Mouse Model for Sex Difference in Autism

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Abstract

Sex differences in the brain may influence the incidence rates of autism. Using two different strains of mice, one social (Black 6) and one asocial (BTBR), it is possible to compare the expression of different genes in certain areas of the brain suspected to be effected by autism. Using real-time PCR the target gene Neuroligin 3 (Nlgn3) showed a statistical difference in expression between the two strains. In addition, we are generating a mouse model which has four genotypes (Male (XY-SRY), Male (XX-SRY), Female (XX), Female (XY-)). Using this model, a sexually dimorphic phenotype can be analyzed between different hormonal conditions (testosterone versus estrogens) and between different sex chromosomal complements (XX versus XY).

Acknowledgements

This project has enabled the learning of laboratory skills as well as the patience it takes to work in a laboratory to obtain desired results. Over the past year, many people have contributed to the overall success of this project.

The staff at Tufts Cummings School of Veterinary Medicine, Biomedical Department, played a huge role in the successes of this project. More specifically, this project could not have happened without Jun Xu and Elizabeth Byrnes. Jun Xu served as the PI of the project and without his guidance, the project would not have happened. His patience and valuable insight every step of the way was an immeasurable amount of help. Elizabeth Byrnes also played a valuable role in this project. She spent countless hours working to extract brain punches as well as developing protocols as needed.

Dr. Daniel Gibson was also a great help during the process of the project. His willingness to advise this project was wonderful. Without his belief that this project would be successful, it would not have been possible.

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Introduction

Autism is the most common form of disability in a group of developmental disorders known as the autism spectrum disorders (ASDs).[1] It is a developmental disability and is believed to be influenced by both environment and genetic makeup. Through various studies using both monozygotic and dizygotic twins, ASDs have been found to be largely genetic. It has been found that 60-92% of monozygotic twins show concordance while only 0-10% of dizygotic twins show concordance.[2] It usually appears in early childhood, affects the ability to communicate, strains social interactions and results in restrictive interests and repetitive behaviors. Other common symptoms are lower sensitivity to pain, higher sensitivity to sound and touch, and an extreme negative reaction to sudden change. Effecting 1 in every 150 American children, and almost 1 in 94 males, it is one of the most prominent disabilities.[3] The disorder usually becomes visible in the first 2-3 years. Common symptoms of autism in infants are a lack of visual attention (non-responsive to something new being added to a room), lack of motor control and very low social response (i.e. showing a response when hearing their name or keeping eye contact). Overall, the symptoms of ASDs stem from abnormalities in brain structure and function.

The brain of an ASD patient has an increased volume during childhood.[4] In the amygdala, there is an increase in neuronal density and a decrease in neuronal size.[5] ASDs are believed to be caused by functional changes in the neural circuitry and an imbalance between excitatory and inhibitory synaptic transmission.[6] Although the exact mechanisms and location(s) of the roots of autism are unknown, it is believe that a number of brain regions may be involved. Among these are the hippocampus, amygdale and the

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[1] National Institute of Neurological Disorders and Stroke. "Autism Facts Sheet." http://www.ninds.nih.gov/disorders/autism/detail_autism.htm April 6, 2008
[2] Klauck, Sabine. "Genetics of autism spectrum disorder". *European Journal of Human Genetics*. 14 (2006) 714
[3] Autism Society of America. "About Autism". http://www.autism-society.org/site/PageServer?pagename=about_home. March 16, 2008
[4] Hardon et al. "Brain Volume in Autism". *Journal of Child Neurology*.16 (2001) 421-424
[5] Schumann C & Amaral D. Stereological analysis of amygdala neuron number in autism. *The Journal of Neuroscience*. July 19, 2006. 26(29):7674-7679.
[6] Tabuchi, Katsuhiko et al., "A Neuroligin-3 Mutation Implicated in Autism Increases Inhibitory Synaptic Transmission in Mice". *Science*. 318, 71 (2007)

