Examining the Role of CELSR3 Cadherin Domain Mutation R774H in Axon Guidance

By

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To: Mom & Dad, Loukas, Sofia, Eli, Catie, Yiayia & Pappou,
Grandma & Grandpa, and, of course, the Lodge

ABSTRACT OF THE THESIS

Examining the Role of CELSR3 Cadherin Domain Mutation R774H in Axon Guidance

by NICOLAS LEONIDAS CARAYANNOPOULOS

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Tourette Syndrome (TS) is a neurodevelopmental disorder that is relatively common among children between school age and adolescence. Characterized by involuntary verbal and motor tics, the condition can range from barely noticeable to severely affecting the quality of life of the patient and their ability to interact with the world and others around them. The purpose of this project was to elucidate a potential physiological cause of TS using a C57BL/6 mouse model engineered to express a human missense mutation identified in TS. Due to the broad and complex spectrum of phenotypes seen in TS patients, it is hypothesized that circuits in the cortical and thalamic regions of the brain, as well as the basal ganglia, are affected in the condition. The Tourette International Collaborative Genetics Consortium identified a mutation (known as R774H) that is highly likely to be associated with TS, and is present in a protein, CELSR3, known to be crucial in the prenatal development of the aforementioned neural circuits. For this thesis proposal, mice were selectively bred to express the R774H mutation and their brains were examined for any gross, macro-scale, abnormalities in the structures of interest. Collectively, the data obtained seem to indicate that R774H does not grossly impact

the development of cortical-basal ganglia-thalamocortical circuit axon tracts. This suggests that mutations in CELSR3 may instead affect development at the level of synapses and/or the regulation of circuit-wide network activity.

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CHAPTER 1

Introduction

1.1 Tourette Syndrome

In 1881, the French neurologist Gilles de la Tourette reviewed and translated a case series of an unusual disorder afflicting a few residents of Moosehead Lake, Maine. The affected individuals had a propensity for jumping at odd times and in response to even the mildest stimuli. After observing these "jumpers" he sought out similar cases of this strange behavior – eventually describing and classifying "une affection nerveuse caracterisee par de l'incoordination motrice accompagnee d'echolalie et de coprolalie" in 1885 **[Lajonchere et al. 1996]**. His mentor Jean-Martin Charcot later dubbed the condition "Le Maladie de Gilles de la Tourette", or Tourette Syndrome **[Lajonchere et al. 1996]**.

Currently, the Diagnostic and Statistical Manual of Mental Disorders V (DSM-V) provides the following diagnostic criteria for Tourette Syndrome (TS) (307.23 DSM-V **[American Psychiatric Association 2013]**): (i) both multiple motor and one or more vocal tics are present at some time during the illness, although not necessarily concurrently; (ii) the tics may wax and wane in frequency but have persisted for more than one year since first tic onset; (iii) onset occurs before the age of 18 years; and (iv) the disturbance is not attributable to the physiological effects of a substance (e.g. cocaine) or other medical condition (e.g. Huntington's Disease, post-viral encephalitis). A neurodevelopmental disorder, TS is hypothesized to derive from improper development of the basal ganglia, causing

dysfunction in their output to the thalamus and subsequent communication with the cortex **[Stern JS 2018]**. The resultant multiple motor and/or phonic tics include constant readjusting of hair, excessive facial movements, and/or involuntary vocalizations, among many others. Initially, TS was believed to be a very rare disorder, as oftentimes only the more outlandish tics were recognized as being pathologic; however, current estimates of TS prevalence are as high as 1% in children between school age and adolescence. Typical onset occurs in early childhood, is more common in boys **[Bloch et al. 2009, Chowdhury et al. 2004]**, and has frequent co-morbidity with attention-deficit hyperactivity disorder (ADHD), obsessive-compulsive disorder (OCD), autism spectrum disorders, and depression **[Kumar et al 2016]**.

The exact cause of TS remains poorly defined, though it appears to be largely genetic in origin. A large family cohort study by Mataix-Cols et al. in 2015 found the heritability of TS to be approximately 77%. Other studies have also provided supporting evidence that the majority of TS cases are inherited, with one study finding a concordance of over 50% in monozygotic twins compared to only 8% in dizygotic **[Price et al. 1985]**. Overall, there appears to be about a 50% chance of TS patients passing the condition on to their children, suggesting that some forms may be inherited from a single dominant allele.

The pathophysiology of tics in TS is also not well understood. Tics are believed to originate from dysfunction in the basal ganglia, cortical, and thalamic regions of the brain, and intercommunications thereof, as these are the regions associated most with executive function and regulation of voluntary movements.

Within these pathways, dopaminergic signaling is heavily implicated but overall, the precise mechanisms by which tic behaviors arise still remains unclear. **[Mink JW 2001]**.

Currently, the treatments available for TS are somewhat limited and their efficacy is inconsistent. Cognitive behavioral therapy is generally the first option to be employed as it is non-medicinal, instead relying on teaching the patient to monitor their behavior and training them to practice behaviors that reduce tic expression. The long-term benefits have not been heavily studied, however, and some concerns exist that younger children (the primary group affected by TS) will see their attention suffer if they are constantly focusing on tic suppression. Atypical antipsychotics are the most common medicinal treatment for TS (as the only FDA approved medications for that purpose), but their efficacy is variable and comes with a host of side effects, including depression and weight gain. In more extreme cases, deep brain stimulation (DBS) therapy has been utilized, which involves surgically implanting electrodes into the brain that electrically stimulate the targeted region. Though significant improvement has been seen in some patients, this method of treatment is still not well understood. **[Kurlan RM 2014]**

Though research into the effects of TS on lifespan is limited, its impact on quality of life has been well documented. Because onset of TS is most common in early childhood, and the majority of patients still expressing tics are children or adolescents, the effects it has on development can be significant. Almost a third of TS patients interviewed in a cohort study reported difficulties making friends and maintaining even familial relationships **[Eeapen et al. 2016]**, which can have an

impact on self-esteem and long-term mental health. In addition, parents of TS patients were found to be significantly more likely to be frustrated with their child and consider them to be more difficult than other children **[Eeapen et al. 2016]**, which could further contribute to difficulties related to social development in the patient. In addition, many patients suffering from TS describe suffering from "premonitory urges", or a "feeling of mounting inner tension" that can only be alleviated by expression of a tic **[Cavanna et al. 2017]**. Upwards of 82% of TS patients claim to suffer from this sensation and, of those that do, over half describe the feeling as more bothersome than their tics and many describe it as the primary symptom of TS **[Cavanna et al. 2017]**. Beyond the developmental impact of the disease, some patients suffering from TS display tics that can directly cause them bodily harm. Thus, it is imperative that the mechanistic root of TS be elucidated so that more efficacious treatments can be developed and existing treatments improved.

1.2 Direct and Indirect Pathways of Movement and Basic Neurophysiology

The brain constantly receives and processes myriad stimuli. As such, it possesses a number of mechanisms to manage the responses to these stimuli – assigning them each a priority, and even ignoring some. Without these mechanisms, an individual would easily become overloaded or rendered ineffective trying to react to near-simultaneous stimuli with competing instructions from the brain. One of these regulatory mechanisms exists as a pair of complementary circuits in the striatum, called the direct and indirect pathways, that project to the basal ganglia to control

the voluntary execution of movement in response to salient sensory stimuli or environmental contingencies. To understand how these two pathways collaborate in the execution of voluntary action, a brief summary of the basic process and the main neuroanatomical players follows.

Sensory stimuli are received from the various organs of the body and routed to the thalamus. The thalamus acts as a relay hub that routes information between subcortical brain structures and the prefrontal cortex. The stimuli received by the thalamus are sent to the corresponding regions of the cerebral cortex to be processed (e.g visual information sent from the retinas is sent to the visual cortex). Once information has been received and processed by cortical subunits, the prefrontal cortex makes decisions based on the information that has been received because this cortical region is tasked with decision-making, planning of complex behavior, and overall executive function. When the prefrontal cortex has decided on a desired voluntary action in response to the information it has received, it sends excitatory inputs to the striatum, which is the "primary input structure of the basal ganglia" and is involved in regulating stimulus response and motor function **[Kandel ER 2013]**. The striatum then inhibits the activity of basal ganglia circuits, which are GABAergic and send inhibitory projections to the motor thalamus (which is itself subdivided into various regions that correspond to specific muscle groups). The net result is thalamic disinhibition and the activation of the motor cortex, resulting in voluntary movements. The direct and indirect pathways are integrated into this neural loop because they directly receive cortical inputs that are then passed along to the basal ganglia (globus pallidus and the substantia nigra). These

nuclei participate in regulating motor function, and together with the cortex and thalamus, form what is known as cortical-basal ganglia-thalamocortical circuitry. In addition to motor planning, this circuit is critical for decision-making, reward, and motivation aspects of cognition. **[Kandel ER 2013]**

The direct pathway is a GABAergic pathway that promotes voluntary movements. At baseline, the motor thalamus is innately inhibited by the globus pallidus. The globus pallidus, one of the aforementioned basal ganglia, has a primarily inhibitory function. Like the motor thalamus, the globus pallidus likely also has specific sub-regions that are associated with the muscle group-specific regions of the motor thalamus. When a desired action is to be carried out, the direct pathway uses GABA signaling to inhibit the globus pallidus region associated with the muscle group of interest. By antagonizing the inhibitory action of the globus pallidus, the motor thalamus is now free to send its excitatory signal to the motor cortex, which allows the desired action to be executed. The direct pathway requires that a sufficient activation threshold be met before firing, and many stimulatory factors can combine to reach this threshold. These can include motivational factors (such as the prefrontal cortex deciding that carrying out the desired action will prevent harm) and emotional factors, among many others. The axon projections of this pathway can be seen connecting the globus pallidus to the substantia nigra. The substantia nigra (also mentioned above) has been specifically tied to motor planning and reward seeking behaviors. **[Young et al. 2018]**

The indirect pathway is also a GABAergic signaling pathway, but it serves to inhibit unnecessary or competing movements in tandem with the activity of the

direct pathway. When a desired movement is being carried out, the indirect pathway ensures that the activation threshold for competing actions remains unmet -- by maintaining inhibition of the motor thalamus. As stated above, the brain is constantly receiving and processing stimuli. The indirect pathway is one of the regulatory measures preventing the brain from responding to too many stimuli simultaneously **[Young et al. 2018]**. It also serves as a sort of switch between actions that are to be performed in a series. This function is not as well understood, but once the activation threshold of an action in a sequence falls below the requirement for firing, the indirect pathway is hypothesized to supply an additional inhibitory effect that helps to transition to the next action in the sequence **[Jin et al. 2015]**.

As previously stated, one of the primary models for the underlying cause of TS is improper functioning of the basal ganglia. Abnormal responses to stimuli and/or dysregulated action selection strategies could very well be the source of the tics seen in the disorder. As such, it is possible that dysfunction in the direct and/or indirect pathways may lead to the spontaneous expression of tics.

