Investigation of schizophrenia factors in human neurons

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ABSTRACT: Schizophrenia is a prevalent neuropsychiatric disorder with a high heritability rate. This heritability rate is attributed to genetic risk factors. The most influential risk factor that has been identified so far is the gene NRXN1. Defects in this gene are found in much higher rates in schizophrenia patients, identifying it as a risk factor. The neurexin protein is involved in neurotransmission, cellular recognition, and neuron development. To investigate the effects of schizophrenia in neurons, cells were used from established schizophrenic lines. These cells were cultured into developed neurons and studied for neuronal migration, astrocyte ratios, and NRXN1 isoform expression. Gene expression was studied through RNA sequencing, and cells were imaged with confocal laser microscopy. Neuronal migration changed significantly along with ratios of astrocytes to neurons, and isoform expression was shown to be altered in schizophrenic lines as well. Observations of these specific functional changes offer more insight into the mechanics of schizophrenia as well as the reliability of cell-based disease studies.

KEYWORDS: Biology; Schizophrenia; Stem Cells; Neurons; Neuronal Migration

Introduction. Schizophrenia is a debilitating and chronic mental disorder that results in a wide variety of symptoms including hallucinations, delusions, and paranoia. It affects large numbers of people with a global prevalence rate of 1% and a rate of 1.2% in the US with 3.2 million Americans having the disorder.[1] In addition to this, current treatment options for schizophrenia are insufficient with very unfavorable side effects.[2] These issues stem from the overall lack of understanding concerning the causes of the disorder. Genes related to schizophrenia such as *NRXN1* have been shown to influence neuronal migration as well as the other types of cells in the brain, but it is unknown how those changes relate to the disorder.[3] In addition to *NRXN1*, many of the other potential genes associated with increased risk for schizophrenia require further functional studies to determine their specific relation to the disorder. The goals of this research were to investigate the changes in neuronal migration and cell type composition that occur in patients with schizophrenia harboring heterozygous deletions in *NRXN1*, as well as investigate changes in the mRNA expression of *NRXN1*. This work may help determine what cellular phenotypes change and may provide more insight towards the actual causes of the disease.

Review of Literature. Past schizophrenia research has identified a set of specific changes that may be related to the development of the disorder. Previously established changes occur within the processes of neuronal migration4 and the ratios of neurons to supporting cells.[5] Both processes are altered in schizophrenic patients and may relate to the disorder.

Neuronal Migration and Glial Cells. Neuronal migration is a vital part of the development of the brain that contributes to growth and later function. Supporting cells such as astrocytes and oligodendrocytes greatly contribute to this process. As the brain develops, neurons can migrate radially or tangentially to form the various structures in the brain. This process is vital for the proper functioning of the brain as it is the driving force behind early development and establishment of proper neuronal circuits.[4,6] Previous animal studies have shown that neuronal migration is defective in models of schizophrenia, but the mechanism has not been identified yet. The relationship between neuronal and supporting glial cells is further implicated as the genes related to these cells have altered expression in patients with schizophrenia compared to controls, which could contribute to differences in the ratios of each cell type in the brain.[6]

NRXN1 Gene. Deletions in *NRXN1* have been shown to be associated with schizophrenia through a greatly increased rate of deletions in patient populations.[7,8] Previous studies investigated copy number variations (CNV) which are duplications or deletions over 100kb in the genome. Patients carrying CNVs in this gene have been shown to have a much higher risk for developing neurological disorders such as schizophrenia. This gene has a number of alternate splicing sites which allows the

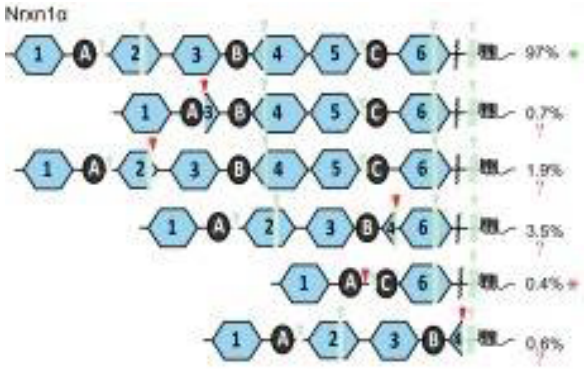

Figure 1. A chart showing isoforms of the gene and their relative prevalences[7].

