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CHARACTERIZATION AND TREATMENT OF A NOVEL MOUSE MODEL OF TSC-ASSOCIATED AUTISM

by

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CHARACTERIZATION AND TREATMENT OF A NOVEL MOUSE MODEL OF TSC-ASSOCIATED AUTISM

A

DISSERTATION

Presented to the Faculty of
The University of Texas
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DOCTOR OF PHILOSOPHY

by

Rachel Michelle Reith, B.S.
Houston, Texas

August 2012

“You will be enriched in every way so that you can be generous on every occasion, and though us your generosity will result in thanksgiving to God.”

2 Corinthians 9:11

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CHARACTERIZATION AND TREATMENT OF A NOVEL MOUSE MODEL OF TSC-ASSOCIATED AUTISM

Publication No._____*

Rachel Michelle Reith, B.S.

Supervisory Professor: Michael J. Gambello, M.D., Ph.D.

Tuberous sclerosis complex (TSC) is a dominant tumor suppressor disorder caused by mutations in either *TSC1* or *TSC2.* The proteins of these genes form a complex to inhibit the mammalian target of rapamycin complex 1 (mTORC1), which controls protein translation and cell growth. TSC causes substantial neuropathology, often leading to autism spectrum disorders (ASDs) in up to 60% of patients. The anatomic and neurophysiologic links between these two disorders are not well understood. However, both disorders share cerebellar abnormalities. Therefore, we have characterized a novel mouse model in which the *Tsc2* gene was selectively deleted from cerebellar Purkinje cells (Tsc2f/-;Cre). These mice exhibit progressive Purkinje cell degeneration.

Since loss of Purkinje cells is a well-reported postmortem finding in patients with ASD, we conducted a series of behavior tests to assess if Tsc2f/-;Cre mice displayed autistic-like deficits. Using the three chambered social choice assay, we found that Tsc2f/-;Cre mice showed behavioral deficits, exhibiting no preference between a stranger mouse and an inanimate object, or between a novel and a familiar mouse. Tsc2f/-;Cre mice also demonstrated increased repetitive behavior as assessed with marble burying activity. Altogether, these results demonstrate that loss of *Tsc2* in Purkinje cells in a haploinsufficient background lead to behavioral deficits that are characteristic of human autism. Therefore, Purkinje cells loss and/or dysfunction may be an important link between TSC and ASD.

Additionally, we have examined some of the cellular mechanisms resulting from mutations in *Tsc2* leading to Purkinje cell death. Loss of *Tsc2* led to upregulation of

mTORC1 and increased cell size. As a consequence of increased protein synthesis, several cellular stress pathways were upregulated. Principally, these included altered calcium signaling, oxidative stress, and ER stress. Likely as a consequence of ER stress, there was also upregulation of ubiquitin and autophagy.

Excitingly, treatment with an mTORC1 inhibitor, rapamycin attenuated mTORC1 activity and prevented Purkinje cell death by reducing of calcium signaling, the ER stress response, and ubiquitin. Remarkably, rapamycin treatment also reversed the social behavior deficits, thus providing a promising potential therapy for TSC-associated ASD.

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Abbreviations:

4E-BP1	eukaryotic translation initiation factor 4E-binding protein 1
4-PBA	4-phenylbutyrate
AMPK	5'AMP-activated protein kinase
ASD	Autism spectrum disorder
ATF6	activating transcription factor 6
CC3	cleaved caspase 3
CHOP	CCAAT-enhancer-binding protein homologous protein
CNV	copy number variation
CSF	cerebrospinal fluid
DISC1	disrupted in schizophrenia 1
DSM	Diagnostic and Statistical Manual
eIF4E	eukaryotic translation initiation factor 4E
ER	endoplasmic reticulum
ERAD	ER-associated degradation
FMR1	Fragile X Mental Retardation gene
HDAC	histone deacetylase
IHC	immunohistochemistry
IP3R	inositol 1,4,5-trisphosphate receptor
IRE1α	inositol-requiring kinase
IRS1	insulin receptor substrate 1
LAM	lymphangioleiomyomatosis
LKB1	liver kinase B1
LOH	loss of heterozygosity
mPFC	medial prefrontal cortex

mTORC1	mammalian target of rapamycin complex 1
NS	not significant
pcd	Purkinje cell degeneration mice
PCP	Purkinje cell promoter
PDI	protein disulfide isomerase
PERK	protein kinase RNA-like endoplasmic reticulum kinase
PI3K	phosphatidylinositol 3-kinase
PTEN	phosphatase and tensin homolog
Rheb	Ras homolog enriched in brain
ROS	reactive oxygen species
SOD	Superoxide dismutase
TSC	Tuberous Sclerosis Complex
Tsc2f/-;Cre	*Tsc2*$^{flox/ko}$*;Pcp2-Cre* mice
Tsc2f/f;Cre	*Tsc2*$^{flox/flox}$*;Pcp2-Cre* mice
SEGA	sub-ependymal giant cell astrocytomas
UPR	unfolded protein response

Chapter One:

TSC Background

History of TSC:

In 1779, just south of Paris, 15-year old Marie was dying in her hospital bed of an unknown disease. Spurred by her death, and compelled to understand this disease, the French neurologist Désiré-Magloire Bourneville performed an autopsy on Marie. He noted thickened regions on the brain that he labeled “tuberous” (meaning bumps) and “sclerosis” (meaning hard). He concluded that these lesions were the origins of Marie’s seizures and untimely demise (5, 6).

Bourneville’s anatomic descriptions of this disorder brought him notoriety as the disease was first named after him. Later, Bourneville’s Disease was renamed, but his original descriptions of the disorder provided the basis for the new name. We now know this disorder by the name of Tuberous Sclerosis Complex (TSC).

TSC Pathology:

TSC affects about 1 in 6,000 people (7), causing benign growths throughout the body – in almost any organ. Many different skin lesions are associated with TSC, leading it to be classified as neurocutaneous disorder. As such, this is often one of the first clues that a child has TSC. These skin lesions can include: hypopigmented macules (white spots on the skin), facial angiofibromas (red bumps on the nose and cheeks similar in appearance to acne), periungual fibromas (growths under the toenails or fingernails), and shagreen patches (rough skin usually found on the back) (8). The kidneys can be marked by renal cysts called angiomyoplipomas. These tumors are a combination of vascular (angio), smooth muscle (myo), and fat (lipoma) origins (9). In fact, these kidney lesions are the leading cause of death in patients with TSC (10). Astrocytic hamartomas are growths that can affect the eyes of TSC patients, though they normally do not affect vision (11). Female patients can also develop lung cysts called lymphangioleiomyomatosis (LAM). This occurs later in life (well after puberty) and can also lead to premature death (12). Cardiac rhabdomyomas can occur in the heart. As these lesions form in utero, detection via ultrasound is a strong indication that a child may have TSC (13).

Finally, one of the most common and debilitating sites for tumor formation is the brain. Not only is it the second highest cause for mortality in TSC patients (10), the brain lesions lead to the most morbidity associated with the disorder. The thickened lesions

that were first described by Bourneville are dubbed “tubers,” meaning bumps or growths in the brain. These can occur in any location in the brain including cortical and subcortical regions. Also, sub-ependymal nodules can form along the ventricles. It is hypothesized that these can later degrade and become sub-ependymal giant cell astrocytomas (SEGAs). The SEGAs can grow to block the ventricle and consequently the flow of cerebrospinal fluid (CSF). Furthermore, approximately 30% of patients have cerebellar abnormalities (14, 15). White matter abnormalities can also be detected in both the cortex and cerebellum of patients (16).

These brain lesions can contribute to seizure disorder in approximately 95% of patients, intellectual disability in about 50% of patents (17), and autism spectrum disorders (ASD) in anywhere between 25-60% of patients (18-22). The first account of patients with TSC being described to have autistic-like behavior came in 1932, 11 years before the term “autism” was even utilized. British neurologists MacDonald Critchley and Charles J.C. Earl analyzed 29 patients with TSC who were in mental institutions. They described the patients to have unusual behavior including odd hand movements, bizarre attitudes, and repetitive movements (23). Understanding the link between these two disorders will help in our understanding of both disorders.

Genetics of TSC:

Historically, J. Kirpicznick was the first to recognize that TSC was a genetic condition. He studied twins with TSC (both monozygotic and dizygotic) as well as a family with three successive affected generations (24). Building upon this work, we now know that TSC is an autosomal-dominant disorder caused by mutations in either *TSC1* or *TSC2* (7). The products of the two genes hamartin (*TSC1*) and tuberin (*TSC2*) form a heterodimer (25).

Patients with mutations in *TSC2* tend to have a more severe phenotype. This has been noted for many of the features associated with TSC, including: intellectual disability and learning disabilities, hypopigmented macules, renal angiomyoplipomas, and sub-ependymal nodules (26, 27).

Fifty to seventy-five percent of TSC cases are due to sporadic mutations (7, 28). Of these sporadic cases, about 80 percent occur as a result of a mutation in *TSC2* (27). TSC2 may be a more common site for mutations because it is the larger of the two

genes and encodes the larger of the two proteins (29). Conversely, familial TSC has an equal distribution of *TSC1* and *TSC2* mutations (27). Since *TSC2* patients tend to display a more severe phenotype (26, 27), they may be less likely to reproduce and therefore less likely to pass on a familial *TSC2* mutation.

Pathogenesis:

Many TSC lesions develop as a result of somatic cell loss of the second allele. This two-hit hypothesis was first proposed by Alfred Knudson in 1971. To develop this model, Knudson first studied 48 patients with genetic retinoblastoma, hypothesizing that the cancer was caused by two separate mutational events. The first mutation was inherited in the germline cells, followed by a second mutation in somatic cells (30). To study the mutational pattern in TSC, Knudson examined the Eker rat model of TSC, first bred in 1954 by a Norwegian pathologist named Reidar Eker. These rats developed renal tumors through an autosomal dominant inheritance (31, 32). Knudson determined that the tumor formation followed his hypothesis of two-hits in the Tsc2 gene (33-35). A heterozygous germline mutation occurred in *TSC1* or *TSC2* (36). Then, loss of heterozygosity (LOH) occurred at the cellular level, altering the remaining copy of *TSC1/2.* This mechanism of pathogenesis has been detected in numerous TSC lesions (36-39). Conversely, LOH has been difficult to demonstrate in cortical tubers (40-42). This could mean: 1.) that haploinsufficiency is sufficient to induce cortical tubers, 2.) that haploinsufficiency combined with LOH in a few cells can cause tuber formation, and/or 3.) that the second-hit events involve some other form of gene silencing (43).

TSC and mTOR

Once the two genes were identified, they were discovered to have important functions in the mammalian target of rapamycin complex 1 (mTORC1) pathway (44-47). The mTORC1 kinase is an important regulator of protein translation and cell growth.

The mTORC1 complex consists of mTOR as well as several other regulatory proteins including: Raptor, mLST8/GβL, and PRAS40 (48-51). It is unclear, however, whether Raptor inhibits or facilitates mTOR activity. In vitro, Raptor is required for mTOR activity, acting as a scaffold protein for mTOR and its downstream substrates including S6K1 and 4E-BP1 (52-55). Conversely, other studies show in the context of amino acid withdrawal, tight binding of Raptor and mTOR inhibit the kinase activity of

mTOR (48). The role of mLST8/GβL in the mTORC1 pathway is unclear, as studies suggest mLST8/GβL-independent phosphorylation of mTOR downstream targets (56, 57). The final protein in the complex, PRAS40, directly hinders the ability of mTORC1 to phosphorylate its downstream targets (58, 59).

The downstream targets of mTORC1 regulate protein translation and cell growth. Active mTORC1 leads to the activation of S6 kinase (S6K) (60) which then phosphorylates ribosomal protein S6 (pS6), an important component of the 40S ribosomal subunit (61, 62). Activate mTORC1 also stimulates eukaryotic translation initiation factor 4E (eIF4E) activity (60). eIF4E recognizes the 5' cap of nuclear-transcribed mRNAs allowing for translation (63). When translation is inactive, eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) is bound to eIF4E, preventing its interaction with other members of the translation initiation complex (63). mTORC1 phosphorylates 4E-BP1, reducing its affinity for EIF4E, and allowing for translation to commence (63).

The TSC1/TSC2 complex inhibits the mTORC1 pathway through direct inhibition of the Ras homolog enriched in brain (Rheb) (64, 65). Rheb is a potent activator of mTORC1 (66). Tuberin (TSC2) has a GTPase activating domain (67) that targets Rheb (68-71). The GTPase domain catalyzes the hydrolysis of GTP-bound (active) Rheb to GDP-bound (inactive) Rheb (68, 71, 72). There are conflicting studies about the GTPase domain of tuberin, suggesting either that it is independent of hamartin (TSC1) (70, 71), or it requires binding to hamartin for activation (60, 68, 73). Nevertheless, hamartin is necessary for the stability of tuberin; otherwise it is readily degraded through the activity of HERC1 ubiquitin ligase (74). Thus, for full activity, both protein products are required, and the net effect is inhibition of Rheb and subsequent inhibition of mTORC1.

The TSC1/2 complex is regulated through inhibitory phosphorylation by Akt on TSC2 (at Ser 939, Ser 1130, and Thr 1462) (44, 75). Once this phosphorylation occurs, the TSC1/TSC2 complex dissociates, thus relieving the inhibition on mTORC1 (44, 76). However, Akt can also bypass TSC2, activating mTORC1 directly (58). Therefore, regulation of mTORC1 can occur even in *Tsc1* or *Tsc2* deficit cells (77-79).

Activation of Akt occurs through phosphatidylinositol 3-kinase (PI3K), which in turn is regulated by insulin, nutrients, and growth factors (80-84). PI3K is activated either in

response to insulin by the insulin receptor substrate 1 (IRS1) (85-87), or through Ras in response to growth factors (88, 89). PI3K is inhibited by the tumor suppressor phosphatase and tensin homolog (PTEN) (90, 91).

mTORC1 is also regulated by cellular ATP levels (92). The ratio of AMP/ATP is detected by 5'AMP-activated protein kinase (AMPK). AMPK is active under low energy states through binding of AMP (93, 94). Activation of AMPK decreases the activity of mTORC1 (95), partially by activating TSC2 (96). However, AMPK can also phosphorylate mTOR directly at Thr 2446, restricting the ability of Akt to activate mTOR on Ser 2448 (97).

The finding of AMPK in the mTORC1 pathway, connected TSC to another disorder: Peutz-Jeghers syndrome (PJS) (98). PJS is another tumor suppressor syndrome caused by the mutation of liver kinase B1 (LKB1), which is known to activate AMPK (99-101). Therefore, a mutation in LKB1 leads to constitutive mTORC1 activity similar to that seen in TSC patients (102, 103). Indeed, the tumors in the two disorders share many similar histological features (103, 104).

Further regulation of the mTORC1 pathway occurs due to negative feedback loops. S6K1, the downstream target of mTORC1, initiates a negative feedback loop by phosphorylating IRS1 and promoting its degradation (105-108). This leads to decreased Akt signaling (109). In addition to phosphorylating IRS1, S6K1 has also been shown to directly phosphorylate mTORC1 (110). However, the function of this phosphorylation is not known.

In addition to the mTORC1 complex, mTOR functions in another distinct complex called mTORC2 (111). mTORC2 consists of mTOR plus Rictor, mLST8/GβL, and Sin1 (49, 111). Rictor is necessary for the complex and Rictor knockout mice die around E10.5, possibly because of abnormal vascular development (112). mLST8/GβL is also critical for mTORC2 function and if knocked out in mice, also results in early embryonic lethality (56). The final member of the complex, Sin1, is also important for mTORC2 assembly and function (113, 114). mTORC2 is thought to regulate the actin cytoskeleton through association with protein kinase C (PKC) (56, 111, 115). mTORC2 leads to increased Akt phosphorylation (116, 117). Interestingly, the TSC1/2 complex can directly activate mTORC2 activity, resulting in a negative feedback loop (118, 119).

The complex interplay of TSC1/2 in the mTORC1 and mTORC2 pathways is highlighted in Figure 1.1.

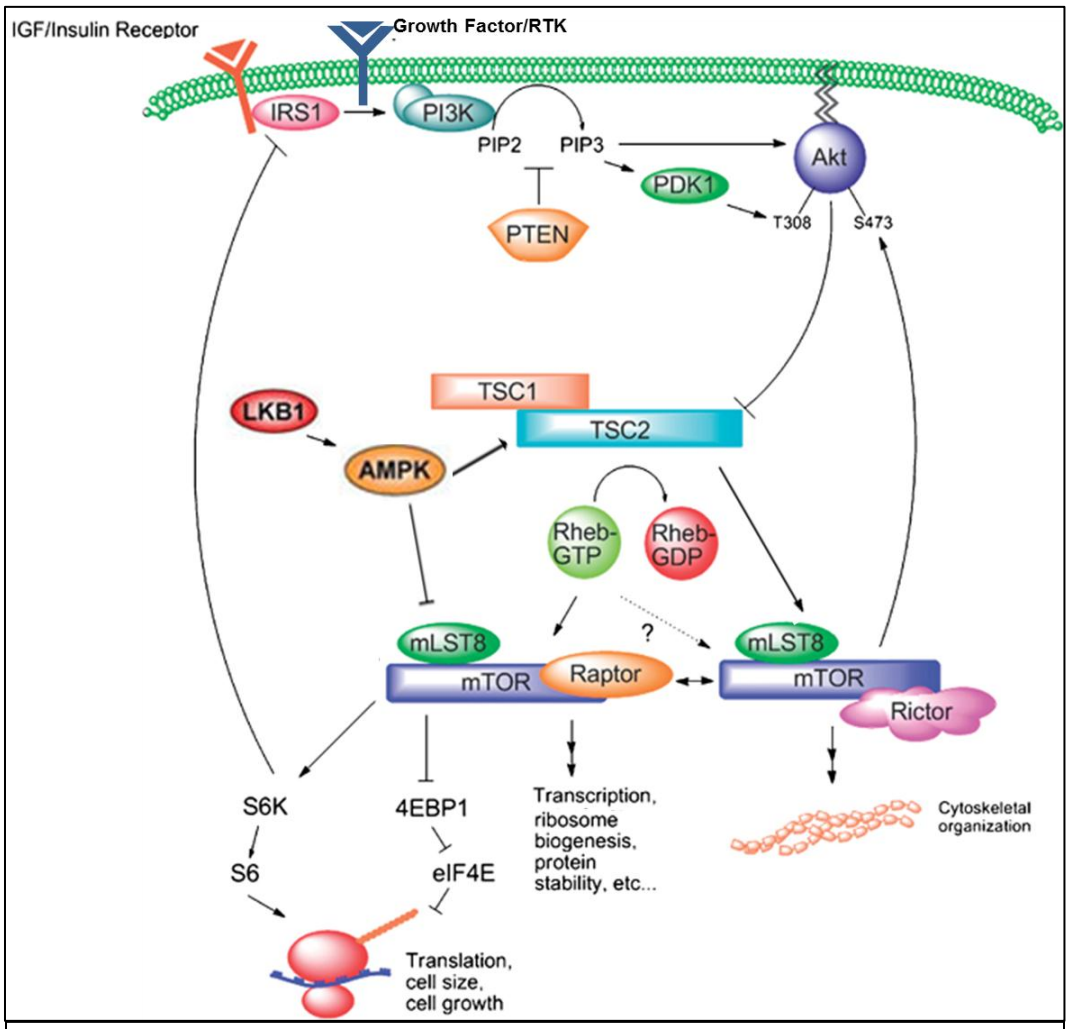

***Figure 1.1:** The interplay of TSC1 and TSC2 in the mTORC1 and mTORC2 pathways is illustrated here.

* Figure modified from (1). Permission to reproduce this figure in this dissertation has been received from Nature Publishing Group to Rachel Michelle Reith, license number 500678965.

The TSC1/TSC2 complex acts as a molecular rheostat, regulating translation and cell growth in response to a multitude of signals. Loss of this regulation leads to aberrant translation and cell growth, regardless of the cell's energy and nutrient status.

Chapter Two:

Autism Spectrum Disorders

History of Autism

The term autism (derived from the Greek autos, meaning self) was coined in 1910 by the Swiss psychiatrist Eugen Bleuler. He was describing the tendency of autistic patients to withdrawal into their own world, not tolerating any outside disturbances (120). The term autism, however, was not widely applied until 1943, when Leo Kanner used it to describe early infantile autism at Johns Hopkins Hospital (121).

Now, autism is just one subset of what is referred to as autism spectrum disorders (ASDs). The occurrence of ASDs is about 1 in every 110 people, with a higher incidence in boys than in girls (4.5:1) (122). Since the emergence of the disorder in the 1950s, it has gained much notoriety. In fact, every April since the 1970s, the Autism Society supports national autism awareness month. Because of the prevalence of this disorder in today's media, much speculation has occurred about historical figures that may have had ASD. Leading psychiatrist at Trinity College in Dublin, Michael Fitzgerald, as well as others have speculated on the mental underpinnings of some leading figures. Such names that have been proposed to have an ASD are: Lewis Carroll (123, 124), Herman Melville (123), Sir Arthur Conan Doyle (123), George Orwell (123), Ludwig van Beethoven (123), Wolfgang Amadeus Mozart (123), Michelangelo (125, 126), Vincent van Gogh (123, 126), Thomas Jefferson (126, 127), Adolf Hitler (128), Isaac Newton (126), and Albert Einstein (126). All of these are based on conjecture and are consequently very debatable.

Behaviors Associated with ASD

ASDs are usually diagnosed before the age of three. Some of the criteria for diagnosis and classifications will be revised in the new Diagnostic and Statistical Manual (DSM) that will be released in May 2013. However, currently diagnosis is based on behavioral evaluations with a focus on three core components: social interactions, communication, and stereotyped repetitive movements (129, 130). There is varying severity of these symptoms which indicates where on the autism spectrum a patient lies. Severity in one component also does not necessarily reflect severity in another component.

Infants with ASD pay less attention to social stimuli, respond less to their own name, and don't frequently smile or look at others. Toddlers with ASD do not have the same

level of eye contact or turn taking as a neurotypical. Furthermore, they do not even use simple movements (like pointing) to communicate their desires (131). To visualize what ASD may look like in a child, this is a personal account: “I was six months old when Mother noticed that I was no longer cuddly and that I stiffened up when she held me. When I was a few months older, Mother tried to gather me into her arms, and I clawed at her like a trapped animal” (132). Often, once an autistic child ages, they still have delayed communication (even nonverbal), they are less likely to respond to emotions, they do not imitate others, they lack social understanding, and they do not approach others in a social scenario (133). Children with high-functioning autism report to have more frequent bouts of loneliness compared to their non-autistic peers (134). Therefore, it is not necessarily that autistic children prefer to be alone; making and maintaining friendships is just more difficult for them.

Probably the most well-known person with autism today is Temple Grandin who was born in 1947 in Boston, Massachusetts and is now a professor of animal science at Colorado State University. In an account she gave to the popular author and British neurologist Oliver Sacks, Grandin described she felt “like an anthropologist on Mars” when she was interacting with neurotypical people (135). This is not all bad, however, because as Grandin points out, “the really social people are not the people who make computers” (136).

To explain lack of communication seen in patients with autism, Grandin describes words as her “second language” and that she primarily thinks in visions (137, 138). She states that her “mind is similar to an Internet search engine that searches for photographs” (138). In fact, it has been shown that autistic people process word-based tasks in the visual cortex (139). However, not all people with autism are visual thinkers like Grandin. Some are pattern thinkers while others are fact thinkers (138). She also described her thinking as associative and she categorizes the pictures in her mind to form concepts. In a humorous account, Grandin states: “When I was a child, I categorized dogs from cats by sorting the animals by size. All the dogs in our neighborhood were large until our neighbors got a Dachshund. I remember looking at the small dog and trying to figure out why she was not a cat” (138). People with autism often have difficulties forming new categories (140). Amazingly, Grandin was finally able to form a new category for dogs grouping them based on shape of their nose (138), though I am not sure how she will deal a pug if she ever sees one.

There are different categories of repetitive behaviors that can be associated with ASD and they are defined in the Repetitive Behavior Scale (141). Autistic patients will often practice stereotypies, which are repetitive movements such as hand flapping, head rolling, or body rocking. They also tend to engage in compulsive behaviors such as stacking or arranging objects, anything that follows a particular set of rules. Patients also tend to have a resistance to change. This means that seemingly small things like moving the couch could send them into an emotional fit.

Anxiety levels are often increased in patients with ASD (142-144). Therefore, some of the repetitive behaviors of ASD: spinning, head banging, repeating phrases (known as echolalia), have been suggested to be more of a coping mechanism (145, 146). Most patients with ASD have hypersensitivity to sensory input potentially leading to increased anxiety. Some have even suggested that this marks another core component of ASDs (147-150). Grandin invented the squeeze machine when she was 18 to counteract her own hypersensitivity (151). She describes her extreme sensitivity to sounds "like being tied to the rail and the train's coming" (136).

Currently there are no treatments for ASDs. However, behavioral therapies tend to help, especially if early intervention is achieved (152, 153). Even with behavioral therapy, only 4-12% of autistic patients can achieve independence upon adulthood (154, 155). Furthermore, these treatment costs are extreme, reaching a lifetime cost of an estimated $3.2 million per patient (156). Therefore, it is estimated that the annual cost for ASD cases in America is more than $35 billion (156). About 10% of this is medical care, 30% extra education and behavioral therapies, and 60% loss of economic productivity (156). Even parental employment is affected due to the time associated with caring for an autistic child (157). Because of this significant burden on the patient, the families, and society, understanding this disorder is paramount so that we can develop more effective treatments.

Etiologies of ASD

In the 1950s, the cause of ASD was attributed to bad parental skills. This view was proposed by Kanner and embraced by the medical field. It was thought that the mothers were cold-hearted, lacked warmth toward their children, and had a distant rejecting demeanor (158). This was popularly named the "refrigerator mother theory."

Numerous association studies have been performed trying to link the rate of ASD with a cause. In the 1980s, it was thought that autism was due to watching too much television. This theory was proposed because the increasing rates of autism correlated with the growth of television between the 1970s and 1980s (159). ASDs have also been found to occur more often in families with engineers, physicists, mathematicians, and scientists (160). Other studies have showed similar results (161), leading to the coinage of the term "geek syndrome" (162). Even rain has been associated as the "environmental trigger" for ASDs (163).

Since the 1980s, the incidence of ASDs has greatly increased (164). Part of the reason for this may be due to increased awareness and diagnosis, but there are likely other environmental factors leading to the rise in occurrence (165, 166).

Since the 1990s, foods are fortified with folic acid to reduce the occurrence of neural tube defects (167). Studies have supported the effectiveness of this dietary alteration (167-171). However, since folic acid supplementation, the rates of autism have been increasing, leading some to speculate folic acid increases might be correlated with ASDs (172, 173). However, a review of the folic acid studies shows conflicting results, making this hypothesis inconclusive (174).

Even today, the public views vaccination of children as a likely environmental risk factor. It was an attractive theory because autistic behaviors are often noticed around the time that a child gets vaccinated. This view is more likely to be held by parents who do not fully understand the safety of vaccines and who do not understand the risk or potential reality of contracting the various diseases (175). Furthermore, the research leading to this theory has been refuted (176), and even been called "an elaborate fraud (177)." Consequently, the original paper has now been retracted (178).

Other controversial proposed environmental factors are the use of heavy metals and pesticides (179). However, the more popularly accepted (though still inconclusive) environmental risk factors include: maternal gestational diabetes, parental age over 30 (both maternal and paternal), and use of medication during pregnancy (180).

One of the most conclusive environmental triggers associated with ASD is prenatal activation of the maternal immune system (181-184). This has been described as the principal, non-genetic cause of ASD (185). Additionally, neuroinflammation has been

seen in numerous postmortem samples of ASD and this occurred across a broad range of ages (5-44 years) (186). However, even this environmental trigger does not account for the majority of cases of ASD. What accounts for the remaining cases? It is likely that there are genetic and epigenetic factors that interact with environmental factors to cause ASD (187, 188). This complex mixture of risk factors forms the gene by environment interaction model (GxE).

Genetic Causes for ASD

The heritability of autism was first demonstrated in twin studies. The concordance rate between monozygotic twins is much higher than dizygotic twins (189-191). Recent studies suggest a concordance rate for ASDs as high as 90% for monozygotic twins (192). Therefore, in order to investigate the principal genetic causes of ASDs, two different approaches have been used. One approach has been to study idiopathic forms of autism, while the other approach focuses on syndromic forms of autism.

Genetic studies of idiopathic autism have consistently revealed alterations in copy number variations (CNVs) (193-199). CNVs represent either small or large duplications or deletions on segments of the chromosome. However, CNVs were first found in healthy controls with an average of 11 CNVs per person (200). This suggests that the presence of CNVs alone does not necessarily confer disease risk. CNVs are typically benign either because of dosage compensation, or because the CNV does not reside in a segment of the chromosome containing any genes. In addition to the large number of CNVs typically present in controls, aneuploidy occurs more frequently in neurons than in other non-brain cell types (201, 202). Therefore, it is believed that CNVs have very little functional importance in neurons (203). That being said, their increased prevalence in ASDs merits further investigation.

ASD has been also been associated with chromosomal rearrangements (204). However, lesions that can be detected using cytogenetics only account for 6-7% of cases of ASD (196). To investigate further causes of ASDs, linkage studies and genome wide association studies have been performed (205-210). Through the information compiled on the Autism Genome Project database, linkage studies and CNV data connect ASD susceptibility loci to every human chromosome (199, 211). Only a small subset of the susceptibility genes are listed in Table 2.1.

Gene	Function	Refs
AVPR1A	Arginine vasopressin receptor 1A	(212)
CDH9/10	Cell adhesion (cadherin genes)	(210)
DISC1	Involved in schizophrenia, depression, and bipolar	(213)
MET	Brain development and immune system (oncogene)	(214)
Neurexin1	Cell adhesion	(199, 215)
OXTR	Regulation of sexual behavior (oxytocin receptor)	(216, 217)
PRKCB1	Cell signaling (associated with protein kinase C)	(218, 219)
Reelin	Neuronal migration	(220, 221)

Table 2.1: A small subset of ASD susceptibility genes listed along with their known functions.

While numerous, none of these mutations can account for more than 1%-2% of cases with ASD (211, 222). Therefore, the current hypothesis is that mutations across many loci confer risk for developing ASD (223). This is known as oligogenic heterozygosity. A notable study of oligogenic heterozygosity examined the etiology of high-functioning, non-syndromic autism (n=339). Schaaf et al. sequenced 21 genes involved in syndromic autism. Based on their findings, they suggested that severe mutations in certain genes lead to syndromic autism. However, idiopathic autism may act like a balance, where simultaneous mutations in multiple genes (the same genes leading to syndromic autism) can tip the scale leading to idiopathic autism (223).

Syndromic Forms of ASD

Another tactic for studying the genetics of ASDs is to focus on well-known genetic syndromes associated with ASD. Such studies may yield insight into the cause of more general forms of ASD. Probably the most well-known ASD-associated syndrome is Fragile X syndrome, resulting from a trinucleotide repeat expansion in the promoter region of the Fragile X Mental Retardation (FMR1) gene (224). This expanded repeat sequence leads to silencing of FMR1 (225, 226). FMRP, the protein encoded by the FMR1 gene, is an RNA-binding protein that acts as a negative regulator of protein translation (227, 228). FMRP has also been suggested to have a role in synaptic plasticity (229, 230). Interestingly, about 15%-60% of patients with Fragile X also

develop ASD (231-235). This accounts for about 5% of total patients with ASD (192, 236).

Tuberous Sclerosis Complex (TSC) (see Chapter 1) is another ASD-associated Mendelian disorder. About 25%-60% of children with TSC have ASD (21, 237, 238), accounting for 0.5%-2.9% of total patients with ASD (239, 240). However, TSC can account for up to 14% of patients with a comorbidity of ASD and seizures (241). In their paper describing oligogenic heterozygosity, Schaaf et al. found a small deletion in the *TSC2* gene of a patient with ASD, but interestingly no diagnosis of TSC (223). Therefore, perhaps the *TSC1* and *TSC2* genes play a larger role in ASD than previously realized.

Neurofibromatosis type 1, due to a heterozygous mutation in NF1, has also been linked to ASD (242, 243). NF1 is a GTPase activating protein involved in inhibition of the Ras/MAPK signaling pathway (244). Similar to TSC, NF1 is also a tumor suppressor disorder that leads to benign growths and a myriad of subsequent complications (245, 246).

Cortical dysplasia-focal epilepsy syndrome (CDFE) is associated with epilepsy, hyperactivity, language abnormalities, mental retardation, and in 67% of cases: ASD (247). This syndrome is caused by recessive mutations in CNTNAP2, contactin associated protein-like 2. CNTNAP2, a transmembrane protein, is a member of the neurexin family. It functions both in neuron-glia interactions as well as clustering of potassium channels in myelinated axons (248, 249). However, CNTNAP2 is also expressed embryonically, suggesting a role in early brain development outside of myelination (248, 250, 251). In addition to syndromic ASD, CNTNAP2 has been linked to sporadic ASD through linkage, association, and gene expression studies (251-254).

The 15q11-13 duplication/deletion syndrome is also associated with ASD (255-257). (257-259). Not only is 15q11-13 a CNV, but several of the genes in the 15q11-13 region have also been separately linked to ASD. Mutations in the maternal copy of 15q11-13 (specifically UBE3A) cause Angelman syndrome, while mutations in the paternal copy of this region result in Prader-Willi syndrome. Both of these are associated with autistic-like behavior. Other 15q11-13 region genes implicated in ASD are: GABRA5, GABRG3, and GABRB3 (255-257). Another deletion syndrome associated with ASD is 22q13,

also known as Phelan-McDermid Syndrome (260, 261). The main ASD associated gene in this region in SHANK3, which is associated with neuronal synapses (262-264).

There are many different genetic mutations and genetic syndromes associated with ASD. However, these can only account for about 20 percent of all the cases of ASD (265), suggesting there are still mechanisms of ASD that need to be explored. Therefore, where does research go from here? Though ASD has a strong genetic component, it seems to involve the interaction of many genes (266). However, there may be common molecular/cellular mechanisms resulting from these different mutations.

Molecular Mechanisms of ASD

Altered brain connectivity is thought to be one putative mechanism for ASD (153, 267, 268). Neuronal development is first achieved by mitotic division from a neural progenitor cell. The newly formed neurons then migrate along radial glial cells to their proper locations. At this point, neuronal differentiation occurs to specify neuronal subtypes. Next, process outgrowth occurs through the extension of the growth cones toward cellular signals. Finally, after the axon reaches the correct target, synaptogenesis proceeds to establish proper connections. During this process, many synapses are pruned to allow for the most efficient signaling possible (269). All of these processes could be disrupted in autism. Increased proliferation has been thought to give rise to excess neurons in autism (270). Migration (266, 271), differentiation (266), axonal pathfinding (268), synaptogenesis (266), and pruning through apoptosis (266) are also thought to be altered. Abnormalities in protein synthesis are also thought to lead to synaptic dysfunction (272, 273). These alterations could lead to an imbalance between excitatory and inhibitory connections (266).

