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Qiaochu (George) Gu April 4, 2016

Investigation of the disruption of miRNA-137 and its effects on the Nrg/ErbB4 pathway in schizophrenia

by

Qiaochu (George) Gu

Gary J. Bassell
Adviser

Department of Biology

Gary J. Bassell

Adviser

Kristen Frenzel

Committee Member

Roger Deal

Committee Member

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Qiaochu Gu

Gary J. Bassell

Adviser

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Abstract

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By Qiaochu (George) Gu

Schizophrenia is a mental disorder that affects over 51 million people worldwide. The disease is characterized by delusions and has a molecular mechanism that is poorly understood. The etiology of schizophrenia has both a genetic and environmental background that could be attributed to interferences of certain protein ligands and different signaling pathway. Neuregulin-1 (Nrg1) is an epidermal growth factor that is implicated as a key candidate in schizophrenia. Research has shown that the dysregulation of Nrg1 interaction with ErbB4, a receptor tyrosine kinase, results in neurodevelopmental defects as well as impaired synaptic connectivity in the hippocampus. Findings also show the significant roles that miRNAs play in manipulating the expression of target genes as well as their importance in proper developmental processes in neurons. To date, there has been some data showing the regulatory effect of a specific miRNA-137 on protein expression and dendritic morphology under the Nrg/ErbB4 pathway, but the mechanism by which this happens is still unclear. We designed an experiment utilizing western blots to look at the expression levels of GSK3β under the disruption of miRNA-137. We hypothesize that miRNA-137 overexpression will cause a disruption in GSK3β protein expression levels through a direct mechanism. In addition, we also used immunofluorescence to assess the effect of miRNA-137 overexpression on dendritic morphology. We believe that overexpression of miRNA-137 would disrupt neuregulin signaling and ultimately inhibit dendritic morphology. Our third aim incorporates a PLA assay to look at the effect of neuregulin stimulation on MAP2 protein synthesis. We hypothesize MAP2 protein synthesis to increase under the influence of neuregulin. Results show that miRNA-137 overexpression decreases the phospho and total protein expression levels of GSK3β. A similar trend was also observed in morphology, in that miR-137 overexpression decreased dendritic arborization in nucleofected neurons (nonsignificant). Neuregulin also stimulated the synthesis of MAP2 proteins in the dendritic region, although more trials are required to replicate these results. Taken together, this study not only provides support that miR-137 is an important modulator of specific signaling pathways implicated in schizophrenia, but also paves the way for a novel approaches toward understanding the etiology of this disease.

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Introduction:

Schizophrenia is a severe brain disorder with poorly understood etiology. About 0.3-0.7% of people are affected by schizophrenia during their lifetime (Van Os et al., 2009). People who are diagnosed with the disorder typically report having symptoms such as delusions, hallucinations, reduced social engagement as well as unclear or confused thinking (Andreasen et al., 1991). Causes of schizophrenia can be both genetic and environmental in nature. The greatest risk for developing schizophrenia is having a first degree relative with the disease, with more than 40% of monozygotic twins of those with schizophrenia also being affected (Picchioni et al., 2007). Many genetic candidates have also been proposed, including specific copy number variations, NOTCH4, and histone protein loci (Mclaren et al., 2010).

Current treatments for schizophrenia are entirely symptom based. However, clinical trials for cures are ongoing. The primary treatment for schizophrenia is antipsychotic medications, often in combination with psychological and behavioral support (Van Os et al., 2009). Assertive community treatment, supported employment and cognitive remediation also play roles in ameliorating certain disease symptoms and may help reduce the likelihood of disease progression (Medalia et al., 2009). Current research has shown promising results in using minocycline, a broad spectrum tetracycline antibiotic, to treat schizophrenia (Dean et al., 2012). Nidotherapy, or efforts to change the environment of people diagnosed with schizophrenia, is also being studied (Chamberlain et al., 2013).

Differences in brain structures have often been noted in patients who have developed schizophrenia. Studies using neuropsychological tests and brain imaging technologies such as fMRI have shown most of these differences to be localized in the frontal lobes, hippocampus and temporal lobes

(Kircher et al., 2005). One of the most common hypotheses used to explain the link between altered brain function and schizophrenia is the dopamine hypothesis, which attributes hallucinations and delusions as the brain's faulty interpretation of the misfiring of dopaminergic neurons. Neurochemical findings are consistent in showing that schizophrenia, in its acute psychotic state, is associated with an increase in dopamine synthesis, dopamine release, and resting-state synaptic dopamine concentrations (Van Os et al., 2009)

Although conventional antipsychotics act by blocking dopamine receptors, there is considerable recent evidence linking defects in other signaling ligands and their receptor pathways. Numerous studies indicate alterations in different aspects of brain development as a result of this signaling dysregulation, which includes deficits in neuronal migration, neurotransmitter receptor expression and myelination (Corfas et al., 2004). The genes that encodes neuregulin-1 (NRG1) and one of its receptors, ErbB4, have been identified as potential susceptibility genes for schizophrenia (Stefansson et al., 2001; Silberberg et al., 2006). Stimulation of ErbB4 on neuronal cell surfaces or postsynaptic membranes has been implicated to alter the expression of glutamatergic receptors such as the AMPA and NMDA receptors (Abe et al., 2011).

Neuregulin-1 (NRG-1)

Neuregulin-1 (NRG-1) is a member of a family of four growth factor, the neuregulins, which are structurally related to the epidermal growth factor genus of cell-cell signaling molecules (Law et al., 2004). In the brain, roles of neuregulin-1 include synapse formation, activity dependent synaptic plasticity, regulation of *N*-methyl-D-aspartate and acetylcholine receptor subunit expression (Stefansson et al., 2002). Neuregulin also influences the differentiation, proliferation and migration of different cell

types such as neurons (Law et al., 2004). Results of a genome wide scan for linkage to schizophrenia and a fine mapping at a locus on chromosome 8p identified neuregulin-1 (NRG1 [MIM 142445]) as a candidate gene for schizophrenia (Stefansson et al., 2002). Transgenic mice that were hypomorphic for NRG1 also exhibited behavioral abnormalities and had fewer functional NMDA receptors than do control mice, similar to what is seen in the brains of schizophrenic patients (Stefansson et al., 2002).

Receptor tyrosine-protein kinase erbB4 (ErbB4)

Receptor tyrosine-protein kinase erbB-4 is a member of the epidermal growth factor receptor subfamily. ErbB4 is a single-pass type I transmembrane protein with multiple furin-like cysteine rich domains, a tyrosine kinase domain, a phosphatidylinositol-3 kinase binding site and a PDZ domain binding motif (HUGO Gene Nomenclature Committee). There are four ErbB receptors expressed in the brain, and ErbB4 is the only receptor isoform that can both directly bind NRG1 and be catalytically active (Cahill et al., 2013). Recent studies have suggested the likelihood of ErbB4 to be a key candidate in schizophrenia (Silberberg et al., 2006). Loss of NRG1/ErbB4 signaling has been shown to impair dendritic spine maturation and perturb the interactions of postsynaptic scaffolding proteins with glutamate receptors in mice (Barros et al., 2009). In addition, ErbB2/B4-deficient mice also showed increased aggression and reduced prepulse inhibition (Barros et al., 2009).

miRNAs and Schizophrenia

microRNAs (miRNAs), which are small non-coding RNA molecules, have emerged as significant regulatory factors in the nervous system (Ambros et al., 2004). These RNAs are usually 18-26 nucleotides

long and associate with a large multi-protein effector complex known as the RNA-induced silencing complex (RISC). The RISC, which is also responsible for the RNA interference pathway, interacts with its specific target mRNAs through complementary base-pairing, effectively inhibiting translation (Gebauer et al., 2004; Lewis et al., 2005; Pillai et al., 2005). They are often expressed in a developmental and tissue-specific manner and are thought to regulate the majority of human genes (Lewis et al., 2005). The widespread expression and activity of miRNAs in the brain (Cao et al., 2006) could support their implications in different neurological diseases. Since miRNAs can elicit broad effects on the expression of genes and their functional pathways, it is likely for their dysregulation to have important consequences in neuropsychiatric disorders such as schizophrenia.

