Role of Antidepressants in Altered Synapse Formation in Autism Spectrum Disorders

by

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Major Department: Engineering

Autism is a genetically complex neurodevelopmental disorder in which patients exhibit social deficits in both verbal and non-verbal forms of communication and display restricted and repetitive behaviors. Approximately 1 in 68 children are diagnosed with Autism in the United States[2]. The prevalence of Autism in North Carolina is even greater where 1 in 58 children are diagnosed[3]. Autism is thought to be influenced by both genetic and environmental factors. Complex interactions between these factors make the creation of therapeutic treatments difficult to achieve. One environmental factor that is being studied in relation to Autism is the anti-depressant Fluoxetine. Fetal exposure to Fluoxetine through maternal ingestion of the drug or consumption of drinking water where the drug is present is thought to interrupt normal fetal brain development[5,6]. Fluoxetine has previously been show to increase dendritic spine formation, the main location of excitatory synapse development[14-17]. However, the exact mechanism that causes this dysregulation of the actin cytoskeleton is not fully understood. Post-mortem samples from individuals with Autism also display increased dendritic spine levels[10]. We hypothesize that Fluoxetine acts through the Rac1 pathway to increase dendritic spine density. To examine the impact of Fluoxetine on fetal synapse formation human cortical organoids, or ‘mini-brains’, were created to recapitulate the second trimester fetal brain. Once the ‘mini-brains’ reached the appropriate time point in development they were treated either acutely with Fluoxetine, chronically

with Fluoxetine, with the Rac1 inhibitor NSC23766 or a combination of Fluoxetine and NSC23766. After 90 days in culture, the ‘mini-brains’ were harvested, fixed, cryosectioned and stained for pre- and post-synaptic markers. Using ImageJ excitatory synapse density and morphology was analyzed. It was determined that Fluoxetine caused enlargement of synapses that were irregular in shape. The effects of Fluoxetine on synapse formation were reduced when combined with the Rac1 inhibitor NSC23766. In addition to examining excitatory synapse formation, the effects of Fluoxetine and NSC23766 on electrical signal transmission was also observed using micro-electrode technology. Both Fluoxetine and NSC23766 were shown to decrease neuronal activity.

Role of Antidepressants in Fetal Synapse Formation in Autism Spectrum Disorders

Thesis

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Master of Science in Biomedical Engineering

by

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List of Abbreviations

5-HT - 5-hydroxytryptamine

ASD – Autism Spectrum Disorder

DMSO - dimethyl sulfoxide

GFP – green fluorescence protein

MEA – multi-electrode array

iPSC – induced pluripotent stem cells

PBS – phosphate buffer saline

PSD-95 – post synaptic density protein 95

SERT - serotonin reuptake transporter

SSRI – selective serotonin reuptake inhibitor

vGLUT-1 - vesicular glutamate transporter

1. Introduction

Autism is a genetically complex neurodevelopmental disorder in which patients exhibit social deficits in both verbal and non-verbal forms of communication and display restricted and repetitive behaviors. Autism is considered a spectrum disorder due to the ranging levels of Autism severity. Each case of Autism is phenotypically and etiologically different[1]. Because of these differences, it is challenging to understand the fundamental genetics and pathophysiology of Autism. Approximately 1 in 68 children are diagnosed with Autism in the United States[2]. The prevalence of Autism in North Carolina is even greater where 1 in 58 children are diagnosed[3]. Autism is thought to be influenced by both genetic and environmental factors. Complex interactions between these factors make the creation of therapeutic treatments difficult to achieve. One environmental factor that is being studied in relation to Autism is the anti-depressant Fluoxetine. Fluoxetine is a selective serotonin reuptake inhibitor (SSRI). During normal synaptic transmission, neurotransmitters, such as serotonin, are released by synaptic vesicles from the axon terminal located in the pre-synaptic neuron. The neurotransmitters then pass through the synaptic cleft to ligand-gated channels which have receptors for specific neurotransmitters. The class of receptors pertaining to serotonin are referred to as 5-hydroxytryptamine (5-HT) receptors. These metabotropic receptors are coupled with specific G-proteins. Once serotonin binds to a 5-HT receptor the corresponding G-protein is activated. The activation of the G-protein causes the induction of a signal cascade specific to the type of 5-HT receptor. For example, activation of 5-HT_{2A} receptors drives excitatory network activity[4.] Typically, after serotonin activates a receptor it is then taken back up by the pre-synaptic neuron to be reused for another synaptic event. Fluoxetine prevents the re-uptake of the neurotransmitter serotonin by blocking the serotonin reuptake transporter (SERT) on the presynaptic neuron (Figure 1).

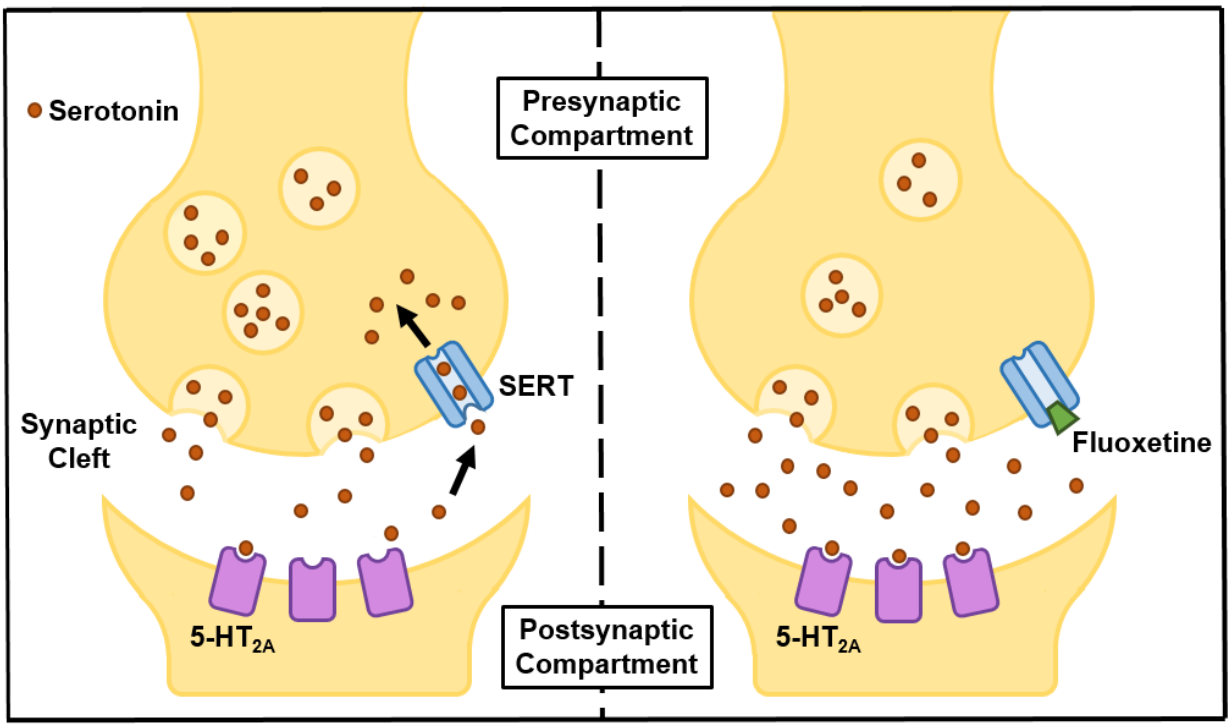

Figure 1. Fluoxetine mechanism of action. Fluoxetine blocks the serotonin reuptake transporter (SERT), causing increased levels of serotonin in the synaptic cleft. The increased amount of serotonin subsequently causes increased activation of serotonin (5-HT) receptors.

By preventing the reuptake of serotonin, Fluoxetine causes increased activation of serotonin receptors. Fetal exposure to Fluoxetine through maternal ingestion of the drug or consumption of drinking water where the drug is present is thought to interrupt normal fetal brain development[5,6]. The specific interactions of Fluoxetine with Autism-associated genetic pathways is currently not completely understood.

One of the main molecular pathways that is disrupted by copy number variations and mutations associated with Autism are actin regulatory pathways[7,8]. The neuronal actin cytoskeleton is responsible for regulating multiple processes critical to brain development,

including neuronal migration, neurite extension and synapse formation and refinement[9]. In Autism, synaptic refinement is disrupted resulting in increased levels of excitatory synapses. These increased levels have previously been observed in post-mortem patient samples from individuals with idiopathic Autism[10]. In the human brain, most excitatory synapses form on dendritic spines. Dendrites are long, highly branched protrusions that extend from the neuronal cell body. Dendritic spines are small membranous protrusions located on the dendritic shaft. These spines emerge from the dendritic shaft as filopodial structures and become mature once they come into contact with a pre-synaptic compartment. This stabilization is the result of interactions between adhesion and scaffolding proteins. Loss of pre-synaptic input subsequently causes loss of stabilization, leading to simplification of the spine structure[11]. Dendritic spines are composed of F-actin, which allows for activity-dependent remodeling[12]. Throughout the human lifespan the number of dendritic spines present in the human brain changes. This structural plasticity allows the brain to remodel the neural connectivity underlying cognitive function, such as learning and memory formation. Figure 2 displays the change in dendritic spine number throughout human lifespan and alterations associated with neuropsychiatric disorders[13].

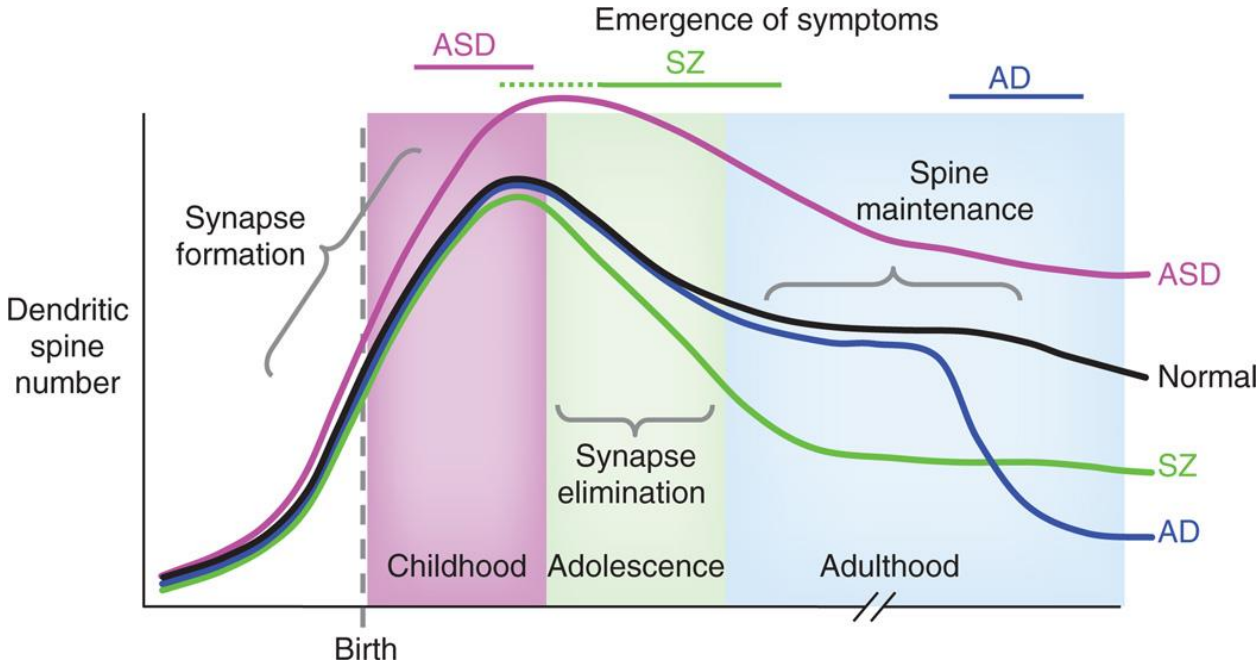

Figure 2. (Copied from Penzes et al.[13]) Trajectory of dendritic spine number throughout human lifespan in individuals with Autism, schizophrenia and Alzheimer's. Increased levels of dendritic spines are observed in individuals with Autism Spectrum Disorder (ASD). Incomplete spine pruning or exaggerated spine formation during childhood is thought to contribute to increase dendritic spine levels in individuals with ASD[13].

In normal individuals, spine number increases before and after birth. During this time synapse formation occurs. Spines that are not actively contributing to synaptic transmission are typically pruned away during later childhood and adolescence. The brain strategically removes synapses that are no longer used by the individual to establish adulthood dendritic spine levels. During childhood the symptoms of Autism usually emerge. Post-mortem brain samples from children with Autism Spectrum Disorder (ASD) display increased levels of dendritic spines[13]. The increased levels of dendritic spines could potentially be attributed to exaggerated spine formation, incomplete pruning of inactive spines during childhood or a combination of both factors[13]. Like Autism, Fluoxetine also increases dendritic spine density[14-17]. While the exact mechanism by

which Fluoxetine increases spine density is unknown, the Rho-GTPase Rac1 may play a role in this process. Following the activation of 5-HT_{2A} serotonin receptors, Rac1 has been shown to initiate dendritic spine enlargement[18]. In addition to altering dendritic spine density, SSRIs, like Fluoxetine, have previously been shown to disrupt normal cognitive function in rodents. Once exposed to a SSRI, the rodents expressed deficient social behaviors characteristic of Autism[19]. Fluoxetine has also been shown to increase fetal serotonin blood levels[20]. Similarly, increased serotonin levels have been observed in individuals with Autism[21,22]. The combination of this evidence suggests that Fluoxetine can impact fetal brain development, possibly leading to Autism. To determine the effects of Fluoxetine on human prenatal synapse formation and function, the anti-depressant needs to be tested in a physiological relevant model. Human cortical organoids, or 'mini-brains', offer a patient specific model that recapitulates the second trimester fetal brain[23]. To better understand the mechanisms by which Fluoxetine disrupts normal dendritic spine formation and also alters cortical network function, the anti-depressant can be tested using human-derived cortical organoids.

2. Background

2.1 Modeling Autism using Human Cortical Organoids

Environmental and genetic factors are thought to work in concert to contribute to Autism pathology. The complex interactions between these factors can be difficult to examine, especially since each case of Autism varies genetically. According to the Simons Foundation Autism Research Initiative, there are currently 881 monogenetic mutations and 2,170 genetic copy number variations that are linked to Autism[24-25]. One way to account for the genetic heterogeneity associated with Autism is to use patient-specific models. Organoids are three-dimensional, patient-specific models that are generated from human induced pluripotent stem cells (iPSCs). To study neuronal development, organoids can be produced by terminally differentiating patient stem cells into neurons. Cortical organoids or 'mini-brains' are currently being used in the Litwa lab to recapitulate fetal brain development and examine brain circuitry associated with Autism. The transcriptomes associated with the 'mini-brains' are like those of the second trimester fetal brain[23]. The 'mini-brains' display similar features to the *in vivo* cerebral cortex, including neural progenitor cells, both excitatory and inhibitory neurons, and supporting glial cells. Preliminary data from the Autism-derived 'mini-brains' show increased area of the excitatory pre-synaptic marker VGLUT-1. Figure 3 displays images of control and Autism-derived 'mini-brains' that are immunostained with the VGLUT-1 marker.

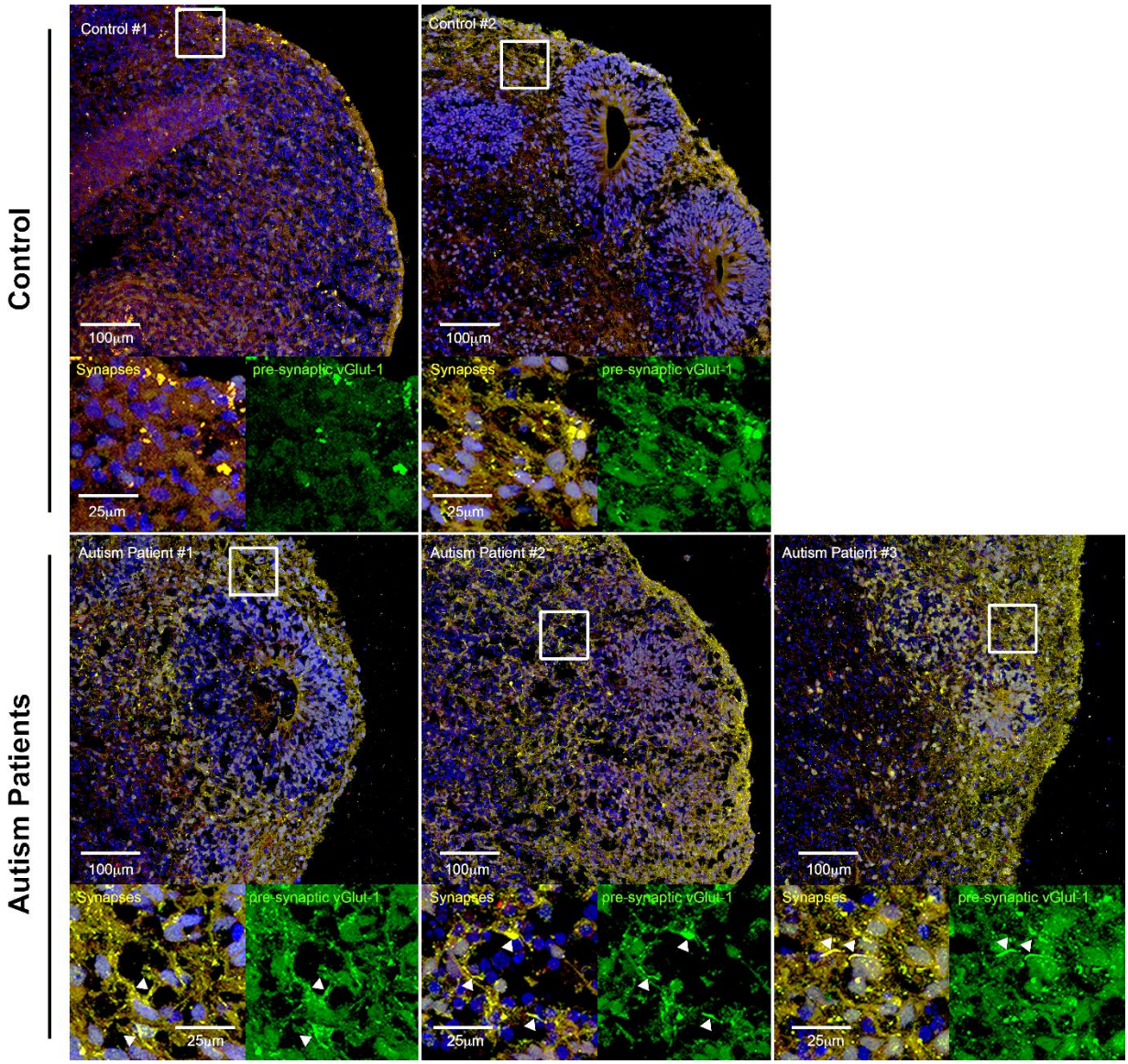

Figure 3: Florescent microscopy images of control and Autism-derived ‘mini-brains’ immunostained with markers for nuclei (blue), excitatory pre-synaptic marker (green) and synapses (yellow).

Credit: Karen Litwa. This figure has not been published.

These images were evaluated to determine the area of the excitatory pre-synaptic marker VGLUT-1 normalized to nuclei. Figure 4 displays the VGLUT-1 areas determined from neurotypic and Autism-derived ‘mini-brains’.

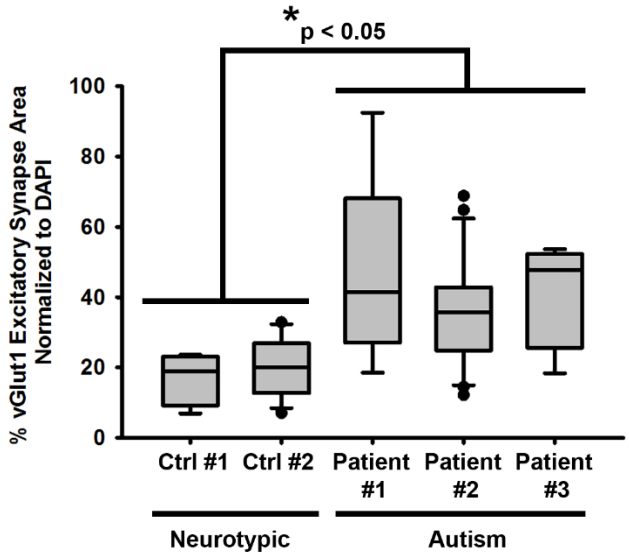

Figure 4: Pre-synaptic VGLUT-1 area normalized to DAPI (nuclei) in neurotypic versus Autism-derived ‘mini-brains’.

Credit: Karen Litwa and Litwa Lab Members, Haroon Dar, Storm Davis, and Adrienne Orbita. This figure has not been published.

A significant increase in VGLUT-1 area was observed in the Autism-derived ‘mini-brains’. In addition to using the ‘mini-brain’ model to study representative areas of synaptic markers, this model can also be used to determine the effects of various environmental factors, such as Fluoxetine, on synapse formation and neuronal electrical signaling.

2.2 Role of Rac1 in Actin Cytoskeleton Regulation

As previously mentioned, the actin cytoskeleton regulates crucial processes in brain development, including the migration of neurons, neurite extension and synapse development and refinement[9]. Actin structures are present in multiple places across excitatory pyramidal neurons (Figure 5).

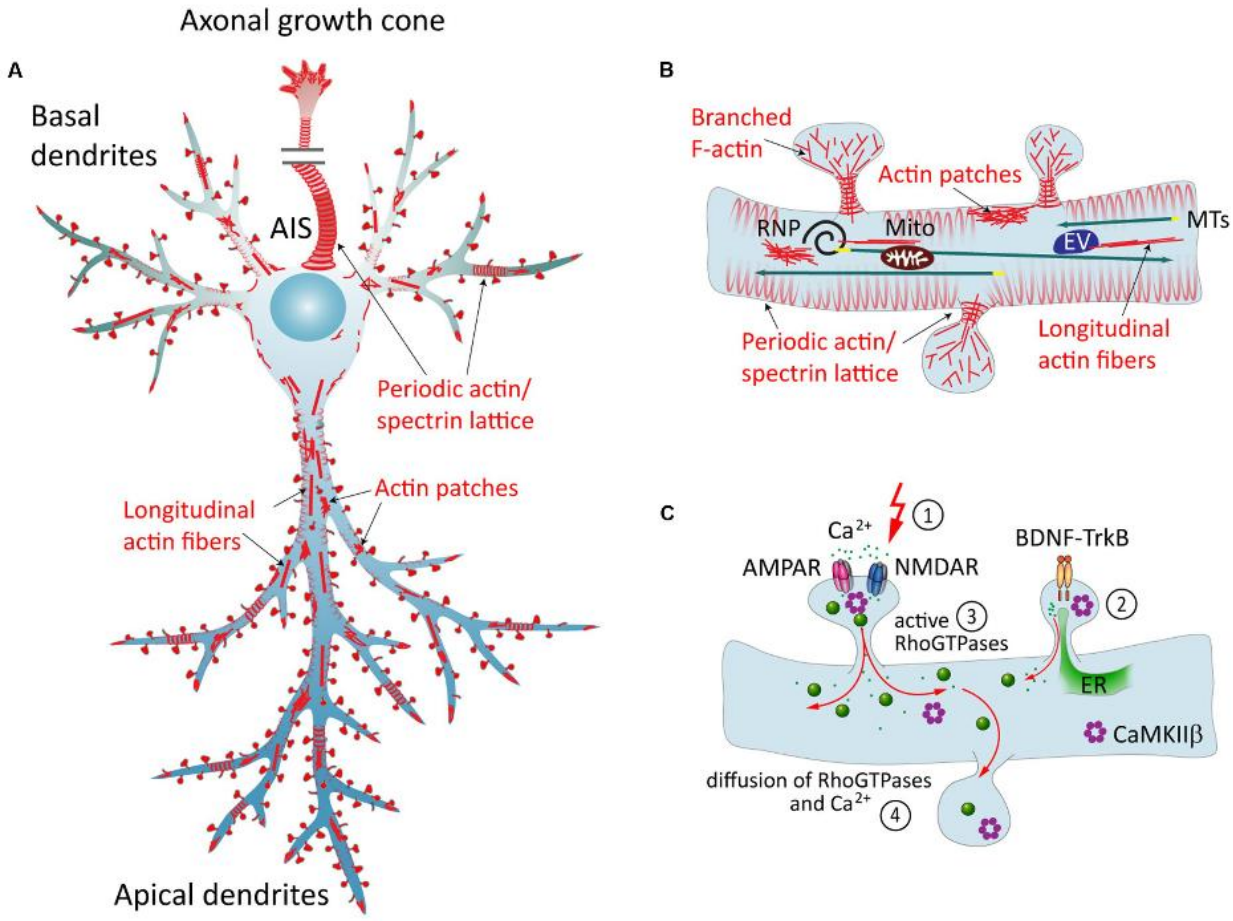

Figure 5: (Copied from Konietzny, Bär and Mikhaylova) Neuronal actin cytoskeleton. (A) Summary of various actin structures found in pyramidal neurons. (B) Actin structures in dendritic spines. (C) External cues that regulate actin cytoskeleton dynamics[12].

Actin is present in both the axonal and dendritic portions of pyramidal neurons. Regulation of the actin cytoskeleton is a dynamic process. Dendritic spine morphology is thought to play a key role in synaptic plasticity[26]. Branched F-actin is located within dendritic spines and influences spine head shape and size (Figure 4B). The shape and size of a dendritic spine head is important, because it affects the number of postsynaptic receptors present on the spine surface. Spines that are larger in size are expected to have more postsynaptic receptors than spines that are smaller in size[23]. Previously it has been determined that the morphology of dendritic spines is altered after long-term potentiation (LTP) occurs[26]. LTP refers to the persistent interactions between neurons that leads to synapse strengthening. LTP causes the synapse between the neurons to become stronger by inducing morphological changes of the dendritic spine. Spine morphology changes resulting from LTP include; enlargement of the dendritic spine head and shortening of the spine neck[26]. In addition to strengthening of the current dendritic spines, spinogenesis can also occur leading to the generation of new spines.