cerebellum.[7] Studies using fMRI have shown diminished activation in the amygdala of autistic patients during social tasks. Researchers believe that ASDs are not caused by a single gene mutation but rather a group of genes being mutated is associated with the disorder. A target gene, Neuroligin 3 (Nlgn3), which is found in the amygdala, has been identified as an autism candidate gene whose mutations cause autism in carriers. Nlgn3 encodes a protein, which regulates the formation of GABAergic synapses in the brain. A defect in Nlgn 3 may prevent the formation or recognition of specific synapse needed for the communication process which is impaired in individuals on the spectrum.[8]

Research is slow in the area due to the lack of an adequate animal model. Recently, mice have been identified as a model for research for autism[9]. BTBR mice have been proposed as the best-suited model. In comparison tests through various studies with other strains of mice, BTBR mice show autism-like tendencies such as no preference for another mouse over inanimate object, increased self-grooming (repetitive behavior), and fewer social play behaviors. BTBR mice were the only strain, of ten tested, "that failed to show significant sociability while showing high open field exploration and a lack of anxiety-like behavior…".[10] These same tests showed many possible models for a "normal" mouse. In this study, we choose to use C57BL/6J (Black 6 or BL6) mice. In comparison with BTBR mice, Black 6 showed higher levels of social interaction, and scored relatively normal scores on various maze and social tests. [11]

As previously mentioned, there is a large statistical difference between the number of males versus females affected with autism. This is due to at least two sources: sex specific hormones and specific genes on the sex chromosomes. In order to separate the effects of sex hormones from sex chromosomes in a mouse model, four genotypes (XY, XY-, XY- SRY, and XX) are produced through breeding. This allows for the affect of the sex chromosomes and sex hormones to be observed independently.

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[7] Bolivar, Valerie et al., "Assessing autism-like behavior in mice: Variations in social interactions among inbred strains" Behavioral Brain Research 176 (2007) 21.

[8] Jamain et al., "Mutations of the X-linked genes encoding neuroligins Nlgn 3 and Nlgn4 are associated with autism". 34 (2003) 27-29

[9] Bolivar, Valerie et al., "Assessing autism-like behavior in mice: Variations in social interactions among inbred strains" Behavioral Brain Research 176 (2007) 21-26.

[10] Moy et al., "Mouse behavioral tasks relevant to autism: Phenotypes of 10 inbred strains". Behavioural Brain Research. 176 (2007) 16

[11] Moy et al., "Mouse behavioral tasks relevant to autism: Phenotypes of 10 inbred strains". Behavioural Brain Research. 176 (2007) 17

Materials and Methods

Breeding

A major part of this project was the breeding of the various strains and genotypes of mice. A Black 6 XY-SRY mouse was obtained from Dr. Arthur P. Arnold (UCLA) and six female mice were obtained from Jackson Laboratory (Bar Harbor, Maine). The mice were housed in one section of a three-section room where only mice used in this project were kept. The light cycle of the room was 12 hours light (7am-7pm) and 12 hours dark (7pm-7am). The food and water were checked and replenished on a daily basis and the cages were cleaned on a set schedule, which did not allow for undesirable living quarters. The temperature and moisture in the room could be controlled and the temperature ranged from 65ºC-71ºC while the moisture was always within 37%-42%. Each mouse was chipped with a unique microchip ID that can be read with a scanner.

Initially, a XY-SRY BL6 male was mated with a wild-type BTBR female to produce an F1 generation of XY-*Sry* BTBR mice. The litter was genotype by using a small piece of tail and extracting the DNA via the DNeasy Kit (Qiagen ©). PCR reactions were performed using three primers, *Sry*, *Ssty* (specific for the Y chromosome) and Myogenin (control gene).

Next, a XY-*SRY* F1 male was mated with a wild-type BTBR female to produce an F2 generation. These offspring were then genotyped to select XY-*Sry* offspring. Currently we have obtained the F4 generation. The process will be repeated to obtain generation F10.