1.3 CELSR3

Identification of mutations associated with TS have consistently proven difficult to identify. However, recent whole exome sequencing studies done in TS trio pedigrees have identified genes containing multiple de novo mutations highly linked to the disorder, with one of the most significant being a cell adhesion molecule called CELSR3 **[Willsey et al. 2017]**. “Cadherin EGF LAG Seven-Pass G-Type Receptor 3” (CELSR3) is a cadherin superfamily protein. Cadherins are cell

adhesion molecules that rely on the presence of calcium to carry out their function ("cadherin" is a portmanteau of "calcium dependent adhesion"). CELSR3 belongs to the subfamily of cadherin molecules known as "flamingo". Most cadherin molecules are used in adherens junctions between adjacent cells to form or stabilize various tissues via interactions with the extracellular matrix. These connections typically rely on interactions between cadherins and another set of proteins called catenins. However, members of the flamingo subfamily of cadherins do not rely on interaction with catenins to carry out their functions, instead relying exclusively on homophilic or heterophilic binding interactions between cadherin proteins **[Usui et al. 1999]**. This distinction is important for explaining the function of CELSR3.

CELSR3 is heavily implicated in the prenatal development of various neural pathways and components, being necessary for proper neuron migration and axon guidance **[Feng et al. 2016, Ying et al. 2009]**. Specifically, it appears to be required for formation of the direct and indirect pathways in addition to thalamocortical projections **[Tissir et al. 2005]**. CELSR3 is expressed on the cell membranes of so-called "pioneer axons" during development. These pioneer axons are directed to their proper destination in the early stages of development and serve as guideposts for subsequent axons targeting to the same location. CELSR3 pioneer axons will engage in both homophilic and heterophilic binding interactions (which is why they do not require catenins) with CELSR3 or other cadherin proteins expressed on the cell surface of the next wave of developing axons that follow the route of the pioneer (hereafter referred to as "follow-up axons"). When this interaction occurs, it serves as a positive signal to the follow-up axons that they are on the correct path and to

continue along. When follow-up axons do not receive this signal they undergo apoptosis, thus preventing improper neural connections from accumulating.

The direct and indirect pathways are not the only regions of the brain whose development is dependent upon CELSR3. The globus pallidus, anterior commissure, internal capsule, and parts of the prefrontal cortex also seem to be dependent on the protein, as do the connections between them. CELSR3 is also implicated in the proper migration of inhibitory interneurons from the ganglionic eminences to the cortex. Notably, most of these neuroanatomical structures are associated with the circuits likely associated with motor tics in TS.

1.4 CELSR3 Cadherin Domain Mutation R774H

The mutation R774H is a nonsynonymous amino acid substitution in the fifth cadherin domain of CELSR3. The mutation was identified by the Tischfield lab when doing genetic analyses of children with TS along with their parents. Whole exome sequencing (sequencing of just the protein encoding regions of the genome) was used to look for de novo mutations in children with TS who had unaffected parents.

The reasons why this mutation is significant, and thus worthy of study, are fourfold. First, it is considered a "rare" genetic variant and is not normally found in the general population. Second, the amino acid substitution was predicted to be significant and damaging *in silico* (when tested using a computer simulation). Third, CELSR3 has been found to have "multiple de novo damaging variants" associated with TS in addition to R774H **[Willsey et al. 2017].** Finally, the nature of the mutation makes it a prime candidate for study when considered in the context of what is understood about TS and CELSR3. CELSR3 is implicated in the development

of cortical-basal ganglia-thalamocortical circuits, which, as described above, are postulated to be affected in TS. Furthermore, the R774H amino acid substitution resides in a cadherin repeat, which is central to the ability of this protein to make homophilic and heterophilic interactions with itself and other cadherin proteins.

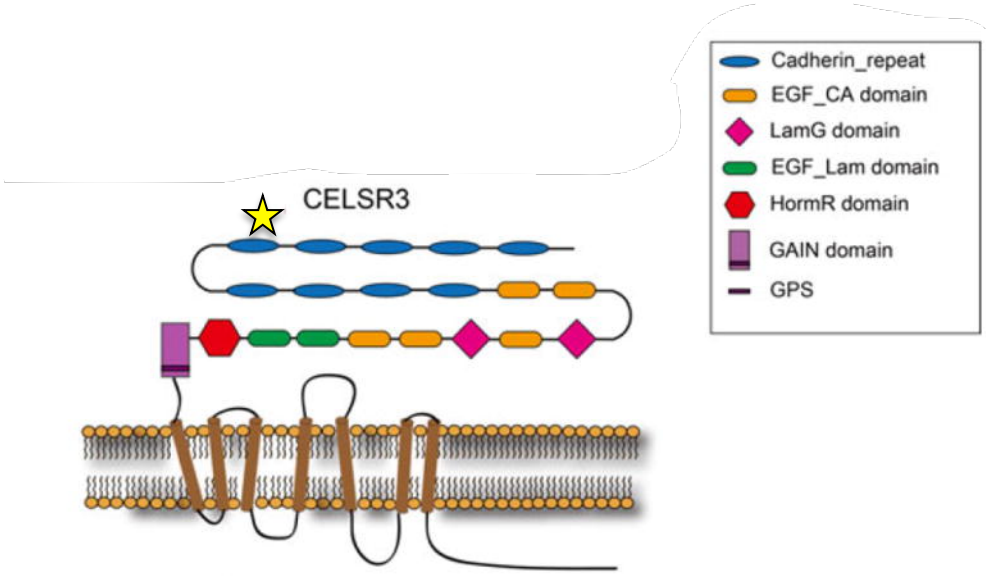


Figure 1 **[Wang et al. 2014]**
Structure of CELSR3 protein
The mutation R774H is found in the fifth cadherin repeat domain (marked with a star) of the protein (indicated with blue ovals in the figure above). The binding interactions described above that occur between CELSR3 molecules are carried out by their cadherin domains and occur much more strongly in the presence of calcium **[Wang et al. 2014].**

CHAPTER 2

Hypothesis and Justification

2.1 Hypothesis

In the presence of the cadherin domain mutation R774H, CELSR3 dysfunction in axon guidance during pre-natal development leads to observable abnormalities in the axon tracts that comprise the direct and indirect pathways, commissural fibers, and projections between the cortex and striatum.

2.2 Justification for Experiment

The Tischfield Lab's goal, with regard to our research into TS, is to determine whether or not the R774H amino acid substitution cause perturbations to brain development that underlie TS. We have chosen to focus on the effects of R774H because, as stated above, it is found in a protein that is heavily involved in the development and function of the axon tracts that comprise the cortico-basal ganglia-thalamocortical circuits. In addition, R774H was identified as a de novo mutation in a TS patient whose parents do not suffer from TS. These two factors together provided us a high level of confidence that this mutation may indeed play a significant role in affecting the neural pathways that underlie behaviors indicative of TS.

The lab is investigating the effects of R774H on brain development by examining possible defects in cell migration, axon guidance, circuit formation, and the regulation of neuronal firing activities. My research specifically looks at the mutation's possible effects on axon guidance. Anatomical abnormalities are a good

place to start when looking into the cause of a disease, as any defects identified would present a rich vein to pursue. Conversely, ruling out a more obvious neuroanatomical root allows more time and resources to be spent on more complex causes that may be found at the biochemical or synaptic level.

CHAPTER 3

Materials and Methods

3.1 Materials

- PBS – 10X stock
- TAE – 10X stock
- Water, purified using a Millipore Milli-Q device
- 4% PFA
- Ethidum Bromide
- Agarose
- Kapa Biosystems 2G ready mix
- Kapa Biosystems Express Extract
- Tris-HCl (pH 8.0-8.5)
- Ethidium Bromide
- 100 Base Pair Ladder
- DNA Primers
- Agarose Powder
- Fluoromount G
- Ketamine
- Xlyazine
- Surgical Scissors
- Micro Scissors
- Butterfly Needles

- IV solution Bags and Tubing
- Micropipette and Pipette Tips
- Microscope Slides and Slide Covers
- R774H Mouse (Created by Rutgers Genome Editing Shared Resource)
- Ai14 Mouse (Jackson Laboratory stock number 007908)
- Emx1-Cre Mouse (Jackson Laboratory stock number 005628)
- Drd1-Cre Mouse (Mouse Biology Program stock number 030989)
- A2a-Cre Mouse (Mouse Biology Program stock number 036158)

Mating Pair Genotypes

Listed below are the genotypes of the parent mice that produced litters used for data collection (organized by the Cre gene present in the offspring)

- Drd1-Cre – Male (R774H/WT CELSR3: Drd1-Cre: Ai14) x Female (R774H/WT CELSR3: Ai14/Ai14)
- A2a-Cre – Male (R774H/WT CELSR3: DA2a-Cre: Ai14) x Female (R774H/WT CELSR3: Ai14/Ai14)
- A2a-Cre – Male (R774H/WT CELSR3: A2a-Cre: Ai14) x Female (WT CELSR3/WT CELSR3: Ai14/Ai14)
- Emx1-Cre – Male (R774H/WT CELSR3: Emx1-Cre: Ai14) x Female (R774H/WT CELSR3: Ai14/Ai14)

Note: Two different mating pairs were used to produce A2a-Cre positive litters because close to the end of the project no litters from the first A2a-Cre mating pair had produced any pups positive for A2a-Cre and homozygous for the CELSR3 wild type allele. Thus a second mating pair was created to increase the likelihood of the desired genotype being present in offspring.

The mice from which data were collected in this experiment were bred to express the CELSR3 mutation R774H along with a specific fluorescent reporter, so that potential neuroanatomical changes could be identified using fluorescence microscopy without needing to apply exogenous stains. To do this, transgenic mice were engineered to express the R774H mutation using CRISPR technology and subsequently bred to produce offspring that had the mutation as well as a combination of genes encoding the fluorescent reporter and the complimentary enzyme needed to activate the reporter in a tissue specific manner.

3.2 Reporter Mice

In this experiment a fluorescent reporter allele was used to visualize the various neural structures that were to be examined. When expressed in mice, this reporter allele causes specific tissues to fluoresce depending on which series of "partner genes" was present with the reporter. This fluorescence provided significant contrast between the tissues of interest and the rest of the brain.