A related hypothesis is that the circuitry in autistic brains favors local connections at the expense of distant connections (274, 275). This hypothesis may offer an explanation of why there are deficits in complex social behaviors, which involve coordination from many aspects of the brain, but why patients have increased sensory perceptions (276-278).

Another compelling common cellular mechanism for ASD is calcium signaling (279). Calcium is involved in early development for processes such as neuronal survival, migration, differentiation, and synaptogenesis (280-285). The calcium hypothesis

combined the knowledge gleamed from mutations associated with idiopathic ASD as well as a syndromic form of ASD known as Timothy syndrome. Timothy syndrome, due to a mutation in L-type voltage-gated calcium channel (CACNA1C), causes multisystemic problems including cardiac abnormalities and ASD (286, 287). However, mutations in other voltage-gated calcium channels have also been associated with ASD (286, 288-290). These mutations prevent the voltage-dependent inactivation of the calcium channel and are therefore predicted to result in excessive calcium influx (279). In addition, other ASD susceptibility genes may be regulated by calcium levels or could themselves affect intracellular calcium levels. Also, some of the environmental factors that are associated with ASD might alter calcium signaling pathways (279).

In addition to these mechanisms, cell signaling pathways have also been implicated in the development of ASD. Numerous ASD candidate genes as well as ASD-associated disorders converge on the same cellular pathway: mTORC1 (291). The most obvious example of this is in the case of TSC, where the *TSC1* and *TSC2* genes act to suppress mTORC1 (21, 64, 65, 237, 238). However, the implication of mTORC1 in ASD extends beyond TSC. Mutations in the gene PTEN, an upstream inhibitor of mTORC1, have been associated with ASD and macrocephaly (91, 292). Downstream products of the mTORC1 pathway have also been found to be associated with ASD. In particular, activating mutations in eIF4E have been linked to ASD in two different families (293).

mTORC1 is also implicated in some other disorders associated with ASD. For example, mutations in the neurofibromatosis gene can result in aberrant mTORC1 activity (294). The NF1 gene is an inhibitor of MAPK (295), which then acts to inhibit TSC2 through its effects on AKT (296), thereby increasing the expression of mTORC1 (294). Furthermore, association studies link the chromosomal region of the MAPK gene with ASD (196, 297, 298). Fragile X syndrome is also related to aberrant mTORC1 signaling. Mutations in FMR1 are associated with mTOR-dependent abnormalities in protein synthesis (299) and knockout of the Fmr1 gene is associated with increased mTORC1 activity (300). Furthermore, the downstream effector of mTORC1, S6K, has been shown to phosphorylate FMRP, regulating the mRNA-binding site (301).

Additionally, altered mTORC1 signaling during development could also lead to abnormal brain connectivity and altered synaptic function, two popular models implicated in ASD (268). Therefore, since the mTORC1 pathway stands at the node of numerous

causes of ASD, and TSC is the prototypical mTORopathy with the most direct regulation of mTORC1, studying TSC might shed light on the genetic and cellular mechanisms involved in ASD and provide us with a model to develop possible therapeutic strategies.

Chapter Three:

Brain Regions Implicated in ASD

Cortical Regions in ASD

Many different anatomical brain regions have been implicated in the pathophysiology of ASD including those involved in language processing. Known for its role in the genesis of speech, Broca's area (located in the left inferior frontal gyrus) (302) is thought to mediate the communication deficits seen in ASD. In functional MRI studies (fMRI), patients with ASD show decreased activation of Broca's area relative to controls (303). Similarly, Wernicke's area (located in the left superior temporal gyrus) has been implicated in ASD due to its role in speech processing. Paradoxically, fMRI studies show that patients with ASD have increased activation of Wernicke's area (303). These results can be explained by the fact that patients with ASD have difficulty integrating individual words into meaningful, complex sentences (function of Broca's area). However, they have a heightened response to the meaning of individual words (function of Wernicke's area) (303, 304).

The inferior parietal lobule and the inferior frontal gyrus are known to be activated in response to imitation or observation of behaviors (activities involved in empathy) (305). The neurons in this area, named mirror neurons, form a network of connections identifiable in both animals (306) and humans (307). Due to the inability of patients with ASD to understand other people, this region of the brain has been investigated. Several studies have shown decreased activation of the mirror neuron network in patients with ASD (308, 309), seemingly correlating with the severity of autistic symptoms (310).

ASD-behaviors have also been correlated to the fusiform face area (FFA). Located in the lateral fusiform gyrus, this area of the brain is engaged during processing of human faces (311-313). Hypoactivation of this brain region has been consistently demonstrated in patients with ASD (314-316). This finding is not surprising given that patients with ASD pay less attention to human faces (317, 318). Therefore, hypoactivation of this brain region may not be the cause of autistic-like behaviors, rather a result of disinterest in facial recognition (319).

In one model, developmental abnormalities of the amygdala are thought to lead to autistic behaviors and subsequent hypoactivation of the FFA (320). The amygdala, located in the medial temporal lobes of the brain, has a crucial role in face processing since it recognizes eye gaze, lip movement, and expression (321). It acts to quickly process emotional stimuli and is involved in emotional learning (322, 323). Postmortem

studies of ASD patients show diminished neuronal arborization in the amygdala (324) and fMRI studies have shown hypoactivation of the amygdala compared to controls during face perception (314, 315, 325).

Another area of the brain implicated in ASD is the prefrontal cortex due to its role in executive functions (i.e. memory, inhibition, organization, planning, and cognitive flexibility), language, and social understanding (326-329). Imaging studies suggest decreased activation and disorganization of both the ventral and medical prefrontal cortices in patients with ASD in response to various social tasks (330-334).

The Cerebellum in ASD

While cortical regions are likely very important anatomical locations for autistic symptomatology, the first neuroanatomical abnormalities in ASD were reported in the cerebellum (Latin meaning “little brain”) (335-337) and this is the most consistent location of anatomical pathology seen in patients (338-342). Some of the abnormalities observed are: hypoplasia and hyperplasia of the vermis and cerebellar hemispheres as well as abnormalities in the deep cerebellar nuclei (339-341). Functional MRI studies of ASD patients also show abnormally low activation of the cerebellum in a selective attention task (338).

Furthermore, many of the ASD-associated syndromes are also marked by similar cerebellar abnormalities. These include: Fragile X Syndrome (specifically the patients with Fragile X and ASD) (341, 343, 344), TSC (14), 22q13 deletion syndrome (345, 346), Joubert syndrome (347-349), Smith-Lemli-Opitz syndrome (350), Rett syndrome (351, 352), neurofibromatosis 1 (353, 354), and Cowden disease (associated with mutations in PTEN) (355, 356). Furthermore, some patients with Dandy-Walker syndrome (a congenital cerebellar malformation syndrome) are reported to have ASD (357).

Cerebellar Circuitry

The cerebellar circuit is a complex network of cells consisting of 200 million mossy fiber inputs onto 40 billion granule cells, that converge through their parallel fibers onto 15 million Purkinje cells that then project to the less than 50 deep cerebellar nuclei cells (358). Each Purkinje cell can receive thousands of inputs from the parallel fibers of the granule cells, suggesting each parallel fiber exerts a very weak connection (358). However, only one climbing fiber (input from the olivary nucleus) will synapse with a

Purkinje cell, suggesting a very strong connection (358). The other circuitry in the cerebellum that affect Purkinje cells are: mossy fibers from the pontine and vestibular nuclei and stellate and basket cells, which are both interneurons residing in the molecular layer (358). The Purkinje cells integrate this information, and provide the sole output of the cerebellar cortex to the deep cerebellar nuclei (359). Since Purkinje cells utilize the neurotransmitter GABA, they elicit an inhibitory effect on the deep cerebellar nuclei (358).

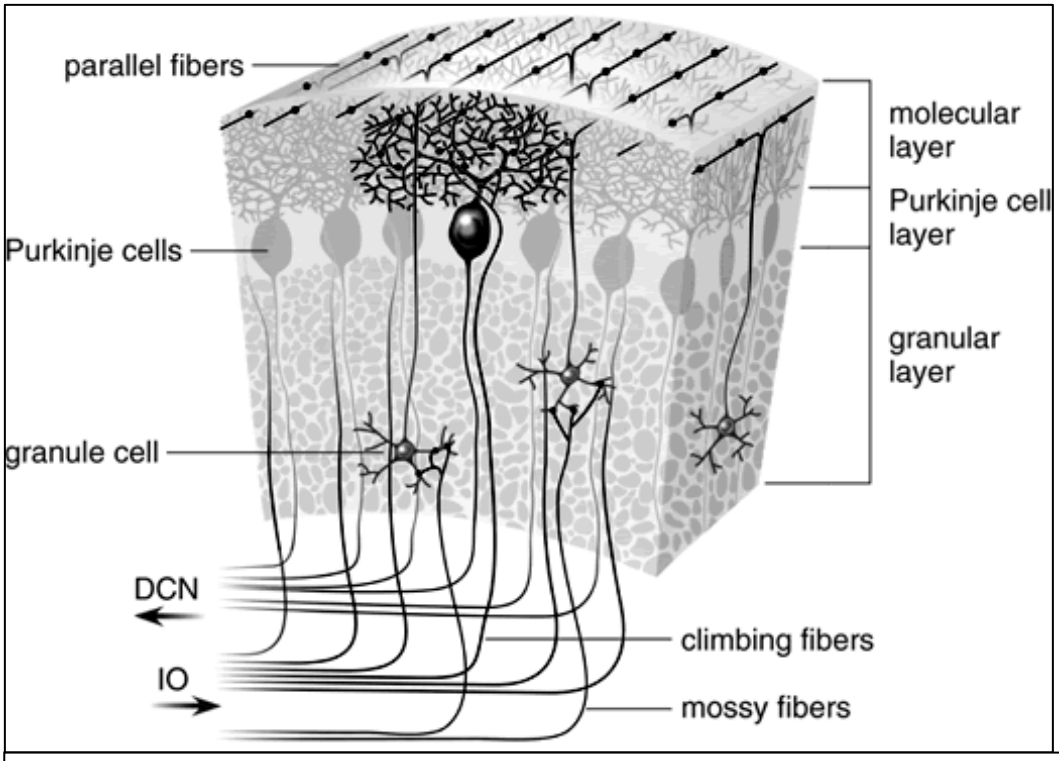

***Figure 3.1:** Cerebellar circuitry. Mossy fibers from the pontine and vestibular nuclei synapse onto the granule cells. Granule cell projections form parallel fibers, synapsing onto the Purkinje cell. Climbing fibers from the inferior olive (IO) synapse directly onto the Purkinje cell. Purkinje cells then provide output to the deep cerebellar nuclei (DCN).

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The cerebellum acts as a feedforward controller receiving sensory input from the mossy fibers. When an unexpected stimulus arises, error signals are generated from climbing fibers. This complex circuitry is thought to help create a complex representation of the sensory information for complex behavioral responses. Error signals from the climbing fibers trigger calcium release in the Purkinje cell – altering the

strength in connections between the Purkinje cells and parallel fibers - decreasing the likelihood of a repeated event and facilitating learning (359).

Since Purkinje cells are the central cell in the cerebellar circuit cortex, it is important to note that in addition to the other cerebellar lesions seen in ASD, Purkinje cell loss is widely reported in autopsy studies of ASD (approximately 75% of the reports) (360-366). This suggests that loss of Purkinje cells may be an important contributor to ASD pathology.

ASD Risk Factors Associated with the Development of the Cerebellum

Many of the ASD candidate genes and environmental influences also have important functions in the proper development of the cerebellum. Engrailed 2, a transcription factor important for embryonic development, has been shown to confer a risk for developing ASD (367). Engrailed 2 is expressed in the developing cerebellum (368) and mutations in engrailed 2 lead to abnormal cerebellar development (369, 370) and Purkinje cell loss (371). Decreased expression of the MET proto-oncogene has also been associated with risk for ASD (214) and is known to be expressed in the granule cells of the cerebellum (372). A mouse model of a MET mutation revealed decreased proliferation of the granule cells which led to abnormal foliation and reduced volume of the cerebellum (373). Neurexin is another ASD susceptibility gene (199, 215) and is required for proper synapse formation between the granule cells and Purkinje cells (374). Patients with the ASD-associated allele in contactin-associated like protein-2 (CNTNAP2) show decreased cerebellar volume (375). Mutations in reelin can cause cerebellar hypoplasia and abnormal axonal connectivity (376, 377). Several GABA receptor genes are in the 15q11-13 region implicated in ASD (255-257, 378) and mutations in these genes leads to cerebellar vermal hypoplasia (379). The 22q13 region also contains the genes: PLXNB2 and MAPK8IP2, which are both expressed in the developing cerebellum (380).

Furthermore, some of the environmental factors might contribute to ASD by affecting cerebellar circuitry. Activation of immune markers has been observed in the cerebellum of patients with ASD (186). Oxidative stress markers are upregulated in many post mortem studies of ASD, particularly in the cells in the cerebellum (363, 381-385). The brain is particularly sensitive to oxidative stress because it has a higher energy requirement, limited antioxidant capacity, and high levels of unsaturated lipids

and iron. Prenatal valproic acid exposure is another well-known risk factor for ASD that may act by inducing cerebellar abnormalities (386).

Cerebellum Involved in Motor Coordination

Though cerebellar abnormalities have been observed in ASD for over a quarter century (335-337), they were initially discounted because of the cerebellum's known role in motor-related functions (359, 387). The cerebellum receives input from sensory systems to fine tune motor activity (359). Therefore, following cerebellar lesions, patients are still able to generate motor activity, but the timing and coordination of their movements are severely altered (388, 389).

From the first reports by Kanner, autistic patients have been noted to have awkward motility and clumsiness (390). More recent studies have reported some ASD patients to have classical cerebellar abnormalities such as coordination of movements, posture, balance, and motor dexterity (150, 391-393). In fact, the motor impairments are associated with the severity of the autistic behavior (391). These studies suggest that cerebellar abnormalities can be linked with ASD.

Cerebellum Involved in Non-motor Cognitive Functions

While the role of the cerebellum in motor coordination is the most recognized, it controls a vast number of functions (340, 394-405). The early reports of the cerebellum on non-motor function were largely anecdotal and were consequently dismissed (401). However a complete neurological study was conducted on 20 patients with isolated cerebellar lesions (401). While motor abnormalities were definitely observed, this group also observed a wider array of defects involving cognitive functions. Some of these deficits included: abnormalities in spatial cognition, impaired executive functions, personality changes (including inappropriate and disinhibited behaviors), and language deficits. Therefore, to describe these observations, they coined the term: "cerebellar cognitive affective syndrome" (401).

Further studies have corroborated these findings suggesting a role of the cerebellum in emotion, cognitive behaviors, language, and social functions (340, 395-407). A PET study was performed on normal individuals given a theory of mind task. The largest area of activation during the task was in the cerebellum (408). These studies provide validation that the cerebellum could play a larger role in ASD.

There are also some characteristics of speech seen in autistic patients that can be linked to the cerebellum including: phrasing, stress, rate, pitch, resonance, and loudness (409). Additionally, the cerebellum may play a role in sensory processing, deficits often noted in patients with ASD (410). In a seminal study, the cerebellar vermis of cats was stimulated eliciting a hypersensitivity to sound and touch (411). Further studies have suggested that the cerebellum likely processes incoming sensory information modulating both motor and non-motor functions to effect relevant behaviors (412-416).

How the cerebellum is able to process higher order cognitive functions is still up for debate. One proposed theory is that: analogous to its role in sensory processing for motor coordination (feedforward mechanism), the cerebellum processes sensory information for cognitive functions (417, 418). Because damage to the cerebellum does not abolish movement, but causes it to be uncoordinated, cerebellar abnormalities would cause “uncoordinated” cognitive functions (419).

Integration of Brain Regions in ASD

Components of the brain, however, do not operate in a vacuum and neither does the cerebellum stand alone in regulation of complex behaviors. The cerebellum forms multiple connections with other parts of the brain including the cerebral cortex. A few of these connections are mentioned in the following section.

Studies have shown that the cerebellum has connections with the limbic system (420-423), an important observation since alterations with the amygdala are associated with ASD (314, 315, 324, 325). There are also connections between the cerebellum and hypothalamus (424). Additionally, the cerebellum projects to the parietal cortex (425, 426), one of the supposed locations for mirror neurons. It is hypothesized that in order to decode another person’s actions (speech and social behavior), sub-threshold activation of your own mirror actions is required (427, 428). This hypothesis can thus integrate the mirror neuron theory with the motor functions of the cerebellum.

Moreover, the cerebellum seems to play a role in inhibiting the frontal lobe in order to delay or abolish certain behavioral responses (429). Functional MRI studies have detected alterations of the medial prefrontal cortex (mPFC) in patients with ASD (331). PET studies have also shown reduced dopamine levels in the mPFC in patients with

ASD (430). However, interestingly the degree of abnormality in the frontal cortex is correlated with the severity of the abnormality in the cerebellum (431).

The output of the cerebellum is the deep cerebellar nuclei: composed of the dentate, interpositus, and fastigial nuclei (359). Stimulation of the dentate nuclei elicits dopamine release to the medial prefrontal cortex (mPFC) and this could lead to autistic behavior (432). Other studies have shown connectivity between the cerebellum and prefrontal cortex (401, 425, 426, 433), and that these connections are reduced in patients with ASD (434). The complex circuitry was worked out by Rogers et al. who showed that mPFC dopamine release was mediated by two distinct circuits both originating in the cerebellum and ending in mPFC (432).

The first circuit incorporates originates with the dentate nuclei, travels to the tegmental pontine reticular nucleus, progresses to the pedunculopontine nucleus, moves to the ventral tegmental area, and finally concludes in the mPFC (435-437). The second circuit also originates in the dentate nuclei, but then traverses to the dorsomedial and ventral lateral thalamic nuclei, and then terminates in the mPFC (438, 439).

The role of the thalamic nuclei in this proposed circuit is noteworthy because MRI studies on patients with ASD show reduced thalamic volume (440-442) and other studies indicate decreased thalamic volume can be associated with repetitive behaviors (443). A diffusion tensor imaging study also showed decreased connectivity between the thalamus and frontal cortex in patients with ASD (444).

Loss of Purkinje cells, as has been associated with ASD, would lead to decreased inhibition on the deep cerebellar nuclei, thus leading to abnormally strong connectivity in the cerebello-thalamo-cortical circuit. The increased cortical excitation may lead to altered patterning which could explain abnormal motor function (445), altered face processing (315), and frontal lobe overgrowth (431). Indeed the dopamine response in mPFC following dentate nucleus stimulation is completely dependent on functional Purkinje cells (446). An image representing the cerebellum to mPFC circuitry is shown in Figure 3.2.

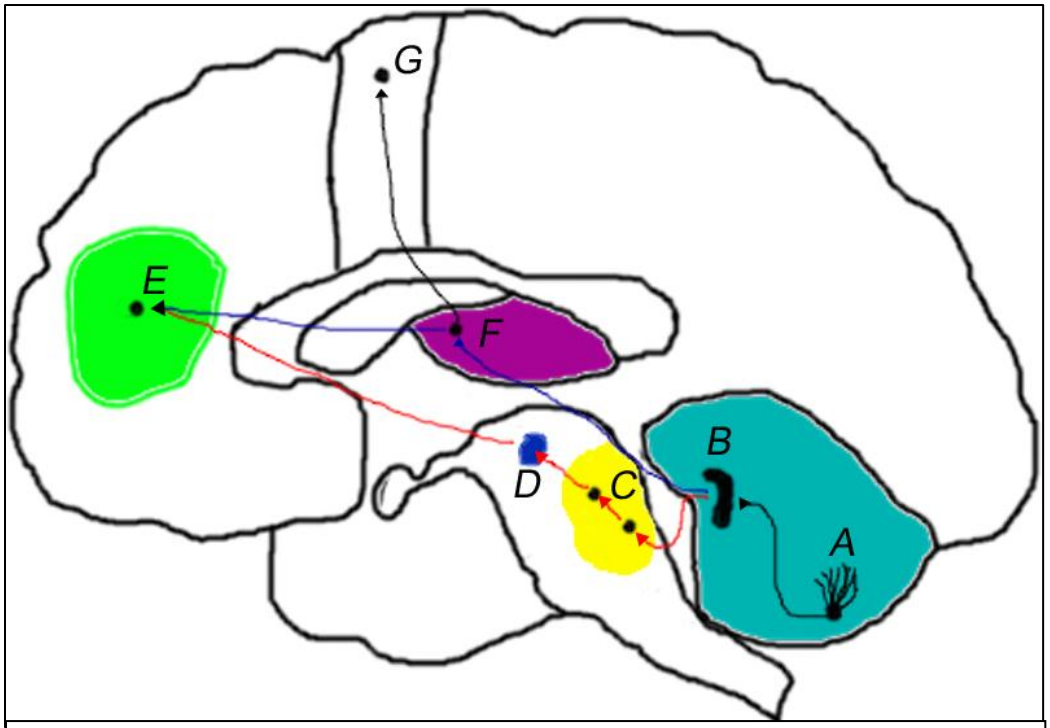

Figure 3.2: Proposed pathway from the Purkinje cell (A) to the deep cerebellar nuclei (B) two one of 2 circuits. 1.) tegmental pontine reticular nucleus to the peduculopontine nucleus (C) to ventral tegmental area (D) to medial prefrontal cortex (E). 2.) Dentate nuclei (B) to the thalamus (F) to medial prefrontal cortex (E). Thalamus (F) also relays projections to premotor cortex (G).

Indeed the cerebellum has been shown to play a pivotal role in the mechanism of ASD. As mentioned previously, TSC is an ideal syndrome to study the genetics and cellular mechanisms of ASD. Therefore, it is crucial to point out that approximately 30% of patients with TSC have cerebellar abnormalities (14, 15). In addition, studies have shown that the severity of autistic behavior in patients with TSC is associated with the severity of cerebellar lesions (14, 447). Furthermore, PET studies have shown increased activation of the deep cerebellar nuclei in patients with TSC associated ASD (15). Since the deep cerebellar nuclei are inhibited by Purkinje cells, this finding could be consistent with loss of Purkinje cells. In fact, loss of Purkinje cells has been observed in a patient with TSC (448). Therefore, we hypothesize that cerebellar lesions in patients with TSC are one possible link for ASD-associated behaviors.

Chapter Four:

Characterization of Tsc2f/f;Cre mice

Introduction:

Since cerebellar lesions in TSC correlated with severity of ASD (14, 447), it is remarkable to note that in addition to Purkinje cell loss in patients with ASD (360-366), a recent paper also demonstrated Purkinje cell loss in a 32-year old man with TSC (448). Since PET studies in patients with TSC-associated ASD show increased activation of the deep cerebellar nuclei (15), this could be a result of a loss of the inhibitory inputs of the Purkinje cells. Moreover, in situ studies have shown that Purkinje cells express high levels of tuberin, suggesting an important function of the *TSC2* gene in Purkinje cell function (449). Therefore, we hypothesized that loss of TSC can predispose patients to Purkinje cell loss and that this can lead to autistic behaviors.

In order to investigate this hypothesis, we generated a mouse model with Purkinje cell specific deletion of *Tsc2* (2). To create this, a Purkinje cell specific Cre mouse (Pcp2-Cre), which expresses Cre recombinase almost exclusively in Purkinje cells and in retinal bipolar cells was used (Figure 4.1) (4). *Tsc2*$^{flox/ko}$*;Pcp2-Cre* mice were generated, mimicking human TSC patients with one germline mutation in *TSC2*. Loss of heterozygosity, or the "second hit", was achieved when Cre recombinase, driven by the Purkinje cell promoter (PCP), began expressing at postnatal day 6 (P6) in Purkinje cells.

When I joined the laboratory for my doctoral studies, the Gambello lab had already created the *Tsc2*$^{flox/ko}$*;Pcp2-Cre* mice. Therefore, my role was to characterize this mouse on a molecular and behavioral level to establish its usefulness as a TSC-associated ASD model. In the following chapter, I will focus on the histological findings of these mice and discuss how this mouse adds to our knowledge of TSC.

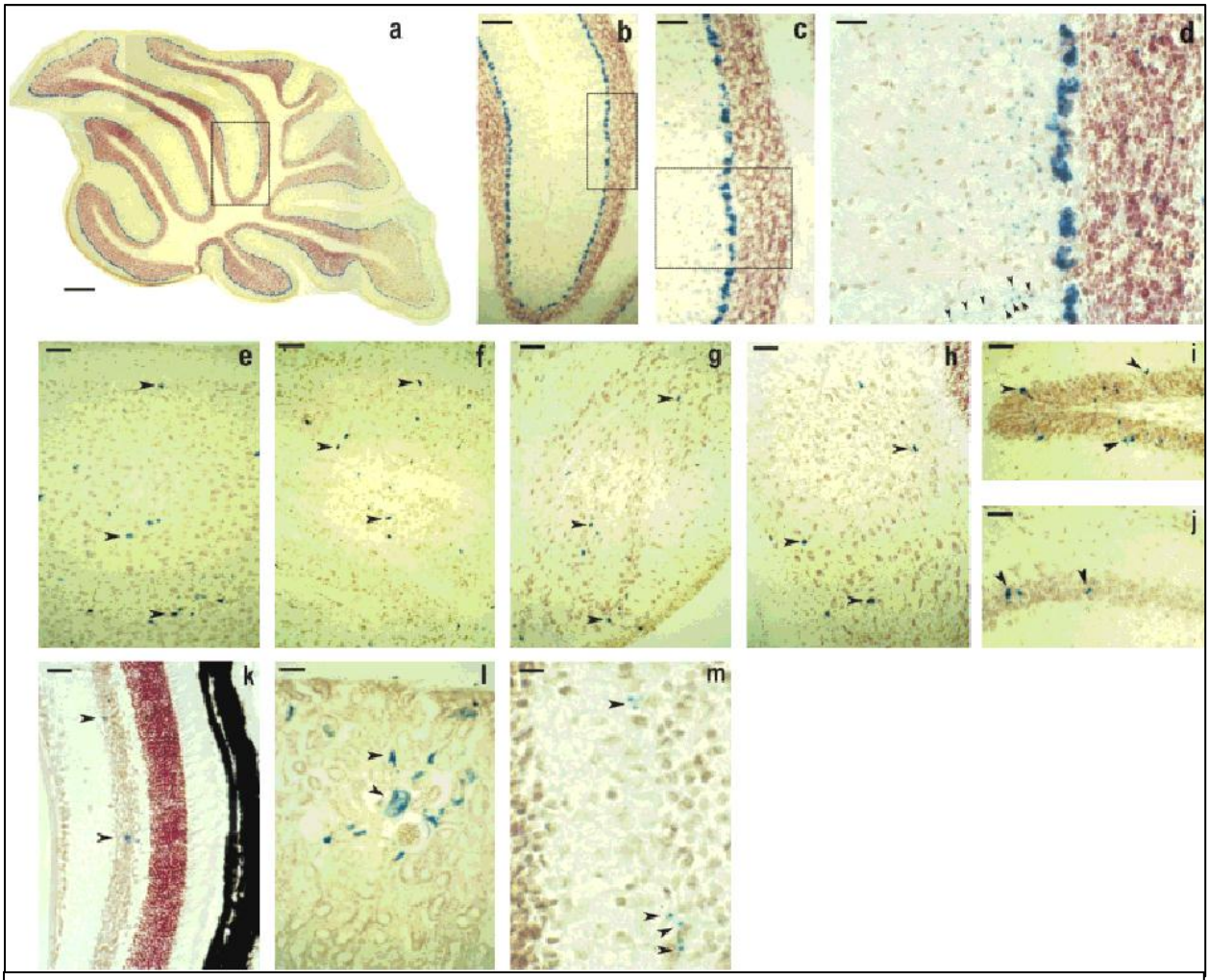

***Figure 4.1**. X-gal staining of adult (**a**)-(**l**) and early postnatal (**m**) *L7Cre-2+;GtROSA26/+* mice. (**a**) Midsagittal section of the cerebellum, bar=5mm. (**b**)-(**d**) Higher magnification of the framed areas. (**b**) bar=100µm (**c**) bar=50µm (**d**) bar=25µm, staining product granules in the molecular layer of the cerebellar cortex are indicated by arrowheads. (**e**)-(**l**) β-galactosidase-positive cells (arrowheads) in : (**e**) parietal cortex, (**f**) inferior olive, (**h**) deep cerebellar nuclei, (**i**) dentate gyrus, (**j**) CA1 area of the hippocampus, (**k**), retina, (**l**) kidney, (**m**) stained Purkinje cells at postnatal day 6. (**e-j, l**) bar=50µm, (**k**) bar=25µm, (**m**) bar=10µm.

*Figure and legend taken from (4). Permission to reproduce this figure in this dissertation has been received from John Wiley and Sons to Rachel Michelle Reith, license number 2898400514385.

Materials and Methods:

Mouse Model

All of the animal experimentation was approved by the UTHSC Animal Welfare Committee. Mice were on a combined C57BL/6J and 129 background. Generation of the $Tsc2^{+/flox}$ and $Tsc2^{+/ko}$ have been previously described (450). The expression of Cre recombinase was controlled by the Purkinje cell protein (PCP-2) specific promoter as previously described (4). Mice were genotyped for the expression of either the *Tsc2*^{flox} and *Tsc2*^{ko} alleles using these primers in a PCR reaction: KO1, 5'-GCAGCAGGTCTGCAGTGAAT-3'; KO2, 5'CCTCCTGCATGGAGTTGAGT-3'; WT2, 5'CAGGCATGTCTGGAGTCTTG-3'. Product sizes were: *Tsc2*^{ko} (547bp), *Tsc2*^{flox} (434bp), and WT (390bp). Cre primers were: RapA, 5'-AGGACTGGGTGGCTTCCAACTCCCAGACAC-3'; RapB, 5'AGCTTCTCATTGCTGCGCGCCAGGTTCAGG-3'; CreF, 5'GGACATGTTCAGGGATCTCCAGGC-3'; CreR, 5'CGACGATGAAGCAGGGATCTCAGGGC-3'. Product sizes were: Rap (590bp) as a positive control band and Cre (219bp).

Histology

Mice were first anesthetized with 2.5% Avertin and then transcardially perfused with PBS and then 4% paraformaldehyde (PFA). Brains and eyes were extracted, post fixed overnight in 4% PFA, stored in 70% EtOH, dehydrated, embedded in paraffin cassettes, and sectioned at 5µm. Slides were rehydrated and either processed for hematoxylin and eosin (H&E) staining or immunohistochemistry (IHC). For IHC, sections were microwaved in 10mM sodium citrate buffer, pH6.0 for antigen retrieval. Sections were then blocked with 10% goat serum, 0.5% Triton X-100, and 1xPBS for 20 min and then incubated in solution of primary antibody overnight at 4^{o}C. Slides were washed in 1xPBS and secondary antibody was applied 1hour at room temperature. For fluorescence, slides were washed in 1xPBS and incubated with 1:1000 Hoechst 33258 (Invitrogen, Carlsbad, CA) for 10 min and then coverslipped using Fluoromount-G (SouthernBiotech, Birmingham, AL). For DAB staining, sections were incubated in 0.3% hydrogen peroxide in methanol for 20 min before adding the primary antibody. Secondary antibody was biotinylated and then slides were incubated with Vectastain ABC working reagent (Vector Laboratories, Burlingame, CA). For visualization, DAB

with or without metal enhancer was used (Sigma-Aldrich, St. Louis, MO). TUNEL staining was performed using the in-situ cell death detection system (Roche, Indianapolis, IN) overnight at 37^{o}C. Slides were then processed for IHC as above. Imaging was performed with an Olympus IX81 microscope through a Qimaging RETIGA-200RV camera and processed with Adobe Photoshop (San Jose, CA). Confocal images were obtained using TCS SP5 confocal laser microscope (Leica, Wetzler, Germany).

The primary antibodies for IHC were: Calbinidn (1:250; Abcam, Cambridge, MA), Calbindin (1:250; Sigma-Aldrich, St. Louis, MO), Cleaved Caspase-3 (CC3) (1:200; Cell Signaling, Bedford, MA), Cone Arrestin (1:200; Connie Cepko, Harvard Medical School, Boston, MA), GS (1:300; BD Biosciences, Franklin Lakes, NJ), IP3R (1:100; Millipore, Billerica, MA), Nitrotyrosine (1:500; Millipore, Billerica, MA), Pax6 (1:200; Covance, Emery Ville, CA), Phospho S6 (Ser 240/244) (1:100; Cell Signaling, Bedford, MA), PKCa (1:500; Millipore, Billerica, MA), R4D2 (1:200; Molday, 1983), and Superoxide dismutase (SOD) (1:250; Abcam, Cambridge, MA).

Secondary antibodies (1:250, Invitrogen, Carlsbad, CA) were: Alexa Fluor 488 (anti-rabbit) (anti-mouse igG_1), Alexa Fluor 594 (anti-rabbit) (anti-mouse IgG_1), Alexa Fluor 555 (anti-rabbit) (anti-mouse IgG_1) (anti-mouse IgG_{2a}).

In Situ Analysis

A Tsc2 BAC clone (ATCC 9895683) was used to make RNA probes. Using PCR, exons 2-4 were amplified and then ligated into a pGEM-T Easy Vector (Promega, Madison, WI) using manufacturer's instructions. T7 RNA polymerase and digoxigenin labeling mix (Roche) were used to synthesize sense and antisense RNA probes. Mice were injected with 2.5% avertin and then transcardially perfused with PBS and then 4% PFA. Brains were removed and post-fixed in 4% PFA overnight, then washed in PBS and transferred into 30% sucrose. Brains were then embedded into OCT and sliced on a cryostat at 14µm and stored at -20^{o}C until ready to be processed for in situ. For the in situ hybridization, slides were washed with 1x PBST (PBS with 0.1% Tween-20) and underwent an acetylation step (acetic anhydride and triethanolamine) for ten minutes. Slides were washed with 1x PBST and incubated with RNA probe overnight at 65^{o}C. Slides were then washed in saline-sodium citrate (SSC) buffer, incubated with 50% formamide for 30 min at 65^{o}C, treated with RNase A (20ug/ml, Roche), and washed with successively decreasing concentrations of SSC at 65^{o}C. Slides were rinsed in MABT

(maleic acid buffer with Tween-20) and blocked with 20% HISS/MABT for one hour. Slides were then incubated with AP-conjugated anti-digoxigenin Fab fragment (1:2000, Roche) overnight at 4^{o}C. Slides were then rinsed in MABT followed by NTM (sodium chloride, Tris, and magnesium chloride). Slides were developed with NBT and BCIP (1:200 in NTM) for six hours in the dark. Slides were rinsed in NTM followed by PBS then fixed in 4% PFA and coverslipped with Cytoseal 60 (Richard-Allan).