The dynamics of the neuronal actin cytoskeleton are regulated by factors including; signals transduced from cell surface receptors that are coupled with Rho-GTPases[12]. Rac1 is a small GTPase of the Rho family that contributes to dendritic spine morphogenesis by regulating actin. Rac1 promotes actin polymerization in its active GTP-bound form. Inhibition of Rac1 has previously been shown to decrease dendritic spine head growth and reduce the number of spines[27]. Rac1 can be activated via activation of 5-HT_{2A} serotonin receptors, resulting in spine enlargement[18]. It may be possible that the anti-depressant Fluoxetine increases dendritic spine density by causing the activation of the Rac1 pathway. Dysregulation of Rac1 is thought to lead to mental retardation, abnormal synaptic plasticity and unusual spine morphology [28]. Rac1 is currently being studied in relation to many cognitive disabilities, including Autism, schizophrenia,

Down syndrome, fragile X syndrome, epilepsy, Rett syndrome and Tuberous sclerosis[28]. While therapeutic agents exist to treat behaviors associated with some cognitive disabilities, very few therapeutic agents addressing dysfunctional synapse formation exist. Therapeutic agents that target Rac1 signaling pathways could potentially be used to correct abnormal synapse formation[25]. NSC23766 is a chemical compound that inhibits Rac1 signaling. NSC23766 can be used to determine whether Fluoxetine acts through the Rac1 pathway to regulate excitatory synapse formation and function.

2.3 Electrophysiological Effects of Fluoxetine

In addition to impacting synapse morphology, Fluoxetine also affects synaptic transmission. One way to evaluate the electrical events occurring at the synaptic compartment is to record the resulting field potentials produced from the neuronal signaling. These recordings can then be evaluated for patterns relating to specific physiological conditions. Field potentials are extra-cellular voltage recordings that are composed of the electrical contributions of multiple neurons resulting from action potentials and ion fluctuations. The field potential is usually determined by measuring electrical activity of a specific area of neurons with respect to a reference point[29]. One method that can be used to evaluate neuronal electrophysiological activity is to utilize micro electrode array (MEA) technology. MEA technology incorporates the growth of excitable cells directly on recording electrodes. Figure 6 displays a representative sample of neurons on an MEA chip and the resulting recording measured from one electrode.

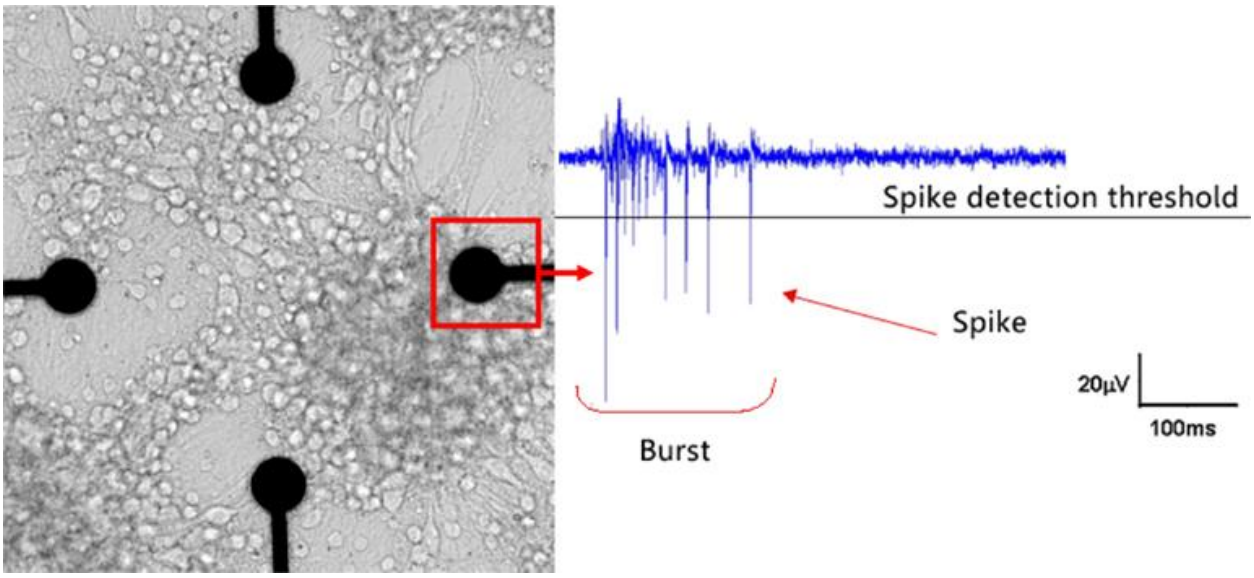

Figure 6: (Copied from Novellino et al) Rat cortical neurons on an MEA chip (left) and resulting recording from one electrode (right)[30].

During the recording process, certain standards are implemented to detect "spikes" of activity. A spike is an increase in electrical activity above a set threshold. This threshold is usually set to 6 times the standard deviation of average electrical activity to prevent the detection of noise as a spike. Regarding neuronal activity, a spike represents an action potential. Multiple spikes within a certain period of time are considered a "burst". A burst is composed of at least five spikes that are separated by an inter-spike interval (ISI) of less than 100 ms[31]. Previously, the toxicity of Fluoxetine has been tested in rat cortical neurons using MEA technology and it was determined that the number of spikes and bursts decreased from control levels once the cells were exposed to the anti-depressant[30]. When exposed to 10 to 20 μM Fluoxetine rat cortical neurons experience complete inhibition. A similar study examined the acute effects of Fluoxetine on cortical murine neuron signaling in different regions of the cortex. It was determined Fluoxetine terminates

electrical activity in both the frontal and auditory cortex. However, rescue of electrical activity was possible[32].

The main two serotonin receptors present in the pre-frontal cortex are the 5-HT_{1A} and 5-HT_{2A} receptors. These receptors play a role in neuronal signaling inhibition (5-HT_{1A}) and excitation (5-HT_{2A}). Serotonin has is thought to have a higher affinity for 5-HT_{1A} receptors than 5-HT_{2A} receptors[33]. Fluoxetine is an anti-depressant that prevents the reuptake of serotonin by the pre-synaptic compartment. The exact down-stream mechanisms through which Fluoxetine treats depressive symptoms is currently unknown. It is hypothesized that combined excitatory and inhibitory responses occur in the same microcircuits in the presence of Fluoxetine. Typically in mouse models, increased serotonin resulting from Fluoxetine treatment causes inhibitory responses mediated by 5-HT_{1A} receptors. This explains why complete inhibition was observed in MEA recordings from mouse and rat cortical neurons. Based on current research, the electrophysiological effects of Fluoxetine have been primarily examined in rat cortical neurons. Further research needs to be completed using human-derived neurons.

3. Specific Aims

The main goal of this project is to examine how Fluoxetine impacts human fetal brain development through the regulation of the actin cytoskeleton. Currently the effects of Fluoxetine on human prenatal synapse formation is not known. To study the consequences of the anti-depressant on neuronal development, cortical organoid models will be used to recapitulate the second trimester fetal brain[23]. These organoids are composed of human iPSCs endogenously tagged with actin green fluorescence protein (GFP). The actin GFP marker allows for visualization of the actin cytoskeleton so the structural impacts of Fluoxetine can be observed. The cells used throughout this project are referred to as ActBmEGFP cells. These cells were obtained under Material Transfer Agreement (MTA) from the Coriell Institute and developed by the Allen Institute for Cell Science.

3.1 Evaluation of Excitatory Synapse Density and Shape in Fluoxetine Treated 'Mini-Brains'

Specific Aim #1-A: Test the hypothesis that Fluoxetine increases excitatory synapses formation in early human cortical development.

The goal of this aim is to determine the effects of Fluoxetine on excitatory synapse formation in human fetal brain development. Throughout the course of this project four treatment groups will be examined, including chronic Fluoxetine (final concentration 1.5 μg/ml), acute Fluoxetine (final concentration 1.5 μg/ml), acute NSC23766 (final concentration 10 μM), and acute Fluoxetine (final concentration 1.5 μg/ml) treatment combined with NSC23766 (final concentration 10 μM). Once the 'mini-brains' have reached the appropriate point in fetal development (second trimester/cultured for 90 days), they will be fixed, cryosectioned, stained

with excitatory synaptic markers and imaged using fluorescence confocal microscopy. Fluoxetine has previously been shown in mouse models to increase dendritic spine density, the main site of excitatory synapse formation. Therefore, it is hypothesized that 'mini-brains' treated with Fluoxetine will display increased levels of excitatory synapses compared to the control.

Fetal exposure to Fluoxetine can either be chronic, from the mother taking the antidepressant, or acute, from short-term exposure through the environment. Both conditions will be examined in this experiment. While it is anticipated that the number of excitatory synapses will increase for the 'mini-brains' treated acutely with Fluoxetine, it is anticipated that the chronically treated 'mini-brains' will display less excitatory synapses. Chronic Fluoxetine was previously administered to rats to determine the effect of the drug on synaptic plasticity. It was determined that the chronic Fluoxetine treatment prevented the enhancement of LTP in neurons from the auditory cortex[34]. In acute Fluoxetine treated 'mini-brain' groups it would be expected that LTP enhances synapse formation since the drug has previously been shown to increased spine formation and head size. In contrast, the frequent doses of the chronic Fluoxetine would most likely cause the cells to become desensitized towards the drug, leading to a new baseline level of synapse plasticity and formation.

Specific Aim #1-B: Test the hypothesis that the Rac1 inhibitor NSC23766 decreases excitatory synapse formation in early cortical development.

Rac1 has been previously shown to increase dendritic spine density[18]. The drug NSC23766 is a Rac1 inhibitor that will be used to treat 'mini-brains' in this experiment. NSC23766 has been

shown to decrease the number of dendritic spines and spine head size. It is hypothesized that the NSC23766 treated ‘mini-brains’ will display decreased excitatory synapse size and density.

Specific Aim #1-C: Test the hypothesis that Fluoxetine acts through the Rac1 pathway to increase excitatory synapse formation in early cortical development.

To determine whether Fluoxetine acts through the Rac1 pathway, Fluoxetine and NSC23766 will be used in combination to treat one of the groups of the ‘mini-brains’. If Fluoxetine does act through the Rac1 pathway it is expected that the NSC23766 drug will prevent increased excitatory synapse formation from occurring in the ‘mini-brains’ treated with both drugs. If Fluoxetine does not act through the Rac1 pathway, the Rac1 inhibitor NSC23766 will not reduce excitatory synapse formation.

After each group of ‘mini-brains’ have been imaged, co-localized pre- and post-synaptic markers will be used to determine excitatory synapse formation. The resulting synapses will then be analyzed using specific synapse shape descriptors including; size, perimeter, circularity and axis ratio. These descriptors will provide information that can be used to characterize the resulting synapse formation in the ‘mini-brains’. Excitatory synapses are typically formed with dendritic spines and the shape of these spines can impact signal transmission. Evaluation of the shape descriptors will provide insight into the morphology of the dendritic spines.

3.2 Evaluation of the Effects of Fluoxetine on Neuronal Electrical Activity.

Specific Aim #2: Test the hypothesis that Fluoxetine will transiently increase excitatory neurotransmission.

The second goal of this project is to evaluate the electrophysiological effects of Fluoxetine on synaptic transmission. To examine the electrical signaling of second trimester 'mini-brains', the cortical organoids will be dissociated and then plated on 24-well MEA plates. After two weeks in culture, the mature neurons will then be tested using the Maestro Edge by Axion BioSystems. Prior to drug treatment, a baseline recording of the cells will be taken. The neurons will then be treated with the control solvent dimethyl sulfoxide (DMSO) or one of three concentrations of Fluoxetine (final concentrations; 0.5 μg/ml, 1.0 μg/ml or 1.5 μg/ml). The resulting changes in electrical activity will be recorded. Previously, the effects of Fluoxetine on electrical activity has been examined in rat and mouse cortical neurons use MEA technology[30-31]. In both experiments inhibition of electrical activity was observed. The inhibition was thought to be mediated by 5-HT_{1A} receptors. The primary receptor found in the human 'mini-brains' that will be used for this project are 5-HT_{2A} receptors (RNAseq performed by Hudson Alpha). Initially, it would seem since 5-HT_{2A} receptors are the most prevalent in this model that neuronal excitation would occur, causing an increase in electrical activity. However, 5-HT_{2A} receptors have also been shown to modulate feed-forward inhibition[33]. Based on this information it is hypothesized that Fluoxetine will cause an initial increase in electrical activity that is followed by inhibition the activity. It is expected that greater concentrations of Fluoxetine will cause a more drastic decrease in electrical signaling. Once the effects of Fluoxetine on electrical activity have been examined, the effects of the Rac1 inhibitor NSC23766 will then be observed. In this portion of the experiment the neurons will be treated with either the solvent dimethyl sulfoxide (DMSO), NSC23766 (final

concentrations; 5 μM or 10 μM) or a combination of Fluoxetine (final concentrations; 0.5 μg/ml, 1.0 μg/ml or 1.5 μg/ml) and NSC23766 (final concentrations 10 μM). The subsequent electrical activity will be recorded using the Maestro Edge (Axion Biosystems). It is hypothesized that the neurons treated with solely NSC23766 will exhibit decreased electrical activity in comparison to the control. This result is expected, because NSC23766 causes the reduction of dendritic spine size, leading to decreased excitatory synapse formation and subsequently reduced electrical signaling. It is hypothesized that the combination of Fluoxetine with NSC23766 will cause a greater inhibitory effect on electrical signaling. This combination is expected to decrease electrical activity, because the inhibition of Rac1 will prevent spine enlargement typically associated with Fluoxetine, thereby reducing the area of excitatory synapse formation.

4. Methods

4.1 Maintenance of Human iPSC

To produce cortical organoids, or 'mini-brains', ActBmEGFP human iPSCs first were cultured on hESC-certified Matrigel coated plates. To ensure the stability of the cells only iPSCs from the 10^{th} passage or greater were used to make the 'mini-brains'. The iPSCs were passaged using Dispase every 4 to 5 days. During the first 48 hours in culture, the ROCK inhibitor Y-27632 was incorporated in the Essential 8 media (Gibco). Once a month the cultures were evaluated to ensure they were free of mycoplasma.

4.2 Suspension of iPSC

To detach the iPSC colonies that were grown in matrigel-coated 6-well plates, the cells were incubated with 0.35 mg/ml Dispase that was dissolved in the Essential 8 media. 1 mL of dispase was added per well and then incubated at 37°C for 3 minutes. A cell scraper was utilized to ensure the detachment of the cells. The cell colonies were transferred to a 50 mL tube using a 25 mL pipette. Multiple wells were combined using DPBS. The contents of the tube were centrifuged for 5 minutes at 300 rotations per minute (RPM). The DPBS supernatant was aspirated from the cell pellet. The pellet was re-suspended in ES/DMEM media containing; ES/DMEM, 10% KnockOut Serum, 1% GlutaMAX, 1% NEAA, and 1% Penn/Strep. Next the suspended colonies were transferred into ultra-low-attachment 6-well plates (Corning™ Costar™). The transfer of the iPSC colonies into the 6-well ultra-low-attachment plates marked the beginning of neural induction.

4.3 Neural Induction and Differentiation

The induction process was initiated by removing FGF2 and adding the two SMAD inhibitors Dorsomorphin (DM, also known as compound C; 5 μM final dissolved in DMSO, BioVision) and SB-431542 (SB, 10 μM final, dissolved in ethanol, StemMACS™ SB431542 by Miltenyi Biotec) to the ES/DMEM media. These two components inhibit bone morphogenetic protein and the transforming growth factor beta (TGF – β). These two components were added to the ES/DMEM media during the first 5 days of culture in the ultra-low-attachment plates. The media was changed daily by gently transferring the spheroids to a Falcon tube and aspirating the media once the spheroids settled to the bottom of the tube. The spheroids were transferred back into the ultra-low-attachment dish. The maximum number of spheroids placed in one well was 12 spheroids to maintain healthy environmental conditions within the each well. After 6 days in suspension, the floating spheroids were transferred into 2mL/well of Gibco® Neurobasal™- A media supplemented with 2% of Gibco® B-27™ without vitamin A, 20ng/mL of PeproTech® bFGF, and 20ng/mL of PeproTech® epidermal growth factor (EGF). This media was used for 19 days. Daily media changes were performed the first 10 days and the remaining 9 days the media was changed every other day. Starting at day 25 in culture FGF2 and EGF were replaced with 20ng/mL of brain-derived neurotrophic factor (BDNF) and 20ng/mL of neurotrophic factor 3 (NT3) from Shenandoah Biotechnology Inc™ to promote the differentiation of neural progenitor cells into neurons. This media was subsequently changed every other day, for 18 days. After day 43 in suspension the 'mini-brains' are given Gibco® Neurobasal™ media without growth factors. At this point in the process the media was only changed every 4 days. Throughout the entirety of the cell culture period, the cells were incubated at 37°C and 5% carbon dioxide. After 90 days in culture the 'mini-brains' were used in live experimentation or harvested and cryopreserved.

4.4 Drug Treatment of 'Mini-Brains'

To evaluate the effects of Fluoxetine on synapse formation and morphology, 'mini-brains' from multiple sets were treated with the anti-depressant either acutely or chronically. The chronically treated 'mini-brains' were treated with Fluoxetine (final concentration 1.5 μg/ml) every four days after the initial 44 days in culture. The Fluoxetine was added to the neuronal media used to regularly feed the 'mini-brains'. The acutely treated 'mini-brains' were not treated with Fluoxetine (final concentration 1.5 μg/ml) until 24 hours prior to fixation (day 89). 'Mini-brains' from four different sets were used in this experiment including; sets R, S, T, and U. To determine whether Fluoxetine works through the Rac1 pathway, some of the 'mini-brains' were treated with either the Rac1 inhibitor NSC23766 (final concentration 10 μM), or a combination of Fluoxetine (final concentration 1.5 μg/ml) and NSC23766 (final concentration 10 μM). The 'mini-brains' treated solely with NSC23766 were treated with the drug (final concentration 10 μM) 24 hours prior to fixation. The 'mini-brains' treated with the combination of Fluoxetine (final concentration 1.5 μg/ml) and NSC23766 (final concentration 10 μM) were treated 24 hours prior to being fixed.

4.5 Immunofluorescence Staining of 'Mini-Brains' for Excitatory Synaptic Markers

Post-drug treatment, the 'mini-brains' were harvested, cryopreserved and sectioned. The resulting slides were specific to each treatment group and featured two slices of multiple 'mini-brains'. To stain the 'mini-brain' samples, the Sequenza rack method was used. To prepare the slides for staining, they were first submerged in 50 ml of Phosphate Buffer Saline (PBS) in a Coplin jar and allowed to sit for five minutes. The slides were removed and assembled with a coverplate using 150 μL of 0.2% Triton X-100/PBS and placed in the Sequenza Rack. An additional 150 μL of 0.2% Triton X-100/PBS was added to each slide in the rack to ensure proper

adhesion between the slides and the coverplates. Next the samples were incubated at room temperature with 150 μL of the blocking buffer containing 5% Normal Goat Serum (Vector Laboratories) in PBS for 30 minutes. During this incubation period, the primary antibodies were diluted in 2% Normal Goat Serum (Vector Laboratories). The primary antibodies used in this experiment included the pre-synaptic excitatory marker vesicular glutamate transporter 1 (vGLUT-1) (guinea pig) and the post-synaptic excitatory marker post synaptic density protein 95 (PSD-95) (mouse). The primary antibodies were diluted in 2% Normal Goat Serum (Vector Laboratories) in PBS at the following concentrations: 1:50 for PSD-95 and 1:1000 for vGLUT-1. Post-incubation with 5% Normal Goat Serum in PBS, 120 μL of the primary antibody solution was added to each slide. The samples were incubated overnight with the primary antibody solution in a 4°C refrigerator. The next day the Sequenza rack was removed from the refrigerator and three, five minute 150 μL PBS washes were performed. While the washes were being performed, the secondary antibodies were simultaneously diluted in 2% Normal Goat Serum (Vector Laboratories) in PBS. The secondary antibodies used in this experiment were guinea pig 647 and mouse 568. Each secondary antibody was diluted 1:500. The secondary antibody solution was centrifuged at 12,000xg for 3 minutes to accumulate any antibody clumps at the bottom of the tube. 120 μL of the secondary antibody solution was added to each sample. The samples were incubated at room temperature with the secondary antibody solution for one hour. The samples were covered to prevent light from exciting the fluorophores. Post-incubation three, five minute 150 μL PBS washes were performed. The slides were rinsed with de-ionized water and carefully removed from the Sequenza rack one at a time. Once removed from the rack, more de-ionized water was used to detach the coverplate from the slide. A glass coverslip was affixed to the slide

with Fluorogel II with DAPI. Each slide was placed on a slide warmer to allow the fluorogel to dry. The samples were stored in a slide box in a 4°C refrigerator.

4.6 Confocal Imaging of Immunofluorescence Stained 'Mini-Brains'

The immunofluorescence stained samples were imaged on the Zeiss LSM 700 confocal microscope. Four channels were utilized on the microscope for each of the following markers; DAPI (nuclei), vGLUT-1 (excitatory pre-synaptic marker), PSD-95 (post-synaptic marker) and the endogenously-expressed ActBmEGFP (actin). The corresponding laser specifications and filter settings for each 'mini-brain' set can be found in the Appendix B section of this paper. The 40x Plan-Apochromat/1.4 Oil DIC M27 objective was used to image the 'mini-brains'. Three, 4x4 tile images were taken from each slide. Each image consisted of five 0.156 μm thick slices in the z-direction. The images were subsequently analyzed using ImageJ to examine the effect of the various drug treatments on synapse formation.

4.7 Dissociation of Human Cortical Spheroids for Electrophysiological Experiments

After 90 days in culture, 'mini-brains' from sets V and W were dissociated so they could subsequently be plated on multi-electrode array (MEA) plates. To dissociate the 'mini-brains', they were first incubated at 37°C for 45 minutes in 3 ml of a papain solution containing; Earle's balanced salts (EBSS, Sigma, E7510), D-(+)-glucose (22.5 mM), NaHCO3 (26 mM), DNase (125 U/ml, Worthington, LS002007), papain (30 U/ml, Worthington LS03126), and L-cysteine (1 mM, Sigma, C7880). Post-incubation the 'mini-brains' were washed three times with an inhibitor buffer containing; BSA (1.0 mg/ml, Sigma A-8806) and ovomucoid (also known as trypsin inhibitor, 1.0 mg/ml, Roche Diagnostics Corporation 109878). The 'mini-brains' were broken apart via trituration. Once the cells were dissociated they were layered on top of high concentration inhibitor

solution (5 mg/ml BSA and 5 mg/ml ovomucoid) and centrifuged for five minutes. The resulting cell pellet from centrifuging was re-suspended in Dulbecco's phosphate-buffered saline (DPBS, Invitrogen, 14287) with 0.02% BSA and 12.5U/ml DNase. After the cells were adequately re-suspended, they were plated onto MEA 24-well plates.

All methods pertaining to the generation and dissociation of the 'mini-brains' were adapted from Pasca et al[23].

4.8 MEA Plate Preparation

Prior to plating neurons from dissociated 'mini-brains', the MEA plates were first treated with polyethylenimine (PEI). In a 24-well MEA plate, the bottom of each well features 16 recording electrodes. To coat the recording electrodes, a pipet tip was used to carefully apply a 10 µl droplet of PEI directly onto the electrodes. Figure 7 displays the correct PEI drop placement.

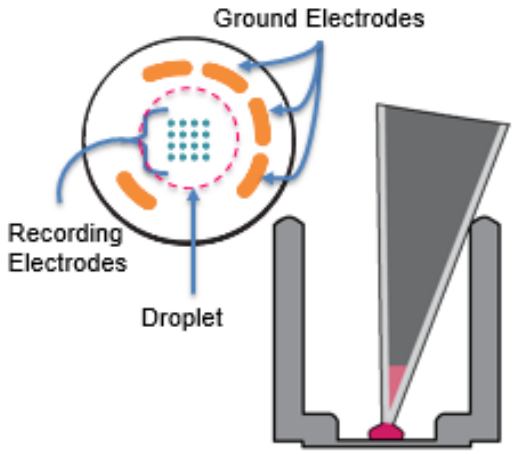

Figure 7: (Used with permission from Axion Biosystems) Correct placement of PEI drop onto recording electrodes[35].

After adding a droplet of the PEI solution to each well, MEA plates were incubated for one hour at 37°C. Post-incubation, three washes were performed with deionized water. The plates were allowed to sit overnight in a culture hood to air dry.

4.9 Plating Dissociated 'Mini-Brains' onto MEA plates

For this experiment two sets of 'mini-brains' were dissociated (Set W and Set V) and each set was plated on a separate 24-well MEA plate. The cells were plated at a concentration of 200,000 cells per well. The plates were incubated for one hour at 37°C. Post-incubation, 300 µl of fresh neuronal media was added to each well. The media was added carefully onto the side of each well to prevent the cells from lifting off of the electrodes. Figure 8 shows how the neuronal media was added to each well.

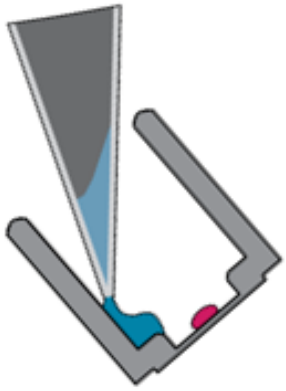

Figure 8: (Used with permission from Axion Biosystems) Application of media after cell plating[35].

An additional 300 µl of fresh neuronal media was then added to each well, bringing the total well volume to 600 µl. The neurons were fed every four days by removing 350 µl of old media and adding 350 µl of fresh neuronal media. The cells were cultured for two weeks prior to experimentation to allow the neurons to become established on the recording electrodes.

4.10 Maestro Edge Set Up

The Maestro Edge incorporates multi-electrode array (MEA) technology to record electrical signals from excitable cells. This device is able to amplify electrical signaling between neurons, allowing for detailed analysis. The Maestro Edge incorporates a plate reading system that uses MEA 24-well plates. Each plate features a barcode that allows for specific recognition in the AxIS software associated with the device. Once a plate is placed inside of the Maestro Edge, the barcode is scanned and the experimental information associated with the plate is expressed in the AxIS Navigator software. AxIS Navigator is the program used to control specific features of the Maestro Edge including; incubation parameters, recording features, stimulation capabilities and signal processing. The Maestro Edge incorporates an incubation system that allows for induction of certain environmental conditions and for prolonged electrical signal recording. For the purposes of recording electrical activity from neurons the gain is set to 1000X and bandwidth is set to 200-4000 Hz. While recording, the AxIS Navigator software displays electrical activity in three capacities including; a continuous waveform, spike plot and activity map. Figure 9 displays the continuous waveform window in AxIS Navigator.

