

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