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gene to be expressed in different sequences called isoforms,[9] and mouse studies have shown a very large number of *NRXN1* isoforms that are expressed normally. Figure 1 shows some of the possible configurations for the exons in *NRXN1*.

Some of these isoforms have already been identified in mice but many possible isoforms have not been identified in humans. In addition to this, past research has failed to compare the isoforms found in schizophrenia patients to those found in controls. Investigating a link between changes in isoform expression with neuronal migration and cell type composition may provide more information relating to the development of schizophrenia and new pathways to target for treatment.

Current issues with schizophrenia research are mostly due to the lack of information relating specific mechanical changes and the psychological symptoms that occur in the disease. This result in difficulty creating new and effective treatment options for schizophrenia. This project investigated three phenotypic changes that occurred in patients exhibiting psychosis who harbor deletions in the gene *NRXN1*, using a variety of established cell lines side-by-side in comparisons. By looking at the functional changes that occur in these patient cell lines it may be possible to form a more complete view of the disease, possibly leading to new and effective methods of treatment and diagnosis.

The issues addressed in this study are:

- The relationship between neuronal migration and patients with *NRXN1* deletions has not been established.
- Imbalances in the ratios between neural and supporting cells are related to schizophrenia but the cause behind this relationship has not been identified.
- The gene *NRXN1* has multiple isoforms and the expression of these isoforms has not been studied in relation to schizophrenia.

The goals of this study are:

- Investigate changes in neuronal migration that occur within patient versus control lines.
- Quantify the ratios of neural and supporting cells that are found in control and patient cell lines.
- Identify various isoforms of *NRXN1* found in patient versus control cell cultures.

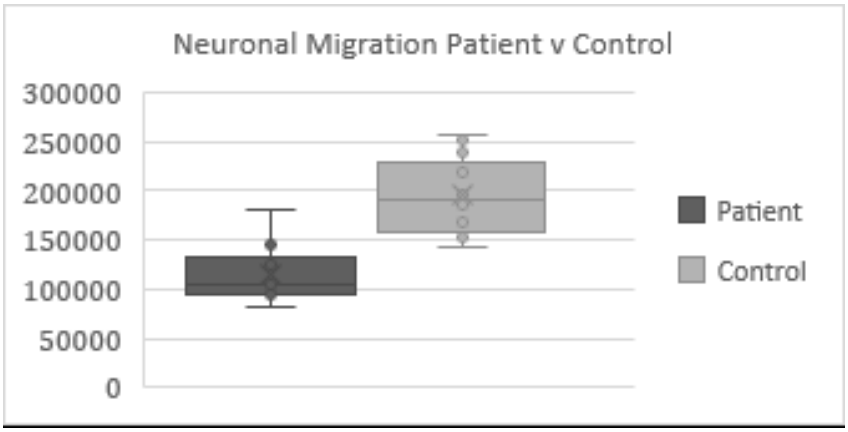

Figure 5. Box-and-whisker chart showing reuronal migration data in square pixels.

Results.

Neurosphere Assay. The measured neurospheres were processed using ImageJ and the resulting figures were analyzed using Excel and organized into data tables. The data set included three patient lines (972, 973, 641) and two control lines (2607, 553) with twelve analyzed neurospheres from each line. Average neuronal migration increased from 114,700 square pixels in patient lines to 196,300 square pixels in control lines ($p < .001$) (Figure 5). This significant 71% increase indicates that neuronal migration is decreased in patient line neurospheres. P values also reached significance when comparing cell lines directly instead of grouped as patients and controls showing a stronger relationship between lines. There were a small number of discarded results due to abnormal growth, multiple neurosphere placement, and placement close to the plate wall. In addition, variance in neurosphere size may have been another source of error. The method of statistical analysis used was a 2-sample t-test.

Astrocyte Quantification. This experiment measured the percentage area covered by a thresholded stain of an astrocyte marker, a neuron marker, and a marker for all genetic material used for normalization. Measurements were normalized to the DAPI blue stain and subjected to a two-sample t- test. There was a significant difference in the astrocyte-neuron results for patient versus control lines. ($p=.048$). Normalization to DAPI blue ensures that the ratio is not influenced by the number of cells in the image, and each of the 32 slides were imaged in two separate areas to ensure accuracy. However, the actual difference in means was not extremely large with an average difference of -.39 ± .20 (Figure 6).

