BTBR-FVB chromosome 4 congenic displays rescue of behavior and neuro-anatomical phenotypes

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BTBR-FVB CHROMOSOME 4 CONGENIC DISPLAYS
RESCUE OF BEHAVIOR AND NEURO-ANATOMICAL
PHENOTYPES

By

Jessica Gracias

A Thesis
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Abstract

Autism is commonly called the most genetic of neuropsychiatric disorders. Currently, the only diagnostic test is a behavioral evaluation. There is an urgent need for biomarkers to diagnose autism instead of just relying on behavior and there is also a need to understand the basis of this disorder. One of the theories is that malformation of the corpus callosum is one of the factors that lead to the behavior deficits seen in autism. This theory is based on the fact that human MRI studies have shown that the corpus callosum is absent or malformed in some autistic individuals. The exact genetic cause of the malformation is, however, unknown. Mice are used to investigate neuroanatomical and behavioral phenotypes related to autism because of the ease by which their genome can be manipulated.

Previous studies conducted found a gene region on the distal end of Chromosome 4 for the corpus callosum phenotype between BTBR T(+)tf/J (a mouse strain displaying an absence of the corpus callosum as well as autism-like social and communication deficits) and FVB/NJ(a mouse strain displaying a fully formed corpus callosum and does not show behavior deficits). A congenic mouse was then made, in which the normal gene region from an FVB mouse was inserted into a BTBR mouse background. The aim of this thesis was to investigate whether this gene region plays a role in corpus callosum development and if it is related to the autistic-like behavior phenotypes observed in BTBR. The last part of the thesis examined whether the best candidate gene in the region, Draxin, was expressed developmentally at time points that are important for corpus callosum development.

The findings from this thesis are three fold. First, the gene region is important for normal corpus callosum formation. Second, behavior tests indicated that the gene region has an effect on self-grooming behavior and may be responsible for an anxiety phenotype, but may have little effect on social behavior. Third, Draxin protein expression is observed at embryonic time points similar to those that are important for crossing of the corpus callosum across the midline.

This thesis project is novel because the mutation in the Draxin gene identified in BTBR is novel and the congenic mouse has not been investigated previously. By investigating if this gene region influences corpus callosum development and examining whether it is related to the repetitive movements and social deficits in the BTBR mouse, we get closer to finding gene markers for autism. This has implications for devising a diagnostic test for autism, which will allow for early intervention and treatment.
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Introduction
Autism Spectrum Disorders

Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders characterized by abnormal social interactions, communication deficits (verbal and non-verbal) and repetitive stereotyped patterns of behavior and interests (Diagnostic and Statistical Manual of Mental Disorders 2000). Currently, there are no objective biomarkers for ASD. Instead, subjective behavior evaluations are used like the DSM 5 (the Diagnostic and Statistical Manual of Mental Disorders), ADI-R (Autism Diagnosis Interview, Revised) and ADOS (Autism Diagnostic Observation Schedule) (Le Couteur et al 1989, Autism Speaks 2013).

The Center for Disease Control (CDC) has estimated that 1 in 88 children in the US have been diagnosed with ASD and it is 5 times more prevalent in boys (1 in 54) than girls (1 in 252) (Center for Disease Control, 2013). ASD has a substantial economic cost to society. A 2005 study by the CDC (Peacock, 2012) showed that, on average, medical costs for families with autistic children was about six times higher ($10,709 per child) compared to families without autistic children ($1812 per child). Besides medical costs, there are also costs related to early behavioral interventions, special education services and residential services which amount to about $60,000 - $128,000 a year (Amendah et al 2011).

Epidemiology and genetics studies conducted in patients with ASD indicate a strong genetics component compared to other neuropsychiatric disorders such as depression or addiction (Verhoeven et al 2010). Autism twin studies have indicated that in monozygotic twins and same-sex dizygotic twins, the heritability for autism is >90% (Freitag et al 2007). However, besides twin studies, a number of candidate genes identified for ASD in the population have shown conflicting data on chromosomal susceptibility regions. Some of the most promising genes are reelin, serotonin transporter (SLC6A4), Neuroligin (NLGN), oxytocin receptor (OXTR), met proto-oncogene hepatocyte growth factor receptor (MET), SH3 and multiple ankyrin repeat domains 3 (SHANK3), and contactin associated protein-2 (CNTNAP2). Reelin (RELN) is a membrane glycoprotein important for positioning of neurons in the cortex and cerebellum during development. Genome wide scans in autistic individuals showed a mutation in the reelin gene on chromosome 7 (7q22) (Li et al 2012). Elevated blood serotonin levels
observed in patients led to the discovery of the SLC6A4 gene mutation. Neuroligin genes are found on the sex chromosome and encode cell adhesion molecules important for synapse formation. So far, NLGN1, NLGN2, NLGN3, NLGN4 and NLGN4Y have been identified in humans (Li et al 2012). Oxytocin is involved in repetitive and social behaviors in animals and humans; hence investigators studied the OXTR gene in individuals with autism (Green and Hollander, 2010). Studies in different ethnic groups have confirmed that there is decreased expression of oxytocin mRNA as well as the OXTR in autistic individuals (Green et al 2010, Liu et al 2010, Suma et al 2009). MET was first studied as an oncogene, but recently it has been shown to have a critical role in neural development. Mutations in the MET gene causes impaired MET signaling, which disrupts migration of interneurons and leads to a decrease in granule cell division and proliferation (Li et al 2012). A lot of recent focus has also been on the SHANK family, especially SHANK 3. SHANK 3 is a scaffolding protein that is located at the excitatory synapses and is believed to function at the postsynaptic site, forming complexes with postsynaptic receptors, signaling molecules and cytoskeletal proteins present in the dendritic spine (Durand et al 2007). There are a lot more genes that play a role in making a person susceptible to autism because this spectrum disorder is complex and it is still unknown if it is a result of multiple gene interactions or rare mutations in the genome that have huge effects (Abrahams et 2008).

Another approach to studying ASD, is by relating brain structure abnormalities of individuals with ASD relative to controls (individuals without ASD). Neuroimaging studies using MRI in humans with autism have shown abnormalities in total brain volume, cerebellar volume, caudate nucleus, thalamus, amygdala and the corpus callosum (Verhoeven et al 2010). Macrocephaly (abnormal head circumference) is observed in 15% of autistic individuals (80% of these form postnatally). In the cerebellum, hypoplasia of vermian lobules VI and VII was reported in autistic individuals (Verhoeven et al 2010). Conflicting results have been obtained by investigators looking at amygdala volumes; some studies show increased volume, while others show decreased amygdala volume (Kim et al 2010, Aylward et al 1999). The anterior cingulate area is significantly smaller in autistic individuals complementing earlier findings that there is an increase in cell density and reduced dendritic fields in autistic patients (Verhoeven et al 2010). The
caudate (which has inhibitory roles in behavior) is enlarged in patients with autism and this has been correlated with repetitive behaviors (Verhoeven et al 2010).

There are many human MRI studies (some of which are described below) that have shown a strong correlation between autism and an impairment in connectivity between the hemispheres, specifically a decrease in the size of the corpus callosum in autistic individuals compared to age matched controls. Prigge et al (2013) studied the area of the corpus callosum developmentally over a 30-year range in autistic individuals and compared them with normally developing controls (68 males with autism ranging from 3 to 36 years of age). The control group showed an anterior to posterior increase in CC area from early childhood till early adulthood, while the autistic group showed a decreased area of the CC (which may be due to slower increase in its size during late childhood and early adulthood). The autistic group showed smaller areas of the whole CC, the splenium and the isthmus. The study also correlated areas of different parts of the CC and symptoms and severity of individuals with autism. They found that a smaller area of the anterior mid-body was related to more severe social impairment. In the control group, smaller area of the rostrum is associated with higher verbal IQ, but, interestingly, in autistic individuals it was found that smaller rostrum was associated with lower verbal IQ. And finally, in the control group, there was a linear relationship between the CC area and processing speed and in the autistic group, slower processing was linked to smaller isthmus area (Prigge et al 2013).

