Unraveling the Function of *GABRB3* and Establishing a possible Connection between Abnormal *GABRB3* Concentration and the Onset of Autism Spectrum Disorder

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Abstract:

Autism Spectrum Disorder (ASD) is a prominent neurodevelopmental disorder that afflicts 1 in 59 children worldwide, as found in a 2018 study, and has risen by up to 15% in the last two years. While ASD contains a diverse array of different symptoms, common effects include impaired motor and communication skills, epilepsy, and sleep disorders. Over the years, research has pointed to a subunit gene of Gamma-aminobutyric acid (GABA) which had been shown to be the primary inhibitory neurotransmitter present in the brain. This gene is *GABRB3* and while various studies have established a link between the gene's presence and the expression of abnormalities associated with ASD symptoms, the true function of *GABRB3* in brain development and function is still largely unknown. Thus, the aim of this study is to observe if a relationship exists between GABRB3 abnormalities and common ASD symptoms and to discover what role GABRB3 plays in the development of the brain. Specifically, we look to the corpus callosum, primary mode of connection between the left and right cerebral hemispheres of the brain, and the border between the primary somatosensory cortex (S1) and secondary somatosensory cortex (S2), regions involved in processing and integrating of incoming sensory signals to motor signals. *We expect to find that the absence of* GABRB3 *leads to a decrease of axons across both the corpus callosum and between the S1-S2 borders, indicative of a decrease in pyramidal neuron activity in the brain. This in turn will provide a possible hypothesis at the*

function of GABRB3 within the neocortex of developing mice and establish a link between GABRB3 deficit (or in this case, complete absence) and the onset of ASD.

A. Research Rationale:

Autism Spectrum Disorders (ASD) is a neurodevelopmental disorder that negatively impacts communication and behavior; specifically it has been found to lead to impaired motor skills, sleeping disorders, and epilepsy (Mostofsky et al., 2009). Diagnosis for this disease averaged around 4 years of age, despite studies which have indicated that neural defects arise at even earlier developmental stages. Furthermore, early diagnosis and developmental intervention is believed to yield better clinical outcomes (Webb et al., 2014). However, behavioral manifestations of ASD symptoms as the primary mode of diagnosis has been shown to result in a delay of 3.6 years between when a parent initially contacts a health professional to the time the child receives a formal diagnosis (Crane et al., 2015). Thus, research towards developing mechanistic insight proves to be especially valuable.

Over the years, research has implicated rare variants of *GABRB3* (subunit gene of $GABA_A$ receptor which encodes for subunit β3) to be associated with ASD, as increased *GABRB3* expression has been theorized to contribute to the pathogenesis of ASD (Sutcliffe et al., 2003 and Chen et al., 2014, respectively). One reason for *GABRB3*'s association with ASD is due to its location on chromosome 15q12, a prominent region of genomic DNA deletions and duplications commonly associated with ASD among other developmental disorders (Hogart et al., 2010). Additionally, it is believed that ASD and other behavioral defects may be caused by an imbalance between GABAergic inhibition and excitation leaning towards more excitation.

Additionally, since GABA, Gamma-aminobutyric acid, is the primary inhibitory neurotransmitter present in the brain, that GABAergic signaling during development may underlie ASD pathophysiology. (Chao et al., 2010, Hunt et al., 2012, Gant et al., 2009, Sutcliffe et al., 2003). Due to this, further research regarding GABAergic signaling is .

Over the years, research has indicated that *Gabrb3* induces abnormalities similar to symptoms of ASD. One study illustrated that mice without the *Gabrb3* gene expressed the epilepsy phenotype in addition to behavioral abnormalities, including deficits in learning and memory, poor motor skills, and a disturbed rest-activity cycle (DeLorey et al., 1998). Another study done by DeLorey et al. showed that *Gabrb3* deficient mice demonstrated various neurochemical, electrophysiological, and behavioral abnormalities similar to traits associated with ASD (DeLorey et al., 2005). Furthermore, in a followup study, DeLorey et al. discovered that *Gabrb3* deficient mice manifested impaired social and exploratory behaviors among other indicators which establish that the phenotype of *Gabrb3* deficient mice to be a potential representative model of ASD (Delorey et al., 2008).

