SEROTONIN RECEPTOR MODULATION AS A POSSIBLE TREATMENT FOR AUDITORY HYPERSENSITIVITY IN AN AUTISM SPECTRUM DISORDER

By

Ayden Grace Osorio

A capstone project submitted for Graduation with University Honors

05/10/2024

University Honors

University of California, Riverside

APPROVED

Dr. Khaleel Abdulrazak

Department of Psychology

Dr. Richard Cardullo, Howard H Hays Jr. Chair

University Honors

ABSTRACT

Fragile X syndrome (FXS) is a neurodevelopmental disorder and the leading known genetic cause of autism, resulting from the mutation of the *Fmr1* gene which leads to a lack of production of Fragile X Messenger Ribonucleoprotein (FMRP), a key protein in neuronal development and maintenance. This lack of FMRP results in cognitive deficiencies and sensory processing issues, notably auditory sensitivity which, in its most robust presentation, can induce seizures upon exposure to intense stimulus: Audiogenic seizures (AGS). Previous studies in *Fmr1* knock-out mice demonstrated that administering NLX-101–a postsynaptic serotonin 1A ($5HT_{1A}$) agonist reduced AGS. To determine if the specificity of NLX-101 is necessary for reducing AGS, we tested two drugs that more broadly modulate serotonin activity: 8-OH-DPAT, a less specific serotonin 1A agonist that also acts on presynaptic receptors, and Fluoxetine, a serotonin reuptake inhibitor (SSRI) which broadly and non-specifically increases serotonin activity throughout the brain. Through exposing groups of combined drug-treated and untreated *Fmr1* knockout mice to a loud (100-110 dB) modulating sound capable of triggering AGS in untreated knockout mice, we found no significant differences in the severity of or the latency of onset for AGS between untreated and drug-treated mice in both 8-OH-DPAT and Fluoxetine treatments. Our findings indicate that the unique specificity of NLX-101 in modulating serotonin activity due to only targeting postsynaptic $5\text{-}HT_{1A}$ receptors is necessary to reduce AGS. These results suggest the use of NLX-101, and other specific $5HT_{1A}$ receptor agonists, as a therapeutic avenue to treat sensory hypersensitivity in FXS and other autisms.

ACKNOWLEDGEMENT

I would like to thank Dr. Khaleel Razak for being my mentor. He created a such welcoming lab environment that I never felt too nervous to reach out for help and ask questions. He served as both a source of knowledge and comfort, and as both an instructor and a mentor, he will always have my respect. I would also like to thank the PhD student who helped me learn the necessary lab skills, Xin Tao. I wish the best for her after graduation and know she will do great things. I would also like to thank my family and friends who supported me and had to endure my ramblings about this project. They served as the perfect test audience for my many presentations and gave me confidence in my knowledge and ability.

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Introduction

Neuroethology is a term most would find unfamiliar but would recognize the concept it represents: It is a field of study that seeks to understand the neurological basis of behaviors. Early in the life of the field, studies focused on animals with less complex nervous systems such as that of crayfish or cockroaches. Through such studies, we can expand our understanding of the behaviors of said animals from a neurological perspective. One might question why neurologists who seek an understanding of the human brain would investigate the nervous systems of animals so simple that they lack a centralized brain; however, under appropriate conditions, findings from even the most basic life forms can be translated to other species, including humans. Rather than trying to parse how the complex circuitry of neurons gives rise to the very behaviors and experiences we participate in every day, animals with only a fraction of our neurons can provide knowledge of the kind that the greatest minds could never surmise without it. Understanding of how we, as humans, determine the location of sounds, learn to speak, and undergo long-term neurological changes can be attributed to fundamental studies in animal models; however, this field can be used to not only understand functional nervous systems but dysfunctional ones as well. Through researching disorders of the nervous system in animals, we can investigate the mechanism of the dysfunction and treatments, eventually translating our findings from bench table to bedside. We seek to use this avenue to examine possible treatments for the potentially fatal symptoms of one such form of neurological dysfunction: Fragile X Syndrome.