In this case, the reporter that was used is called Ai14, a so-called "Cre reporter gene". Cre-dependent reporters utilize a site-specific recombinase (Cre recombinase) to control where and/or when they are expressed. Cre recombinase recognizes short target sequences known as "LoxP sites" and recombines them, allowing genes to be activated, repressed, or exchanged based on the organization of the Lox sites **[Li et al. 2018, Sauer B 1998]**. Ai14 (developed by the Allen Brain Institute and sold commercially through the Jackson Laboratory) is derived from a constitutively active locus known as ROSA26. In Ai14 mice, this locus has been genetically modified to contain a stop cassette, which prevents transcription of the

locus, flanked by LoxP sites upstream of coding sequence for a red fluorescent protein (RFP) known as tdTomato. The stop cassette prevents constitutive transcription from occurring. However, because LoxP sites flank the stop cassette, when a Cre recombinase is present, the stop cassette is removed and expression of RFP occurs in all tissues that express Cre. This is where the aforementioned "partner genes" come in to play. By pairing Ai14 with engineered Cre recombinase genes designed to be expressed in a tissue specific manner, activation of Ai14 fluorescence is limited to only specific tissues of interest with relatively low levels of leakage (unintended expression of Ai14 outside the expected tissue types). These Cre genes are engineered by taking tissue-specific promoters and recombining them with a sequence that encodes for a common Cre recombinase enzyme.

Three of these engineered Cre genes were used in this experiment: Drd1-cre, A2a-cre, and Emx1-cre. The prefix indicates which promoter the Cre enzyme was attached to. Drd1 is a dopamine receptor primarily found in the striatum and neurons of the direct pathway, A2a is an adenosine receptor abundant in the basal ganglia and neurons of the indirect pathway, and Emx1 is a transcription factor found in almost all excitatory cortical and hippocampal cells.

Thus, when a mouse has both an Ai14 and an A2a-cre present, it will express RFP in the axon tracts of the indirect pathway and label the inputs onto the basal ganglia, allowing for gross neuroanatomical observation of these structures.

3.3 Genotyping

After litters were born, their genetic profiles were assessed for the presence of R774H and appropriate Cre genes. Cre specifically must be present in conjunction

with Ai14 for any specific structures to be visible under the microscope. However, as only one parent is heterozygous for the Cre allele, only approximately 50% of the pups will be Cre positive and thus must be genotyped. The presence of R774H is also determined via genotyping assays, however the presence of Ai14 is not normally tested for, as one parent in a breeding pair is homozygous for the reporter allele and only one copy is required for strong fluorescence signals in the offspring.

Genotyping was done primarily via Polymerase Chain Reaction (PCR). A few days after a litter of pups was born, they would have their toes clipped for identification purposes. Those toes would also provide the tissue sample from which their genetic profile would be determined. To extract DNA from mouse toes, they were suspended in a mixture made by diluting an extraction mixture from Kapa Biosystems ten fold in a 10 mM Tris-HCl buffer (pH 8.0-8.5), and then placed in a PCR cycle for at least 15 minutes. The extraction mixture from Kapa contained a buffer and a novel, thermostable protease. After incubation in the PCR machine, the mixture remaining in the PCR tubes could be used as a source of DNA.

Once DNA had been extracted from the tissue sample, the target sequence needed to be amplified. When testing for the presence of a Cre, a small sample of extracted DNA was added to a master mixture of Kapa Biosystems fast genotyping mix (which contained a specially designed DNA polymerase, dNTP's and a buffer), mili-q water, and primers corresponding to the sequence of interest. After thermal cycling, if the target sequence (i.e. Cre) had been present in the original sample, then that sequence would have been heavily amplified during the PCR cycle. The mixture that had been cycled in the PCR machine would then be run through gel

electrophoresis for 30 minutes, in a 3% agarose gel containing ethidium bromide (which binds to DNA fragments and allows them to be seen under UV light as a band on the gel). The presence of any band at all indicated the presence of the Cre being tested for, while no band indicated a lack of Cre.

The process of testing for R774H began the same way; with an extracted DNA sample being added to master mix containing a DNA polymerase along with the appropriate primers. However, an additional step was required before the sample could be run through gel electrophoresis. When the R774H mice were created, a restriction fragment length polymorphism (RFLP) was also introduced nine base pairs downstream. This RFLP was specific to the restriction enzyme Afe1. After the DNA amplification was completed, Afe1 and it's associated buffer is added to the mixture for 30 minutes. Based upon the pattern of DNA digestion, I could determine if a specific mouse had a wild-type allele or was heterozygous or homozygous for the R774H substitution. In an R774H homozygote, the RFLP would be present on both copies of the gene and thus all the DNA fragments present would be cut at that site when exposed to Afe1, leading to the presence of two different sized DNA segments that would segregate during gel electrophoresis and form 2 distinct bands. In the case of a CELSR3 wild type homozygote, the RFLP was not present at all, so only a single larger strand would be present leading to only one band forming higher up on the gel than the two bands found in an R774H homozygote. If the mouse were heterozygous for R774H and wild type CESLR3, then around half of the DNA fragments present would be expected to contain the RFLP leading to the formation of 3 bands on the gel, two that correspond with the segments that were

cut by Afe1, and one that corresponds to the fragments that were not cut. In all gels run, a 100 base pair ladder is used as a frame of reference.

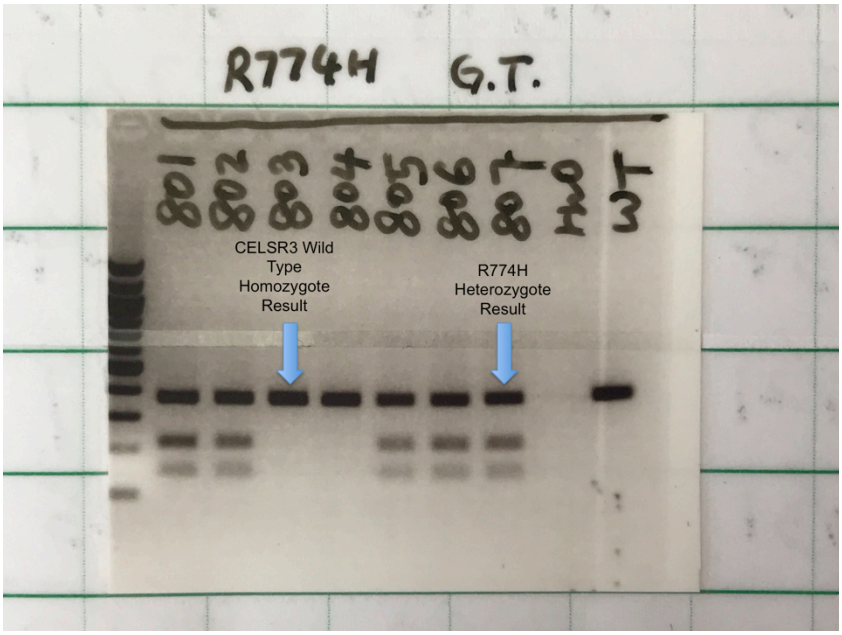


Figure 2
Example of R774H genotyping gel electrophoresis result (courtesy of Dr. Junbing Wu)
Genotyping results for wild type and R774H heterozygote animals are shown. R774H homozygotes would produce just the bottom two bands of the heterozygote animals (bands would be darker as well)

3.4 Perfusion-Fixation and Brain Extraction

Mice are heavily sedated with Ketamine/Xylazine until they are unresponsive to painful stimuli (this is verified by squeezing the sensitive tip of the mouse's tail with forceps). Next, the chest cavity is opened using surgical scissors, with care being taken not to damage any of the vital organs. A small needle is then inserted into the left ventricle at the apex of the heart, pointing upward towards the aorta. Care must again be taken when inserting the needle to not perforate any

other walls of the ventricle. The needle is then secured for the remainder of the procedure. Using micro scissors, an incision is then made at the base of the right atrium near the venae cavae and 1X phosphate-buffered saline (PBS) is allowed to run through the needle into the left ventricle. Because the vascular circuit has been cut at the right atrium and PBS is introduced in the left ventricle, the flow of saline will flush most of the blood out of the mouse's circulatory system. The marker for a well-cleared circulatory system is the mouse's liver going from a deep red to a washed out, pale yellow-brown color. Once the body has been sufficiently cleared of blood, the flow of saline is stopped and replaced with a mixture of 4% paraformaldehyde (PFA).

The PFA is used to fix the tissues of the brain so that they are preserved for extraction. The fixation process increases the rigidity of the brain due to crosslinking of proteins by PFA, making it easier to remove without damage, and also prevents breakdown of proteins prior to examination. It also makes the brain easier to cut into thin sections that can then be mounted on slides. The crosslinking effect of PFA also works on proteins that are present in the blood stream, which is why the mouse must first be cleared of blood. If this is not accomplished, the proteins will form clots in the vasculature and obstruct the flow of formaldehyde throughout the body, potentially preventing proper fixation of the brain.

The PFA is allowed to run for around 6 minutes (too much exposure to PFA may "over-fix" the brain, making it unusable for data collection). Once the 6 minutes have elapsed, PFA flow is cut off and the needle is removed from the left ventricle. The mouse skull is then removed from the carcass and the brain extracted using

surgical scissors and bone cutting tools. The brain is then suspended in a solution of 4% PFA for an additional 24 hours to finalize the fix. Suspending the brain in PFA is not as potent as fixing it from the inside out which is why 24 hours are required as opposed to the 6 minutes required during the perfusion stage.

3.5 Histology

Once the brain has been fixed and removed, it is suspended in 3% agarose gel using a cube-shaped mold. Once suspended, it is cut into 120-micron (μm) sections on a Leica VT1000s vibratome, in either the sagittal or coronal planes. Sections were then mounted on microscope slides with Fluoromount-G (a mounting medium that does not fluoresce but helps to reduce photobleaching, lengthening the amount of time the slides can be kept). Mounted brain sections were then imaged on a Leica M165FC stereomicroscope.

CHAPTER 4

Results

4.1 Assessment of Neuroanatomy in Mice with CELSR3 Mutation R774H

In order to examine the impact of CELSR3 cadherin-domain mutation R774H on prenatal neuroanatomical development of axon tracts, the brains of transgenic mice harboring the mutation (both heterozygotes and homozygotes) were compared to those of mice who possessed only the wild type allele. Brains were cut into 120-micron sections and compared side by side under a fluorescence microscope, looking for any consistent abnormalities in anatomy. Three different Cre-recombinase alleles in conjunction with Ai14 were used to visualize different structures potentially impacted by the mutation. For each Cre (Emx1-Cre, Drd1-Cre, and A2a-Cre), two to three R774H brains were compared against two to three wild type CELSR3 brains. Sections were taken in either the sagittal or coronal plane and were matched for comparison based on depth.

4.2 Drd1-Cre and the Direct Pathway

The first structures examined were the axon tracts of the direct pathway, along with the globus pallidus, striatum, and substantia nigra – through which these tracts course (Figure 2). Using mice possessing both the Ai14 gene and Drd1-Cre, which is expressed primarily in cells of the striatum and direct pathway, brain sections were examined for any gross abnormalities. After comparing a total of 7 brains (three that possessed wild type CELSR3 loci, two that were heterozygous for

R774H, and two that were homozygous for R774H), no significant differences could be found (Figure 3).