In this study, we sought to build on the existing literature and reveal the effects *Gabrb3* on neurotransmission in specific regions within the brain. One region that is of particular interest is the corpus callosum as it is primary mode of connection between the left and right cerebral hemispheres of the brain. Specifically, the corpus callosum functions to integrate and transfer information from both hemispheres in order to process sensory, motor, and high-level cognitive signals (Goldstein et al., 2019). Additionally, it plays a major role in transferring visual, auditory, and somatosensory information in posterior regions (Goldstein et al., 2019). In regards to ASD, studies have established that the corpus callosum provides an inhibitory effect that normally

deters uncoordinated hand-motor behavior, a common symptom expressed in patients with ASD (Tzourio-Mazoyer et al., 2016). Thus through investigating the effect *Gabrb3* on intracortical communication, we aim to discover if the presence or complete absence of *Gabrb3* may play a role in inducing abnormalities in neuron transmission across the corpus callosum.

The second region we chose to investigate was the border between the primary somatosensory cortex (S1) and the secondary somatosensory cortex (S2). The function of S1 ranges from processing incoming sensory input to the integration of both sensory and motor signals required for skilled movement. Throughout the years, numerous studies have illustrated a relationship between abnormal S1 activity, specifically the processing of somatosensory information, and motor dysfunction, similar to those expressed in those diagnosed with ASD (Elbert et al., 1998; Jacobs et al., 2012; Konczak and Abbruzzese, 2013). Furthermore, emerging research indicates that inaccurate processing and translation of sensory inputs contributes to deficits observed in neurological disorders, similar to ASD, which are characterized by imparied motor functions (Hummelsheim et al., 1988; Rub et al., 2003; Wolpert et al., 2013). S2 is a region whose functions remain largely unknown, however, a recent study indicates that there is a link present between the perception and processing of touch and S2 activation (Lamp et al., 2019). While Lamp et al. did not look exclusive on the effect of tactile stimulation of S2 activation, their results suggest that the S2 plays an important role in sense perception in that the S2 region was activated for both right and left hand tactile stimulation (Lamp et al., 2019). Thus the border between the two somatosensory cortices proves to be especially of interest.

B. Methodology:

B.1 Immunohistochemistry [Cell Staining]

Taking advantage of the relationship between antigens and antibodies, in that antibodies bind only to specific corresponding antigens, Immunohistory (IHC) is utilized to help visualize the distribution of cellular components in tissue.

Essentially, IHC is used to stain specific cells to be viewed under the microscope. Staining is important as it is essential to the identification and differentiation of one gene from another.

IHC Protocol (Adapted from Immunohistochemistry (IHC) Fundamental Principle):

I. Tissue Preparation
    A. The tissue had been previously cryostated and prepared by a supervisor for her ongoing experiment and placed in the freeze.

II. Solution Preparation
    A. All solutions used for this process were either pre-prepared by a supervisor or pre-purchased.

III. Incubation after removal from Freezer
    A. For incubation, the slides of tissue were kept at room temperature in a transparent enclosed humidified chamber (which was achieved by placing a wet paper towel below the slides and elevating the slides above the wet paper towel using a cut plastic pipette such that the two would not touch).

B. The slides were left in this condition for approximately 30 minutes to ensure that the tissue fully thawed out/defrosted.

IV. Rinsing the slides

A. A phosphate buffer solution (PBS) with 0.025% Triton X-100 was used to rinse the slides.

B. The slides were rinsed by soaking the slides in PBS and allowing them to sit for 2 minutes at room temperature (with an aluminum cover to prevent overexposure to the light) before the solution was drained.

1. This step was repeated 3 times.

V. Blocking the slides

A. Blocking was achieved by pipetting serum from the same species as a secondary antibody (the blocking serum was pre-purchased from a certified laboratory supplier).

B. After the serum was evenly distributed throughout the tissue, an aluminum cover was placed over it and it was allowed to sit at room temperature for an hour before the blocking solution was drained (allowing it to sit in the solution for too long would result in excessive blocking).

1. This was a crucial step in the IHC process as blocking significantly lowers the chances of obtaining false positive results that may occur when the residual sites on the cryostat tissue binds to secondary antibodies.

VI. Applying the Primary

A. Before applying anything, the slides were rinsed again (3x) to ensure that no blocking solution lingered on the slides.

B. A primary was applied onto the slides and the slides were transported to the freezer (approx. 10°C) and allowed to incubate overnight

    1. This is in order to allow for the optimal binding of the antibodies to tissue targets to in turn reduce nonspecific background staining.

