Tufts
UNIVERSITY
Cummings School of
Veterinary Medicine

Sex Difference in Expression of Autism Candidate Gene *Gabrb3* in Mouse Amygdala

A Major Qualifying Project
Submitted to the faculty of the
Worcester Polytechnic Institute
in partial fulfillment of the requirements for the
Degree of Bachelor of Science

by:

Briana Dougherty

Date: April 24, 2008

Approved:

Dr. Daniel Gibson III, Major Advisor

Table of Contents

Acknowledgements

I would like to thank Dr. Jun Xu of Tufts University Cummings School of Veterinary Medicine for allowing me the use of his lab and always pushing me in the right direction.

I would also like to thank Dr. Elizabeth Byrnes of Tufts University Cummings School of Veterinary Medicine for her expertise with mouse brains.

Thanks to Dr. Daniel Gibson of Worcester Polytechnic Institute for his support and guidance.

Abstract

This project targeted gene expression in specific brain regions known to play a role in autism spectrum disorders (ASD). Due to ASD affecting males four times more often than females, we compared the male and female gene expression in different brain regions. Specific brain regions were extracted and analyzed using a PCR panel containing 84 neurotransmitter genes to identify differences between males and females in gene expression. Gabrb3 was found to be significantly higher in females than males. siRNA gene silencing was tested in the second part of the project for studying functionality. In the trial, the injection of gene specific siRNA, Mapk1, was shown to completely silence expression of the targeted gene. This would be the next step in identifying which genes are specific to autism.

Table of Figures

Table of Tables

1 Introduction

Autism spectrum disorders (ASD) are neurodevelopment disorders that are characterized by impaired social interaction, deficits in communication, and repetitive behaviors and restricted interests. One out of 166 newborns is affected by ASD, likely caused by both genes and environment. Parents are usually the first to notice a problem with the child, usually noticing that the child is unresponsive to people and/or focusing on one thing intently for an extended period of time (National Institute of Mental Health). The developmental disorders can range from mild to severe. There is no single best treatment plan for all affected children. Early intervention is vital since individuals respond to highly specialized programs.

Genetic factors are known to play a role in the etiology, as indicated by the difference in concordant rates for ASD between identical twin (ranging from 36% to 96%) and dizygotic twin (0%). The focus has been brought to specific chromosomal regions that possibly contain autism-related genes (National Alliance for Autism Research). These "autism susceptible genes" make certain individuals more vulnerable by influencing their brain development and/or function. One such gene is Gabrb3 which has an important role in neuronal communication (see below).

In neuropathologic examinations, anatomical changes have been consistently found in autism patients in the cerebellum, cerebral cortex, and limbic system. Based on recent technologies, scientists have been able to study the structure and function of specific brain regions. Some of these technologies include positron tomography (PET), computerized tomography (CT) and magnetic resonance imaging (MRI). Using these new advances, major brain structures associated with autism have been found, including the cerebellum, cerebral cortex, corpus

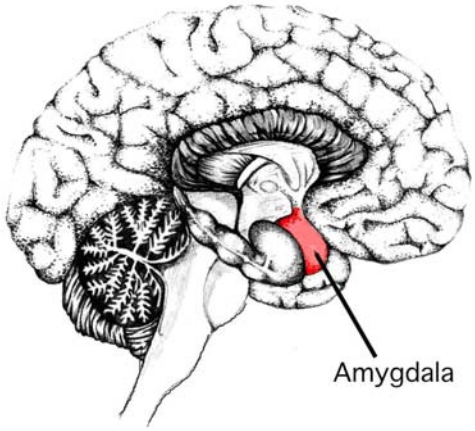

Figure 1: Location of amygdala.

callosum, brain stem, hippocampus, and the limbic system (National Institute of Mental Health).

One important component of the limbic system is the amygdala, which was chosen as the focus of our initial analysis. The amygdala plays an important role in appraising emotionally relevant information in social interaction. It is also responsible for emotional responses, including aggressive behavior (National Institute of Mental Health). Altered neural activities in the amygdala were revealed by fMRI and PET scans in people with ASD while they were engaged in social perception tasks. In the amygdala, GABAergic neurons play an essential role in emotion perception and memory formation. Specifically GABA-A receptor genes including *Gabrb3* have been identified as autism loci in genome scans.

$GABA_A$ receptors are important for proliferation, migration, and differentiation of precursor cells that coordinate development of the embryonic brain. $GABA_A$ receptors consist of eight different types of subunits: α1-6, β1-4, γ1-3, δ, ρ1-2, π, ε, and θ. Protein subunits co-assemble with other protein molecules to form an oligomeric protein. Amongst GABA subunit genes, there is a cluster consisting of Gabrb3, Gabra5, and Gabrg3. Together, the proteins coded by this gene cluster have unique regional and distribution in the central nervous system. *Gabrb3* is vital for both mature brain function and correct brain development (DeLorey, 208). Based on a study by DeLorey, et. al., it has been found that $gabrb3^{-/-}$ mice exhibit social deficits in behavior, similar to that of ASD. This leads us to believe that the gene *Gabrb3* has a part to play in the development of ASD.

Boys are four times more likely to be diagnosed with ASD than girls. The underlying biological mechanism is unclear. One possibility is that gene expression, and in turn neuronal activity differs between the two sexes in autism-related brain regions such as the amygdala. Mice and humans show similar gene expression patterns in the brain and therefore we performed gene expression analysis in the amygdala between adult male and female mice.