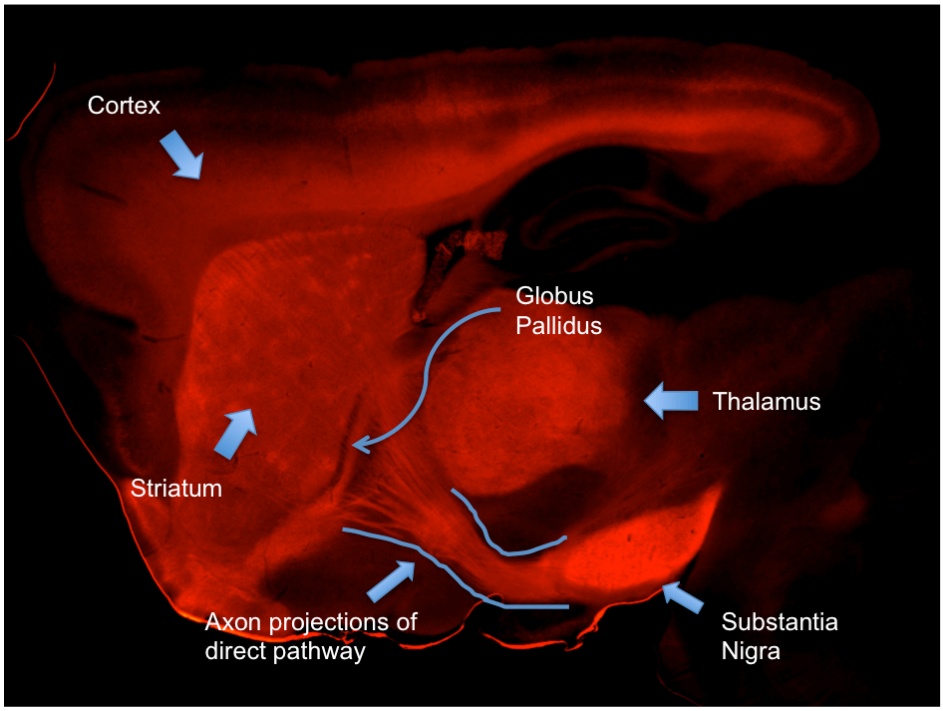
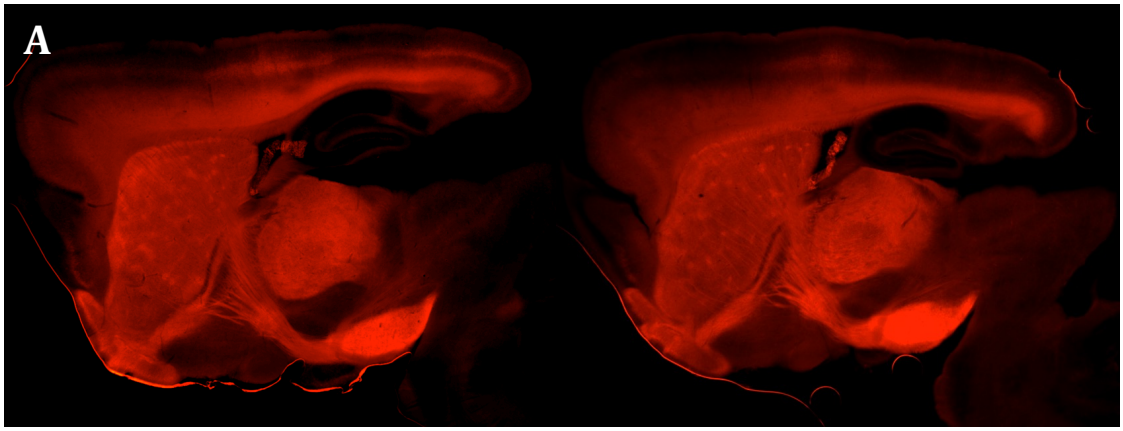


Figure 3
A representative section taken from a mouse homozygous for the wild type CELSR3 allele
All the structures of interest highlighted by Drd1-Cre labeled in blue.



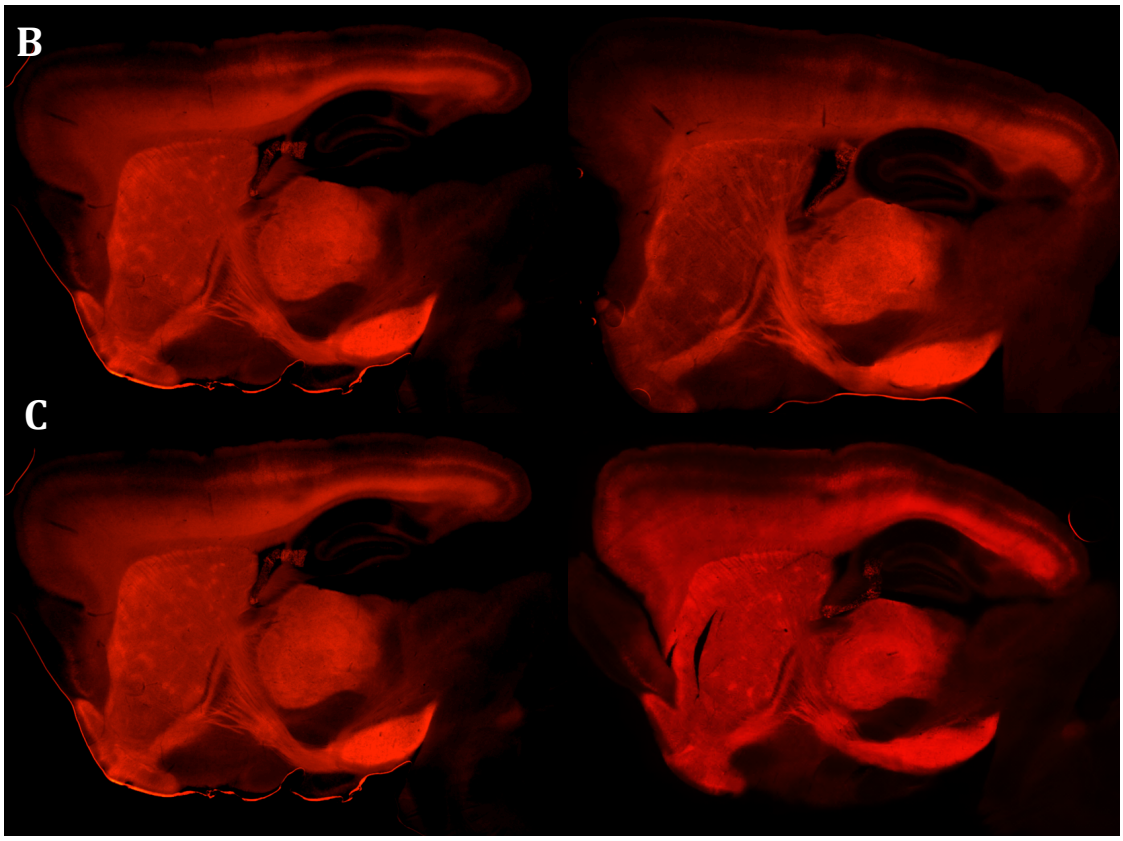


Figure 4
Representative section of a CELSR3 wild type mouse brain compared to R774H homozygote, heterozygote, and a second wild type (visualized with Ai14 and Drd1-Cre)

(A) Sagittal section of brain from a mouse homozygous for wild type CELSR3 (left) compared to a sagittal section at comparable depth taken from a mouse homozygous for R774H (right). (B) The same section from CELSR3 wild type mouse as in Fig 2A (right) being compared to a section from a mouse heterozygous for R774H at similar depth. (C) The same section from CELSR3 wild type mouse as in Fig 2A (right) being compared to a section from a different mouse homozygous for wild type CELSR3

When Drd1 sections were compared to one another, no apparent deviation in the expected path of the axons of the direct pathway circuit were observed between R774H homozygotes and CELSR3 wild type homozygotes. Variation in the specific appearance of the structures visible in the sections was found, but this can likely be

attributed to natural variation between different animals, as well as slight differences in the angle at which the brains were cut. These angle differences occur because the when brains are set in agarose gel prior to sectioning, they must be adjusted by hand and it is difficult to ensure that every brain is embedded in the exact same orientation. Figures comparing wild type sections to heterozygote and other wild type sections were included to illustrate the lack of an apparent and consistent difference in the appearance of the structures of interest that could be classified as a phenotype attributable to the presence of the R774H mutation. Thus, at the macroscopic level, the direct pathway is intact in animals that possess the R774H amino acid substitution in the heterozygous and homozygous state.

4.3 A2a-Cre and the Indirect Pathway

A2a-Cre positive mice were used to examine the structures of the indirect pathway. The axons made visible by this Cre run largely between the striatum and globus pallidus externa (Figure 4), and sections were once again compared side by side to look for any gross abnormalities between the R774H homozygotes and CELSR3 wild type animals. Due to the A2a-cre breeding pair producing a smaller number of litters with fewer pups, only 6 mice were usable for comparison (two CELSR3 wild type homozygotes and four R774H homozygotes).