In another study linking autism to the agenesis of the corpus callosum (AgCC), Lau et al (2012) investigated a group of 106 individuals ranging from 4 to 74 years of age (consisting of both males and females) that had agenesis of the CC. In children, 45% of the AgCC group and 4.3% of the control group scored in the autism range. In adolescents, it was 35% of AgCC individuals and in adults; it was 18% of AgCC individuals that scored in the autism range. No sex differences were observed. Hence, a significant number of people with AgCC displayed traits that are characteristic with individuals diagnosed with autism (Lau et al 2013).

Anderson et al (2011) investigated differences in interhemispheric connectivity between high functioning autistic individuals and controls during childhood and into adulthood. They found that autistic individuals showed significantly lower
interhemispheric correlations compared to the controls throughout the brain, but some regions showed more difference than others. Besides a significant decrease in the mean total corpus callosum volume in autistic individuals, they also found significant decreases in the primary sensorimotor cortex and the prefrontal cortex, the fusiform gyrus, the frontal insulæ, the superior temporal gyrus and the superior parietal lobule, all of which have been related to symptoms of autism-like motor skill learning impairments, low social interactions, inability to identify novel objects and abnormal auditory processing (Anderson et al 2011). This study supports the theory that autism is a disorder caused by impaired connectivity and hence, a decreased or impaired integration of information. Using Magnetization Transfer Imaging, another study (Gozzi et al 2012) looked at myelination differences in the corpus callosum between children with autism and age matched controls. All 101 autistic children showed a significant decrease in myelination of the mid-sagittal corpus callosum compared to normally developing children used as controls.

In conclusion, there is plenty of evidence from human studies that points to the malformation or agenesis of the corpus callosum in autistic individuals. The best way to fully understand how the CC phenotype is tied with genetic predisposition to autism is by using mouse models. Mouse models provide unique tools to explore a phenotype while keeping the genotype constant. In addition, a single gene manipulation in mouse models is a very valuable tool to study single gene effects. Mice are, therefore, the perfect tools to find candidate genes for Autism.

**BTBR as a mouse model for autism**

In the autism research field, two basic approaches are used to take advantage of mouse genetics and behavioral phenotypes (Pobbe et al 2011). One is to manipulate candidate genes found in humans into the mouse genome and analyze the resultant phenotypes. For example, Neuroligin 3 (Nlgn 3) mutations were found in a study of Swedish families with ASD individuals (Li et al 2012) and subsequently, the NglN 3-knockout mouse was made that displayed impairments in social behavior (Jamain et al 2008, Radyushkin et al 2009). The other is to observe phenotypes in inbred strains that show autistic phenotypes and investigate the genes or mechanisms important for the
phenotypes to occur (Pobbe et al 2011). For example, the BTBR T(+)tf/J mouse strain displayed autism-like behaviors and then genetic studies were performed to understand the basis of the phenotypes.

The BTBR mouse model was initially used for phenylketonuria and insulin studies (Ranheim et al 1997, Hong et al 2004), but recent focus has been on this strain as a model for autism because it displays social deficits, communication deficits and repetitive restricted behavior. As part of the mouse phenome project, Wahlsten et al (2003) did a survey of 21 inbred strains and reported that BTBR had an absent corpus callosum (CC) and a severely reduced HC. In addition to the CC phenotype, the BTBR mouse also shows impaired social interactions, impaired ultrasonic communication and repetitive behaviors (self-grooming) -the hallmarks of autism. Each of these is discussed in more detail below.

Social behavior has been widely studied in BTBR and compared to several different inbred strains of mice. This strain has consistently been shown to have low levels of social behavior compared to most common inbred strains. McFarlane et al (2008) characterized some of the autism-like behavior of BTBR. B6 was used as a control because it shows moderate levels of social behavior and low levels of repetitive behaviors. The social approach test measures the tendency of a mouse to approach a novel mouse compared to a novel object using a three-chambered apparatus (Crawley, 2007). BTBR spent significantly less time in the chamber containing the novel mouse compared to B6, and more time in the chamber with the novel object. Reciprocal social interactions were tested using freely moving B6 and BTBR pairs. Social interaction is calculated by the amount of time spent sniffing, following, mounting, allogrooming, huddling, and wrestling. Total interactions of BTBR were significantly lower compared to B6. Time spent sniffing and following were also much lower in BTBR (McFarlane et al 2008). BTBR mice also engage in significantly less Juvenile play (tests social interactions at a younger age) than B6 (McFarlane et al 2008). In a ‘novel social proximity’ test developed by Defensor et al (2011), to test social anxiety in BTBR, BTBR and B6 mice were closely confined and tests were done under four experimental conditions: same-strain male pairs, different-strain male pairs, same-strain male-female pairs, and same-strain male pairs treated with an anxiolytic. They then recorded different
social interactions like: nose tip-nose tip (NN), Nose-to-head (NH), Nose-to-angiogenital (NA), crawl over (CO), crawl under (CU), upright (U) and jump escape (JE). BTBR mice showed significantly lower NN, NH and upright behavior and increased NA, CU and CO compared to B6 controls. Hence, BTBR shows social avoidance and avoidance of frontal contact, which may be considered parallel to the gaze avoidance shown by individuals with autism.

Rather than social behavior being classifiable as normal or abnormal in mice, it is more on a continuum. Inbred strains differ in the amount of social behavior they engage in. Bolivar et al (2007) conducted an inbred strain survey of social behavior in 129S1/SvImJ, C57BL/J (B6), BTBR, BALBcBy/J, DBA/2J, A/J (A) and FVB/NJ and found that total time spent in social interactions (including mounting, wrestling, following, allogrooming, sniffing and hudding) was significantly higher in FVB than BTBR. In fact, of all the strains tested, FVB spent the most time engaged in social behavior. FVB is another inbred strain that is commonly used as a comparison to BTBR because it shows a fully formed corpus callosum and displays high levels of social behavior. In contrast, there was a smaller difference in behavior between BTBR and B6. Thus, for our genetic investigations we selected FVB and BTBR strains. Our lab uses FVB over B6 for a number of reasons. First, FVB is similar sized compared to BTBR. Second, litter size of FVB and BTBR are comparable. This is important if there is a maternal effect on behavior. Third, the exploratory activity level of FVB is similar to BTBR (Bolivar et al 2006). Lastly, Bolivar et al (2007) found that total sniffing behavior is significantly higher in FVB than B6. Hence, the FVB/NJ mouse strain is a good comparison in behavior tests, for BTBR.