Brain, Blood, Tail and Microchip Extraction

The mice need to be euthanized to obtain the brain, blood sample, piece of tail for DNA analysis, and to extract the microchip. For preparation, to keep the blood from clotting, glass centrifuge tubes were coated with 10 μl Heparin. The mouse was sacrificed using CO_2 in a sealed container. Immediately after, the mouse was decapitated and the body placed in a funnel to aid the blood draining into a glass centrifuge tube. While the blood was draining, the brain was extracted from the skull using surgical tweezers and scissors. Once the brain was extracted, it was placed in Isobutene to freeze the brain then

placed it in a 15 ml conical tube on dry ice. They were stored at -80ºC After the maximum amount of blood was gathered in the tube (0.5 – 1.0 ml), cut a small piece of tail (0.25- 0.5 cm) and place it in a 1.5 ml centrifuge tube. Lastly, the micro ID chip was removed by making an incision in the back of the mouse and pulling out the chip with surgical tweezers. The chip was placed in 100% ethanol for sterilization. All tubes were labeled with the genotype, date of birth, and date of euthanization.

Brain Punching

During the process of brain punching, the brain must be kept relatively frozen. A cryostat machine was used to hold the brain in a suitable cold environment. Using a mouse brain atlas, specific regions of the brain were identified and a 1-millimeter micro punch was taken and placed in a 1.5 ml centrifuge tube. Punches were taken from the cerebellum, hippocampus, hypothalamus, amygdale, and striatum.

RNA Extraction

The RNeasy Plus Mini Kit (Qiagen ©) was used to carry out the RNA extraction from different brain sections. A buffer composed of 10 μl β-mercaptoethanol for every 1 ml Buffer RTL plus was prepared so there was 400 μl per sample. Each sample (two micro punches) was ground with 400 μl buffer in a 1 ml grinder and transferred to a 1.5 ml centrifuge tube. To confirm the quality and quantity of RNA, spectrometer readings and a gel was obtained. The RNA was then made DNA free and converted into cDNA using the QuantiTect Reverse Transcription kit (Qiagen ©).

Real Time RT- PCR

Following the synthesis of cDNA, a Real Time Reverse Transcriptase (RT)-PCR was performed to determine the level of expression of desired genes. A master mix was made for each gene according to the number of templates. Each template required 15 μl of master mix that was composed of 3μl H_2O, 10 μl SYBR Green, and 2 μl primer. After dividing the master mix in the appropriate number of wells, 5 μl of cDNA template was added. To ensure the mix was at the bottom of the well, the 96-well plate was centrifuged for 1 minute at 1010 rpm then placed in the Real Time RT PCT machine.

Results

This project focused on two main objectives. The first was to develop an ideal mouse model and the second was to test target genes using RT-PCR. With the ideal mouse model still in the stages of development at the F4 generation, the results obtained were taken from the initial mice.