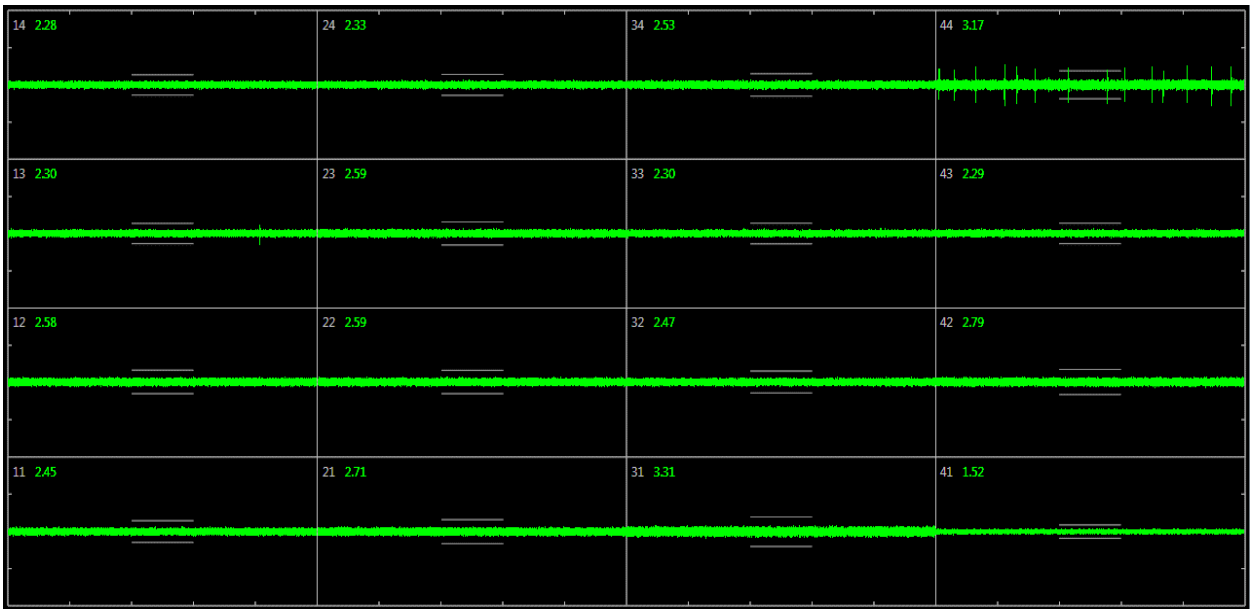

Figure 9. Continuous waveform window in AxIS Navigator.

During a recording this window displays the raw signal being collected from each electrode. In this image, each box corresponds to one electrode in the selected well. For more specific information regarding the spike activity in each well the user can examine this information in the spike plot window in AxIS Navigator. Figure 10 displays the spike plot window in AxIS Navigator.

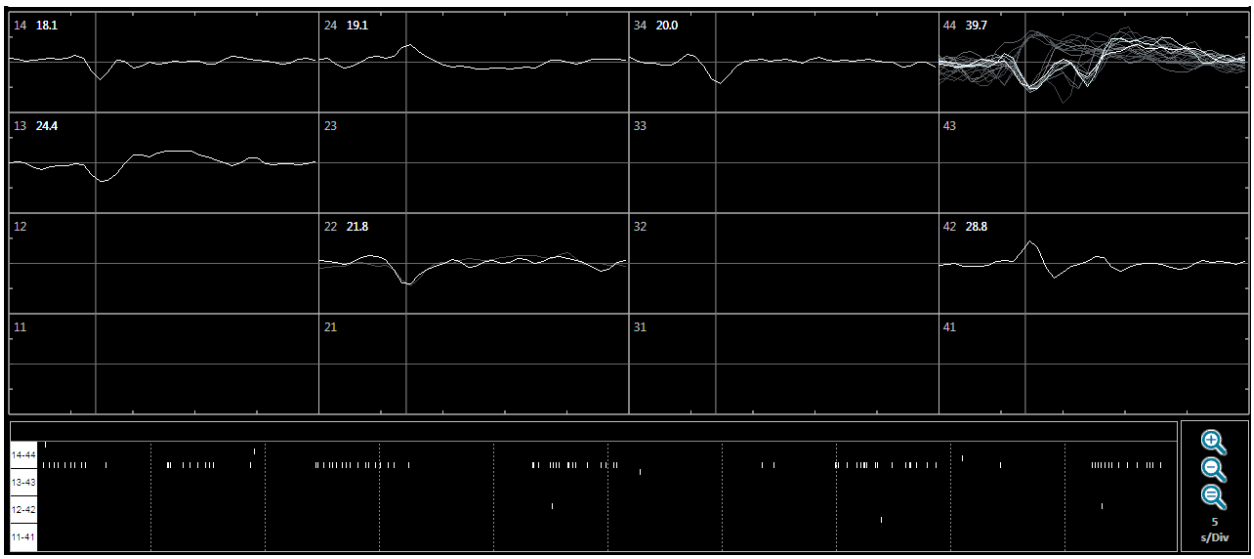

Figure 10. Spike plot window in AxIS Navigator.

In the spike plot window information can be observed including; waveforms and raster plots specific to a single electrode. The raster plot section (located in the bottom portion of the window) also provides information on bursting activity. A burst is defined as at least five spikes occurring within a 100 millisecond interval. To examine a general estimate of plate activity the heat map located in the plate activity window can be examined. Figure 11 displays the plate activity window.

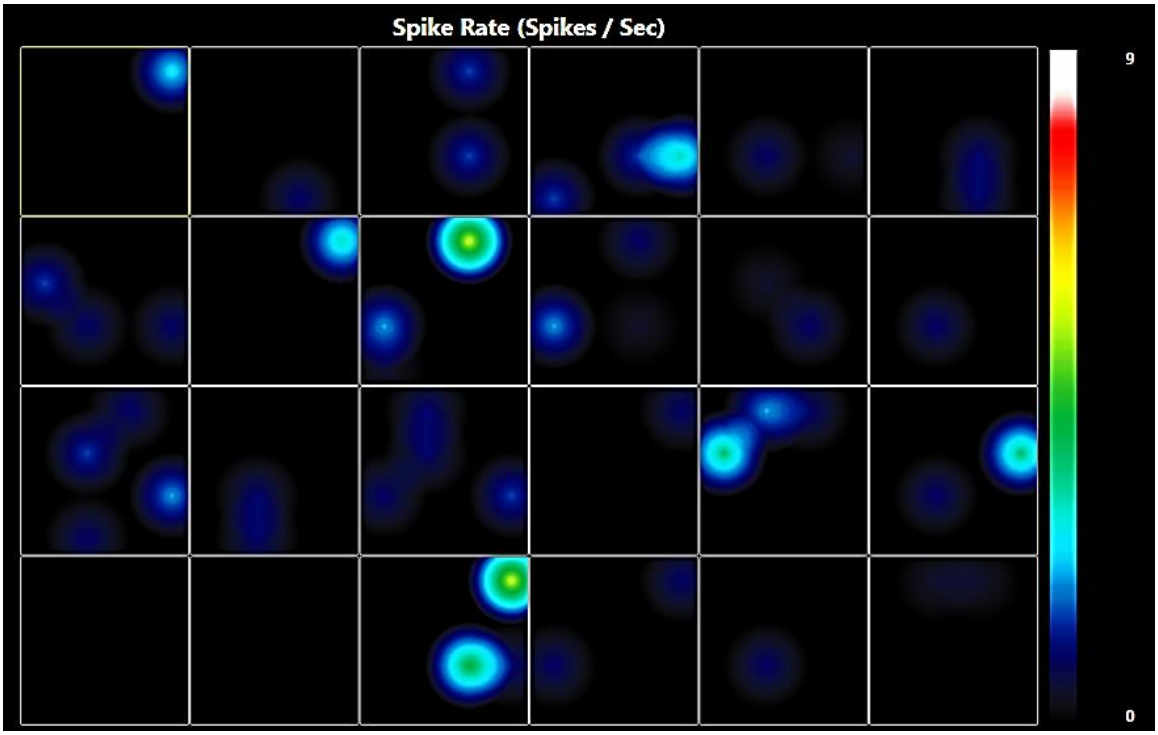

Figure 11. Plate activity window in AxIS Navigator.

As opposed to the other windows available in AxIS Navigator, this window gives a quick overview of the spiking activity across the entire plate. In addition to the AxIS Navigator program, the Maestro Edge also incorporates two other programs crucial to this project including; the AxIS

Metric Tool and the Neural Metric Tool. Once a recording is completed and processed in AxIS Navigator, it is then imported into the Neural Metric Tool for further processing. The Neural Metric Tool allows for specifications relating to active electrode criterion, bursting parameters, synchrony parameters and stimulation parameters to be applied to a recording. Once the recording has been processed in the Neural Metric Tool it can then be imported into the AxIS Metric Tool for statistical analysis.

4.11 Fluoxetine MEA Experiment

To determine the effects of Fluoxetine on electrical signaling, various concentrations of Fluoxetine were applied to dissociated 'mini-brains' and the resulting electrical signals were recorded using the MEA technology. Prior to placing one of the plates inside of the Maestro Edge, the temperature and carbon dioxide levels necessary for incubation were calibrated to be 37°C and 5% carbon dioxide. Once the appropriate gas and temperature levels were reached, the 24-well MEA plate corresponding to 'mini-brain' Set V was placed inside of the machine. Prior to experimentation, a representative plate map was made in the AxIS Navigator program. The plate map provides information regarding which wells correspond to each treatment group. Figure 12 displays the plate map utilized for the Fluoxetine experiment.

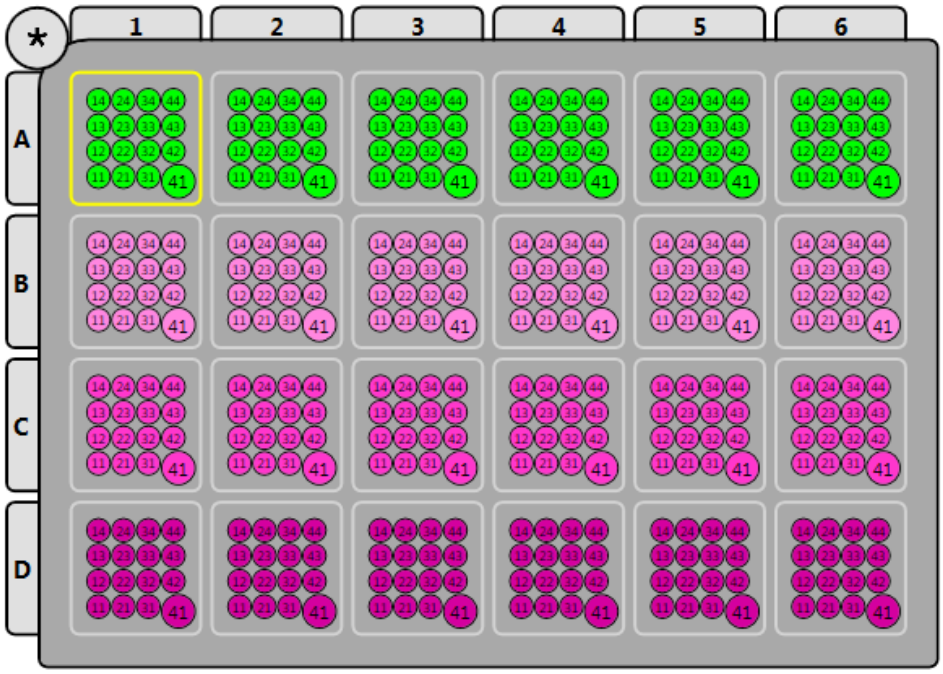

Figure 12: Plate map used in the Fluoxetine experiment.

In this plate map each row corresponds to a different treatment group. Row A (shown in green) represents the control group, which was treated with dimethyl sulfoxide (DMSO). Rows B, C and D (shown in increasingly dark shades of pink) correspond to three treatment levels of Fluoxetine including, 0.5 µg/ml, 1.0 µg/ml and 1.5 µg/ml. In this plate map each box corresponds to one of the 24 wells and each well contains 16 circles representing electrodes. Inactive individual electrodes or entire wells can be turned off using this plate map. For the purposes of this experiment all electrodes were used while recording. Once the plate map pertaining to Set V was created, a baseline recording was taken for 10 minutes. After the baseline was finished the various treatments were prepared in a biological hood. The DMSO and Fluoxetine were diluted in neuronal media without vitamin A. Once the treatments were prepared, the MEA plate was removed from the Maestro Edge and placed in the biological hood. 100 µl of media was removed from each well,

bringing the total well volume to 600 µl. 200 µl of the various treatment medias were added to the corresponding wells. The MEA plate was placed back into the Maestro Edge. The effects of the treatments on electrical activity was recorded for two hours. A recording schedule was set up to record for five minutes, every 15 minutes. After the two hours, all 800 µl of media was removed from each well and 600 µl of fresh media was added. The recovery period was recorded for 1 hour and 30 minutes. Similar to the treatment period, a recording schedule was set so a five minute recording would be taken every 15 minutes. The same procedure was repeated for both plates of dissociated ‘mini-brains’.

4.12 Fluoxetine and NSC23766

Four days following the initial Fluoxetine experiment the same cells were used to evaluate the effects of the Rac1 inhibitor NSC23766. The drug from the prior experiment was washed out and the cells were given four days to recover before completing the NSC23766 experiment. Prior to experimentation a new plate map was created to reflect the new drug treatments being tested. Figure 13 displays the plate map used for this experiment.

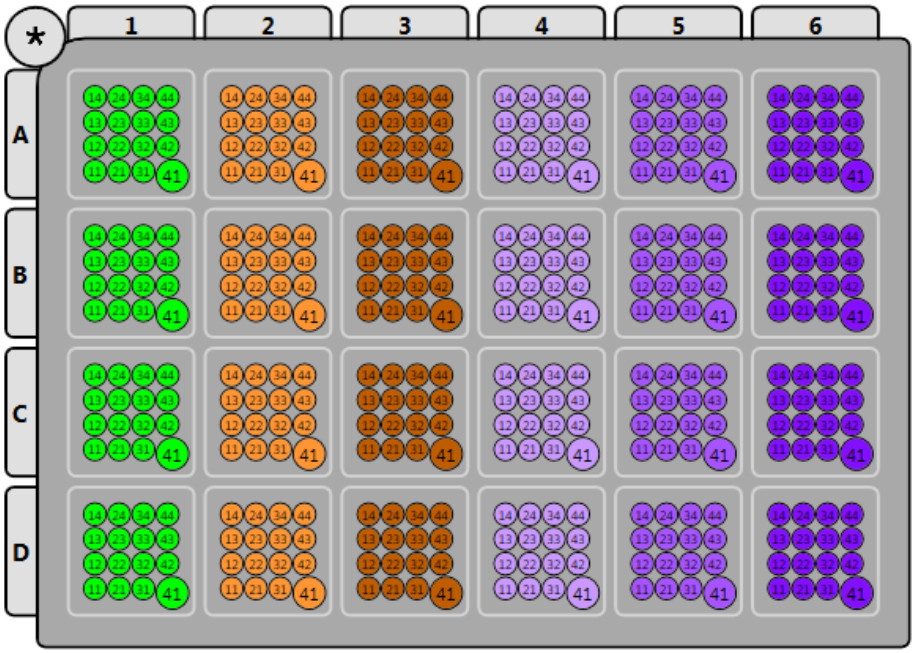

Figure 13: Plate map used in Fluoxetine and NSC 23766 experiment.

In this plate map each column represents a different treatment group. Column 1 (shown in green) corresponds to the control group treated with DMSO. Columns 2 and 3 (shown in darkening shades of orange) correspond to two concentrations of NSC 23766 including; 5 µM and 10 µM. Columns 4, 5 and 6 (shown in increasing shades of purple) represent the various treatments of 10 µM NSC23766 combined with three concentrations of Fluoxetine including; 0.5 µg/ml, 1.0 µg/ml and 1.5 µg/ml. Prior to experimentation the Maestro Edge was calibrated with the following incubation settings; 37°C and 5% carbon dioxide. After the Maestro Edge was equilibrated, the MEA plate corresponding to the 'mini-brain' set V was placed inside of the machine. Baseline electrical activity was recorded for 10 minutes. From the baseline recording it was determined that Row C did not display significant activity, therefore it was excluded from experimentation. A new baseline recording was captured with Row C turned off. Once the baseline data was collected, the

drug treatments were produced in a biological fume hood. The various concentrations of DMSO, Fluoxetine and NSC23766 were diluted in neuronal media without vitamin A. Once the treatment medias were ready, the MEA plate was removed from the Maestro and placed in a biological fume hood. 200 µl of each treatment media was added to the appropriate wells. Immediately following the addition of the treatment medias, the plate was placed back inside of the Maestro Edge and recorded from for 2 hours. The recording schedule initiated a five minute recording every 15 minutes during the 2 hour time period. Post-recording the plate was once again moved to a biological hood, where all 800 µl of treated media was removed. 600 µl of fresh media was added to each well. The recovery period was recorded on the Maestro Edge. The recording schedule lasted for 1 hour and 30 minutes and started a new five minute recording every 15 minutes. The same process was repeated for the MEA plate corresponding to the ‘mini-brain’ set W, however, Row C was included in the experimentation of this plate.

5. Results

5.1 Confocal Images of 'Mini-Brain' Samples

To determine the effects of Fluoxetine on synapse formation, human-derived cortical organoids were treated with the drug either acutely or chronically. In addition to treating 'mini-brains' with Fluoxetine, other 'mini-brains' were treated with either the Rac1 inhibitor NSC23766 or a combination of both compounds. After 90 days of culture, the 'mini-brains' were harvested, fixed, cryopreserved and sliced. Each sample was stained with three immunofluorescent markers including; DAPI, VGLUT-1 and PSD-95. The 'mini-brains' also expressed a GFP actin marker endogenously. The 'mini-brain' samples were imaged on the LSM 700 Zeiss confocal microscope. The resulting images consisted of 5 slices in the z-direction. Prior to analysis, each of the five slices had to be exported as a series, separately for each of the markers. The series of images pertaining to each marker was imported into ImageJ for processing and analysis. A maximum z-projection stack was performed for each series of images. This process allows for the information from multiple image slices to be combined into one image. Figure 14 displays a representative sample of the control 'mini-brain' group.

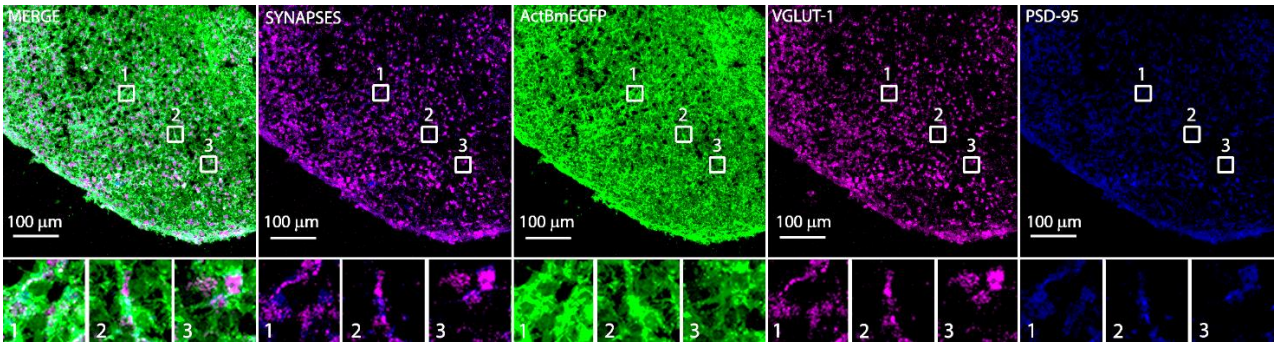

Figure 14: Immunofluorescent images of control 'mini-brain' sample. To examine excitatory synapse formation each 'mini-brain' was stained with a pre-synaptic maker (VGLUT-1) and a post-synaptic marker (PSD-95). These two markers were co-localized to determine the presence of excitatory synapses. In addition to synaptic markers, the ActBmEGFP cells endogenously expressed GFP as an actin marker. Increased magnification of three areas in the 'mini-brain' display neuronal circuitry and localization of relevant markers. A larger version of this image can be examined in Appendix B.

In the images of the control sample, co-localization of the excitatory pre-synaptic marker (VGLUT-1) and post-synaptic marker (PSD-95) can be observed. The co-localization of these markers represents excitatory synapse formation. The same conditions used to image and analyze the control samples were also used for the 'mini-brains' in the treatment groups. Images pertaining to the chronic Fluoxetine, acute Fluoxetine, NSC23766 and Fluoxetine plus NSC23766 treatment groups can be observed in Figures 15-18, located in Appendix B. When viewing the resulting images for each group, a drastic reduction in actin (green) marker expression is seen in the chronically treated 'mini-brains'. Previously, Fluoxetine has been shown to increase dendritic spine density. These spines contain high concentrations of F-actin. The decreased expression of actin in the chronically Fluoxetine treated group suggests that chronic exposure to the anti-depressant reduces dendritic spine formation.

5.2 Analysis of Synapse Formation in Human-Derived 'Mini-Brains'

After the maximum projection of each channel was created, the resulting images were analyzed in ImageJ. The scale for each image was set to be 6.3989 microns/pixel and only particles

in the range of 0.25 to 15 μm^2 were analyzed. The co-localization plugin in ImageJ was used to determine the co-localized points between the pre-synaptic excitatory marker VGLUT-1 and the post-synaptic marker PSD-95. This plugin works by overlaying the maximum projection of the VGLUT-1 and PSD-95 images. The resulting areas that express a pre- and post-synaptic marker are then emphasized in white. Figure 20 displays the co-localized points of a representative control sample.

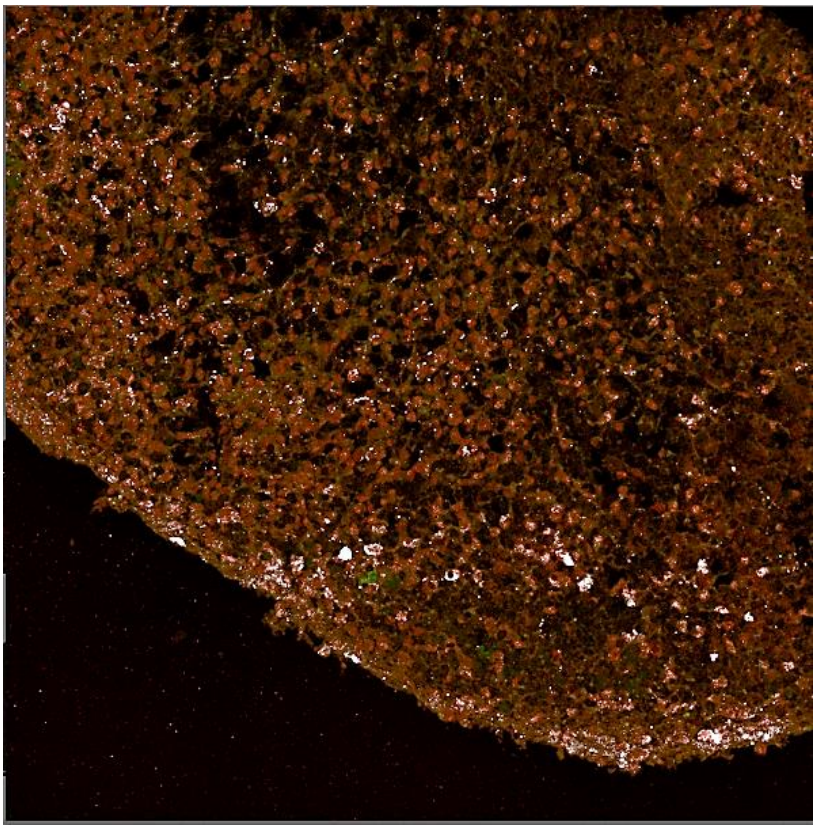

Figure 19: Co-localized points of VGLUT-1 (green) and PSD-95 (red) for control sample. White indicates a co-localized point.

Additional images of the co-localized points pertaining to the 'mini-brains' from other treatment groups can be observed in Figures 20-23 located in Appendix B. The co-localized points

were evaluated using the measure function in ImageJ to determine excitatory synapse size, perimeter, circularity and axis ratio. Excitatory synapse size refers to the area of each co-localized point. Figure 24 displays the resulting synapse size data in the form of percent control for each of the 'mini-brain' treatment groups.

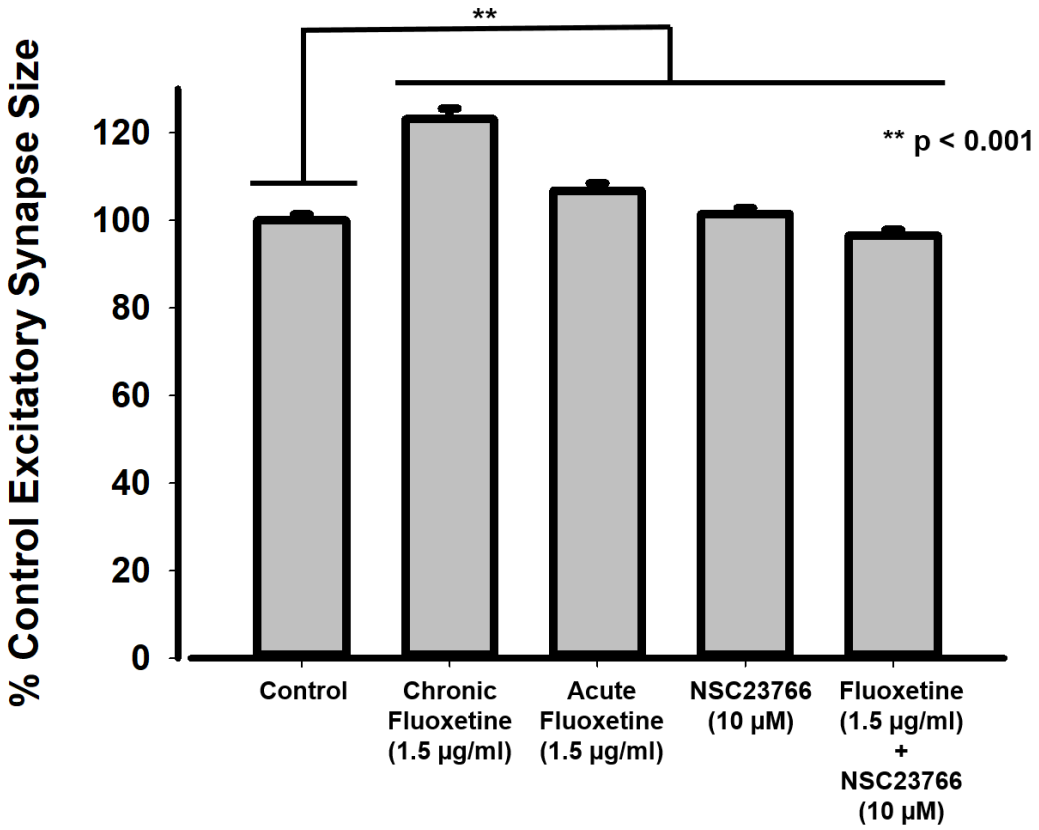

Figure 24: Excitatory synapse size determined for control (n = 10,468), chronic Fluoxetine (n = 7,119), acute Fluoxetine (n = 8,728), NSC23766 (n = 10,745) and Fluoxetine plus NSC23766 (n = 12,097). It was determined each of the treatment groups was significantly different from the control. The significance value for each group was determined to be $p < 0.001$.