The second characteristic of autism that can be modeled in BTBR mice is communication abnormalities. Mice communicate by using olfaction andaudition. Deposition of urine and scent markings are used to demarcate territory, attract the opposite sex, recognize individual mice, and maintain family organization. One of the methods to test communication is by the social transmission of food preference test. In this test, a ‘demonstrator’ mouse eats a novel food and an ‘observer’ mouse interacts with it. The observer mouse is then presented with the choice of eating the food eaten by the demonstrator or another novel food (Wren et al 2004). When given the choice, B6 ate
more food of the type that the demonstrator mouse ate (compared to the other novel food), compared to BTBR (McFarlane et al 2008). Another method for measuring communication deficits includes simultaneously measuring female urine scent markings and ultrasonic vocalizations in adult BTBR mice (Markus et al 2011). The open field test revealed that BTBR deposited fewer scent markings throughout the open field box when female urine was present, compared to B6. BTBR male mice also displayed significantly less ultrasonic vocalizations in the presence of female urine compared to B6 mice. A study by Scattomi et al (2011) studied the ultrasonic communication in BTBR in three social contextual settings: male-female, male-male (resident-intruder) and female-female interactions. This type of approach looked at two aspects of BTBR behavior: social motivation and bioacoustic communication. The B6 control used in this study showed a positive correlation between social investigation and ultrasonic vocalization in all three interactions. BTBR mice, however, only showed a significant positive correlation in the male-female interaction (Scattoni et al 2011). All of these findings indicate major communication deficits in BTBR mice.

The study by McFarlane et al (2008) also investigated self-grooming behavior in BTBR. Across four developmental time points (P18, P28, P38, P68), BTBR showed unusually high levels of self-grooming compared to B6 controls. Repetitive grooming behavior in BTBR was also reported by Amodeo et al (2012). They also used the marble burying test, where BTBR buried significantly more marbles than B6, to further illustrate the repetitive behavior seen in this mouse. Pearson et al (2011) further characterized the repetitive phenotype seen in BTBR by analyzing the microstructure of the self-grooming behavior. They reported an increase in frequency in all grooming subtypes (such as paw licking, head wash, body grooming, leg licking and tail/genital grooming) and an increase in stereotypic bar-biting (using home cage apparatus) in BTBR compared to B6. In the object investigation task, BTBR showed a more patterned sequence of investigations of novel object (Pearson et al 2011). Another interesting study by Reynolds et al (2013) investigated the effect of environmental enrichment on repetitive grooming. They found that after enrichment (which consisted of a dog kennel with two floors and various toys like running wheels and tunnels), the BTBR mice showed significant reduction in grooming time compared to the BTBR mice that did not receive any enrichment.
However, they found no significant changes in grooming pattern. To investigate ‘resistance to change’ behavior that autistic individuals display, BTBR mice were subjected to a probabilistic reversal learning test, which is a test used to study behavioral flexibility in BTBR. Mice are trained through positive reinforcement to choose a ‘correct side’ of two chambers. Once they learn the correct side, reversal learning is conducted where the food cue is on the ‘incorrect side’. BTBR needed significantly more trials to reach the reversal learning criterion compared to B6. Hence, BTBR show impairments in cognitive flexibility comparable to autistic individuals (Dionisio et al 2012).

In conclusion, the BTBR T+tf/J mouse is a good model for autism because it shows low reciprocal social behavior, repetitive behaviors like self-grooming and marble burying, impaired ultrasonic communication and ‘resistance to change’ behavior. It also displays complete agenesis of the corpus callosum. This is important in an animal model of autism because MRI studies have shown partial to complete agenesis of the corpus callosum in humans with autism.

**Development of the corpus callosum**

The corpus callosum (CC) is the major superhighway (190 million axons) of the brain connecting the left and right hemispheres. The anterior part of a fully formed CC is called the genu and the posterior part is called the splenium. The ‘body’ is the part between the genu and the splenium. Posterior and inferior to the genu, is the rostrum. Finally, the isthmus is a very narrow portion of the CC between the body and the splenium (Hofar and Frahm, 2006). Axon guidance is one of the many important steps, including correct midline patterning and formation of telencephalic hemispheres that are very important for proper formation and development of the CC (Lynn et al 2007).

In humans, callosal fibers begin to form at 4 months and continue to mature throughout postnatal development and young adulthood (Paul et al 2011). Figure 1.1 shows similarities between mice and humans at embryonic day 17 and Week 17, respectively. Many of the structures and cell locations are similar, for example, glial wedge, the subcallosal sling, indusium griseum glia and callosal neurons crossing the midline. Mouse models have greatly increased our understanding of the development of the corpus callosum, both embryonically and post-natally. Numerous gene expression
profiles found in mice that are linked to CC development are similar to those found in the embryonic human brain. Hence, mice are a very valuable tool to study the CC and the disorders linked to its malformation or agenesis (Danahoo and Richards 2009).

Even though the anatomy of the development of the CC has been explained, the molecular and cellular mechanisms that govern its proper formation are still not completely understood. When the dorsal walls of the prosencephalon turn in (ultimately forming the hemispheres), very important molecules called ‘morphogens’ are critical for normal midline formation and formation of the commissural plate. Some of the most important forebrain morphogens (signaling proteins) are the BMP (bone morphogenetic protein), Wnt signalling proteins, FGF (fibroblast growth factors), and Shh (sonic hedgehog). Formation of the commissural plate is critical for the CC, HC and AC to cross the midline (Danahoo and Richards 2009). After the commissural plate is formed, the next step is to generate the callosal projecting neurons from progenitor cells. Axons cross the midline after the process of migration and differentiation of these neurons. Callosal projection neurons are formed by differentiation of progenitor cells in the ventricular zone, which is the area around the ventricles from which most neurons of the higher brain neurons originate. These callosal neurons are regulated by very distinct factors like LIM domain only 4 (LMO4), Empty spiracles homolog 1 (Emx1), Emx2, etc. that aid in modification of gene expression in the axons in response to the external environment, thus mediating axon guidance factors in other cells like midline glia (Danahoo and Richards 2009). In fact, axon guidance at the midline is one of the most important factors that help callosal fibers to cross.
Figure 1.1: Representation of a coronal plane of a mouse brain at Embryonic day 17 (E17) and a human brain at week 17 of development. There are very clear similarities in the structures and cell populations like the glial wedge, the subcallosal sling, indusium griseum glia and callosal neurons crossing the midline (pink).

*Figure adapted from Donahoo AL, Richards LJ. ‘Understanding the mechanisms of callosal development through the use of transgenic mouse models.’ Semin Pediatr Neurol. 2009 Sep;16(3):127-42. Review.*
Previous findings in the Bolivar Lab

In trying to understand the development of the corpus callosum, our lab investigated a F2 cross between BTBR (which shows agenesis of the corpus callosum) and FVB (which shows a fully formed corpus callosum), an abnormal distribution was observed for CC index, where, 40 of the 140 animals were acallosal, indicating that the mutation is autosomal recessive (Unpublished data, Bolivar Lab).

Next, we performed a statistical analysis to separate the spike at 0 and the rest of the normal distribution. The two part analysis method gave significance QTL on the distal end of chromosome 4, with a LOD score of 25.9348 and peak of 145.7857, which indicated an extremely significant correlation.

A similar Quantitative trait Loci study by Jones-Davis et al (2013) looked at a F2 cross between BTBR and B6 and identified significant loci on the BTBR genome that could be related to the autism-like phenotypes seen in this mouse. Six significant QTLs on chromosome 1,3,9,10,12 and X were found that were related to the social phenotypes in the BTBR mouse. More interestingly, four significant QTLs were found for commissures on chromosomes 4, 6 and 12. Hence, this study supports our hypothesis that the high significance QTL we found in our lab on the distal end of chromosome 4 may be related to the agenesis of the corpus callosum phenotype in BTBR.

To try to isolate the gene region on chromosome 4, our laboratory made a congenic mouse. Congenic mice strains are very useful to analyze single gene effects. A congenic mouse strain differs from an inbred strain at a short chromosomal section. This type of mouse model allows exploration of a particular genetic locus without the problem of background genetic effects. There are many methods to develop a congenic strain, which include backcrossing, speed congenics and the cross-intercross method (Flaherty, Chapter 10- The mouse in Biomedical Research).