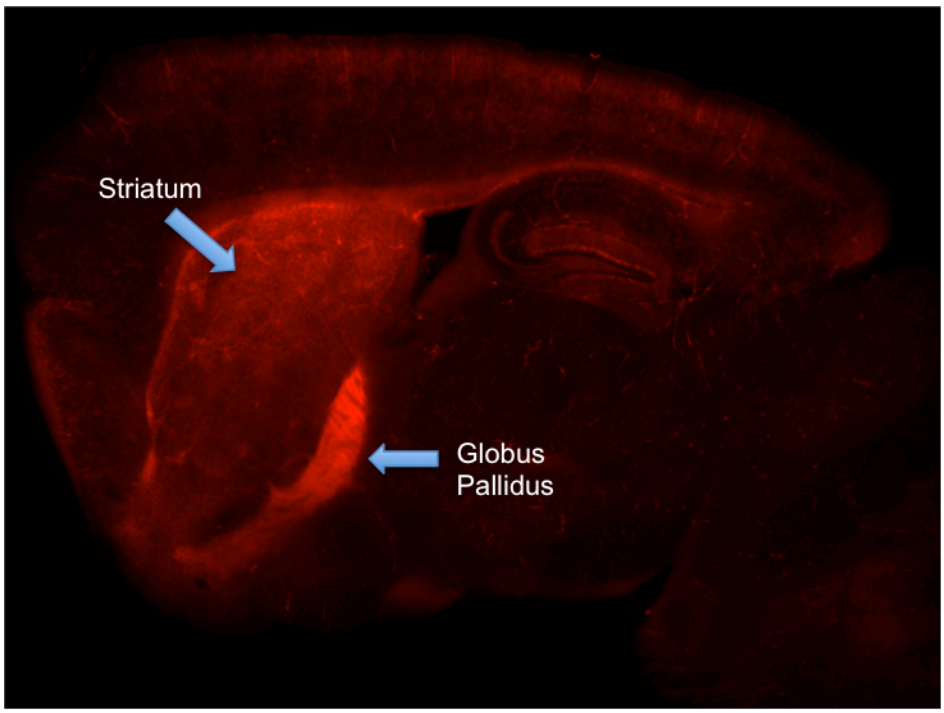
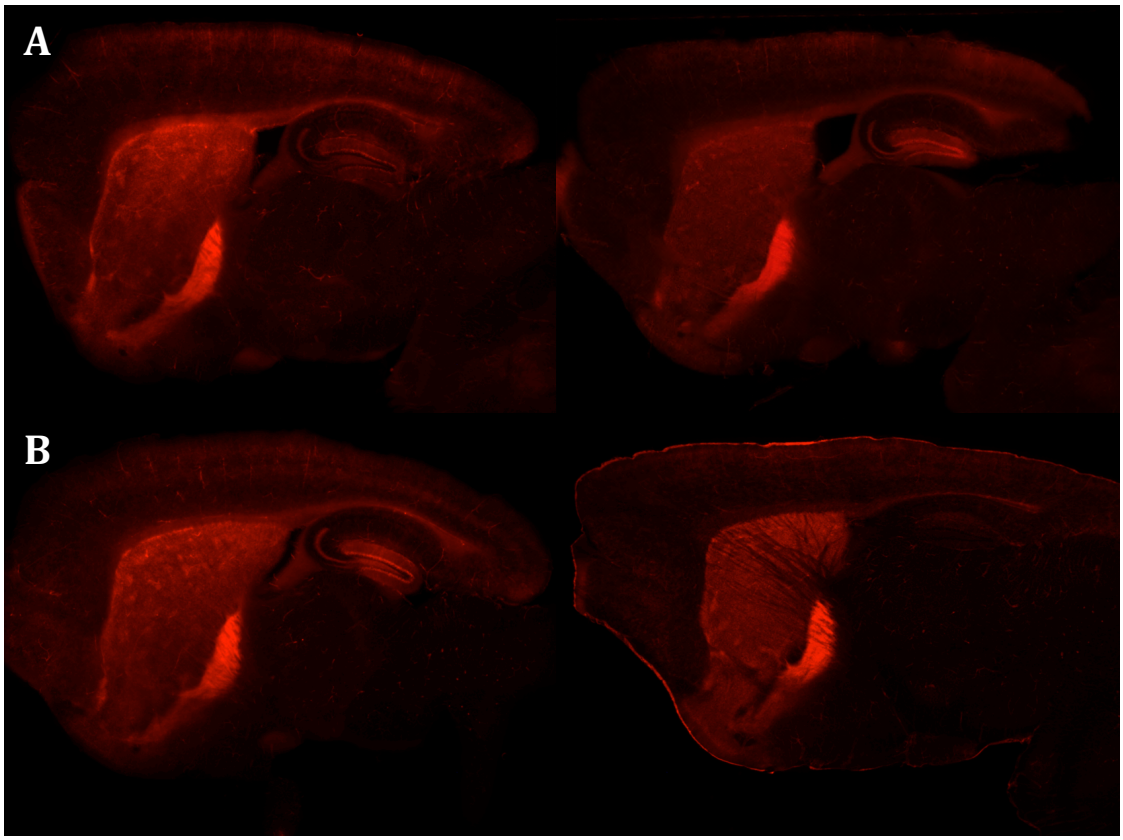


Figure 5
A representative section taken from a mouse homozygous for the wild type CELSR3 allele
All the structures of interest highlighted by A2a-Cre labeled in blue.



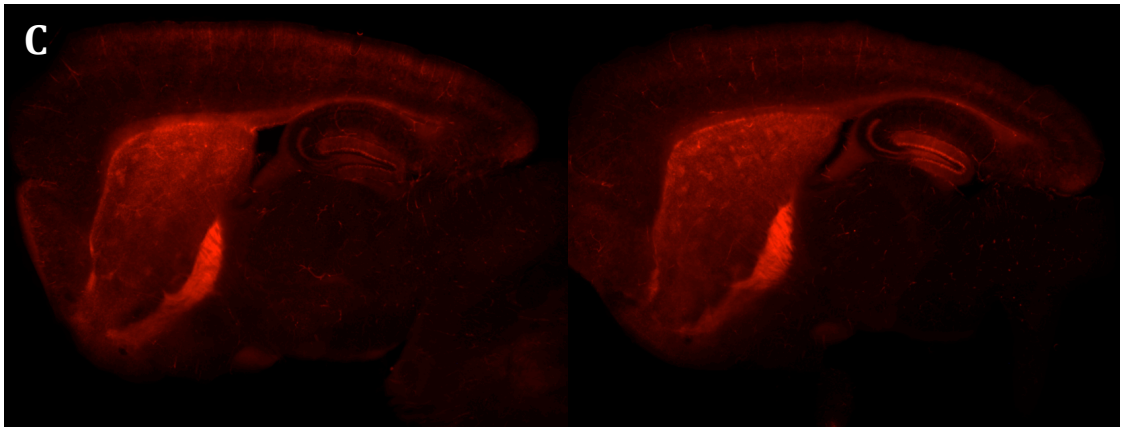


Figure 6
Representative sections of CELSR3 wild type brains being compared to R774H homozygotes and to each other (visualized using Ai14 and A2a-Cre)

(A) Sagittal section of brain from a mouse homozygous for wild type CELSR3 (left) compared to a sagittal section at comparable depth taken from a mouse homozygous for R774H (right). (B) Second CELSR3 wild type compared to a second R774H homozygote. (C) CELSR3 wild type from Fig 4A being compared to CELSR3 wild type from Fig 4B

After comparing sections from mice expressing A2a-Cre, no noticeable deviations in the expected paths of axon tracts were observed. Thus, at the macroscopic level, the indirect pathway is also intact in animals heterozygous or homozygous for the R774H amino acid substitution.

4.4 Emx1-Cre: Supplemental Data on Neuroanatomy

Emx1-Cre is a more diffusively expressed than either A2a or Drd1, being expressed in all cortical cells. Using this Cre driver, other structures that express CELSR3 were examined to supplement the data acquired from investigating the direct and indirect pathways. Specifically, cortical-striatal axon projections, the anterior commissure, the amygdala, the hippocampus, and the mammillary tracts were examined for any abnormalities.

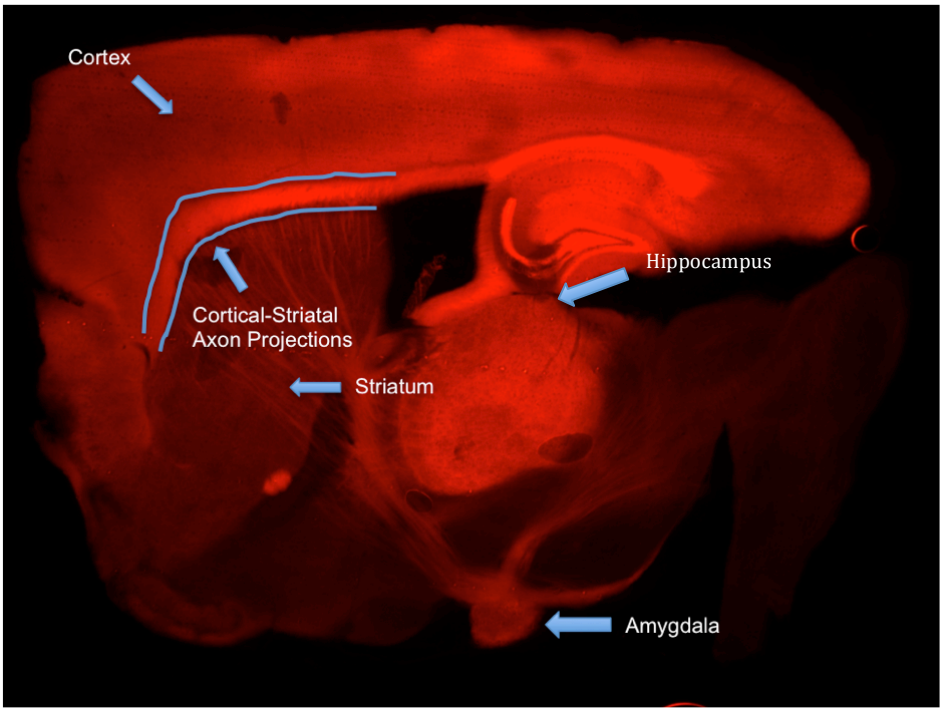
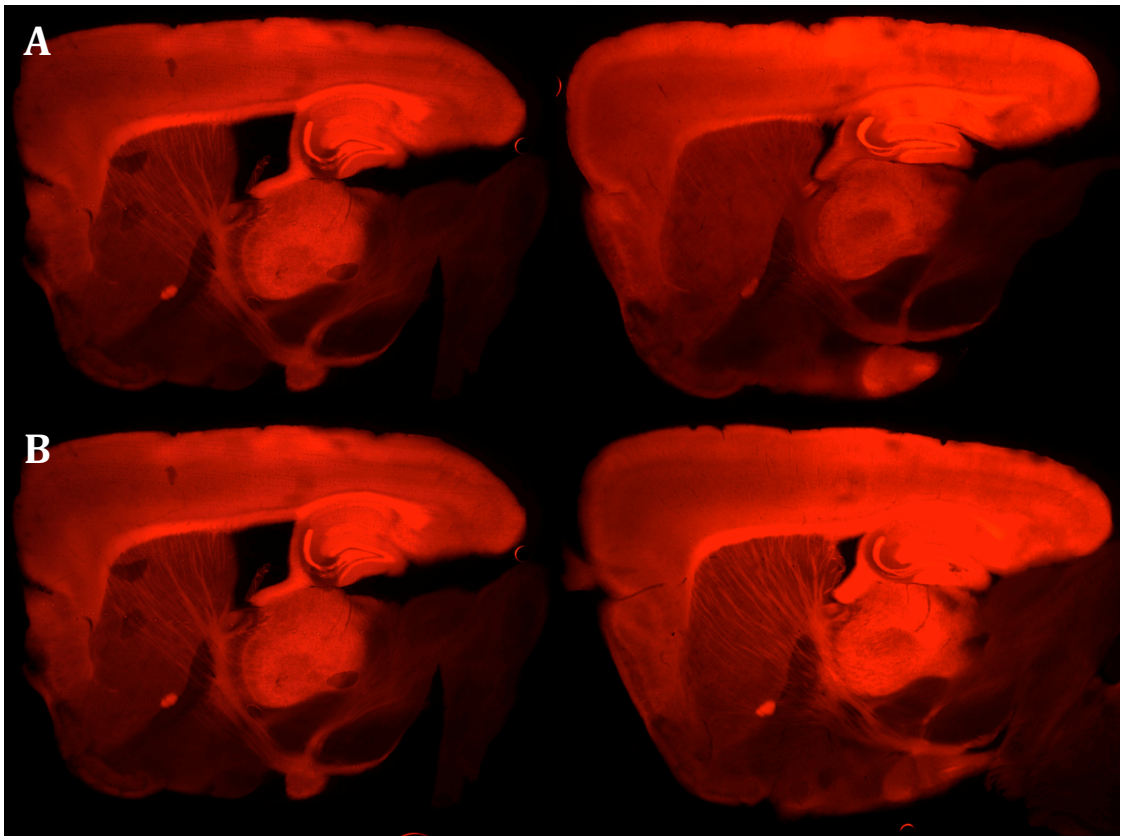