The excitatory synapse size was found to be significantly different compared to the control for each of the treatment groups. The largest difference in excitatory synapse size was the increase in synapse size observed in the chronically Fluoxetine-treated 'mini-brains'. Acute Fluoxetine also significantly increased synapse size compared to the control. These results were expected, because Fluoxetine causes an increase in dendritic spines, the main area of excitatory synapse formation. In contrast to the other treatments involving Fluoxetine, the 'mini-brains' treated with Fluoxetine plus NSC23766 displayed significantly smaller synapse size compared to the control. One of the main goals of this project was to determine whether Fluoxetine acts through the Rac1 pathway. NSC23766 was combined with Fluoxetine as a treatment to see if Rac1 inhibition subsequently inhibits the effects of Fluoxetine. It was determined the combination of both compounds caused this treatment group to display a decrease in excitatory synapses size from the control. A decrease in synapse size was expected, because inhibition of Rac1 has previously been shown to decrease dendritic spine size and number. This data also supports the hypothesis that Fluoxetine acts through the Rac1 pathway as the effects of Fluoxetine on synapse size were inhibited by NSC23766. To determine whether the combination of acute Fluoxetine and NSC23766 rescued the effects of acute Fluoxetine on synapse size, a Mann-Whitney Rank Sum test was performed. The two groups were not found to be significantly different from each other, even though there seems to be decreased synapse size in Fluoxetine plus NSC23766 treatment group. The next shape descriptor examined in each of the 'mini-brain' samples was the synapse perimeter. The perimeter refers to the outside boundary of the co-localized points. Figure 25 displays the resulting perimeter values for each of the treatment groups.

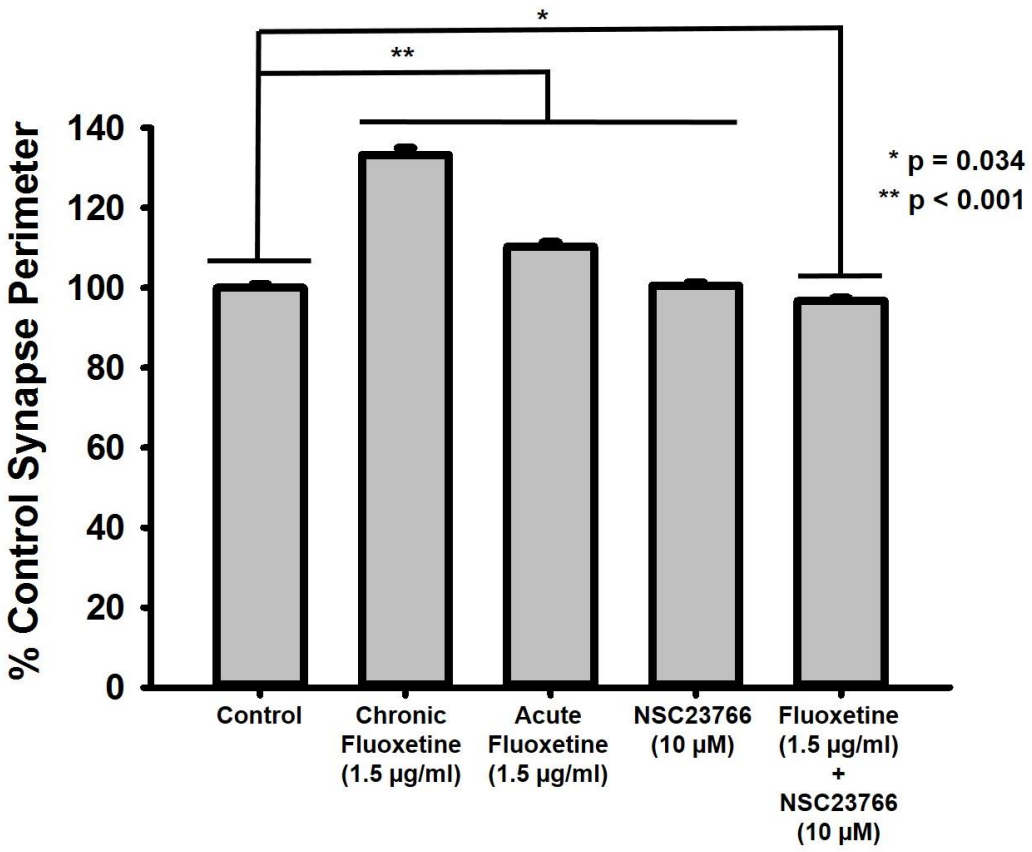

Figure 25: Synapse Perimeter determined for control (n = 10,468) ($p < 0.001$), chronic Fluoxetine (n = 7,119) ($p < 0.001$), acute Fluoxetine (n = 8,728) ($p < 0.001$), NSC23766 (n = 10,745) ($p < 0.001$) and Fluoxetine plus NSC23766 ($p = 0.034$) (n = 12,097). It was determined each of the treatment groups was significantly different from the control except for the NSC23766 treated cells.

The 'mini-brains' treated chronically with Fluoxetine displayed a significant increase in synapse perimeter when compared to the control. Earlier in this experiment it was established that chronically treated 'mini-brains' display increased synapse size compared to the control group, suggesting the presence of enlarged spines. Perimeter can be used to describe spine size and characterize the circularity of a synapse. The combination of Fluoxetine and NSC23766 reduced

the effects of Fluoxetine on synapse morphology by inhibiting the Rac1 pathway. To determine whether the combination of the two substances rescues the effects of acute Fluoxetine on synapse formation a Mann-Whitney Rank Sum test was performed. It was determined Fluoxetine plus NSC23766 significantly reduced the perimeter of synapses in comparison to those present within the acutely treated 'mini-brain'. Similarly to the trend observed in the synapses size, the NSC23766 treated ' mini-brains' data looks very similar to the control 'mini-brain' data. The resulting perimeter data for each 'mini-brain' group was used to calculate circularity, another synapse shape descriptor. The circularity of the co-localized points was determined using the following equation.

$$4\pi \times \frac{Area}{Perimeter^2}$$

The value corresponding to circularity can only range between 0.0 and 1.0. A circularity of 1.0 indicates a perfect circle. A circularity of 0.0 means the shape of the synapse is more elongated than circular. The resulting circularity for each treatment group is displayed in Figure 26.

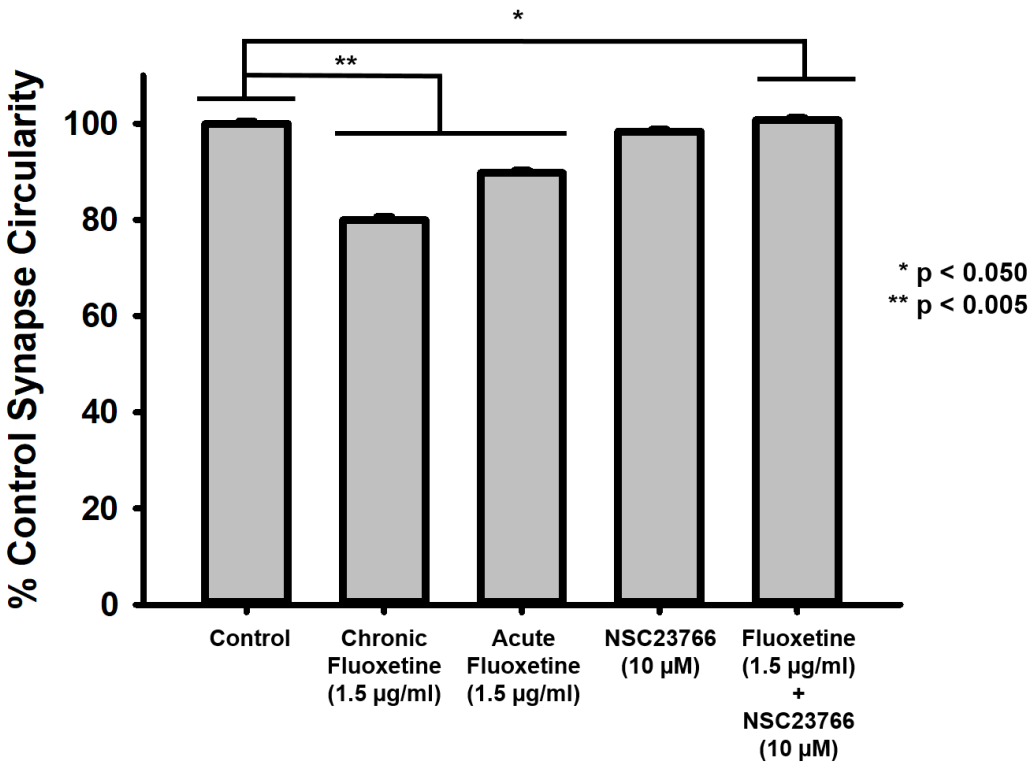

Figure 26: Synapse circularity determined for control (n = 10,468) ($p < 0.001$), chronic Fluoxetine (n = 7,119) ($p < 0.001$), acute Fluoxetine (n = 8,728) ($p < 0.001$), NSC23766 ($p = 0.134$) (n = 10,745) and Fluoxetine plus NSC23766 (n = 12,097) ($p = 0.042$). It was determined each of the treatment groups was significantly different from the control except for the NSC23766 only treated cells.

In comparison to the control group, the 'mini-brains' treated with Fluoxetine chronically, displayed a significant decrease in synapse circularity. This suggests that the synapses formed in these 'mini-brains' were irregular in shape. The irregularity in shape is further supported by the increased perimeter observed in the chronically treated 'mini-brains'. The acutely treated 'mini-brains' also displayed a significant decrease in circularity in comparison to the control 'mini-brains'. However, the chronic exposure to Fluoxetine seems to have a greater effect on irregular

synapse formation. In contrast to the effect of the acute and chronic Fluoxetine treatments, when Fluoxetine is combined with the Rac1 inhibitor NSC23766 less irregular synapse formation seems to occur. This supports the hypothesis that the effects of Fluoxetine on synapse formation are inhibited when combined with a Rac1 inhibitor. Since the Rac1 inhibitor seems to have an effect on Fluoxetine, this provides evidence that Fluoxetine alters synapse formation through the Rac1 pathway. Another descriptor that was used to analyze the shape of the co-localized points was the axis ratio. The axis ratio refers to the ratio of the major axis to the minor axis. The major axis refers to the primary axis of the best fitting ellipse and the minor axis corresponds to the secondary axis. Figure 27 displays the axis ratio for each of the 'mini-brain' treatment groups.

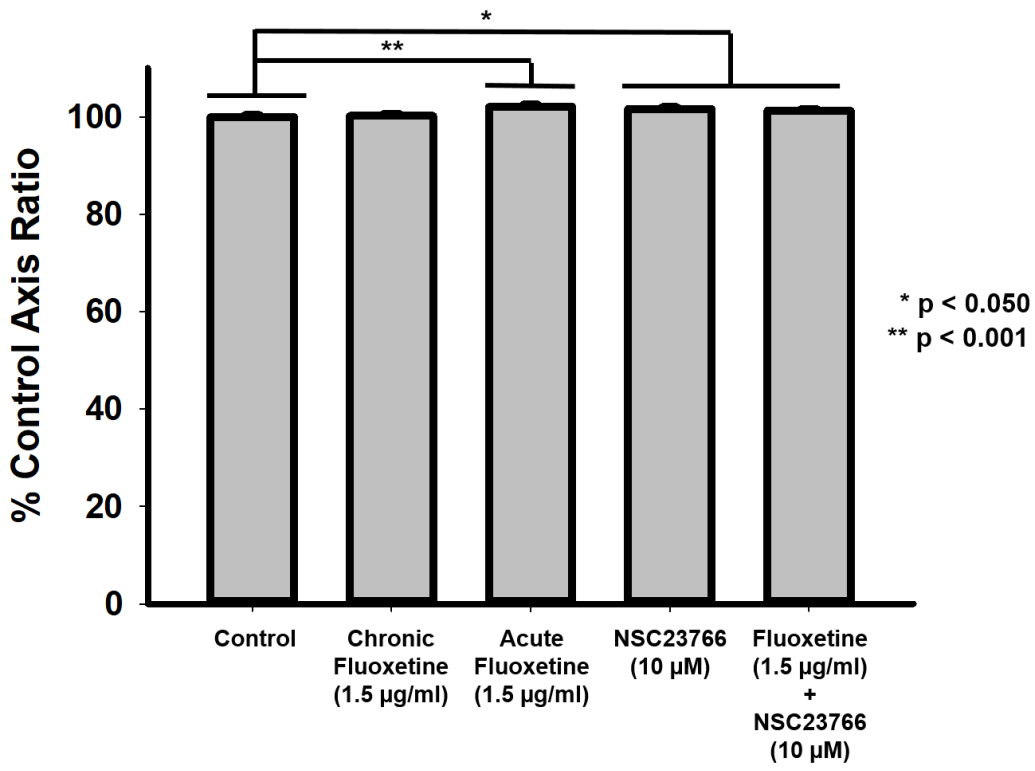

Figure 27: Axis ratio of excitatory synapses in 'mini-brain' model treated with actin cytoskeleton altering substances. The acute Fluoxetine ($p < 0.001$), NSC23766 ($p = 0.006$) and Fluoxetine plus

NSC23766 (p = 0.038) treated ‘mini-brains’ displayed significant differences compared to the control.

The axis ratio for the acute Fluoxetine treated group, group treated with NSC23766 and the ‘mini-brains’ treated with a combination of both substances, were all determined to be significantly higher than the control. The ‘mini-brains’ treated chronically with Fluoxetine were not found to be significantly different than the control.

In addition to evaluating shape descriptors of the excitatory synapses present in each ‘mini-brain’, the excitatory synapse density normalized to nuclei was also calculated (Figure 28). To determine synapse density, the area of the nuclei (stained with DAPI) was calculated in ImageJ. The number of co-localized points was then divided by the nuclei area.

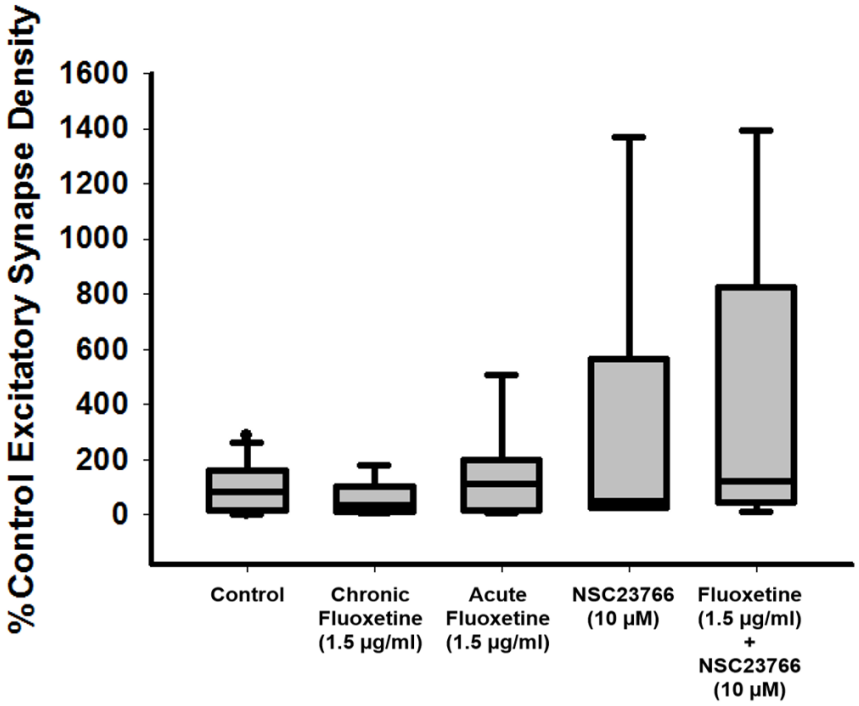

Figure 28: Excitatory synapse density in 'mini-brain' samples belonging to the following groups; control (n = 12), chronic fluoxetine (n = 9), acute Fluoxetine (n = 9), NSC23766 (n = 9) and Fluoxetine plus NSC23766 (n = 9). None of the treatment groups were significantly different from the control.

While none of the groups were determined to be significantly different from the control, a trend in excitatory synapse density can be observed. Out of all of the treatment groups, the chronically treated 'mini-brains' seem to display the least excitatory synapse density. While chronic Fluoxetine seems to decrease excitatory synapse density, it appears that acute Fluoxetine causes a slight increase in excitatory synapse formation. Both NSC23766 and the combination of NSC and Fluoxetine seems to increase excitatory synapse density. In the future, more samples need to be examined to gain a better understanding of the impacts of the various treatments on excitatory synapse density. While the synaptic density was not determined to be significant due to low sample size, all of the synapse shape descriptor data displayed significant differences.

5.3 Analysis of the Effect of Fluoxetine on Electrophysiological Activity

To determine the effects of Fluoxetine on electrical neuronal activity, MEA technology was used to record the effects of the drug. For this portion of the project the Maestro Edge (Axion BioSystems) was used to record the changes in electrical field potentials that resulted from the application of various concentrations of Fluoxetine. Prior to experimentation a baseline recording was taken to establish normal electrical activity levels. Following the baseline recording, the solvent DMSO and three concentrations of Fluoxetine (0.5 μg/ml, 1.0 μg/ml and 1.5 μg/ml) were individually added to each row on the 24-well MEA plate. After the treatment medias were added

to their respective wells, the effect of the drug was examined for 2 hours. A recording schedule was set so that a five minute recording would occur every 15 minutes. Following the two hour recording period, the drug was washed out. 30 minutes post-washout a new recording schedule was started that initiated a five minute recording every 15 minutes. The resulting data was analyzed using the statistical software SigmaPlot13.0. Both the mean firing rate and burst frequency were evaluated. Mean firing rate corresponds to the number of spikes that occur over the duration of the recording. Figure 29 displays the mean firing rate over the course of the experiment for the group treated with the solvent DMSO.

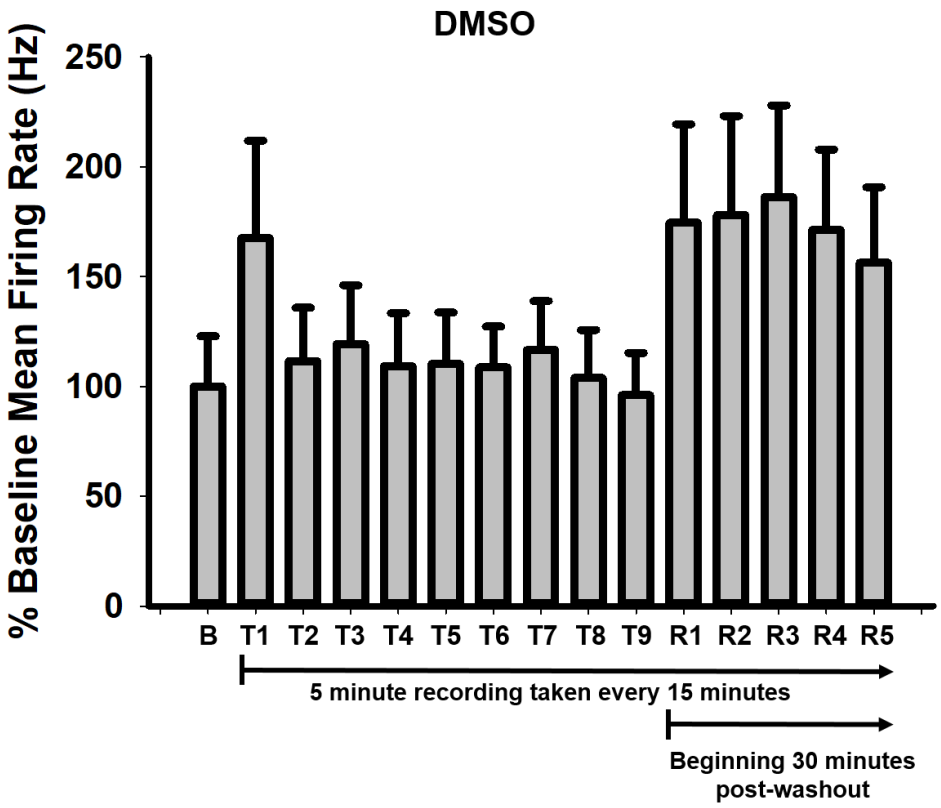

Figure 29: Mean firing rate of DMSO treated cells in Fluoxetine MEA experiment. Data is displayed as % Baseline. Prior to experimentation a baseline recording (B) was taken. The cells were then treated with DMSO and the five minute treatment recordings (T) were taken every 15 minutes for two hours. The recovery (R)

period was recorded starting 30 minutes after the drug was washed out for a total time of one hour and 30 minutes.

The mean firing rate for the control treatment group DMSO was not found to be significantly different during any of the treatment or recovery recordings when compared to baseline. Since DMSO was chosen as the control, it was expected that it would express electrical activity similar to baseline throughout the recording period. DMSO was chosen as the control, because it was used to dilute Fluoxetine to the final concentrations needed for treatment. In addition to mean firing rate, the burst frequency was also examined for DMSO (Figure 30). Burst frequency refers to the number of bursts that occur during a recording, divided by the duration of the recording. In this project the burst criteria was set to be a minimum of five spikes per burst, with an inter-spike interval of less than 100 ms.

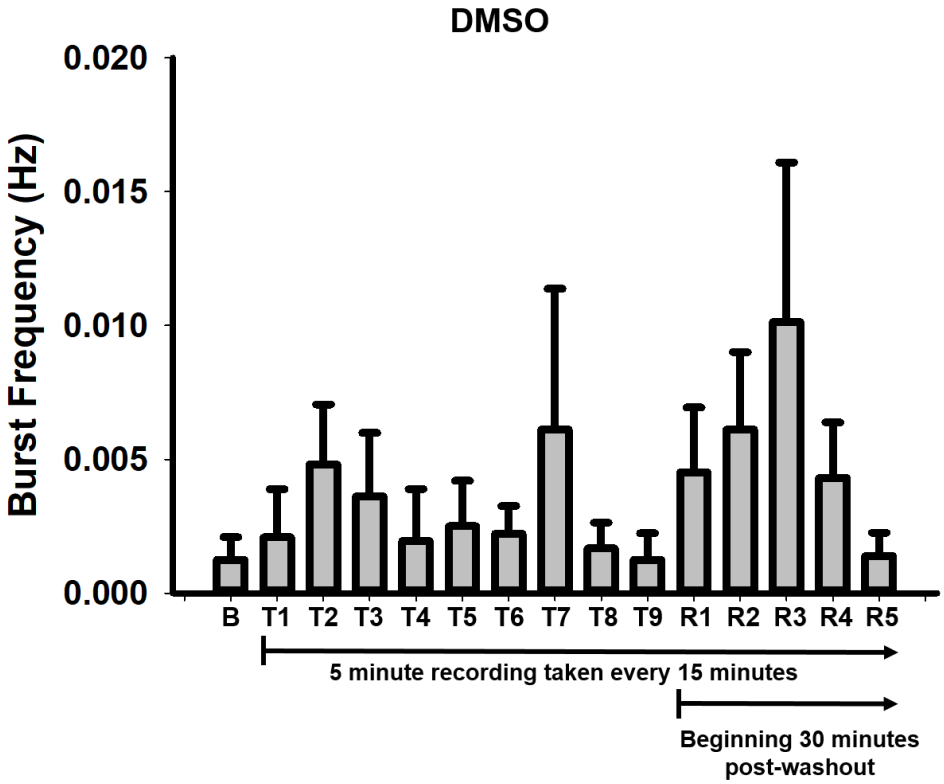

Figure 30: Burst frequency of DMSO treated cells in Fluoxetine MEA experiment. Prior to experimentation a baseline recording (B) was taken. The cells were then treated with DMSO and the five minute treatment recordings (T) were taken every 15 minutes for two hours. The recovery (R) period was recorded starting 30 minutes after the drug was washed out for a total time of one hour and 30 minutes.

The burst frequency varied across both the treatment and recovery portions of this experiment. The bursts were recorded spontaneously (without the application of a stimulus). Compared to baseline none of the burst frequencies were consider significant. Throughout each of the MEA experiments the burst frequency was not statistically significant compared to the control. The mean firing rate

was next examined for the neurons treated with Fluoxetine (final concentration of 0.5 µg/ml) (Figure 31).

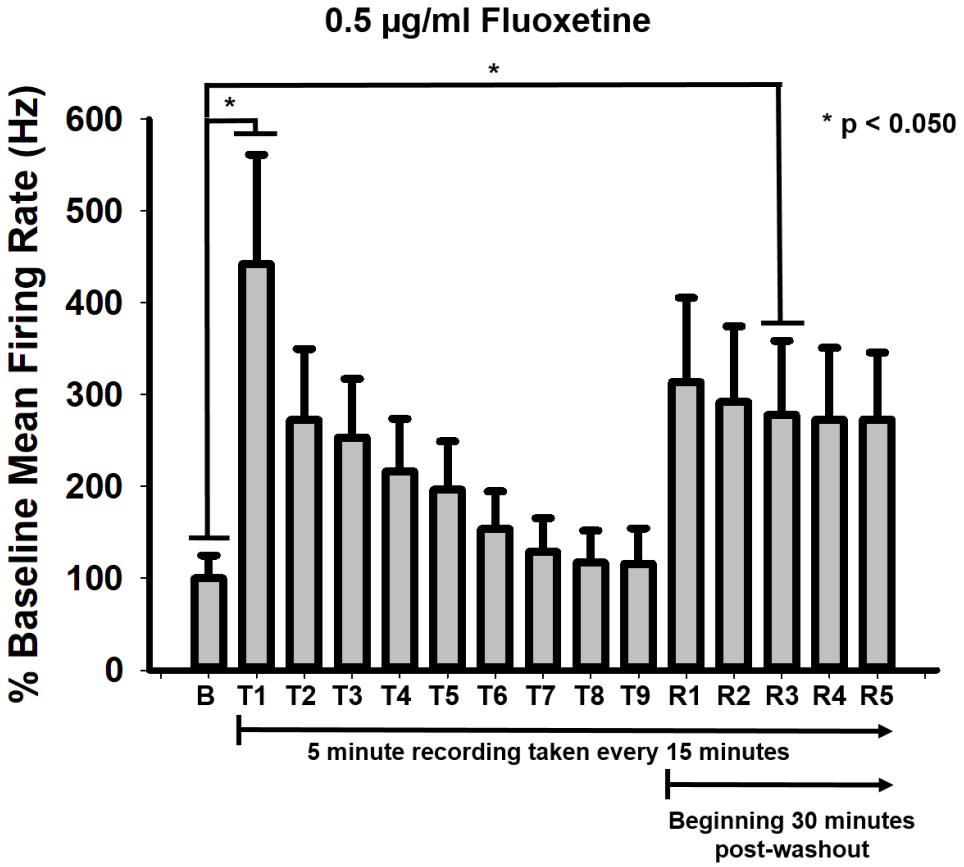

Figure 31: Mean firing rate of Fluoxetine treated cells (final concentration 0.5 µg/ml) in Fluoxetine MEA experiment. Data is displayed as % Baseline. Prior to experimentation a baseline recording (B) was taken. The cells were then treated with 0.5 µg/ml Fluoxetine and the five minute treatment recordings (T) were taken every 15 minutes for two hours. The recovery (R) period was recorded starting 30 minutes after the drug was washed out for a total time of one hour and 30 minutes. T1 ($p = 0.009$) and R3 ($p = 0.047$) were determine to be significantly different from baseline.