Quantitative Analysis

Two to three mice (each with three to four sections) were used for quantitation. Cerebellum was sliced sagittally along the midline. From there, 5µm sections were sliced. Purkinje cell numbers were determined from folium II, IX, and X. The perimeter of Purkinje cell layer was measured using ImageJ v1.38 x (Rashband, National Institutes of Health, Bethesda, MD). Purkinje cell density is therefore reported as Purkinje cell number per distance (mm). Cell size was determined by measuring area of consecutive neurons in folium II using ImageJ v1.38 x (Rashband, National Institutes of Health, Bethesda, MD) and then averaging those numbers.

Motor function analysis

Motor coordination was examined by determining latency to fall (180s cap) using an accelerating (4-40 rpm over 200s) ENV-576M RotaRod (Med Associates, Georgia, VT). Mice were given two trials on the same day with approximately two hours rest between trials. The mean of the two trials was used in the analysis.

Gait was analyzed by using inkblot testing. Non-toxic ink was used on the fore (red) and hind (black) paw of the mouse. The mouse was permitted to walk down a tunnel. Gait width (cm) was measured from the left step to the adjoining right step.

Statistical Analysis

When appropriate, statistical analysis was done using a two-tailed Student's t-test. Statistical significance was denoted at an alpha of 0.05. Graphs are represented with standard error of the mean.

Human Tissue

Human cerebellum was obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD. The right side of the cerebellum was fixed in 10% formalin and sectioned in 0.5cm intervals radiating out from the midline by the NICHD Brain and Tissue Bank. The second section was ordered for all cerebellum sections to keep anatomical location consistent. The matched folia were embedded in paraffin and processed as above. Some descriptions of patients were obtained from NICHD Brain and Tissue Bank. TSC patient 1' was a 56-year-old female. In addition to TSC, she had diffused interstitial lung disease. Her list of medications was: albuterol, calcium, hydrochlorothiazide, inhaled tiotropium, ipratropium, moxifloxacin, prednisone, valsartan, and vitamin D. TSC patient 2' was a 31-year-old female. As a part of TSC pathology, she had lung and kidney involvement. She had epilepsy since 9 months of age which was initially treated with phenobarbital, which only increased seizure frequency. Consequently, she was treated with phenytoin and diazepam for many years. Several years before her death, she was treated with carbamazepine and valproic acid. TSC patient 3' was a 47-year-old female with concurrent mental retardation. She also had seizures which were treated with phenytoin and valproic acid. As a part of her TSC pathology, she had multiple bilateral kidney cysts and angiomyolipomas as well as masses in her lungs. In addition to TSC, she also had chest pains, chronic renal insufficiency, hypothyroidism, pyelonephritis (which was treated with IV antibiotics), and recurrent pleural effusions and chylothorax. TSC patient 4' was a 58-year-old male who had no additional available information.

Results:

TSC patients show Purkinje cell loss

Whether Purkinje cell loss is a common pathologic feature of TSC is unclear. There has only been one report of TSC associated Purkinje cell loss in the literature (448). To assess if loss of Purkinje cells is a more common feature of TSC, cerebellum samples from four TSC patients and age matched controls were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland. Purkinje cell counts were determined following H&E staining of the sections (Figure 4.2a-c). Two of the four samples were noted to have reduced Purkinje cell densities than their aged-matched controls. mTORC1 activity was also determined using phosphorylated

ribosomal protein S6 (pS6). Two of the four samples were noted to have increased pS6 levels compared to their aged-matched controls (Figure 4.2d-e).

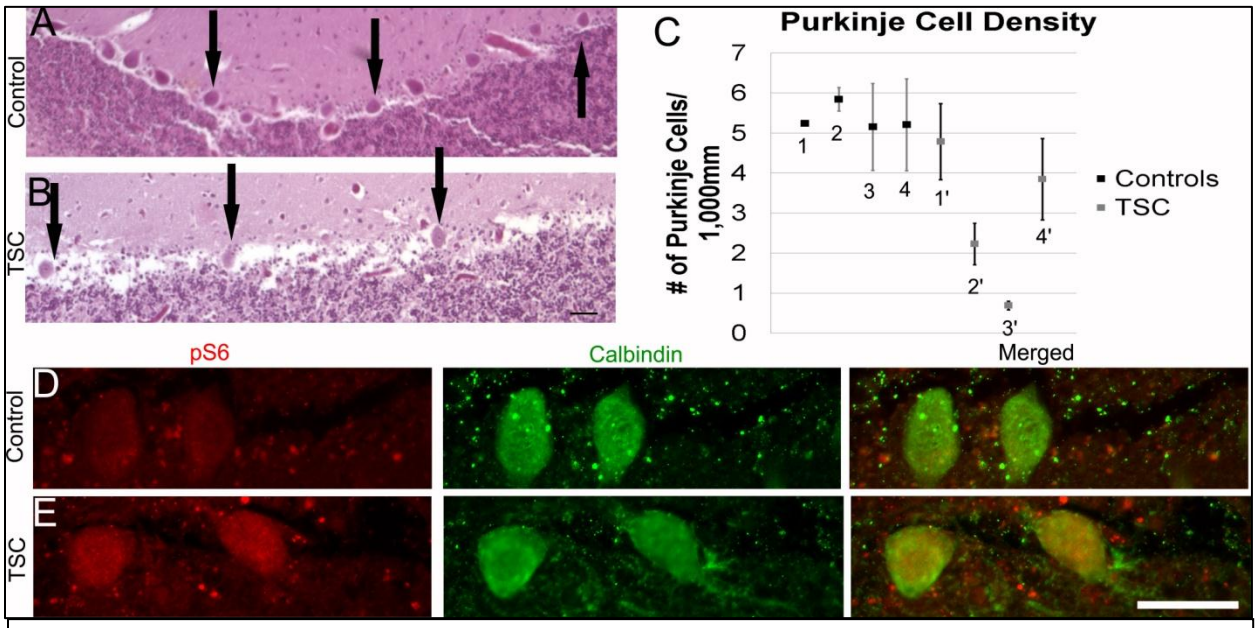

***Figure 4.2:** Purkinje cell pathology in TSC patients. (A-B) Human cerebellum sections were H&E stained. Representative TSC patient (2') shown in (B) compared to aged matched control patient (A). Black arrows indicate Purkinje cells. Scale bar, 200µm. (C) Lower density of Purkinje cells in some of the TSC patients compared to age-matched controls. (D-E) pS6 staining of human cerebellum sections showing increased pS6 in TSC patient 1' (E) compared to age matched control (D). Scale bar, 50 µm.

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Loss of Tsc2 in the Purkinje cells in mice causes progressive cell death

In *Tsc2$^{flox/ko}$;Pcp2-Cre* (Tsc2f/-;Cre) mice, PCP drives Cre expression beginning at postnatal day 6 (P6) and Cre expression is fully established after 1-2 weeks (4). Tsc2f/-;Cre mice were observed in the normal Mendelian ratio, though approximately 25% died before one month of age. While the cause of death is not known, some mice were observed to have seizures. Surviving mice, however, were healthy and fertile. To confirm successful *Tsc2* deletion, an in situ was performed at six weeks revealing complete loss of the *Tsc2* message in Tsc2f/-;Cre mice (Figure 4.3a-b). Purkinje cells showed increased mTORC1 activity as detected through pS6 staining (Figure 4.3c-d). Tsc2f/-;Cre Purkinje cells were also larger than control Purkinje cells (Figure 4.3e-f,k). Notably, Purkinje cell loss was detected at one month of age (Figure 4.3g-h) and loss progressed with age (Figure 4.3h-j). Folium X was initially spared from degeneration, but by seven months of age, it too had significant Purkinje cell loss (Figure 4.3j).

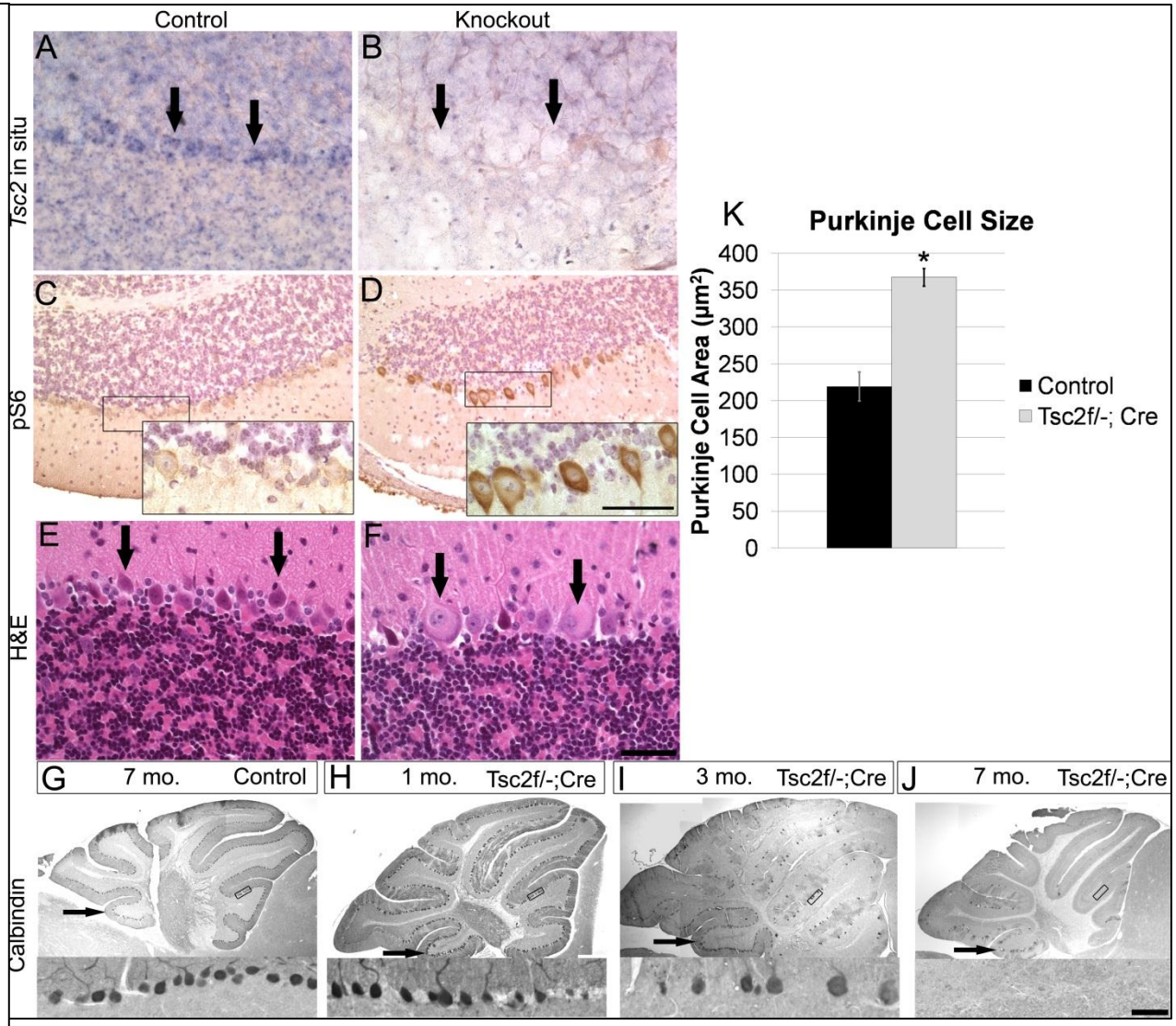

***Figure 4.3**. Analysis of Purkinje cells in Tsc2f/-;Cre and Tsc2f/f mice. (A-B) In situ hybridization showing deletion of Tsc2 mRNA in a 1.5-month-old Tsc2f/f;Cre cerebellum (B) compared to control (A). (C-D) Immunohistochemistry shows increased phosphor-S6 (pS6) indicative of mTORC1 activation in a 1-month old Tsc2f/-;Cre animal (D) compared to control (C). (E-F) H&E staining shows large dysplastic Purkinje cells of a 1 month Tsc2f/-;Cre animal (F) compared to control (E). (G-J) Calbindin immunohistochemistry shows progressive Purkinje cell loss in Tsc2f/-;Cre animals at different ages: (H) 1 month, (I) 3 months, and (J) 7 months of age. Arrow indicates folium X that was somewhat resistant to Purkinje cell loss. (K) Comparison of cell area shows larger cells in 3-month-old Tsc2f/-;Cre (n=3) compared with controls (n=3; *p=0.01). Scale bars, 50µm.

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Tsc2-mediated Purkinje cell loss is largely cell-type-specific

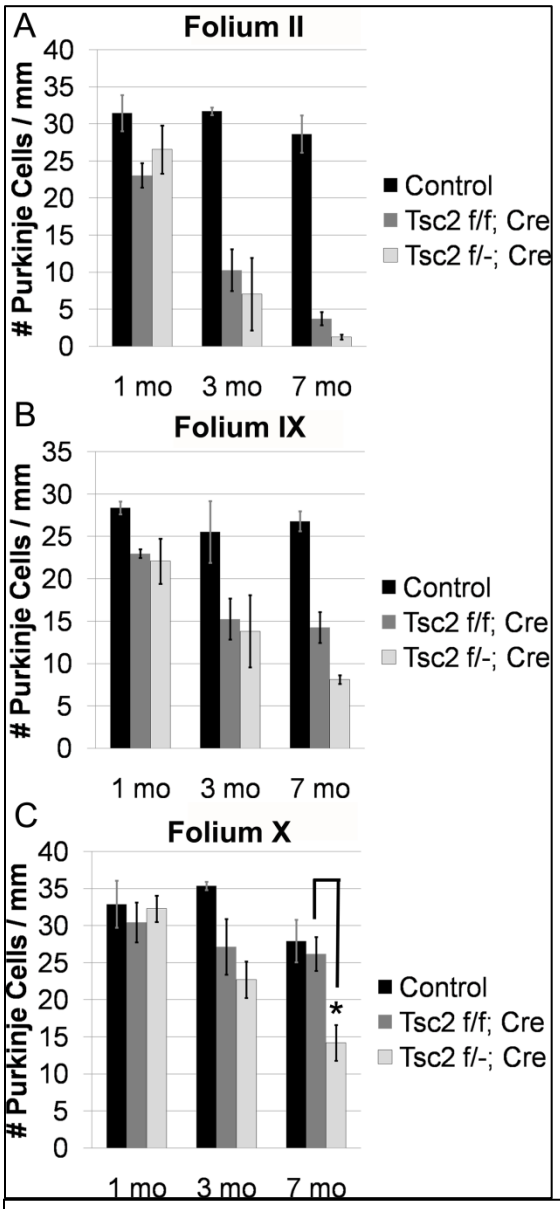

*Figure 4.4. Comparison of age-dependent Purkinje cell loss between Tsc2f/-;Cre and Tsc2f/f;Cre mice. (A-C) Quantitation of Purkinje cells (Purkinje cells/mm) among control (black), Tsc2f/-;Cre (light gray), and Tsc2f/f;Cre (dark gray) mice in folium (A) II, (B) IX, and (C) X. Only in folium X at 7 months of age (C) was there a statistical difference (*p<0.05 in the number of remaining Purkinje cells between Tsc2f/-;Cre and Tsc2f/f;Cre mice.

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Though the Tsc2f/-;Cre mice are a good genetic replication of TSC patients (one inactivated germline *Tsc2* allele and somatic loss of the floxed allele in Purkinje cells), this scheme makes it complicated to determine exact cause of Purkinje cell death. Either the genotype of the Purkinje cell ($Tsc2^{-/-}$) or the haploinsufficient effect of neighboring input cells ($Tsc2^{f/-}$) could contribute to cell death. Therefore, to determine if Purkinje cell loss was cell-type specific, only mediated by loss of *Tsc2* in Purkinje cells, *$Tsc2^{flox/flox}$;Pcp2-Cre* (Tsc2f/f;Cre) mice were generated. Tsc2f/f;Cre mice were observed in the normal mendelian ratio, and were healthy and fertile with 100% survival to one month of age. However, the rate of Purkinje cell loss was almost identical to that of Tsc2f/-;Cre mice (Figure 4.4). This suggests that Purkinje cell loss is not due to haploinsufficiency, but mainly due to loss of *Tsc2* specifically in Purkinje cells. However, by 7 months of age, Tsc2f/-;Cre mice had greater Purkinje cell loss than Tsc2f/f;Cre mice (Figure 4.4), suggesting that a haploinsufficient

environment can mitigate the progression of Purkinje cell death at later time points. However, as most analysis was conducted before three months of age, Tsc2f/f;Cre mice were used for further studies to isolate mechanisms of Purkinje cell death.

Tsc2f/f;Cre mice have normal retinal phenotype

Since Pcp2-Cre expression also occurs in retinal bipolar cells (4), retinal cell type-specific staining was performed at five months of age. No differences were detected in Tsc2f/f;Cre mice across the seven cell types of the retina including the bipolar cells (Figure 4.5).

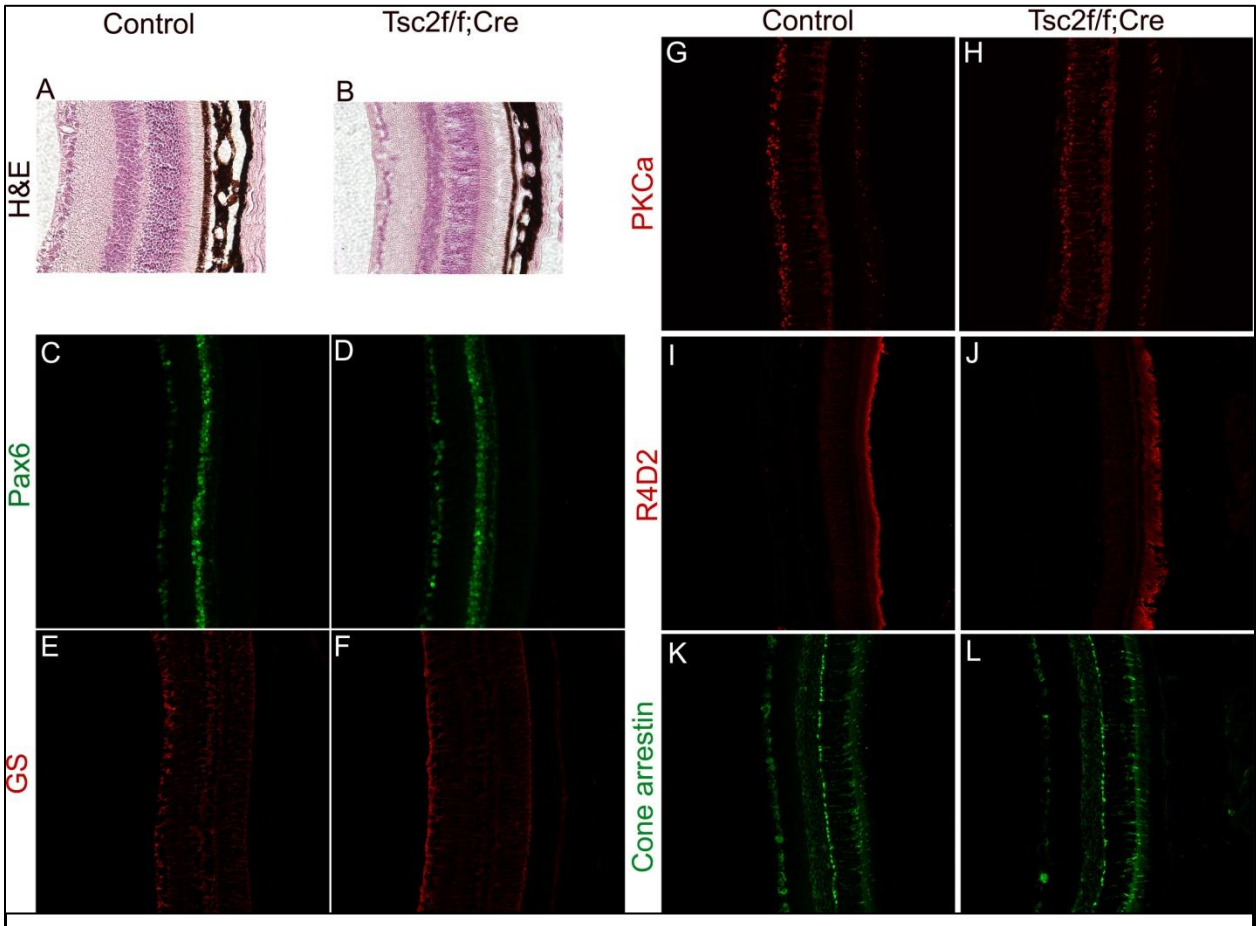

***Figure 4.5.** Layer specific staining of Tsc2f/f;Cre retina at 5 months of age. (A-B) H&E staining of control (A) and Tsc2f/f;Cre retina (B). (C-D) Pax6 staining for amacrine, ganglion, and horizontal cells in the control (C) and Tsc2f/f;Cre (D). (E-F) GS staining for muller glia cells in the control (E) and Tsc2f/f;Cre (F). (G-H) PKCa for rod bipolar cells in the control (G) and Tsc2f/f;Cre (H). (I-J) R4D2 staining for rod photoreceptor cells in the control (I) and Tsc2f/f;Cre (J). (K-L) Cone arrestin staining for cone cells in the control (K) and Tsc2f/f;Cre (L).

*Samples were processed and stained by Seo-Hee Cho, Ph.D.

Tsc2f/f;Cre mice display ataxia

To determine if *Tsc2*-mediated Purkinje cell loss resulted in motor deficits, the gait of three-month-old mice was analyzed. The fore and hind paws of each mouse were inked before placing the mouse into a narrow tunnel (Figure 4.6a-b). Tsc2f/f;Cre mice took significantly wider (p=0.05) steps than control mice indicating ataxia (Figure 4.6c). Furthermore, RotaRod testing revealed that Tsc2f/f;Cre mice tended to have a decreased latency to fall compared to controls (p=0.066)(Figure 4.6d). Taken together, these data demonstrate mild ataxia in Tsc2f/f;Cre mice.

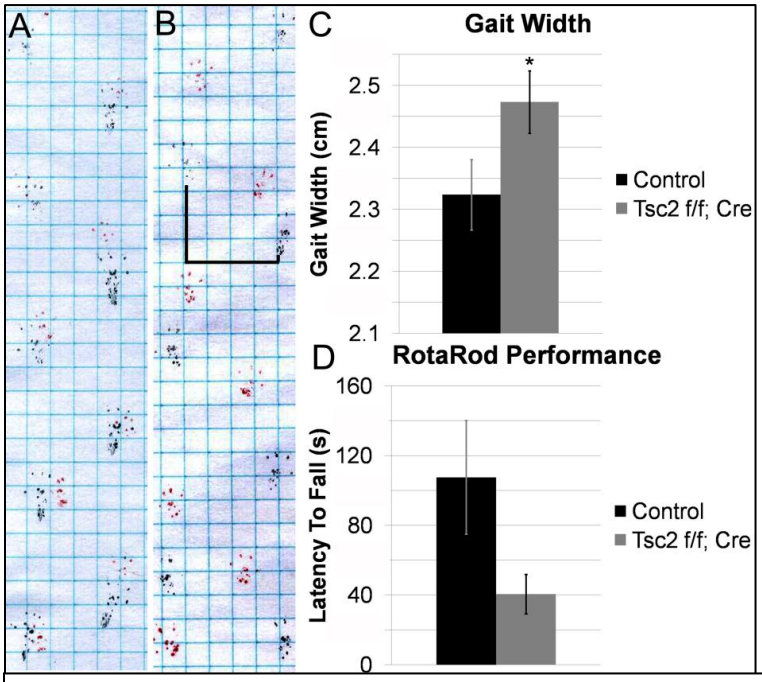

***Figure 4.6.** Abnormal motor function in Tsc2f/f;Cre mice. (A-C) Abnormal gait as indicated by the inked paw print analysis. Representative images are shown for three month control (A) and Tsc2f/f;Cre (B) mice. Tsc2f/f;Cre mice (n=29) had a significantly wider gain (*p=0.05) than control mice (n=21) (C). Representative gait width measurement in shown in (B). (D) RotaRod analysis showed that Tsc2f/f;Cre mice (n=6) tended to fall off the RotaRod sooner than controls (n=5; *p=0.066).

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Tsc2-null Purkinje cells are predisposed to apoptotic cell death

Apoptosis is the best characterized mechanism of cellular death and plays a large role in neurodegenerative diseases (451). A cell undergoing apoptosis is characterized by DNA fragmentation, nuclear pyknosis, membrane blebbing, rupture of the nuclear membrane, cell shrinkage, and ultimate removal by phagocytes (452). Therefore, in effort to characterize the type of cell death leading to Purkinje cell degeneration, we examined markers of apoptosis. Both cleaved caspase 3 (CC3) and TUNEL staining were increased in one-month-old Tsc2f/f;Cre mice compared to controls, indicative that Purkinje cells were undergoing apoptosis (Figure 4.7a-d).

By seven months of age, there are few surviving Purkinje cells (Figure 4.3j, Figure 4.4). Therefore, the cerebellar circuit is disrupted. In effort to understand the consequences to Purkinje cell degeneration, other cells in the cerebellar circuit were examined. CC3 positive cells were detected in both the granule cell layer and molecular cell layer of seven-month-old Tsc2f/f;Cre mice (Figure 4.7e). This suggests that granule and molecular cells degenerated after losing their cellular input (the Purkinje cell).

Furthermore, Purkinje cells project to the deep cerebellar nuclei (358). Therefore, we examined CC3 staining in the deep cerebellar nuclei after degeneration of Purkinje cells. We detected CC3 staining in eight month old Tsc2f/-;Cre mice (Figure 4.8).

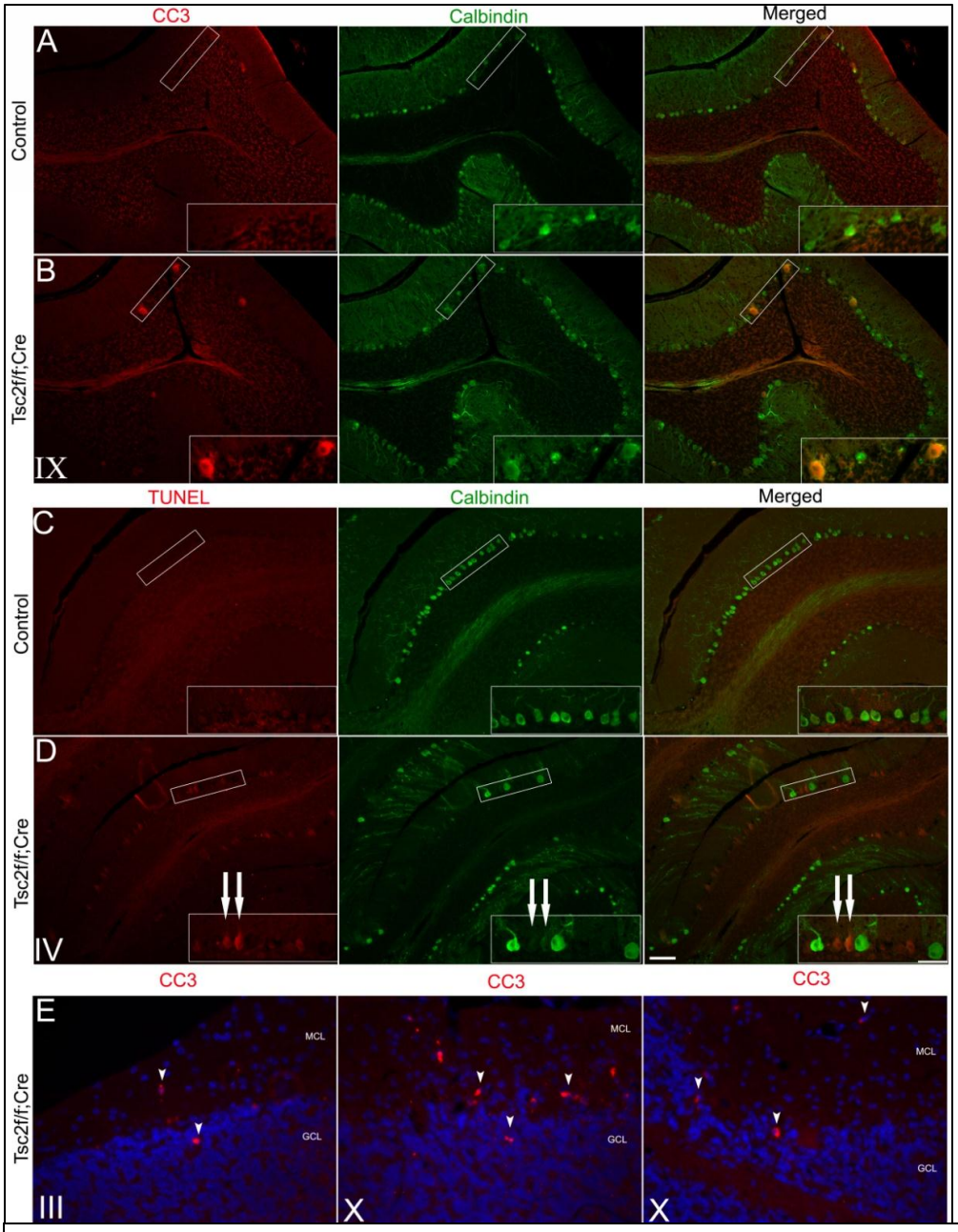

***Figure 4.7:** Apoptotic Purkinje cell death. (A-B) Immunohistochemistry for cleaved caspase 3 (CC3) shows increased apoptotic cell death in 1 month Tsc2f/f;Cre mice (B) compared to control (A). (C-D) TUNEL analysis confirms apoptotic cell death in 1 month Tsc2f/f;Cre mice (D) compared to control (C). Arrows show TUNEL positive Purkinje cells. Purkinje cells are strained green with calbindin antibody. (E) CC3 staining (indicated by arrow heads) shows subsequent target-related apoptotic cell death of molecular cells (MCL) and granule cells (GCL) in 7 month old Tsc2f/f;Cre mice. Folia are shown in Roman numerals. Scale bars, 50µm.

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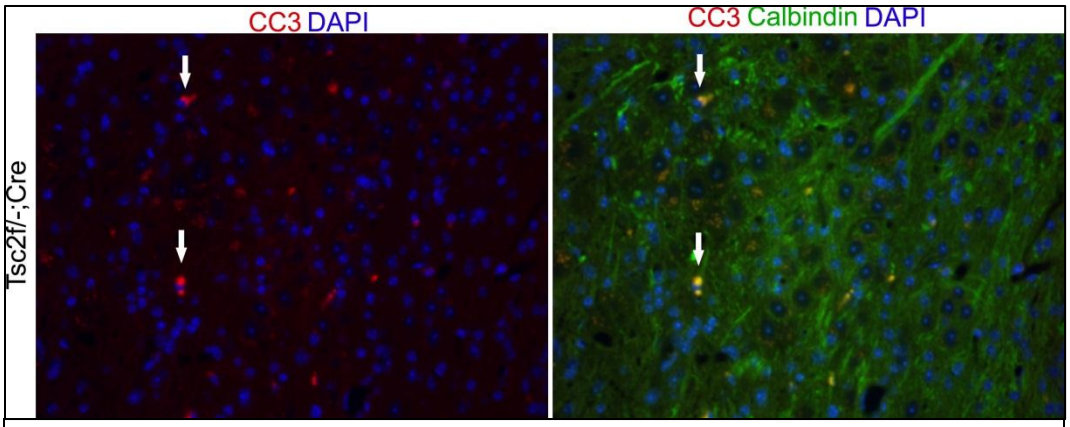

Figure 4.8. Apoptotic death in deep cerebellar nuclei. CC3 positive cells were detected in 8 month old Tsc2f/-;Cre mice. Slides were colabeled with calbindin. Note the white arrow heads indicating that the CC3 positive cells are also calbindin positive, suggesting apoptotic death of the deep cerebellar nuclei cells in contact with the Purkinje cell axon.

Tsc2 loss in Purkinje cells leads to increased calcium receptor expression and oxidative stress

Some of the cellular processes that can mediate apoptosis were examined in Tsc2f/f;Cre mice. Calcium signaling is a critical messenger system regulating a number of cellular events including: proliferation, cell division, development, learning and memory, behavior, neuronal signaling, and apoptosis (453). The inositol 1,4,5-trisphosphate receptor (IP_3R) mediates intracellular calcium release in response to extracellular signals (454). Expression of IP3R was examined in Tsc2f/f;Cre Purkinje cells and found to be upregulated, suggesting that calcium signaling may be altered following deletion of *Tsc2* (Figure 4.9a-b).

The intrinsic pathway of apoptosis originates in the mitochondria (455) which is also an important site for the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS)- both known to influence apoptosis (456). Reactive nitrogen species such as nitric oxide, lead to the production of pro-oxidant peroxynitrite ($ONOO^-$) (457). This oxidant is then able to induce a cascade of events in the cell including nitrating tyrosine residues (a readout for the presence of RNS in a cell) (458). Immunohistochemistry for nitrotyrosine showed significant increase in *Tsc2*-null Purkinje cells compared to control (Figure 4.9c-d). An indirect readout of ROS in a cell is the overexpression of ROS scavenging enzymes such as superoxide dismutase (SOD).

SOD is responsible for converting superoxide (O_2^-) into a less toxic hydrogen peroxide and molecular oxygen (459). Purkinje cells of Tsc2f/f;Cre mice also had upregulated SOD compared to controls (Figure 4.9e-f).