What is Fragile X Syndrome?

Its name deriving from the X chromosome appearing as if a portion is breaking off, Fragile X Syndrome (FXS) is a leading genetic form of Autism Spectrum Disorder (ASD)[1]. As it is a mutation affecting the X chromosome, it is unsurprising that FXS occurs more frequently in the male population (1 in 4000 males) than the female population (1 in 8000 females)[2] as males only need the presence of the mutation in the one X chromosome they typically have compared to females who typically have 2 X chromosomes, permitting a wider range of symptom severity than that seen in males. FXS is caused by an expansion of the CGG trinucleotide in the Fragile X Messenger Ribonucleoprotein 1 gene (*Fmr1*)—previously known as Fragile X Mental Retardation 1 gene[3]—silencing the gene due to the unregulated methylation of the CGG trinucleotide, with the full mutation expressing at 200 repeats of the CGG sequence. As a result of the gene being silenced, the protein encoded by the *Fmr1* gene—Fragile X Messenger Ribonucleoprotein (FMRP)—can no longer be produced. FMRP is known to be vital in the translation of proteins involved in neurological development via regulating RNA stability and the transport of neural mRNA, contributing to synapse formation and plasticity. Thus, when this protein is no longer produced, it has significant impacts on neurological function: Without key synaptic proteins, a person with this disorder will have such clinical characteristics as cognitive deficits in language and mental development, psychiatric dysfunction demonstrating as social anxiety, aggression, and hyperactivity disorders, motor dysfunction in the form of reduced muscle tone (hypotonia) and involuntary muscle contractions (clonus), and other autistic characteristics.[1] Often neglected in the discussion of symptoms, sensory hypersensitivities are another key set of characteristics present in FXS and ASD.

Despite significant research into ASD, the aspect of hypersensitivity is not well understood. Previous research has found that hypersensitivity in sensory processing during early development can give rise to symptoms associated with FXS such as anxiety, abnormal behaviors, and hyperactivity disorders. Later in life, this hypersensitivity can give rise to seizures. In auditory hypersensitivity, the most common form of sensory hypersensitivity, these seizures take the form of audiogenic seizures (AGS) or seizures caused by intense auditory simlulus.[2] AGS in mice can be separated into three stages of growing severity: Wild running and jumping (WRJ), tonic-clonic seizure, and respiratory arrest (RA). Wild running and jumping involves violent running with intermittent jumping in a manner that indicates forced movements characteristic of a seizure. It is believed to be an intense form of the flight reaction, a mechanism for an animal to escape an auditory stimulus intensified to the point of becoming a seizure. Tonic-clonic seizures involve whole-body muscle stiffening and twitching, believed to be an overactive form of the freeze response.[4] Respiratory arrest is the manner of death seen in the most robust expression of AGS, resulting from the overactive neurological activity present in seizures compromising respiratory control. Neurological structures involved in this process are that of escape behaviors, particularly the brainstem. When it comes to WRJ, the main structure associated with it is the inferior colliculus (IC). Extensive firing was observed in the IC just before WRJ occurred, resulting from the hyperexcitability of the auditory system. It is also responsible for innate defense reactions (flight or freeze reaction), further supporting the idea that WRJ is a hyperactivation of the flight response. As for TCS, the periaqueductal gray (PAG), is the major structure relating to this seizure type. A spike of activity is also found in the region before the initiation of seizure activity. The PAG mediates the startle reaction and has been used to evoke the freezing reaction through its activation. A detail about these key structures that is

worthy of mentioning is that they both experience regulation by the neurotransmitter serotonin.[4] Serotonin serves to modulate the activity of the inhibitory neurotransmitter, GABA, which is a key interaction considering the imbalance of excitation and inhibition present in the nervous system of FXS patients could be corrected with changes in serotonin activity.[3] It was found that activating serotonin receptors in the IC reduced sound-induced reactions, specifically increasing the activity of serotonin 1A receptors ($5HT_{1A}$) using agonists–drugs which bind and increase the activity of the receptor it is an agonist of.[5] This implies that auditory hypersensitivity in FXS could be potentially treated through the administration of drugs which modulate $5HT_{1A}$ receptor activity.