VII. Applying the Secondary

A. Before applying anything, the primary antibodies were drained from the slides and they were rinsed (3x) to ensure that no primary solution remained on the slides.

B. A fluorophore-conjugated secondary antibody was applied to the slides and the slides were allowed to incubate for 1 hour at room temperature (with an aluminum cover).

VIII. Preparing the slide for microscopy

A. In order to ensure that the stained tissue is protected against accidental exposure to other chemicals or weathering, a coverslip was placed on top of the slide and sealed with nailpolish (along the edges).

B.2 Confocal Microscopy [Imaging]:

After IHC is complete, imaging is achieved through utilizing a laser scanning confocal microscope (LSCM). As opposed to the conventional wide-field microscopy, confocal microscopy allows for Z-stacking, or the viewing of the three-dimensional structure of the

specimen. If such a sample were to be viewed using wide-field imaging techniques, the image would lack contract due to the present of fluorophores throughout the entire depth of the specimen being illuminated, and not simply at a specific depth. On the other hand, confocal microscopes collect light selectively from an optical section less than 1 μm thick at the plane of focus. Additionally, studies have confirmed that due to its capability of optical sectioning, confocal imaging is opportune for the study of cell structure and function using organic dyes, fluorescence in situ hybridization, and immunofluorescence reagents (Goldstein and Watkins, 2008 and Knoll et al., 2007). Hence confocal microscopy proved to be well matched for this experiment.

The two areas of the brain, I focused on was the corpus callosum and the border between the primary somatosensory cortex and secondary somatosensory cortex. I chose to image two slices per brain for a total of 12 brains, 24 photos, taking a 10x photo of the corpus callosum, 10x generalized photo of the S1-S2 border, and also a 20x photos laterally down the border (from the pia to the end of the somatosensory cortex). Due to the small field of view of the 20X images, they were taken in sections and later pieced together in the step detailed below.

All Confocal setup procedures undertaken were carried out in accordance to the labory's Confocal Setup protocol.

B.3 ImageJ: Fiji

Corpus Callosum

ImageJ, Fiji, was used in order to analyze the images taken by the confocal microscope. Data collected in this area dealt with the average thickness (using layer 1 as a border in

measurements of the left and the right, in addition to also taking a measurement of the middle) of the axons being projected through the corpus callosum from one hemisphere to the other. Specifically, using a measuring tool, the ratio between actual micrometers of the image and the pixels of it was established in order to allow for scaling and measuring of the imaged section.

S1-S1 Border

Using this program, images of the latitudinal view of the S1-S2 border from the same brain was stitched together. This was a necessary step since images of this section were taken at 20X, which limited the field of view and did not allow for the imaging of the length of the S1-S2 border in one picture. Stitched pictures that resulted in crookedness were either retaken or had the edges cropped out, making for a slightly smaller width, which was later corrected for before beginning the data analysis.

For the S1-S2 border, we looked to analysis the percent of the imaged brain covered with axons, an indicator of interneuron activity between the S1 and S1 regions. This was accomplished through splitting the color channels and enhancing the amount of axons visible within the green channel [as the axons were visible due to the prenatal electroporation of green fluorescent protein]. Additionally, a cropping tool was used to exclude all non-somatosensory regions from consideration in order to increase the accuracy of the data collected.

B.4 Mice

The mice used in this sample were Postnatal (P) mice between P8-P12 years old and were sacrificed for an ongoing project, from which this stems as a side-study. Gender was mixed and not all the mice included in this experiment was from the same litter.

All procedures involved in handling and sacrificing the mice was acted in accordance with the Institutional Animal Care and Use Committee (**IACUC**) guidelines.

C. Projected Results:

C.1 Corpus Callosum

We hope to see a significant decrease in pyramidal cell activity (as measured through axonal transmissions) between the two hemispheres, through the corpus callosum as that would be indicative as a decrease in intracortical communication.

C.2 S1-S2 Border

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D. Analysis & Conclusion

To be determined based on results.

E. Discussion

To be determined based on results.

F. Limitations and Future Research

A limitation of this study is the time constraint which prevented a large number of samples to be used.

Future research is to be determined based on results.

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***Addendum: The only major changes added to this project are the result and data portion; no changes were made in regards to methodology, research rationale, or bibliography ***