The congenic developed in the Bolivar lab was formed by the back crossing method. In this method, donor and recipient strains are used. The donor strain will have the allele of interest at the differential locus. In our case, the donor strain was FVB and the recipient strain was BTBR (Figure 1.2). The first step is crossing FVB with BTBR. The resulting F1 generation (also called ‘N1’generation) is then backcrossed against a pure BTBR background. The next step is genotyping the F2 (N2) generation for the gene
region of interest. The mouse with the differential locus is chosen and backcrossed again against a pure BTBR mouse to yield the N3 generation. This process is repeated again until the N10 generation is reached (Flaherty and Bolivar 2007). At each backcrossing stage, a part of the donor chromosomal region near the differential locus is retained. This is called the passenger gene (Flaherty, Chapter 10- The mouse in Biomedical Research). The probability that this passenger gene will be retained in the congenic is calculated as

\[ P_n = (1-c)^{n-1} \]

\( n_1 = F1 \)

Where ‘n’ = number of backcrossed generations and ‘c’ = probability of crossing over between the differential and passenger gene. Using this formula, after 10 generations, \( P \) becomes 0.002 or 0.0005. Hence, backcrossing is stopped and the congenic strain is maintained by a brother sister mating protocol (Flaherty and Bolivar, 2007).

Figure 1.2: A schematic of the process by which the BTBR-FVB chromosome 4 congenic was made.
To further examine genes in the region, we investigated gene expression in BTBR, FVB and C57BL/6J inbred strains. C57BL/6J (B6) is another inbred strain which has normal CC development. Microarray analysis performed at embryonic day 15 of the forebrains of these three inbred strains showed that only one gene in the chromosome 4 region was expressed differently in BTBR than the other two strains (B6 and FVB). It was also observed that this gene (*Draxin*) is expressed 5 times lower in BTBR than FVB or B6. Hence, it was determined that this gene, *Draxin*, was the best candidate from the region that could be responsible for the CC phenotype, and via sequencing we found an 8bp deletion in exon 2 of this *Draxin* gene in BTBR compared to FVB (Flaherty, Chapter 10- The mouse in Biomedical Research). A frame shift mutation produced as a result of the deletion, causes an early stop codon and hence, a truncated protein. We believe that this truncated protein may cause agenesis of the corpus callosum in BTBR mice.

*Draxin* (Dorsal Repulsive Axon Guidance Protein) (Islam et al 2009) or Neucrin (Miyake et al 2009) was first described by Islam et al in 2009 when they were looking for novel secreted and transmembrane proteins involved in nervous system development. It is a novel secreted repulsive axon guidance molecule involved in forebrain commissure, hippocampal, olfactory bulb and spinal cord development (Islam et al 2009, Miyake et al 2009, Zhang et al 2010, Su et al 2010, Ahmed et al 2011). *Draxin* mRNA and protein expression was found in the olfactory bulb, cortex, mid brain, cerebellum and the pontine nuclei in B6 mice. Axons follow very well defined paths during development of the nervous system and growing axons in a developing brain ‘sense’ the surrounding environment which contains a variety of attractants and repellants that guide an axon to its target (Tessier-Lavigne and Goodman, 1996). Hence, it is hypothesized that *Draxin* works by ‘telling’ the axons where not to go, effectively guiding them to the right targets.

There is 76% sequence identity between the mouse and human *Draxin* protein sequence. In mice, *Draxin* contains 343 amino acids and in humans it contains 349 amino acids (Miyake et 2009). The N terminal has a 24 amino acid putative signal sequence and the C terminal has a cysteine rich domain. The position of this domain is similar to that of Dkk, an antagonist to the canonical Wnt signaling pathway. This indicates that *Draxin* may be an antagonist of the Wnt signaling pathway in the developing brain (Miyake et 2009).
To investigate the role of Draxin in the development of the corpus callosum (CC), Hippocampal commissure (HC) and anterior commissure (AC), Islam et al (2009) made a Draxin knockout mouse. They found that the CC axons failed to cross the midline and were directed ventrally in these mice (Zhang et al 2010). Using immunohistochemistry, Draxin expression was seen during development in regions around the CC, HC, and AC in normal mice and was absent in the knockout. In the control mice, Draxin was seen expressed by midline glial cells. They also found that in B6 mice, at embryonic day 17 (when the CC crosses the midline), Draxin repels cortical neurite outgrowth indicating that it is a repulsive axon guidance molecule which was seen expressed at the midline and hence may be important to help the commissural axons cross the midline (Islam et al 2009).

A recent study by Ahmed et al (2012) investigated the possibility that Draxin does not work alone- it may interact with a protein called Tsukushi (TSK), that belongs to the small leucine-rich proteoglycan family) and cause normal commissure formation. The study found that even though Draxin and TSK do not interact biochemically, they interact genetically as observed by malformation of the corpus callosum and agenesis of the anterior commissure in a Draxin/TSK doubly heterozygous mice. Together, these two genes also interact to inhibit cortical and anterior olfactory neurite outgrowth (Hossain et al 2013). This evidence underscores the complexity of commissure development.

It is important to distinguish between the Draxin studies performed by Islam et al (2009) and the Draxin mutation studied in our lab. First, they eliminated the entire second exon of Draxin (in the Draxin KO), while there is part of the second exon in the Draxin of our mouse: the frameshift mutation starts in the middle of exon 2. Second, the Draxin KO made by Islam et al shows a total absence of the HC and the AC, while evidence from the congenic mouse we made shows that the Draxin mutation may cause agenesis of the CC, a reduced HC and a relatively normal AC. Therefore, there may be something about the retention of the small part of exon 2 in the Draxin protein sequence that may still allow some axons to cross the midline.
Study 1
Purpose and Rationale: Previous studies in our lab indicate that Draxin may be the gene important for proper commissure development. The aim of this study was to examine the dosage effect of the gene region containing Draxin on the corpus callosum, hippocampal commissure and anterior commissure in the congenic mouse across the three genotypes: M (mice with 2 copies of the gene region from BTBR), W (mice with 2 copies of the gene region from FVB) and H (Heterogeneous for the gene region-one copy from BTBR and one copy from FVB).

METHODS:

Animals

18 adult BTBR-FVB chr4 congenic mice were examined in this study. They were divided into three groups based on genotype: 3 male and 3 female mice with both copies of the gene region from BTBR (denoted as ‘M’), 3 male and 3 female mice with both copies of the gene region from FVB (denoted as ‘W’), and 3 male and 3 female mice with one copy of the gene region from BTBR and one copy of the gene region from FVB (denoted as ‘H’). All mice were housed and handled according to IACUC-approved protocols. All animals were allowed free access to autoclaved food and water. Mice of the same sex were housed 4-5 to a cage made out of clear Plexiglas (29X112.5 cm) and maintained on a 12 hour light/dark cycle (lights on at 7am).

Corpus Callosum, Hippocampal Commissure and Anterior Commissure size measurement

Gold Chloride was used to stain the Corpus Callosum (CC), Hippocampal Commissure (HC) and Anterior Commissure (AC) based on a method adapted from Wahlsten et al (2003). Briefly, whole brains were removed from mice and fixed in formalin for 48 hours. After removing excess moisture from the brain with a Kim wipe, it was weighed and cut in the mid-sagittal plane with a scalpel blade. The brain halves were then put into well plates containing 0.2% gold chloride solution (Sigma) and PBS. Plates were incubated for about 45 minutes at 37°C. After staining, the brain halves were transferred to a 2.5% sodium thiosulphate and imaged. Using image J, the
area of the CC, HC and AC was traced and measured. Thereafter, the following equations were used in excel to measure the CC, correcting for brain weight.