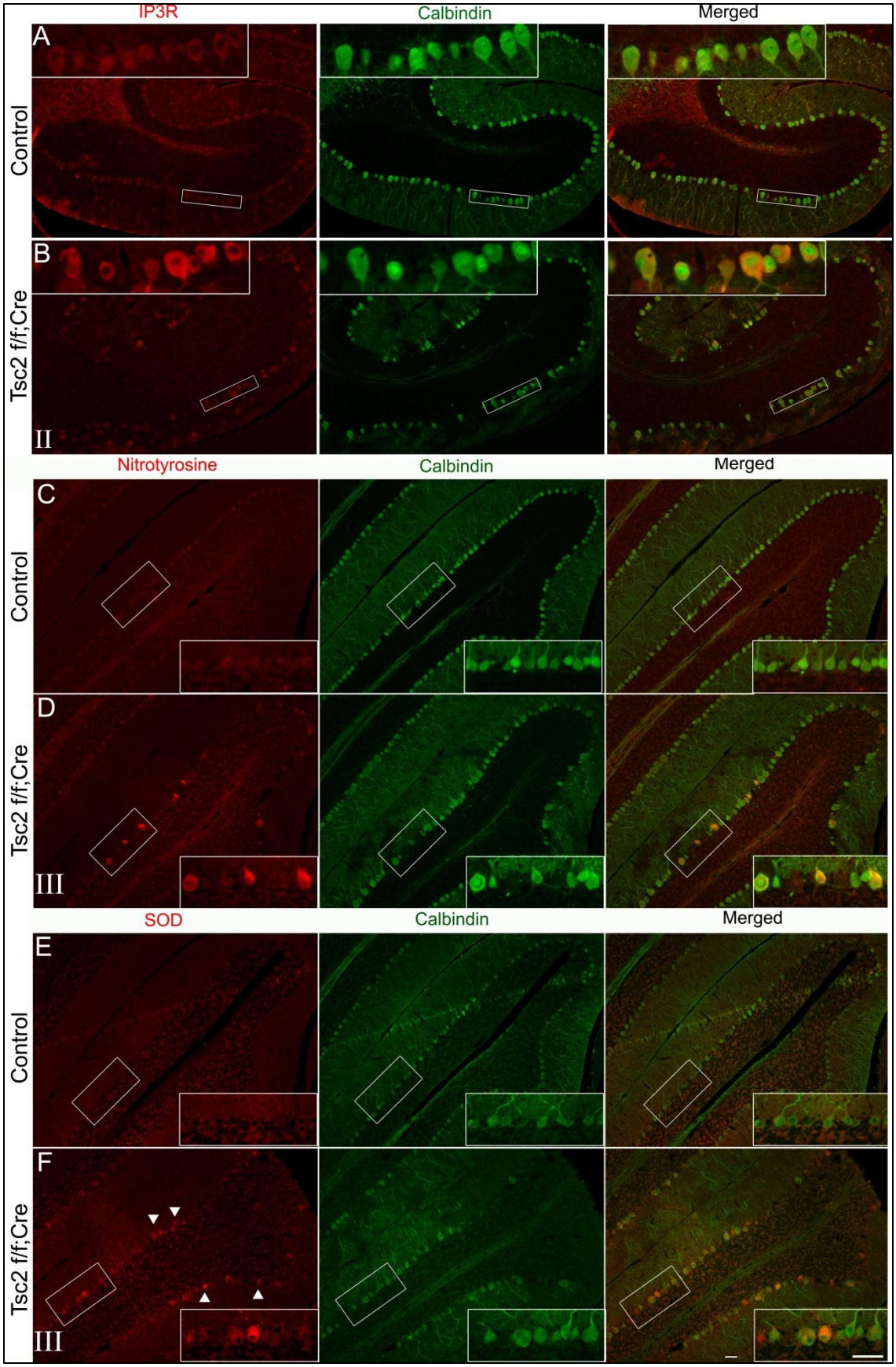
IP3R
Calbindin
Merged
A
Control
B
Tsc2 f/f;Cre
II
Nitrotyrosine
Calbindin
Merged
C
Control
D
Tsc2 f/f;Cre
III
SOD
Calbindin
Merged
E
Control
F
Tsc2 f/f;Cre
III

***Figure 4.9 (previous page).** Calcium signaling and oxidative stress are activated in the Purkinje cells of Tsc2f/f;Cre mice. (A-B) Increased inositol 1,4,5-triphosphate receptor (IP_3R) in 2 month Tsc2f/f;Cre mice (B) compared to control (A) indicate altered calcium signaling. (C-D) Increased expression of nitrotyrosine residues demonstrates elevated oxidative stress in 1 month Tsc2f/f;Cre mice (D) compared to controls (C). (E-F) Elevated oxidative stress is also demonstrated by increased expression of superoxide dismutase (SOD) in 1 month Tsc2f/f;Cre mice (F) compared to controls (E). Purkinje cells are co-labeled with calbindin. Folia are shown in Roman numerals. Scale bars, 50µm.

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Discussion:

Purkinje cell loss is a well-reported phenotype associated with ASD (360-366). I have examined the hypothesis that Purkinje cell loss may also be an important pathology in patients with TSC. In addition to the established report on the 32-year-old TSC patient with Purkinje cell loss, we have demonstrated Purkinje cell loss in two (out of four examined) additional patients with TSC. The predisposition to Purkinje cell loss is likely mediated by multiple influences.

Cerebellar atrophy and Purkinje cell loss have often been reported in patients with seizures (460). However, it is unknown if Purkinje cell loss is due to seizure activity itself or due to the drugs for the treatment of seizures (460). Three common seizure drugs have been shown to cause cerebellar abnormalities: phenytoin (461-463), phenobarbital (464, 465), and valproic acid (386). In the case of the two TSC patients with Purkinje cell loss, both had chronic seizures, and treatment with phenytoin and valproic acid. Patient 2' had also been treated with phenobarbital.

There are a couple of possibilities for the Purkinje cell loss observed in the two TSC patients. First, they could have sustained a "second hit" for a mutation in either *Tsc1* or *Tsc2* in Purkinje cells. To have achieved this in a large population of Purkinje cells, the second hit event would have most likely occurred early in development. The second, explanation is that haploinsufficiency in the Purkinje cells predisposed them to the

toxicity of further perturbations. It is known that Purkinje cells are highly sensitive to stresses (466).

Furthermore, two of the TSC patients had increased pS6, indicative of increased mTORC1. Increased pS6 is observed in patients with TSC even in the absence of a second hit (467). It is important to note, of the two patients with Purkinje cell loss, only one displayed increased pS6 (2'). It possible that the other patient with Purkinje cell loss (3') did not have elevated pS6, but a more likely explanation is that the quality of the tissue and the severe Purkinje cell loss made it difficult to detect immunostaining. Calbindin staining in this sample (3') was also very weak. Also, TSC patient (1') with increased pS6 did not have Purkinje cell loss. This patient was not reported to have seizures and therefore was not reported to have taken any of the anticonvulsant drugs known to be toxic to Purkinje cells. Though the sample size is small, this information, along with the mouse model leads me to hypothesize: patients with TSC have a genetic predisposition to Purkinje cell loss and are therefore sensitive to second hit events. Second hit events can be both genetic LOH events or as a result of an environmental perturbation. In the case of the two patients examine here, I hypothesize that there was either a "pharmacological second hit" or "environmental second hit" leading to Purkinje cell loss.

We also sought to examine the consequences of a genetic second hit in Purkinje cells in mice. Genetic loss of *Tsc2* led to Purkinje cell degeneration. As the rate of Purkinje cell loss was similar in both Tsc2f/-;Cre and Tsc2f/f;Cre mice, this shows that Purkinje cell loss occurred in a cell-type specific manner, not mediated by haploinsufficiency in other cerebellar cells. However, other cerebellar cells (including granule and molecular cells) were affected by loss of Purkinje cells. This is not surprising given the circuitry of the cerebellum since the Purkinje cells are the ultimate target of all other cells in the cerebellar cortex (359). Furthermore, target-related degradation of granule cells has been observed following Purkinje cell degeneration in Lurcher mice (468).

One difference, however, between the Tsc2f/-;Cre and Tsc2f/f;Cre mice was the premature death observed in approximately 25% of the Tsc2f/-;Cre mice. While the cause of this death is unknown, several of the mice were noted to have seizures shortly before death. Tsc2f/f;Cre mice, however, were not observed to have seizures or

premature death. Additionally, Tsc2f/- mice without Cre expression were not observed to have seizures or premature death. This suggests that seizures and premature death are likely mediated by haploinsufficiency of *Tsc2* in combination with Cre expression (either in Purkinje cells or the slight Cre expression in the cerebrum see Figure 4.1 (4)).

Purkinje cell loss in both Tsc2f/-;Cre and Tsc2f/f;Cre mice was folia dependent- with folium X demonstrating initial resistance. Furthermore, Purkinje cell loss did not occur all at one time, but was spread out over the course of months. The differential timing of Purkinje cell death may be due to the gradual expression of Cre between P6 and P21 (4). However, folia-dependent Purkinje cell loss in not a unique finding to this study, but has been observed in other Purkinje cell degeneration mutants such as pcd (469), sticky (470), and woozy (471) mice. The structure of the cerebellum is divided into different independent modules. Though these modules have a similar structure, they respond to different inputs and have different outputs (472). There is also molecular heterogeneity of Purkinje cells (473, 474). Therefore, it is possible that other influences on the Purkinje cells cause them to be either more susceptible or resistant to loss of *Tsc2*. One way to study this modular structure would be to use the markers called “zebrins,” reminiscent of a zebra’s stripes. Zebrins are antibodies that specifically recognize subsets of Purkinje cells (472). Therefore, staining with different zebrin antibodies might indicate which subset of Purkinje cells is more resistant to *Tsc2*-mediated Purkinje cell degeneration.

Neurodegeneration is not typically examined as a feature in TSC, though my study suggests it might have some underappreciated importance. Additionally, upregulation of the mTORC1 pathway has been demonstrated in other neurodegenerative disorders such as Alzheimer’s disease (475, 476) and Parkinson’s disease (477). Furthermore, Purkinje cell degeneration is also a feature reported in Alzheimer’s and Huntington’s disease (478, 479).

Since mTORC1 is important in mediating cell survival (especially in the context of tumor formation) (83, 480, 481), how do we reconcile the neurodegeneration observed in Purkinje cells following upregulation of mTORC1? The role of mTORC1 in cell survival has been characterized in mitotic cells, however, it is suggested that post-mitotic neurons may have a different response to hyperactivation of this signaling cascade (482, 483). Therefore, how might mTORC1 upregulation mediate neurodegeneration in post-mitotic neurons?

One of the mechanisms we examined is through altered calcium signaling. Calcium is involved in early development for processes such as neuronal survival, migration, differentiation, and synaptogenesis (280-285). Cell surface receptors, including receptor tyrosine kinases and G protein-coupled receptors, activate phospholipase C (PLC) leading to hydrolysis of the phospholipid PIP2 to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). IP3 then diffuses to the endoplasmic reticulum (ER) to activate its receptor, IP3R (454). IP3R then mediates intracellular calcium release- acting as a relay for calcium dependent responses including proliferation, cell division, neuronal signaling, and apoptosis (453). Since we found increased levels of IP3R in Tsc2f/f;Cre mice, it would be interesting to investigate subsequent calcium levels in the cells and to determine what role this plays in apoptosis.

Importantly, Akt (the upstream activator of mTORC1), has been shown to phosphorylate IP3R (484-486). Furthermore, mTOR and IP3R co-immunoprecipitate (487) and mTOR has been shown to potentiate calcium release through IP3R (488, 489). Increased calcium response has also been implicated in other neurodegenerative disorders. IP3R was identified to interact with huntingtin (htt) and huntingtin-associated protein 1 (490). Furthermore, inhibition of IP3R reduced mutant htt aggregation (491), suggesting exaggerated calcium release may be leading to neuronal toxcicity in Huntington's disease. Furthermore, mutations in presenilin, responsible for familial Alzheimer's disease, enhanced the activity of IP3R, again suggesting exaggerated calcium release as a mechanism of neuronal toxicity (492). The suggestion that excessive calcium release leads to toxicity, was also proposed in patients with ASD due to mutations in voltage-dependent calcium channels (279). Therefore, altered calcium signaling in Purkinje cells leading to degeneration may provide one mechanism for the link between TSC and ASD.

I also found upregulation of oxidative stress markers in Tsc2f/f;Cre mice. Activation of oxidative stress can also lead to upregulation of calcium response (493, 494). One of the important sites for calcium homeostasis is the mitochondria which is also involved in the release of cytochrome c, which initiates the intrinsic pathway of apoptosis (451). However, the mitochondria are also the site for the electron transport chain, an important generator of cellular ATP, (495), but also the major site of ROS generation (451). The free radicals generated in oxidative stress can cause cellular damage by attacking the chemical bonds of lipids, proteins, and nucleic acids (496, 497). Therefore, excessive

ROS production, beyond the cells ability to cope, leads to oxidative stress. Oxidative stress is thought to be an important component in: Alzheimer's disease (498, 499), atherosclerosis (500), bipolar disorder (501), cancer (502), chronic fatigue syndrome (503), heart failure (500), myocardial infarction (504), Parkinson's disease (498), Schizophrenia (501, 505), and sickle cell anemia (506). In fact, oxidative stress is now considered to be a feature of all neurodegenerative disorders (456, 507), and consequently antioxidants and ROS scavengers are popular therapeutic targets for these disorders (508, 509). However, it is unclear whether oxidative stress causes neuronal cell death or results from the degenerative processes (510, 511). Additionally, oxidative stress (due to genetic susceptibility or environmental factors) is likely important in Fragile X syndrome (7) and ASD (363, 381-383). Oxidative stress has also been implicated in TSC, as it has been shown that *Tsc1*-deficient neurons have elevated levels of ROS (512).

Other genetic models of Purkinje cell degeneration can provide us with more mechanisms. Some of these models are listed in the following section:

In 1976, a mouse model was first described that developed ataxia after 3-4 weeks of age. Upon histological examination, they were noted to have Purkinje cell loss (beginning at p18 and rapidly progressing over a two week period) and were therefore appropriately named Purkinje cell degeneration (pcd) mice. Cells in folium X are initially resistant to degeneration, but eventually succumbed to death by four months of age (469). We now know that pcd mice are the result of a naturally occurring recessive mutation in the *Nna1* gene, the function of which is still being characterized (513). Purkinje cells were determined to degenerate via apoptosis (514, 515). Furthermore, a microarray was conducted on pcd mice and found upregulation of many genes including ribosomal protein S6 and eukaryotic translation elongation factor 1 alpha 2 (516), two downstream targets of mTORC1.

Another model of Purkinje cell degeneration is the Lurcher mouse. This mutation results from an autosomal dominant mutation in the δ2 glutamate receptor (GluRδ2) which causes the death of almost all of the Purkinje cells (not in a folia dependent manner) (517, 518). Homozygous deletion causes lethality. Apoptosis (519-521), necrosis, and autophagy (518) have all been used to describe Lurcher Purkinje cell death. Oxidative stress (through SOD and nitrotyrosine) have also been demonstrated

in the Lurcher mice (522). It is thought that the mutation causes cell death through excitotoxicity (523). Interestingly, mutations in another glutamate receptor, glutamate receptor ionotrophic kainite 2 (GRIK2), have been associated with ASD (524-526) and are also associated with abnormal cerebellar development (527).

Nervous mice, due to a naturally occurring mutation arising at Jackson labs, were first described by Story Landis in 1973. Around P9, Landis noticed that some of the Purkinje cells had altered mitochondrial morphology- becoming round and enlarged. By P19, many of the organelles had lysed presumably leading to cell death (528). The majority of Purkinje cell death occurred between three to four weeks of age in a non-random pattern in the cerebellum (529). The molecular pathway of the nervous mice is only just now beginning to be elucidated. Nervous mice have a ten-fold increase in expression of tissue plasminogen activator (tPA) which is involved in mitochondrial function and dendritic growth (530). Though it is not specified in the original report, perhaps the altered morphology of the mitochondria is a result of or induces oxidative stress.

Homozygous mutations in the sticky gene, an editing defect in a tRNA synthetase, leads to Purkinje cell loss beginning at three weeks of age. Purkinje cell loss progresses slowly over the course of a year and acts in a folia dependent manner (470). Degenerating cells were positive for both TUNEL and CC3, indicating apoptotic cell death (470). However, it was also demonstrated that the sticky mutation led to accumulation of misfolded proteins and subsequent increase of ubiquitin as well as the ER stress response (470).

Another Purkinje cell degeneration model is the woozy mouse, which occurs as a result of a spontaneous recessive mutation in the Sil1 gene which is a cochaperone of BiP. Apoptotic Purkinje cell death begins around 10 weeks of age and reaches its peak around three to four months of age. Progression is slow and continues even in 18 month old mice. Purkinje cell degeneration again shows a folia dependent effect (471). Since the *SIL1* gene is involved as a protein chaperone, it important in the ER stress response (531, 532). Therefore, it is also important to note that woozy mice had upregulated ubiquitin as well as the ER stress response (471).

Many of these models of exhibit apoptosis of Purkinje cells, frequently in a folia-dependent manner. However, the processes leading to apoptosis are varied in the

different models. Some may be the result of oxidative stress. However, these models of Purkinje cell degeneration suggest that there are likely other mechanisms that perturb Purkinje cells leading to their degeneration. Therefore, I will focus on some of these in the next chapter, with particular focus on the ER stress response.

Chapter Five:

Loss of *Tsc2* in Purkinje cells leads to the ER stress response

Introduction:

The ER is an important cellular site for the synthesis, folding, and processing of secretory proteins (495). Alterations in this process can lead to a buildup of unfolded or misfolded proteins, which are detrimental to the cell (533); and a buildup of these misfolded proteins can lead to a cellular event called ER stress or the unfolded protein response (UPR) (534). There are three ER receptors designed to recognize ER stress: ATF6 (activating transcription factor 6), IRE1α (inositol-requiring kinase), and PERK (protein kinase RNA-like endoplasmic reticulum kinase). In a steady state/non-stressed, these three receptors are held in an inactive conformation due to their association with the chaperone protein, BiP (535). Stress inducing situations induce dissociation of BiP from the ER stress receptors (535).

The ER stress pathway first acts to mediate translation arrest through PERK-dependent phosphorylation of eIF2α (536). The second step of the ER stress pathway acts to increase the folding capacity of the ER through selective activation of chaperone proteins (536). This is achieved, in part, due to IRE1α-mediated cleavage/activation of XBP1 (xbox binding protein 1) (537). XBP1 is involved in transcriptional activation of chaperone proteins as well as proteins involved in ER biogenesis (538). The third step of the ER stress pathway is to function in ER-associated degradation (ERAD) to remove misfolded proteins (536).

The three steps of the ER stress pathway are survival steps attempting to ameliorate the stressful conditions; however, prolonged ER stress can lead to apoptosis (539, 540). One critical protein for ER-stress induced apoptosis is CHOP (CCAAT-enhancer-binding protein homologous protein), a 29kDa protein also known as GADD153 (growth-arrest and DNA damage inducible gene 153) (540-542). CHOP is primarily induced through ATF4 (543, 544), which is generated through the PERK/pEIF2α pathway (543, 545). However, maximal CHOP expression requires all three receptor-mediated signaling pathways (546). CHOP is involved in apoptosis by its repression of pro-survival Bcl2 and it also sensitizes cells to ER stress-inducing agents (537, 547).

ER stress-mediated apoptosis is an important biological event in several disorders, including: diabetes, ischemic diseases, and neurodegenerative diseases (536, 548). Protein misfolding is an important component in the pathogenesis of Alzheimer's,

Parkinson's and Huntington's diseases (549, 550). For example, it was found that mutations in presenilin-1, which causes the familial form of Alzheimer's disease, induced CHOP expression (551). The reason ER stress is so widespread in pathological conditions, is because it can be triggered by many events in the cell including: altered calcium regulation, glucose deprivation, viral infection, increased protein synthesis, misglycosylated proteins, protein misfolding, or abnormalities in protein degradation (552-554).

Not only can altered calcium signaling induce ER stress, but CHOP is also involved in activating calcium signaling (555). CHOP mediates expression of ERO1α (ER oxidase 1 alpha), which is then responsible for activating IP3R (inositol 1,4,5-triposphate receptor) (556). In fact, this is one of the ways in which CHOP induces apoptosis, because the calcium release from the ER then triggers apoptosis through activation of CaMKII (calcium/calmodulin- dependent protein kinase II) (556).

Furthermore, ER stress also has a unique relationship with oxidative stress. First, hypoxia can induce ER stress through activation of PERK mediated phosphorylation of eIF2α (557-561). However, reactive oxygen species (ROS) are also formed as a byproduct of ER stress (562). Part of the increase in reactive oxygen species is due to disulfide bond formation which require oxidation/reduction reactions (563). Moreover, CHOP can also lead to increased production of ROS (512, 540, 542).

Examination of both sticky (470) and woozy (471) mice show that alterations in the endoplasmic reticulum (ER) stress response can lead to Purkinje cell degeneration Additionally, studies suggest that loss of the TSC1/TSC2 complex leads to upregulation of the ER stress pathway (512, 564, 565). Therefore, since ER stress is responsive to increased protein synthesis, aberrant calcium signaling, oxidative stress, and increased mTORC1, I investigated the role of the ER stress pathway in Tsc2f/f;Cre mice.

Materials and Methods:

Mouse model, histology, statistical analysis, and human tissue methods were performed the same as in Chapter 4.

Antibodies

The primary antibodies for immunohistochemistry (IHC) were: Beclin (1:100; Cell Signaling, Bedford, MA), Beclin (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), Calbinidn (1:250; Abcam, Cambridge, MA), Calbindin (1:250; Sigma-Aldrich, St. Louis, MO), CHOP (GADD153)(1:100; Santa Cruz Biotechnology, Santa Cruz, CA), ERp57 (GRP58) (1:100 Assay Designs, Ann Arbor, MI), phosphorylated S6 235/236 (1:100; Cell Signaling, Bedford, MA), Superoxide dismutase (SOD) (1:250; Abcam, Cambridge, MA), and ubiquitin (1:250; Millipore, Billerica, MA).

Secondary antibodies (1:250, Invitrogen, Carlsbad, CA) were: Alexa Fluor 488 (anti-rabbit) (anti-mouse igG_1), Alexa Fluor 594 (anti-rabbit) (anti-mouse IgG_1), Alexa Fluor 555 (anti-rabbit) (anti-mouse IgG_1) (anti-mouse IgG_{2b}).

Results:

Tsc2f/f;Cre mice show increased ER stress

To assess for ER stress, IHC was performed for a chaperone protein, ERp57/GRP58, which is a member of the protein disulfide isomerase (PDI) family that acts as a thiol oxidoreductase- facilitating in the formation and breakage of disulfide bonds during protein folding (566, 567). Tsc2f/f;Cre mice had increased expression of ERp57 (PDI) compared to controls (Figure 5.1a-b). To further assess for ER stress, we also examine expression of the ER-stress specific apoptotic inducer, CHOP. Levels of CHOP were also upregulated in Tsc2f/f;Cre mice compared to controls (Figure 5.1c-d). Since CHOP can induce the formation of ROS (540, 542), Purkinje cells were co-labeled manganese superoxide dismutase (MnSOD) showing that CHOP and MnSOD co-localized (Figure 5.1e).

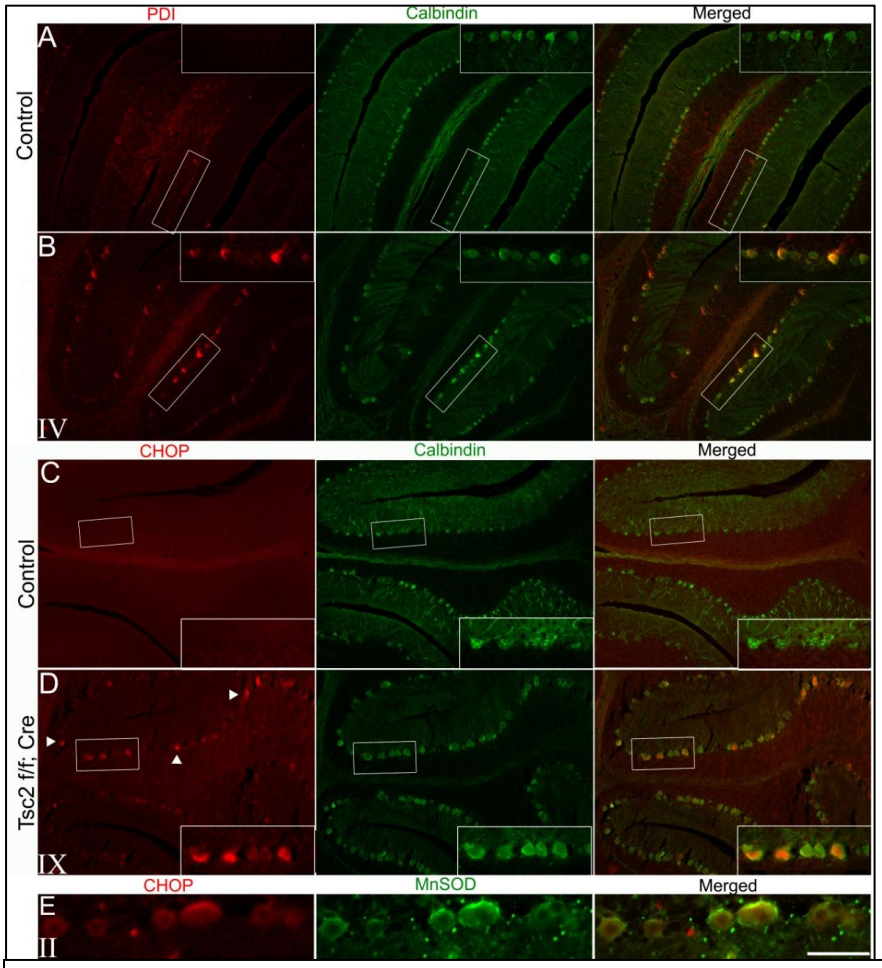

***Figure 5.1:** ER stress is activated in the Purkinje cells of Tsc2f/f;Cre mice. (A-B) Increased expression of ERp57, a protein disulfide isomerase (PDI), in Purkinje cells of 1 month Tsc2f/f;Cre mice (B) compared to control (A). (C-D) CHOP positive (GADD153) Purkinje cells demonstrate activation of ER stress in 1 month Tsc2f/f;Cre mice (D) compared to controls (C). Purkinje cells are co-labeled with Calbindin. (E) Co-localization of CHOP and MnSOD shows both ER stress and oxidative stress in Tsc2f/f;Cre mice. Folia are shown in Roman numerals. Scale bars, 50µm.

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Detection of ER stress in the Purkinje cells of TSC patients

To investigate whether ER stress might contribute to pathogenicity in TSC patients, expression of the ER stress marker PDI in Purkinje cells from TSC patients was examined by IHC. Two out of our four patient samples had increase PDI expression

(Figure 5.2), suggesting elevated ER stress as a possible mechanism of pathogenesis in TSC patients.

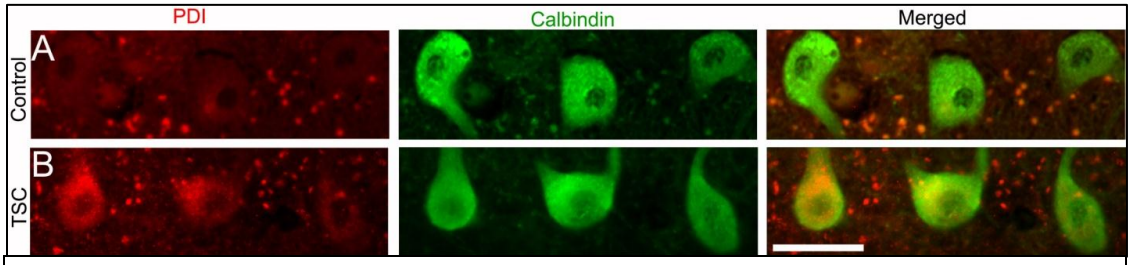


Figure 5.2: Human TSC patients show increased ER stress. (A-B) Increased PDI expression in representative TSC patient 1' (B) compared to age-matched control (A). Purkinje cells are co-labeled with Calbindin. Scale bar 50µm.

Tsc2f/f;Cre mice have increased production of ubiquitin

The third step of the adaptive ER stress pathway is ER-associated degradation (ERAD) (536), which acts to clear misfolded proteins through the ubiquitin-proteasome pathway (568-570). Therefore, IHC was performed to detect the amount of ubiquitin in the cell and found upregulation of ubiquitin in Tsc2f/f;Cre mice compared to controls (Figure 5.3).

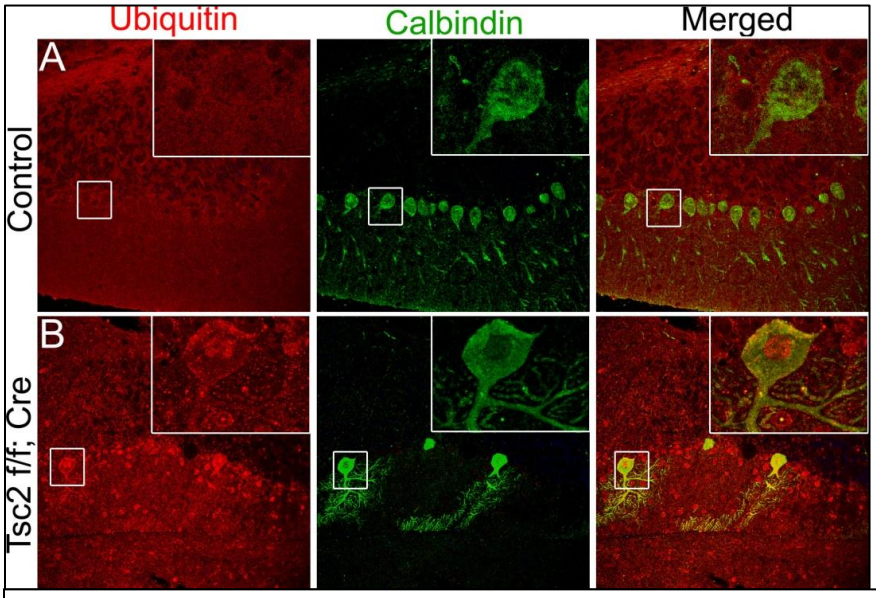


Figure 5.3: (A-B) Increased expression of ubiquitin in 2 month old Tsc2f/f;Cre mice (B) compared to controls (A). Purkinje cells are co-labeled with Calbindin.

Tsc2f/f;Cre Purkinje cells show evidence of autophagy

One of the well-known consequences of ER stress is induction of autophagy (571-577). Though traditionally thought of as another programmed cell death pathway (578), the paradigm is now shifting to also view autophagy as a mechanism of cell survival (578-580). In the context of ER stress, autophagy can be used to degrade misfolded proteins or to degrade the damaged ER (581). Therefore, we examined the presence of autophagy in Tsc2f/f;Cre mice following ER stress. Purkinje cells of Tsc2f/f;Cre mice had increased levels of Beclin-1, a protein required for the formation of the autophagasome (Figure 5.4a-b). H&E staining also revealed the presence of autophagic-like vacuoles in the Tsc2f/f;Cre mice (Figure 5.4e-f).

Since mTORC1 is a well-known inhibitor of autophagy (582), we examined Purkinje cells to see if mTORC1 was inhibited in the cells undergoing autophagy. Paradoxically, pS6 expression was still upregulated in the Beclin-expressing Purkinje cells (Figure 5.4c-d).

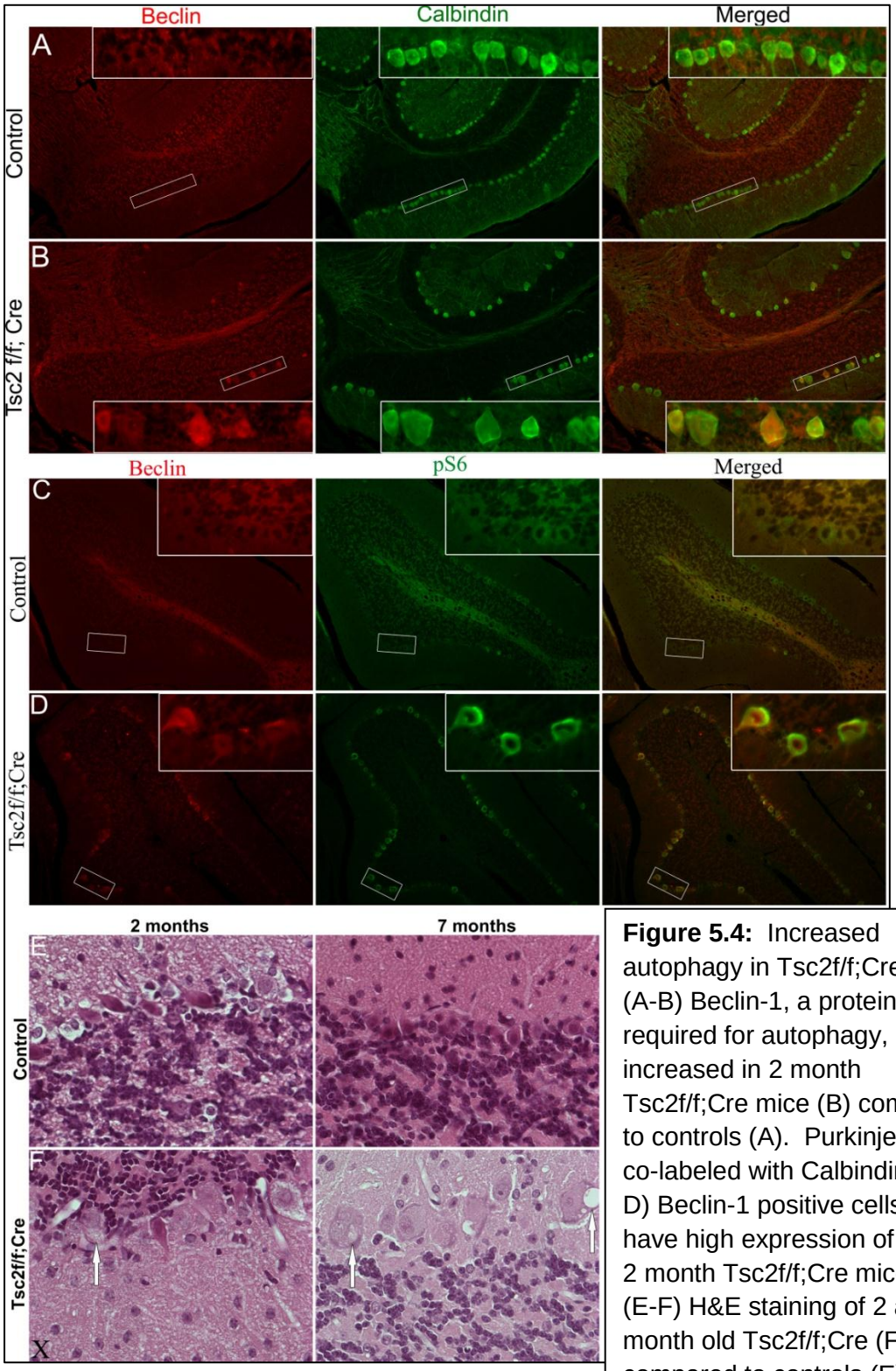


Figure 5.4: Increased autophagy in Tsc2f/f;Cre mice. (A-B) Beclin-1, a protein required for autophagy, is increased in 2 month Tsc2f/f;Cre mice (B) compared to controls (A). Purkinje cell are co-labeled with Calbindin. (C-D) Beclin-1 positive cells also have high expression of pS6 in 2 month Tsc2f/f;Cre mice (D). (E-F) H&E staining of 2 and 7 month old Tsc2f/f;Cre (F) mice compared to controls (E) indicates presence of autophagic-like vacuoles indicated by the white arrows.