Once a gene is found to be expressed differently between males and females, its involvement in amygdala function as well as social behavior can be further analyzed by RNA interference method, which allows brain-region specific gene silencing.

RNA interference, or RNAi, is a mechanism for RNA guided regulation of gene expression where double stranded RNA (dsRNA) inhibits the expression of genes with complementary nucleotide sequences. The pathway can be seen in Figure 2. The RNAi pathway is initiated by dicer, an enzyme that cleaves dsRNA to short fragments about 20-25 base-pairs in length. The guide strand is incorporated into the RNA-induced silencing complex, or RISC, where it base-pairs with its complementary sequence.

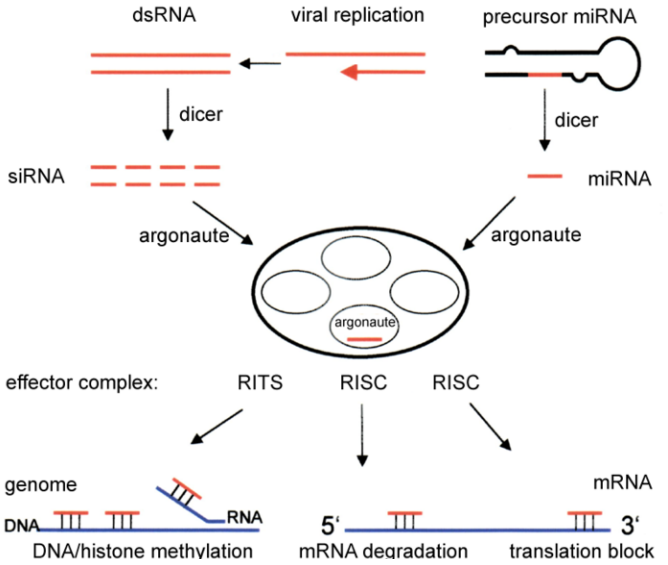

Figure 2: RNAi pathway. The enzyme dicer trims double stranded RNA, to form small interfering RNA or microRNA. These processed RNAs are incorporated into the RNA-induced silencing complex (RISC), which targets messenger RNA to prevent translation (Hammond, S)

The most well known outcome of this is post-transcriptional RNA gene silencing. This occurs when the guide strand base-pairs with mRNA to induce degradation of the mRNA by the catalytic component of the RISC complex. The results of this are short RNA fragments and are commonly known as small interfering RNA (siRNA) or microRNA (miRNA). The difference between siRNA and miRNA are dependant upon whether the fragments are from exogenous sources, siRNA, or when they are produced endogenously from RNA-coding genes, miRNA.

2 Materials and Methods

2.1 Mouse Model

The animals used in this study were two month old adult black 6 mice. The brain was freshly dissected from a two month old mouse and then frozen. The frozen brain was then placed in the cryostat and a 1 mm micropunch was used to isolate specific brain regions such as amygdala.

2.2 RNA Samples

An RNA extraction was performed using Qiagen's RNAeasy Mini Kit to isolate the RNA (Qiagen, Valencia, CA). The protocol followed was the one detailed in the manual found on the Qiagen website, (http://www1.qiagen.com/literature/handbooks/literature.aspx?id=1000291). The total RNA obtained was then run in the spectrophotometer to measure the concentration of the RNA. A 1.5% agarose gel was also run at 90 volts to assess the quality of the RNA. The quality of the RNA was confirmed by the presence of two bands, 18S and 28S bands which can be seen in Figure 3.

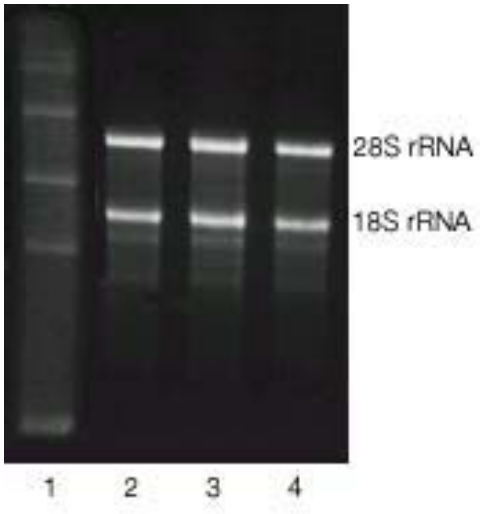

Figure 3: RNA integrity can be viewed by gel electrophoresis

2.3 SuperArray Analysis

Neurotransmitter Receptor SuperArrays (SuperArray, Frederick, MD) were used which are 96-well plates allowing simultaneous measurements of 84 genes on a real time PCR machine. The protocol followed was the one that was detailed in the manual online, (http://www.superarray.com/Manual/pcrarrayplate.pdf). These genes encode receptors for neurotransmitters such as acetyl-choline, dopamine, and GABA. Four pooled samples, 2 male and 2 female samples, were tested. Each sample contained amygdala RNA derived from 3 mice.