Figure 7
A representative section taken from a mouse homozygous for the wild type CELSR3 allele (sagittal plane)
All the structures of interest highlighted by Emx1-Cre labeled in blue



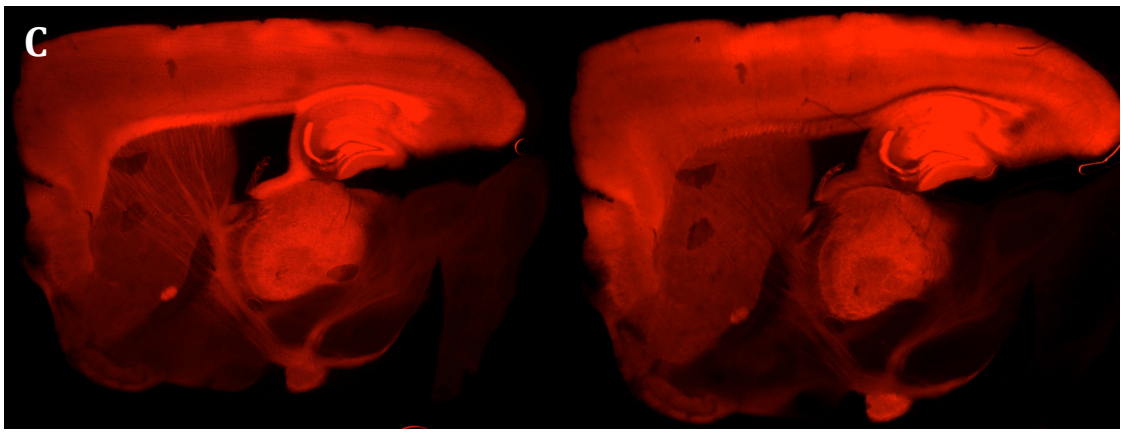


Figure 8
Representative sagittal sections of CELSR3 wild type brain being compared to R774H homozygote, R774H heterozygote, and a second CELSR3 wild type homozygote (visualized with Emx1-Cre)

(A) Sagittal section of brain from a mouse homozygous for wild type CELSR3 (left) compared to a sagittal section at comparable depth taken from a mouse homozygous for R774H (right). (B) CELSR3 wild type from Fig 6A compared to an R774H heterozygote. (C) CELSR3 wild type from Fig 6A being compared to a second CELSR3 wild type homozygote. These sections are a representative sample of the many sections taken and imaged, and were used to compare the appearance of the cortical-striatal projections, amygdala, and hippocampus in mice with the R774H point mutation to those in mice homozygous for the wild type CELSR3 allele.

The initial reason Emx1-Cre positive mice were included in this experiment was to examine the descending neural fibers of the cortex that innervate the striatum. These fibers have been shown to be missing when CELSR3 is knocked out and thus may have been affected by the R774H point mutation. The resolution and visibility of these fibers when marked using Emx1-Cre and Ai14 were limited upon imaging and thus any judgment that can be made, regarding whether any abnormalities are present in mice positive for the point mutation, is limited. The limited views in the collected sections appeared largely comparable to the CELSR3 wild type homozygotes, with any differences present likely being due to natural, expected variation.

During imaging of the sagittal brain sections it was discovered that the amygdala and hippocampus also expressed enough tdTomato for fluorescence visualization (Figure 8). Since these two structures have previously been implicated in the presentation of verbal tics in TS **[Mccairn et al. 2016]**, they were also examined for any developmental malformations, but no significant differences were found. Because the anterior commissure is also known to be absent in mice that have CELSR3 completely knocked out, sections in the coronal plane were taken to see if the R774H point mutation contributed to any abnormalities in that structure as well (Figure 9).

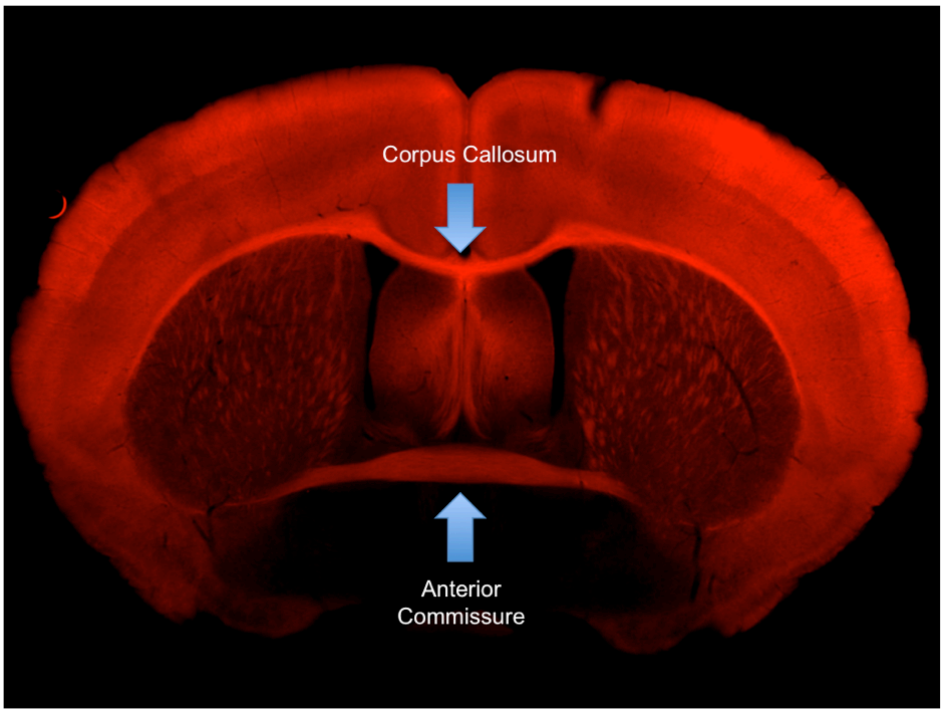


Figure 9
A representative section taken from a mouse homozygous for the wild type CELSR3 allele (coronal plane)
All the structures of interest highlighted by Emx1-Cre labeled in blue

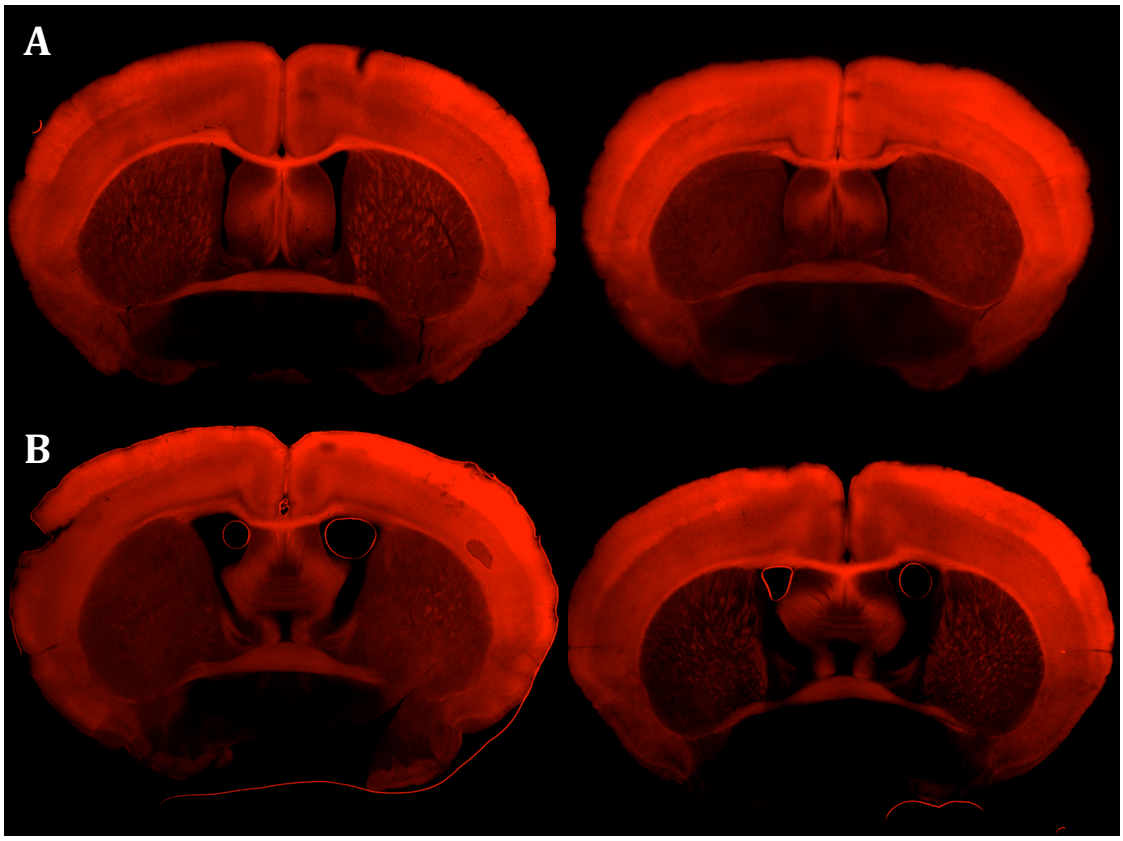


Figure 10
Representative coronal sections of 2 CELSR3 wild type brains being compared to two R774H homozygote brains (visualized with Emx1-Cre)

(A) Coronal section of CELSR3 wild type homozygote (left) being compared to a coronal section of R774H homozygote (right). (B) A second CELSR3 wild type homozygote (left) being compared to a second R774H homozygote (right). These images were taken to look for any abnormalities in the morphology of the anterior commissure that may be caused by R774H. Note: apparent difference in brain sizes is simply due to different magnifications being used to capture the images.

Upon inspection, the anterior commissure of mice possessing the R774H point mutation showed no signs of deformity or abnormal development when compared to the brains of mice homozygous for the wild type CESLR3 allele.

As with the inspection of the amygdala and hippocampus in the sagittal sections, axon fibers of the mammillary tract (or mammillothalamic tract) were also visible due to expression of Emx1-Cre and Ai14. This tract interconnects the

amygdala, hippocampus, and thalamus -- all structures implicated in the presentation of TS; so additional brain sections were taken to search for any gross malformations in this structure as well.