Discussion

ER stress in human disease

ER stress plays an important role in a variety of human diseases including normal aging and diseases associated with aging (583-585). However, it has also been shown in other diseases including atherosclerosis (586-588), autoimmune disease (589-591), bipolar disorder (current medications are targeting the induction of UPR) (592-594), cancer (595-599), glomerulonephritis (acute kidney injury) (589-591, 600, 601), heart disease (602-607), type 1 diabetes (545, 608-613), type 2 diabetes (614), and stroke (615-618). Furthermore, it has also been shown to play a role in numerous neurodegenerative diseases including: Alzheimer's disease (551, 619-622), amyotrophic lateral sclerosis (ALS) (623), and Parkinson's disease (624-628).

I have shown that ER stress is part of the pathophysiology in the neurodegeneration of Purkinje cells in Tsc2f/f;Cre mice. Induction of PDI, a molecular chaperone, is part of the adaptive arm of the ER stress pathway. However, the presence of CHOP positive cells indicates that the ER stress pathway is culminating in apoptotic cell death of Purkinje cells. These data are interesting in light of other models of TSC. Upregulation of ER stress occurred in vitro following deletion of either *Tsc1* or *Tsc2* (512, 564, 565). These data, in addition to that presented here, suggest that activated mTORC1 increases protein translation beyond the folding-capacity of the ER, leading to a progressive accumulation of misfolded/unfolded proteins.

Kang et al. found that ATF4, ATF6, and CHOP expression were abolished in *Tsc1* deficient MEFs and *Tsc2* deficient LEFs (derived from kidney tumors of Eker rats), suggesting that *Tsc1/Tsc2* deficient cells have an incomplete ER stress response and are therefore more sensitive to ER-stress induced apoptosis (564). They did find increased apoptosis through markers for cleaved caspase 3, 9, and 12 (564). I, however, disagree with the interpretation of their data. Although caspase 12 can also participate in ER-stress induced apoptosis (629), ATF4 induction of CHOP is also a potent inducer of apoptosis (540-542). Therefore a lack of ATF4 and CHOP expression is does not merely represent a shortened ER stress response that leaves the cells more susceptible to ER-stress induced apoptosis, as Kang et al. suggested (564).

However, they did present convincing data that CHOP expression was not induced upon activation of ER stress in *Tsc1/Tsc2* deficient cells (564). In light of our conflicting data, I think that it is interesting to compare our two models. To determine the role of *Tsc1/Tsc2* in regulation of ER stress, Kang et al. used an in vitro model, while we chose to use an in vivo mouse model. Furthermore, induction of ER stress by Kang et al. was achieved by pharmacological treatment of either MG132 (a proteasome inhibitor), thapsigargin (a calcium ATPase inhibitor), or tunicamycin (blocks protein gycosylation). These drugs likely have other toxic effects on the cells other than strict induction of ER stress. Furthermore, our results show that *Tsc2*-deficiency alone can lead to increased ER stress. So, to treat *Tsc2*-deficient cells with an ER stress inducing agent may change the known kinetics and effects of the drug. Instead of an pharmacologic inducer of ER stress, ER stress in Tsc2f/f;Cre mice is a natural response to deal with a genetic mutation. While there are also other events going on in Purkinje cells contributing to their demise (presented in Chapter 4), this is a more physiologically relevant model. Finally, Purkinje cells are very different from MEFs and LEFs and show an extraordinary sensitivity to metabolic perturbations (466, 630). The presence of ER stress in other Purkinje cell degeneration mutants (470, 471, 515, 631) also indicate the sensitivity of the Purkinje cells to perturbations in ER homeostasis.

Another important finding presented by Di Nardo et al. is the relationship between ER stress and oxidative stress (512). This group found that both ER and oxidative stress were induced in *Tsc2*-deficient cells and specifically that inhibition of CHOP reduced the incidence of oxidative stress (512). Therefore, they concluded that CHOP induced oxidative stress in *Tsc2*-deficient cells (512). However, it is also known that oxidative stress can induce ER stress (557-561). The Purkinje cells in the Tsc2f/f;Cre mice co-labeled with both CHOP (ER stress) and SOD (oxidative stress) suggesting an interplay between these two cellular events. The co-labeling, however, does not indicate which of the two stresses occurs first. To sort out the order of cellular stresses, rescue experiments could be performed. If attenuated ER stress (either through CHOP deletion or ER stress protective agents) reduced levels of oxidative stress, it would suggest that ER stress was important in causing oxidative stress. However, if antioxidants reduced ER stress, it would indicate that oxidative stress was the initiator.

It is also important to link what we find in animal models back to the human condition. Ozcan et al. found upregulation of ER stress using peIF2α in a cortical tuber

from a patient with TSC (565). My results also indicate that ER stress is upregulated in the Purkinje cells of some patients with TSC. The two patients with increased PDI (2' and 3') were the same two patients with upregulated pS6 levels (see Chapter 4). This suggests that activation of mTORC1 in human patients can lead to activation of the ER stress response. The resulting question is whether this directly leads to Purkinje cell degeneration in these patients. This, however, is not known as one of the two patients with upregulated ER stress did not have Purkinje cell loss in the sample analyzed (1'). Furthermore, Purkinje cells in patient 3' did not show staining of PDI. However, as mentioned in Chapter 4, the Purkinje cell staining in this particular patient sample was poor and may be the cause of the "negative" results.

Ubiquitin

In addition to activation of ER stress, my results show increased staining of ubiquitin in Tsc2f/f;Cre mice. While these results indicate activity of the ubqituitin/proteasome pathway to clear misfolded proteins, it does not prove that this is the case. The antibody used for IHC recognizes all ubiquitin molecules, both conjugated and unconjugated. Therefore, while expression of ubiquitin is increased in Tsc2f/f;Cre mice, the ubiquitin molecules may not be attaching to misfolded proteins and targeting them for degradation. Furthermore, the presence of ubiquitin itself is not necessarily pathologic since monoubiquitination often plays an important role in cell signaling and does not always lead to degradation (632). However, proteins with a polyubiquitin chain are delivered to the proteasome for degradation (495). Therefore, a better measure of protein degradation activity would be to examine the enzymatic activity of the proteasome (633). However, I hypothesize that there is function behind the increased expression of ubiquitin in Tsc2f/f;Cre mice. Given that the ubiquitin/proteasome pathway plays a role in ER-associated degradation pathway (ERAD) following ER stress (568-570), I hypothesize the increased expression of ubiquitin in Tsc2f/f;Cre mice serves a similar role in the ER stress pathway. Moreover, accumulation of ubiquitin molecules in a punctate pattern is also evidence of a pathological condition and is also seen in Alzheimer's, Huntington's, and Parkinson's diseases (634, 635). We also noted increased staining of ubiquitin in the molecular cell layer of Tsc2f/f;Cre mice. The dendritic tree of Purkinje cells extends into the molecular cell layer. Therefore, these cells are likely closely linked with the *Tsc2*-deficient Purkinje cells. A follow up study

should address the effect of Purkinje cell degeneration on the remaining cell populations in the cerebellum.

Autophagy

Another intriguing finding in Tcs2f/f;Cre Purkinje cells is the induction of autophagy seen through expression of Beclin-1. Autophagy involves the sequestration of cytoplasmic organelles and their delivery to the lysosome (636). It would be ideal to confirm the induction of autophagy by another method. For example, it is common to demonstrate the conversion of LC3I to LC3II (578). LC3 is required for the formation of autophagosomes (579), but is normally kept in a cytosolic form (LC3I) (580). However, upon activation of autophagy, LC3 is cleaved into LC3II which tightly associates with the membrane of the autophagosome (580). Therefore, a Western blot comparing the levels of LC3II to LC3I is a good indicator of autophagy activity (582). The limitation of Western blotting in this model, however, is that Purkinje cells comprise such a small percentage of the total number of cells in the cerebellum (358). Therefore, Western blots on whole cerebellar lysates have not been sensitive enough to detect even known differences including TSC2 and pS6. Thus far, I have not been able to purify Purkinje cells for immunoblot analysis.

However, autophagy is a natural response resulting from the ER stress pathway (571-577). Autophagy can occur to eliminate ubiquitinated proteins (637). Interestingly, demonstrating the unique interplay between ER and oxidative stress, ROS can also induce autophagy (638, 639). ER stress can induce autophagy both through the PERK receptor signaling cascade and the IRE1α signaling cascade (571, 573, 574, 576, 581). One of the mechanisms by which ER-stress induced autophagy can occur is through inactivation of mTOR (640). One study found that *Tsc2*$^{-/-}$ MEFs had a deficient autophagy response to ER stress (640), suggesting that mTOR suppresses autophagy. However, Kang et al. actually showed induction of autophagy following ER stress in *Tsc1*$^{-/-}$ MEFs (564). Both groups were again using pharmacologic agents to induce ER stress and different results could be achieved using a naturally occurring condition in vivo, specifically in very vulnerable Purkinje cells. Following ER stress, we found upregulation of autophagy in addition to continued upregulated mTORC1 activity, determined by levels of phosphorylated S6. This suggests an mTOR-independent method of autophagy regulation.

Despite the conflicting nature of the two previous reports, autophagy is regulated via mTORC1-dependent and mTORC1-independent mechanisms (641). However, much less is known about mTORC1-independent regulation of autophagy. One of the known mTORC1-depent regulators of autophagy is through activation of AMP activated protein kinase (AMPK) (642). However, recent work suggests that AMPK can induce autophagy in a manner that is independent of mTORC1, possibly through direct interaction with the mitochondria (643).

Therefore, an alternative pathway of autophagy induction is through decreased levels of IP3, which seems to have very little crosstalk with the mTORC-1 dependent regulation of autophagy (644, 645). Studies show inhibiting the IP3R can induce autophagy (646-648). However, other contradictory studies indicate that IP3R is required for autophagy (574, 649). Calcium is likely to be a complex regulator of autophagy. Alterations in either direction can perturb normal cell function. Since ER stress can lead to calcium release (650) and autophagy (571-577), and since both are upregulated in Tsc2f/f;Cre Purkinje cells (see also Chapter 4), I hypothesize that altered calcium signaling induced by ER stress may lead to autophagy in the Tsc2f/f;Cre mice. However, these mechanisms need to be further explored.

Lam et al. showed IP3R is required for autophagy induction. They examined *Dictyostelium* cells, which lack apoptotic machinery. Therefore, they were able to associate all of the cell death in this model to the induction of autophagy. When they knocked out the IP3R gene, they found inhibition of autophagy (649). Therefore, perhaps IP3R activity is required specifically for autophagy associated with cell death. Whether autophagy is occurring as a protective mechanism or as a part of the degenerative process of Purkinje cells in Tsc2f/f;Cre mice is unknown and needs to be addressed. Treatment with autophagy inhibiting agents may help address this issue.

There are two distinct autophagic processes in the cell: starvation/nutrient induced and quality control autophagy (651). The role of mTORC1, as an energy sensor, in starvation induced autophagy is well-known (636). Perhaps quality control autophagy, like that induced by ER stress, is mediated by an alternate pathway.

Another pathway for quality control autophagy might be due to histone deacetylase 6 (HDAC6). HDAC6 activity is required for the fusion of the autophagosome to the lysosome in quality control autophagy (652). HDAC6 has a unique ability among other

HDACs to bind to ubiquitin molecules (653). Following accumulation of ubiquitinated proteins, HDAC6 can then induce autophagy (654). Therefore, although studies are still preliminary, as staining has only been performed on one animal, it is intriguing to note that HDAC6 levels were increased in a one month old Tsc2f/f;Cre mouse compared to control (Figure 5.5). Dysregulated HDAC activity may be a common mechanism seen in other neurodegnererative diseases such as amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Fridreich ataxia, Huntington's disease, and stroke (655).

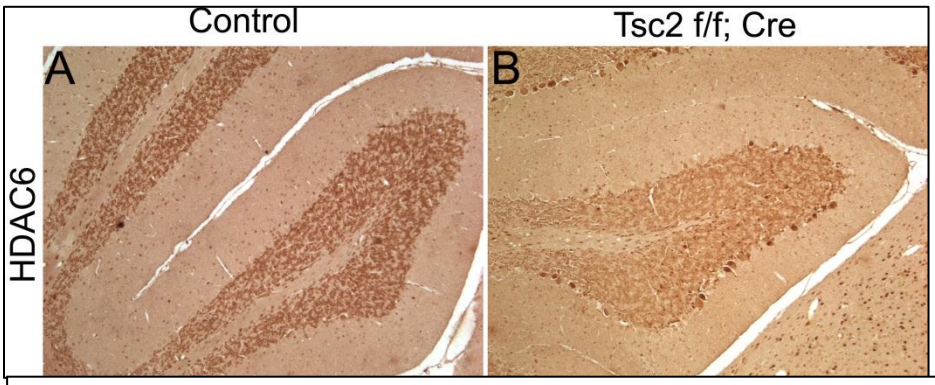


***Figure 5.5:** Increased expression of HDAC6 in one month Tsc2f/f;Cre mouse (B) compared to control (A).

*Slides were stained in the lab of Jason Ericksen at the University of Houston.

Other Purkinje cell models

Now that we have established a novel model of Purkinje cell degeneration, we can compare it to other Purkinje cell degeneration models. These similarities can elucidate the overall mechanisms that lead to Purkinje cell degeneration and thus establish therapeutic targets. The Purkinje cell degeneration models that have been presented in this chapter and in Chapter 4 are compared in Table 5.1.

Mouse	Timing	Pattern	Apoptosis	ROS	ER	Autophagy	Ubiquitin	Refs
Tsc2f/f;Cre	1 month – 7 months	Yes	Yes	Yes	Yes	Yes	Yes	(2)
Lurcher	1 week – 4 weeks	No	Yes	Yes	_	Yes	_	(517-522)
PCD	2 weeks – 4 weeks	Yes	Yes	Yes	Yes	Yes	_	(469, 514, 515)
Woozy	2 months – 18 months	Yes	Yes	_	Yes	Yes	Yes	(471)
Sticky	3 weeks – 3 months	Yes	Yes	_	Yes	_	Yes	(470)

Table 5.1: Other mouse models of Purkinje cell degeneration and the known molecular mechanisms of degeneration. Columns indicate timing of Purkinje cell degeneration, whether degeneration is folia-dependent (pattern), and presence of apoptosis, oxidative stress (ROS), ER stress (ER), autophagy, and increased ubiquitin.

Though not much is known about the gene involved in the Purkinje cell degeneration mice (pcd) (513), it is interesting that the molecular mechanisms associated with Purkinje cell death are almost identical to what was observed in the Tsc2f/f;Cre mice. The noticeable exception is the timing of Purkinje cell degeneration which occurs much earlier in pcd mice. However, the pcd mutation is natural occurring, not relying on the timing of Cre expression, which is postnatal in Tsc2f/f;Cre mice. Though the function of the pcd gene is not elucidated, the microarray data showing upregulated S6 and eIF2α (516) suggest involvement of the mTORC1 pathway. Given this knowledge, the similarities between these mice are not surprising.

Lurcher mice are unique in that they do not have patterned Purkinje cell degeneration; but rather rapid, non-selective Purkinje cell death (517, 518). Understanding the variations in Tsc2f/f;Cre mice that lead to differential Purkinje cell sensitivity would reveal insight into the mechanics of Purkinje cell degeneration and possibly reveal therapeutic strategies to prevent Purkinje cell death. The Lurcher phenotype is due to a gain-of-function mutation in the δ2 glutamate receptor (GluRδ2) which is thought to lead to excitotoxicity (523). Interestingly, studies suggest that activation of metabotropic glutamate receptors leads upregulation of mTORC1 (632). Though not previously explored, it would be interesting to examine downstream targets of mTORC1 in Lurcher mice to see if they are indeed activated. If so, this could provide a molecular link between Purkinje cell loss in Tsc2f/f;Cre mice and Lurcher mice. Furthermore, the resulting question would be why ER stress and increased ubiquitin have not been reported in Lurcher mice. Are these processes not part of Lurcher pathology, or have they simply not been examined?

Sticky mice occur as a result of an editing defect in a tRNA synthetase, causing misincorporation of amino acids into putative proteins, leading to accumulation of misfolded proteins and ER stress (470). Woozy mice occur as a result of a mutation of a cochaperone of BiP, which therefore reduces the folding capacity of the ER and leads to subsequent ER stress (471). Since ER stress and oxidative stress are linked, these mice should be examined to see if there is increased production of ROS as a result of increased ER stress. Also, since we show induction of autophagy as one possible mechanism of protein clearance, this pathway should also be examined in sticky mice to see if it acts as a mechanism to degrade the altered proteins.

Potential therapies

Examination of these mouse models as a group suggests that activation of ER stress leads to Purkinje cell degeneration in multiple models, and is therefore worth pursuing as a possible cause in other mouse models of Purkinje cell degeneration. Furthermore, though many cellular processes can regulate ER stress, activation of mTORC1 target genes can lead to subsequent alterations in the ER stress pathway. Therefore, since ER stress is such a central regulator of Purkinje cell viability, the next step of research should be to explore pharmacological agents targeted at reducing or alleviating ER stress.

One potential agent is sodium 4-phenylbutyrate (4-PBA) which is a small molecule that acts as an HDAC inhibitor (656). 4-PBA increases histone acetylation and leads to increased transcription of several chaperone proteins (657). It has few side effects and is penetrable to the blood brain barrier (658). Interestingly, 4-PBA has been used for a wide array of applications. 4-PBA treatment has been found effective for disorders including: acute promyelocytic leukemia (659), Alzheimer's disease (660), ALS (661), cerebral ischemic injury (662), Huntington's Disease (663, 664), multiple sclerosis (665), Parkinson's disease (666), and spinal and bulbar muscular atrophy (a motor neuron disease and neurodegenerative disease due to an expanded polyglutamine repeat sequence) (667, 668). 4-PBA is also used to treat sickle cell anemia and thalassemia due to its HDAC activity increasing transcription of β- and γ-globin (669, 670). 4-PBA was found to increase levels of heat-shock proteins. As such, 4-PBA has also been found to promote trafficking of the DeltaF508 mutation (the mutation in the CFTR protein that results in cystic fibrosis)(671) which allows the protein to mature and insert into the membrane, thus rescuing the pathology seen in cystic fibrosis (656). 4-PBA treatment was also shown to dramatically increase the life span of *Drosophila,* possibly by increasing histone acetylation (657).

4-PBA can also lead to increased production of antioxidants and increased activity of superoxide dismutase to clear ROS (672). Treatment with 4-PBA increased the activity of the ubiquitin-proteosome pathway and decreased expression of CC3 (663). 4-PBA has also been shown to act as a chemical chaperone, able to reverse the aggregation of misfolded proteins (673, 674). Furthermore, 4-PBA has been shown to decrease ER-stress induced apoptosis though its inhibition of eIF2α phosphorylation, induction of CHOP, and subsequent caspase-12 activation (662). Ozcan et al. treated Tsc1/Tsc2 deficient cells with 4-PBA and found rescue of ER stress, though mTORC1 levels were still elevated (565). Given the chaperone properties of 4-PBA (673, 674) and subsequent inhibition of CHOP (662), I hypothesize that treatment in Tsc2f/f;Cre mice will be able to rescue Purkinje cell degeneration. Furthermore, the effects of 4-PBA on ROS (672) and its inhibitory effects on HDACs (656), may yield further benefit in this model.

To assess whether 4-PBA can rescue Purkinje cell degeneration, we treated Tsc2f/-;Cre mice with intraperitoneal (i.p.) injections of 200mg/kg/day 4-PBA. This treatment led to a small, but not statistically significant reduction in CHOP positive cells. However,

4-PBA has a short physiological half-life, breaking down into phenylacetic acid after 0.8 hours (635). Therefore, daily treatment may not provide a sufficient window for therapeutic benefit. Some groups have even delivered 4-PBA through the drinking water (675). Future experiments in Tsc2f/f;Cre mice should seek to establish a dosing regimen where sufficient amounts of the drug are maintained for the maximal effect. Then, markers for ER stress, oxidative stress, and cell death should be examined to determine if 4-PBA can rescue Purkinje cell death.

Because of the limitations with the short half-life of 4-PBA, it may also be wise to consider other therapeutic agents designed to reduce ER stress. Therefore, another HDAC inhibitor known to reduce ER stress is valproic acid (646, 650). Valproic acid has been shown to increase the induction of chaperone proteins (650), therefore, decreasing ER-stress associated apoptosis (676). Valproic acid has also been shown to block low threshold calcium currents in neurons (647). Therefore, I hypothesize that treatment with valproic acid might be able to rescue Purkinje cell degeneration.

Another possible way to target reduction in ER stress-induced apoptosis is through genetic means. *CHOP*$^{-/-}$ mice have normal lifespan, development, and are fertile (611, 677). Even in the presence of ER stress, *CHOP*$^{-/-}$ mice have reduced apoptosis (611, 612, 677). Therefore, I hypothesize that creation of Tsc2f/f;CHOP-/-;Cre+ mice will at least partially rescue Purkinje cell death.

ER stress and ASD

The role of ER stress in ASD is relatively unexplored. However, there are a few reports suggesting a link between this stress pathway and ASD. One indirect link between ASD and ER stress is through inflammation. Prenatal activation of the immune system is a risk for developing ASD (181-184). However, neuroinflammation is also seen in postmortem studies of ASD patients (186). Chronic inflammation can lead to ER stress, but ER stress can also trigger inflammation (654, 678).

Furthermore, several cell adhesion molecules have been associated with autism, including neuroligin (NLGH) 3 and 4, contacting-associated protein-like 2, and cell adhesion molecule-1 (CADM1) (253, 679, 680). Several of these mutations impair cellular transport of the protein (681). Furthermore, when these ASD-associated mutations were knocked into mice, they induced upregulation of the ER stress pathway

seen with peIF2α and CHOP (681). These mice also demonstrated behaviors associated with ASD (681). Since ASD-like behaviors have been associated with at least one mouse model of increased ER stress, I sought to examine behaviors characteristic of ASD in my mouse model.

Chapter Six:

Examination of ASD-Associated Behavior

Introduction:

Studies examining Tsc2f/-;Cre and Tsc2f/f;Cre mice indicate that loss of *Tsc2* in Purkinje cells leads to Purkinje cell degeneration. Histological examination of some TSC patients further corroborates the notion of Purkinje cell susceptibility (see Chapter 4). Given that Purkinje cell loss is one of the most common anatomical abnormalities seen in autopsy studies of autistic patients (360-366), analysis of mouse models of Purkinje cell degeneration will provide further insight into a mechanism for this association. Studies of the Lurcher mouse (Purkinje cell degeneration model) revealed decreased anxiety-related behaviors, increased activity levels, and increased repetitive behaviors (682, 683). As repetitive behaviors are a hallmark of ASD (133), the Lurcher mouse provides a good model to study the association of Purkinje cell loss and ASD. This current study investigates whether there is an association between *Tsc2* mediated Purkinje cell loss and ASD-like behavior.

Materials and Methods:

Tsc2f/-;Cre mice were generated and immunohistochemistry was performed the same as described in Chapter 4.

Behavioral Testing:

To assess for autistic-like behaviors, behavior testing was performed beginning at one month of age to three months of age. To assess for social interaction abnormalities, the social preference and social novelty test were performed (684). To assay for repetitive behaviors, the marble burying task was performed (685). Anxiety-like behaviors were assessed through open-field and light/dark box testing (686). The order of testing was done from the least stressful to the most stressful. In general, two tests per week were conducted. The timeline is shown in Table 6.1. The experimenter was blind to the genotypes of the mice for all behavior testing.

Age	Test
P30-31	General observation and reflexes
P37	Nest Building
P40	Response to Social Cues
P44	Marble Burying
P47	Open-field
P49-54	Buried Food
P58	Social Behavior
P61	Inkblot
P65	RotaRod
P68	Light/Dark Box
P72-77	Water Maze
P85-89	Reverse Water Maze
P90	Vision Water Maze

Table 6.1: Timeline of testing.

Home Cage Behavioral Video Recording:

Each cage used in behavior testing was videotaped for 20 minutes at 8:00AM, 12:00PM, and 4:00PM for a total of one hour of video.

Reflexes:

Before behavior testing, mice were tested for intact neurological reflexes. Reflex testing included eye blink, ear twitch, forepaw extension, grasping, and whisker twitch (686). Mice were also examined to make sure that hind limb clasping, indicating a significant neurological/motor impairment, was not observed.

Nest Building:

Nest building was performed by placing mice individually in cages and allowing then to acclimatize for ten minutes. A 5" x 5" cotton piece of nesting was then placed in the cage and the thickness of the nest was measured at 30, 45, 60, 90, 120, 180, and 240 minutes. At the end of the four hour period, the ratio of the weight of the nest built compared to remaining nestlet was also determined.

Response to Social Cues:

Mice were placed individually in a clean cage and allowed to acclimatize. Then a cotton tip applicator swabbed with different scents (water, lemon, vanilla, social cage 1, and social cage 2) was presented to the mice. Scents from social cages were obtained by wiping the cotton swab through a dirty cage of a mating pair of mice. Each scent was presented three different times and the time spent sniffing the applicator was recorded (684).

Marble Burying:

Repetitive behaviors were assessed by marble burying activity. Mice were placed in a clean cage with 4.5cm corncob bedding with 20 black glass marbles (15mm diameter) arranged in a grid on top of the bedding. Mice were allowed to explore the cage for 30 minutes. At the end of the experiment, the number of marbles buried (>50% of the marble covered by the bedding) was recorded (685).

Open-Field Activity:

Exploratory locomotor activity was measured in an open field (16 x 16 inch) plexiglass chamber with photobeams (Photobeam Activity System, San Diego Instruments). Mice were placed in the chamber for 30 minutes. Total distance traveled as well as average speed was measured. To assess for anxiety related behaviors, the percent of time in the center of the chamber was also recorded. Mice spending more time in the center are generally described as less anxious (686).

Buried Food:

To assess olfaction, a buried food test was performed (687). Two days prior to testing, mice were placed on a food restricted diet (0.5g of mouse chow/mouse/day). On each of the four days of testing, mice were placed in standard housing cage with 3 cm of bedding. Latency to find a buried 0.5g pellet in the bedding was recorded. Food pellet location was changed for each trial.

Social vs. Inanimate Preference:

The social test apparatus consisted of a 60 x 40 x 35(h) cm plywood chamber lined with white contact paper and a plexiglass bottom. The chamber was evenly divided into three sections by plexiglass partitions with a 5 x 8 cm opening in the center. On one side of the chamber, a non-familiar female mouse was placed in an inverted wire mesh cage (stranger mouse). An empty inverted wire mesh cage (inanimate object) was on the opposite side of the chamber. A weight was placed on the top of each of the cages to prevent the test mice from tipping the cage over. The test mouse was placed in the center chamber with the partitions closed off to the other chambers all allowed to acclimatize for ten minutes. At the initiation of the test, the partitions were removed and the mouse was allowed to freely explore all three chambers. Mice were video-recorded for ten minutes and the time spent in each chamber was recorded using ANY-maze software (Stoelting Wood Dale, IL) (688).

Preference for Social Novelty:

The preference for social novelty test immediately followed the social vs. inanimate preference test. In the chamber with the empty wire mesh cage (inanimate), a novel unfamiliar female mouse was place in the mesh cage (novel). The previous stranger mouse remained in the opposite chamber (familiar). Mice were video-recorded for ten minutes and the time spent in each chamber was recorded using ANY-maze software

(Stoelting Wood Dale, IL). The chamber was wiped down with 95% ethanol between each test mouse (688).

Inkblot:

Gait was evaluated by using inkblot analysis. Non-toxic ink was placed on the fore (red) and hind (black) paw of the mouse. The mouse was made to walk down a dark tunnel. The average length and width of the steps were measured.

RotaRod:

Motor deficits were evaluated by measuring latency to fall (180s max) on an accelerating (4-40 rpm over 200s) ENV-576M RotaRod (Med Associates, Geogia, VT). Two trials were conducted on one day with approximately two hours between trials. The average of the two trials was used in the analysis.

Light/Dark Box:

The light/dark box was a 60 x 40 x 35(h) cm plywood chamber with a plexiglass bottom and line with contact paper. The chamber was divided by a plexiglass partition with a 5 x 8 cm opening in the center. The light side was 40 x 40 cm and lined with white contact paper. The dark side was 20 x 40 cm, enclosed, and lined with black contact paper. Mice were placed in the light side and allowed to freely explore for ten minutes. ANY-maze software (Stoelting Wood Dale, IL) tracked the mice (689).

Morris Water Maze:

Spatial memory was assessed using the standard hidden platform Morris water maze. Mice were given four trials a day for five days. Each trial began from each of four random starting positions. Mice were given a maximum of 60 seconds to find the platform. If a mouse failed to find the platform after 60 seconds, it was lead there. Mice were allowed to remain on the platform for ten seconds before being placed in a 37^{o}C warming cage between trials. The intertrial interval was four minutes. 24 hours following the end of the hidden platform testing, the platform was removed and a probe trial was given for 60 seconds. Latency to first platform location and total number of platform crossings were recorded using tracking software (Ethovision, Noldus Information Technology, Leesbury, VA, USA).

Reverse Water Maze:

To measure resistance to change, the reverse Morris water maze was performed one week after the Morris water maze. The location of the platform was changed with respect to the original Morris water maze. Mice were given four trials a day for four days to learn the new location of the platform. 24 hours following the end of the hidden platform testing, the platform was removed and a probe trial was given for 60 seconds. Latency to first platform location and total number of platform crossings were recorded.

Vision Water Maze:

Vision was assessed using a visual Morris water maze. Upon completion of the reverse water maze, a white brick was placed on the platform to make it visible. Mice were given three trials to find the visible platform.

Statistical Analysis:

Statistical analyses were conducted using analysis of variance (ANOVA) followed by Tukey post-hoc comparisons to compare the results of the Tsc2f/+, Tsc2f/-, and Tsc2f/-;Cre genotypes. For social preference and social novelty, a t-test was conducted to examine the difference between time spend in the social and inanimate object chambers. Statistical significance is claimed when $p<0.05$. However, data reported with a $p<0.1$ is also reported as possibly relevant. Error bars are shown as standard error of the mean.

X-gal staining

For x-gal staining, *Rosa26* reporter mice were crossed with *Pcp2-Cre* mice. Mice were first anesthetized with 2.5% avertin and then transcardially perfused with PBS and then 4% PFA. Brains were extracted, post fixed for 1 hour in 4%PFA, then washed in 1x PBS overnight at 4^{o}C. Brains were then transferred to 30% sucrose overnight at 4^{o}C and then embedded in OCT and stored at -80^{o}C. Sections were sliced sagittaly at 18µm (3 per slide) using a cryostat. Slides were washed in Rinse Buffer (100mM sodium phosphate, pH7.3, 2mM $MgCl_2$) and then incubated in Rinse Buffer Plus (Rinse buffer plus 5mM potassium ferricyanide, 5mM potassium ferrocyanide, and 1mg/ml x-gal) for 2 hours at 37^{o}C. Slides were then rinsed in 1x PBS, couterstained with eosin, dehydrated, and coverslipped.

Results:

General Health Assessment:

Behavioral testing was conducted on control (Tsc2f/+), Tsc2f/-, and Tsc2f/-;Cre mice. At one month of age, the mice were given a physical examination (686). All mice were healthy and had normal reflexes (eye blink, ear twitch, forepaw reach, grasping, and whisker twitch). At six weeks of age, olfaction was assessed using a buried food test. The latency to find a buried food pellet was measured once a day for four days (Figure 6.1A). There was no difference in latency to find food demonstrating intact olfaction in all genotypes.

Since Pcp2-Cre expression also occurs in retinal bipolar cells (4), it was important to assess vision and retinal histology. We performed a vision dependent Morris water maze at three months of age to determine if the animals could navigate to a visible platform. The latency to locate the visible platform was measured across three trials (Figure 6.1B). All mice found the platform in a similar time demonstrating comparable visual acuity for the specific test. There were no histological abnormalities in the retinas of Tsc2f/f;Cre mice (see Chapter 4).

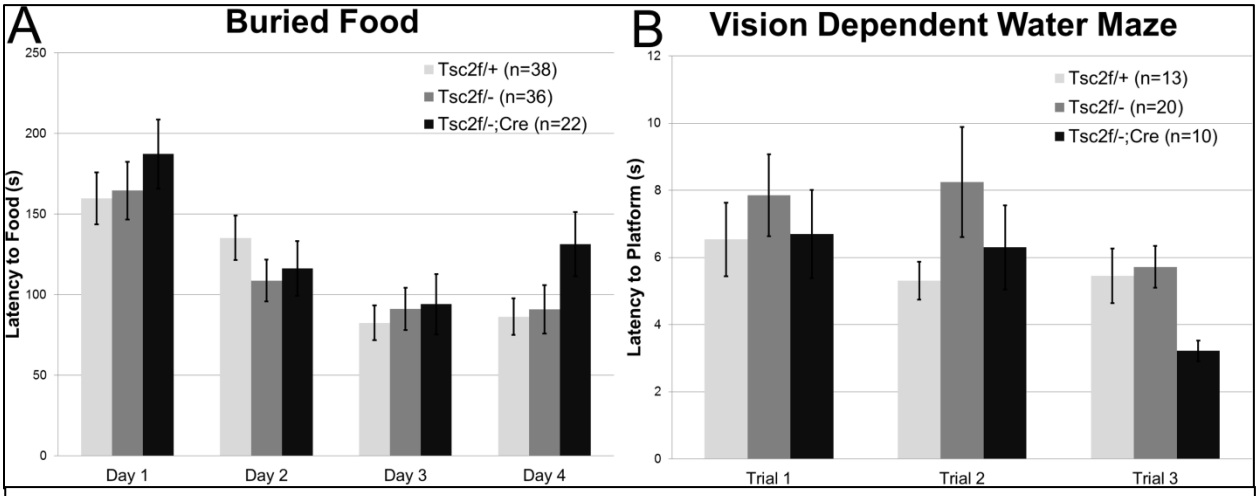
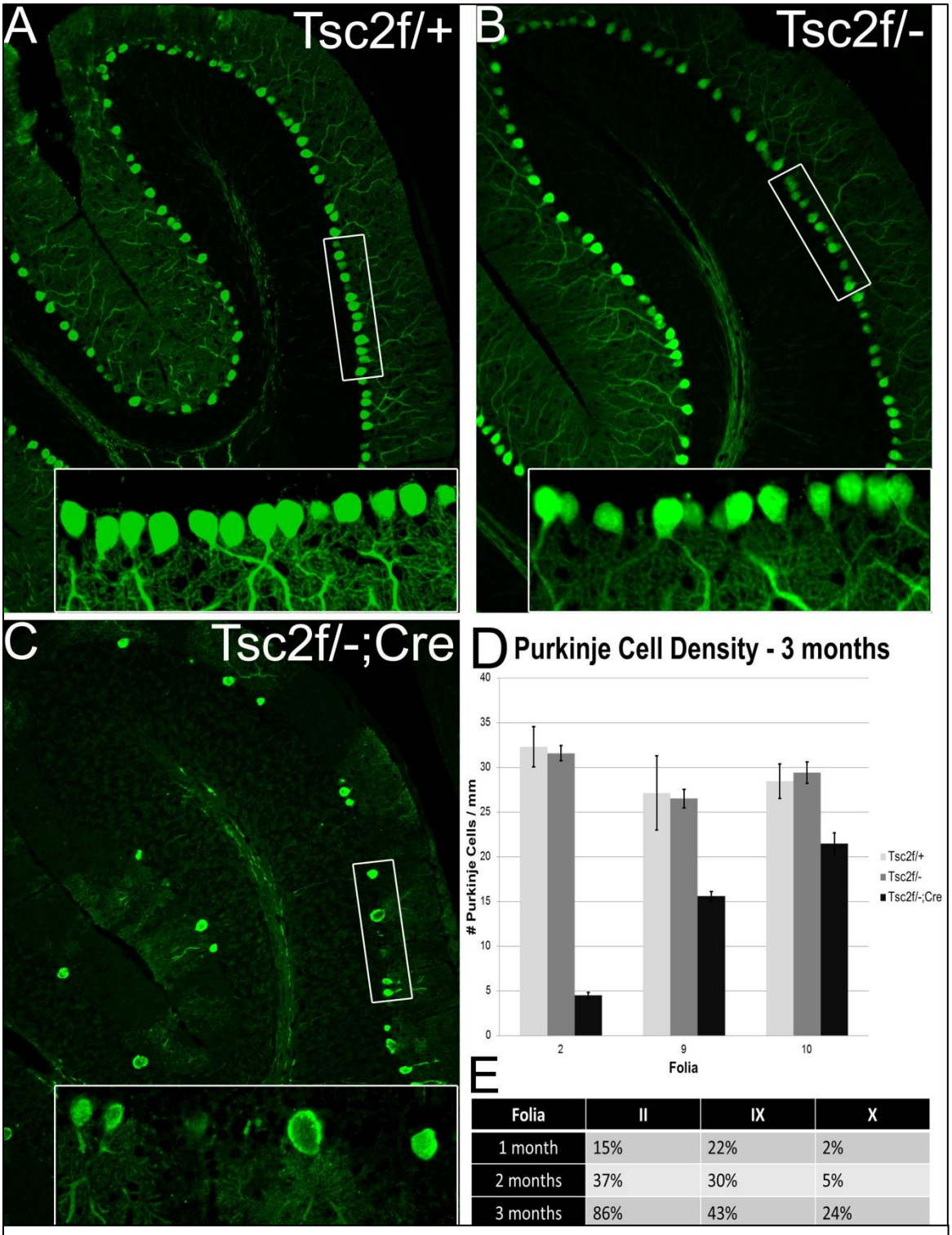


Figure 6.1: Assessment of olfaction and vision in Tsc2f/-;Cre mice. (A) Latency to locate buried food as a measure of olfaction. There was no significant difference in latency to the food across any of the trials. (B) Vision dependent water maze was conducted to determine the ability of the mice to see. There was no significant difference in latency to the visible platform across any of the trials.