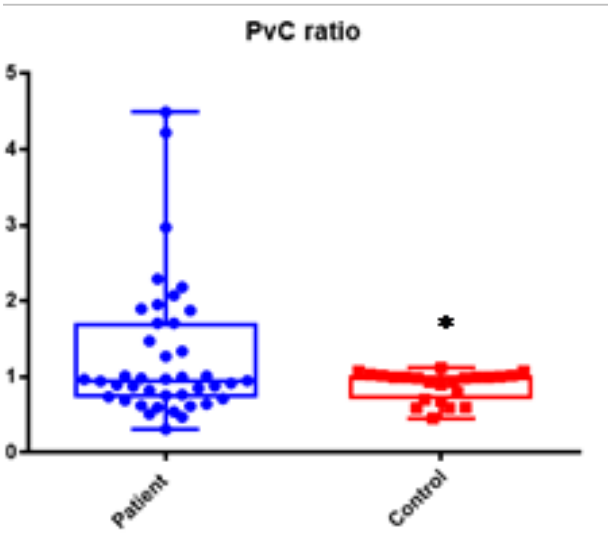

Figure 6. Ratio data points in patients and control groups.

Isoform Identification. In this section of the experiment we sequenced RNA from a number of cell lines and compared the data to a database sample taken from NCBI of *Homo sapiens NRXN1* alpha (Accession number: NM_004801.) Snapgene was then used to align the sequences and identify exons missing from the sequenced

samples. The sequencing process used multiple primers due to the length of RNA samples. Identified isoforms differed from the comparison sequence in numerous ways including deletions of exon 2 and various other insertions. There were four separate isoforms that were identified with certain isoforms only appearing in the RNA samples taken from the patient cell cultures and not present in the control cultures, showing a change in genetic structure between the schizophrenia patients and healthy controls. Although the sample size is relatively small, these findings still contribute to the observed changes in the structure of this gene in humans with schizophrenia.

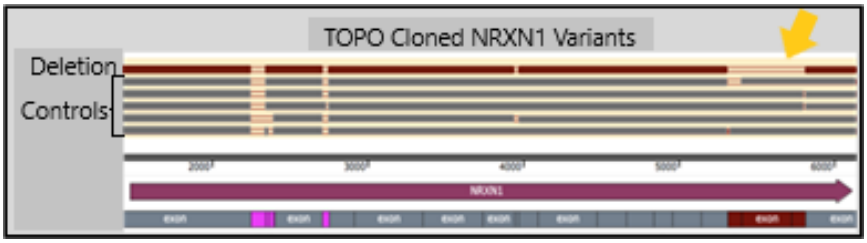

Figure 7. Sequencing data with highlighted deletion of exon 7, figure provided by mentor.

Conclusion. The two-sample t-test related to the neuronal migration experiment demonstrated a highly significant change in the distance migrated radially by each neurosphere. Such a significant increase in neurosphere migration is indicative of a change in the functioning of neuronal migration, a key aspect of neural development that is vital for proper functioning of the brain. These results mirror what has been observed in past animal and postmortem studies using models of schizophrenia as well as schizophrenic cells.[4] The consistent results confirm these findings as a mechanical symptom of schizophrenia as well as reinforcing the consistency of using stem cell cultures in order to study aspects of schizophrenia. Defects in this process have already been associated with other neuropsychiatric disorders in the past, and this experiment reinforces the relationship between schizophrenia and neuronal migration. The results support the original hypothesis that neuronal migration would be much more pronounced and functional in the control lines when compared to the patient lines.

The results for astrocyte quantification showed a significant increase in the ratios found in patient lines when compared to controls. The results indicate that the sampled cell cultures decreased in ratio of astrocytes to neurons in controls which refutes previous studies.[6] However, there were numerous sources of error such as issues in culture setup, staining method, and imaging technique that could contribute to these results. In addition to this, the results barely reached significance but there is not enough information to definitively refute or support the original hypothesis that the ratio would decrease in patient cell lines compared to controls. There is also variance between types of schizophrenia concerning change in astrocyte ratios which could have contributed to the inconsistent results.