For CC: \( \frac{\text{CC area}}{-1.1+2.2(\text{brain weight})} \)

For HC: \( \frac{\text{HC area}}{-1.1+.40(\text{brain weight})} \)

For AC: \( \frac{\text{AC area}}{-0.02+3.3(\text{brain weight})} \)

**PCR Analyses:**

Genomic DNA was isolated from tail samples by digesting them in PBND and proteinase K. PCR analysis was performed using Draxin primers designed by Kevin Manley (Wadsworth Center). The primers were designed to take advantage of FVB-BTBR SNPs in exon 2 of the Draxin sequence. There is an 8 base pair deletion in exon 2 of Draxin in BTBR. The forward primer compliments a sequence identical in both strains (BTBR and FVB). Two reverse primers were used: the BTBR reverse primer was designed to extend from a G/C SNP (B6-G, BT-C) and the B6 Draxin reverse primer extends from the sequence in the BTBR 8bp deletion. Thus, BTBR reverse primer amplified only BTBR gDNA and the B6 reverse primer amplified only B6 (or FVB) gDNA.

The primer sequences are as follows:

- Draxin Forward Primer: TCCTGATCCTCCTGCTGTTTCC
- BT Draxin Reverse primer: GGAAGCCTGCTTGGTAGCAc
- B6 Draxin Reverse primer: TCGCAGACGGTCCctgc
RESULTS

Rescue of corpus callosum phenotype in the congenic mouse

All mice that were genotyped as having the mutant Draxin in the gene region (denoted as ‘M’) had no corpus callosum and a malformed Hippocampal commissure (Figure 2A). Congenic mice that had normal Draxin in the gene region (denoted as ‘W’) and the heterozygous mouse (‘H’) had a fully formed corpus callosum and hippocampal commissure. (Figure 2B and 2C)

Next, the corpus callosum, hippocampal commissure, and anterior commissure were traced, measured and adjusted for brain weight across the three different genotypes: M, W and H. Using T-test, there is no significant difference between the CC indices for mice of H and W genotype. Mice with mutant Draxin in the region (M) have complete absence of the corpus callosum (Figure 3) and are therefore, significantly different than W or H mice (Using one way ANOVA p<0.0001). The hippocampal commissure indices in the M group were significantly lower than the H and W group (p<0.01). The anterior commissure, however, is not significantly different between the three genotypes.

The results indicate that there is a complete rescue of the corpus callosum phenotype in the congenic mice containing the normal Draxin sequence in the gene region. Since the mutation is recessive, both the heterozygous group and the wildtype group (W and H) of mice display a fully formed corpus callosum and hippocampal commissure. The mutant Draxin group (M), similar to BTBR, displays a total absence of the corpus callosum and malformation of the hippocampal commissure. Hence, these results indicate that the gene region containing a normal Draxin sequence influences the corpus callosum phenotype.
Figure 2: Representative images of brains cut in the mid-sagittal section and stained with gold chloride. A. Brain image of a mouse 2 copies of the region from BTBR (‘M’) B. Brain image of a mouse with 2 copies of the gene region from FVB (‘W’) C. Brain image of a mouse with one copy of the gene region from BTBR and one copy of the gene region from FVB.
Figure 3: Corpus Callosum indices for the three genotypes: W, M and H. There is no significant difference between H and W. p value between H and M and W and M is <0.001, which is very significant.
Figure 4: Hippocampal Commissure indices for the three genotypes: H, M and W. There is no significant difference between H and W. p value between H and M and W and M is <0.01, which is very significant.
Figure 5: Anterior Commissure indices for the three genotypes: H, M and W. There is no significant difference between the three genotypes.
III

Study 2
**Purpose and rationale:** The aim of this study is to investigate the role of the Chromosome 4 gene region on autism-like behaviors. As discussed in detail in the introduction, BTBR display some of the hallmark characteristics of autism: low social behavior, robust repetitive self-grooming and low communication. With all this information from previous studies, the four behavior tests were chosen to be performed on our congeneric mouse (Open field, Zero maze, sociability and repetitive self-grooming) to investigate if the gene region had an influence on any of these behaviors.

**METHODS**

**Animals**

Twelve males and twelve females of each genotype (M, H and W) were considered for this study. All mice were housed and handled according to IACUC approved protocols. All animals were allowed free access to autoclaved food and water. Mice of the same sex were housed 4-5 to a cage made out of clear Plexiglas (29X112.5 cm) and maintained on a 12 hour light/dark cycle (lights on at 7am) in temperature controlled rooms (68-70°).

**Behavior Tests**

Behavior tests were conducted with strict compliance to IACUC protocols and guidelines. All mice were put through a battery of four behavior tests: Open field, elevated zero maze, three chamber social box, and self-grooming. All mice tested were between 60 to 70 days old and went through the four tests in order, with at least a 24-hour gap between tests. Mice were allowed to acclimate to the testing room for one hour before the testing started. All tests were performed in the afternoon between 1pm-6pm.

*Open Field test*- Exploratory activity is accessed through the open field test. The open field test is carried out in an automated Versamax system (40cm x 40 cm x 30 cm) equipped with photocells that track mouse horizontal and vertical movements. After the mice were acclimated to the behavior room, they were moved to a holding cage and allowed to acclimate for 15 minutes. The test mouse was then placed in the
testing chamber equipped with infrared beams that automatically detected and traced movements of the mouse in the 15 minute testing period using the Versamax system. Parameters that were automatically analyzed include horizontal activity, total distance travelled, and time spent in the center versus time spent at the margins (Peacock et al 2012)

_Elevated Zero Maze_ - Zero maze testing is done to measure anxiety in mice. The maze (Accuscan Instruments, Columbus, OH) consists of four quadrants 21cm long: 2 open unprotected arms and 2 enclosed protected arms (walls 40 cm in height), all elevated to about 1m from the floor. The test was performed in the dark. At the beginning of the test, the test mouse is placed in one of the open sections (9.5 cm X 9.5 cm) and given a choice to spend time in either the open or closed space. Photo detectors in the apparatus measured how much time the mouse spent in open or closed quadrants, number of entries and total distance moved in each quadrant (Amendah et al 2011).

_Sociability_ - The sociability assay tests the behavioral reactivity between a test and a stimulus mouse (Li et al 2012). Photocells in the apparatus detected movement between the chambers. One chamber contained the stimulus mouse, the second chamber contained a novel object and the middle chamber connected the two (Abrahams et al 2008). After the test mouse has been acclimated to the room, it was placed in the center chamber and allowed to explore the three chambers for the first 10 minutes. This is called the habituation phase. Photocell detectors tracked how much time the test mouse spends in each of the chambers. At the end of habituation, the test mouse was placed in the center chamber and the two doors leading to the other two chambers are shut. The stimulus mouse (129S1/SvImJ strain that is age and sex matched) was placed on either the right or the left side. The doors were then reopened and mice were tested for 10 minutes. The sessions were videotaped and the amount of time that the test mouse spent sniffing the stimulus mouse or the novel object was coded. An automated system monitored the total time the test mouse spent in the three chambers as well as the number of transitions from one chamber to another.
**Self-grooming**- A clear 2000ml glass beaker under normal fluorescent lighting was used to assess self-grooming behavior. The mouse was acclimated to the chamber for 15 minutes. Each testing session lasts 15 minutes and was videotaped (Freitag et al 2007). Total time spent for grooming all body parts was scored from the videotapes.