Serotonin Modulatory Drugs on Auditory Hypersensitivity

The most abundant and widely expressed of the serotonin receptors, $5HT_{1A}$ receptors are metabotropic receptors, meaning they are G-protein-coupled receptors (GPCR), which use signal cascades to amplify signaling. These receptors are subject to down-regulation upon extensive exposure to serotonin, resulting in a decreased sensitivity to serotonin signaling. This receptor can be divided into two populations by their location in the synapse: The post-synaptic and the pre-synaptic receptors. Post-synaptic receptors act in inhibitory processes, serving to suppress the generation of action potentials in the case of cortical pyramidal cells by acting on the axon hillock. Structures with these receptors tend to be related to mood and emotion, such as the cortex, hippocampus, and amygdala. Alternatively, pre-synaptic receptors are known as inhibitory autoreceptors, meaning they regulate neurotransmitter release by inhibiting the pre-synaptic release of serotonin when an excessive concentration of serotonin is present at the synapse. This negative feedback system serves to regulate the serotonin system, with autoreceptors found in the nucleus responsible for serotonin release: the raphe nucleus.[6] Drugs

that can increase the activity of these receptors are referred to as $5HT_{1A}$ receptor agonists, of which includes the biased agonist NLX-101. As a biased agonist, it is highly specific in where it acts, demonstrating a preference for post-synaptic $5HT_{1A}$ receptors and a low preference for the pre-synaptic $5HT_{1A}$ receptors. This high specificity is one of the main reasons it was tested in reducing auditory sensitivity in FXS, providing the means to narrow down how these receptors impact different regions of the brain when it comes to modulating neuronal activity to reduce auditory sensitivity. In this study, mice with the *Fmr1* gene knocked out, known as *Fmr1* KO mice, were exposed to an auditory stimulus known to cause AGS in untreated KO mice. NLX-101 was found to reduce the frequency and severity of AGS. As implied from the results, NLX-101 could serve as a potential treatment for auditory hypersensitivity, and the specificity of NLX-101 may be significant in reducing hypersensitivity; however, providing further evidence towards this conclusion requires experimentation with less specific serotonin-modulatory drugs.[7]

We seek to determine the necessity of the specificity provided by NLX-101 by determining the efficacy of less specific targeting drugs in reducing AGS. Starting with a drug that is close in the function of NLX-101, 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) is a $5HT_{1A}$ agonist that acts on both the post-synaptic receptors and the pre-synaptic autoreceptors as well as having a minor affinity for $5HT_7$ receptors.[8] Should the specific targeting of post-synaptic receptors prove true, we should expect this drug to have little to no effect. To further investigate the role of these receptors in reducing auditory sensitivity, fluoxetine was also tested. As a selective serotonin reuptake inhibitor (SSRI), fluoxetine, a common antidepressant known more commonly as Prozac, causes a broad and widespread increase in serotonin activity by blocking serotonin reuptake transporters (SERT) and thus delaying the removal of serotonin from the

synapse. By delaying the reuptake mechanism, serotonin activity is allowed to continue for a longer period of time than normally expected.[9] Through testing the efficacy of 8-OH-DPAT and fluoxetine, we hope to determine if the unique specificity provided by NLX-101 in receptor targeting is necessary to treat audiogenic seizures. We hypothesize that this difference is significant; thus, we expect our drug treatments to have a reduced efficacy in treating AGS. Through our study, we hope to expand our understanding of the mechanism underlying AGS in FXS and to provide potential treatment opportunities for those who experience AGS.

Methodology

All procedures were based on previous studies dealing with the acute drug treatment of auditory hypersensitivity in FXS with modifications made to accommodate for the different drug treatments.[7] Procedures have undergone approval from the Institutional Animal Care and Use Committee (IACUC) to ensure ethical research methodology.