Compared to baseline, it was determined that the first treatment recording (T1) and the third recovery recording (R3) were significantly different. After the initial application of the Fluoxetine onto the cells, an increase in electrical activity was observed. This initial increase in neuronal activity is thought to be a result of increased activation of 5-HT_{2A} receptors from the increased levels of serotonin caused by Fluoxetine. These receptors typically produce an excitatory response in the post-synaptic neuron. The downstream effects of 5-HT_{2A} receptors is thought to cause inhibition of electrical activity. This trend can be seen in the treatment recordings of the 0.5 μg/ml Fluoxetine. However, the effects of the treatment were not statistically significant. After the drug was washed out of each well, complete recovery of neuronal signaling was seen. The burst frequency was also examined for the 0.5 μg/ml Fluoxetine treatment group. The burst frequency data (Figure 32) can be observed in Appendix C.

The burst frequency was not determined to be statistically significant for any of the recordings when compared to baseline. However, it does appear that during the later recordings of drug treatment (T6 – T9) the burst frequency seems to decrease. Next the mean firing rate (Figure 33) and burst frequency (Figure 34 – Appendix C) were analyzed for the Fluoxetine treated cells (final concentration 1.0 μg/ml).

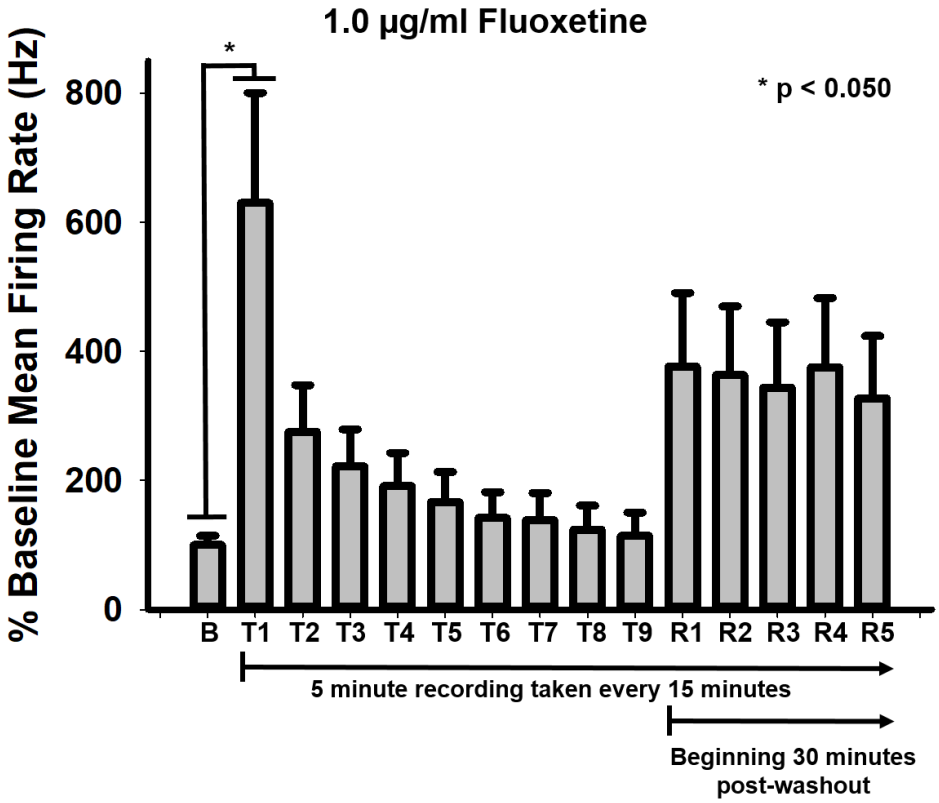

Figure 33: Mean firing rate of Fluoxetine treated cells (final concentration 1.0 µg/ml) in Fluoxetine MEA experiment. Data is displayed as % Baseline. Prior to experimentation a baseline recording (B) was taken. The cells were then treated with 1.0 µg/ml Fluoxetine and the five minute treatment recordings (T) were taken every 15 minutes for two hours. The recovery (R) period was recorded starting 30 minutes after the drug was washed out for a total time of one hour and 30 minutes. When compared to baseline T1 was determined to be statistically significant ($p = 0.014$).

Similarly to what was observed in the 0.5 µg/ml Fluoxetine data, the mean firing rate initially increased once the drug was applied to the cells and then continued to decrease electrical activity throughout the treatment portion of the experiment. The mean firing rate of T1 was found to be significantly different from baseline. While the electrical activity decreased during the treatment

period, it recovered once the drug was washed out and replaced with new media. Once the 1.0 µg/ml Fluoxetine was added to the cells a significant increase in bursting activity was observed. Following this initial increase in bursting, the bursting frequency was then reduced. The mean firing rate was next observed in the neurons treated with the highest concentration of Fluoxetine (final concentration 1.5 µg/ml) (Figure 35).

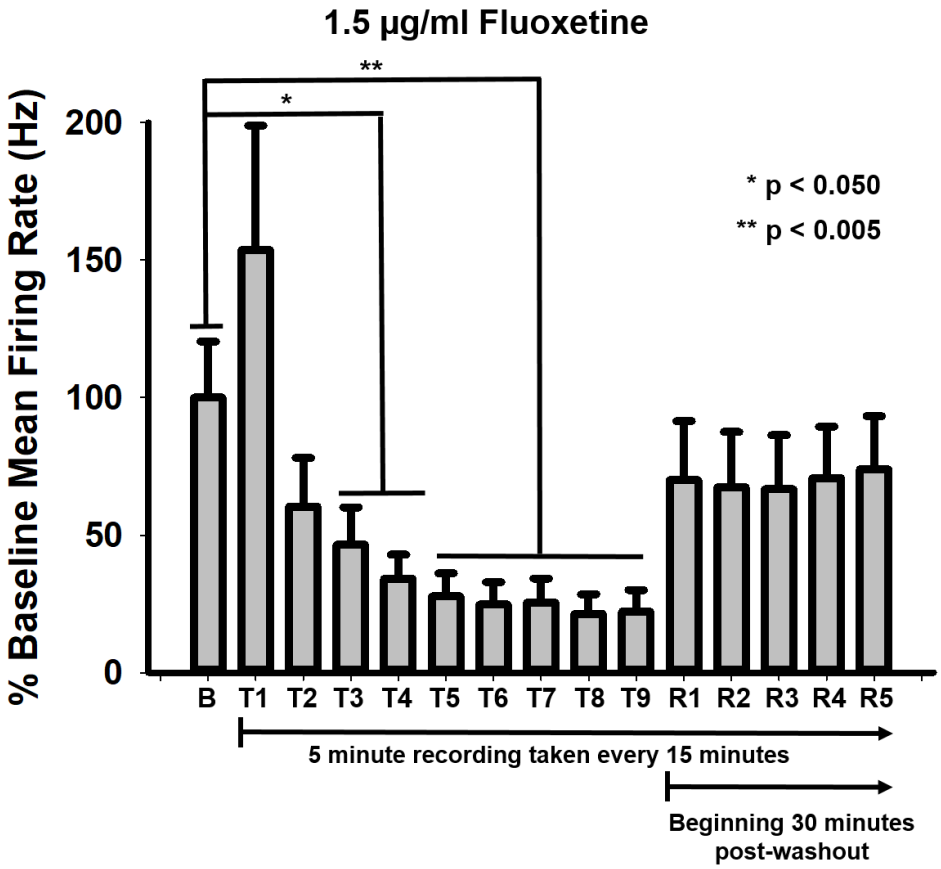

Figure 35: Mean firing rate of Fluoxetine treated cells (final concentration 1.5 µg/ml) in Fluoxetine MEA experiment. Data is displayed as % Baseline. Prior to experimentation a baseline recording (B) was taken. The cells were then treated with 1.5 µg/ml Fluoxetine and the five minute treatment recordings (T) were taken every 15 minutes for two hours. The recovery (R) period was recorded starting 30 minutes after the drug was washed out for a total time of one hour and 30 minutes. When compared to baseline the recordings T3 (p =

0.040), T4 (p = 0.010), T5 (p = 0.003), T6 (p = 0.002), T7 (p = 0.002), T8 (p = 0.001) and T9 (p = 0.002) were determined to be statistically significant.

During the treatment portion of this experiment, the 1.5 μg/ml Fluoxetine treated cells displayed significant decreases in mean firing rate for recordings T3 through T9. The cells did however recover once the drug was washed out. From the three concentrations of Fluoxetine, the highest concentration seemed to have the greatest effect on the electrical activity. The bursting frequency was also examined for the 1.5 μg/ml Fluoxetine treated cells (Figure 36 – Appendix C). While some bursting activity did occur, there were no significant differences observed regarding any of the recordings when compared to baseline. Originally it was hypothesized that the application of Fluoxetine to the neurons would cause an initial increase in electrical activity followed by inhibition of the activity. Across each treatment group an initial spike in activity was recorded which was followed by a subsequent decrease in activity throughout the rest of the treatment recording period. This increase in activity could possibly be due to the activation of 5-HT_{2A} receptors, which are serotonin receptors that usually cause an excitatory response. In some cases these receptors initiate an excitatory post-synaptic potential that eventually downstream leads to inhibition of certain neurons. This mechanism would explain the initial increase in neuronal activity that is later inhibited. In contrast to the initial hypothesis, significant decrease in neuronal activity was not observed in the wells treated with 0.5 μg/ml and 1.0 μg/ml of Fluoxetine. A significant decrease in mean firing rate was however recorded in the neurons treated with 1.5 μg/ml Fluoxetine. This supports the hypothesis that larger concentrations of Fluoxetine should have a greater inhibitory effect on electrical activity. Regarding burst frequency, from the initial baseline recording only variable burst activity was examined. The neurons used throughout this experiment

displayed spiking activity, but only sporadic bursting. The lack of burst activity is possibly due to the fact that the cells were not stimulated.

5.4 Analyzing the Effect of Fluoxetine Combined with NSC23766

After performing the initial MEA experiment to observe the effects of various concentrations of Fluoxetine on neuronal activity, Fluoxetine was combined with the Rac1 inhibitor NSC23766 to determine the effect of these substances on subsequent activity. It was hypothesized that NSC23766 would reduce signal transmission as it has been shown to decrease dendritic spine size. Decrease in spine size subsequently would cause reduction of serotonin receptors, leading to less serotonin-induced signal transmission. The effects of the combination of NSC23766 and Fluoxetine were also examined in this portion of the experiment. It was hypothesized that the combined effects of the Rac1 inhibitor and Fluoxetine would cause greater inhibition of signal transmission than they do separately. Six treatment groups were examined in this experiment including; the solvent DMSO, 5 μM NSC23766, 10 μM NSC23766, and three concentrations of Fluoxetine (0.5 μg/ml, 1.0 μg/ml and 1.5 μg/ml) combined with 10 μM NSC23766. Prior to treatment, a baseline recording was recorded to characterize the electrical activity of the cells. Post-treatment a recording schedule was created that initiated a five minute recording every 15 minutes, for a two hour period. After the treatment period ended, the cells were washed out with new media. The recovery period was recorded for five minutes, every 15 minutes, for two hours. The mean firing rate and burst frequency were examined for each recording specific to the treatment groups. The mean firing rate of the cells treated with DMSO is displayed in Figure 37.

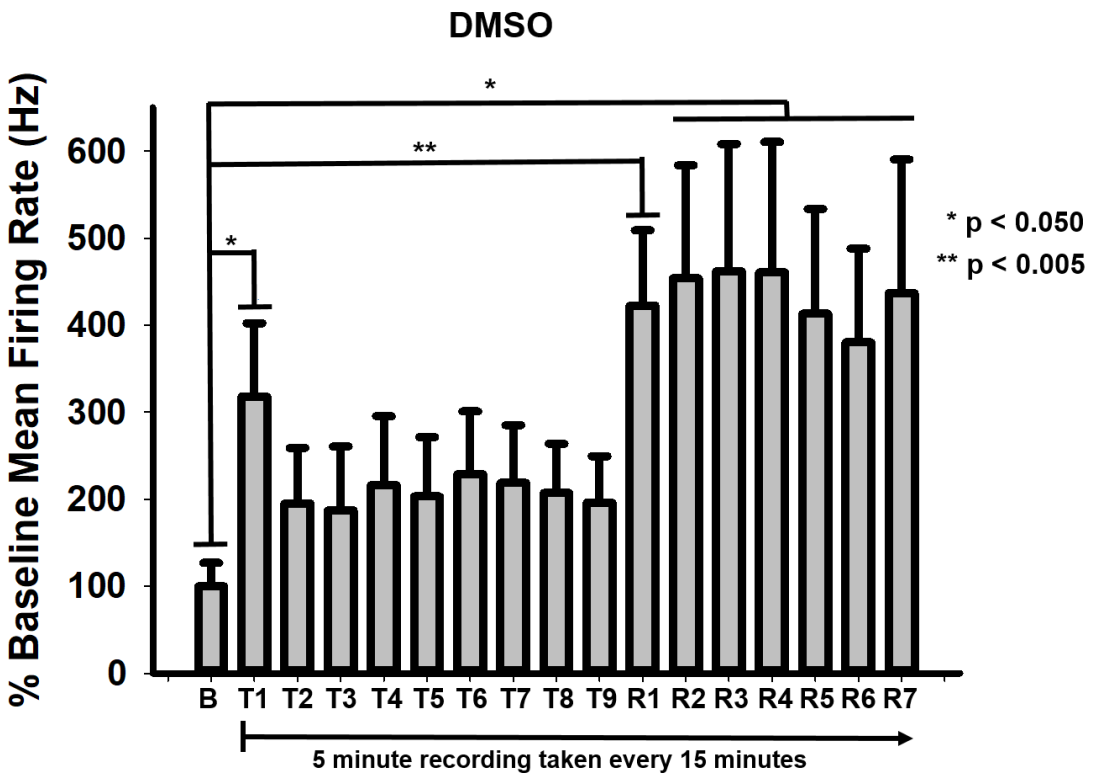

Figure 37: Mean firing rate of DMSO treated cells in the Fluoxetine + NSC23766 MEA experiment. Data is displayed as % Baseline. Prior to experimentation a baseline recording (B) was taken. The cells were treated with DMSO and the five minute treatment recordings (T) were taken every 15 minutes for two hours. The recovery (R) period was recorded beginning after the treatment was washed out. A five minute recording was take even 15 minutes, for two hours. When compared to baseline the recordings T1 ($p = 0.029$), R1 ($p = 0.004$), R2 ($p = 0.007$), R3 ($p = 0.031$), R4 ($p = 0.007$), R5 ($p = 0.026$), R6 ($p = 0.027$) and R7 ($p = 0.038$) were determined to be statistically significant.

The DMSO treated cells initially displayed an increased mean firing rate during the first treatment recording. The remaining treatment recordings were determined to be similar to the baseline recording. After the drug treatment time period was completed, the treatments were removed and

new media was added to each well. During the recovery period the mean firing was determined to be significantly higher than that of the control. This suggests that the change in media induced greater electrical activity in the neurons. Next the burst frequency was determined for the DMSO treated cells (Figure 38 – Appendix C). No bursting activity was detected in the baseline recording of the DMSO cells. After the application of DMSO to the neurons, an increase in burst frequency was observed. Next the mean firing rate (Figure 39) and burst frequency (Figure 40 – Appendix C) was determined for the 5 µM NSC23766 treated cells.

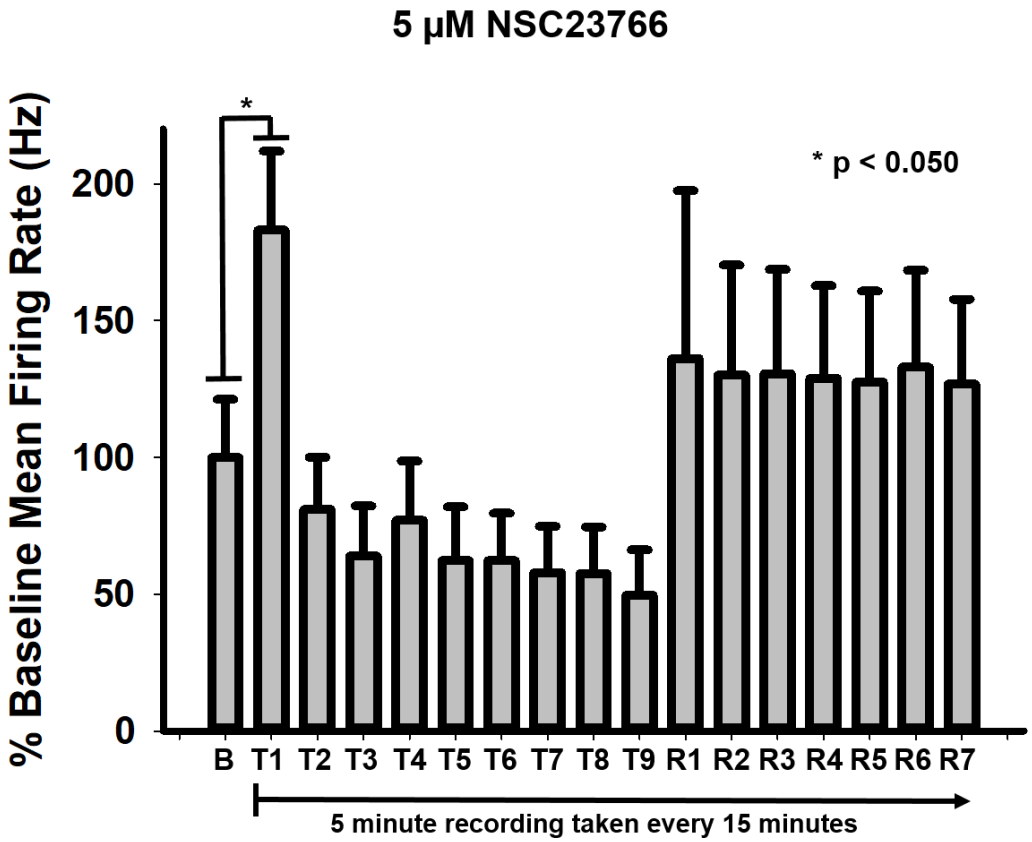

Figure 39: Mean firing rate of 5 µM NSC23766 treated cells in the Fluoxetine + NSC23766 MEA experiment. Data is displayed as % Baseline. Prior to experimentation a baseline recording (B) was taken. The cells were then treated with 5 µM NSC23766 and the five minute treatment recordings (T) were taken every 15 minutes

for two hours. The recovery (R) period was recorded beginning after the treatment was washed out. A five minute recording was take even 15 minutes, for two hours. T1 was determined to be statistically different from the baseline ($p = 0.039$).

The mean firing rate for the first treatment recording for the 5 μM NSC23766 treated cells was significantly different compared to baseline. The 5 μM NSC23766 treatment did not appear to have a significant effect on the remaining treatment recordings. Post-washout the recovery recordings were also not significant compared to the baseline. This suggests that 5 μM NSC23766 does not really have an effect on neuronal activity. In addition to examining the mean firing rate of the 5 μM NSC23766 treated cells, the burst frequency was also observed (Figure 40 – Appendix C). The burst frequency of the cells that were treated with 5 μM NSC23766 was not determined to be significant. Interestingly, the burst frequency was determined to be the same for each recording that expressed bursts. In addition to testing the 5 μM NSC23766, the effects of 10 μM NSC23766 on electrical activity was also examined (Fig 41).

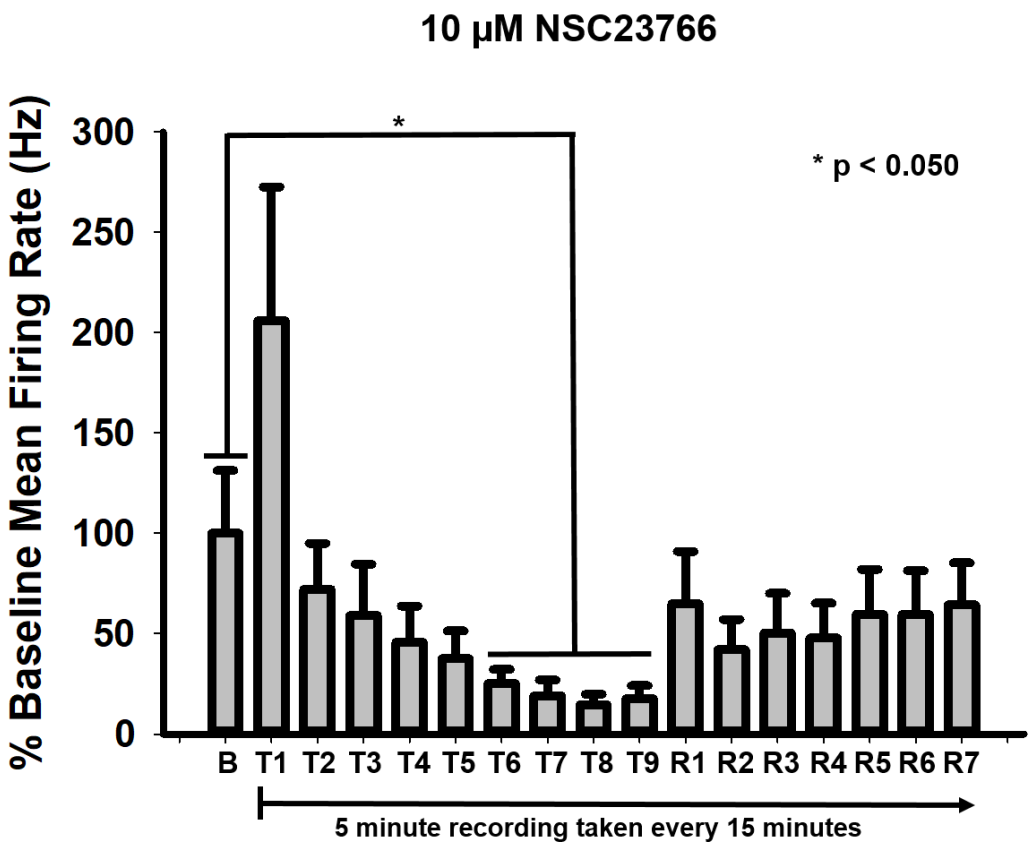

Figure 41: Mean firing rate of 10 µM NSC23766 treated cells in the Fluoxetine + NSC23766 MEA experiment. Data is displayed as % Baseline. Prior to experimentation a baseline recording (B) was taken. The cells were then treated with 10 µM NSC23766 and the five minute treatment recordings (T) were taken every 15 minutes for two hours. The recovery (R) period was recorded beginning after the treatment was washed out. A five minute recording was take even 15 minutes, for two hours. T6 ($p = 0.038$), T7 ($p = 0.038$), T8 ($p = 0.017$) and T9 ($p = 0.017$) were determined to be significantly different from the control.

In contrast to the 5 µM concentration, the 10 µM NSC23766 caused a significant decrease in the mean firing rate. It was expected that the Rac1 inhibitor would decrease neuronal activity, since it is known to cause a reduction in dendritic spines. Post-washout the electrical activity of the cells

recovered to similar levels of baseline activity. Cells treated with 10 μM NSC23766 did not display significant burst frequencies (Figure 42 – Appendix C). To further evaluate the relationship between Fluoxetine and Rac1 signaling, NSC23766 and Fluoxetine were combined and applied directly to human dissociated 'mini-brains'. In the previous MEA experiment three concentrations of Fluoxetine were examined including, 0.5 μg/ml, 1.0 μg/ml, and 1.5 μg/ml. It was determined that for each concentration an initial increase in electrical activity occurred that was followed by inhibition of neural activity. The highest concentration of Fluoxetine (1.5 μg/ml) had the greatest effect on electrical activity. After evaluating the effects of the various concentrations of Fluoxetine on neuronal signaling, the same concentrations were combined with 10 μM NSC23766 and applied to dissociated 'mini-brain' neurons. It was hypothesized that the addition of NSC23766 to Fluoxetine treatments will cause a greater decrease in electrical activity than the. This decrease in electrical activity is expected as a result of inhibited spine formation via NSC23766. Fluoxetine has previously been shown to increase dendritic spine formation. Previous MEA experiments completed in this study have shown that Fluoxetine decreases electrical activity. Therefore, it is expected the combination of the two substances will further inhibit electrical signaling. The mean firing rate for 0.5 μg/ml Fluoxetine combined with NSC23766 is displayed in Figure 43.