**Corpus callosum measurement**

After the last behavior test was completed, mice were euthanized and the brain was removed quickly and put in Formalin for at least 48 hours. The brain was then cut in the mid-sagittal plane, stained and imaged as described in Study 1. The area of corpus callosum was traced and measured using ImageJ. The measurements were corrected for brain weight using the formula:

CC index= (CC area)/[−.1+2.2(brain weight)]

**DNA analyses**

Tails taken after euthanizing mice that completed behavior tests were taken and DNA analyses was done as previously described in Study 1 to confirm the genotype.

RESULTS

**Exploratory Behavior**

Examining exploratory behavior allows us to rule out the influence of hypo- and hyper-activity in the social three chamber test. One of the main parameters that the open field test is designed to investigate, is the total distance traveled by the mouse in a 15 minute test session. The analyses are further broken up into three time groups: the first 5 minutes (T1), the middle 5 minutes (T2) or the last 5 minutes (T3) as shown in Figure 6. This enables us to measure intra-session habituation, or the mouse’s ability to adapt to the novel environment. No significant difference was found across the three genotypes (M, W and H) in the three time periods.

The open field box also automatically tracks mouse movement to give the ratio of the distance travelled in the center compared to the margins and the total time spent in the center (Figure 7 and 8). It could be an indicator of increased anxiety if the mouse
spends more time at the margins compared to the center. The analyses indicated that the W group spent significantly more time in the center and travelled less in the center compared to the H group. Therefore, this could mean that the gene region is involved in an anxiety phenotype. A more robust test for anxiety is the elevated zero maze test.
Figure 6: Exploratory behavior in the congenic mouse across the three genotypes: M, W and H. The chart shows total distance travelled by the mouse in the first 5 minutes (T1), the middle five minutes (T2) and the last five minutes (T3). No significant difference was found across the genotypes at the three time points. The 3 genotypes are color coded as Blue=M, Red=W and Green=H.
Figure 7: Distance travelled in the center relative to the distance travelled in the center and the margins. ‘Center dist. 1’ refers to the distance travelled in the first 5 minutes, ‘Center dist. 2’ is the distance travelled in the middle 5 minutes and ‘Center dist. 3’ is the distance travelled in the last 5 minutes. The 3 genotypes are color coded as Blue=M, Red=W and Green=H. There was a significant difference in Center dist. 2 and Center Dist. 3 between H and W groups (p<0.03).
Figure 8: Time spent in the center: ‘Center time 1’, ‘Center time 2’ and ‘Center time 3’ refer to the time spent in the center of the box in the first 5 minutes, middle 5 minutes and last 5 minutes respectively, in a 15 minute session. The 3 genotypes are color coded as Blue=M, Red=W and Green=H. A significant difference was seem in time spent in the center in the last 5 minutes between the H and W groups (p=0.027).
Elevated Zero Maze

The elevated zero maze test investigates whether certain strains of mice are inherently more anxious. Figure 9 shows that the group with mutant Draxin in the region (M) spent less time in the closed space compared to the group with wildtype Draxin in the region (W). This indicates that the M group is less anxious than is the wildtype group. Previous studies in BTBR (McFarlane et al 2008) have shown that BTBR mice spend more time in the open spaces than the closed spaces. Hence, the gene region may be responsible for the anxiety phenotype and more investigation into this region will be required to pinpoint the gene connected to this phenotype.
Figure 9: Zero maze test: ‘A’ shows total time spent in the closed arms of the elevated zero maze test in a 5 minute test period. ‘B’ shows total time spent in the open parts of the maze in the same 5 minute test session. No significant difference was found between H and M groups. There was a significant difference between M and W group in time spent in closed and open space (p=0.034). There was also a significant difference found between H and W group. (p=0.003) in time spent in open and closed space.
Three chamber social box

This test is designed to investigate whether the test mouse spends more time with a stranger mouse or a novel object. An important parameter to look at in this test is the total time spent in chamber 2 (the center chamber). If the test mouse spends significantly more time in the center, it could disrupt the interpretation of the social box results. No significant difference was found in time spent in Chamber 2 across the 3 groups (Figure 10).

The next parameter analyzed by the social box is total time spent on the novel object side and total time spent on the stimulus mouse side. In this study, no significant difference was found across the three genotypes for the time spent on the object side (Figure 11) or the time spent by the test mouse on the social side (Figure 12). A more robust parameter to examine sociability is time spent sniffing, which was coded from videotapes. This parameter provides a better insight into social behavior because it counts the time the mouse sniffs the container or the stimulus mouse rather than just counting the time spent in each chamber. Surprisingly, this study showed that there was no significant difference in time spent sniffing the novel object or time spent sniffing the stimulus mouse between mice that had normal Draxin in the gene region (W) and mice that had mutant Draxin in the region (M) [Figure 13 and Figure 14]. Interestingly, however, the H group sniffed the novel mouse more than the W (p=0.04) or the M groups (p=0.01). It is not clear why this is, and more studies will need to be done to investigate this further. It could be some kind of gene interaction when the mouse receives one copy of the gene region from BTBR and one copy of the gene region from FVB or it could be due to a phenomenon called ‘transgressive segregation’ which refers to the presence of extreme phenotypes in the heterozygous progeny compared to either of the parental lines.
Figure 10: Time spent in Chamber 2, the middle chamber, in the three chamber social box across the three genotypes. No significant difference was found.
Figure 11: Time spent on the novel object side (in minutes) across the three genotypes was not significantly different from each other.
Figure 12: Total time spent (in min) on the stimulus mouse side across the three genotypes were not significantly different.
Figure 13: Total amount of time the test mouse spent sniffing the novel mouse in the three chamber test across the three genotypes: M, W and H. The H group differed sniffed the novel mouse significantly more than the M group (p=0.018) and the W group (p=0.044).
Figure 14: Total amount of time the test mouse spent sniffing the novel object in the three chamber test across the three genotypes: M, W and H. No significant difference was observed between the genotypes.
**Repetitive self-grooming**

It has been well established that BTBR mice are known to spend significantly more time grooming than B6 or FVB mice (McFarlane et al 2008, Pearson et al 2011, Dionisio et al 2012, Reynolds et al 2013). This study found that mice which received both copies of the gene region from BTBR spent significantly more time grooming than mice that received both copies of the gene region from FVB \(p<0.02\), figure 15]. Furthermore, an analysis with a pure BTBR and pure FVB inbred strain along with the three genotypes (Figure 16) showed a similar trend between BTBR and FVB and the congenic. The H group that received one copy of the gene region from FVB and one copy of the gene region from BTBR, however, acted differently than expected: they spent almost the same time grooming as the M group. These results indicate that gene region may play a role in grooming behavior.
Figure 15: Total time the test mouse spent grooming across the three genotypes. The Wildtype group spent significantly less time grooming compared to the H group (p=0.045) and the M group (p=0.02). No significant difference was found between H and M groups.
Figure 16: Comparison between the congenic (M, H and W) and 12 BTBR and 12 FVB mice in total time spent grooming. A similar trend is seen between the W and M group and the FVB and BTBR group of mice, indicating that the gene region may be involved in grooming behavior.
**Corpus Callosum Measurement**

The brains of all the mice that went through the behavior tests were analyzed for the area of the corpus callosum across the three genotypes (M, W and H). As expected, the corpus callosum is absent in the M group and is normal in the W and H group (p<0.001 by one way ANOVA) [Figure 17].
Figure 17: Corpus callosum measurements across the 3 genotypes: W, H and M. The M group differs significantly from the H and W group (p<0.001), while there is no significant difference between the H and W group in corpus callosum indices.
IV

Study 3
**Purpose and Rationale:** Our lab chose Draxin as the best candidate in the gene region that is important for crossing of the callosal axons across the midline. It was chosen for a number of reasons as discussed in the introduction. The aim of this study is to investigate Draxin protein expression at three developmental time points: E15, E17 and P0. Embryonic day 15 (E15) was chosen because it is the time point before the corpus callosum is formed. At embryonic day 17, the fibers start to cross and at postnatal day 0 (P0) the corpus callosum is fully formed. Since the hypothesis is that the Draxin gene region is important for the development of the corpus callosum, these three time points were best to investigate anti-Draxin antibody staining qualitatively across the three genotypes.