Ache	**Anxa9**	**Brs3**	**Cckar**	**Cckbr**	**Chat**	**Chrm1**	**Chrm2**	**Chrm3**	**Chrm4**	**Chrm5**	**Chrna1**
A01	**A02**	**A03**	**A04**	**A05**	**A06**	**A07**	**A08**	**A09**	**A10**	**A11**	**A12**
Chrna2	**Chrna3**	**Chrna4**	**Chrna5**	**Chrna6**	**Chrna7**	**Chrnb1**	**Chrnb2**	**Chrnb3**	**Chrnb4**	**Chrnd**	**Chrne**
B01	**B02**	**B03**	**B04**	**B05**	**B06**	**B07**	**B08**	**B09**	**B10**	**B11**	**B12**
Chrng	**Comt**	**Drd1a**	**Drd2**	**Drd3**	**Drd4**	**Drd5**	**Gabra1**	**Gabra2**	**Gabra3**	**Gabra4**	**Gabra5**
C01	**C02**	**C03**	**C04**	**C05**	**C06**	**C07**	**C08**	**C09**	**C10**	**C11**	**C12**
Gabra6	**Gabrb2**	**Gabrb3**	**Gabrd**	**Gabrg1**	**Gabrg2**	**Gabrp**	**Gabrq**	**Gabrr1**	**Gabrr2**	**Gad1**	**Galr1**
D01	**D02**	**D03**	**D04**	**D05**	**D06**	**D07**	**D08**	**D09**	**D10**	**D11**	**D12**
Galr2	**Galr3**	**Glra1**	**Glra2**	**Glra3**	**Glra4**	**Glrb**	**Gpr103**	**Npffr1**	**Prokr1**	**Prokr2**	**Npffr2**
E01	**E02**	**E03**	**E04**	**E05**	**E06**	**E07**	**E08**	**E09**	**E10**	**E11**	**E12**
Gpr83	**Grpr**	**Htr3a**	**Maoa**	**Mc2r**	**Nmur1**	**Nmur2**	**Npy1r**	**Npy2r**	**Npy5r**	**Npy6r**	**Ntsr1**
F01	**F02**	**F03**	**F04**	**F05**	**F06**	**F07**	**F08**	**F09**	**F10**	**F11**	**F12**
Pgr15l	**Ppyr1**	**Prlhr**	**Slc5a7**	**Sstr1**	**Sstr2**	**Sstr3**	**Sstr4**	**Sstr5**	**Tacr1**	**Tacr2**	**Tacr3**
G01	**G02**	**G03**	**G04**	**G05**	**G06**	**G07**	**G08**	**G09**	**G10**	**G11**	**G12**
Gusb	**Hprt1**	**Hsp90ab1**	**Gapdh**	**Actb**	**MGDC**	**RTC**	**RTC**	**RTC**	**PPC**	**PPC**	**PPC**
H01	**H02**	**H03**	**H04**	**H05**	**H06**	**H07**	**H08**	**H09**	**H10**	**H11**	**H12**

Table 1: Gene Table of Mouse Neurotransmitter Receptors and Regulators

A detailed list of the genes can be found in Appendix A on page 24 of this document.

Expression of these genes was shown based on comparative expression of Gapdh expression. These results were calculated using the following calculations.

The relative expression of each gene was calculated using the formula:

$$\Delta Ct = Ct^{GOI} - Ct^{AVG(HKG)}$$

Where *CtGOI* is the Ct value of gene of interest determined on the real time PCR machine indicating abundance of specific mRNA and *CtAVG(HKG*) is the average Ct value of five housekeeping genes.

The relative expression (Exp) of each gene was calculated using the formula,

$$Exp = 2^{-\Delta Ct} \ (\Delta Ct = Ct^{GOI} - Ct^{Gapdh})$$

To determine statistical significance ($p < .05$) t-tests were used to compare males and females. Six samples were included for each sex.

The SuperArray kit simultaneously measures 84 genes in a 96 well plate. The remaining 12 wells contain a combination of controls. Wells H1-H5 contain the housekeeping genes. These genes are control genes that should definitely show expression. These genes include: Gusb, Hsprt1, Hsp90ab1, Gapdh, and Actb. The housekeeping genes are used to normalize expression in the other 84 genes.

Another important gene of the control genes is the MGDC, or mouse genomic DNA contamination. It can be found in well H6. Since there is no purification method that can guarantee that RNA is completely DNA free, it is vital to know if there is any residual DNA that is still present with the RNA. The MGDC tests specifically for genomic DNA contamination in each sample. A MGDC threshold cycle less than 30 indicates a significant presence of genomic DNA contamination.

2.4 Individual Gene Analysis

2.4.1 cDNA Synthesis

Complementary DNA (cDNA) was synthesized from RNA. When synthesizing cDNA, it is necessary to remove any DNA contaminants. This was done with a genomic DNA elimination step. In order to make DNA-free RNA, a kit by Ambion (Austin, TX) was used. The protocol followed was the one given by the company (http://www.ambion.com/techlib/prot/bp_1906.pdf).

Using the DNA free RNA, cDNA was synthesized using Invitrogen's SuperScript III Platinum Two-Step qRT-PCR Kit (Invitrogen, Valencia, CA). The protocol followed was the one detailed in the manual. The cDNA synthesized was later used to test specific gene primers to identify any sex differences using real-time PCR.

2.4.2 Real-time PCR

cDNA was synthesized with reverse transcriptase (Invitrogen) followed by real time PCR quantifications using gene-specific primers. Primer sequences were obtained from the PrimerBank website and primers were ordered from IDT (Coralville, IA).