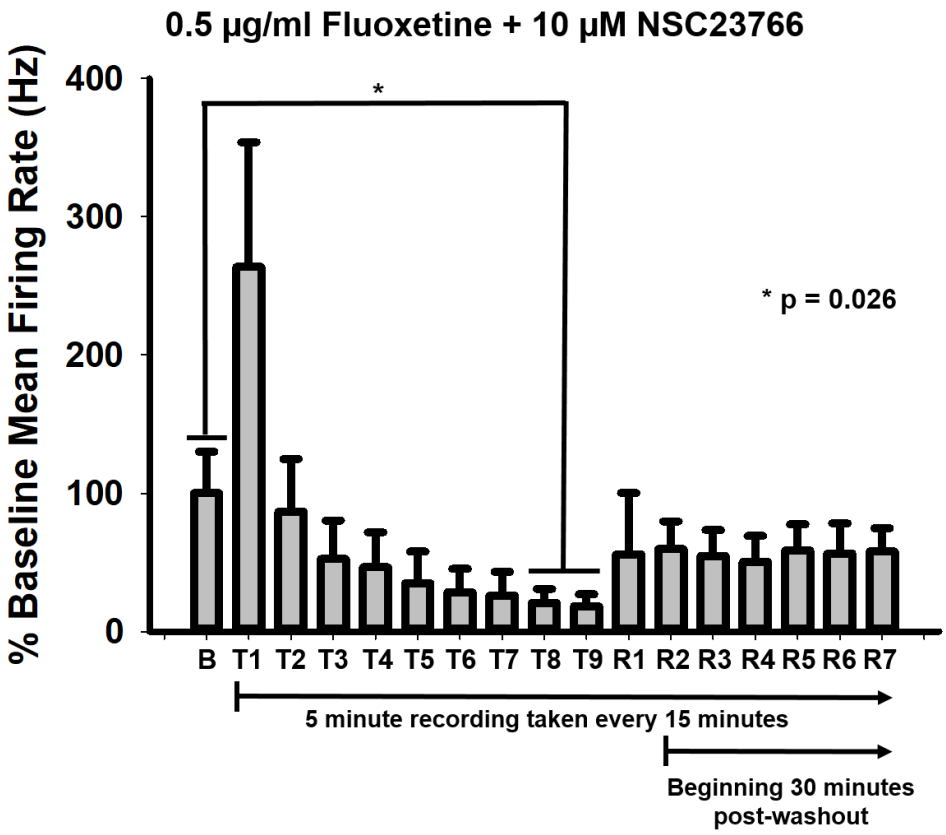

Figure 43: Mean firing rate of 0.5 µg/ml + 10 µM NSC23766 treated cells in the Fluoxetine + NSC23766 MEA experiment. Data is displayed as % Baseline. Prior to experimentation a baseline recording (B) was taken. The cells were treated with 0.5 µg/ml + 10 µM NSC23766 and the five minute treatment recordings (T) were taken every 15 minutes for two hours. The recovery (R) period was recorded beginning after the treatment was washed out. A five minute recording was take even 15 minutes, for two hours. T8 ($p = 0.026$) and T9 ($p = 0.026$) were determined to be significantly different from the control.

0.5 µg/ml Fluoxetine combined with 10 µM NSC23766 caused a significant decrease in the mean firing rate in the last two treatment recordings collected from human dissociated 'mini-brain' neurons. Post-washout the treated neurons made a full recovery. In the first MEA experiment the

0.5 µg/ml Fluoxetine without added NSC23766 did not significantly affect the mean firing rate. The burst frequency for 0.5 µg/ml Fluoxetine plus NSC23766 can be observed in Appendix C (Figure 44). Similar to the findings of the majority of the other burst frequencies in this study, the burst frequencies associated with the 0.5 µg/ml Fluoxetine plus 10 µM NSC23766 treatment were determined to not be statistically significant. Next the mean firing rate and burst frequency were analyzed for the 1.0 µg/ml Fluoxetine plus 10 µM NSC23766 treatment.

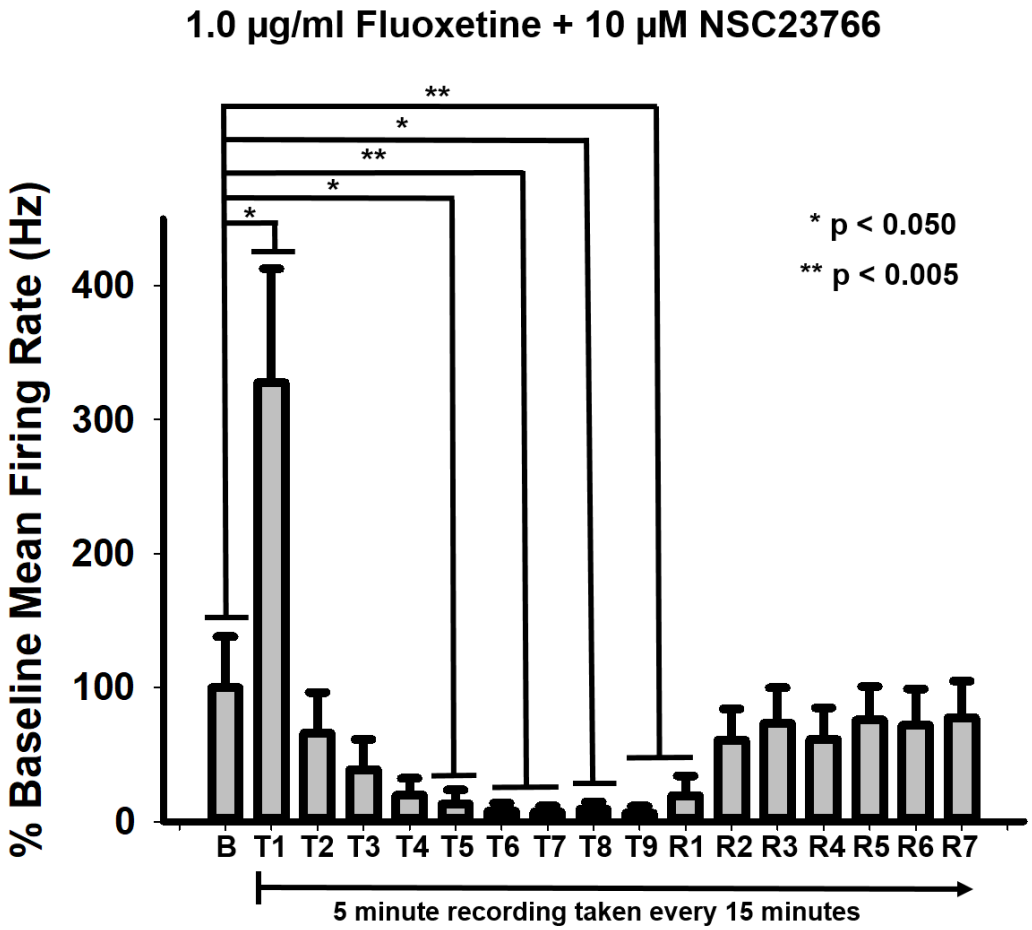

Figure 45: Mean firing rate of 1.0 µg/ml Fluoxetine + 10 µM NSC23766 treated cells in the Fluoxetine + NSC23766 MEA experiment. Data is displayed as % Baseline. Prior to experimentation a baseline recording (B) was taken. The cells were treated with 1.0 µg/ml + 10 µM NSC23766 and the five minute treatment

recordings (T) were taken every 15 minutes for two hours. The recovery (R) period was recorded beginning after the treatment was washed out. A five minute recording was take even 15 minutes, for two hours. T1 ($p = 0.031$), T5 ($p = 0.047$), T6 ($p = 0.007$), T7 ($p = 0.007$), T8 ($p = 0.035$), T9 ($p = 0.007$) and R1 ($p = 0.007$) were determined to be significantly different from the control.

The neurons that were treated with 1.0 μg/ml Fluoxetine plus 10 μM NSC23766 displayed a significant decrease in neuronal activity. Post-washout the cells did not recover until 30 minutes into the recovery period. In the previous MEA experiment, the mean firing rate of the Fluoxetine treated neurons always recovered as soon as the media was changed post-washout. This suggests that the combination of Fluoxetine and NSC23766 has a more drastic effect on neuronal signaling. It is to be expected that greater concentrations of Fluoxetine will elicit a larger inhibition response in electrical activity. In addition to affecting the mean firing rate of the treated neurons, burst activity was also impacted by the drug treatment (Figure 46 – Appendix C). It was determined that there were no significant differences between the experimental burst frequencies and the baseline burst frequency.

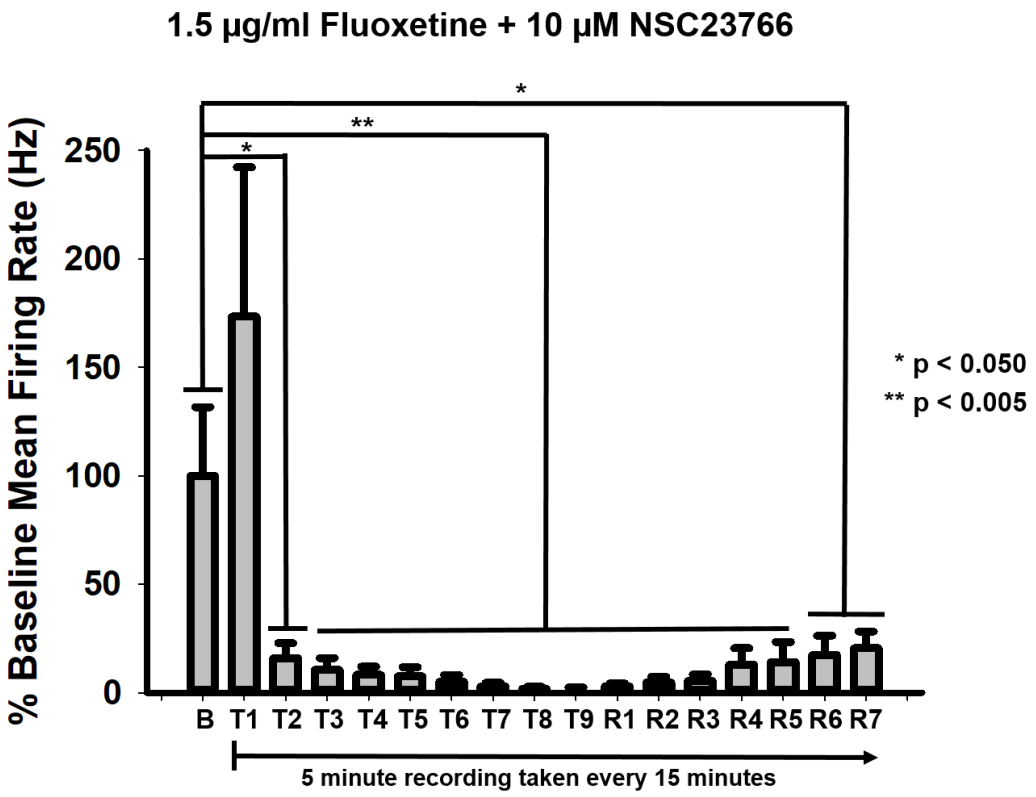

Figure 47: Mean firing rate of 1.5 µg/ml + 10 µM NSC23766 treated cells in the Fluoxetine + NSC23766 MEA experiment. Data is displayed as % Baseline. Prior to experimentation a baseline recording (B) was taken. The cells were treated with 1.5 µg/ml + 10 µM NSC23766 and the five minute treatment recordings (T) were taken every 15 minutes for two hours. The recovery (R) period was recorded beginning after the treatment was washed out. A five minute recording was take even 15 minutes, for two hours. T2 ($p = 0.007$), T3 ($p = 0.002$), T4 ($p = 0.002$), T5 ($p = 0.002$), T6 ($p = 0.001$), T7 ($p < 0.001$), T8 ($p < 0.001$), T9 ($p < 0.001$), R1 ($p < 0.001$), R2 ($p = 0.001$), R3 ($p = 0.001$), R4 ($p = 0.004$), R5 ($p = 0.004$), R6 ($p = 0.007$), and R7 ($p = 0.011$) were determined to be significantly different from the control.

1.5 µg/ml Fluoxetine plus 10 µM NSC23766 was the final concentration of Fluoxetine that was tested in the human dissociated 'mini-brain' neurons. A significant decrease in neuronal activity

was observed throughout both the treatment and recovery periods of the experiment (Figure 47). This concentration of Fluoxetine combined with NSC23766 had the greatest effect on the mean firing rate. The neurons never completely recovered during the 2 hour recording period. The burst frequency was also evaluated for this concentration (Figure 48 – Appendix C). The values were not determined to be significant from the control.

6. Discussion

Autism Spectrum Disorders (ASD) have become increasingly more prevalent in recent years. In 2000, 1 in 150 children in the United States were diagnosed with Autism[36]. This number has since increased and was reported in 2012 as 1 in 68 children being diagnosed with the disorder[36]. The increased prevalence in Autism is thought to result from a combination of environmental and genetic factors. Complex interactions between these factors are difficult to study. Due to the complexity of these interactions and also the genetic heterogeneity associated with Autism, patient specific models are necessary to take into account all factors contributing to Autism pathology. In this project patient specific human cortical organoid, 'mini-brain', models were used to examine synapse formation. While the exact cause of Autism is unknown, dysregulation of the actin cytoskeleton is thought to play a role in Autism pathology. The actin cytoskeleton is responsible for regulating multiple processes critical to brain development, including neuronal migration, neurite extension and synapse formation and refinement[9]. Synapses are connections between neurons that allow for electrical and chemical communication. Synapses can either be excitatory or inhibitory in nature. Increased levels of excitatory synapses have previously been observed in post-mortem patient samples from individuals with idiopathic Autism[10]. In the Litwa lab increased excitatory synapse levels have also been observed in patient derived 'mini-brains' from individuals with idiopathic Autism (Figure 4). In the human brain most excitatory synapses are formed on dendritic spines, which protrude from dendrites surrounding the neuronal cell body. Dendritic spines form in response to pre-synaptic input. Only spines that are actively receiving input will become and remain mature. Spines that are no longer receiving input become destabilized and are pruned away. The increased excitatory synapse levels seen in Autism

are thought to result from increased levels of dendritic spines. Post-mortem brain samples from children with Autism Spectrum Disorder (ASD) display increased levels of dendritic spines[13]. The increased levels of dendritic spines could potentially be attributed to exaggerated spine formation, incomplete pruning of inactive spines during childhood or a combination of both factors[13].

One environmental factor that is thought to play a role in Autism pathology is fetal exposure to the antidepressant Fluoxetine. Similar to what is observed in Autism, Fluoxetine has previously been shown to increase dendritic spine formation[14-17]. While the exact mechanism by which Fluoxetine increases spine density is unknown, the Rho-GTPase Rac1 may play a role in this process. Fluoxetine is a selective serotonin reuptake inhibitor that increases the amount of serotonin available at the synaptic cleft. This increase in serotonin leads to increased activation of serotonin receptors. Following the activation of 5-HT_{2A} serotonin receptors, Rac1 has been shown to initiate dendritic spine enlargement[18]. To test the hypothesis that Fluoxetine alters fetal synapse formation in Autism through the Rac1 pathway, human 'mini-brains' were created to recapitulate the second trimester fetal brain. Once the 'mini-brains' reached the appropriate stage in development (day 89 in culture) they were treated acutely with either Fluoxetine, the Rac1 inhibitor NSC23766, or a combination of both substances. Some 'mini-brains' were treated chronically with Fluoxetine prior to day 89. After 90 days in culture the 'mini-brains' were harvested, fixed, cryosectioned and stained with excitatory synapse markers. The 'mini-brain' samples were then imaged on the LSM 700 Zeiss confocal. The resulting images were analyzed using ImageJ. For this portion of the study it was hypothesized that acute Fluoxetine treatment would cause increased excitatory synapse formation in early human cortical development. It was also hypothesized that synapse enlargement would occur as a result of Fluoxetine's activation of the actin polymerization. Acute Fluoxetine was shown to significantly increase synapse size and

perimeter. This result was expected as Fluoxetine is thought to increase dendritic spine size through actin polymerization. The circularity of the synapses formed in the acutely Fluoxetine treated 'mini-brains' was determined to be significantly higher than the control. The chronic Fluoxetine treatment also was determined to increase synapse size (Figure 24) and perimeter (Figure 25). However, in contrast to these shape descriptors, the circularity (Figure 26) of both the acutely and chronically Fluoxetine treated 'mini-brain' synapses were significantly decreased from the control. This suggests that the synapses in the Fluoxetine treated 'mini-brains' were irregular in shape. The circularity of a synapse is affected by the shape of the structure that it is formed on. Since only excitatory synapses were examined in this project, it is likely that the majority of the synapses were formed on dendritic spines or spine precursors along the dendrite itself or on filopodia-like projections. This suggests that spine formation was altered in the presence of Fluoxetine. To examine whether Fluoxetine affects actin polymerization through the Rac1 pathway, some 'mini-brains' were acutely treated with a combination of Fluoxetine and the Rac1 inhibitor NSC3766. It was initially hypothesized that this combination of treatment would reduce the effect of Fluoxetine on synapse formation. Evidence supporting this hypothesis resulted from this experiment. The combination of Fluoxetine and NSC23766 was shown to decrease synapse size and perimeter in comparison to the synapses seen in 'mini-brains' treated with just Fluoxetine. In contrast, the 'mini-brains' treated with both substances displayed increased circularity compared to the synapses resulting from the Fluoxetine treatments. This suggests that the addition of NSC23766 prevents the synapses from becoming irregular. The effects of the Rac1 inhibitor when combined with Fluoxetine supports the idea that Fluoxetine may affect actin polymerization through the Rac1 pathway. The effects of NSC23766 without Fluoxetine added were also examined in this experiment. Out of all of the treatment groups, NSC23766 resembled the

characteristics of the control group the most. It was initially expected that NSC23766 would decrease synapse size and formation. However, this was not the case. Future experiments need to be conducted to analyze the effects of chronic NSC23766 on synapse formation. The resulting synapse size and density would be expected to decrease in 'mini-brains' chronically treated with NSC23766. This would be expected as a result of prolonged Rac1 inhibition. Furthermore, we could evaluate the effects of drug treatment at later stages of cortical organoid development coinciding with increased synapse formation on actin-enriched dendritic spines, rather than spine precursors. Throughout this project excitatory synapse formation was examined. Future studies need to be conducted to evaluate the effect of the Fluoxetine on inhibitory synapse formation. While excitatory synapses mainly form on dendritic spines, inhibitory synapses typically form directly on the shafts of dendrites[37]. Since Fluoxetine has been shown to impact dendritic spines, the primary sites of excitatory synapse formation, we would expected that Fluoxetine would have less of an effect on inhibitory synapse morphology. However, this is based on the assumption that Fluoxetine acts through the Rac1 pathway. It is possible that Fluoxetine could affect inhibitory synapse formation through a different mechanism.

After synapse formation was characterized in the 'mini-brain' models, the electrical synapse transmission was then evaluated. To evaluate the electrical activity, two experiments were performed using micro-electrode array technology. Prior to experimentation the 'mini-brains' were dissociated and plated on MEA plates. The neurons were then given two weeks to stabilize on the plates. Two experiments were completed to evaluate the effects of Fluoxetine on neuronal electrical activity. The first experiment tested three different concentrations of Fluoxetine on the dissociated 'mini-brain' neurons including; 0.5 μg/ml, 1.0 μg/ml and 1.5 μg/ml. It was hypothesized that Fluoxetine would cause an initial increase in electrical activity. This was

expected to happen as a result of increased activation of 5-HT_{2A} receptors resulting from the increased levels of serotonin in the synaptic cleft. It was also hypothesized that Fluoxetine would cause a decrease in activity as a result of downstream inhibitory effects of the 5-HT_{2A} receptors. It was determined that Fluoxetine did cause an initial increase in activity that was followed by inhibition of electrical activity. The concentration of 1.5 μg/ml Fluoxetine had the greatest effect on neuronal signaling (Figure 35). Following each treatment, the Fluoxetine was washed out and replaced with new media. In each case the neuronal activity made a full recovery back to baseline levels. After the effect of Fluoxetine on electrical signaling was characterized, the addition of NSC23766 to dissociated 'mini-brain' neurons was then examined. In this experiment the neurons were treated with either 5 μM NSC23766, 10 μM NSC23766, or a combination of Fluoxetine (concentrations; 0.5 μg/ml, 1.0 μg/ml and 1.5 μg/ml) and 10 μM NSC23766. It was hypothesized that NSC23766 would cause a decrease in neuronal activity. This is anticipated, because the Rac1 inhibitor has been shown to decrease dendritic spine size. Previously, in the first MEA experiment Fluoxetine was determined to cause an increase in neuronal activity that was subsequently inhibited by downstream factors. Based off of these findings it was hypothesized that the combination of Fluoxetine and NSC23766 would cause increased inhibition of neuronal activity compared to the effects of Fluoxetine alone. It was determined that the combination of both substances did cause a greater effect on electrical signaling. This was seen in the recordings taken during both the treatment and recovery recording periods. In the treatment recording period, more of the mean firing rates were significantly decreased from the control in contrast to the neurons treated with Fluoxetine alone. Also in contrast to the neurons only treated with Fluoxetine, it took longer for the neurons treated with Fluoxetine and NSC23766 to recover. The neurons treated with 1.5 μg/ml Fluoxetine and 10 μM NSC23766 never returned back to baseline. Overall it was

determined that Fluoxetine impacted both synapse formation and transmission in human 'mini-brains'.

This project has displayed the capabilities of 'mini-brains' to be used as patient-specific models to examine the effects of environmental factors on altered fetal synapse formation in Autism. To further investigate the effects of Fluoxetine on fetal synapse formation, other experiments can be completed to expand upon the results of this project. Throughout this project only excitatory synapse formation was examined. Excitatory synapse formation was chosen to be observed in this experiment due to the increased excitatory synapse levels that have been seen in relation to Autism. It is possible that Fluoxetine could also affect inhibitory synapse formation. In future experiments, the effect of Fluoxetine on inhibitory synapses also needs to be tested. Additionally, the effect of chronic Fluoxetine on electrical signaling needs to be characterized through MEA recordings. In this experiment only the effects of acute Fluoxetine on mean firing rate were examined. It is expected that chronic Fluoxetine treatment would have a different effect on signaling that acute Fluoxetine. Since chronic Fluoxetine treatment seems to decrease excitatory synapses density, it would be expected that the baseline neurotransmission would be decreased compared to the control. While the Rac1 inhibitor NSC23766 was examined in this project, in the future the effect of chronic NSC23766 also needs to be evaluated regarding synapse formation and transmission. It is expected that the chronic application of the Rac1 inhibitor would cause decreased excitatory synapse density and size. Through this project it was determined that NSC23766 when combined with Fluoxetine, reduced the effect of the anti-depressant on synapse formation. To further examine the role of Rac1 in altered synapse formation in Autism, Autism-derived 'mini-brains' can be grown and then treated with NSC23766 to determine whether the drug can restore normal synapse formation.

References

[35] Axion BioSystems. *Neuron Cell Plating Technique for MEAs*. Instructional Presentation. 2018.

[16] Bessa, J. *et al*. The mood-improving actions of anti-depressants do not depend on neurogenesis but are associated with neuronal remodeling. *Mol. Psychiatry* 14, 764-773 (2008).

[5] Boukhris, T. *et al.* Antidepressant Use During Pregnancy and the Risk of Autism Spectrum Disorder in Children. *JAMA Pediatr.* 170, 117 (2016).

[29] Buzsáki, G., Anastassiou, C. A., & Koch, C. (2012). The origin of extracellular fields and currents — EEG, ECoG, LFP and spikes. Nature Reviews. Neuroscience, 13(6), 407–420. http://doi.org/10.1038/nrn3241

[33] Castañé, A., Kargieman, L., Celada, P., Bortolozzi, A., & Artigas, F. (2015). 5-HT2A receptors are involved in cognitive but not antidepressant effects of fluoxetine. European Neuropsychopharmacology, 25(8), 1353-1361. doi:10.1016/j.euroneuro.2015.04.006

[36] CDC. *Autism Spectrum Disorders – Data and Statistics.* April 2018. https://www.cdc.gov/ncbddd/autism/data.html

[3] CDC. *Snapshot of Autism Spectrum Disorder in North Carolina.*

[2] Christensen, D. L. *et al*. Prevalance and Characteristics of Autism Spectrum Disorder Among Children Aged 8 Years – Autism and Developmental Disabilities Monitoring Network, 11 sites, United States, 2012. MMWR. *Surveill. Summ.* 65, 1-23 (2016).

[24] “Copy Number Variant Database.” *SFARI Gene*, Simons Foundation Autism Research Initiative, gene.sfari.org/database/human-gene/.

[37] Dobie, F. A., & Craig, A. M. (2011). Inhibitory synapse dynamics: Coordinated presynaptic and postsynaptic mobility and the major contribution of recycled vesicles to new synapse formation. The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 31(29), 10481-10493. doi:10.1523/JNEUROSCI.6023-10.201

[34] Dringenberg, H. C., Branfield Day, L. R., & Choi, D. H. (2014). Chronic fluoxetine treatment suppresses plasticity (long-term potentiation) in the mature rodent primary auditory cortex in vivo. Neural Plasticity, 2014, 571285. doi:10.1155/2014/571285

[22] Gabriele, S., Sacco, R. & Persico, A.M. Blood serotonin levels in autism spectrum disorder: A systematic review and meta-analysis. *Eur. Neuropsychopharmacol.* 24, 919-929 (2014).

[4] Guiard, B. P., & Giovanni, G. D. (2015). Central serotonin-2A (5-HT2A) receptor dysfunction in depression and epilepsy: the missing link? Frontiers in Pharmacology, 6, 46. http://doi.org/10.3389/fphar.2015.00046

[15] Guirado, R. *et al.* Chronic fluoextine treatment alters the structure, connectivity and plasticity of cortical interneurons. *Int, J. Neuropschopharmacol.* 17, 1635-1646 (2014).

[17] Hajszan, T. *et al*. Short-term treatment with the antidepressant fluoxetine triggers pyramidal dendritic spine synapse formation in rat hippocampus. *Eur. J. Neurosci.* 21, 1299-1303 (2005).

[25] “Human Gene Model.” *SFARI Gene*, Simons Foundation Autism Research Initiative, gene.sfari.org/database/human-gene/.

[18] Jones, K.A. *et al*. Rapid modulation of spine morphology by the 5-HT_{2A} serotonin receptor through kalirin-7 signaling. *Proc. Natl. Acad. Sci. U.S.A.* 196, 19575-80. (2009).

[12] Konietzny, A., Bär, J., & Mikhaylova, M. (2017). Dendritic actin cytoskeleton: Structure, functions, and regulations. Frontiers in Cellular Neuroscience, 11, 147. doi:10.3389/fncel.2017.00147

[8] Li, J. *et al*. Genes with de novo mutations are shared by four neuropsychiatric disorders discovered from Npdenovo database. *Mol. Psychiatry* 21, 290-7 (2016).

[11]Lin, Y.-C., & Koleske, A. J. (2010). Mechanisms of Synapse and Dendrite Maintenance and Their Disruption in Psychiatric and Neurodegenerative Disorders. Annual Review of Neuroscience, 33, 349–378. http://doi.org/10.1146/annurev-neuro-060909-153204

[1] Marchetto, MC. et al. “Altered proliferation and networks in neural cells derived from idiopathic autistic individuals.” *Molecular Psychiatry*, vol. 0, 2016, pp. 1–16.

[14] McAvoy, K. *et al*. Fluoxetine induces input-specific hippocampal dendritic spine remodeling along the septotemporal acis in adulthood and middle age. *Hippocampus* 25, 1429-1446 (2015).