Considering that each miRNA is possibly regulating the expression of hundreds to thousands of target genes, the clinical implications of a disturbance of such a system is substantial (Beveridge et al., 2012). Apart from their roles in silencing translation, the levels of miRNA in disease individuals is variable. miRNA expression has been shown to be altered in the midbrain of Parkinson's patients, suggesting that miRNA dysregulation could contribute to some aspects of this disease (Kim et al., 2007). In fragile X mental retardation, loss of FMRP, which is a part of the RISC, has also been shown to alter synaptic development (Jin et al., 2004). Additionally, multiple studies have also suggested a link between miRNA dysregulation and Alzheimer's disease, although the specific molecular mechanism is still under investigation (Herbert et al., 2009; Wang et al., 2010; Nelson et al., 2010).

In schizophrenia, the dysregulation of miRNA transcription and processing have been evident in postmortem analysis of cortical miRNA expression (Beveridge et al., 2008). Expression profiling studies in postmortem gray matter of individuals with schizophrenia have shown numerous miRNAs to be altered in the disorder (Beveridge et al., 2008). A variety of microarray platforms and quantitative RT-PCR techniques have also shown the upregulation of different miRNA species in the superior temporal

gyrus and hippocampus of the brain (Beveridge et al., 2010; Beveridge et al., 2008; Stark et al., 2008). Postmortem brain studies suggest that altered miRNA biogenesis could potentially serve as a mechanism for miRNA dysregulation in their patient cohorts (Perkins et al., 2007). With respect to this influence, there is increasing evidence that dysregulation of cellular miRNA processing and maturation can contribute to the pathophysiology of schizophrenia (Beveridge et al., 2012). In this context, it is interesting that copy number variation and polymorphisms affecting genes in the miRNA biogenesis pathway could be possibly overrepresented in schizophrenia (Beveridge et al., 2012). The best characterized of these associations is the 22q11.2 microdeletion syndrome known as DiGeorge Syndrome (Beveridge et al., 2012), where the most common deletions, ranging across 2 Mb and 1.5Mb, contain a number of schizophrenia-associated genes (Beveridge et al., 2012). Approximately 30% of children with a deletion will develop schizophrenia as a disorder (Bassett et al., 2003; Murphy et al., 1999).

MicroRNA-137

Apart from the broad roles that miRNAs play in silencing different gene transcripts, some research has indicated the dysregulation of certain miRNAs to be more potent than others. Studies have increased evidence that hsa-miR-137, microRNA-137, is candidate miRNA implicated in schizophrenic. A recent Genome Wide Association Study (Ripke et al., 2011) with a sample size of 21,856 participants has shown 5 loci within the MIR137 gene to be possibly associated with schizophrenia. The strongest association was the SNP rs1625579 within an intronic region of the MIR137HG gene, the miR-137 host gene (non-protein coding), that includes miR-137 (Swami et al., 2011). This miRNA has also been functionally shown to regulate genes that play a role in schizophrenia such as CACNA1C (calcium

channel, voltage-dependent, L-type, alpha 1c subunit) and transcription factor 4 (TCF4) (Swami et al., 2011).

In addition, miR-137 has certain roles in brain development as well. Sun et al. has shown the miRNA to be highly expressed in embryonic and adult brains and its overexpression to cause decreased proliferation of mouse embryonic neural stem cells leading to their premature neuronal differentiation. Similar effects were also seen in adult mouse neural stem cells derived from the subventricular zone or from brain tumors (Silber et al., 2008). Conversely, overexpression of miR-137 in the adult neural stem cells derived from the subgranular layer of the dentate gyrus was found to disrupt the expression of stage specific differentiation markers such as DCX and NeuN (Smrt et al., 2010). This difference could be due to miR-137 playing different roles in regulating the different stages of neuronal differentiations. Szulwach et al. has also shown that overexpression of miR-137 decreases neuronal maturation and increases proliferation while antagonism of miR-137 in mature neural stem cells increased neuronal differentiation and reduced proliferation. From these studies, one can see the delicate balance that miRNAs play in regulating proper neuronal development.

Apart from being implicated in neurodevelopment, miR-137 has also been shown to have a role in synaptic plasticity. In vivo, miR-137 gain-of-function has been shown to result in changes in synaptic vesicle pool distribution, impaired mossy fiber-LTP induction and deficits in hippocampus-dependent learning and memory. Furthermore, miRNA-137 gain of function was observed as a result of 4 different disease-associated SNPs on the gene which has also been shown to cause downregulation of presynaptic target genes such as Complexin-1, Nsf, and Synaptotagmin-1 (Siegert et al., 2015). The localization and enrichment of miR-137 within the dentate gyrus (DG) of the hippocampus also play functional roles in the formation of neuronal connectivity (Smrt et al., 2010). Specifically, researchers have looked at this phenomenon by identifying the mRNA targets of miR-137 such as Mind-Bomb-1, a

ubiquitin ligase known to be important for neurodevelopment (Choe et al., 2007) and Ezh2, a histone H3 lysine 27 methyltransferase (Boyer et al., 2006). The impact this dysregulation of miR-137 plays in compromising the control of different protein targets at the synapse not only results in differential synaptic alterations, but also brain connectivity (Weinberger et al., 1999).

Effect of neuregulin 1 signaling on dendritic morphology and protein expression through a miR-137 mediated mechanism

Dysregulation of both the Nrg/ErbB4 signaling pathway and miR-137 expression have been shown to be integral in proper development of brain function (Barros et al., 2009; Wright et al., 2013). In further investigating some of the targets of miR-137 that could potentially play a role in this development, our lab has used a TargetScan to predict potential mRNA targets of miR-137. Our lab then conducted a DAVID analysis to identify these targets of miR-137 within the Nrg/ErbB4 pathway. One motivation for the scan came from prior observation of how miR-137 targets were significantly enriched within the Nrg/ErbB4 pathway (Wright et al 2013). We seek to investigate whether or not miRNA-137 can affect the Nrg/ErbB4 pathway by regulating proteins downstream of Nrg/ErbB4 signaling and whether or not this mechanism of regulation is through the phosphorylation of these proteins. Out of all of the different proteins downstream of Nrg/ErbB4 signaling, we looked at ERK, mTOR and GSK3β. We hypothesize that miRNA-137 overexpression will decrease the phospho and total levels of these proteins by regulating their expression levels.