Mouse Model

As our animal model, mice were used due to previous research determining mice are an appropriate model to study the auditory system in FXS such that it is translatable to humans.[2] In this study, mice obtained from Jackson Laboratory were bred in-house using FVB breeding pairs. Mice were weaned (offspring are removed from their parents and separated by male and female) at postnatal (P)21 (21 days old), with mice being tested at P21-P23. This age is used due to being a key neuronal developmental period, meaning AGS can be observed during this time period of life. All mice used were male due to being more susceptible to AGS, increasing the probability of AGS occurring in control mice during trials.[7] Considering the goal of this study is to compare the severity of AGS in untreated and drug-treated mice, it is vital that untreated mice

undergo AGS to properly elucidate the effect of the drug treatment on AGS severity. As previously stated, *Fmr1* KO mice are mice with the *Fmr1* gene knocked out to silence the gene. This results in the same neurological condition as seen in FXS. Only *Fmr1* KO mice were used as our study does not focus on differences between FXS mice and wild-type mice (mice who are not KOs). Mice were kept in clear cages with 2-4 in a cage at a time to prevent socially isolating or overcrowding mice. Each cage was divided into a drug treatment group and a control group. This is done so each trial involves mice of each treatment group undergoing the same conditions throughout the experimental procedure, limiting any factors that may result in one of the experimental groups becoming more susceptible to AGS than the other. The identity of each mouse was determined by applying different colors to the fur of the mouse using non-toxic markers. Each color was placed on a different region of the mice, so an individual could be known by the location and color of the identifying mark. After the procedure, if mice survived, they were humanely euthanized using a CO_2 chamber.

Experimental Procedure

The dosages and drug onset period of 8-OH-DPAT and fluoxetine were determined by reviewing literature of studies investigating related systems also dealing with acute dosages of drug treatments to determine effective values for each criterion. 8-OH-DPAT was administered at a dosage of 1.5 mg/kg (milligrams of drug per kilogram of specimen) with a drug onset period of 15 minutes.[10,11] Fluoxetine was administered at a dosage of 25 mg/kg with a drug onset period of 30 minutes.[12] Each drug was dissolved into a saline solution such that the dosage would be 1 ml of drug-treatment solution per kilogram of weight of the specimen or 0.01 milliliter per gram of weight of the specimen (0.01ml/g). This permits the dosage of each mouse to be determined by weighing each with an electronic scale and quickly determining the volume of solution to be

administered. For control mice, the dosage of saline is determined using the same method as described above.

As previously discussed, AGS is a robust form of defensive behavior, so these procedures reflect the need to control the amount of stress experienced by the mice during the procedure so as to not exacerbate AGS susceptibility. There is also the ethical aspect of not wanting to cause unnecessary distress to animal subjects. For each trial, the auditory stimulus is checked and adjusted as necessary to ensure it is at 100-110 dB, then the intensity is recorded. This range is known to be sufficient to induce AGS in untreated KO mice. Then, mice are given an intraperitoneal (IP) injection of either the drug treatment or saline as a control. These injections occur at the lower underbelly of the mouse. Any cases of potential puncture of internal organs or leakage of the treatment from the injection site would be noted; however, it did not occur over the experimental period. Control treatment mice are also given injections to ensure no discrepancies occur due to only drug-treated mice enduring the stress of being handled, increasing the susceptibility of the drug-treated group to seizures. Before injection, each mouse is anesthetized by placing them in a closed container with isoflurane until signs of unconsciousness are present. Mice are then color-labeled and placed into a clean cage which will serve as their cage for the trial. Once all mice are injected, they are left for the drug onset period to ensure the drug treatment has fully taken effect before the induction of the AGS procedure. Then for 5 minutes, mice will be moved to and remain in the attenuation booth where the auditory stimulus for AGS will be presented. This period allows the mice to get used to–or habituate to–the novel environment to ensure that unnecessary stress is not present in mice before the auditory stimulus begins. At this point, a camcorder is started to record the activity of

the mice, and the booth is closed. After the habituation period, the auditory stimulus is started: For 15 minutes, a 100-110 dB siren is played via a speaker placed on top of the cage, modulating from 2-8 kHz. Following this procedure, video and data analysis takes place.