Motor Function

Purkinje cell specific homozygous deletion of *Tsc2* caused Purkinje cell loss as described in Chapter 4 (see also Figure 6.2A-D). All behavior testing was performed between one and three months of age. The percent of Purkinje cell loss in the Tsc2f/-;Cre mice across this time frame is shown (Figure 6.2E). Interestingly, *Tsc2* haploinsufficiency did not affect Purkinje cell viability, as Tsc2f/- mice did not have any Purkinje cell loss at three months of age (Figure 6.2B). Gross motor coordination was examined using an open-field arena. There was no difference in average speed over a 30 minute interval in any of the groups (Figure 6.3A). At two months of age, gait analysis, using inkblot testing, indicated that Tsc2f/-;Cre mice had a slightly wider gait compared to controls ($p=0.079$)(Figure 6.3B) suggesting a mild ataxia. However, there was no significant difference in latency to fall among any of the groups on an accelerating RotaRod at two months of age (Figure 6.3C). Some of the mice, though, were re-tested on the RotaRod at five months of age, after more Purkinje cell loss had occurred. At five months of age, they did develop ataxia – having a significantly shorter latency to fall ($p=0.029$)(Figure 6.3D).



Folia	II	IX	X
1 month	15%	22%	2%
2 months	37%	30%	5%
3 months	86%	43%	24%

Figure 6.2: Loss of *Tsc2* causes Purkinje cell loss. (A-C) Calbindin staining at 3 months of age shows loss of Purkinje cells in the Tsc2f/-;Cre (C) compared to the Tsc2f/+ (A) and Tsc2f/- (B). (D) Quantitation of Purkinje cell density at 3 months across Folia 2, 9, and 10 shows Purkinje cell loss in Tsc2f/-;Cre mice but not in Tsc2f/- mice with respect to Tsc2f/+ mice. (E) Percent cell loss in Tsc2f/-;Cre mice relative to controls across the different folia and throughout the timespan of testing.

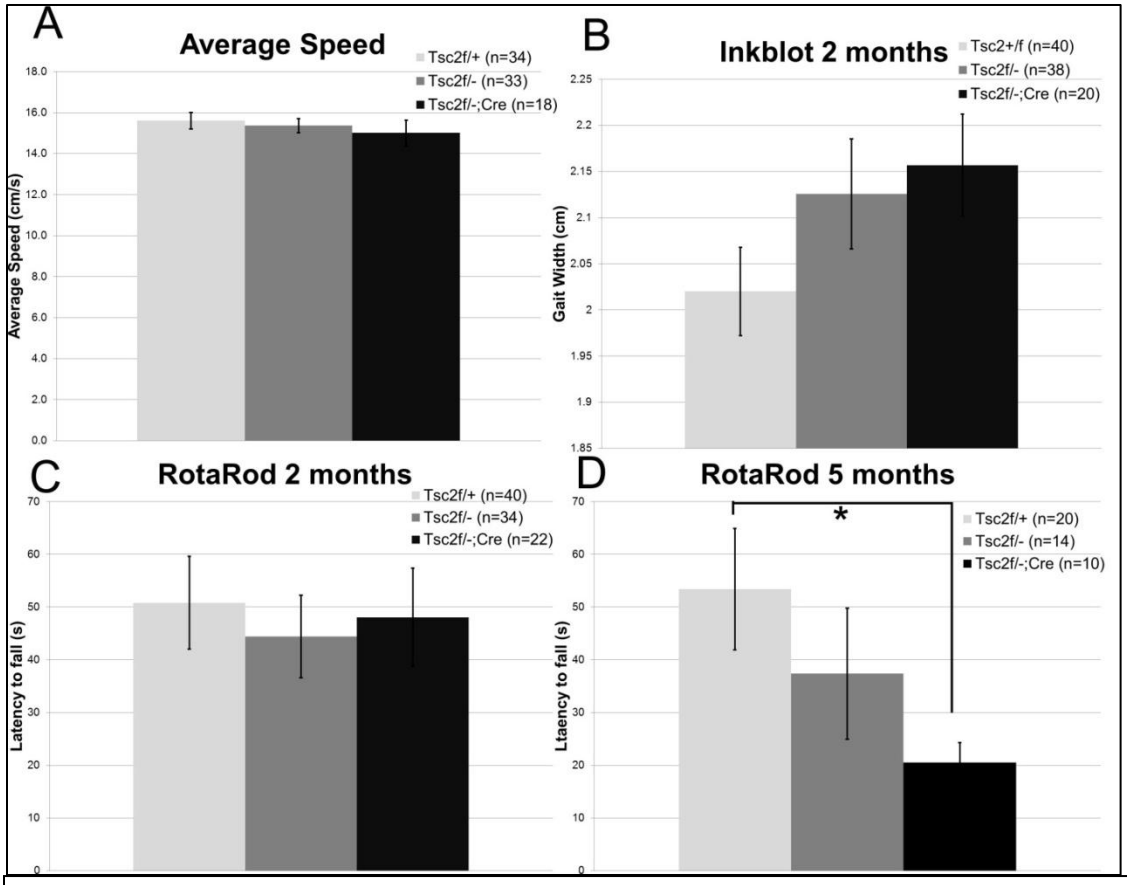


Figure 6.3: Motor function. (A) Average speed in an open-field did not differ among Tsc2f/+; Tsc2f/-; and Tsc2f/-;Cre mice. (B) Gait width in Tsc2f/-;Cre mice was slightly increased (p=0.079) compared to Tsc2f/+. (C) RotaRod performance at 2 months of age shows no difference in latency to fall among Tsc2f/+; Tsc2f/-; and Tsc2f/-;Cre mice. (D) RotaRod performance at 5 months of age indicates that Tsc2f/-;Cre have a significantly (p=0.029) shorter latency to fall that Tsc2f/+ mice, indicating a time-dependent development of ataxia.

Social Behavior Testing

Impaired social behavior is a prominent feature of ASD (133). A number of behavioral paradigms have been developed to assess social interactions in mice (690). One widely used assay is the three chambered apparatus which has been used to detect social deficits in multiple mouse models of autism (651, 653, 691). The three chambered apparatus was used to determine sociability and social novelty preference of the mice at two months of age. We tested males and females separately to detect sex specific differences similar to that seen in human ASD. The stranger mouse was female. Male Tsc2f/+ mice spent significantly more time ($p<0.001$) in the chamber with

the stranger mouse than the chamber with the inanimate object (Figure 6.4A). Male Tsc2f/- mice also spent significantly more time (p=0.047) in the chamber with the stranger mouse than the chamber with the inanimate object. Male Tsc2f/-;Cre mice, however, did not show a preference for the stranger mouse (Figure 6.4A), Tsc2f/+ female mice spent more time (p=0.057) in the chamber with the stranger mouse than the chamber with the inanimate object. However, both Tsc2f/- and Tsc2f/-;Cre females did not show a preference for either chamber (Figure 6.4B). These data suggest abnormalities in sociability in the Tsc2f/- mice that increase upon deletion of the second copy of *Tsc2* in Purkinje cells.

When social novelty was assessed, male Tsc2f/+ mice spent significantly (p=0.014) more time with the novel mouse than the familiar mouse (Figure 6.4C). Male Tsc2f/- mice showed a slight preference for the novel mouse than the familiar mouse, but this was not statistically significant. However, male Tsc2f/-;Cre mice spent about equal time with the novel mouse as with the familiar mouse (Figure 6.4C). Tsc2f/+ and Tsc2f/- female mice spent significantly (p=0.018, p=0.0008, respectively) more time with the novel mouse than with the familiar mouse. Female Tsc2f/-;Cre mice did not show significant preference for the novel mouse compared with the familiar mouse. The social novelty data are in agreement with the social preference testing, supporting a social behavioral deficit in Tsc2f/- mice that is exaggerated in Tsc2f/-;Cre mice.

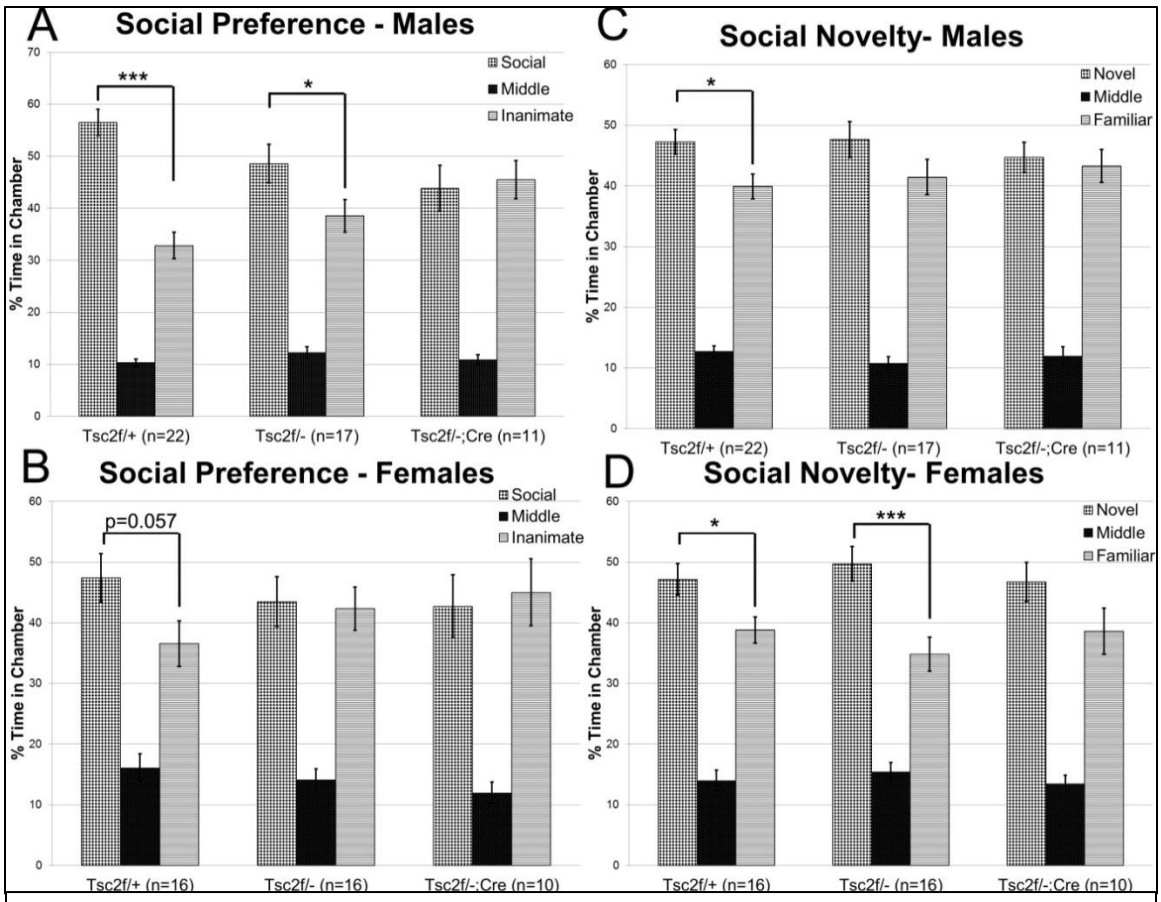


Figure 6.4: Social behavior deficits in Tsc2f/-;Cre mice. (A) Male Tsc2f/+ mice spent more time ($p<0.001$) in the social chamber than in the inanimate object chamber. Tsc2f/- spent more time ($p=0.047$) in the social chamber. However, Tsc2f/-;Cre mice spent equal time in both chambers. (B) Female Tsc2f/+ mice spent more time ($p=0.057$) with the stranger mouse than with the inanimate object. Conversely, both Tsc2f/- and Tsc2f/-;Cre mice spent equal time in both chambers. (C) Male Tsc2f/+ mice spent more time ($p=0.014$) more time with a novel mouse than with a familiar mouse. Tsc2f/- mice showed a slight preference (NS) for time spent with the novel mouse. However, Tsc2f/-;Cre mice spent equal time in both chambers. (D) Female Tsc2f/+ ($p=0.018$) and Tsc2f/- ($p=0.0008$) mice spent more time with the novel mouse than the familiar mouse. Tsc2f/-;Cre mice also spent slightly more time (NS) with the novel mouse than with the familiar mouse.

These results showed that loss of Purkinje cells (or loss of *Tsc2* in Purkinje cells) in a haploinsufficient environment led to social behavior deficits. A second hit event in a haploinsufficient environment mimics the LOH hypothesis for TSC patients. However, to isolate the effects of the Purkinje cells in the role of social behavior, Tsc2f/f;Cre (only Purkinje cells affected by loss of *Tsc2*) mice were examined along with their littermate

controls. In this case, male Tsc2f/+ mice showed a slight preference (NS) for time spent in the social chamber than time spent in the inanimate object chamber (Figure 6.5A). However, male Tsc2f/f;Cre mice spent equal time in both chambers (Figure 6.5A). When assessing social novelty, male Tsc2f/+ mice spent significantly (p=0.0029) more time in the chamber with the novel mouse than the familiar mouse (Figure 6.5B). However, Tsc2f/f;Cre mice spent equal time in both chambers (Figure 6.5B). These results suggest that it is the Purkinje cell pathology contributing to social deficits.

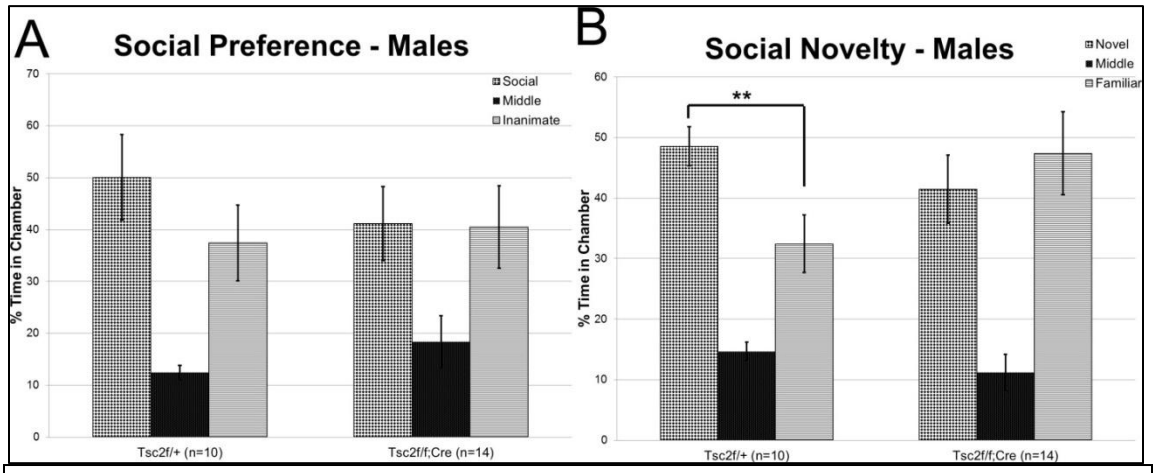


Figure 6.5. Social behavior deficits in Tsc2f/f;Cre mice. (A) Male Tsc2f/+ mice spent slightly more time (NS) in the social chamber than in the inanimate object chamber. However, Tsc2f/f;Cre mice spent equal time in both chambers. (B) Male Tsc2f/+ mice spent more time (p=0.0029) more time with a novel mouse than with a familiar mouse. However, Tsc2f/-;Cre mice spent equal time in both chambers.

To examine if ectopic Cre expression in ASD-associated brain regions could be contributing to social behavior deficits, we further examined Cre expression in the cerebral cortex using a Pcp2-ROSA26 cross. The ROSA26 mice have a *lacZ* gene flanked by a STOP sequence. When crossed with a Cre mouse, Cre expression deletes the STOP sequence and allows expression of the *lacz* gene (692). Therefore, we performed x-gal staining on PCP-ROSA mice at two months of age, the same age we detected social deficits. Examination of the frontal cortex and thalamus revealed no ectopic Cre expression. As indicated in the original report (4), there were some Cre positive cells in the dentate gyrus. As a positive control, x-gal staining was also detected in the Purkinje cells (Figure 6.6).

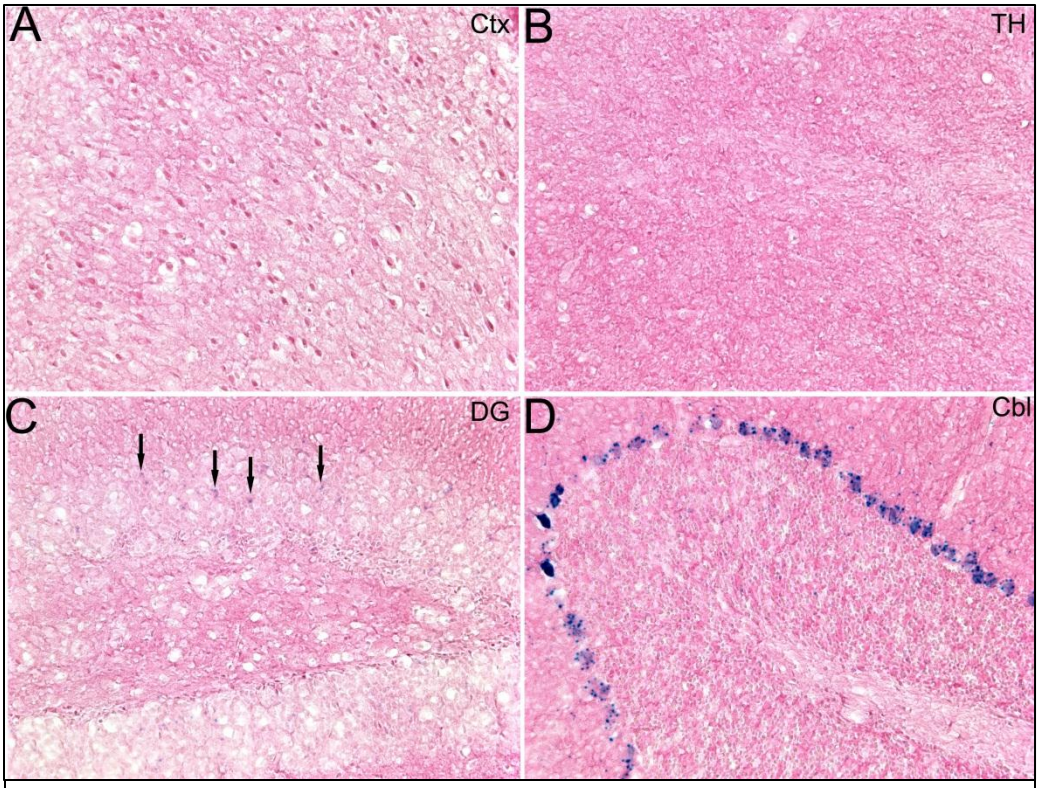


Figure 6.6: Detection of Cre expression. (A-B) No ectopic Cre expression was detected in the cortex (Ctx)(A) and thalamus (TH)(B) (C) Scattered Cre expressing cells were detected in the dentate gyrus (DG) and are labeled with arrows. (D) X-gal staining in Purkinje cells of the cerebellum (Cbl) are a positive control for PCP-2 Cre expression.

Response to Social Cues:

Another assay designed to detect autistic-like behavior in a mouse, is olfactory response to social cues (690). This tests aims to examine the habituation/dishabituation of both non-social and social cues. All mice showed habituation to repeated presentations of a scent, but dishabituation (heightened response) upon the presentation of a novel scent (Figure 6.7A). Male Tsc2f/-;Cre mice, however, showed a diminished preference for the first social scent (Cage 1A)($p=0.063$) than Ts2f/+ mice indicating a decreased response to social cues (Figure 6.7A-B). Female mice did not differ by genotype in this test (data not shown).

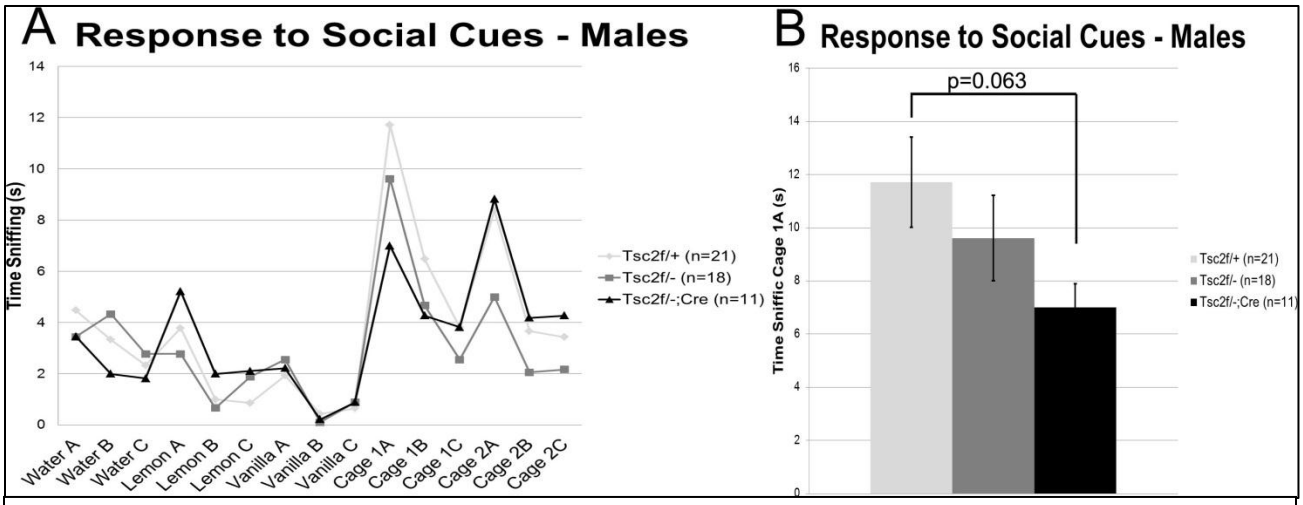


Figure 6.7: Response to social cues. (A) Mice were presented with non-social and social scents. Each scent was presented three separate times to measure habituation to the scent. All mice show habituation/dishabituation. Male Tsc2f/-;Cre mice showed decreased preference for the first social scent (Cage1A)(p=0.063) compared to controls. (B) Different visual representation of response to first social scent, isolating just that response and indicating standard error of the mean.

Repetitive Behavior and Anxiety

Repetitive behavior is a well described feature of ASD (133). The number of marbles that a mouse will bury in a specific time period is an established assay for repetitive behavior (685). We determined the number of marbles buried in a 30 minute period by male and female mice of all genotypes. Male Tsc2f/-;Cre mice buried significantly more marbles than either Tsc2f/+ (p=0.042) or Tsc2f/- (p=0.016) mice (Figure 6.8A). Similarly, female Tsc2f/-;Cre mice also buried more marbles than either Tsc2f/+ (p=0.070) or Tsc2f/- (p=0.028) mice (Figure 6.8A). There was no difference between Tsc2f/+ and Tsc2f/- of either sex. These data show an increase in repetitive behavior associated with the loss of *Tsc2* in Purkinje cells.

Anxiety levels are often increased in patients with ASD (142-144). To determine anxiety levels, mice were place in an open-field arena and the percentage of time spent in the middle was determined. Anxious animals spent more time along the perimeter of the chamber rather than the middle. Male Tsc2f/-;Cre mice spent slightly (NS) more time in the middle of the chamber than Tsc2f/+ and Tsc2f/- (Figure 6.8B), possibly suggesting decreased anxiety levels. Conversely, female Tsc2f/-;Cre mice spent slightly less time (NS) in the middle than Tsc2f/+ and Tsc2f/- mice, possibly suggesting increased anxiety levels (Figure 6.8B). These data suggest sex-specific differences in anxiety-related behaviors.

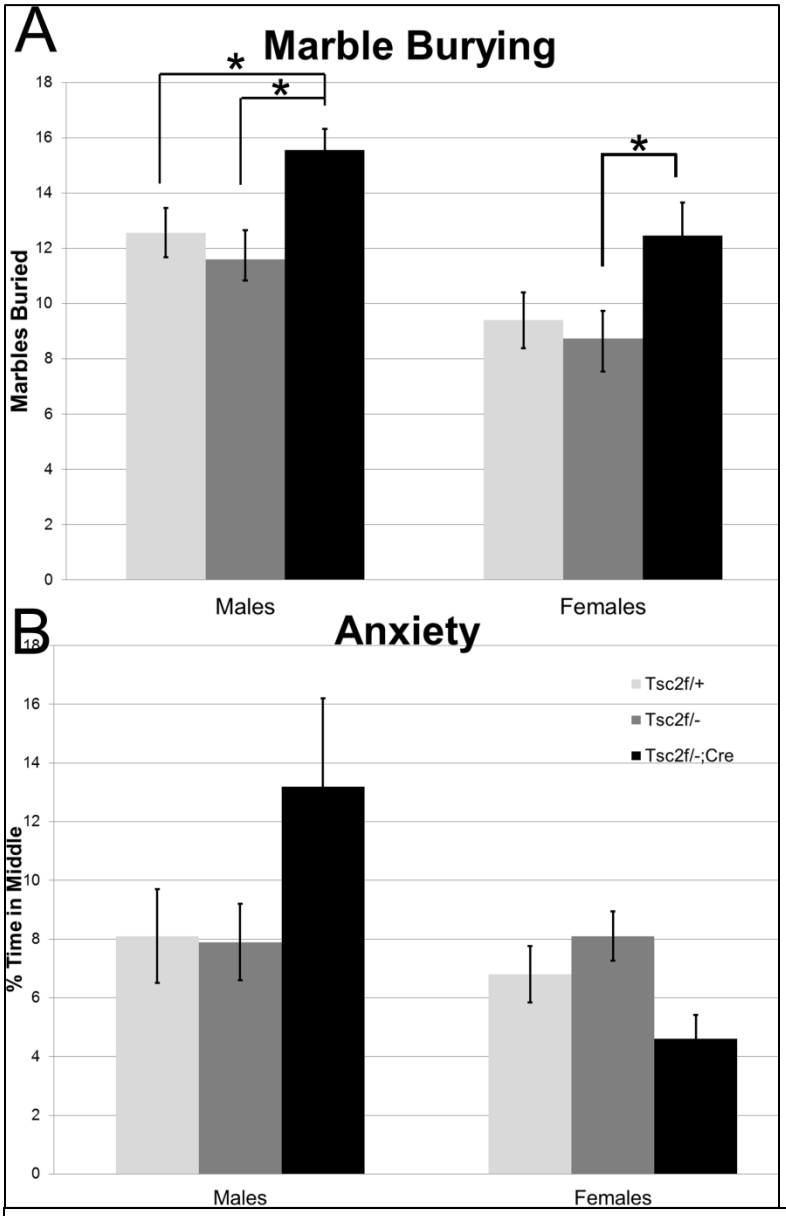


Figure 6.8: Repetitive behaviors and anxiety. (A) Male Tsc2f/-;Cre (n=11) mice buried significantly more marbles than either Tsc2f/+ (n=23) (p=0.043) or Tsc2f/- (n=20) (p=0.016) mice. Female Tsc2f/-;Cre (n=11) mice buried more marbles than either Tsc2f/+ (n=20) (p=0.070) or Tsc2f/- (n=19) (p=0.028) mice. (B) In an open-field arena, male Tsc2f/-;Cre (n=7) mice spent slightly (NS) more time in the middle than either Tsc2f/+ (n=17) or Tsc2f/- (n=17) mice. Female Tsc2f/-;Cre (n=11) mice, however, spent less time in the middle (NS) than either Tsc2f/+ (n=18) or Tsc2f/- (n=16) mice.

Spatial Learning and Memory

There is a high association of intellectual disability and ASD, and the severity of ASD tends to associate with the severity of intellectual disability (693). Therefore, to assess for deficits in spatial learning and memory, mice were trained on a Morris water maze. Tsc2f/-;Cre mice did not show any deficits in spatial learning over a five day training interval (Figure 6.9A). One aspect of restricted behaviors often seen in patients with ASD is resistance to change (694, 695). To test this, we measured reversal learning on the Morris water maze by changing the location of the hidden platform after the acquisition phase. Tsc2f/-;Cre mice did not show any deficits in reversal learning (Figure 6.9B-C).

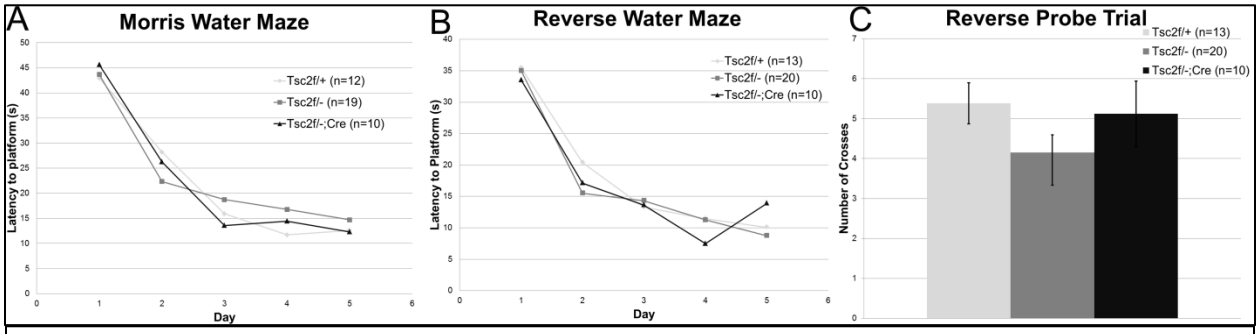


Figure 6.9: Morris water maze assessment of spatial learning and reversal learning. (A) Tsc2f/+, Tsc2f/-, and Tsc2f/-;Cre mice did not show any differences in spatial learning. (B) There were no differences in reversal learning once the platform was moved. (C) A probe trial was performed 24 hours after the reverse water maze and did not show any difference among Tsc2f/+, Tsc2f/-, and Tsc2f/-;Cre mice.

Summary

A summary of the major findings is listed in Table 6.2.

Table 6.2.

Behavior	Test	Tsc2f/-	Tsc2f/-;Cre
Social Behavior	Social Preference	Mild	Yes
	Social Novelty	Mild	Yes
Repetitive Behavior	Marble Burying	No	Yes
Anxiety	Open-field	No	Sex specific

Discussion:

Between 17%-60% of patients with TSC have ASD (21, 237, 238), a much higher prevalence than in the general population. This high comorbidity underscores the importance of understanding how mutations in either *TSC1* or *TSC2* are linked to the ASD phenotype. Genetically altered mice have been important tools in dissecting out the link between these two disorders (696-700). In this chapter, the behavior of Tsc2f/-;Cre mice was characterized. The results demonstrate a novel mouse model of TSC with ASD-associated behaviors. More importantly, this data supports the hypothesis that loss of heterozygosity of *Tsc2* in cerebellar Purkinje cells and/or frank Purkinje cell loss contributes to ASD-like behavior.

Before behavior testing was initiated, all mice were assessed for general health. None of the mice were noted to have impaired reflexes or senses. Interestingly, despite displaying Purkinje cell loss, Tsc2f/-;Cre mice did not show evidence of significant ataxia. As presented earlier (see Chapter 4), Tsc2f/f;Cre mice did show signs of ataxia at three months of age. Purkinje cell loss progresses with age, so perhaps RotaRod deficits only occur after a threshold of Purkinje cell loss. Supporting this hypothesis, some of the Tsc2f/-;Cre mice were retested at five months of age with significant differences noted on the RotaRod, suggesting a progression of motor coordination abnormalities. However, even at two months of age, inkblot results were somewhat suggestive of a mild ataxia in Tsc2f/-;Cre mice. Other, more sensitive analysis, like the Catwalk system (an automated apparatus for sensitive quantitative gait analysis)(701) might be better able to detect subtle differences that may be present in Tsc2f/-;Cre mice at two months of age.