In addition to the implications miRNAs can have on protein expression, it has also been shown to regulate dendritic morphology along with neuregulin. Krivosheya et al. has demonstrated that perturbations of neuregulin/ErbB4 signaling inhibits specific synapse developments and dendritic

outgrowth. Previous works from the Bassell lab have focused on investigating the underlying mechanism of miR-137 dysregulation in affecting neuregulin signaling. Unpublished data from our lab has also found that depletion of miRNA-137 can reverse the effects of neuregulin-1 on dendritic morphology (Figure 4). Although this is still under active investigation, we believe that miRNA-137 regulates the effects of neuregulin stimulation on dendritic morphology through the mTOR pathway. To further understand the effects of miRNA-137, we seek to investigate the effects of miR-137 overexpression on dendritic morphology. We hypothesize that miRNA-137 dysregulation under neuregulin stimulation will inhibit dendritic arborization of neurons.

Although some studies have shown that dendritic development is dependent upon miRNA regulation and neuregulin/ErbB4 signaling, the downstream cascade of how this occurs is still not well understood (Giusti et al., 2014; Krivosheya et al., 2008). Krivosheya et al. suggested that neuregulin signaling could play different roles in regulating neural development and dendritic arboization. Furthermore, studies done by Diez-Guerra et al. showed that dendritic arborization in hippocampal neurons depends on the phosphorylation of MAP2. Our final area of investigation is to examine whether or not neuregulin-1 increases MAP2 protein levels in dendrites through the stimulation of MAP2 protein synthesis. MAP2, otherwise known as microtubule-associated protein 2, is an enzyme that is often localized to the dendrites of hippocampal neurons (Caceres et al., 1984). MAP2 also serves to stabilize microtubules as well as regulate dendritic morphology during neuronal development (Baas et al., 2009). Preliminary unpublished data from the Bassell lab have shown that the stimulation with neuregulin in hippocampal neurons, increases MAP2 protein levels in dendrites. However, whether or not this is due to increasing MAP2 protein synthesis or the stabilization of existing protein is still unclear. By utilizing a puromycin labeling and a proximity ligation assay, we hypothesize that the increase in fluorescence signal from MAP2 in dendritic regions is due to neuregulin stimulating MAP2 protein synthesis. Our

ultimate goal is to elucidate the mechanism by which neuregulin could stimulate MAP2 protein synthesis

Materials and Methods

Detection of GSK3beta under miRNA-137 overexpression

Cell transfection: A line of neuroblastoma cells called N2a cells were first transfected in preparation for western blot. Plate layout contained 4 different conditions, and the respective cells were transfected with either a Control miR-OE plasmid (Genecopoeia), miR-137 OE plasmid (Origene), miR-137 sponge plasmid (Aschrafi Lab), or a FUGW plasmid (control for overexpression) (Aschrafi Lab). The plasmid type denotes the condition of the neurons. Cells were transfected with lipofectamine 2000 (Invitrogen) per manufacturer's instructions

BCA protein assay: Before performing the western blot, protein concentrations were measured using a BCA assay (Thermo Fisher Scientific). Instructions were followed per the manufacturer's instructions.

SDS-PAGE, Transfer and Western Blotting: After the determination of appropriate concentrations, 4 ug/sample of protein were run on 8% SDS polyacrylamide gels. The resolved proteins were then transferred on 0.45µm nitrocellulose membranes overnight. Primary antibodies used to probe the membranes were specific for phospho and total Gsk3beta (1:1000 Sigma), phospho and total mTOR (1:500 Sigma), phospho and total ERK (1:500 Sigma) and beta III-tubulin (1:10,000 Sigma). Secondary antibodies used to detect primaries were Donkey anti-mouse (IRDye 680LT, Li-COR) and Donkey anti-rabbit (IRDye 800CW, Li-COR). Signals from the blots were detected using an Odyssey CLx Imaging Software (LI-COR Biotech, Lincoln NE). Densitometric analysis was done on ImageJ (NIH) to

detect band signals. Beta III-tubulin was also imaged on the membranes, serving as a loading control for our proteins of interest.

Assessment of dendritic morphology under neuregulin and miR-137 overexpression

Plating of cortical neurons. Glass coverslips in a 6-well plate were coated with 3 beads of poly-L-lysine (1mg/ml) per coverslip at 37 degrees Celsius for 2 hours. The coverslips were washed three times with autoclaved water. Neurons were then plated on the cover slips

Transfection of hippocampal neurons. Neurons were transfected with either a FUGW plasmid with the miR-137 overexpression sequence (Genecopoeia) or the FUGW plasmid (Aschrafi Lab) via nucleofection. Instructions were followed per the manufacturer's directions. After plating, cells were left in an incubator overnight at 37 degrees Celsius.

Cell treatment and fixation. Cells were treated at 2 days in vitro with Nrg1 Alpha (10nM, R&D) or an equal volume of PBS. After treatment, cells were fixed on 4 days in vitro in 4% paraformaldehyde for 15 minutes. Cells were washed 3x in PBS in preparation for blocking.

Immunofluorescence. Each coverslip was incubated at room temperature in IF block (1X PBS, 5% Normal Donkey Serum, 0.1% BSA, 0.1% Triton x-100) on a shaker. Afterwards, primary antibodies of Rabbit anti-MAP2 (Millipore) and Mouse anti-Tau-1 (Millipore) were added to the coverslips (40uL) overnight at 4 degrees C. The next day, coverslips were washed 3x 10 minutes in PBS. Secondary antibodies diluted in IF block (Donkey anti-Rabbit Cy3 (Millipore) and dk-anti Mouse 647 (Millipore)) were then added to the coverslips, and the plate was incubated for 1 hours at room temperature. Subsequent steps were performed in the dark. Coverslips were washed 3X10 minutes in PBS. DAPI

(1:1000) was then added to the coverslips and incubated for 10 minutes at 37 degrees. Coverslips were washed for 2x 5 minutes in PBS. Mounting of the coverslips was performed in a solution of glycerol, polyvinyl alcohol and propyl gallate. Coverslips were dried overnight in a cabinet before viewing.

Fluorescence microscopy. Neurons were visualized on a coverslip using a 40x Plan Neofluar objective (Nikon, Melville, NY) of a Nikon Eclipse inverted microscope with 1x magnification. Images were acquired with a cooled CCD camera (Photometrics, Tucson, AZ) and Nikon Elements software. Exposure times were kept constant and below saturation for quantitative analysis. Immunostaining for MAP2 and tau-1 was used to differentiate dendrites and axons. After splitting the separate channels using ImageJ, dendrites were traced with NeuronJ (NIF). Dendrite complexity was quantified using the Sholl-Analysis plug-in for ImageJ. Complexity was compiled by quantifying the area under the curve and data analysis was performed using Graph Pad.

Detection of newly synthesized MAP2 utilizing puromycin labeling and a proximity ligation assay

Plating of hippocampal neurons: Hippocampal neurons were prepared as previously described in Williams et al. and plated on beaded coverslips coated with poly-L-lysine (Sigma-Aldrich). Neurons were treated with neuregulin-1 at 6 days in vitro.

Cell treatment and fixation. Nrg1 Alpha (100ug/ml, R&D) or 1X PBS was added to each respective well. Coverslips were then incubated for 30 minutes. For puromycylation, neurons were incubated with 0.17uL puromycin (50 ug/ul stock) and emetine (10 ug/ul stock) for 5 minutes in full medium at 37 degrees Celsius in a humidified incubator. Incubation was stopped with 1x 2min cold HBS + digitonin solution (0.0003% digitonin). For the protein synthesis inhibitor control wells, cells were

pretreated with 0.849 uL anisomycin for 1 hour. Cells were fixed immediately for 15 minutes with 4% paraformaldehyde. After fixation, cells were washed 3x with 1X PBS solution.