The isoform identification portion of this experiment succeeded in isolating and sequencing a number of alternative isoforms from a wide number of cell cultures from patient and control lines. There were consistent patterns found in the deletion and insertion of exons and intron sequences of the genes which shows a change in the resulting variable expression that occurs due to the multiple splice sites. By identifying these isoforms this experiment validates the findings that variable expression is affected by the presence of schizophrenia in patient lines and reinforces the findings of past studies concerning isoform expression.[9,11] However, the sample size in this experiment was somewhat reduced due to primer and sequencing errors. The original hypothesis was partially supported by the identification of alternative isoforms, but additional validation is required to accurately categorize the isoforms.

Methodology. This experiment primarily involved three experimental assays used to quantify data related to isoforms, neuronal migration, and astrocyte ratios in cell cultures to investigate changes in patients harboring *NRXN1* deletions. For the identification of isoforms, RNA was extracted using a liquid-liquid technique involving phenol and chloroform. The extracted RNA was sequenced using long-read sequencing and compared to an original *NRXN1* sequence in order to identify different isoforms. The neuronal migration assay was performed by allowing progenitor cells to form neurospheres in culture. These spheres were allowed to migrate across a plate and the resulting spread of cells was quantified and measured across cell lines. The astrocyte ratios were measured by staining cell cultures with markers for astrocytes, neurons, and all cells. The cells were imaged in three colors in order to quantify the ratios of each type of cell in the culture in order to compare the areas taken up on the image by each stain separately.

Neurosphere assay. Neurospheres are clumps of a few hundred cells that form naturally when neurons are grown in free-floating culture. The starting cells are taken from a stock of pre-modified pluripotent cells and plated. They begin to differentiate naturally and consist of a heterogeneous mix of cell types and stages of development. These neurospheres are utilized to measure the natural movement of neurons as they grow. In order to generate useable neurospheres, the mentor plated groups of neural progenitor cells in non-adherent plates and allowed them to aggregate for 48 hours. The student prepared a 96-well

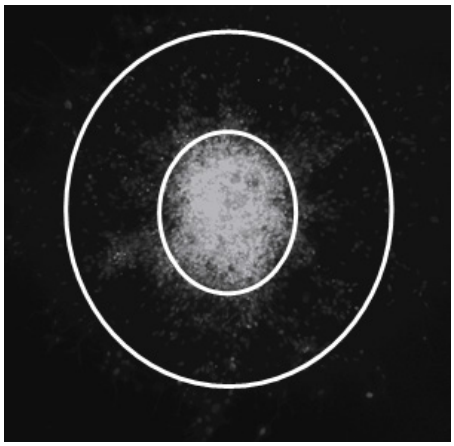

Figure 3. Example neurosphere assay with circles showing approximate inner and outer measurements.

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plate with a Matrigel coating and picked similar sized neurospheres into the plate using a pipette. Each neurosphere was centered in the plate by the student and checked for a single neurosphere in each well. Migration occurred for 48 hours and the cells were fixed with a 4% paraformaldehyde solution and stained using DAPI, a nuclear DNA stain, to allow for imaging.[10] Cells were imaged using an epifluorescence microscope and the resulting images were quantified using ImageJ, an image processing software. This allows for quantification of the original size of the neurosphere compared to the size after the 48-hour migration period.[10] Figure 2 shows the approximate points of measurement used for the inner and outer boundaries. This method allows for the comparison of distance covered by the cells migrating, obtained by subtracting the original distance from the migrated distance. This method effectively quantifies and indicates the presence of neuronal migration. [10]