[9] Menon, S. & Gupton, S. L. in *International review of cell and molecular biology* 322, 183-245 (2016).

[20] Morrison, J.L., Riggs, K. W. & Rurak, D. W. Fluoxetine during pregnancy: Impact on fetal development. *Reprod. Fertil. Dev.* 17, 642 (2005)

[30] Novellino A, Scelfo B, Palosaari T, Price A, Sobanski T, Shafer TJ, Johnstone AFM, Gross GW, Gramowski A, Schroeder O, Jügelt K, Chiappalone M, Benfenati F, Martinoia S, Tedesco MT, Defranchi E, D'Angelo P and Whelan M (2011) Development of micro-electrode array based tests for neurotoxicity: assessment of interlaboratory reproducibility with neuroactive chemicals. Front. Neuroeng. 4:4. doi: 10.3389/fneng.2011.00004

[23] Pasca, A. M. *et al*. Functional cortical neurons and astrocytes from human pluripotent stem cells in 3D culture. *Nat. Methods* 12, 671-8 (2015).

[13] Penzes, P., Cahill, M. E., Jones, K. A., VanLeeuwen, J.-E., & Woolfrey, K. M. (2011). Dendritic spine pathology in neuropsychiatric disorders. *Nature Neuroscience*, *14*(3), 285–293. http://doi.org/10.1038/nn.2741

[7] Pinto, D. *et al*. Functional impact of global rare copy number variation in autism spectrum disorders. *Nature* 466, 368-72 (2010).

[31] Sanchez, K. R., Mersha, M. D., Dhillon, H. S., Temburni, M. K. Assessment of the Effects of Endocrine Disrupting Compounds on the Development of Vertebrate Neural Network Function Using Multi-electrode Arrays. J. Vis. Exp. (134), e56300, doi:10.3791/56300 (2018).

[19] Simpson, K. *et al.* Perinatal antidepressant exposure alters cortical network function in rodents. doi: 10.1073/pnas.1109353108.

[27] Tashiro, A. & Yuste R. Regulation of dendritic spine motility and stability by Rac1 and Rho kinase: evidence for two forms of spine motility. *Mol Cell Neurosci*. 429-40. (2004). 10.1016/j.mcn.2004.04.001

[28] Tejada-Simon, M. V. Modulation of actin dynamics by Rac1 to target cognitive function. *Journal of Neurochemistry*. 113, 6. (2015). doi: 10.1111/jnc.13100.

[6] Thomas, M. A., Klaper, R.D., Kim, K., Steinberg, M. & Sarachana, T. Pyschoactive Pharmaceuticals Induce Fish Gene Expression Profiles Assiociated with Human Idiopathic Autism. *PloS One* 7, e32917 (2012).

[10] Vanleeuwen, Jon-Eric, et al. "Dendritic Spine Pathology in Neuropsychiatric Disorders." Nature Neuroscience, vol. 14, no. 3, 2011, pp. 285–293., doi:10.1038/nn.2741. Accessed 30 Aug. 2017.

[21] Weiss, L.A. *et al* Variation in ITGB3 is associated with whole-blood serotonin level and autism susceptibility. *Eur. J. Hum. Genet.* 14, 923-931 (2006)

[32] Xia, Y., Gopal, K. V., & Gross, G. W. (2003). Differential acute effects of fluoxetine on frontal and auditory cortex networks in vitro. Brain Research, 973(2), 151-160. doi:10.1016/S0006-8993(03)02367-9

[26] Yuste, R., & Bonhoeffer, T. (2001). Morphological changes in dendritic spines associated with long-term synaptic plasticity. Annual Review of Neuroscience, 24(1), 1071-1089. doi:10.1146/annurev.neuro.24.1.1071

Appendix A

Treatment Media Components – Fluoxetine MEA Experiment

Treatment Type	Treatment Amount	Media Amount
DMSO	11.2 µl	1400 µl
Fluoxetine (0.5 µg/ml)	5.6 µl	1400 µl
Fluoxetine (1.0 µg/ml)	11.2 µl	1400 µl
Fluoxetine (1.5 µg/ml)	16.8 µl	1400 µl

Treatment Media Components – Fluoxetine + NSC23766 MEA Experiment

Treatment Type	Treatment Amount	Media Amount
DMSO	9.6 µl	800 µl
NSC23766 (5 µM)	1.6 µl	800 µl
NSC23766 (10 µM)	3.2 µl	800 µl
Fluoxetine (0.5 µg/ml) + NSC23766 (10 µM)	3.2 µl (Fluoxetine) 3.2 µl (NSC23766)	800 µl
Fluoxetine (1.0 µg/ml) + NSC23766 (10 µM)	6.4 µl (Fluoxetine) 3.2 µl (NSC23766)	800 µl
Fluoxetine (1.5 µg/ml) + NSC23766 (10 µM)	9.6 µl (Fluoxetine) 3.2 µl (NSC23766)	800 µl

Appendix B

Confocal Images Settings

'Mini-Brain' Set	**Set R**		**Set S**		**Set T**		**Set U**	
Lasers	**Power**	**Gain**	**Power**	**Gain**	**Power**	**Gain**	**Power**	**Gain**
639	5.00%	700	5.00%	625	2.80%	700	3.50%	750
555	8.00%	700	8.00%	625	8.00%	700	8.00%	750
488	3.00%	625	3.00%	625	2.80%	625	4.50%	700
405	1.00%	625	1.20%	625	2.00%	625	0.90%	700

Confocal Images of 'Mini-Brain' Samples

Representative samples of 'mini-brain' images taken on the Zeiss LSM 700 confocal microscope. Each series of images includes individual images of ActBmEGFP, PSD-95 and VGLUT-1. An image showing the co-localization of the pre- (VGLUT-1) and post-synaptic (PSD-95) markers and a merge image of all three markers is also included.

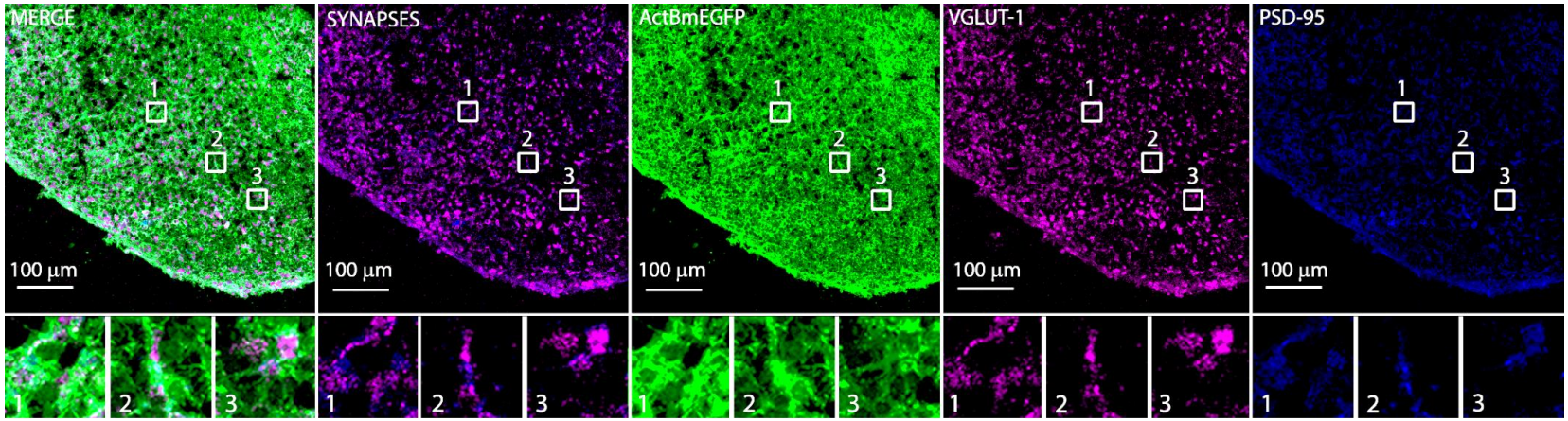

Figure 14: Immunofluorescent images of control ‘mini-brain’ sample. To examine excitatory synapse formation each ‘mini-brain’ was stained with a pre-synaptic maker (VGLUT-1) and a post-synaptic marker (PSD-95). These two markers were co-localized to determine the presence of excitatory synapses. In addition to synaptic markers, the ActBmEGFP cells endogenously expressed GFP as an actin marker. Increased magnification of three areas in the ‘mini-brain’ display neuronal circuitry and localization of relevant markers.

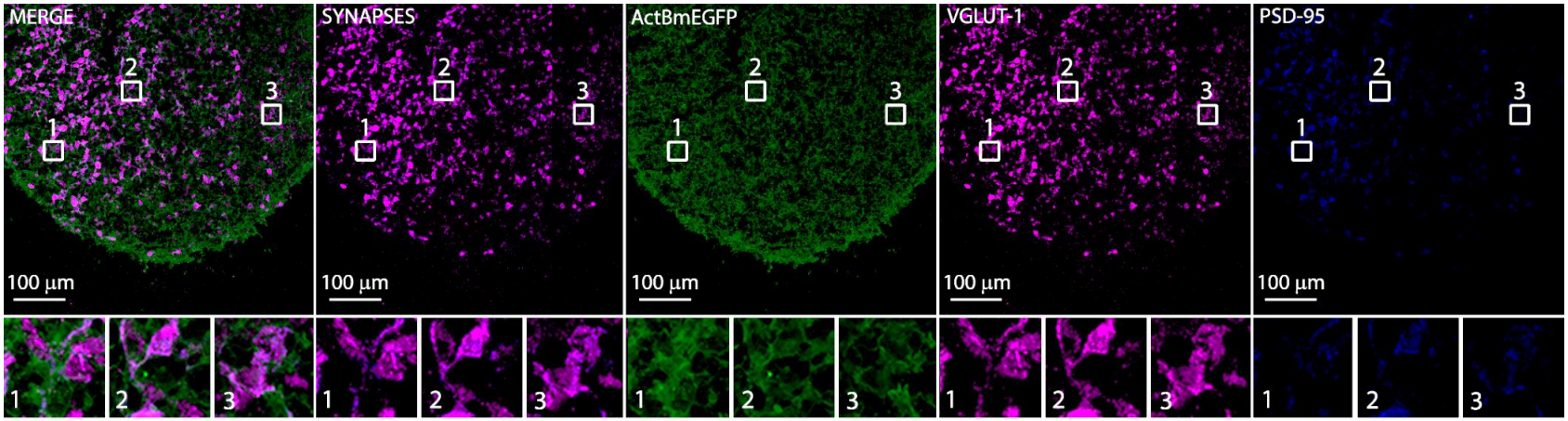

Figure 15: Immunofluorescent images of chronically Fluoxetine-treated ‘mini-brain’ sample. To examine excitatory synapse formation each ‘mini-brain’ was stained with a pre-synaptic maker (VGLUT-1) and a post-synaptic marker (PSD-95). These two markers were co-localized to determine the presence of excitatory synapses. In addition to synaptic markers, the ActBmEGFP cells endogenously expressed GFP as an actin marker. Increased magnification of three areas in the ‘mini-brain’ display neuronal circuitry and localization of relevant markers.

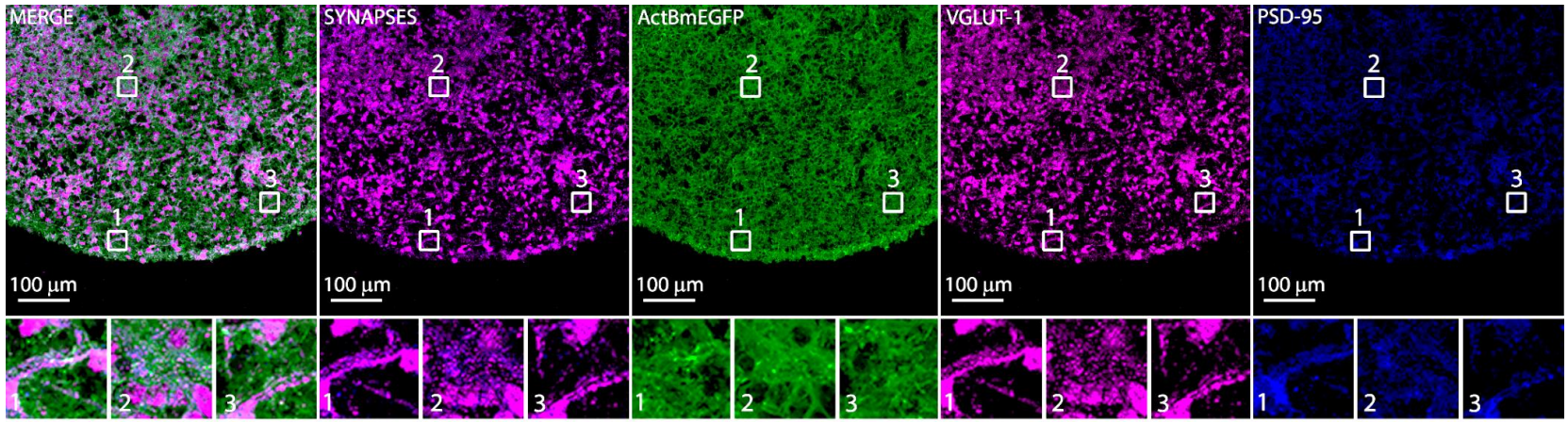

Figure 16: Immunofluorescent images of acutely Fluoxetine-treated 'mini-brain' sample. To examine excitatory synapse formation each 'mini-brain' was stained with a pre-synaptic maker (VGLUT-1) and a post-synaptic marker (PSD-95). These two markers were co-localized to determine the presence of excitatory synapses. In addition to synaptic markers, the ActBmEGFP cells endogenously expressed GFP as an actin marker. Increased magnification of three areas in the 'mini-brain' display neuronal circuitry and localization of relevant markers.

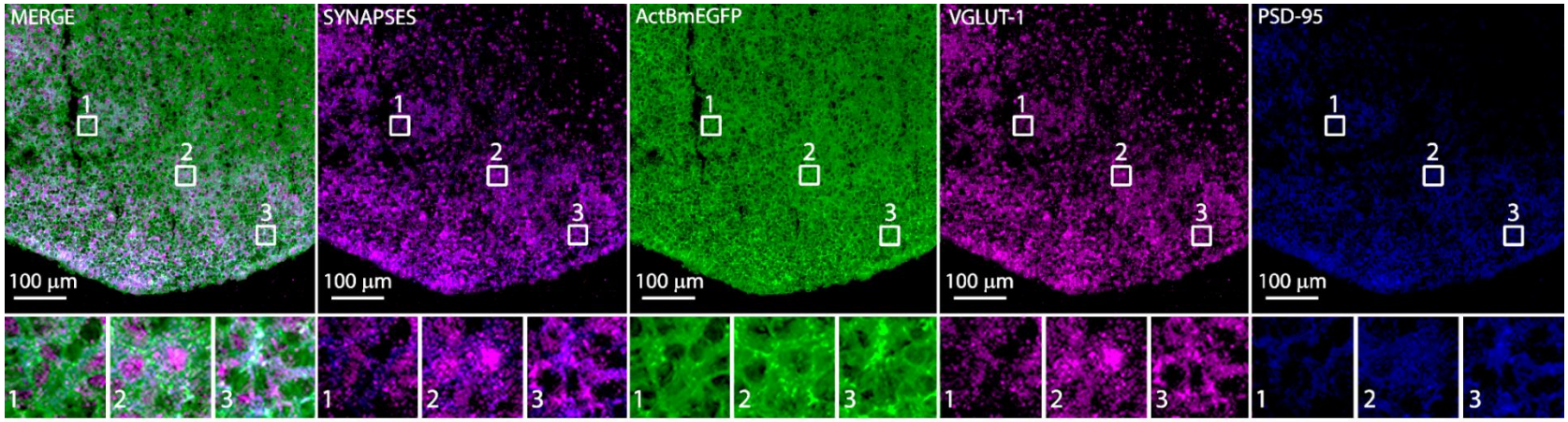

Figure 17: Immunofluorescent images of NSC23766 treated 'mini-brain' sample. To examine excitatory synapse formation each 'mini-brain' was stained with a pre-synaptic maker (VGLUT-1) and a post-synaptic marker (PSD-95). These two markers were co-localized to determine the presence of excitatory synapses. In addition to synaptic markers, the ActBmEGFP cells endogenously expressed GFP as an actin marker. Increased magnification of three areas in the 'mini-brain' display neuronal circuitry and localization of relevant markers.

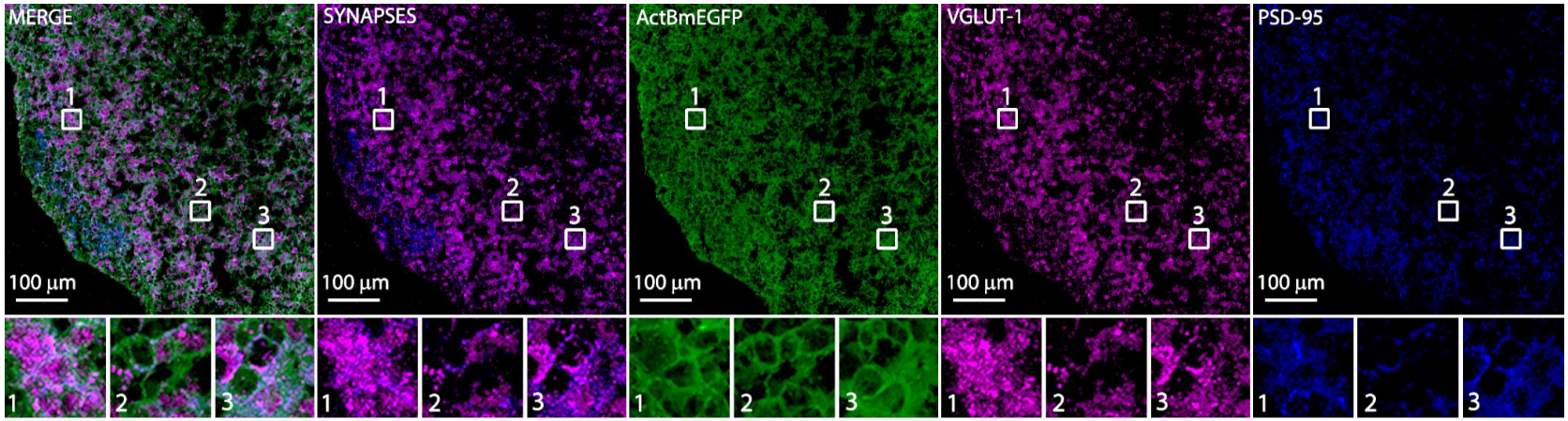

Figure 18: Immunofluorescent images of 'mini-brain' sample treated with Fluoxetine and NSC23766. To examine excitatory synapse formation each 'mini-brain' was stained with a pre-synaptic maker (VGLUT-1) and a post-synaptic marker (PSD-95). These two markers were co-localized to determine the presence of excitatory synapses. In addition to synaptic markers, the ActBmEGFP cells endogenously expressed GFP as an actin marker. Increased magnification of three areas in the 'mini-brain' display neuronal circuitry and localization of relevant markers.

Co-localization of VGLUT-1 and PSD-95

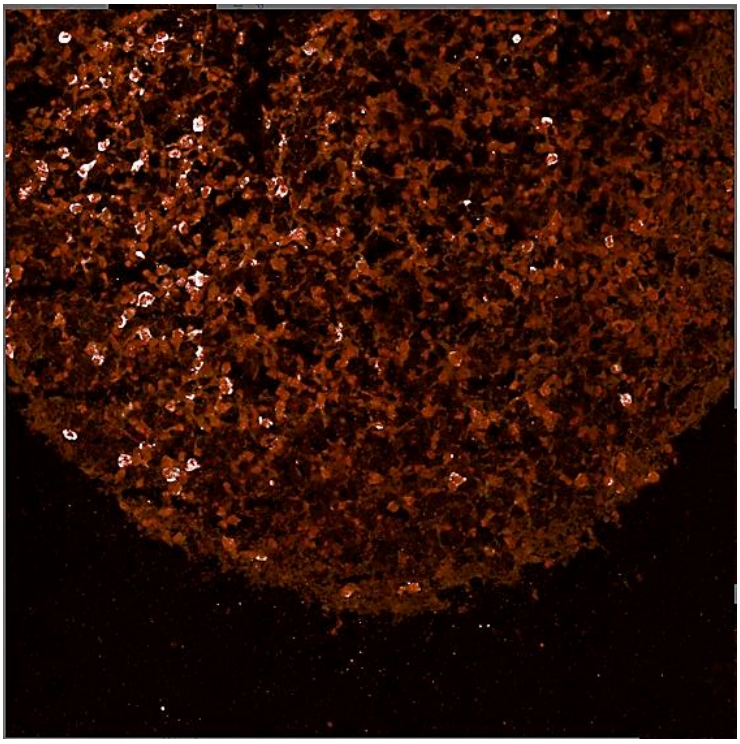

Figure 20: Co-localized points of VGLUT-1 (green) and PSD-95 (red) for chronic Fluoxetine sample.

White indicates a co-localized point.

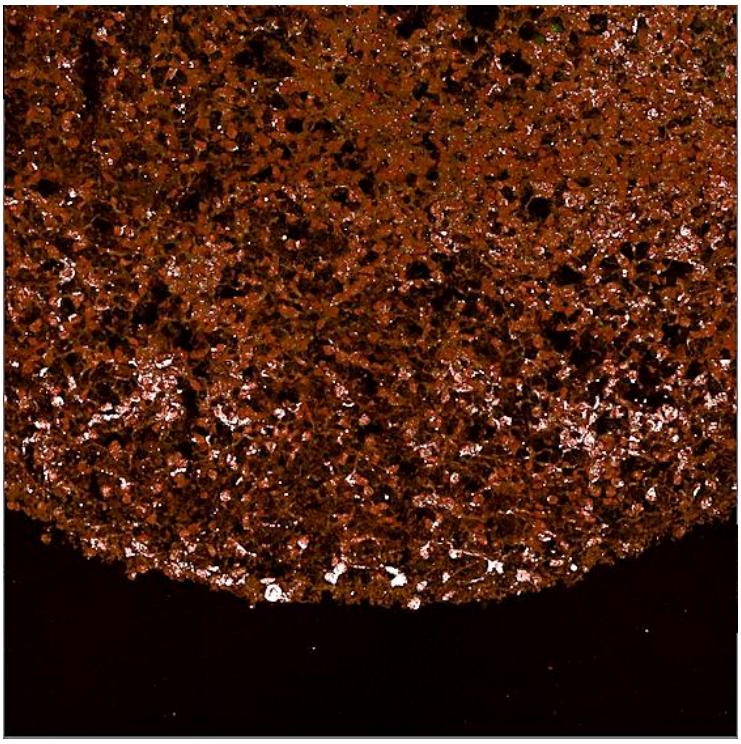

Figure 21: Co-localized points of VGLUT-1 (green) and PSD-95 (red) for acute Fluoxetine sample. White indicates a co-localized point.

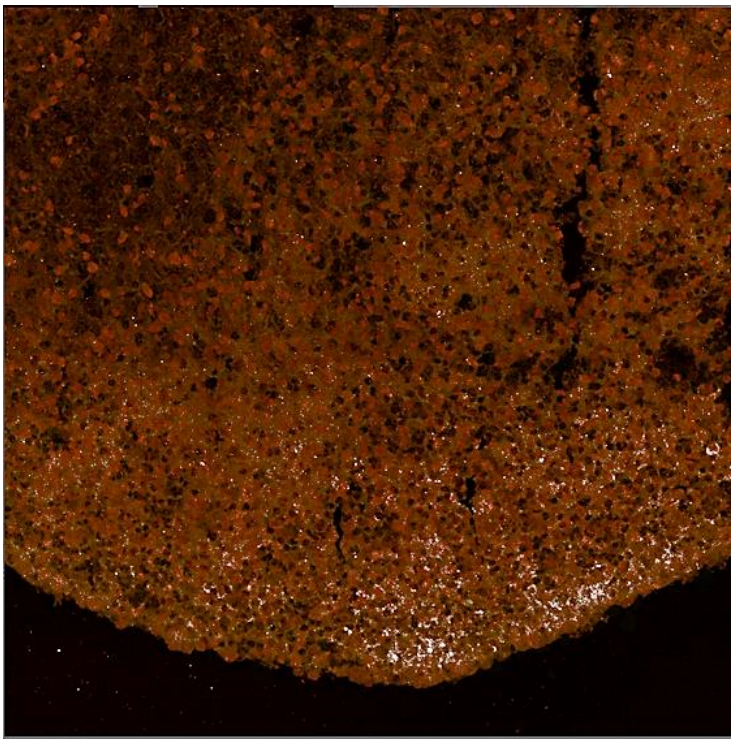

Figure 22: Co-localized points of VGLUT-1 (green) and PSD-95 (red) for NSC23766 sample. White indicates a co-localized point.

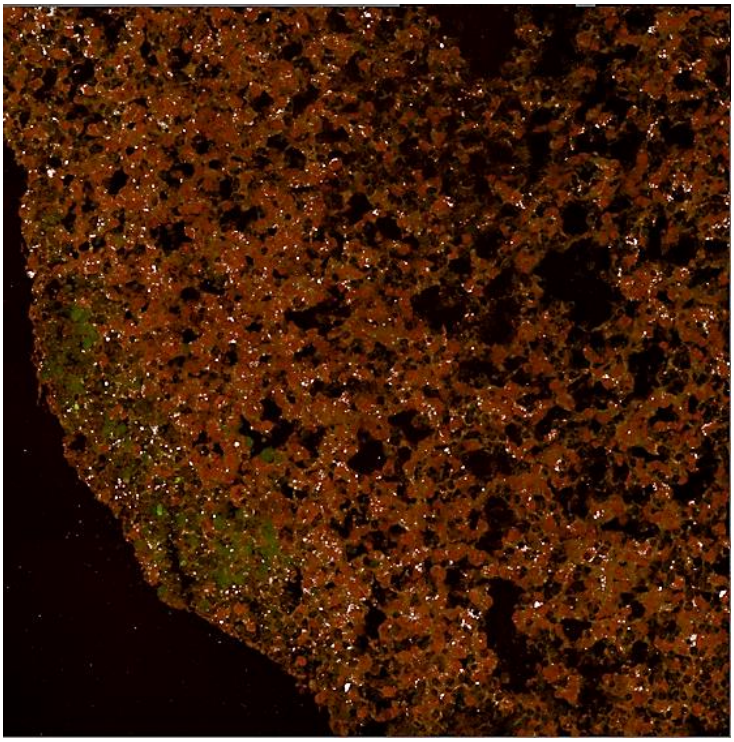

Figure 23: Co-localized points of VGLUT-1 (green) and PSD-95 (red) for Fluoxetine + NSC23766 sample. White indicates a co-localized point.