Proximity Ligation Assay. Procedures were adapted from Dieck et al., and modified according to our experiment (Figure 6). In preparation for incubation with primary antibodies, coverslips were incubated for one hour in IF block (1X PBS, 5% Normal Donkey Serum, 0.1% BSA, 0.1% Triton x-100, milliQ water). Detection of newly synthesized proteins by proximity ligation was carried out using an anti-puromycin antibody from mouse species (1:500 DSHB) in combination with an anti-MAP2 (1:500 Millipore) antibody from rabbit species. Coverslips were incubated overnight at 4 degrees Celsius. Rabbit (rb) PLA PLUS (Duolink In Situ.) and mouse (ms) PLA MINUS (Duolink In Situ.) probes were diluted in IF block and used as secondary antibodies (1:1:3 probe to probe to block dilution). "Duolink Detection Reagents Red" (Sigma) kit was used for ligation, amplification and label probe binding. After incubation, coverslips were washed for 2x 10 minutes in Wash Buffer B (5.84g NaCl, 4.24g Tris Base, 26.0g Tris-HCl, 500mL high purity water, pH = 7.5, Duolink In Situ.) at room temperature. Coverslips were then incubated in fluorescein Phalloidin (1:20 dilution in 1x PBS, Sigma) for 30 minutes at room temperature, and then washed 3x 5min in PBS (1:1000). Finally, mounting was performed with Duolink In Situ Mounting Medium with DAPI (Sigma) and the edges were sealed with nail polish.

Imaging and analysis. Cells were visualized with a 60x Plan-Neofluar objective (Nikon, Melville, NY) on a Nikon Eclipse inverted microscope at 1x magnification. Images were acquired using a cooled CCD camera (Photometrics, Tucson, AZ) and Nikon Elements software. Exposure times were unchanged and below saturation for quantitative analysis. Image deconvolution was performed using AutoQuant X (Media Cybernetics, Bethesda, MD). Images were then analyzed with Imaris (Bitplane). Cell volume was measured by creating a contour surface ranging from 30-50um. The MAP2 channel was then masked and a new channel was created. Puncta were selected within the masked channel, and their puncta

volume and intensity were measured. PLA puncta were thresholded using the same value for all samples within one experiment. The sum of the PLA puncta volume or the integrated intensity of the PLA signal within the dendritic region of interest (ROI) was divided by the total volume of the ROI.

Results

Previous unpublished data from the Bassell Lab has shown that miRNA-137 inhibition disrupted Nrg1α signaling. This work was done with the S6 protein, which is involved in regulating translation in cells. Normally, neuregulin causes an increase in S6 phosphorylation. However, in the presence of a miRNA-137 sponge, the effects of neuregulin were inhibited (Data not shown). Although we saw this disruption, we did not know which specific miRNA-137 targets were responsible for this effect. Therefore, we've decided to examine GSK3β as a possible candidate. GSK3β, otherwise known as glycogen synthase kinase 3 beta, is an enzyme involved as a phosphorylating and inactivating agent of glycogen synthase (Plyte et al., 1992). We decided to study this protein based on the important role GSK3β plays in neuronal cell development and its potential implications in serving as a therapeutic for schizophrenia (Nadri et al., 2003).

A primary goal of this study is to investigate whether or not GSK3β protein expression is affected by the overexpression of miRNA-137. We did this by transfecting cortical neurons with a miRNA-137 overexpression plasmid, and then seeing its effect via the utilization of Western Blots. We hypothesized that miRNA-137 overexpression will ultimately decrease the phospho and total protein expression of GSK3β.

miRNA-137 overexpression causes a decrease in phospho and total GSK3β expression levels

We first examined the effect of phospho and total GSK3β expression levels by transfecting the Neuro2a cells with either a control or a miRNA-137 overexpression plasmid. The control miRNA-OE plasmid overexpresses a small control RNA of miRNA-137 that does not have any predicted mRNA targets, whereas the miR-137OE plasmid overexpresses the precursor of miRNA-137 that eventually gets processed into the mature form of miRNA in the neurons. Upon analysis after five separate trials, we saw a statistically significantly decrease in Phospho (S9)-GSK3β protein expression for the miRNA-137 OE samples as compared to the control, ($P<0.01$, Paired T-test, $N=5$) (Figure 2A). Similarly, total GSK3β protein expression, when normalized to βIII-tubulin, was also seen to decrease with respect to the control ($P<0.01$, Paired T-test, $N=5$) (Figure 2A). There was no significant effect seen in comparing the phospho (S9)-GSK3β protein levels normalized to the total protein levels for the control and miRNA-137 overexpression samples ($P = 0.34$, Paired T-test, $N=5$).

miRNA-137 overexpression does not affect ERK or mTOR protein levels

In addition to GSK3β, we also looked at the protein levels of ERK and mTOR under miRNA-137 dysregulation. In contrast to previous lab data that has shown that miRNA-137 overexpression reduced mTOR levels in primary cortical neurons, we did not see an overall convincing trend that miRNA-137 regulated mTOR. However, we did see a non-significant trend with regards to ERK phosphorylation under the condition of miRNA-137 sponge condition. The “miR-137 sponge” contains four partially complementary miRNA-137 binding sites, and is used to selectively reduced miRNA-137 interaction with its endogenous targets (Ebert et al., 2007). Phospho ERK protein expression levels, when normalized to

βIII tubulin, decreased in the miRNA-137 sponge samples in comparison to the control (Figure 3). There was also a non-significant decrease for the treated samples when phospho ERK is normalized to total ERK. This seemingly contrasting result with how miRNA-137 overexpression affects GSK3β brings up the evidence that miRNA-137 regulates different targets through very different mechanisms.

miRNA-137 overexpression blocks the effect of neuregulin on dendritic morphology

Previous unpublished work from the Bassell Lab have shown that in the presence of miRNA-137 sponge, dendritic outgrowth is disrupted in neurons (Figure 4). Specifically, miRNA-137 sponge samples changed how the neurons responded to neuregulin and saw a statistically significant increase in dendritic complexity as compared to the control (6 biological replicates, two-way ANOVA with Tukey's planned comparison, $p<0.05$). This led us to hypothesize that different proteins involved in dendritic outgrowth, could be dependent on miRNA-137 regulatory mechanisms. To see how dendritic complexity in neurons would respond to neuregulin upon the overexpression of miRNA-137, we transfected neurons at 4 days in vitro with the miRNA-137 OE plasmid. Upon analysis, we identified that miR-137 overexpression blocked the effect of neuregulin on dendritic arborization for the experimental sample (3 biological replicates, two-way ANOVA with Sidak's unmatched comparison, $p>0.05$) (Figure 5). Surprisingly, we did not identify a significant increase in the FUGW samples treated with neuregulin, which suggests that this experiment needs to be replicated (3 biological replicates, two-way ANOVA with Sidak's unmatched comparison, $p>0.05$). To ensure that there was no bias upon analyzing the different replicates (n=3), we also blinded the conditions of each slide (FUGW and miRNA-137 OE) before we proceeded to perform fluorescence microscopy. The reasons for a faulty positive control, which in this case was the effect of neuregulin on the FUGW transfected cells, could be due to technical

difficulties involved in plating the cells as well as the date of the original neuregulin stock. In addition, a contamination in our cell incubator for several weeks could have led to less than optimal neuron samples, which also can affect our outcomes. We hope to investigate the mechanism of how miR-137 disrupts the effect of neuregulin on dendritic morphology in a future follow up study.