**METHODS**

**Animals**

The BTBR-FVB Chr4 congenic mice were bred as [Heterozygous X Heterozygous] brother-sister mating and hence their progeny had all three genotypes: mice containing mutant Draxin (denoted as “M”), mice containing normal Draxin in the region (denoted as “W”) and mice containing one copy of the mutant Draxin and one copy of normal Draxin (denoted as “H”). In order to obtain embryos, male and female heterozygous brother-sister mice were mated in a 1:1 ratio. They were allowed to be in the same cage overnight and were separated the next day. That day is counted as embryonic day 1. In this study, embryonic day 15 (E15) and embryonic day 17 (E17) mice were obtained in this fashion. P0 pups were obtained by checking the cages of breeder mice every morning.

**Fixing, embedding and cutting the brain**

**Fixing:**

*For E15 and E17:* Timed pregnancies were set up as described above. At E15 or E17, the female mouse was anesthetized using CO2 and fetuses were removed. Brains were dissected out of the skull using a dissection microscope and placed in 4% paraformaldehyde in 0.1M phosphate buffer for 48 hours at 4°C. The brains were then transferred to a solution of 15% sucrose in phosphate buffer for 24 hours.
For P0 pups: Newly born pups were decapitated and the brain was quickly removed and placed in 4% paraformaldehyde in 0.1M phosphate buffer for 48 hours at 4°C. The brains were then transferred to a solution of 15% sucrose on phosphate buffer for 24 hours.

Embedding:

After the brains were allowed to remain in 15% sucrose for at least 24 hours, the brainstem and hindbrain were cut out coronally. The rest of the brain was embedded with egg yolk and hardened by exposure to formalin for 4 hours. After this, they were put back in sucrose.

Cutting:

The egg yolk-embedded brains were allowed to stay in the 15% sucrose solution for at least 8 hours before cutting. A sliding microtome was used to cut 40 micron coronal sections from frozen brains. The sections were stored serially in a 6X6 well container containing 0.1 M phosphate buffer containing Sodium Azide.

Immunohistochemistry Assay

Immunohistochemistry assays were performed as free floating sections using the ABC- peroxidase procedure. Sections were chosen that represent the area where the corpus callosum is located. The selected sections were first washed with 0.1M PBS (Phosphate Buffered Saline) and then blocked with 5% normal horse serum (NHS) in 0.1% Triton in PBS. The sections were then incubated with the primary antibody, sheep anti-Draxin antibody (R&D systems 1:1000), overnight at 4°C. The next day, sections were washed in PBS for a few rinses and incubated for one hour in the secondary antibody: biotinylated anti-sheep antibodies (Vector Labs; 1:200). Sections were then rinsed again in PBS and incubated in ABC peroxidase solution (Vector). The last step consisted of rinses with PBS and finally reacted with 0.05% DAB (diaminobenzidine)/0.25% nickel ammonium sulfate/0.0015% H2O2 for 2 minutes, rinsed in PBS again and then mounted on chrom-alum gelatin slides. The slides were allowed to dry overnight at room temperature. The next day, they were dehydrated with ascending alcohols and xylene and Micromount (Surgipath) was used to apply coverslips.
**Imaging**

After immunostaining, sections were viewed on a Leica microscope. Images were captured with a digital camera at 2X, 4X, 10X and 20X magnification. The light level and exposure time was the same for all images captured. Images were cropped and rotated to highlight the midline using ImageJ software. The brightness was changed by the same amount for all the images of the same magnification. The intensity of staining was compared between the three genotypes (M, H and W) at the three different developmental time points (E15, E17 and P0).

**DNA analyses**

DNA analyses on tails taken from embryos and P0 pups were performed as described in Study 1.

**RESULTS**

Figure 18 shows an example of a coronal slice of a P0 brain. The area in the box is the midline and that will be the focus of this study. The arrow points to a corpus callosum that has crossed the midline. Draxin is an axon guidance molecule and is believed to be important for the crossing of the callosal fibers. Therefore, this study investigated Draxin protein expression at 3 time points: E15, E17 and P0.

At E15, robust Draxin staining is observed in the W and H groups that have normal Draxin in the gene region (Figure 19). There is very little staining in the M group that has the Draxin mutation in the gene region (Figure 19). At embryonic day 17 (figure 20), Draxin staining is very strong in the W and H groups compared to the M group. The no primary section refers to the sections that did not receive any antibody and hence is a control. Postnatal day 0 shows a similar trend with robust Draxin expression in W and H groups and virtually none in the M group (Figure 21). At P0, corpus callosum fibers can be clearly seen crossing the midline in both W and H groups. Hence, this indicates that Draxin may be one of the important molecules that have to be expressed in the midline during brain development for the fibers of the corpus callosum to cross.
Figure 18: An example of a coronal section of a mouse brain on post-natal day 0. The solid box encloses the midline and the arrow points to the corpus callosum. The immunostaining in the following figures focuses on the midline.
Figure 19: E15 images of 40 micron brain slices with Draxin immunostaining. A, D and G are images of a brain of W genotype at 5X (A), 10X (D) and 20X (C) magnification. B, E and F are images of a brain of H genotype at 5X (B), 10X (E) and 20X (F) magnification. C, F and I are images of a brain of M genotype at 5X (C), 10X (F) and 20X (I) magnification. Robust Draxin staining is seen in the W and H genotypes, while the M genotype shows very little staining.
Figure 20: E17 images with Draxin immune-staining. A and E are images from a M genotype brain. B and F are images from a H genotype brain and C and G are images from a W genotype brain. D and H are brain slices with no primary antibody present. E, F and G are higher magnification (20 X) of the boxes in A, B and C (10X).
Figure 21: Post natal day 0 images of 40 micron brain slices with Draxin immunostaining. A and B are images of a W genotype brain. B is at 4X and A is at 2X. D, E, F is images of brains of M, W and H genotype at 5X magnification. C and G are brain slices with no anti-draxin antibody. NOTE: A, B and C were taken with a different microscope and at different brightness settings than the rest of the images.
Summary and Future Directions
Corpus callosum abnormalities have been linked to autistic individuals by a number of studies (Lau et al 2012, Prigge et al 2013, Anderson et al 2011, Gozzi et al 2012, Casanova et al 2011). By investigating genes that may be involved in this process of corpus callosum development and autistic behaviors, we are a step closer to finding a much needed biomarker for autism. Mice are a particularly valuable tool to investigate genetic, behavioral and neuroanatomical aspects of this disorder because of the ease by which its genome can be manipulated. This thesis project revolved around a gene region which is about 6MB long containing Draxin-the best candidate gene in the region. The aim of this thesis was to confirm that the gene region containing the Draxin gene is important for normal corpus callosum formation and to investigate if the region is linked to some of the autistic-like behaviors in the BTBR mouse. The thesis was divided into 3 studies: Study 1 investigated the effect of the gene region on the corpus callosum, Study 2 investigated whether the gene region had any effect on behavior and Study 3 looked at expression of Draxin (the best candidate in the gene region) developmentally.