Appendix C

Burst Frequency Data

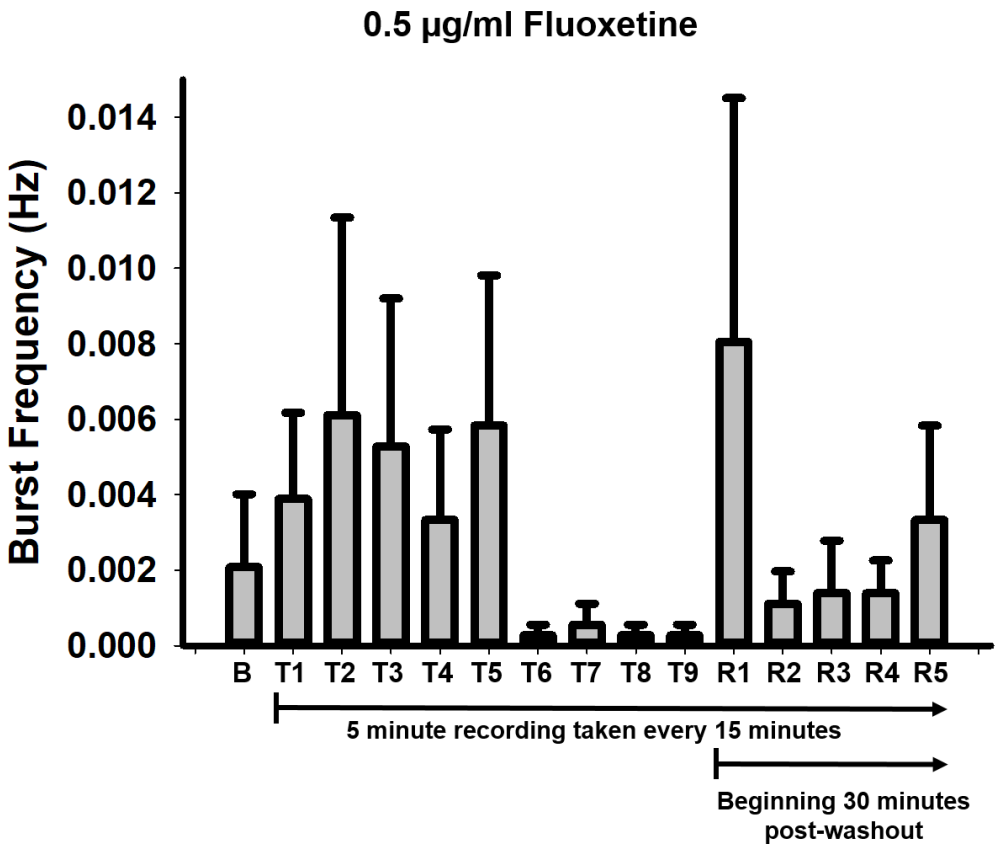

Figure 32: Burst frequency of Fluoxetine treated cells (final concentration 0.5 µg/ml) in Fluoxetine MEA experiment. Prior to experimentation a baseline recording (B) was taken. The cells were then treated with 0.5 µg/ml Fluoxetine and the five minute treatment recordings (T) were taken every 15 minutes for two hours. The recovery (R) period was recorded starting 30 minutes after the drug was washed out for a total time of one hour and 30 minutes.

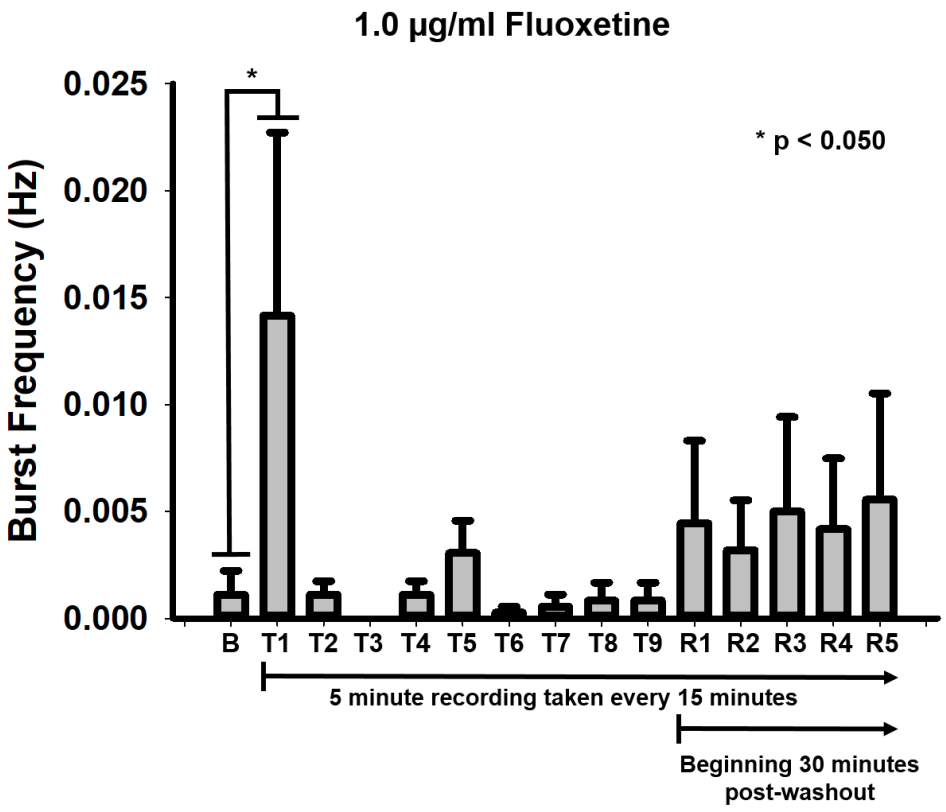

Figure 34: Burst frequency of Fluoxetine treated cells (final concentration 1.0 µg/ml) in Fluoxetine MEA experiment. Prior to experimentation a baseline recording (B) was taken. The cells were then treated with 1.0 µg/ml Fluoxetine and the five minute treatment recordings (T) were taken every 15 minutes for two hours. The recovery (R) period was recorded starting 30 minutes after the drug was washed out for a total time of one hour and 30 minutes. When compared to baseline T1 was determined to be statistically significant ($p = 0.019$).

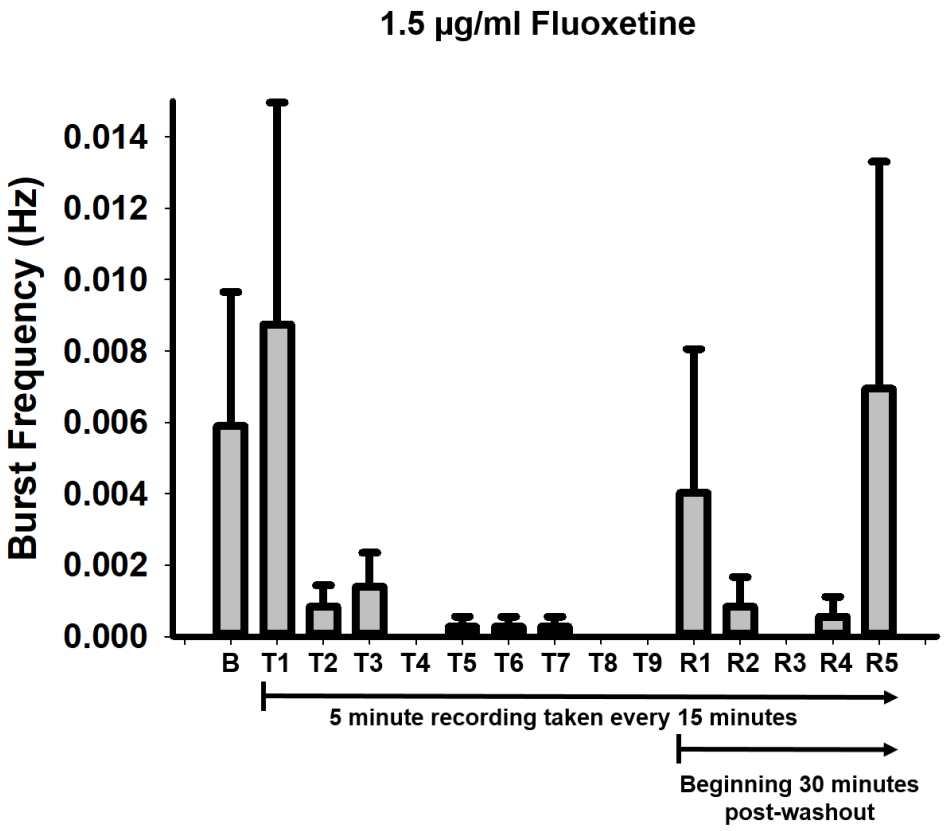

Figure 36: Burst frequency of Fluoxetine treated cells (final concentration 1.5 µg/ml) in Fluoxetine MEA experiment. Prior to experimentation a baseline recording (B) was taken. The cells were then treated with 1.5 µg/ml Fluoxetine and the five minute treatment recordings (T) were taken every 15 minutes for two hours. The recovery (R) period was recorded starting 30 minutes after the drug was washed out for a total time of one hour and 30 minutes.

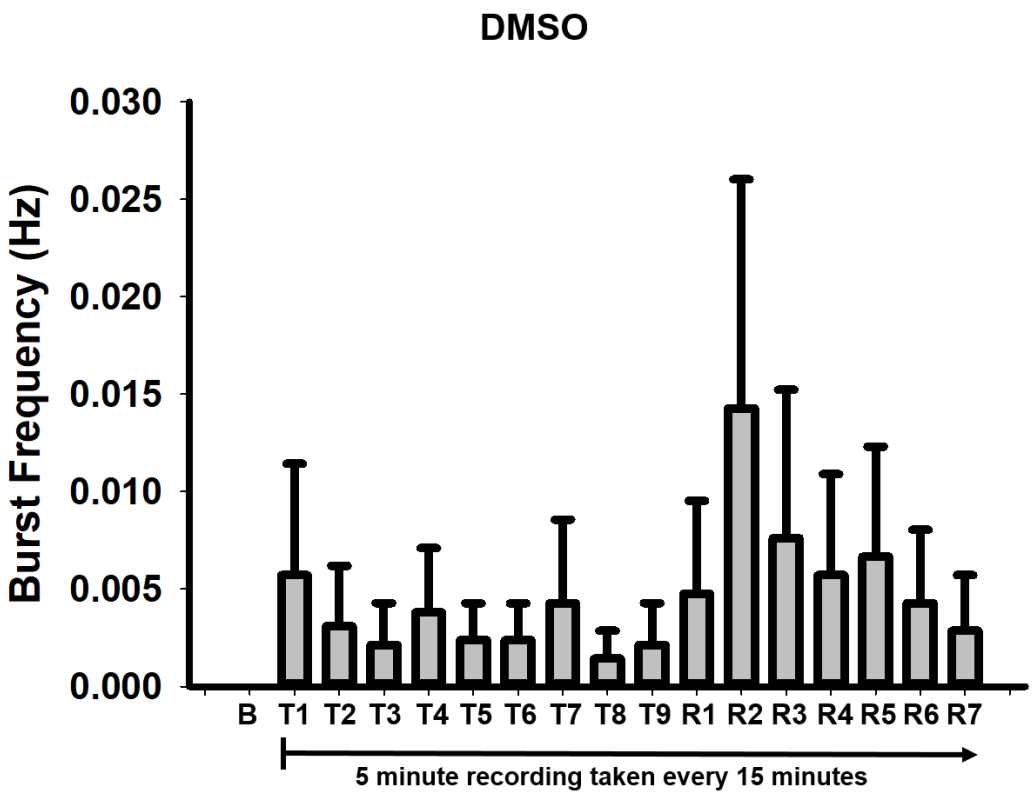

Figure 38: Burst frequency of DMSO treated cells in the Fluoxetine + NSC23766 MEA experiment. Prior to experimentation a baseline recording (B) was taken. The cells were then treated with DMSO and the five minute treatment recordings (T) were taken every 15 minutes for two hours. The recovery (R) period was recorded beginning after the treatment was washed out. A five minute recording was take even 15 minutes, for two hours.

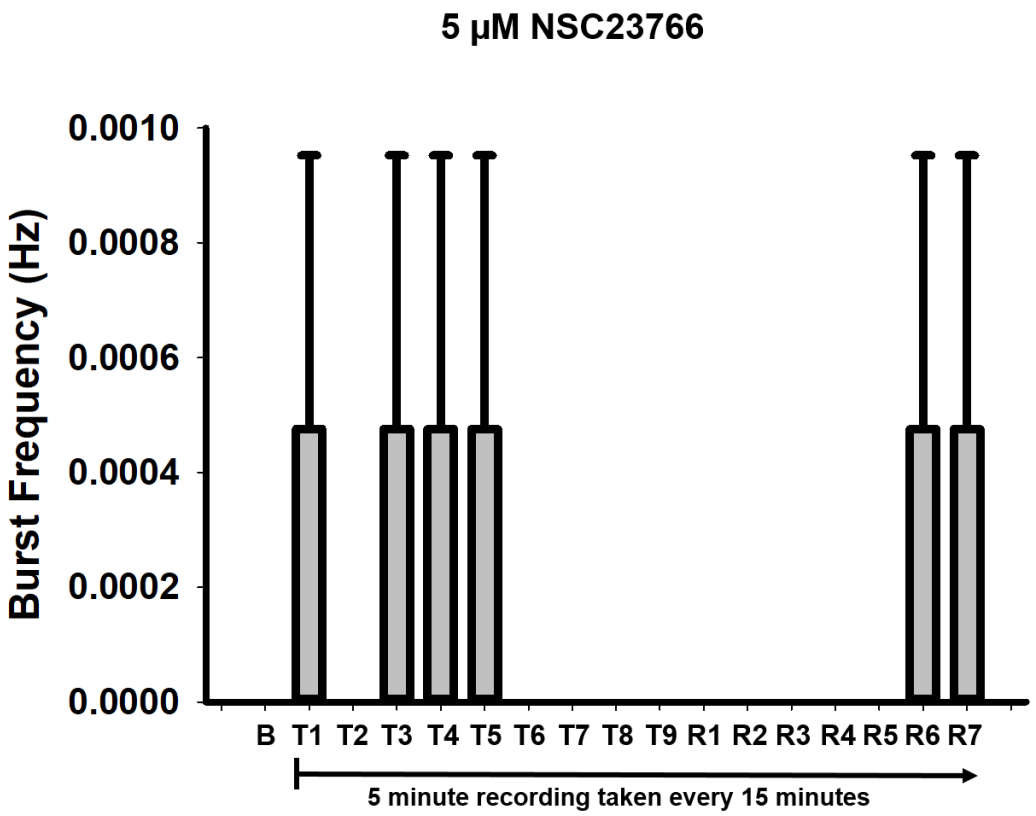

Figure 40: Burst frequency of 5 µM NSC23766 treated cells in the Fluoxetine + NSC23766 MEA experiment. Prior to experimentation a baseline recording (B) was taken. The cells were then treated with 5 µM NSC23766 and the five minute treatment recordings (T) were taken every 15 minutes for two hours. The recovery (R) period was recorded beginning after the treatment was washed out. A five minute recording was take even 15 minutes, for two hours.

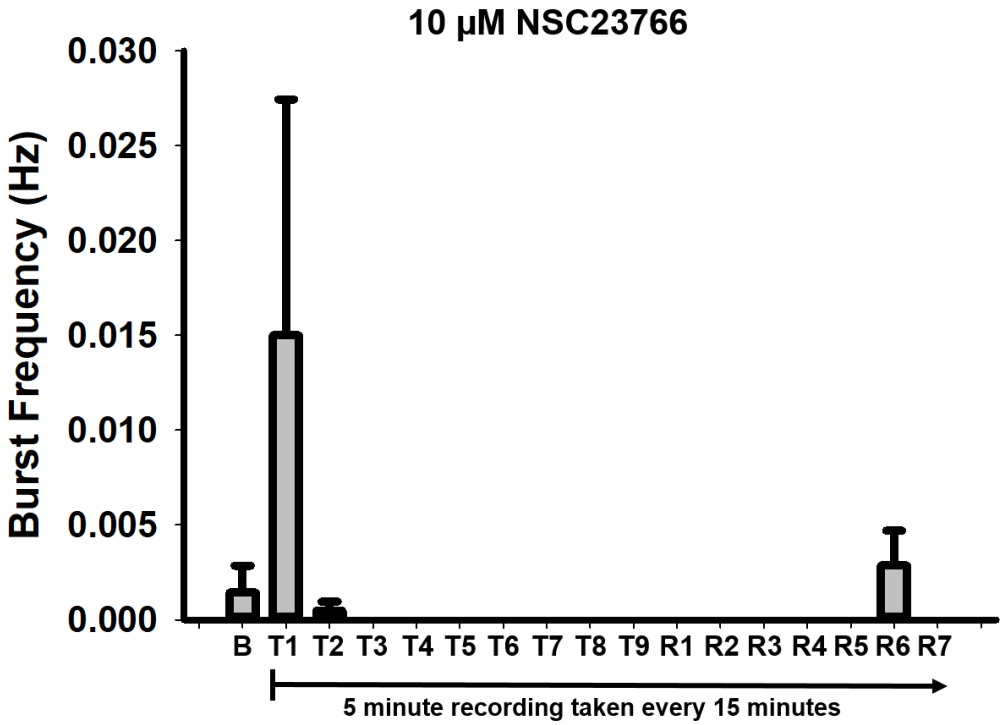

Figure 42: Burst frequency of 10 µM NSC23766 treated cells in the Fluoxetine + NSC23766 MEA experiment. Prior to experimentation a baseline recording (B) was taken. The cells were then treated with 10 µM NSC23766 and the five minute treatment recordings (T) were taken every 15 minutes for two hours. The recovery (R) period was recorded beginning after the treatment was washed out. A five minute recording was take even 15 minutes, for two hours.

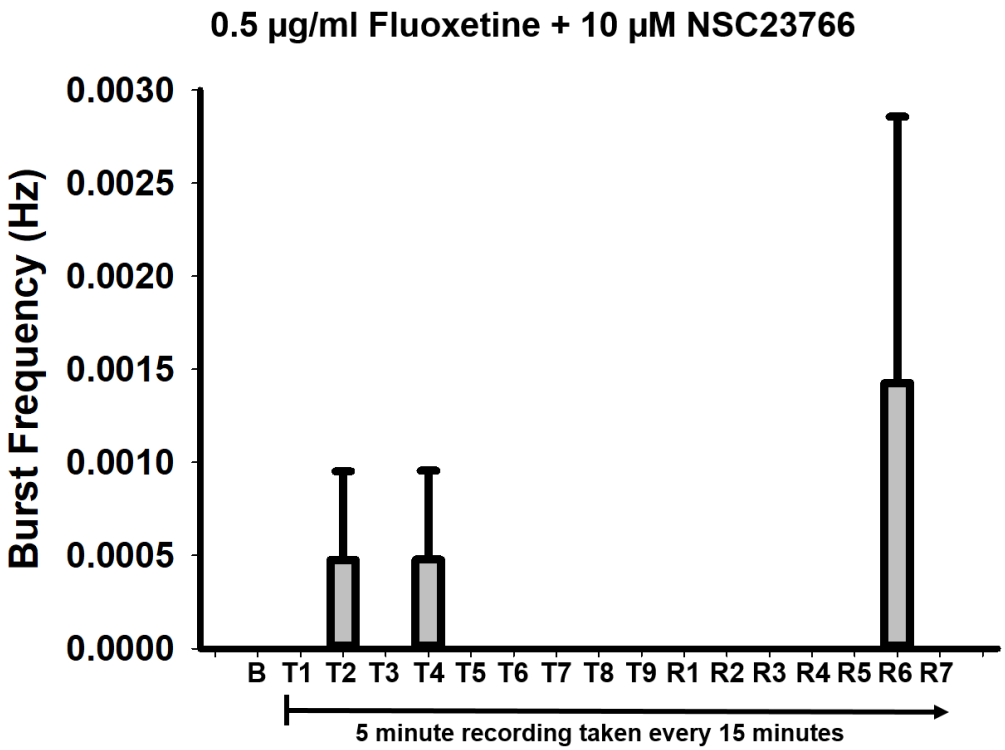

Figure 44: Burst frequency of 0.5 µg/ml + 10 µM NSC23766 treated cells in the Fluoxetine + NSC23766 MEA experiment. Prior to experimentation a baseline recording (B) was taken. The cells were then treated with 0.5 µg/ml + 10 µM NSC23766 and the five minute treatment recordings (T) were taken every 15 minutes for two hours. The recovery (R) period was recorded beginning after the treatment was washed out. A five minute recording was take even 15 minutes, for two hours.

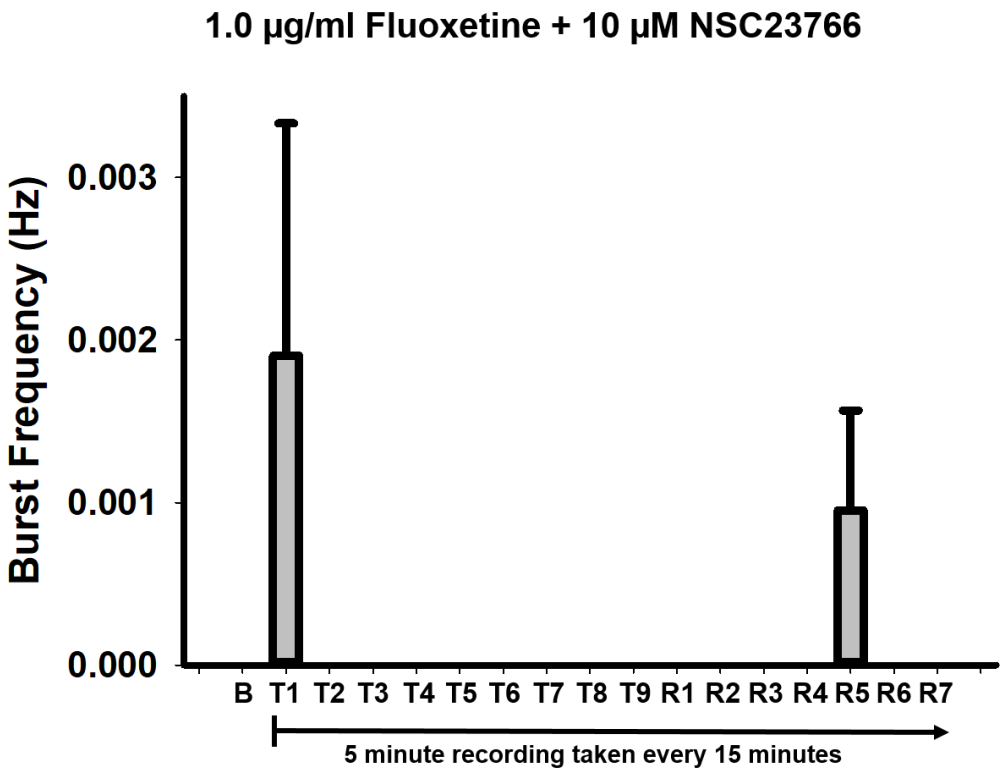

Figure 46: Burst frequency of 1.0 µg/ml + 10 µM NSC23766 treated cells in the Fluoxetine + NSC23766 MEA experiment. Prior to experimentation a baseline recording (B) was taken. The cells were then treated with 1.0 µg/ml + 10 µM NSC23766 and the five minute treatment recordings (T) were taken every 15 minutes for two hours. The recovery (R) period was recorded beginning after the treatment was washed out. A five minute recording was take even 15 minutes, for two hours.

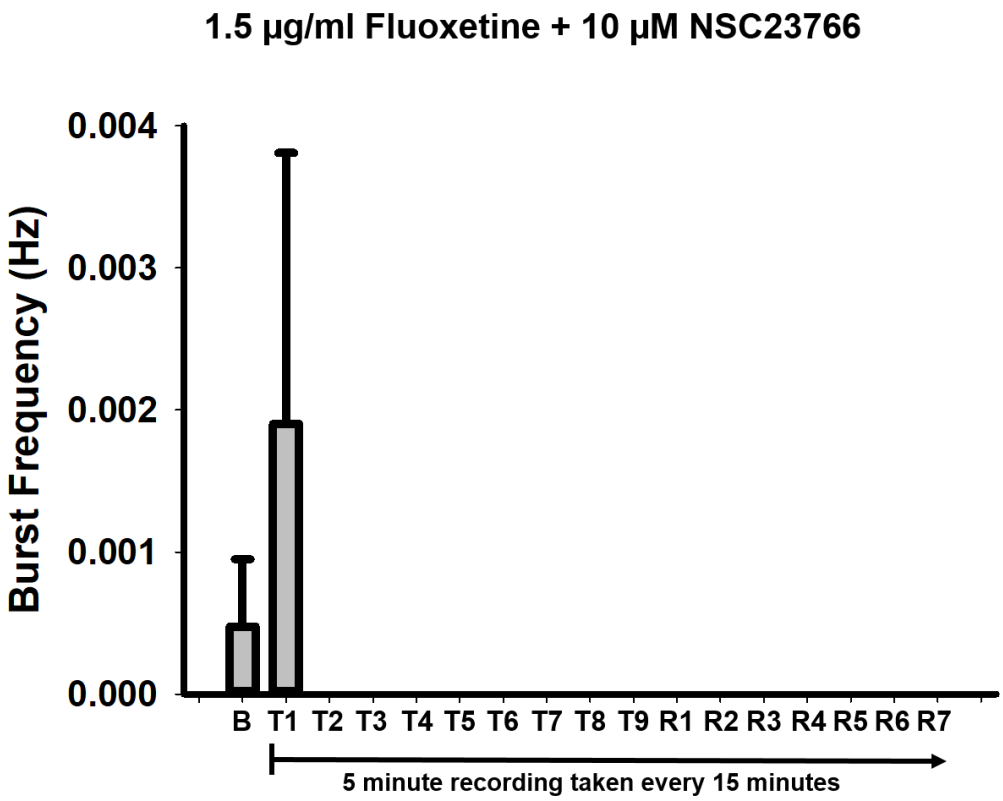

Figure 48: Burst frequency of 1.5 µg/ml + 10 µM NSC23766 treated cells in the Fluoxetine + NSC23766 MEA experiment. Prior to experimentation a baseline recording (B) was taken. The cells were then treated with 1.5 µg/ml + 10 µM NSC23766 and the five minute treatment recordings (T) were taken every 15 minutes for two hours. The recovery (R) period was recorded beginning after the treatment was washed out. A five minute recording was take even 15 minutes, for two hours.