Nrg1α may increase dendritic MAP2 protein synthesis

Previous studies have shown the presence of neuregulin-1 and its receptor ErbB4 to play important roles in dendritic outgrowth (Krivosheya et al., 2004; Mei et al., 2008). We therefore examined neuregulin-1's effect on a specific protein known to play a role in determining and stabilizing dendritic shape during different stages of neuronal development. The red "puncta" in the different regions of the cell represent locations where newly synthesized MAP2 proteins are located.

A key goal of this particular experiment was to determine whether neuregulin stimulated the increased synthesis of MAP2 or its stabilization in hippocampal neurons. Based on preliminary data (not shown), we saw an increase in MAP2 protein levels in dendritic regions of the neuregulin treated samples. Although we initially saw this effect, we did not know whether this was through increasing MAP2 protein synthesis or the stabilization of existing MAP2 proteins. We predicted that neuregulin is stimulating MAP2 protein fluorescence through increased translation of the protein itself. To validate this, we utilized a puromycin labeling, proximity ligation assay method. What this entails is that puromycin, a protein synthesis inhibitor, gets incorporated into the growing polypeptide chain during active translation. In turn, puromycin inhibits the synthesis of the protein at the stage of elongation or termination. After fixing the cells, antibodies specific for our protein of interest (MAP2) and puromycin were incorporated. A ligation reaction was then added, which ligates the oligonucleotides on both

antibodies only when they are in close proximity to one another. Upon analysis of our data, we did see an increase in the levels of MAP2 PLA signal for the neuregulin treated samples as compared to the control treated with PBS (Figure 7). This suggests that instead of the stabilization of current MAP2 protein levels in the dendritic regions, neuregulin stimulates the synthesis of new MAP2 proteins through an indirect mechanism that causes the fluorescence signal intensities to increase in our FUGW Nrg treated samples.

Discussion

Neurodevelopmental deficits, improper synaptic connections and alterations in neuronal morphology are all characteristics of a schizophrenic brain (Jaaro-Peled et al., 2009; Agarwal et al., 2014; Kriyosheva et al., 2009). Although many different physiological and neurological characteristics have been identified with the disease, their molecular mechanisms are poorly understood. miRNA-137 plays an integral role in regulating many of the pathways that lead to these deficits (Beveridge et al., 2011; Wright et al., 2013; Collins et al., 2014). A recent study supported that miRNA-137 played a role in modulating neuronal maturation and was a top target in the ephrin receptor signaling pathways (Wright et al., 2013). Our study might provide further insight into the different mechanistic effects of miRNA-137 in schizophrenia.

Here, we show that miRNA-137 overexpression causes the reduced expression of phospho and total GSK3β protein expression (Figure 2). This novel finding correlates with my model that miRNA-137 regulates different target kinases in the neuregulin/ErbB4 signaling pathway through a distinct mechanism (Figure 1). It's conceivable to say, however, that GSK3β is regulated by a direct mechanism. The reason we propose this is because of the fact that we did see a statistically significant decrease in both the phospho and total GSK3β protein levels. If miRNA-137 was to inhibit the synthesis of GSK3β indirectly, one would expect to see a regulation only in the activity level of GSK3β and not its expression. However, we seek to perform future experiments to confirm our speculation. Another possible explanation for this decrease that we see could be attributed to the potential miRNA-137 has in mRNA

degradation of GSK3β transcripts directly. For a future study, we would be able to confirm this by utilizing RT-PCR and assess the transcript levels in between controls and miRNA-137OE groups.

Studies have shown the role of GSK3β in being indirectly inhibited via the regulation of several intracellular signaling cascades, including the canonical Wnt, Reelin and tyrosine kinase receptor (Trk)-PI3K-Akt (Koros et al., 2007). However, this is the first time evidence has shown its regulation under miRNA-137. In the developing CNS, Nrg/ErbB4 signaling is implicated in proper proliferation of neuronal progenitor cells as well as neuronal migration in different areas of the brain (Peled et al., 2009). In neuronal progenitor cells, DISC1 interacts directly with GSK3β, which reduces phosphorylation of β-catenin and increases its stability (Mao et al., 2009). From this, one can see the direct role that GSk3β plays in neuronal development as well as the different ways in which it can be expressed. By utilizing the accessible TargetScans and different databases, we were able to compile potential targets of miR-137 over a broad range of genes with a variety of functions. ERK and mTOR were two of the proteins that were also looked at that were part of the neuregulin/ErbB4 pathway. Although we saw no significant change in the protein levels of ERK and mTOR, we did see that miRNA-137 sponge actually reduced the level of ERK phosphorylation in comparison to the control sample. In contrast to what we observed with miRNA-137 overexpression in GSK3β, we can infer that miRNA-137 regulates kinases through different mechanistic manners and pathways. In addition, Meares et al., has identified a shuttling mechanism of GSK3β in between the nuclear and cytoplasmic compartments of the cell-body, which suggests that GSK3β could play different roles throughout the neuron. If this were to be true, it could confirm the finding of Smrt et al., that miRNA-137 act on different signaling pathways depending on the developmental timeline of a neuron. However, since we were utilizing a very fast growing neuroblastoma cell-line, it is possible that the effect miRNA-137 exerts on GSK3β is different from that in neurons.

Dendritic morphology is also a feature that has been implicated in schizophrenia. Neuregulin signaling plays a role in regulating dendritic morphology via trans-synaptic and tyrosine kinase activity (Krivosheya et al., 2008). Recent data from our lab has shown that Nrg1α stimulation of dendritic outgrowth depends on endogenous miR-137 activity (Figure 4). Given that specific miRNAs are enriched in the dentate gyrus (DG) and hippocampus, where neuronal growth is continually taking place, it is reasonable to suggest that miRNA-137 dysregulation could contributes to a disturbance in morphology. The trend that we saw in our data (Figure 5) highlights what could potentially happen to dendritic complexity in the presence of miRNA-137. We hypothesize that outgrowth of neurons could depend partly on the mTOR pathway, which serves as a central hub for different protein kinases that regulates dendritic complexity (Kumar et al., 2005). Furthermore, an unpublished study done by the Bassell lab also saw that Nrg1α increased dendritic complexity through the mTOR pathway, in that samples treated with DMSO showed an increase in normalized dendritic complexity for a 2 day neuregulin treatment (data not shown). We believe that miRNA-137 regulates certain kinases within this pathway, ultimately leading to the inhibition of dendritic outgrowth reflective of the non-significant trend that we see in our experiment.

Something unexpected that we saw with our experiments was that neuregulin did not stimulate morphology to a significant extent for the FUGW condition (control). This could be due to issues with the sample itself or the quality of the neuregulin stock. In the future, we wish to troubleshoot as well as replicate the experiment under stricter conditions with regards to time sensitivity in the hopes that we can see a statistically significant difference and better understand the mechanism by which dendritic morphology is regulated by miRNA-137 and neuregulin signaling.