The results from Study 1 showed that, in adult mice, the group of mice that had normal Draxin in the gene region (H and W group) showed a fully formed corpus callosum and hippocampal commissure while the mice that had the mutated Draxin in the gene region showed an absence of the corpus callosum and a reduced hippocampal commissure. Hence, these results are in accordance with the hypothesis from previous studies in our lab, that the gene region containing Draxin is autosomal recessive for the mutation between BTBR and FVB.

The aim of Study 2 was to investigate if this gene region had an effect on four behaviors: open field, zero maze, sociability and self-grooming. The open field tests exploratory activity by investigating total distance travelled, distance travelled in the center compared to margins and time spent in the center. The congenic did not differ in total distance travelled across the three genotypes. This is a positive result because if there was a difference between the genotypes in exploratory activity, it could affect the interpretation of the results in the sociability test. An interesting result was obtained when distance travelled in the center compared to margins and time spent in the center was analyzed: the wildtype group seemed to spend significantly less time and explore significantly less in the center of the box compared to the margins, especially in the last 5
minutes of the test. This points to a possible increase in anxiety in the W group compared to the H and M group. However, a more robust test for anxiety is the zero maze test.

The zero maze investigates how much time the mouse spends in the open space compared to the closed space on an elevated circular disc. The results show that the W group spent significantly more time in the closed space (more anxious) compared to the H and M groups. This was a surprising result because when the BTBR and FVB data were analyzed, no significant difference was found (Unpublished Bolivar Lab data). Hence, this indicates that the gene region may be involved in an anxiety phenotype.

In the sociability test, surprisingly, the gene region does not seem to have an effect on social behavior in the congenic mouse as investigated by the three chamber social box. No significant difference was found between the three genotypes in time spent in the novel mouse chamber, time spent in novel object chamber and time spent sniffing the novel object. In the analysis of time spent sniffing the novel mouse, the H group interestingly spent more time sniffing the stimulus mouse compared to the W and M groups. Finally, the self-grooming test revealed that the W group spent significantly less time grooming than the M or the H group. This indicates that the gene region has an effect on grooming behavior.

In short, the behavior analyses gave confusing results on most tests, especially since the H group acted differently than the W group. This could be due to a number of reasons including a phenomenon called ‘transgressive segregation’ which refers to the presence of extreme phenotypes in the heterozygous progeny compared to either of the parental lines. Another reason for this type of result in the H group could be gene interactions when the mouse has one copy of the gene region from FVB and one copy of the gene region from BTBR.

Draxin was chosen to be the best candidate among the eight protein coding genes in the region because it is expressed five times more in FVB than BTBR and it is an axon guidance molecule said to be important in neural development. Hence, in Study 3, Draxin protein expression was investigated developmentally by immunohistochemistry experiments at 3 different time points: Embryonic day 15, embryonic day 17 and postnatal day 0. As expected, Draxin protein expression was observed in the midline during fetal development and postnatal day 0 in the mice that had normal Draxin in the
gene region (W and H groups). Very little staining was found in the mice that had mutant Draxin in the gene region group (M group). This further supports the hypothesis that Draxin is important in development of the corpus callosum.

There are a few limitations to this study. First, a whole genome scan needs to be done on the congenic mouse to make sure that there is no residual FVB DNA in parts of the genome other than the gene region (about 6MB long). Second, the results of the behavior tests point to a possibility of another gene in the region that could be involved in the behavior phenotypes. Our laboratory’s microarray data from a previous study will have to be revisited to look for expression differences in other genes in the region. Third, because of time constraints, this thesis project could only focus on a few behavior tests and more tests need to be done to further investigate and characterize the congenic mouse. These tests will be mentioned in the coming paragraphs.

There are several directions that this project could go in the future and are divided here into genetics, behavior and immunohistochemical directions.

Future directions: Genetics: Study 1 and Study 3 results taken together give strong evidence that Draxin is involved in the development of the corpus callosum and Study 2 gives some evidence of the gene region’s involvement in grooming and anxiety behaviors. Therefore, it will be interesting to further investigate the role of Draxin by making a targeted deletion of the normal Draxin gene in a FVB mouse and a targeted insertion of a normal Draxin gene in a BTBR mouse. According to the hypothesis, the targeted deletion of normal Draxin in FVB would be expected to lead to an absence of the corpus callosum in the FVB strain and targeted insertion of normal Draxin in BTBR would be expected to lead to a fully formed corpus callosum in these mice. Mice would then be put through the same set of behavior tests as the congenic and pure BTBR and FVB inbred strains to further explore and dissect the genetics behind the autism-like behaviors in BTBR mice and investigate if these behaviors are related to the corpus callosum.

Future directions: Behavior: The open field and zero maze tests point out to a possible anxiety phenotype due to the gene region. One possible future direction is to perform behavior tests that investigate anxiety phenotypes in mice like light-dark exploration test (Crawley et al 1980), fear conditioned startle and light enhanced startle
test (Davis et al 1979, Kehne et al 1988, Walker et al 2002) and the Vogel conflict test (Vogel et al 1971). The home cage social interaction behavior test is a more robust and in depth way of investigating social behavior. Even though the three chamber test did not yield results, it would be worthwhile to look at home cage behavior because it allows the investigator to look at very detailed social behaviors like nose-to-nose sniff, nose-to-body sniff, nose-to-angiogenital sniff, pawing, follow, push crawl, and front approach -- all of which cannot be accessed through the social three-chamber box. The self-grooming test revealed that there is some effect of the gene region on total time spent self-grooming. Investigating grooming patterns and testing mice with the marble burying test (test for repetitive behaviors) to look for differences would be one possible future direction here. BTBR mice are known to have very rigid grooming patterns (Scattoni et al 2013) and hence it would be interesting to look at whether this gene region has an effect on this pattern. The hypothesis here would be that the mice with mutant Draxin in the gene region will have a very rigid and inflexible pattern of grooming compared to the H and W groups.

**Future directions: Neuroanatomical:** It is still unclear if Draxin is secreted by neuronal or glial cells in the midline. Draxin and GFAP (a glial marker) double immunostaining and Draxin and NeuN (a neuronal marker) double immunostaining would be a good way to look Glia and neuron expression respectively. While the most significant finding was that Draxin was expressed in the midline in the W and H groups, there was also some Draxin expression in the hippocampus in the congenic mouse. This could mean that Draxin has a role to play in hippocampal development. This finding is similar to the findings of Zhang et al 2010 in which Draxin mRNA was detected widely in the hippocampus at E17 till P0. The hippocampus is involved in memory consolidation, spatial motor learning and emotional responses to stimuli. Hence, behavior tests related to studying hippocampal function like the Morris water maze, rotarod, and fear conditioning need to be performed in the congenic mouse. In brief, the Morris water maze is designed to test motor and spatial learning and memory, where mice are placed in a pool and have to find a visible platform, a hidden platform or a hidden platform at a new location. (Moy et al 2007). The rotarod tests motor coordination and fear conditioning tests memory consolidation (Contextual and Cued memory). Contextual
memory, in particular, has been well studied and linked to the hippocampus (Park et al 2013, Yang et al 2013, Pan et al 2013, Wagner et al 1985, Kailisch et al 2006).

If the results of the behavior, genetic and neuroanatomical directions show a positive correlation between the corpus callosum development and the Draxin gene, the next possible step is to screen for the gene in human samples and relate it to individuals who developed Autism. Hence, we become one step closer to a biomarker for this disorder and this will allow for early intervention.
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