Mild social deficits were detected in heterozygous Tsc2f/- mice suggesting that haploinsufficiency of *Tsc2* is sufficient to cause a behavioral phenotype. Many other behavioral deficits have been reported in mice haploinsufficient for *Tsc1* or *Tsc2* (696-698, 700). Goorden et al. detected social behavioral deficits in a $Tsc1^{+/-}$ model even in the absence of seizures and cerebral lesions (700). It is known, however, that patients with mutations in *TSC2* have a more severe neurological phenotype including an increased risk for autism (26, 702). Therefore, it is important to note that animals haploinsufficient for *Tsc2* showed social behavioral deficits when combined with seizures (698) or gestational immune activation (696). The association of haploinsufficiency of

Tsc1/2 and behavioral deficits is compelling, though the precise cellular mechanisms remain obscure. Haploinsufficiency of *Tsc1* alters dendritic spine structure in vitro by increasing dendritic length and decreasing spine density (703), possibly leading to altered cellular input. Also, haploinsufficiency of *Tsc2* leads to growth cone collapse and subsequent abnormalities in axonal pathfinding (704)- leading to altered cellular output. These effects could possibly induce social behavioral deficits. Furthermore, complete loss of *Tsc1/2* leads to cell migration abnormalities and reduced myelination (450, 705-707). Haploinsufficiency might cause subtle abnormalities in the position or signaling of neurons, possibly affecting connectivity and leading to abnormal behavior.

Contradictory to the data presented here, Ehninger et al. reported that $Tsc2^{+/-}$ mice do not have social deficits in the three chambered apparatus (696, 708). Their model, however, was on a pure C57BL/6 background. Interestingly, Goorden et al. did detect social deficits in a C57BL6 $Tsc1^{+/-}$ model (700). Perhaps, however, there are modifier genes in the different strains that contribute to the behavioral effect. This would not be unexpected, as ASD is not 100% penetrant in patients with TSC, suggesting additional influences in the development of behavioral deficits.

Behavioral deficits were detected in a novel *Tsc2* dominant/negative mouse model. A dominant negative mutation is more severe than just the haploinsufficient mutation. In this case, Tsc2 could still bind to Tsc1, but had a mutated C-terminus, so it could not inhibit Rheb. The *Tsc2* dominant/negative mutation was driven in all tissues by the cytomegalovirus promoter (709). These mice showed increased anxiety (710) and social behavioral deficits (699). Noteworthy is that these dominant negative animals had no histologic cortical defects, but cell aggregates in the cerebellum (709). However it is not clear if the cerebellar aggregates had anything to do with the phenotype.

Since there is mounting evidence that cerebellar abnormalities play a role in ASD, particularly Purkinje cell loss (186, 341, 360, 366, 711, 712), Tsc2f/-;Cre mice with Purkinje cell specific *Tsc2* loss were assessed for ASD-like deficits. Tsc2f/-;Cre mice loose the remaining copy of *Tsc2* in all Purkinje cells by one month of age, when Purkinje cells begin to progressively die. Tsc2f/-;Cre mice showed more severe social deficits than the haploinsufficient Tsc2f/- animals. These data reveal a role for *Tsc2* in Purkinje cell survival that is important for normal murine sociability. The mechanism of this observation is unclear and warrants further study. The cerebellum is thought to be

involved in higher order processes similar to its role in motor coordination. It has been postulated that in order to decode someone else's actions (like in social behavior), sub-threshold activation of your own actions is required (427, 713). This behavior is believed to be modulated by the connections of the cerebellum to the prefrontal cortex (432, 433, 714). Purkinje cells are the sole inhibitory output of the cerebellum, synapsing on the deep cerebellar nuclei (715). The deep cerebellar nuclei then relay projections through the thalamus to various cortical regions including the prefrontal cortex, a region important in autism pathology (438, 716-718). This circuit is altered or abolished with loss of *Tsc2* in Purkinje cells or frank Purkinje cell loss. It is unclear if complete loss of Purkinje cells per se and/or dysfunctional *Tsc2*-null remaining Purkinje cells are important for this autistic-related phenotype. The assessment of sociability at different time-points may reveal whether dysfunctional Purkinje cells and/or Purkinje cell loss per se cause the behavioral deficits.

Cell specific transgenic mice can often be "leaky." Through the PCP2 promoter is predominantly active in Purkinje cells, the original characterization of these animals showed Cre expression in the cortex and hippocampus (4). To assess the specificity of Cre expression in these studies, the Pcp2-Cre mice used in these experiments were crossed with the ROSA26 reporter mice. The results were congruent with the original report of the Pcp2-Cre mouse, indicating ectopic Cre expression in the dentate gyrus (4). However, we further explored other areas specifically implicated in ASD-related behaviors, namely: thalamus and prefrontal cortex (330-334, 444). In these regions, we did not find β-galactosidase-positive cells, suggesting that Cre recombinase was not active. Though we cannot exclude the possibility that ectopic Cre expression in the cortex may be contributing to ASD-like behaviors in the mice, Cre expression is largely focused in the Purkinje cells.

The behavioral deficits in Tsc2f/-;Cre mice are associated with *Tsc2* haploinsufficiency in all cells and Purkinje cell loss. To test whether Purkinje cell loss in a wildtype background could also lead to social deficits, Tsc2f/f;Cre mice were subjected to behavioral testing. Tsc2f/f;Cre mice only lose *Tsc2* in Purkinje cells, and all remaining cells are Tsc2f/f which is equivalent to $Tsc2^{+/+}$ (719). Tsc2f/f;Cre mice also demonstrated social deficits, suggesting that Purkinje cell loss is sufficient to induce social deficits. However, the Tsc2f/+ littermates also did not show significant social preference (though they did retain preference for social novelty). One plausible

explanation for this behavior is the slight strain difference between Tsc2f/-;Cre mice and Tsc2f/f;Cre mice. Tsc2f/-;Cre mice and respective controls are approximately 74% C57BL/6J and 26% 129X1/SvJ. Tsc2f/f;Cre mice and respective controls are approximately 62% C57BL/6J and 38% 129X1/SvJ. While C57BL/6J mice are noted to have normal social behavior, 129 mice show abnormalities in social behavior tasks (720). Therefore, Tsc2f/+ mice with increased percentage of 129X1/SvJ stain do not show significant social preference.

However, replication of behavioral results are important to establishing their validity (690). The continued presence of behavioral deficits in a different environment, by a different experimenter, decreases the likelihood that the results could have occurred by chance. Therefore, another group of Tsc2f/-;Cre mice should be tested, perhaps even on a pure C57BL/6J background.

Another test for social behavior in mice is nest-building activity (690). Mice are social creatures that build a nest and sleep together in their home cage. Therefore, the nest building test performed here may not be as relevant to social behavior as desired, since the mice were removed from their home cage and isolated for four hours to build a nest. No differences were detected between Tsc2f/+ and Tsc2f/-;Cre mice.

Another core component to autistic behavior are deficits in communication (133). One means of communication for mice is through olfactory cues (690). Upon examining response to olfactory cues in Tsc2f/-;Cre, we noted that they had a decreased preference for the first presentation of a social cue (however, general olfaction was unaffected). This may indicate a deficit in olfactory communication, however, they did perform the same as Tsc2f/+ mice upon presentation of a second social cue. Further testing will be required to better assess whether there is a deficit in response to social cues.

Another test for communication in mice is to examine ultrasonic vocalizations (690). Ultrasonic vocalizations have been examined by Young et al. on $Tsc2^{+/-}$ mice (697). They found that pups from $Tsc2^{+/-}$ mothers had altered ultrasonic vocalizations. Another intriguing study on ultrasonic vocalizations, suggested a deficit in a knockin mutation of Foxp2 (associated with language abnormalities), where the mice also had loss of Purkinje cells (721). Therefore, ultrasonic vocalization testing should also be performed in Tsc2f/-;Cre mice.

Repetitive behavior is another hallmark of ASD (133). We detected increased marble burying activity in Ts2f/-;Cre mice, indicating increased repetitive behaviors. Interestingly, haploinsufficiency of *Tsc2* was not sufficient to cause an increase in repetitive behaviors, suggesting that either complete loss of *Tsc2* in Purkinje cells and/or Purkinje cell loss is required for this phenotype. Interestingly, nervous mice, another model of Purkinje cell loss, show increased perseverative behaviors of nose poking (722). Lurcher mice also demonstrated increased repetitive behaviors through marble burying activity (683). The role of the cerebellum in repetitive behaviors may lie in its role to coordinate motor functions. Since dysfunction and/or loss of GABAergic Purkinje cells leads to decreased inhibitory efferents to the deep cerebellar nuclei and consequently other parts of the brain, this could lead to behavioral disinhibition. It has been hypothesized that autistic patients are constantly in a state of overstimulation (723). Therefore, performing repetitive behaviors may have a calming effect on this overstimulated state (145).

Although autism occurs in a 4:1 male female ratio in the general population (724), TSC-associated autism occurs in a 1:1 male female ratio (21, 725-727). However, a genotype-phenotype study found that male TSC patients had more severe neurological findings (26). In our mouse model correlate, we find autistic-like behaviors in both male and female mice. However, the male mice show the greater increase in both repetitive behaviors and social deficits suggesting that gender does influence the severity of these characteristics. How sex affects the neurologic phenotypes remains unclear.

Though not a core feature of ASD, increased anxiety levels are often noted in ASD patients (142-144). Unexpectedly, male Tsc2f/-;Cre mice were less anxious than Tsc2f/+ mice. However, intriguingly, female Tsc2f/-;Cre mice had increased levels of anxiety. These results were not statistically significant and therefore need to be interpreted with caution. Females are often noted to have an increased risk for developing anxiety disorders than men (728-730). In fact, cortical and subcortical regions of the brain involved in stress responses have been reported to be sexually dimorphic (731). What is intriguing is that gender seems to mediate this effect in the context of the *Tsc2* mutation. This sex-specific effect may be due to hormonal differences between genders and merits further exploration. Another interesting dichotomy is that anxiety levels are increased following a dominant/negative mutation of *Tsc2* (710), but decreased in the Lurcher model of Purkinje cell loss (682).

Finally, we show that there are no learning deficits in Tsc2f/-;Cre mice. This finding was a bit surprising given the reports of learning deficits in *Tsc2*$^{+/-}$ mice (708). Ehninger et al. measured time spent in quadrant as an indicator of learning deficits, while we examined latency to the platform. Perhaps different analyses would yield different results. Another possible explanation behind these conflicting results might be due to strain differences of the mice. Ehninger et al. conducted their studies on a C57BL/6NCrl background. Our studies, however, are on a mixed C57Bl6/129 background. Therefore, there are likely modifier genes contributing to this effect. Interestingly, Eker rats, a heterozygous Tsc2$^{+/-}$ based model, also show no evidence for learning deficits (698).

In summary, I demonstrate that deletion of *Tsc2* in Purkinje cells, leads to social behavior deficits and increased repetitive behaviors. This provides a novel mouse model of TSC-associated autism that will allow for the exploration of cerebello-cortical projections and their ability to modulate autistic-like behaviors. This mouse model also paves the way for potential therapeutic targets aimed at preventing Purkinje cell degeneration and therefore ameliorating behavioral deficits.

Chapter Seven:

Rescue with the mTORC1 Inhibitor Rapamycin

Introduction:

Easter Island, located in the South Pacific Ocean, is best known for its famous stone head statues named moai, which are categorized as the Eighth Wonder of the World. In 1965, on an excursion to Easter Island, scientists brought back a soil sample containing a bacterium called *Streptomycete hygroscopicus* (732). These bacteria secreted an anti-fungal macrolide. As Easter Island, in the native language, is known as Rapa Nui, this new macrolide was named rapamycin. And truly rapamycin has added a new wonder to the world.

The target of rapamycin is mTORC1 (mammalian target of rapamycin complex 1) (733, 734). Rapamycin forms a complex with FKBP12, which binds to the FRB domain of mTORC1 (735). This leads to the dissociation of mTOR and Raptor, ultimately preventing the phosphorylation of mTORC1's downstream targets (48, 736). Because of the broad scope of mTORC1 in the cell, rapamycin has been used for many indications.

In 1977, scientists discovered that rapamycin inhibited the immune system (737). Because it had a lower nephrotoxicity than previously used cyclosporines (738), rapamycin was clinically approved in 1999 for use as an immunosuppressant for kidney transplants. As an immunosuppressant, rapamycin has also been helpful for the treatment of autoimmune disorders such as rheumatoid arthritis (739, 740). In 1983, it was found that rapamycin could inhibit glioma (741), which has led to numerous clinical trials for various cancers (742-744) and the FDA approval of everolimus (a rapamycin derivative) for the treatment renal cell carcinoma.

Amazingly, rapamycin has also shown promise in neurodegenerative diseases such as Alzheimer's (745, 746), Parkinson's disease (747, 748), and Huntington's disease (749, 750). These effects are largely due to rapamycin's ability to increase autophagy thereby degrading aggregated or misfolded proteins. However, there is also evidence that rapamycin's effects in these diseases may be due to decreased protein translation.

It has also been found to increase longevity in yeast (751), *Drosophila* (752), and mice (753). Treatment in genetically heterogeneous mice was even initiated late in life, at 600 days old, but still had beneficial effect (753). However, inhibiting autophagy in the yeast prevents rapamycin's ability to extend lifespan, suggesting that increased autophagy is one of the mechanisms that rapamycin utilizes for increased longevity

(754). In *Drosophila*, it was found that both induction of autophagy and decreased protein translation were required for longevity extension (752).

The effects of rapamycin are so far-reaching that there are currently 1306 clinical trials that involve rapamycin (755)! Therefore, when the *TSC1* and *TSC2* genes were found to be key regulators of the mTORC1 pathway, rapamycin was a logical potential pharmacologic treatment for TSC, a disease characterized by overactive mTORC1.

One of the original human studies, conducted by Franz et al., investigated the effect of rapamycin in four TSC patients with a subependymal giant cell astrocytoma (SEGA) (756). All patients responded to rapamycin therapy with regression of the SEGA. This study, along with others led to the FDA approval of everolimus (a derivative of rapamycin) for the treatment of SEGAs associated with TSC in 2010.

However, Franz et al. also noted that one patient developed an acneiform rash and aphthous ulcers and wished to be removed from treatment. Although her SEGA had regressed while on treatment, cessation of rapamycin allowed the SEGA to regrow. Importantly, once she was placed back on rapamycin, the tumor again shrank (756). This study was the first to indicate that rapamycin therapy would likely need to be continued for lasting clinical effects. Thankfully, this particular patient did not again develop the side effects noted earlier (756). Other side effects of rapamycin treatment include: anemia, hyperlipidemia, immunosuppression, impaired wound healing, interstitial pneumonitis, and thrombocytopenia (757, 758).

Since *TSC2* interacts with *TSC1* to inhibit the mTORC1 pathway, phenotypes observed in loss of *TSC2* might be rescued through the pharmacologic inhibition of mTORC1. Studies of the effectiveness of rapamycin in mouse models of TSC have shown its efficacy for improving astrogliosis, cell size, epilepsy, kidney cysts, learning deficits, median survival, and myelination (708, 759-763). However, postnatal rapamycin treatment was unable to rescue cortical disorganization and neuronal dysplasia (759, 763). These studies have led to clinical trials in patients with TSC for the treatment of: angiofibromas, angiomyolipomas, epilespsy, and LAM (755).

However, with the exception of learning and memory (708, 763), the role of rapamycin in behavior is not as well explored. Furthermore, since limitations in the ability of rapamycin to rescue all the effects of TSC-associated lesions, particularly

neuronal dysplasia (759), and since rapamycin has been shown to enhance susceptibility to apoptosis in the context of cancer (742, 764, 765) we set out to examine the effect of rapamycin treatment on the histology and behavior of Tsc2f/f;Cre and Tsc2f/-;Cre mice.

Materials and Methods:

Mouse model, histology, antibodies, quantitative analysis, and statistical analysis were performed the same as in Chapters 4 and 5. Behavior testing and associated statistical analysis was performed the same as in Chapter 6.

Rapamycin Treatment:

For histological analysis, rapamycin (MP Biomedicals, Solon, OH) was dissolved in methanol and stored in a 1mg/ml stock solution at -20^{o}C. For behavioral analysis, rapamycin (MP Biomedicals, Solon, OH) was dissolved in ethanol and stored in a 1mg/ml stock solution at -20^{o}C. A working solution was diluted in PBS before each use. Beginning at postnatal day 10 (P10), mice were given IP (intraperitoneal) injections three times per week with 2mg/kg rapamycin. Injections continued until 1 month, 3 months, or completion of behavior testing.

Results:

Rapamycin rescues Purkinje cell death:

After treating mice with rapamycin until three month of age, we found significant rescue of Purkinje cell death in treated Tsc2f/f;Cre mice compared to untreated mice ($p=0.004$)(Figure 7.1A-E). Furthermore, we also found that rapamycin decreased the size of the normally enlarged Purkinje cells in treated Tsc2f/f;Cre mice compared to untreated mice ($p=0.08$). This rescue, however, was not complete, as treated Tsc2f/f;Cre mice still had larger Purkinje cells than treated controls ($p=0.03$)(Figure 7.1F). To determine if functional benefit occurred after Purkinje cell rescue, we examined the mice for motor deficits using the inkblot testing. Rapamycin improved gait width, suggesting intact motor function (Figure 7.1G).

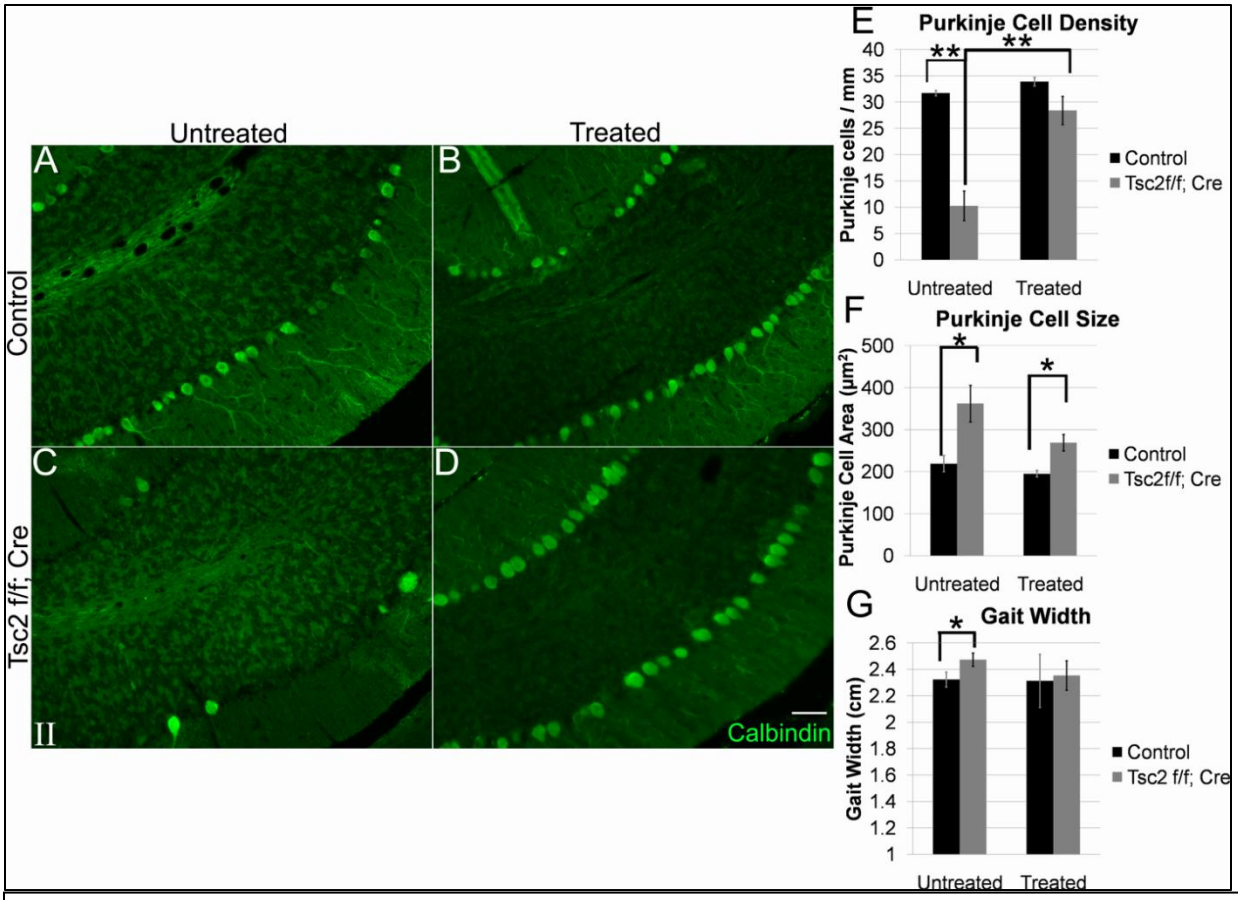


***Figure 7.1:** Rapamycin treatment rescues Purkinje cell degeneration. (A-D) Calbindin staining shows rescue of 3 month treated Tsc2f/f;Cre mice (D) compared to 3 month untreated Tsc2f/f;Cre mice (C). Treated control mice are shown in (B) and untreated control mice are shown in (A). Scale bar, 50µm. (E) Quantitation of Purkinje cell density. Untreated Tsc2f/f;Cre mice lost significantly more Purkinje cells than untreated control mice (**p=0.001). Rapamycin treatment prevented Purkinje cell loss in Tsc2f/f;Cre mice (n=4); **p=0.004). (F) Quantitation of cell size. Untreated Tsc2f/f;Cre mice had significantly larger Purkinje cells than control mice (n=3; *p=0.04). Rapamycin treatment partially rescued the enlarged Purkinje cell phenotype compared with untreated Tsc2f/f;Cre mice (p=0.08). This rescue, however, was only partial as treated Tsc2f/f;Cre mice still had larger Purkinje cells than treated control mice (n=4; *p=0.03). (G) Untreated Tsc2f/f;Cre mice (n=29) also had a wider based gait than control mice (n=21; p=0.05). Rapamycin treatment partially rescued the gait defect (n=3; p>0.05).

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In accordance with the partial rescue in Purkinje cell size, we detected mTORC1 activity by immunohistochemistry to pS6. pS6 levels in untreated Tsc2f/f;Cre mice were higher than control mice (Figure 7.2A-B). Rapamycin treatment decreased pS6 levels in Tsc2f/f;Cre mice, though not to the level of controls (Figure 7.2C-D).

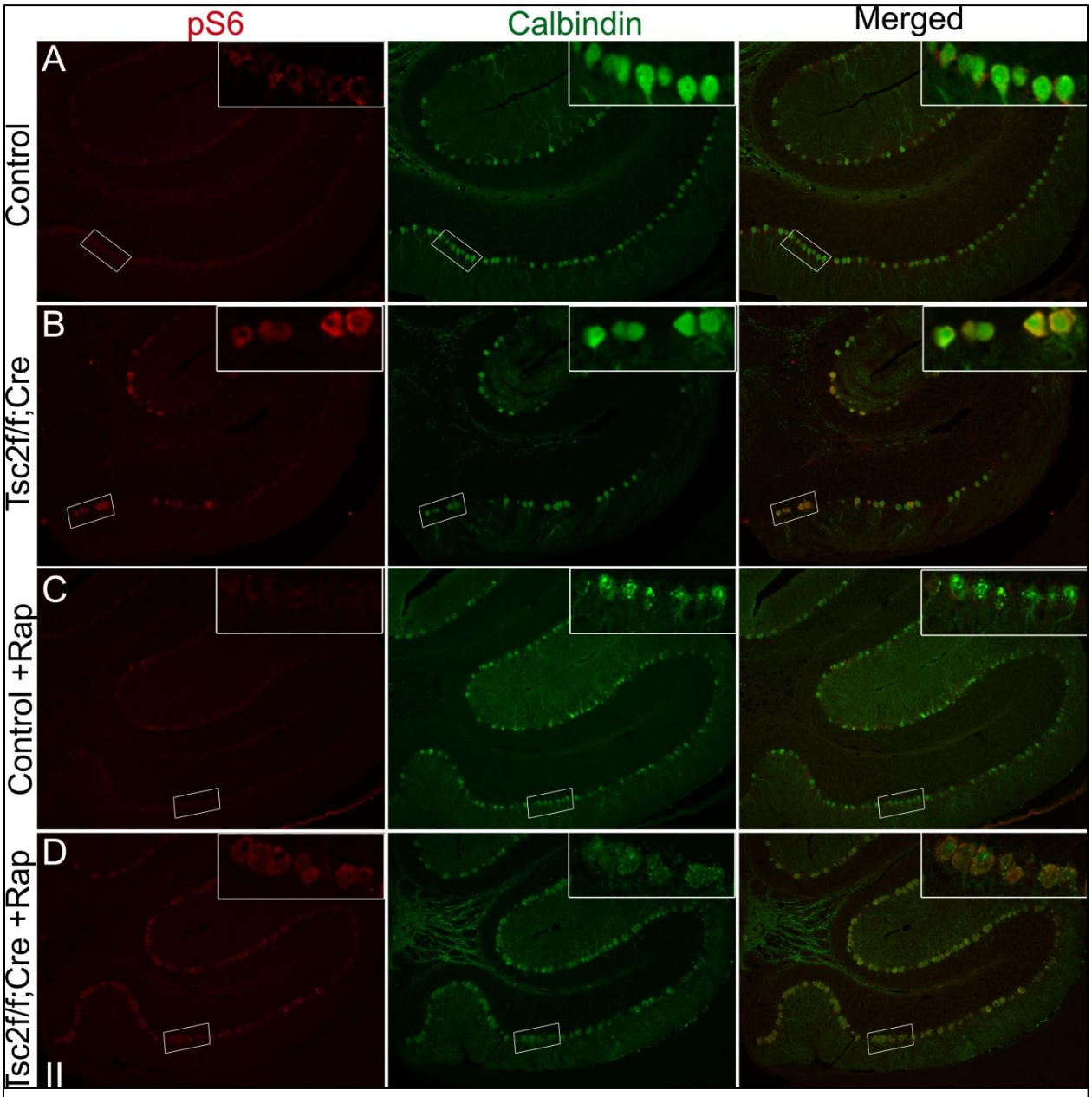

Figure 7.2: Rapamycin reduces mTORC1 activity in Tsc2f/f;Cre mice. (A-D) pS6 expression in a treated Tsc2f/f;Cre mouse (D) shows decreased mTORC1 activity compared to an untreated Tsc2f/f;Cre mouse (B). pS6 levels, however, are still higher in treated Tsc2f/f;Cre mice than in control mice (A,C). Purkinje cells are colabeled with Calbindin. Roman numeral indicates folia.

Rapamycin rescues other downstream consequences of mTORC1 activation

To examine the effects of rapamycin treatment on Tsc2f/f;Cre mice, we also reexamined the pathology that was presented in Chapters 4 and 5. Rapamycin treatment resulted in decreased expression of the inositol 1,4,5-triphosphate receptor

(IP_3R), indicating restored calcium levels (Figure 7.3). Rapamycin also reduced activation of the ER stress response, as there were fewer CHOP positive cells in treated Tsc2f/f;Cre mice than untreated Tsc2f/f;Cre mice (Figure 7.4). Therefore, it is not surprising that rapamycin treatment also reduced the levels of ubiquitin in Purkinje cells (Figure 7.5), indicating a decreased need for misfolded protein degradation.

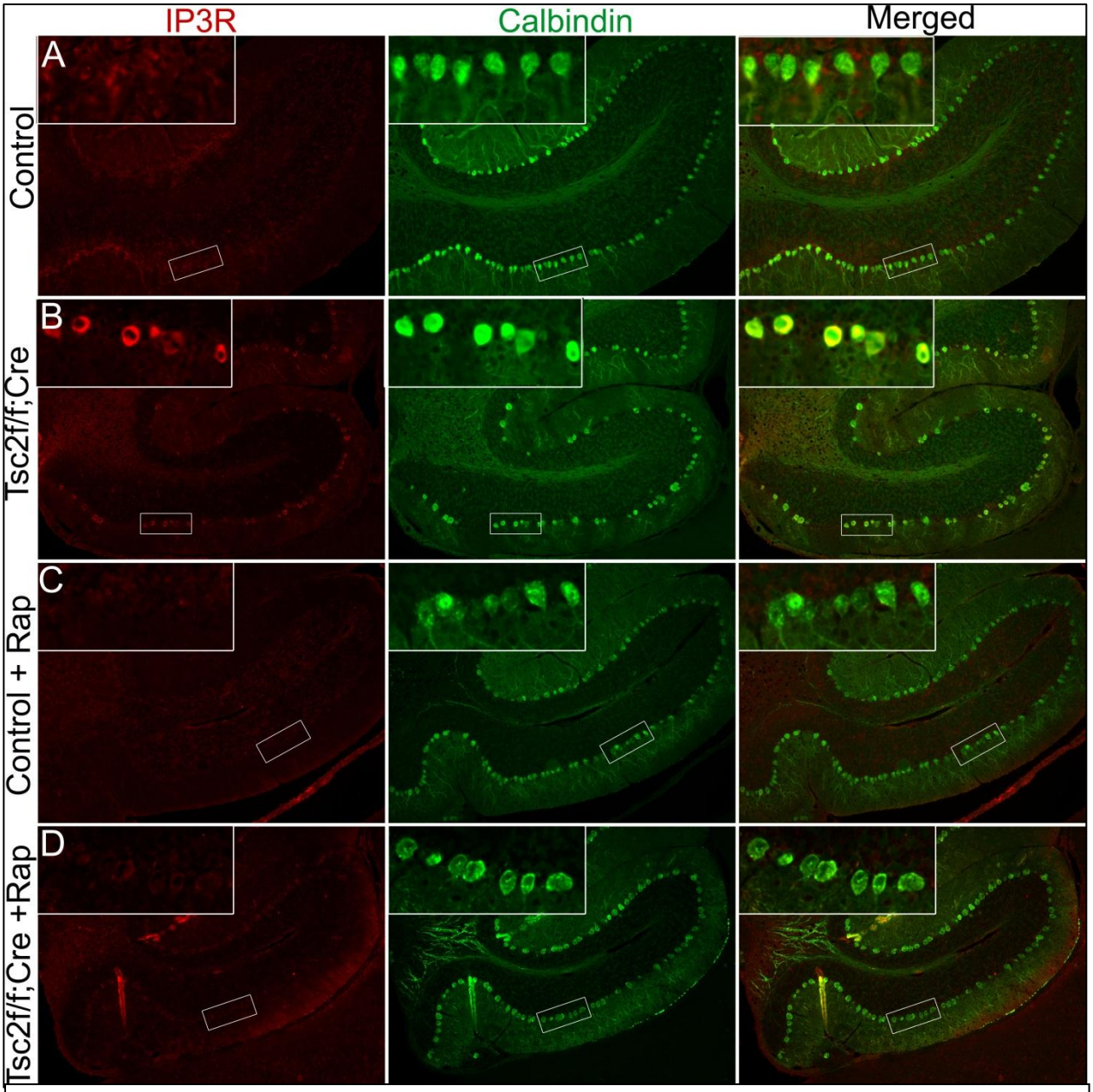

Figure 7.3: Rapamycin reduces calcium signaling. (A-D) Untreated Tsc2f/f;Cre mice (B) had elevated levels of IP3R, but with rapamycin treatment this is reduced (D) and similar to the levels seen in control mice (A,C). Purkinje cells are colabeled with Calbindin.

CHOP
Calbindin
Merged
A
B
C
D
E
Degree of ER Stress Rescue
CHOP Positive Cells
50
40
30
20
10
0
Control
Tsc2f/f; Cre
Untreated
Treated
Control
Tsc2 f/f; Cre
Control +Rap
Tsc2 f/f; Cre +Rap

***Figure 7.4: (Previous page)** Rapamycin reduces ER stress in Tsc2f/f;Cre mice. (A-D) CHOP expression in a treated Tsc2f/f;Cre mouse (D) shows reduced ER stress compared to an untreated Tsc2f/f;Cre mouse (B). Note that CHOP levels in treated Tsc2f/f;Cre mice are still higher than in control mice (A,C). White arrows indicate some CHOP positive cells. Scale bars, 50μm. (E) Quantitation of average number of total CHOP positive cells in the cerebellum of the untreated mice.

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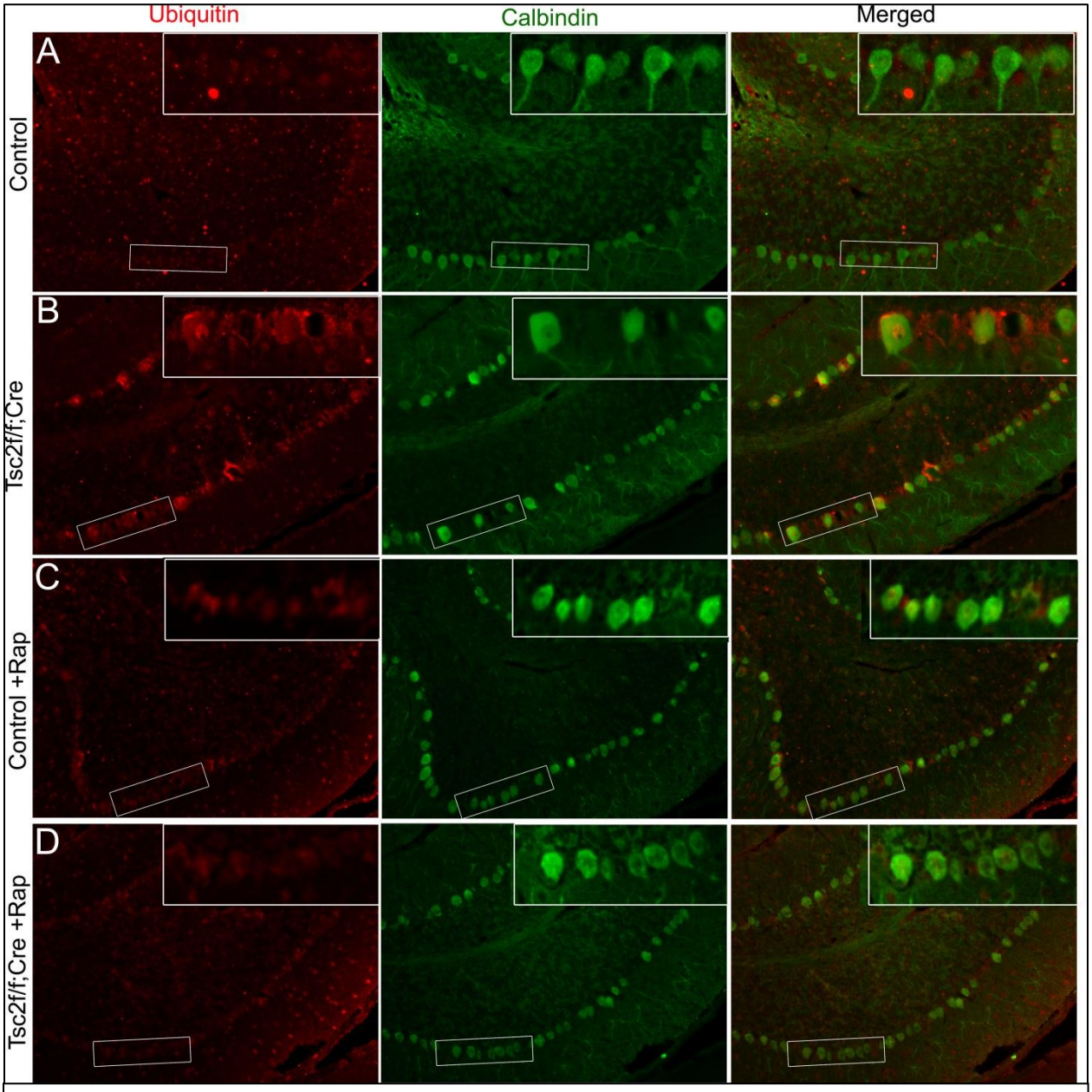

Figure 7.5: Rapamycin reduces levels of ubiquitin. (A-D) Untreated Tsc2f/f;Cre mice (B) had elevated levels of ubiquitin, but with rapamycin treatment this is reduced (D) and similar to the levels seen in control mice (A,C). This suggests reduced ER-associated degradation. Purkinje cells are colabeled with Calbindin.