Data Analysis

Video analysis consists of rating the severity and latency of each instance of AGS for each individual. Latency is measured as the duration between the beginning of the auditory stimulus until the beginning of the AGS, being recorded in seconds. Severity was determined using a numerical rating system from 0-5: A score of 0 indicates that no seizure occurred. A score of 1 means that there was only one occurrence of WRJ with no instances of more severe forms of AGS, and a score of 2 means that there were multiple bouts of WRJ. The initiation of WRJ is determined by observing when rapid running is followed by popcorn-like jumping. Mice can also participate in short sprints across the cage that do not qualify as WRJ, so this distinction of jumping is necessary for properly identifying occurrences of seizures. If there was a single occurrence of TCS, it is given a rating of 3, with multiple bouts of TCS getting a score of 4. TCS is identified by the mouse falling to their side or back with its hindlegs extended and ears pinned back to the body. A score of 5 is indicative of RA, the most severe expression of AGS. The point of death is determined by observing the sudden fall of the abdomen indicative of an exhale and the ears relaxing back to their upright position. Each video is analyzed using the color on each mouse to identify individuals as the chaotic and violent movement of mice undergoing seizures can make it difficult to identify individuals after moments of high activity if no clear indicators are present.

Data is analyzed using Prism 9, graphing the survivability of each stage of AGS for the control and drug-treated groups, using the latency of the first occurrence of each stage if multiple bouts occurred. These survival graphs can be used to observe if the treatment is reducing the occurrence of the seizure and if the treatment is delaying the onset of seizures. A violin graph is also used, depicting the distribution of AGS score data for the control and drug-treated groups. This permits us to observe what scores are most common in the treatment and control groups to more accurately compare the difference in seizure severity present in each experimental group.

Results

For the first 4 trials of experimentation, no mice displayed any signs of AGS. Due to the possibility of genetic drift resulting in the loss of the seizure phenotype, these trials were disregarded in the final results. In the study of 8-OH-DPAT (n = 11) and saline (n = 10) treatment, no significant difference was found at any stage of AGS and no significant difference was found between the AGS values of the two treatments (Figure 1). One potential explanation is that the activation of autoreceptors by 8-OH-DPAT resulted in serotonin activity never reaching the threshold of serotonin activity needed to reduce auditory sensitivity due to pre-synaptic receptors decreasing serotonin release

Similarly, in our study of fluoxetine (n = 12) and saline (n = 10) treatment, no significant difference was found at any stage of AGS and between AGS scores (Figure 2). The non-specific nature of fluoxetine action is likely the cause of this result, as it would fail to specifically target post-synaptic receptors, also affecting autoreceptors and synapses lacking $5HT_{1A}$ receptors. This board modulation of serotonin activity could fail to increase serotonin activity by the amount

necessary to reduce auditory sensitivity or it could activate or inhibit regions that would promote AGS rather than reducing it as a result.

Conclusion

8-OH-DPAT and fluoxetine both resulted in no significant differences being observed when compared to the control sample. This supports the claim that the specificity of NLX-101 is necessary for treating auditory sensitivity. Future research can help further clarify the accuracy of this claim in regard to NLX-101 specificity.