Apart from the role neuregulin plays in dendritic complexity, MAP2 has also been implicated in many neuronal processes such as stabilization and microtubule assembly (Cotter et al., 2000). Although it serves important cellular functions, it has also been studied in the disease pathology of schizophrenia. Agarwal et al., found that dysregulated expression of neuregulin-1 disrupted synaptic plasticity of different neuronal connections. Other studies show that increased dendritic MAP2 expression is present in the hippocampus of patients, and that this effect could cause synapto-dendritic abnormalities in schizophrenia (Cotter et al., 2000). Our results may show an increase in the newly synthesized MAP2 protein in the dendritic regions. In addition, it is also the first time to note that the increase in expression was due to the stimulation of neuregulin. This finding links the work of Agarwal et al., and Cotter et al, in that the dysregulation of neuregulin stimulation could be altering the underling connections of synaptic plasticity in schizophrenic patients through a MAP2 expression mechanism. We seek to optimize the experiment by polishing on our manual dexterity in performing the PLA, adhering more strictly to the time sensitivity of treating and fixing the cells, as well as revising our protocols to better accommodate to the conditions of the experiment. Although the result was not statistically significant, we were able to see a trend in MAP2 protein synthesis with neuregulin stimulation and hope to replicate our results and utilize this assay for future experiments.

Limitations for our results lie in the current number of replicates that have been analyzed thus far. Although we were able to see a trend, a replicate of one does not provide sufficient statistical power. However, given our preliminary data and observation that MAP2 puncta volume increases upon neuregulin stimulation, we believe that this warrants further experimentation in this area. In the future, we hope to increase statistical power by performing more replicates.

Another goal that was previously addressed in the Results section refers to our hypothesis on why we believe that neuregulin is directly stimulating the synthesis of MAP2 and not stabilizing existing

proteins. There are a total of two reasons: firstly, the PLA assay is a robust tool in that it relies on the incorporation of puromycin onto existing chains of polypeptide during the translation process. As translation terminates, polysomes are stabilized and stalled at the level of elongation and/or termination (Graber et al., 2013). This stabilization is also enhanced by emetine, which we added to function as an inhibitor that prevents the puromycylated nascent chain from releasing from the ribosome (Graber et al., 2013). As a result, we are able to assess the change in fluorescence signal as visualization of ribosome bound nascent chains and not the MAP2 proteins that were present previously in the neuron.

Overall, we were able to confirm the effects of miRNA-137 overexpression on phospho and GSK3β protein levels. miRNA-137 overexpression in Neuro2a cells decreased phospho and total GSK3β protein expression in comparison to the control. We also saw a slight decreasing trend for the dendritic morphology upon incorporating a miRNA-137 overexpression plasmid in our nucleofected neurons that were treated with neuregulin. However, results were not statistically significant, and we seek to repeat this experiment. Furthermore, we also seek to determine whether or not GSK3β and MAP2 are possible candidate proteins involved in regulating dendritic morphology under miRNA-137 influence. In addition, we were able to see an increase in MAP2 protein synthesis upon stimulating the hippocampal neurons with neuregulin. We aim to perform further replicates to increase statistical power as well as to assess if MAP2 is a direct target of miRNA-137.

Figures.

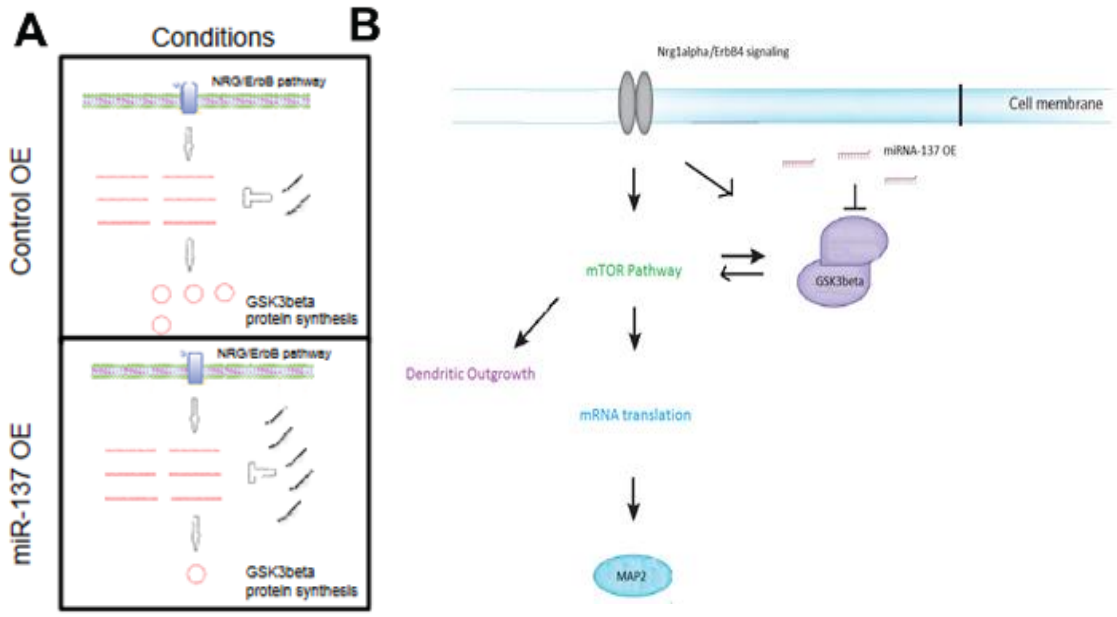

Figure 1: Hypothetical model of the interactions of miRNA-137 in the neuregulin/ErbB4 pathway. For the control OE condition, normal GSK3β protein levels are still being expressed due to a normal and physiological level of miRNA-137 being present. Under miR-137 OE conditions, however, miR-137 is overexpressed in the neurons and represses GSK3β protein synthesis through a direct mechanism in the Nrg/ErbB4 pathway. Arrows denote next step of the cellular process and flathead arrows denote silencing. (B) A hypothetical model of the pathway by which miRNA-137 exerts its effects (Bassell Lab).

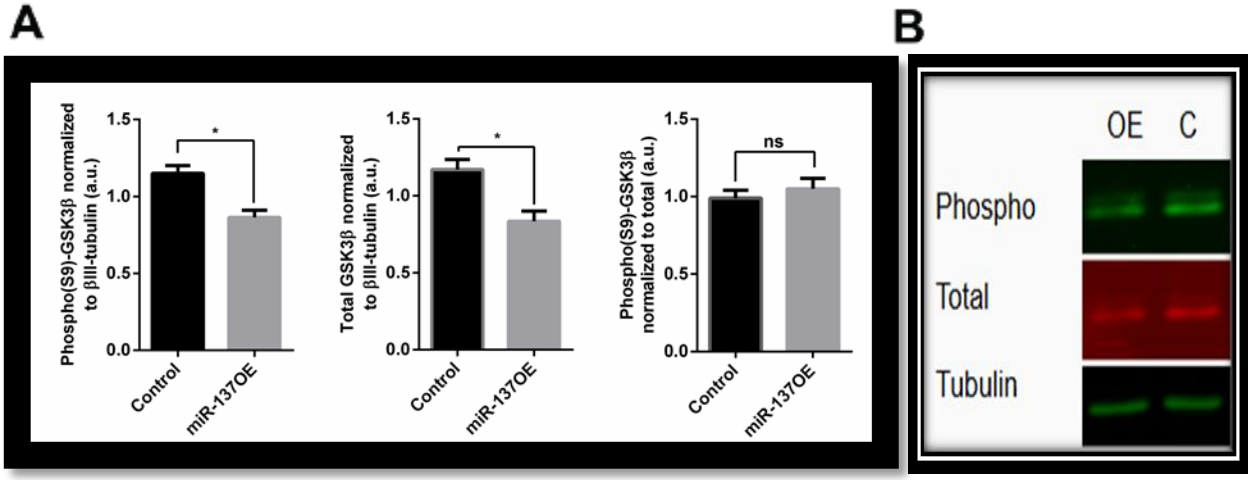

Figure 2. (A) Mean GSK3β protein levels under the different conditions as indicated above. Phospho and total protein levels of GSK3β are normalized to βIII-tubulin levels. Phospho-GSK3β levels, when normalized to control, shows a statistically significant decrease for miRNA-137 overexpression treated samples as compared to the control (n=5; *p<0.05, ratio paired t-test). Similarly, there is a statistically significant decrease for the miR-137 overexpression samples for the total protein, when GSK3β is normalized to βIII tubulin (n=5, *p<0.05, ratio paired t-test). No significant differences were seen for either samples when phospho GSK3β is normalized to total GSK3β (n=5, p>0.05, ratio paired t-test). (B) Western Blot analysis for one of the replicates of phospho GSK3β, total GSK3β and βIII-tubulin. Bands show GSK3β in cultured N2A cells, infected with either a control plasmid or a miRNA-137 OE plasmid. BIII-tubulin was used as a loading control.