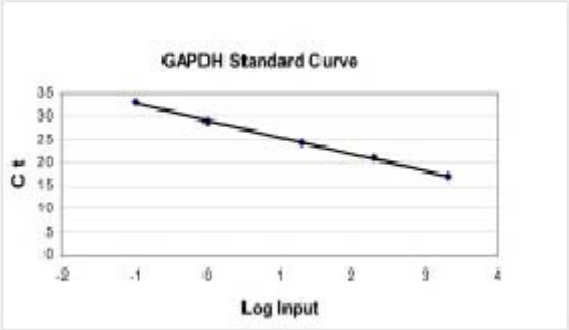

Figure 4: Graph showing linearity of serial dilution.

Each primer pair was tested for linearity with serial diluted samples and specificity which was indicated by aligned disassociation curves across samples. *Gapdh* expression was also quantified serving as a reference for loading.

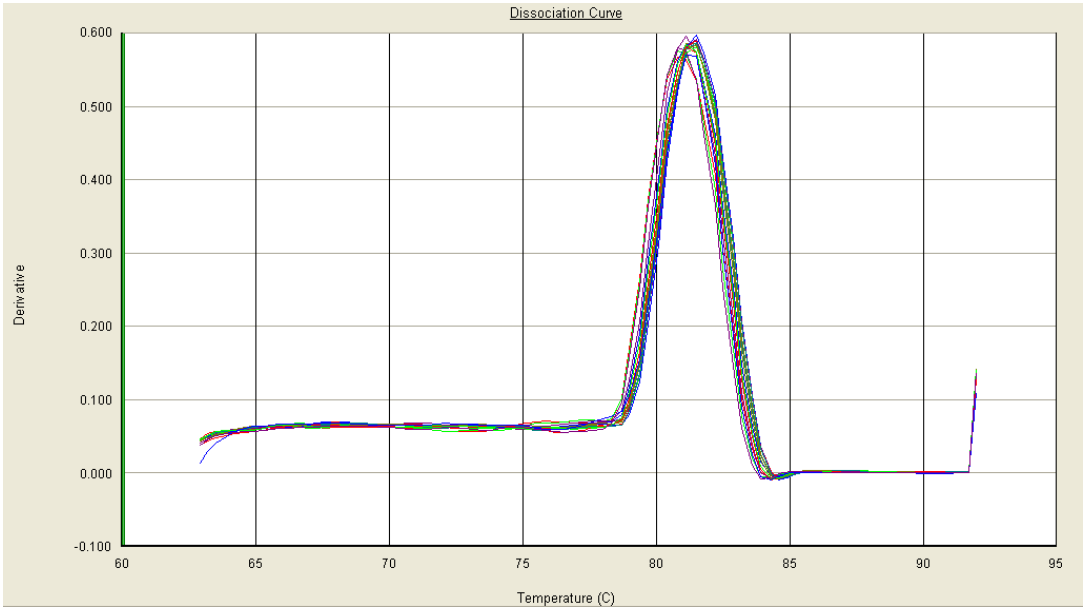

Figure 5: Graph showing dissociation curve

2.5 siRNA Injection

Gene specific siRNA was purchased from Qiagen and mixed with DOTAP (Roche, Penzberg, Germany) which served as transfection vector. Each injection of 3.75µg siRNA in 1µl was delivered with a Precision pump into the brain of adult BL6 mice. Brain region specific expression of the target gene was analyzed two days after the injection.

2.6 Cell Culture

P19 stem cells were used for the cell cultures. Three different plates were used, NUNC plates, Falcon plates, and 12-well plates. The NUNC plates were used for general growth of stem cells. The Falcon plates were used for retinoic acid-induced neuronal differentiation. The 12-well plates were used for siRNA treatments. The medium used for maintaining cells was Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic. Phosphate Buffer Saline was used to wash the cells and trypsin was used to detach the cells. The cells were grown and stored in a 37°C incubator.

2.6.1 Unfreezing the Stock

The stock was frozen in 1 ml of Freezing Medium which is toxic to the cells. In order to prevent cell death, the stock is only partially thawed. When the stock was partially thawed, it was dumped into a 15 ml conical tube containing 10 ml DMEM. The conical tube was then inverted until everything was mixed. The cells were then centrifuged for five minutes. After five minutes, the cells form a pellet at the bottom of the conical tube. The excess medium was then removed and the pellet was resuspended in 2ml of DMEM. The cells were then plated in 10 ml DMEM in a NUNC plate.

2.6.2 Maintaining Growth

The protocol for maintaining growth is pretty consistent among cell culture labs. Once the cells are plated in a NUNC plate they were incubated at 37°C for two days. After the two day incubation period, the DMEM was removed and 5 ml of PBS was added to wash the cells. The PBS was gently swirled and then removed. Then, 1 ml of trypsin was added to the plate to detach the cells from the bottom of the plate. Once the trypsin was added, the plate was placed in the 37°C incubator for three minutes. The plate was gently hit to detach the cells from the plate. In order to stop the trypsinization, 9 ml of DMEM was added to the plate. In order to break up the cells, it was necessary to pipette up and down about ten times. A new plate was labeled and 10 ml of DMEM was placed in the plate. Only 0.5 ml of cells were placed in 10 ml of DMEM leaving us with a 1:20 dilution. The cells were then incubated for two days at 37°C.

2.6.3 Neuronal Differentiation

Retinoic acid (RA) treatment induces P19 stem cells to differentiate into neurons. The RA treated cells were plated in Falcon plates, where the cells did not adhere to the bottom of the plate. The cells were still incubated for 2 days at 37°C.