The preliminary tests were run with four of each type of mouse (4 BL6 males, 4 BL 6 females, 4 BTBR males, 4 BTBR females). The amount was used in the RNA extraction and gene expression experiments to collect consistent, statistically significant data. Using numerous samples allows for higher accuracy in results. The results in this section were done with cDNA created from RNA extracted from the amygdala. Figure 1 below shows the amplification plot produced from the Neuroligin 3 gene.
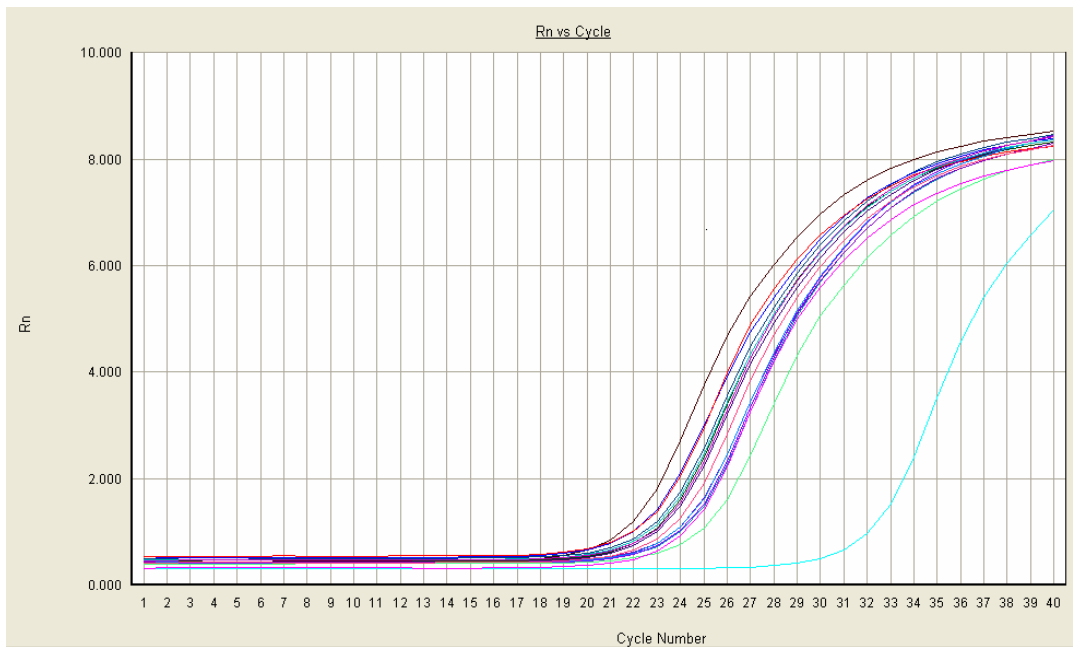

Figure 1: (RT PCR) Amplification plot of Nlgn 3 expression in the amygdala.

The slopes of these curves reflect the amplification efficiency of the reaction. An amplification plot can also be used to identify any irregular amplification. This allows for the sample that shows irregular amplification to be identified and its results discarded.

The second way in which the results of the Real time RT-PCR were examined was by the dissociation curve. Figure 2 shows the dissociation curve of the Neuroligin 3 samples.
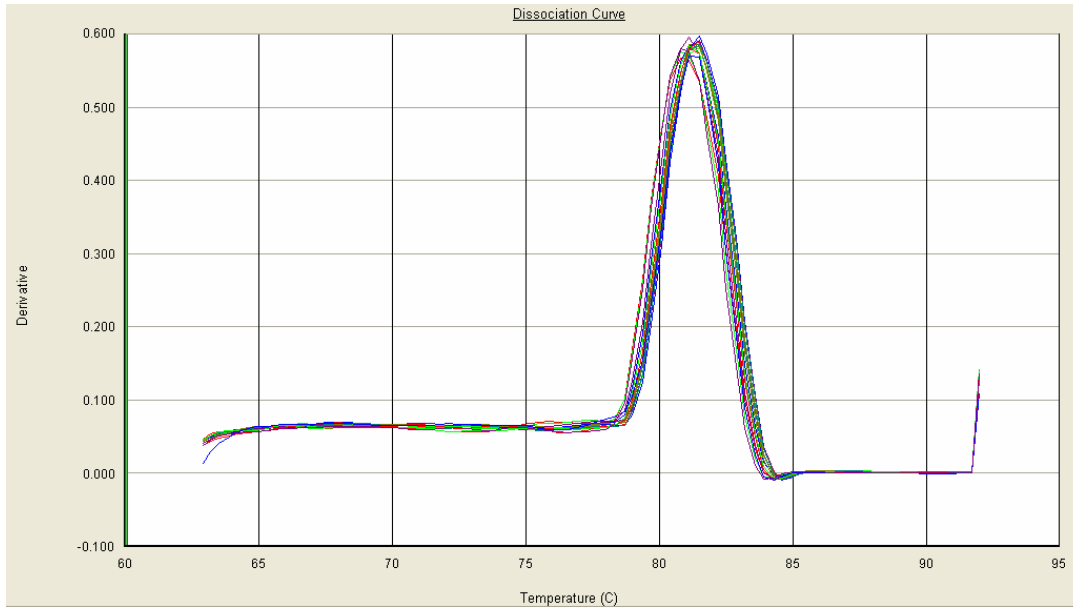

Figure 2: (RT PCR) Dissociation curve of Nlgn 3 in the amygdala.