Only one dosage of each drug present in this study was used, so it would be informative to test the efficacy of more dosages. Potentially, the dosages used were simply outside of the effective range either because a significantly higher dosage than expected is required to modulate serotonin activity to the degree of reducing auditory hypersensitivity or the dosage was high enough to fall outside of the effective range. Alternatively, our drug treatment was given just before the auditory stimulus procedure, so a chronic drug treatment might be necessary to be effective due to the potential neuromodulatory effects of chronic activation of the relevant serotonin receptors involved in reduced auditory responses. Another aspect which may explain possible issues with the dosage is that different strains of mice are used in different studies referenced when determining dosage. By nature of being different strains, there exists the risk of each strain not having the same effective dosage range for a drug. For example, a study investigating serotonin's role in death from seizures used the same fluoxetine dosage of 15-25 mg/kg, but the mice used were DBA/2 mice. It should be noted that fluoxetine was given after AGS occurred to test for reduction of AGS upon repeated events,[13] but the issue of strain

differences is still worth acknowledging. It would also be beneficial to observe the direct comparison between each drug treatment and NLX-101 to reconfirm the effectiveness of NLX-101 as a treatment and to compare the NLX-101 and saline treatment data to the NLX-101 and drug treatment data. We expect these two results to not have significantly different outcomes; however, if there is found to be a significant difference, then this could indicate our drug treatments may have a partial effect on AGS severity.

Although two potential drug treatments are presented in this study, there are also a range of $5HT_{1A}$ agonists that have differing binding affinities that could elaborate on the significance of specificity by potentially elucidating a uniqueness about NLX-101 that makes it highly effective in treating auditory hypersensitivity in FXS. (S)-5-(2′-fluorophenyl)-N,N-dimethyl-1,2,3,4-tetrahydronaphthalen-2-amine (FPT)–a 5HT1A partial agonist–was found to be a potential treatment for seizures in FXS, eliminating lethal seizures and TCS occurrences.[14] This indicates that high specificity might not be as necessary as demonstrated in our results, necessitating further research into the efficacy of 8-OH-DPAT in reducing auditory sensitivity, especially when noting that $5HT_7$ activity is implicated in alleviating seizure severity, a receptor that 8-OH-DPAT is a partial agonist for.[5, 14]

Although the findings of this paper point to NLX-101 as an effective treatment, it is important to discuss the long-term goals of such research: Ultimately, this research has the goal of understanding the mechanism of AGS and finding effective treatments so those who experience auditory hypersensitivity can have effective treatments available for them. As part of this goal, it

would be most favorable for drugs that are already approved by the FDA to be proven effective. The typical process of drug approval for treatment involves a preclinical study to determine the efficacy of a treatment in animal models, clinical trials in humans to determine the safety of the drug, and a new drug application being approved.[15] This is a long process that ensures the safety of treatments before they are permitted to be used on the population, so it is understandable that a drug that has yet to be scrutinized to ensure its safety would take longer to be available as a treatment than a drug which has already gone through the process of FDA approval for other treatments. The faster a treatment is available for patients, the sooner these symptoms can be alleviated for those who experience them.

Complications that arose during the duration of the study included the reliance on the consistency of breeding pairs of mice having litters. There were several periods of time in which no litters were born, meaning no trials could be conducted for this study. Additionally, this study only used male mice, meaning that of the already limited number of mice to work with for trials, only a portion of these mice would be used, usually with males consisting of a minority of the litter. There was also the risk of genetic drift resulting in a loss of the seizure phenotype, meaning that, regardless of the auditory stimulus, the mouse will not undergo AGS. Although this can be resolved by getting a new breeding pair, it takes time for pairs to breed and have litter. Of course, even if the breeding pairs are having litters, there is still the possibility of fetal death occurring. Overall, time was the core issue due to the conduction of the study relying on the consistency of offspring success in breeding pairs. Future complications that could occur in future experimentation which had not occurred during the experimental period include the risk of puncturing the internal organs of the mice while giving IP injections as well as leakage of the

drug treatment from the puncture hole. If the internal organs are pierced, this should not be problematic when it comes to the mouse dying means besides respiratory arrest cause by AGS, but this would put additional stress on the mouse and increase their seizure susceptibility. If leakage of solution were to occur, then we could not confidently state that the mouse received the proper dosage, so any results risk being invalid.