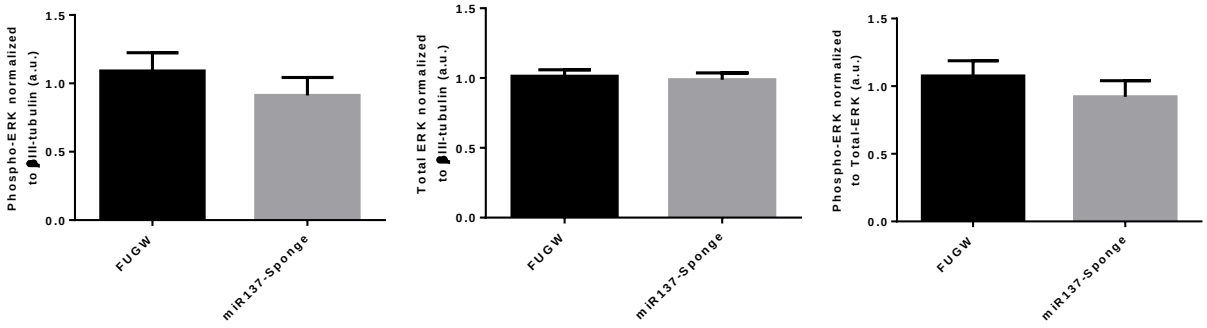

Figure 3. Mean ERK protein levels under the different conditions as indicated above. Phospho and total protein levels of ERK are normalized to βIII-tubulin levels. Results indicate that phospho-ERK levels, when normalized to control, shows a decreasing trend for miRNA-137 sponge treated samples as compared to the control (n=3; $p>0.05$). Similarly, there is a decrease for the miR-137 sponge samples when phosphor ERK is normalized to total ERK (n=3, $p>0.05$). No significant differences were seen for either samples when total ERK is normalized to βIII-tubulin (n=3, $p>0.05$).

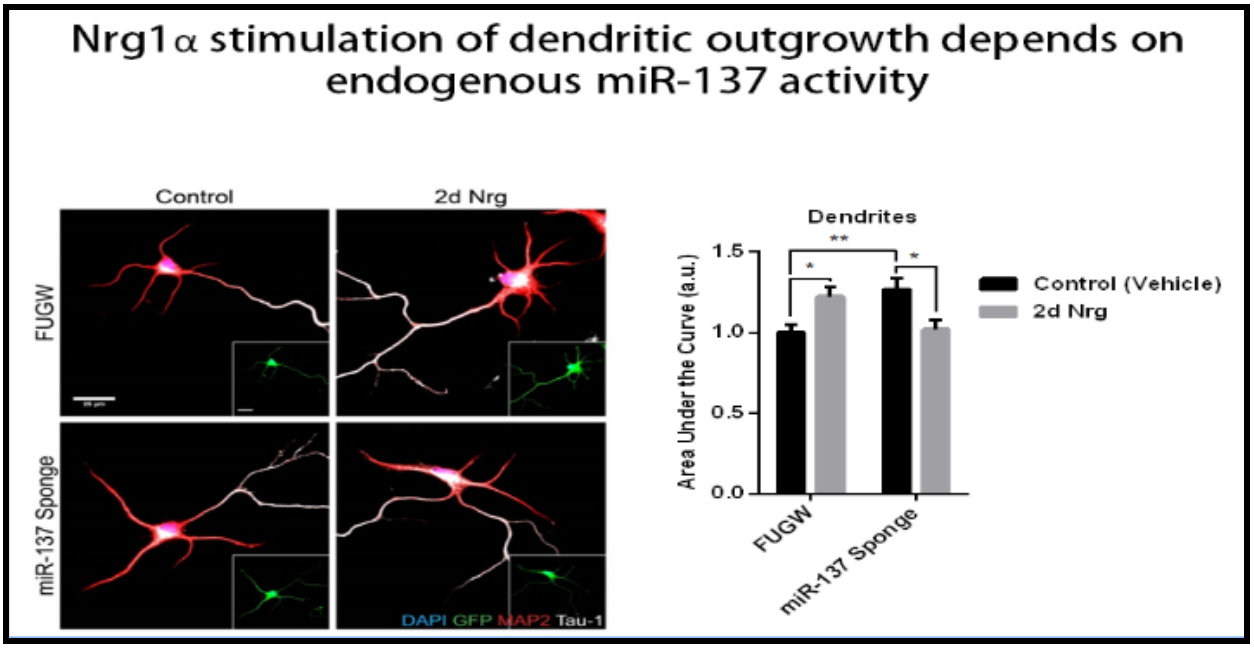

Figure 4. Preliminary results for dendritic outgrowth of primary cortical neurons imaged under regular fluorescence in the presence of miRNA-137 sponge. 10 neurons/condition/replicate were imaged. Data shows an effect whereby miRNA-137 sponge is seen to inhibit the effect of Nrg1α for the miRNA-137 sponge samples (n=60, 6 biological replicates, two-way ANOVA with Tukey's planned comparison, $p<0.05$). For the FUGW samples (control), Nrg1α stimulation increases dendritic morphology as compared to the control (n=60, 6 biological replicates, two-way ANOVA with Tukey's planned comparison, $p<0.05$). Pictures on the left represent marked dendritic morphology for the different neurons under different conditions. MAP2 marks the different dendritic regions in red, white represents Tau-1 which marks the axons, and green represents GFP fluorescence.