After stopping trypsinization of the cells, 1.5 – 3 ml of cells were plated in a Falcon plate containing 10 ml DMEM and 3 µl RA/ 10 ml medium. The plate was then stored at 37°C, this is neuronal differentiation Day 1 (D1). Cells form aggregates in suspension.

After two days, the medium was changed by removing all the media from the plate and transferring it to a 15 ml conical tube. The tube was left to sit for five minutes to allow the cells to settle to the bottom of the tube. During this time, 10 ml DMEM and 3 µl RA were mixed in a new tube. The medium was then removed and the fresh DMEM/RA mixture was used to resuspend the cells. The total volume was transferred to a new Falcon plate and incubated for another two days at 37°C.

On D5, RA treatment is complete. The total volume from the plate is removed and put into a 15 ml conical tube and allowed to sit for five minutes so the cells will settle at the bottom. The medium was removed from the tube and washed with 5 ml PBS, dispersed by flicking tube. After washing, the PBS was removed and 3 ml of trypsin was added and dispersed by flicking the tube. The tube was then incubated for five minutes at 37°C. After five minutes, the trypsin was removed, as much as possible, and 2 ml of medium was added. By pipetting up and down about ten times to be sure the cells were completely dispersed. 1 ml of cells was plated in 10 ml DMEM in NUNC a plate.

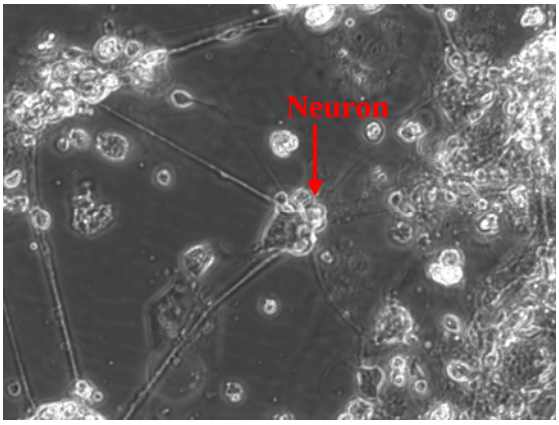

Figure 6: D6 retinoic acid treatment

On D7, Ara-C treatment was started. Ara-C incorporates into the DNA in dividing cells and thus kills any cells that divide (such as glia and fibroblasts as well as undifferentiated stem cells). Therefore, Ara-C kills any stem cells that were not differentiated into neurons. For 1 ml of DMEM, 1 µl of Ara-C is needed. The DMEM present in the plate was removed and 10 ml DMEM plus 10 µl Ara-C were then added gently to the plate. The cells were incubated for two days at 37°C.

2.6.4 siRNA Treatment

siRNA treatment allows for a knockdown of a specific gene of interest. As a trial, we tested knockdowns in mitogen activated protein kinase 1 (MAPK1) and Jarid1c as the gene of interest. Jarid1c mutations have been found to have a part in both X-linked

mental retardation as well as aggression. MAPK1-siRNA is a positive control sold by Qiagen.

Shortly before transfection occurred, 100 µl of cells were seeded in each well of a 12-well plate in 1 ml DMEM. The plate was then incubated under normal growth conditions (37°C). Then, 100 µl of DMEM without FBS was mixed with 3 µl of 2 µM siRNA and 6 µl HiPerfect Transfection Reagent (Qiagen) by vortexing. The mixture was then incubated at room temperature for 5-10 minutes to allow a complex to form. The siRNA was specific for one of three genes, MAPK1, Jarid1c, or a negative control. The complex that formed was added into the wells containing newly seeded cells in a drop-wise fashion, and after gently rocking the plate to ensure uniform distribution; the plate was placed back into the 37°C incubator for two days.

After two days, a photo was taken of each well and then they were harvested. The DMEM was removed and each well was washed with 1 ml of PBS. The PBS was removed and 500 µl of RLT + 5 µl βME were added to each well. It was allowed to sit for two minutes and then the plate was struck to disrupt the cells. Everything was completely removed from each well and placed in a labeled microcentrifuge tube and stored at -20°C. These cells were later used to extract RNA from.

2.6.5 Harvesting Cells

Harvesting the cells allows us to freeze for later use, most likely an RNA extraction.

The DMEM was removed from the plate. The cells were washed with 5 ml of PBS, and the PBS was gently removed. The cells were trypsinized using 1 ml of trypsin, incubating the plate for two minutes at 37°C. The trypsinization process was stopped with 2 ml of DMEM. As long as the cell types are the same, they can be combined into one tube and then centrifuged for five minutes. Then the DMEM + trypsin was removed from the cells and washed with 5 ml PBS by disrupting the pellet. Centrifuge again for five minutes. Removing as much of the supernatant as possible, the cell were then ready to be stored at -80°C.

2.6.6 Freezing Cells

Freezing cells is helpful if you are going to be away from the cells for more than a few days. The DMEM was removed from the plate. The cells were washed with 5 ml of PBS and then the PBS was gently removed. The cells were trypsinized using 1 ml trypsin, incubating the plate for three minutes. The cells were dispersed by hitting the plate. The trypsinization was stopped by adding 5 ml DMEM. Then, the tube was centrifuged for five minutes. Without disturbing the pellet, as much medium as possible was removed. The pellet was then resuspended in freezing medium containing DMSO and 1 ml of the mixture was added to each cryotube. The cells were then stored at -80°C.