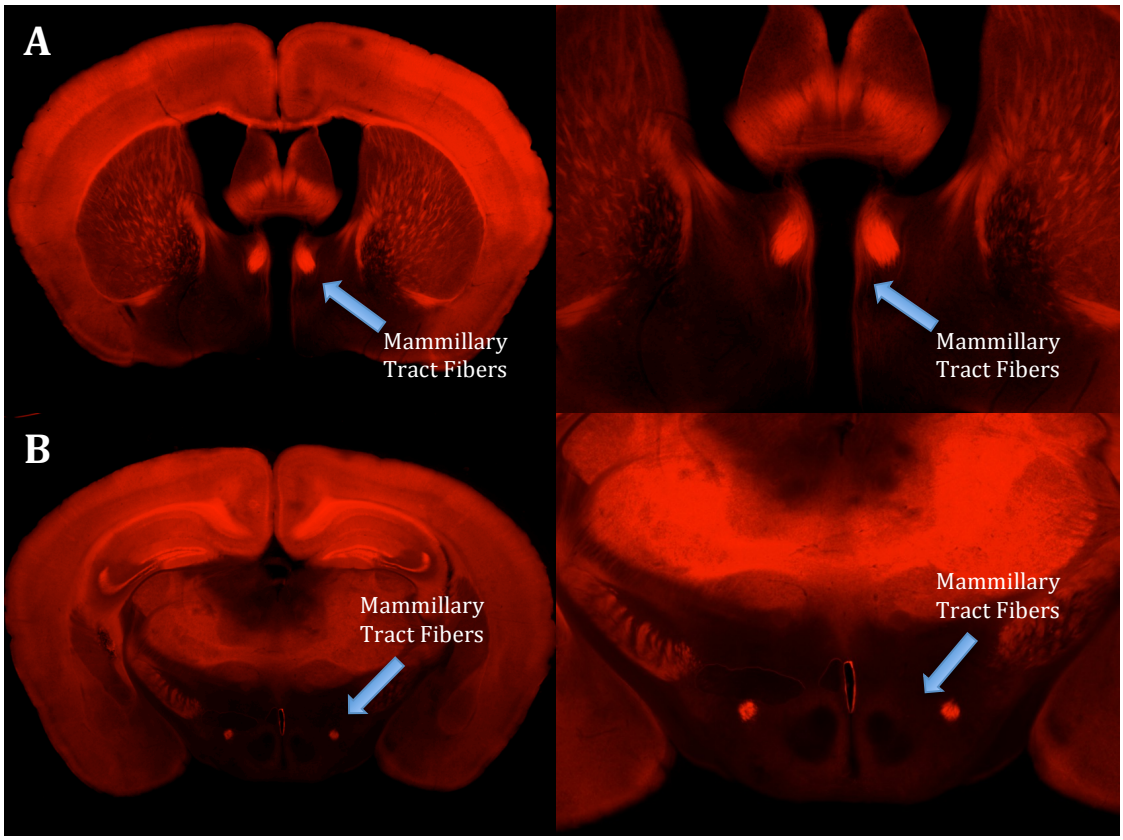


Figure 11
Representative coronal sections of 2 CELSR3 wild type brains showing mammillary tract fibers

(A) Coronal section of CELSR3 wild type homozygote (left) next to the same section zoomed in (right) to show standard morphology of mammillary tract. (B) Coronal Section of CELSR3 wild type homozygote (left) at different depth next to the same section zoomed in (right). These two sections represent the two depths at which the morphology of the mammillary tract was compared in this experiment.

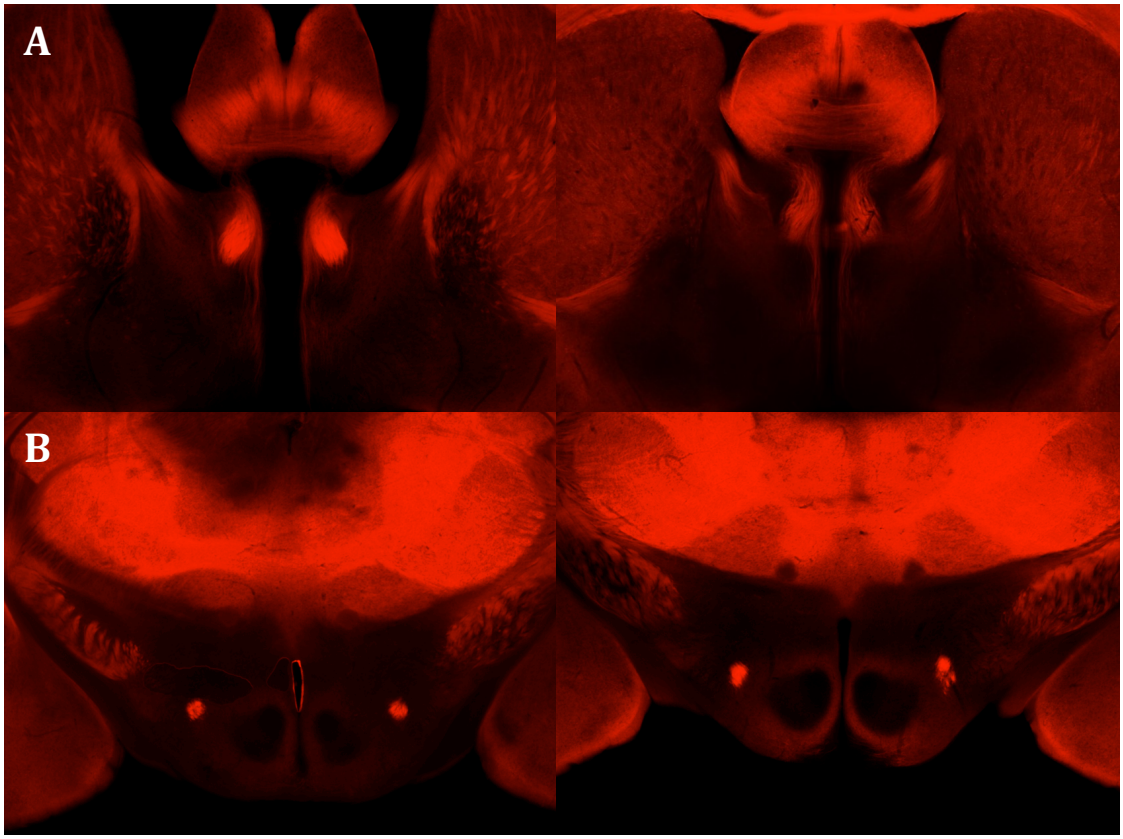


Figure 12
Representative coronal sections of two CELSR3 wild type homozygotes compared against two R774H homozygotes for the purposes of examining mammillary tract morphology

(A) Coronal section of CELSR3 wild type homozygote (left) compared against an R774H homozygote (right). (B) Coronal Section of CELSR3 wild type homozygote (left) at different depth next to a second R774H homozygote (right).

Sections were taken and the morphology of the mammillary tract was compared at two different depths in the brain. No significant differences were found.

CHAPTER 5

Discussion

This work was performed to determine whether CELSR3 mutation R774H was associated with any major neuroanatomical abnormalities on a pure C57Bl/6 background, specifically in structures having CELSR3-dependent development, or those associated with the presentation of TS. These structures included the axon tracts of the direct and indirect pathways of movement, the amygdala, the anterior commissure, and the hippocampus. Opportunistically, the mammillary tract was also included in this investigation as a supplement, since it connects multiple structures of interest and was visible when using Emx1-Cre.

R774H is a novel mutation identified in a human patient with TS. Neither of the patient's parents had TS or the mutation, the mutation is not present in the general population, and it is one of 10 mutations identified in the coding sequence for CELSR3 that have been associated with patients presenting symptoms of TS. Identifying an abnormal phenotype in mice that could potentially be linked to R774H would be a first step in identifying at least one element of the pathophysiology of TS. To do this, mouse lines having the R774H mutation were engineered using CRISPR technology, and subsequently bred with mice that possessed one of three combinations of Cre drivers plus a fluorescent reporter allele that would allow for neuroanatomical focused investigation of the structures in which they were respectively expressed. The results of these experiments are discussed below.

5.1 The Direct and Indirect Pathways of Movement

The direct and indirect pathways are directly tied to the regulation of voluntary movement in response to stimuli. It was hypothesized during the genesis of this experiment that impaired development of these pathways could potentially dysregulate responses to stimuli -- leading to the tics seen in patients with TS. The primary goal of this experiment was thus focused on examining the morphology of the axon tracts making up these pathways in mouse models expressing the R774H CELSR3 mutation. If a morphological phenotype was found in the mice that could potentially be associated with the R774H mutation, then further research could be pursued to explore the possibility that a similar malformation is involved in some cases of TS.

To examine the axons of the direct pathway, mice with and without the R774H mutation were engineered to the express red fluorescent protein tdTomato in the cells of the direct pathway circuit. The brains of these mice were extracted and cut into 120-micron sections and examined under a microscope. Sections at various depths were matched as well as was feasible to analogous sections from each of the other brains and compared, searching for consistent morphologic differences in the axon tracts that may have been attributable to the neurodevelopmental role of CELSR3.

A priori, it was hypothesized that the most likely abnormalities to be encountered would be either gross hypoplasticity in the axon tracts, or a routing error that caused axons to not follow their normal path. The hypoplasticity was expected due to our current understanding of the process of “axon pruning” and its

observed dependence upon CELSR3. During development, axons extend from neuronal bodies and connect the various structures of the brain. If an axon is superfluous or has been routed incorrectly, then it will be removed **[Geden et al. 2019].** If CELSR3 provides a positive signal to encourage axon growth along a specified path, and lack of that signal results in the "pruning" (selective degeneration) of that axon, then a mutation in the cadherin domain of CELSR3 that reduces its ability to carry out this function could result in a reduced number of axons reaching their target destination during development. On the other hand, if the mutation in the cadherin domain altered the behavior of CELSR3 rather than simply inhibiting it, then perhaps axons that had been incorrectly routed, and that would have normally been pruned during development, had been allowed to fully develop. However, after careful examination, no consistent aberrant phenotype could be identified. The axon tracts of the direct pathway in mice either heterozygous or homozygous for R774H showed no major differences when compared to those of mice homozygous for wild type CELSR3. Based on the available data, no significant differences in axon density or route could be identified. As stated above in the results section, the morphology on display in many of the sections is not exactly identical when compared to other sections taken from analogous depths in the brain. However, none of these minor differences in morphology were ever consistently associated with mice of any genotype. It was just as likely for a CELSR3 wild type homozygote to look slightly different from an R774H homozygote, as it was to look different from another wild type. These small changes were attributed to slight variations of the plane in which the brains were

sectioned as well the as natural variation expected to exist between two distinct animals.