The single peak in the curve supports that there was not any non-specific binding of the primer. The program used showed the individual curve of each well. The above curves show all 16 samples had the expected binding and none showed non specific results.

Figure 3 is a graphical representation of the Real Time RT-PCR results for Neuroligin 3.
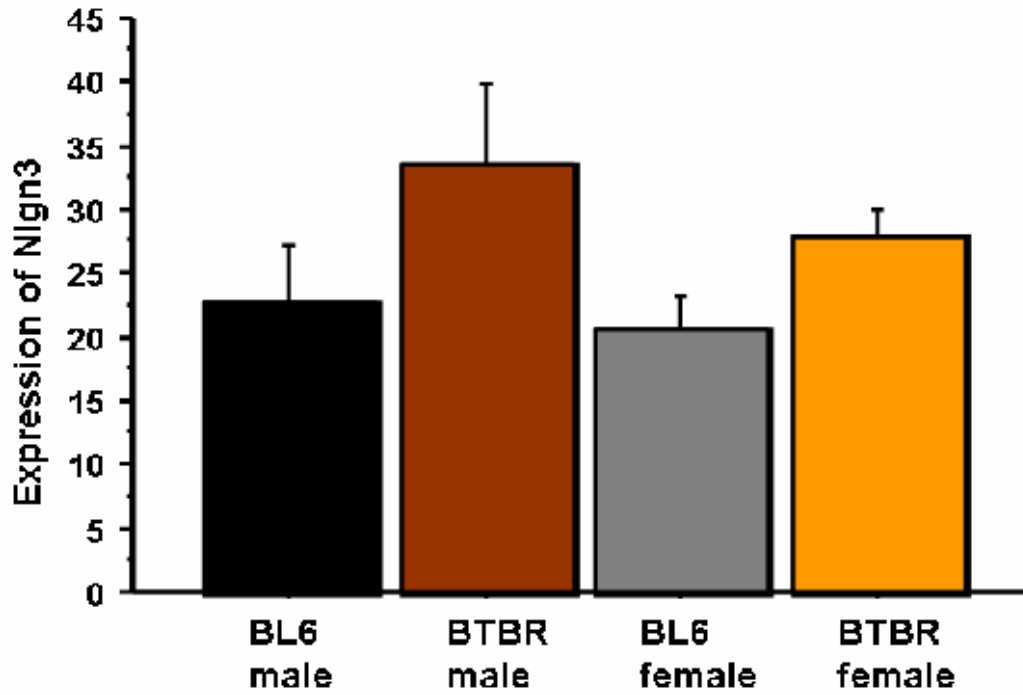
Figure 3: Nlgn 3 expression in the amygdala

Each group has four samples and each sample was measured at least twice. There is a strain effect of Nlgn3 that BTBR mice have a higher level, both males and females, than BL6 mice. The p value was 0.5, which makes these finding marginally significant.

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Conclusions and Discussion

A mouse model is currently being developed which will allow observations of the effect of sex chromosomes and sex hormones on autism-behaviors in BTBR mice. Once the F10 generation is obtained, a more convincing model for autism will be available to compare with normal mice. Using this model, RT-PCR using many different candidate genes can be performed to narrow the field of genes involved. It is believed that there is a group of genes involved in human ASDs and each one produces a different phenotypic response.