3 Results

3.1 SuperArray Results

SuperArray analyses of 84 neurotransmitter receptor genes were performed on 4 biologically independent samples (2 per sex). Some genes were found to have higher expression in females such as *Sstr3* and *Gabrb3*, and some higher in males, such as *Anxa9*. *Gabrb3* is a receptor subunit for the neurotransmitter GABA, a major inhibitory transmitter of the nervous system. Mutations in this gene may be associated with the pathogenesis of Angelman syndrome, Prader-Willi syndrome, and autism. (National Center for Biotechnology Information, Gabrb3). *Sstr3* is somatosation receptor 3. It acts to inhibit the release of hormones and other secretory proteins at many different sites. It is expressed in the highest levels in brain and pancreatic islets. (National Center for Biotechnology Information, Sstr3). *Anxa9* (annexin A9) encodes a divergent member of the annexin protein family having a structural analysis that suggests the conserved putative ion channel that is formed by the tetrad core. (National Center for Biotechnology, Anxa9).

In addition, some genes were found to be more actively transcribed in the amygdala, *Gad1* and *Gabra1*, while some were expressed at much lower levels, *Npy6r* and *Tacr2*. *Gad1*, glutatmate decarboxylase 1, is the rate-limiting enzyme in GABA synthesis and is identified as a major autoantigen in insulin-dependant diabetes and deficiency in this enzyme has been shown to lead to seizures (National Center for Biotechnology Information, Gad1). Like Gabrb3, *Gabra1* is a receptor subunit for the major inhibitory neurotransmitter GABA in the mammalian brain. At least 16 distinct subunits of GABA-A receptors have been identified (National Center for Biotechnology Information, Gabra1). *Tacr2*, tachykinin receptor 2, belongs to a family of genes that functions as receptors belonging to this family are characterized with G proteins and 7 hydrophobic transmembrane regions.

Our results such as the female-bias in expression of Sstr3 are consistent with previous findings including the up-regulatory effect of estrogens on Sstr3 expression.

3.2 Individual Gene Results

Gabrb3 and *Sstr3* were verified to be expressed more highly in the female amygdala than in males (*: $p < .05$ in both cases). Another GABA receptor gene, *Gabra5*, showed no sex difference in expression therefore the sex difference appears to be specific for certain GABA receptor genes but not others. No sex difference was found in expression of *Anxa9* when 6 male and 6 female amygdala samples were tested, in spite of the SuperArray result.

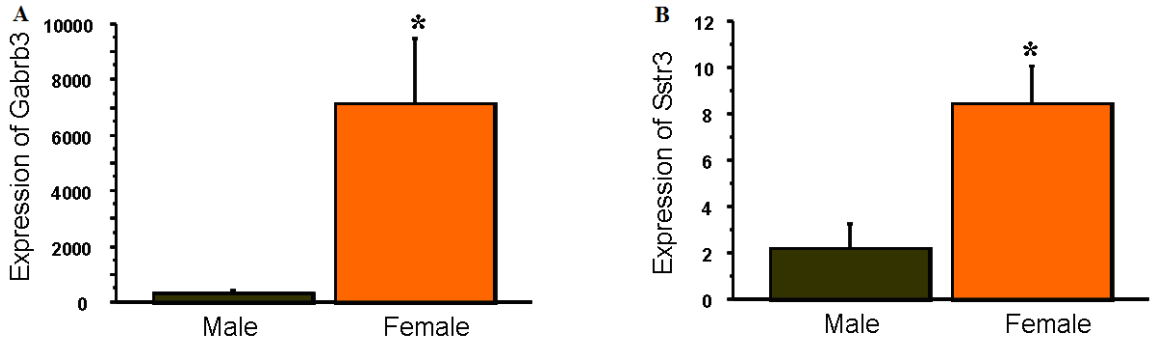

Figure 7: Graph A compares expression of Gabrb3 between males and females which shows that females had a higher level of Gabrb3 mRNA in the amygdala than males. Graph B shows that the female bias in expression is consistent with up-regulatory effects of estrogen on Sstr3 expression.

Figure 7A shows the difference in *Gabrb3* expression. As it can be seen, the expression of *Gabrb3* shows a significant difference, * = $p < 0.5$ with a higher expression in females. Figure 7B shows the difference in *Sstr3* expression, and it also shows a significant difference, * = $p < 0.5$.

3.3 siRNA Results

As a test for methodology, siRNA specific for *Mapk1* was injected into the dorsal hippocampus and quantitative real time PCR was performed 48 hr later. *Mapk1* was found to be specifically silenced as can be seen in Figure 8.

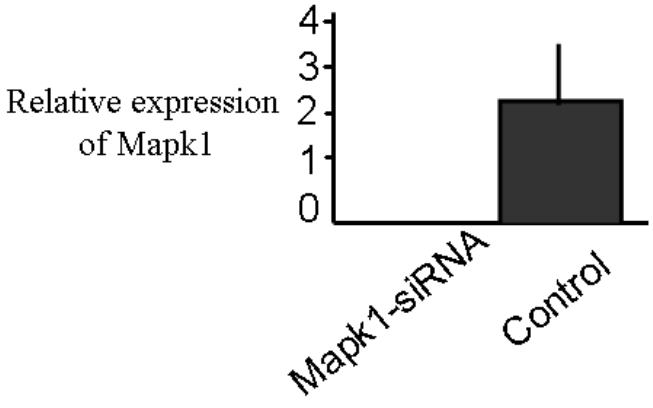

Figure 8: Expression of Mapk1 siRNA implying that Mapk1-siRNA treated brains the expression of Mapk1 was silenced.