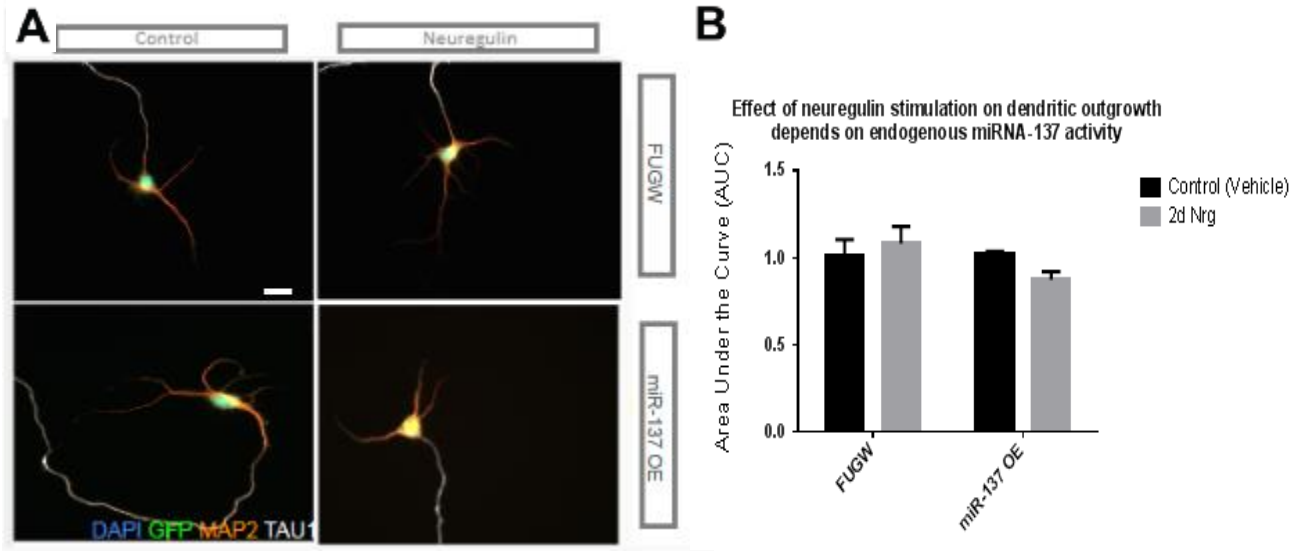

Figure 5. (A) Representative microscopy images showing dendritic morphology of primary neurons transfected with a FUGW plasmid (control) or miRNA-137 overexpression plasmid (10 cells/well, 3 biological replicates). Neurons were either treated with a 2 day neuregulin treatment or vehicle (1XPBS). Scale bars, 10µm (top left image) for all images. (B) Effect of neuregulin stimulation on dendritic outgrowth. Dendritic morphology was measured by taking the Area Under the Curve using NeuronJ. For the FUGW samples, neuregulin slightly increased dendritic outgrowth (3 biological replicates, two-way ANOVA with Sidak's unmatched comparison, $p>0.05$). For the miR-137 OE samples, a non-statistically significant decrease was seen for the 2d neuregulin treated samples (3 biological replicates, two-way ANOVA with Sidak's unmatched comparison, $p>0.05$)

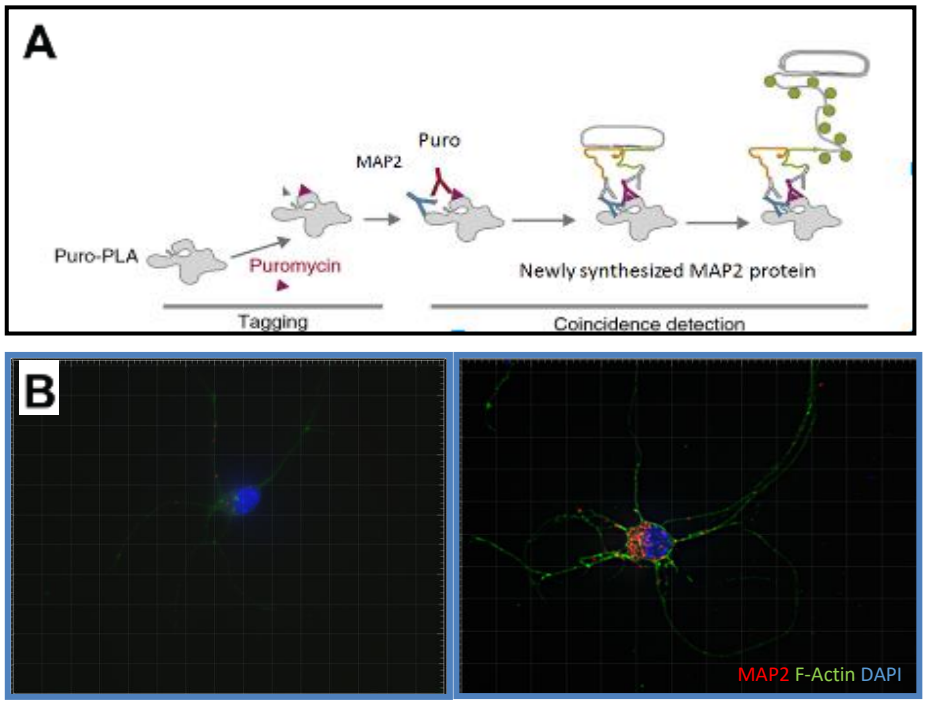

Figure 6: (A) Mechanism of the Proximity Ligation Assay for our experiment. Newly synthesized MAP2 proteins incorporate puromycin, which actively inhibits translation. Antibody recognition of the newly synthesized MAP2 protein as well as the recognition of the puromycin tag happens when PLA^{MINUS} and PLA^{PLUS} oligonucleotides (yellow and green squiggles) are coupled to secondary antibodies (gray Y). When the puromycin and MAP2 are close enough, they serve as the template arranging linker oligonucleotides such that subsequent formation of a circular product by ligase and rolling-circle amplification is possible. Signal is obtained by the binding of fluorescently coupled detection probes (green). (B) Representative images showing PLA signal. The neuron on the left denotes a very weak PLA MAP2 signal. The neuron on the right denotes a stronger PLA MAP2 signal (MAP2 is shown in red, DAPI in blue, and F-Actin in green).

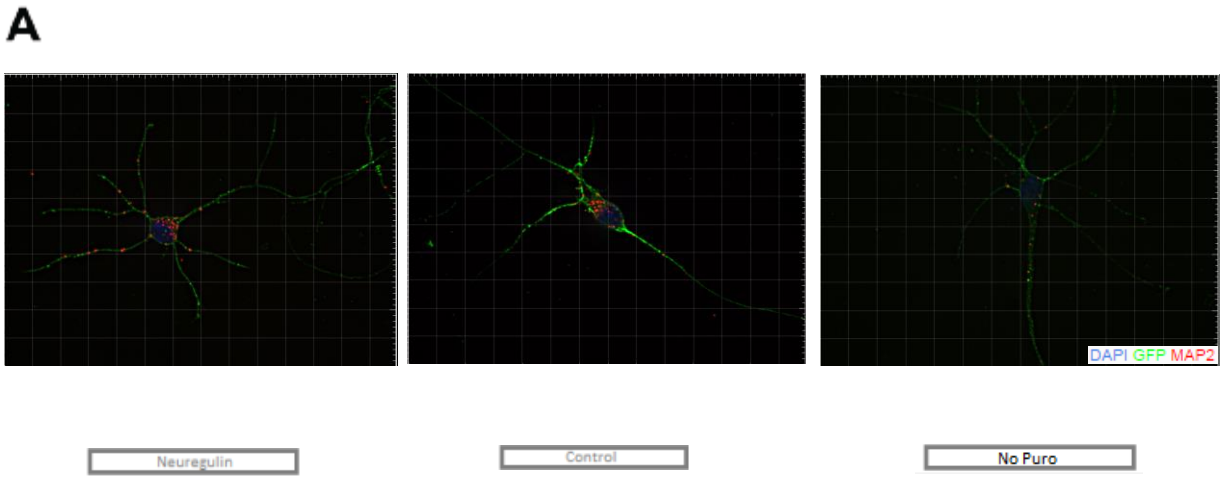

Figure 7. (A) Images taken of hippocampal neurons under three different conditions. Left most image represents one of the neuregulin treated neurons. Middle image represents one of the control neurons treated with PBS. Right most image represents one of the neurons not treated with puromycin (negative control). (B) Graph representing one replicate of our data comparing the three different conditions outlined above (10 cells/condition, 1 biological replicate).

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