Figures

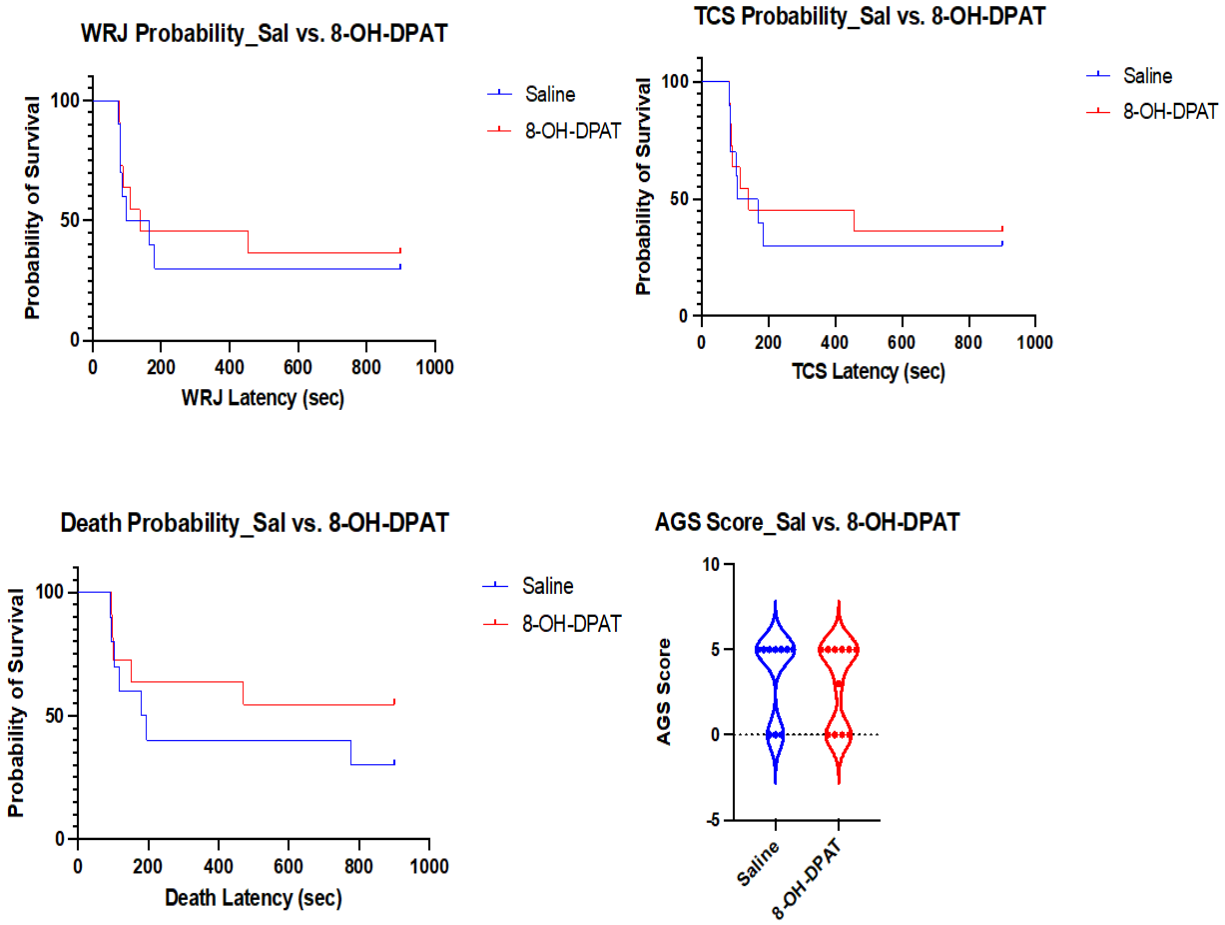

Figure 1 : The graphical analysis of 8-OH-DPAT (n = 11, control n =10) treatment study is presented here. The survival graphs (top left, top right, bottom left) depict the latency to occurrences of each stage of seizure. WRJ data is represented in the top left graph, TCS data in the top right, and RA or death data in the bottom left graph. All are found to have no significant difference. The violin graph (bottom right) depicts the distribution of the AGS scores of the treatment and control groups. No significant difference was found between the AGS scores.