4 Discussion

Gabrb3, a gene which is implicated in autism in humans, was found to be expressed more highly in female amygdala than in males in mice. It is unclear whether a similar sex difference will be identified in humans and whether such a sex difference contributes to the difference in vulnerability in autism. If *Gabrb3* is also expressed more highly in human females than males, we can make the connection that *Gabrb3* has some affect on blocking autism in humans. In the future we will compare *Gabrb3* expression in BTBR mice. BTBR mice are so called "autistic mice". These mice exhibit autistic physical behaviors, such as anti-social behavior, and not playing as much with other mice. In addition, using siRNA brain region-specific gene silencing, we will delineate in the future whether *Gabrb3* gene plays a role in sex differences in behavior such as mouse social play behavior. Our goal for the future would be to inject *Gabrb3* siRNA into the amygdala.

Sstr3, a gene encoding somatosatin receptor subtype, was also found to be expressed higher in females than in males. This sex difference has been previously shown to be due to the effect of estrogen. Based on this information we cannot deduce what effect *Sstr3* has regarding autism. The next step would be to do comparative analysis between male and female mice, as well as between males with no testes and females with testosterone.

Based on our findings, we cannot make any definitive conclusions on what genes affect human autism. However, this evidence will hopefully lead us in the correct direction to isolate the genes responsible for autism in humans.

5 Works Cited

Arnold, Arthur P., et al. "Minireview: Sex Chromosomes and Brain Sexual Arnold, Arthur P., et al. "Minireview: Sex Chromosomes and Brain Sexual Differentiation." Endocrinology 145.3 (2004): 1057.

Baron-Cohen, Simon, Rebecca C. Knickmeyer, and Matthew K. Belmonte. "Sex Differences in the Brain: Implications for Explaining Autism." Science 310 (2005): 819.

Bolivar, Valerie J., Samantha R. Walters, and Jennifer L. Phoenix. "Assessing Autism-Like Behavior in Mice: Variations in Social Interactions among Inbred Strains." Behavioral Brain Research 176 (2007): 21-6.

DeLorey, Timothy M., et al. "Gabrb3 Gene Deficient Mice Exhibit Impaired Social and Exploratory Behaviors, Deficit in Non-Selective Attention and Hypoplasia of Cerebellar Vermal Lobules: A Potential of Autism Spectrum Disorder." Behavioral Brain Research 187 (2008): 207-20.

Hammond S, Bernstein E, Beach D, Hannon G (2000). "An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells". *Nature* **404** (6775): 293–6. PMID 10749213.

National Alliance for Autism Research. "Exploring Autism." Brilliant Content. 2002. <http://www.exploringautism.org/autism/index.htm>.

National Center for Biotechnology Information. "Entrez Gene: Anxa9." March 13 2008. <http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=Retrieve&dopt=Graphics&list_uids=8416>.

---. "Entrez Gene: Gabra1." April 6 2008. <http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=Retrieve&dopt=Graphics&list_uids=2554>.

---. "Entrez Gene: Gabrb3." April 13 2008. <http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=Retrieve&dopt=Graphics&list_uids=2562>.

---. "Entrez Gene: Gad1." April 13 2008. <http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=Retrieve&dopt=Graphics&list_uids=2571>.

---. "Entrez Gene: Sstr3." April 6 2008. <http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=Retrieve&dopt=Graphics&list_uids=6753>.

National Institute of Child Health and Human Development. "Autism Overview: What We Know." National Institute of Health. May 2005. <http://www.nichd.nih.gov/publications/pubs/upload/autism_overview_2005.pdf>.

National Institute of Mental Health. "Autism Spectrum Disorders." National Institute of Health. April 3 2008. <http://www.nimh.nih.gov/health/publications/autism/complete-publication.shtml>.

Pachernik, J., et al. "Retnoic Acid-Induced Neural Differentiation of P19 Embryonal Carcinoma Cells is Potentiated by Leukemia Inhibitory Factor." Physiological Research (2005) . April 10, 2008 <http://www.biomed.cas.cz/physiolres/pdf/54/54_257.pdf>.