Isoform identification. For the identification of RNA isoforms, the mentor prepared a culture of hiPSC neurons in 6-well plates and stored them in an incubator. The cell cultures were lysed using 1mL TRIzol per well, a solution containing phenol. The TRIzol and cells were mixed with 150 µL chloroform and centrifuged in order to separate the cellular components from RNA. Figure 3 shows the phase separation after centrifuging. The aqueous phase that contains RNA in solution was transferred to another container with isopropanol and centrifuged to pellet the RNA. The resulting pellet was washed using ethanol and resuspended in water for storage. This method was taken from the TRIzol Reagent User Guide from Thermo Fischer Scientific. The purified RNA was used to create a complimentary cDNA. This cDNA was used in a PCR with a forward and reverse primer targeting the first and last exons of *NRXN1*, respectively. The PCR products were separated through gel electrophoresis, extracted, and purified. The resulting sample was ligated with a TOPO vector that resulted in a complete plasmid ring that could be transformed into bacteria for culturing. The bacteria were then harvested, and RNA purified with a Thermo-Fischer miniprep. This purified RNA was sent to GENEWIZ for Sanger sequencing using their proprietary method. A gene sequence software called GENEWIZ was then used to compare the resulting sequences and identify differing isoforms by aligning them to a comparison sequence and identifying missing exons or inserted exons which indicates a differing isoform.[9]

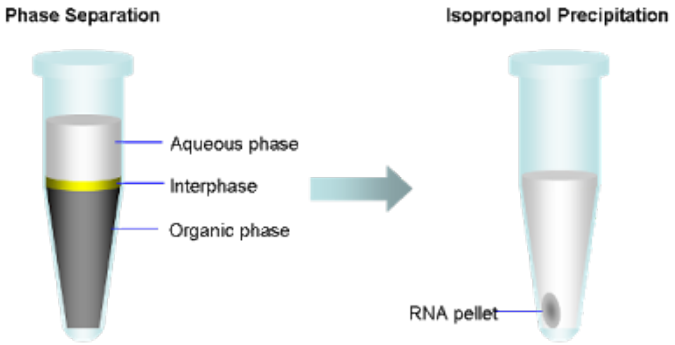

Figure 3. Showing phase separation of reagents, the aqueous phase contains RNA from molecularhub.blogspot.com

Astrocyte quantification. The method for quantification of astrocyte ratios utilizes three markers that stain for astrocytes, neurons, and all nuclei. A primary antibody was used to target s100b and MAP2 followed by a secondary antibody with an attached fluorescent probe for either GFP or RFP, two fluorescent proteins. The dyes used were GFP, RFP and DAPI blue which all fluoresce under differing wavelengths targeting s100b (glial-specific protein), MAP-2 (neural specific protein) and DAPI (all genetic material) respectively (Figure 4). The prepared cultures were grown from different neural progenitor lines by the mentor and placed on microscope slides for imaging. Each slide was imaged on a Zeiss LSM 780 confocal microscope using an automated z-stack program that allows for the imaging of multiple layers of cells simultaneously. Each section of cells was imaged under three wavelengths (395 nm, 588nm, and 358nm for GFP, RFP, and DAPI respectively) in order to measure each dye separately. Methods for this section were provided by the mentor and developed independently in the lab. This allowed for three separate images of the same section of cells each highlighting either all neurons, all astrocytes, and all genetic material in both types of cells. The resulting images were quantified using ImageJ and normalized to the DAPI dye in order to obtain a ratio of neurons to astrocytes which was compared across cell lines. Each image was separated into channels and thresholded to produce a black-and-white image to quantify the surface area occupied by each channel. The surface areas were then normalized to the DAPI blue and analyzed in Prism Graphpad.

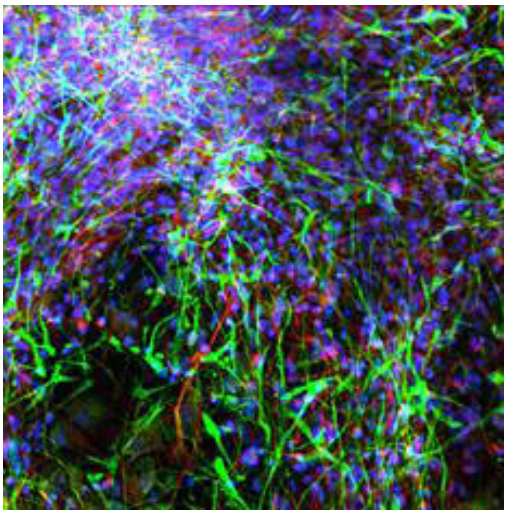

Figure 4. A composite image showing the three channels before separation and thresholding.

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