The autism candidate gene Neuroligin 3 shows preliminary difference in expression between BL6 and BTBR mice in the amygdala, which may be related to behavioral differences between the two strains. Further research needs to be conducting using the ideal F10 generation. Through Real Time RT-PCR and observational data, the significance of the effect of Neuroligin 3 in autism can be further determined.

Since it is believed that autism is not caused by a single gene, further research with candidate genes such as Nlgn4 and Nrxn1 should be conducted. Hopefully, using Real Time RT-PCR and mouse models, the etiology of autism can slowly be uncovered.

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3. Autism Society of America. "About Autism". http://www.autism-society.org/site/PageServer?pagename=about_home. March 16, 2008

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8. Jamain et al., "Mutations of the X-linked genes encoding neuroligins Nlgn 3 and Nlgn4 are associated with autism". 34 (2003) 27-29

10,11. Moy et al., "Mouse behavioral tasks relevant to autism: Phenotypes of 10 inbred strains". Behavioural Brain Research. 176 (2007) 1-20

Appendix A: Raw Real Time PCR Data

Sample	MEA Nlgn 3-1	MEA Nlgn 3-2	MEA Nlgn 3-3-1	MEA Nlgn 3-3-2	MEA Nlgn 3 Avg
BL6 Male 1	21.482	21.04	20.303	20.891	20.929
BL6 Male 2	23.406	22.003	21.976	22.06	22.36125
BL6 Male 3	25.052	23.278	23.256	23.056	23.6605
BL6 Male 4	23.476	21.764	22.137	21.543	22.23
BTBR Male 1	23.075	22.011	22.232	23.037	22.58875
BTBR Male 2	23.233	21.521	21.539	21.84	22.03325
BTBR Male 3	24.392	23.629	23.326	23.492	23.70975
BTBR Male 4	21.593	22.106	22.096	22.385	22.045
BL6 Female 1	24.827	23.043	23.432	23.294	23.649
BL6 Female 2	23.695	23.08	23.272	23.375	23.3555
BL6 Female 3	25.801	23.964	25.249	24.524	24.8845
BL6 Female 4	32.329	30.318	31.557	30.828	31.258
BTBR Female 2	24.338	22.593	22.69	24.511	23.533
BTBR Female 3	23.243	21.748	22.187	21.713	22.22275
BTBR Female 4	23.584	21.629	22.54	22.712	22.61625
BTBR Female 5	23.927	22.26	23.016	24.673	23.469

Sample	MEA Gapdh 1	MEA Gapdh 2	MEA Gapdh 3	MEA Gapdh Avg.
BL6 Male 1	16.455	16.365	16.263	16.361
BL6 Male 2	17.143	16.355	16.48	16.659333
BL6 Male 3	18.279	17.407	17.494	17.726667
BL6 Male 4	16.738	16.116	16.147	16.333667
BTBR Male 1	17.883	16.896	16.716	17.165
BTBR Male 2	17.349	16.505	16.468	16.774
BTBR Male 3	18.15	17.93	18.02	18.033333
BTBR Male 4	17.424	16.734	16.692	16.95
BL6 Female 1	18.074	17.085	17.224	17.461
BL6 Female 2	18.201	17.299	17.461	17.653667
BL6 Female 3	19.294	18.11	18.191	18.531667
BL6 Female 4	25.468	24.323	24.522	24.771
BTBR Female 2	18.128	17.13	17.365	17.541
BTBR Female 3	17.167	16.325	16.363	16.618333
BTBR Female 4	17.702	16.873	16.831	17.135333
BTBR Female 5	17.985	33.064	17.145	22.731333

MEA BL6 Male Averaage		MEA BTBR Male Average	
Nlgn3	22.29519	Nlgn3	22.59419
Gapdh	16.77017	Gapdh	17.23058

MEA   MEA
BL6 Female Average   BTBR Female Average
Nlgn3   25.78675   Nlgn3   22.96025
Gapdh   19.60433   Gapdh   18.5065