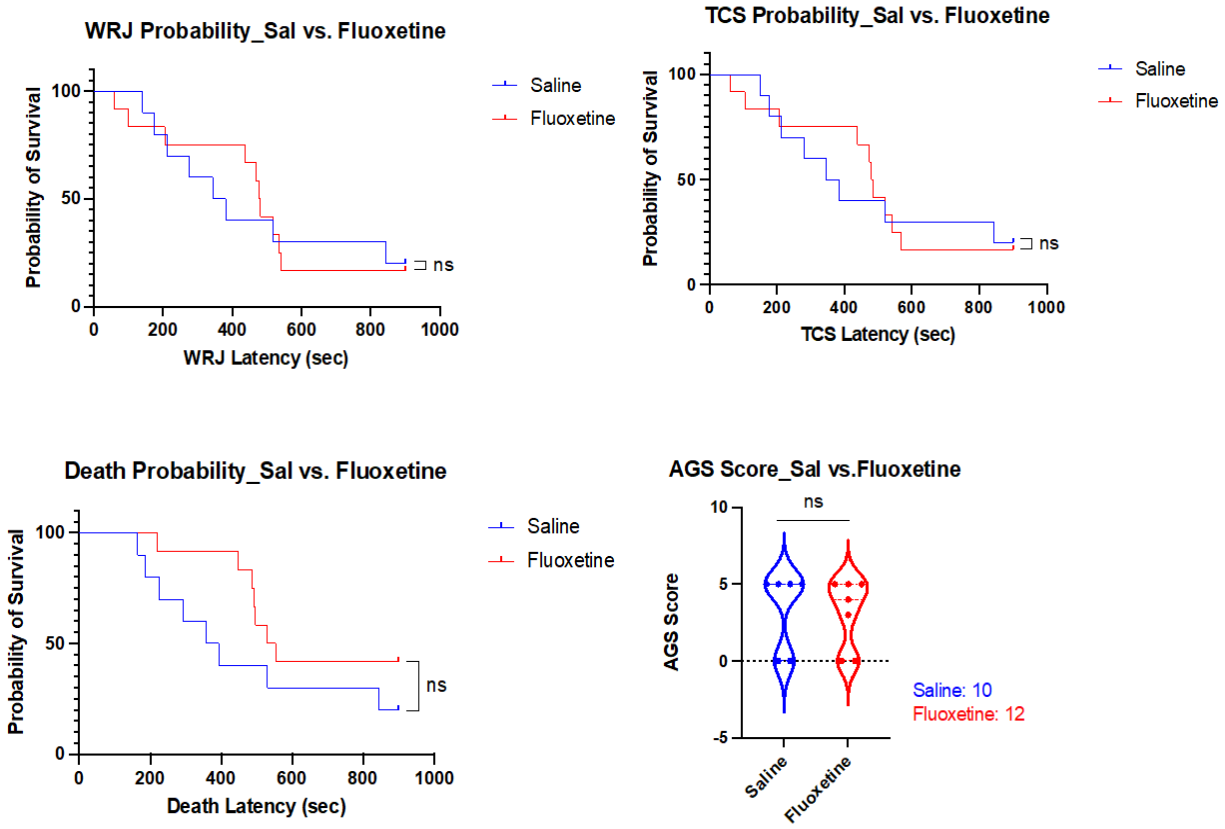

Figure 2 : Figure 1 : The graphical analysis of Fluoxetine (n = 12, control n =10) treatment study is presented here. The survival graphs (top left, top right, bottom left) depict the latency to occurrences of each stage of seizure. WRJ data is represented in the top left graph, TCS data in the top right, and RA or death data in the bottom left graph. The violin graph (bottom right) depicts the distribution of the AGS scores of the treatment and control groups. No significant difference was seen in any of the survival graphs or the violin graph.

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