6 Appendices

6.1 Appendix A: Gene Table of Mouse Neurotransmitter Receptors and Regulators

Position	Symbol	Description
A01	Ache	Acetylcholinesterase
A02	Anxa9	Annexin A9
A03	Brs3	Bombesin-like receptor 3
A04	Cckar	Cholecystokinin A receptor
A05	Cckbr	Cholecystokinin B receptor
A06	Chat	Choline acetyltransferase
A07	Chrm1	Cholinergic receptor, muscarinic 1, CNS
A08	Chrm2	Cholinergic receptor, muscarinic 2, cardiac
A09	Chrm3	Cholinergic receptor, muscarinic 3, cardiac
A10	Chrm4	Cholinergic receptor, muscarinic 4
A11	Chrm5	Cholinergic receptor, muscarinic 5
A12	Chrna1	Cholinergic receptor, nicotinic, alpha polypeptide 1 (muscle)
B01	Chrna2	Cholinergic receptor, nicotinic, alpha polypeptide 2 (neuronal)
B02	Chrna3	Cholinergic receptor, nicotinic, alpha polypeptide 3
B03	Chrna4	Cholinergic receptor, nicotinic, alpha polypeptide 4
B04	Chrna5	Cholinergic receptor, nicotinic, alpha polypeptide 5
B05	Chrna6	Cholinergic receptor, nicotinic, alpha polypeptide 6
B06	Chrna7	Cholinergic receptor, nicotinic, alpha polypeptide 7
B07	Chrnb1	Cholinergic receptor, nicotinic, beta polypeptide 1 (muscle)
B08	Chrnb2	Cholinergic receptor, nicotinic, beta polypeptide 2 (neuronal)
B09	Chrnb3	Cholinergic receptor, nicotinic, beta polypeptide 3
B10	Chrnb4	Cholinergic receptor, nicotinic, beta polypeptide 4
B11	Chrnd	Cholinergic receptor, nicotinic, delta polypeptide
B12	Chrne	Cholinergic receptor, nicotinic, epsilon polypeptide
C01	Chrng	Cholinergic receptor, nicotinic, gamma polypeptide
C02	Comt	Catechol-O-methyltransferase
C03	Drd1a	Dopamine receptor D1A
C04	Drd2	Dopamine receptor 2
C05	Drd3	Dopamine receptor 3

C06	Drd4	Dopamine receptor 4
C07	Drd5	Dopamine receptor 5
C08	Gabra1	Gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 1
C09	Gabra2	Gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 2
C10	Gabra3	Gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 3
C11	Gabra4	Gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 4
C12	Gabra5	Gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 5
D01	Gabra6	Gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 6
D02	Gabrb2	Gamma-aminobutyric acid (GABA-A) receptor, subunit beta 2
D03	Gabrb3	Gamma-aminobutyric acid (GABA-A) receptor, subunit beta 3
D04	Gabrd	Gamma-aminobutyric acid (GABA-A) receptor, subunit delta
D05	Gabrg1	Gamma-aminobutyric acid (GABA-A) receptor, subunit gamma 1
D06	Gabrg2	Gamma-aminobutyric acid (GABA-A) receptor, subunit gamma 2
D07	Gabrp	Gamma-aminobutyric acid (GABA-A) receptor, pi
D08	Gabrq	Gamma-aminobutyric acid (GABA-A) receptor, subunit theta
D09	Gabrr1	Gamma-aminobutyric acid (GABA-C) receptor, subunit rho 1
D10	Gabrr2	Gamma-aminobutyric acid (GABA-C) receptor, subunit rho 2
D11	Gad1	Glutamic acid decarboxylase 1
D12	Galr1	Galanin receptor 1
E01	Galr2	Galanin receptor 2
E02	Galr3	Galanin receptor 3
E03	Glra1	Glycine receptor, alpha 1 subunit
E04	Glra2	Glycine receptor, alpha 2 subunit
E05	Glra3	Glycine receptor, alpha 3 subunit
E06	Glra4	Glycine receptor, alpha 4 subunit
E07	Glrb	Glycine receptor, beta subunit
E08	Gpr103	G protein-coupled receptor 103
E09	Npffr1	Neuropeptide FF receptor 1
E10	Prokr1	Prokineticin receptor 1
E11	Prokr2	Prokineticin receptor 2
E12	Npffr2	Neuropeptide FF receptor 2
F01	Gpr83	G protein-coupled receptor 83
F02	Grpr	Gastrin releasing peptide receptor
F03	Htr3a	5-hydroxytryptamine (serotonin) receptor 3A
F04	Maoa	Monoamine oxidase A

F05	Mc2r	Melanocortin 2 receptor
F06	Nmur1	Neuromedin U receptor 1
F07	Nmur2	Neuromedin U receptor 2
F08	Npy1r	Neuropeptide Y receptor Y1
F09	Npy2r	Neuropeptide Y receptor Y2
F10	Npy5r	Neuropeptide Y receptor Y5
F11	Npy6r	Neuropeptide Y receptor Y6
F12	Ntsr1	Neurotensin receptor 1
G01	Pgr15l	G protein-coupled receptor 15-like
G02	Ppyr1	Pancreatic polypeptide receptor 1
G03	Prlhr	Prolactin releasing hormone receptor
G04	Slc5a7	Solute carrier family 5 (choline transporter), member 7
G05	Sstr1	Somatostatin receptor 1
G06	Sstr2	Somatostatin receptor 2
G07	Sstr3	Somatostatin receptor 3
G08	Sstr4	Somatostatin receptor 4
G09	Sstr5	Somatostatin receptor 5
G10	Tacr1	Tachykinin receptor 1
G11	Tacr2	Tachykinin receptor 2
G12	Tacr3	Tachykinin receptor 3
H01	Gusb	Glucuronidase, beta
H02	Hprt1	Hypoxanthine guanine phosphoribosyl transferase 1
H03	Hsp90ab1	Heat shock protein 90kDa alpha (cytosolic), class B member 1
H04	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
H05	Actb	Actin, beta, cytoplasmic
H06	MGDC	Mouse Genomic DNA Contamination
H07	RTC	Reverse Transcription Control
H08	RTC	Reverse Transcription Control
H09	RTC	Reverse Transcription Control
H10	PPC	Positive PCR Control
H11	PPC	Positive PCR Control
H12	PPC	Positive PCR Control