The experimental process was repeated for mice bred to express red fluorescent protein in the axons of the indirect pathway circuit. Four R774H homozygotes were compared to two CELSR3 wild type homozygotes and, as with the axons of the direct pathway, no appreciable difference in morphology was found. In fact, even less natural variation was seen between sections when they were well matched for depth and plane of cut.

5.2 Cortical-Striatal Projections, the Amygdala, and the Hippocampus

While the direct and indirect pathways were the primary focus of this experiment, other structures potentially affected by the R774H mutation were also examined. In CELSR3 knockout mice, the descending fibers of the cortex that innervate the striatum (cortical-striatal projections) are missing **[Tissir et al. 2005]**. As such, we decided it would be prudent to examine these axons in R774H positive mice and compare them to those found in CELSR3 wild type mice. Using the same protocol that was used to look at the fibers of the direct and indirect pathways, sections were prepared using mouse brains positive for Emx1-Cre (which results in red fluorescent protein being expressed in all cortical cells). Two CELSR3 wild type homozygotes, three R774H homozygotes, and one R774H heterozygote brains were sectioned and imaged.

Upon comparison, it was discovered that the way in which the cortical-striatal projections were labeled was not optimal for an in depth comparison. At the

resolution used, not as much detail was visible compared to the images of the direct and indirect pathway projections. The tracts that were visible however were determined to be largely comparable across genotypes. However, cells of the amygdala and hippocampus were also found to be labeled and clearly visible in the sagittal plane. Due to the lack of detail visible when examining the cortical-striatal projections, we decided to compare the morphologies of the amygdala and hippocampus as well. These regions of the brain have also been previously implicated in contributing to vocal ticks in TS patients **[Mccairn et al. 2016]**.

Examining the general structure of these two subcortical structures proved much easier than examining the cortical-striatal projections had been. The limited resolution of the camera being used proved to be a much less significant factor because of much more distinctly labeled the two structures were. Again, however, no noticeable differences could be seen when comparing R774H positive brains to brains homozygous for wild type CELSR3.

5.3 The Anterior Commissure and Mammillary Tracts

The final structures examined in this experiment were the anterior commissure and the mammillary tracts. Both are labeled using by Emx1-Cre in conjunction with Ai14. The preparation of sections was largely the same as those described previously, with the one difference being that the brains were sectioned in the coronal plane rather than the sagittal.

The anterior commissure was examined first due to previous studies finding that its development was severely affected (either being underdeveloped or entirely absent) in complete CELSR3 knock out mice **[Tissir et al. 2005]**. While it's exact

function is not entirely understood, the anterior commissure does connect the amygdalae of the left and right hemispheres (which as previously discussed are implicated in verbal tics in patients with TS **[Mccairn et al. 2016]**). As such, examination of the anterior commissure for any malformations potentially attributable to the presence of R774H was potentially valuable.

For this portion of the experiment, two R774H homozygotes were compared against four CELSR3 wild type homozygotes. After side-by-side comparison, no differences were seen in the structure of the anterior commissure in the R774H positive mice compared to the CELSR3 wild type mice. As with every other comparison, any mild differences that were seen were likely attributable to natural variation between animals. With that said, the morphology of the anterior commissure was found to be more conserved, even between animals of the same genotype, to a greater degree than any other structure examined, with the possible exception of the indirect pathway axon tracts.

Similar to the examination of the amygdala and hippocampus, looking at the structure of the mammillary tracts was done to supplement the data collected in this experiment when it was noticed that they were labeled quite clearly by Emx1-Cre. The mammillary tracts either directly or indirectly connect the amygdala and hippocampus, much like the anterior commissure, as well as the thalamus (all structures that have been implicated in TS). The morphology of the mammillary tracts was compared at two different depths between the R774H mice and wild type CELSR3 mice. The resolution of the photographs taken did not provide as much detail as that obtained when examining other structures in this experiment.

However, what could be seen was, once again, largely comparable between the two different genotypes, with no noteworthy abnormalities identified.

5.4 Experimental Limitations

This work was performed to determine whether the R774H mutation in CELSR3 caused gross neuroanatomical abnormalities in genetically engineered mice along pathways postulated to be involved in the pathogenesis of TS. By using mice engineered to express the red fluorescent protein tdTomato in structures of interest, we were able to photograph them and compare their overall appearance in animals with different genotypes. However, one major limitation associated with this approach relates to the resolution of the imaging employed in this initial work. The camera used (Leica DFC7000T) provided clear images showing the general appearance of the sections being examined, but more subtle changes that might be present, at the level of individual axons, cannot be visualized. In addition, the labeling provided by Ai14 in conjunction with a specific Cre was sometimes suboptimal for examining certain neural components, such as the cortical-striatal projections examined with Emx1-Cre. This was often due to the structure simply not being large enough or distinct enough in the plane it was being viewed for sufficient detail to be visible from the fluorescence of tdTomato.

The number of brains examined was also limited by the time frame of this project. When a litter of pups was born, there was no guarantee that the desired combination of alleles would be present, and each litter that failed to provide the desired genotype meant waiting at least another month for a new litter to be born and reach the age at which they could be used for data. Because the number of mice

that were appropriate for breeding was limited, there was no way to increase the number of active breeding pairs while also maintaining a consistent genetic profile in the offspring. Should this experiment be repeated in the future, a greater number of brains comparisons would significantly increase the confidence with which a conclusion can be reached.

The level of fluorescence shown in each individual mouse also seems to be somewhat variable. I saw no consistent pattern in the expression of red fluorescent protein, so in future experiments it may be helpful to standardize the genotype of mice used with regard to Ai14 (e.g. genotype for presence of Ai14 and use only pups heterozygous for Ai14 for data collection). If fluorescent protein expression becomes more consistent with standardized genotypes, then perhaps it would be possible to control for level of fluorescence and use it as an indicator of higher or lower density of axon tracts.

Another limitation that was briefly referred to in previous sections was the matter of setting the brains in agarose for sectioning. Due to the small scale of the brains and the sections into which they were being cut (on the order of microns), even small deviations of a few degrees in the placement of the brain in agarose would accumulate as more sections were taken. As a result, even well matched brain sections were likely not displaying exactly analogous anatomical cross sections. This could result in a confounding effect, where differences found between sections that were actually due to slight differences in the depth at which each structure would normally be found being classified as a potential variation due to the presence of R774H. If this experiment were to be repeated in the future, it could be beneficial to

fabricate some kind of mold to ensure consistent placement of the brains to reduce this uncertainty.

5.5 Conclusion

Comparison of brain sections from mice either heterozygous or homozygous for the R774H allele revealed no gross abnormalities of axon tract development in neural circuits implicated in human TS.

If the CELSR3 R774H mutation does cause neurological dysfunction that translates to mice, it is more likely to be found at the cellular or molecular level, rather than the anatomical.

5.6 Future Directions

The research presented in this thesis was designed to preliminarily assess the likelihood of a potential cause of TS (neuroanatomical abnormalities caused by the novel CELSR3 mutation R774H disrupting proper prenatal development). Though the results of the experiment seem to indicate that if R774H plays any causative role in TS, it is not because of anatomical malformations, it may still be prudent to verify this conclusion in the future. As stated above, this experiment largely focused on comparing the overall appearance of neural structures between mice with and without the R774H mutation. Should the results of this experiment need to be further confirmed, a quantification analysis could be a good step to take. Using higher magnifications and 3D depth projections made possible by a confocal microscope, more detailed pictures can be analyzed and measured using quantification software, as there may be anatomical abnormalities present that are too subtle to be detected by the methods described here. For example, a consistent

reduction in the axon density of the direct pathway that is not clearly visible in the images above might become apparent using this method.

The results of this experiment have provided useful insight in helping to determine the possible effects of R774H that may be contributing to the development of TS. Given such a consistent lack of abnormality found across all structures examined, it seems more likely that the effects of R774H will be at the cellular or molecular level (should R774H have any appreciable effect). As such, future research into the effects of R774H should be focused on these avenues. The Tischfield Lab specifically is interested in examining potential dysfunction in interneuron migration and electrophysiological cell-to-cell communication resultant in the presence of R774H. A 2009 study by Ying et al. found that knocking out the function of CELSR3 prevented proper migration of interneurons into the developing cortex from the striatum. A primary function of interneurons is to allow communication between sensory and motor neurons, which is crucial for higher brain functions **[Kandel et al. 2013]**. If this function were to be disrupted, it is feasible that decision-making pathways could be impacted in such a way that motor or verbal tics arise. By ruling out anatomical abnormalities as the primary effect of R774H that contributes to TS, more resources can be allocated to exploring other promising alternatives.

With regard to other types of experiments that could be done to follow up the results of this research project, I found it pertinent to consider the possible explanations for said results. What were the most obvious reasons that R774H failed to cause any morphological abnormalities in the brains I examined? Upon

consideration of this question I was drawn back to the model I had used to conduct my experiment: a mouse. TS is a human disease and the human brain is significantly more complex than that of a mouse. As such it bears determining if the results above were obtained simply because R774H is a neutral mutation in mice.

Although it would be impossible to recreate the above experiment using human brains, an alternative does exist that could potentially provide a more accurate modeling system. Organoids are miniature, simplified organ systems that can be grown *in vitro* from induced pluripotent stem cells (IPSCs) collected from human subjects. These "mini organs" can be directed to develop in a variety of ways and can be coaxed to develop into a three dimensional structure, making them useful for studying the microanatomy of a variety of different human organs, including the brain [**Lancaster et al. 2014]**. Brain organoids have been used recently as model systems for the study of developmental disorders in humans and can be directed to form tissues "representative of certain brain regions, such as the cerebral cortex, hippocampus and midbrain" **[Qian et al. 2019]**. Using these human brain organoids as a model, it is potentially possible to determine if there are physiological abnormalities attributable to R774H in human cells.

Organoids have been successfully created using both human and mouse stem cells **[Lancaster et al. 2014]**, which allows for the possibility to answer the question I posed above about R774H being neutral in mice but not humans. A potential experiment to determine this could involve comparing human and mouse brain organoids with the R774H mutation (introduced to the IPSCs using CRISPR technology) to analogous organoids without the mutation.

Other experiments could be designed to look for impacts on the developmental process caused by R774H. The development of human brain organoids that have had CELSR3 completely knocked out could be compared to those that have R774H as well those with wild type CELSR3. Alternatively, a mixed culture could be done, made from progenitor IPSCs with and without R774H that are engineered to express different colored fluorescent proteins respectively. By culturing these cells together to produce a single organoid, the developmental process could be monitored and the final composition of the organoid assessed to see if either group of progenitor cells is over or under represented. Pure cultures of mutant and wild type CELSR3 organoids could also be used to compare the rate of development to determine if that factor is negatively impacted. Because using organoids as a model removes many of the time and cost constraints associated with using mice, the possibilities offered by this technology are myriad and could be vastly useful for studying a developmental disease like TS.

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