Effects of rapamycin on behavior

Since rapamycin rescued Purkinje cell degeneration, we next asked whether that led to functional rescue in the ASD-associated behaviors. Male Tsc2f/+ and Tsc2f/-;Cre mice were treated with rapamycin from P10 and tested in a subset of the tests in Chapter 6. Rapamycin treatment was continued throughout testing.

Both rapamycin and vehicle treated mice of all genotypes had intact reflexes. General olfaction was measured using the buried food test and found that there was no difference in latency to food among any of the treatment groups or genotypes on days 1 and 3, suggesting proper olfactory function (Figure 7.6A). To examine general locomotor activity, an open-field test was conducted which indicates that there are no significant differences of average speed in any of the treatment/genotypes (Figure 7.6B). However, vehicle treated knockout mice did trend to move more quickly, though this was not statistically significant. These data, however, indicate that treatment has no adverse effects on locomotor activity.

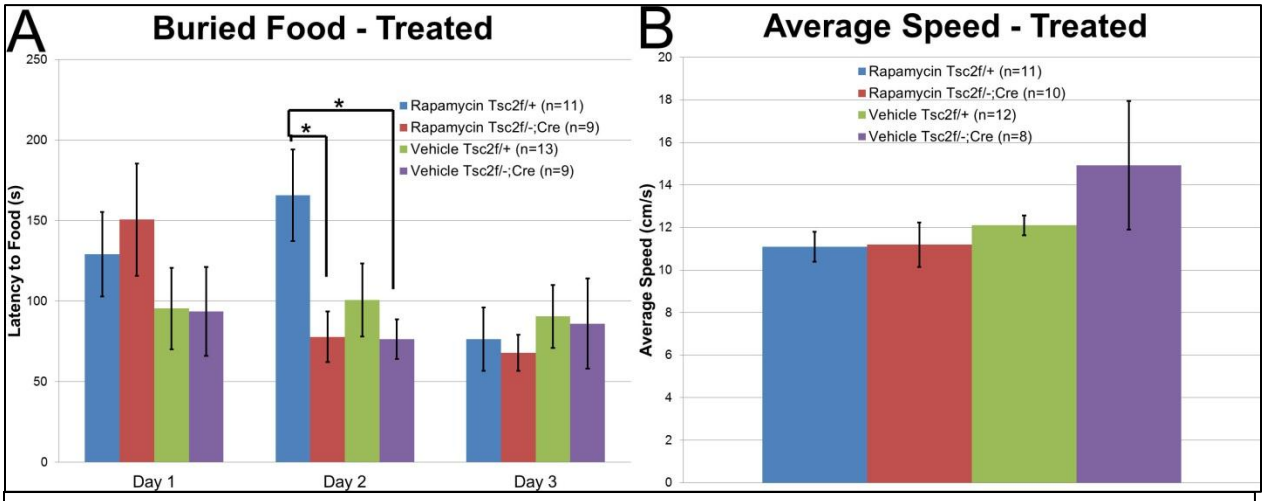

Figure 7.6: General health assessment. (A) Latency to buried food as a measure of olfaction. There was no significant difference in latency to the food across any of the treatment group or genotypes on days 1 and 3. Day 2, rapamycin Tsc2f/+ had a significantly increased latency to the food. (B) Average speed in an open-field did not significantly differ between vehicle and rapamycin treated Tsc2f/+ and Tsc2f/-;Cre mice.

Since Tsc2f/-;Cre mice had impaired social behavior, we also conducted the three chambered apparatus test to determine social preference of treated mice. Importantly, vehicle treated Tsc2f/+ mice retained preference for the chamber with the social mouse than the chamber with the inanimate object (p=0.0076) (Figure 7.7A). Vehicle treated

Tsc2f/-;Cre mice, however, did not show a preference for the social chamber (Figure 7.7A), validating the social behavioral deficit noted in untreated Tsc2f/-;Cre mice (see Chapter 6). Notably, rapamycin treated Tsc2f/-;Cre mice did show preference for the social chamber over the chamber with the inanimate object (p=0.013) (Figure 7.7A), indicating that rapamycin treatment rescued the social behavioral deficit. It was interesting, however, to note that rapamycin treated Tsc2f/+ mice did not show significant preference for the social chamber (Figure 7.7A).

When we tested for social novelty, vehicle treated Tsc2f/+ mice still showed preference for time spent in the chamber with the novel mouse than with the familiar mouse (p=0.031) (Figure 7.7B). Vehicle treated Tsc2f/-;Cre mice, however, did not show preference for social novelty (Figure 7.7B), confirming the results obtained in untreated mice (see Chapter 6). Again, rapamycin treated Tsc2f/-;Cre mice had rescued preference for social novelty, spending significantly more time in the chamber with the novel mouse than in the chamber with the familiar mouse (p=0.022) (Figure 7.7B). Rapamycin treated Tsc2f/+ mice also displayed preference for social novelty, spending more time with the novel mouse than the familiar mouse (p=0.048) (Figure 7.7B).

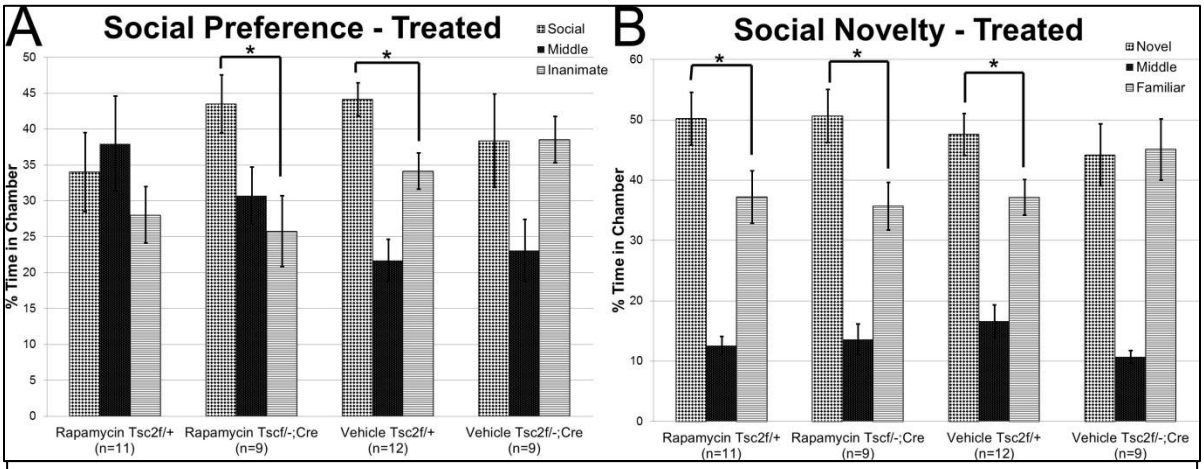

Figure 7.7. Rapamycin rescues social behavior deficits. (A) Vehicle treated Tsc2f/+ mice spent more time (p=0.0076) in the social chamber than in the inanimate object chamber. However, vehicle treated Tsc2f/-;Cre mice spent equal time in both chambers. With rapamycin treatment, Tsc2f/-;Cre mice spent more time (p=0.013) in the social chamber than in the inanimate object chamber. However, rapamycin treated Tsc2f/+ mice did not show a preference for the social chamber. (B) Vehicle treated Tsc2f/+ mice spent more time (p=0.031) with a novel mouse than with a familiar mouse. However, vehicle treated Tsc2f/-;Cre mice spent equal time in both chambers. With rapamycin treatment, Tsc2f/-;Cre mice spent more time (p=0.022) with a novel mouse than with a familiar mouse. Rapamycin treated Tsc2f/+ mice also spent more time (p=0.048) with a novel mouse than with a familiar mouse.

One behavior that was noted during the social behavior testing was the high amount of time that all treated mice spent in the middle chamber (Figure 7.7A). While this resolved over the subsequent testing session (Figure 7.7B), it was still drastically increased from that seen in untreated mice (see Chapter 6). This behavior seemed to indicate increased anxiety in all the treatment groups. Therefore, the percentage of time spent in the middle of an open-field arena was examined as another indicator of anxiety levels. Vehicle treated Tsc2f/-;Cre mice spent more time in the middle of an open-field arena ($p=0.059$) than vehicle treated Tsc2f/+ mice and rapamycin treated Tsc2f/-;Cre mice ($p=0.0078$) (Figure 7.8A). This confirms that Tsc2f/-;Cre males have decreased levels of anxiety which was also noted in untreated mice (see Chapter 6). However, when compared to untreated mice (figure repeated from Chapter 6), all treatment groups (both vehicle and rapamycin) spent less time in the middle, suggesting that added handling and/or injections exacerbated anxiety levels (Figure 7.8A-B).

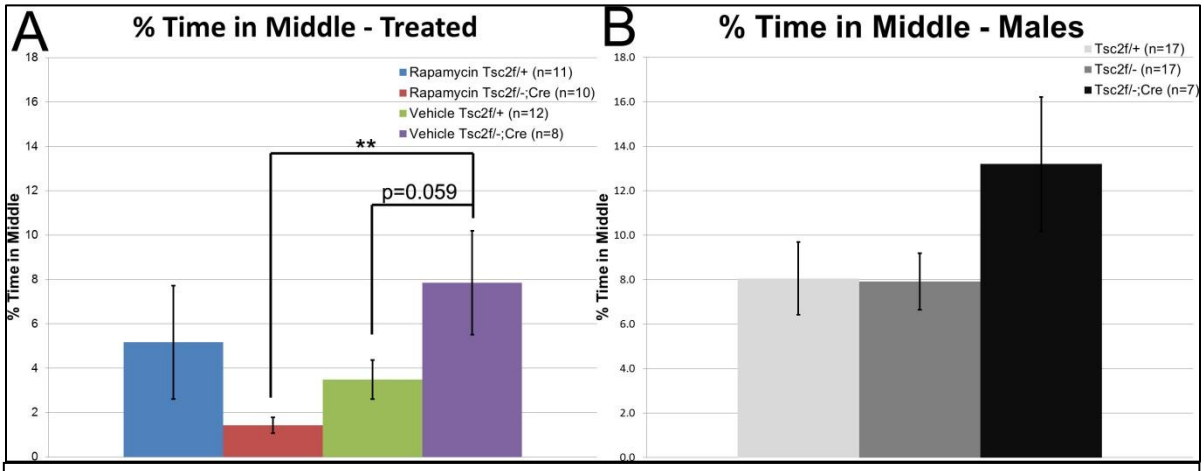

Figure 7.8. Treatment increases anxiety. (A) Vehicle treated Tsc2f/-;Cre mice spent more time in the middle of an open-field chamber than vehicle treated Tsc2f/+ ($p=0.059$) or rapamycin treated Tsc2f/-;Cre ($p=0.0078$) mice. However, both genotypes of both treatment groups spent less time in the middle of an open-field arena than untreated mice (B)(Graph reprinted from Chapter 6).

Discussion:

Rapamycin is a promising therapy for alleviating various aspects of pathology associated with TSC. In this chapter, I showed that rapamycin therapy in Tsc2f/f;Cre and Tsc2f/-;Cre rescued many of the cellular and behavioral phenotypes associated with loss of *Tsc2* in Purkinje cells. In particular, rapamycin treatment rescued Purkinje cell degeneration and many of the events leading up to degeneration such as: increased cell size, overactive mTORC1, increased IP3R, ER stress, and increased ubiquitin levels. Furthermore, rapamycin treatment also rescued social behavioral deficits. This study will offer valuable insight into advancing rapamycin therapy into clinical trials for these various aberrations. However, there are still caveats that need to be explored.

The study conducted by Franz et al. examining rapamycin treatment for SEGAs, indicated that in order for rapamycin to be effective, chronic administration is necessary (756). However, although short-term rapamycin treatment is specific to mTORC1, long-term rapamycin treatment leads to indirect inhibition of mTORC2 (766). The hypothesis is that rapamycin binds to mTOR molecules, which leads to disruption of mTORC2 complex formation because mTOR is no longer free to bind to Rictor (766). It is believed that some of the side effects of rapamycin are due to its indirect effect on mTORC2 (766).

Another consequence of long-term rapamycin treatment may be developed resistance. In a study conducted on the Eker rat model, naturally occurring $Tsc2^{+/-}$, a small percentage of the renal lesions became resistant to rapamycin therapy (762). Subsequent studies have revealed that long-term rapamycin treatment leads to rephosphorylation of 4EBP1 leading to resistance (767-769). This could then lead to resulting upregulation of protein synthesis.

Therefore, there are multiple possibilities why increased cell size, pS6 levels, and ER stress were reduced, but not restored to control levels. First, rapamycin levels may not have been sufficient to fully rescue this pathology, and a higher dosage may be needed. Or, chronic treatment is sufficient to allow for reactivation of downstream components of mTORC1 signaling or more efficient drug clearance. Examination of other mTORC1 substrates, like 4EBP1 should be examined and treating mice at a higher dose should also be attempted.

Although autophagy was enhanced in Tsc2f/f;Cre mice (see Chapter 5), rapamycin did not alleviate autophagy. This, however, was not a surprising finding. In a normal setting, mTORC1 acts to inhibit autophagy (582), and consequently it has been shown that inhibition of mTORC1 through rapamycin causes induction of autophagy (770).

Another finding in Tsc2f/f;Cre mice that was not alleviated by rapamycin was increased oxidative stress (see Chapter 4). This effect could possibly be due to the effect of methanol. The body processes methanol by oxidizing it to formaldehyde and then formate (771). These metabolic processes increase the levels of ROS in the cell (771-773). Because of this known toxicity of methanol, we switched to dissolving rapamycin in ethanol for behavior testing.

The behavior testing conducted on Tsc2f/-;Cre mice revealed that rapamycin treatment ameliorated social behavior deficits. Likely, this is due to the rescue of Purkinje cell loss and subsequent cerebello-thalamo-cortical circuit. Only Tsc2f/+ and Tsc2f/-;Cre males were treated with rapamycin. However, it would be interesting to see if rapamycin rescued the mild social behavioral deficits observed in Tsc2f/- mice (see Chapter 6). These results are particularly important because they provide crucial information that rapamycin can have a positive effect on social behavior deficits associated with TSC. They also provide a great framework to a current clinical trial NCT01289912, examining the effects of rapamycin on neurocognition in patients with TSC. The aims of his study are to examine if rapamycin is able to reduce learning deficits and ASD features in patients with TSC (755).

Rapamycin induced social behavioral deficits in Tsc2f/+ mice. This was only noted on the social preference test, as rapamycin treated Tsc2f/+ mice still retained preference for social novelty. The social preference and social novelty test look at two slightly different components of social behavior, which is why they may be getting seemingly conflicting results. Furthermore, these results may be obscured by the increased time spent in the middle of the chamber in the social preference task.

However, the possibility that rapamycin negatively affects behavior in control mice needs to be further explored. There was one Tsc2f/+ mouse that did not respond well to rapamycin treatment, having health problems throughout his life and succumbing to premature death before the conclusion of the behavior testing. Given that mTOR is an essential gene for proper cellular function (mTOR knockout mice are embryonic lethal

(774, 775)), it is reasonable to expect adverse consequences of mTORC1 inhibition, if starting at physiological levels. However, in the case of Tsc2f/-;Cre mice and in patients with TSC, mTORC1 levels are elevated, and therefore inhibition of mTORC1 back to basal levels is beneficial. Nevertheless, considering the vast implications of rapamycin therapy outside of TSC, further cognitive testing should be conducted both in murine models and patients on rapamycin therapy who do not have elevated mTORC1 levels (like transplant patients).

Treatment seemed to cause increased anxiety. Not only was this detected in the open-field arena, but it was also observed in the general handling of the mice. This effect was not specific to rapamycin treatment, but also occurred as a consequence of vehicle treatment. Although vehicle treated Tsc2f/-;Cre mice were still less anxious than the other treatment groups, there were notably more anxious than their untreated counterparts. Because the effect was not isolated to rapamycin therapy, it is likely to be a generalized effect. Mice were given two behavior tests and three injections per week. Therefore, when they were handled, they did not know which of these two events were about to take place. When they were injected, they also behaved as if the injection caused them physical pain (likely due to the ethanol). Therefore, one hypothesis is that they associated handling with the pain caused from the injection, leading to the increased anxiety.

To offset handling induced anxiety, I suggest attempting alternate rapamycin delivery methods. One potential method is through the use of osmotic pumps which can be implanted in the mouse and would then provide continuous and controllable dosing of rapamycin (776). Another potential method of rapamycin delivery, used by Harrison et al. in their paper showing the life extension in mice, is to use encapsulated rapamycin incorporated into mouse chow (753). The encapsulation of the rapamycin increases the amount that survives the food preparation process and prevents degradation by the stomach acid (753). Food delivery would be a less invasive measure of rapamycin delivery, but would need to be controlled to ensure a set level of rapamycin delivered.

Untreated Tsc2f/-;Cre mice also demonstrated increased repetitive behaviors as demonstrated by an increased number of marbles buried in a 30 minute interval (see Chapter 6). Conversely, vehicle treated Tsc2f/-;Cre mice showed no increase compared to vehicle treated controls. It is possible that the increased anxiety levels are also

affecting this task, as the treated mice are perhaps not as active as untreated mice. However, I plan on conducting the marble burying test on another cohort of untreated mice to see if I can replicate the previous findings.

The buried food test indicated that all treatment groups and genotypes had functioning olfaction. However, on the second day of the test, rapamycin treated control mice took significantly longer to find the food. Likely, this is not biologically relevant because latency to food on the third day was comparable to the other groups. However, as mentioned before, rapamycin could have a detrimental effect on control mice. However, while the location of the pellet was changed each day, it was closest to me on day two. Perhaps their increased anxiety levels kept them from exploring the side of the cage that was nearest me. However, it is unclear why this would have preferentially affected the rapamycin control mice.

In the untreated mice, I extended the buried food test out to four days of trials. This meant that the mice were on a food restricted diet for six days. However, the mice undergoing rapamycin treatment were not able to tolerate food deprivation as long (dropping too much weight too quickly), so the test was only conducted for three days in the treated groups. It is likely that the combinatorial effects of food deprivation and rapamycin (which already causes poor weight gain) were too much for the mice. However, this sensitivity needs to be monitored in patients receiving rapamycin therapy, especially as it may be considered for children with TSC-associated ASD (755).

Though rapamycin does eventually lead to decreased activity of mTORC2 (766), it may be prudent to consider a dual inhibitor of both mTORC1 and mTORC2. One such inhibitor is Torin 1, which is selective for the kinase domain of mTOR (769). Notably, because this has been an issue with rapamycin (767-769), Torin leads to sustained inhibition of 4EBP1 (769).

Rapamycin treatment in Tsc2f/f;Cre and Tsc2f/-;Cre mice has revealed exciting potentials to therapy for Purkinje cell loss and social behavioral deficits associated with TSC. This pre-clinical trial gives valuable knowledge that can be translated into human applications.

Chapter Eight:

Significance and Future Directions

Knowledge of TSC has progressed immensely since first described by Bourneville in 1780. Then, in 1932, Critchley and Earl noted that TSC was associated with bizarre attitudes and repetitive movements (23). This was the first account of TSC being associated with autism, a disorder that wasn't really described until Kanner in 1943 (121). Since its first descriptions, the research in the field of ASDs has exploded. However, even today much remains unknown about these disorders including what causes their association.

Recent studies have led to an increasing appreciation of the role of the cerebellum in both TSC and ASD. In my research, I have added to the existing literature, and uncovered one link between TSC and ASD: the Purkinje cell. Not only is Purkinje cell loss a well-reported finding in autopsy studies of ASD (360-366), but I have presented evidence along with Boer et al. that Purkinje cell loss may represent a pathology associated with TSC (2, 448). Furthermore, I have characterized a novel mouse model of Purkinje cell degeneration resulting from a Purkinje cell specific mutation in *Tsc2* and have shown that they have behaviors associated with ASD. This provides a novel mouse model for the study of TSC-associated ASD.

These results underscore the importance of loss of *Tsc2* in Purkinje cells as a mechanism for ASD-associated behavior, including both repetitive behaviors and social deficits. Additionally, I have described some of the cellular mechanisms resulting from mutations in *Tsc2* leading to Purkinje cell death. This work revealed that loss of *Tsc2* led to upregulation of mTORC1 and increased cell size. As a consequence of increased protein synthesis, several cellular stress pathways were upregulated. Principally, these included altered calcium signaling, oxidative stress, and ER stress. Likely as a consequence of ER stress, there was also upregulation of ubiquitin and autophagy.

The finding that autophagy was upregulated, despite upregulation of mTORC1, was a bit paradoxical since mTORC1 is an autophagy inhibitor (582). This suggests an alternate, mTORC1-independent pathway for autophagy. Induction of autophagy (through rapamycin) has shown promise in the treatment of neurodegenerative disorders such as: Alzheimer's (745, 746), Parkinson's (747, 748), and Huntington's disease (749, 750). Furthermore, lifespan extension in yeast and Drosophila by rapamycin treatment required induction of autophagy (752, 754). Therefore, elucidating the additional

autophagy pathway (mTORC1-independent) in Tsc2f/f;Cre mice may be able to contribute to treatment of these disorders.

Another interesting finding in Tsc2f/-;Cre and Tsc2f/f;Cre mice was the folia-dependent, patterned Purkinje cell loss. While interesting, this is not a unique finding-being demonstrated in other mouse models of Purkinje cell degeneration (469-471). More studies need to be conducted to examine the differences between susceptible and resistant Purkinje cells. Knowledge of the genes associated with Purkinje cell resistance will help develop therapies to slow or inhibit neurodegeneration. One possible way of addressing this would be to perform laser capture of the two groups of Purkinje cells and conduct a microarray from the isolated RNA (777) to reveal differentially expressed genes that confer resistance to perturbations. For example, there is patterned expression of a glutamate transporter in Purkinje cells (778). This could make Purkinje cells in these regions more resistant to excitotoxic damage.

I have also shown exciting data that treatment with rapamycin was able to decrease levels of mTORC1 and rescue Purkinje cell death. Consequently, this led to rescue of IP3R expression, the ER stress response, and levels of ubiquitin. Remarkably, rapamycin treatment also reversed the social behavior deficits, thus providing a promising potential therapy for TSC-associated ASD.

Furthermore, not only is the mTORC1 pathway a central node of many of the genes and syndromes associated with ASD (see Chapter 2), but my results show that it may also have a role in some of the cellular implications in ASD. Oxidative stress, which was upregulated in the Purkinje cells of Tsc2f/f;Cre mice following mTORC1 upregulation, has been seen in many cases of ASD, particularly the cerebellum (363, 381-385).

One of the cellular dysfunction hypotheses of ASD is that excessive calcium release leads to cellular toxicity (279). mTORC1 has been shown to activate calcium release through the inositol 1,4,5-tripohosphate receptor (IP3R) (488, 489). Therefore, since we observed increased IP3R in Tsc2f/f;Cre mice, calcium flux should be further examined to see if it contributes towards Purkinje cell loss and ASD-associated behaviors. If so, this may lead to alternative therapies such as calcium channel blockers.

Moreover, elevated calcium plays a key role in the immune response (779, 780), a well-documented environmental factor associated with ASD (181-184, 186). Activation

of the immune response upregulates the mTORC1 pathway (781). mTORC1 is also a critical regulator of the immune system, particularly T cell production and differentiation (782). A few studies suggest that overactivation of the immune system leads to an inflammatory immune response in TSC patients (783, 784). Since mTORC1 is activated in Tsc2f/f;Cre mice, examination of the inflammatory immune response should be performed. This may indicate another cellular stress event leading to Purkinje cell loss and associated ASD-like behaviors.

Furthermore, ER stress co-occurred with ASD-like behavior in Tsc2f/f;Cre mice. Also, a mouse model of CADM1 mutation links increased ER stress and ASD-like behaviors (681). Additionally, ER stress can be linked to many of these other cellular responses. ER stress can induce oxidative stress (512, 540, 542), calcium release (555, 556), and inflammation (678). Therefore, I hypothesize that ER stress may have a larger role in ASD than has been previously examined. Perhaps examination of ER stress markers in postmortem ASD samples will yield novel results, correlating ER stress and ASD. Then, further examination of animal models could be conducted to determine if induction of ER stress leads to ASD-like behavior. A summary of my findings and the potential relevance to ASD is shown in Figure 8.1.

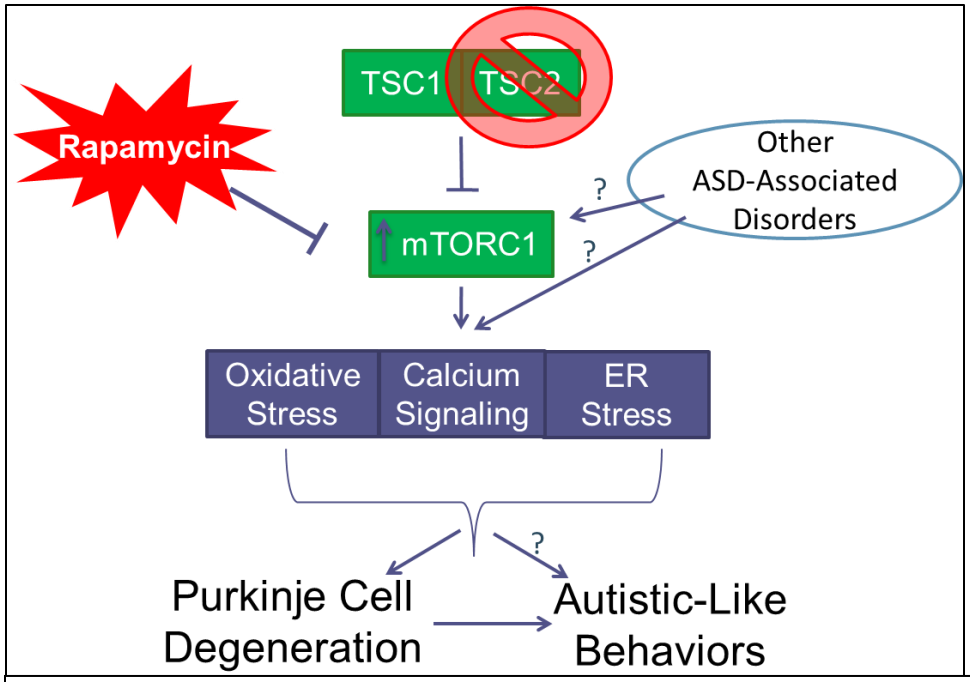

Figure 8.1: Summary. Deletion of *Tsc2* leads to upregulated mTORC1. Subsequently, there is increased oxidative stress, IP3R expression, and ER stress. These contribute to Purkinje cell death. Loss of Purkinje cells is associated with ASD-like behaviors. Other reports indicate altered mTORC1 in some ASD-associated disorders and that these cellular mechanisms may have a broader role in ASD-associated behaviors.

Even haploinsufficiency in *Tsc2* led to mild social behavior deficits. Elucidating the cellular mechanisms by which haploinsufficiency leads to behavioral changes would be very important, as it would be applicable to all patients with TSC, not only those who have undergone a second hit event. Since $Tsc1^{+/-}$ cells have shown altered dendritic spine structures (703), this could change how the neurons associate with each other. In the model, Nie et al. showed increased dendritic length, but decreased spine density (703). In vivo, this could reduce the number of connections a neuron is able to make. These variations change the input received by haploinsufficient neurons, thus potentially altering their signaling pattern. Comparing the dendritic structure of Tsc2f/- mice and Tsc2f/+ mice, would help indicate if this finding translates to a *Tsc2* in vivo model system. Further, comparing Tsc2f/-;Cre would indicate if the pathology worsens upon second deletion of *Tsc2*, and considering the extensive dendritic tree of Purkinje cells, would indicate the altered cerebellar connections even before Purkinje cell death. Labeling of the dendritic structure could be accomplished via golgi staining or carbocyanine dye (DiI) (785).

Another important point is that loss of heterozygosity (LOH) in Purkinje cells is not a proven mechanism of pathogenesis in human TSC patients. LOH has been difficult to prove as a mechanism of pathogenesis for TSC brain lesions (40-42). In a study that conducted deep sequencing of all the coding exons of both TSC1 and TSC2, Qin et al. only detected 1 second hit event in a cortical tuber out of 34 TSC patients (42). For cortical lesions, either haploinsufficiency is sufficient to induce tubers, or second hit events involve some other form of gene silencing (either through epigenetic regulation or mutation in regulatory regions) (43). One hypothesis I formed after examining data from human TSC patients, was that the “second hit” event was an environmental perturbation (maybe pharmacological due to treatment with phenytoin, which can cause cerebellar abnormalities). LOH studies have not been attempted on Purkinje cells. This would likely be complicated to perform and would require laser capture microdissection and subsequent sequencing of isolated Purkinje cells. However, the Purkinje cell would need to be caught in the early stages of degeneration. Nevertheless, the information garnered from Tsc2f/-;Cre mice (mimicking an LOH event in the Purkinje cell) and Tsc2f/- mice gives credence to the LOH or second hit hypothesis. Tsc2f/- mice do not have Purkinje cell loss at three months of age, while Tsc2f/-;Cre mice, with LOH, have

an 86% Purkinje cell loss in folium II (see Chapter 6). It is possible that Purkinje cell loss would eventually occur in Tsc2f/- mice, or that LOH is required for Purkinje cell loss.

Purkinje cell loss was correlated with ASD-like behaviors in Tsc2f/-;Cre mice. Additionally, both nervous and Lurcher mice (models of Purkinje cell loss) exhibit increased repetitive behaviors (683, 722), a common feature associated with ASD (133). Though neurodegeneration is not a classical feature associated with TSC or ASD, it may have unexplored importance. Upregulation of the mTORC1 pathway has been associated with classic neurodegenerative disorders such as Alzheimer's disease (475, 476), which is also characterized by Purkinje cell loss (478, 479). Though the dementia associated with Alzheimer's is certainly the most striking symptom of the disease, it is interesting to note that another common feature is increased repetitive behaviors (786, 787). Therefore, these associations, along with the behavior testing on Tsc2f/-;Cre mice, correlate Purkinje cell loss with ASD-associated behavior. Furthermore, a microarray study of Purkinje cell degeneration (pcd) mice suggests the involvement of the mTORC1 pathway, though these results have yet to be validated (516). Additionally, activation of metabotropic glutamate receptors induces upregulation of mTORC1 (632), potentially suggesting mTORC1 in the pathogenesis of Lurcher mice. Therefore, mTORC1 activation should be explored in other mouse models of Purkinje cell degeneration. Moreover, other testing for ASD-like behaviors should be conducted in these models. There are no reports in the literature of social behavior deficit in a mouse model of Purkinje cell degeneration. In addition, communication deficits could be assessed through ultrasonic vocalizations. These studies would highlight the importance of mTORC1 in Purkinje cell loss and ASD-associated behaviors.

It is still unclear if loss of Purkinje cells per se and/or dysfunctional *Tsc2*-null remaining Purkinje cells are important for this autistic-related phenotype in Tsc2f/-;Cre mice. Comparing the extent of social behavior deficits at different time points will be an important follow-up experiment. If social behavior deficits progress over time, correlating with Purkinje cell loss, it would suggest social behavior was due to loss of Purkinje cells. Furthermore, though labor intensive, generation of chimeric Tsc2f/-;Cre mice (like that was done in Lurcher mice (683)) would indicate if the number of Purkinje cells affected correlated to severity.

Purkinje cells likely do not act alone in causing ASD-like behaviors. Rather, they act in concert with the rest of the cerebellar circuit, and ultimately cortical connections. Since the Purkinje cells project to the deep cerebellar nuclei (358), we examined histology of the deep cerebellar nuclei following Purkinje cell loss. Apoptotic cells were detected in the deep cerebellar nuclei at eight months of age. This cell death is likely disrupting the cerebellar connections to other areas of the brain. Examination of these circuits in Tsc2f/f;Cre mice should be performed. Anterograde tracing can detect the axonal projections from the Purkinje cell, and be used to follow the resulting circuit.

I also noted sex specific effects on anxiety levels. Male Tsc2f/-;Cre mice tended to be less anxious than controls (both untreated and vehicle groups), while female Tsc2f/-;Cre mice tended to be more anxious than controls. This dichotomy may be regulated by hormonal influences. Other aspects of TSC are also thought to be modulated by the action of hormones. First, lymphangioleiomyomatosis (LAM) occurs almost exclusively in women- alluding to hormonal regulation (788). Supporting this notion, estrogen treatment causes disease progression (789). Additionally, the angiomyolipomas of the kidneys occur more frequently in women. Treatment with estrogen exacerbated growth and increased phsorphorylation of Akt (790). A TSC mouse model of liver hemangioma (which loosely models the cellular make-up seen in LAM and angiomyolipomas) also showed exacerbation following estrogen treatment (791). However, treatment with Tamoxifen, an estrogen receptor antagonist, reduced the growth of the hemangiomas (791). Another TSC lesion thought to be hormonally regulated are cardiac rhabdomyomas. Formation occurs in utero but then they regress upon birth, suggesting a role for maternal hormones (792). If infants were treated with corticotrophin as an anti-convulsant, they had a rapid enlargement of the rhabdomyoma (793). Interestingly corticotrophin hormone stimulates the production of estrogen (794, 795). High levels of the estrogen receptor are expressed in the brain, including the limbic regions (important for anxiety) as well as Purkinje cells (796, 797). Furthermore, it has been shown that estrogen can activate mTORC1 (798). Therefore, estrogen might be an important regulator of multiple pathologies associated with TSC, including the behavioral responses. Further studies aimed to examine if estrogen exacerbates Purkinje cell loss and ASD-like behaviors may lead the way for alternate therapies including estrogen antagonists such as Tamoxifen.

In conclusion, I have characterized a novel mouse model of TSC-associated ASD. Rapamycin therapy shows promising potential for alleviating behavioral and cellular abnormalities in the mice. However, histological analysis demonstrates other possible therapeutic targets that should be considered. Finally, just as cancer therapies are targeting dual treatments (rapamycin + another agent) (799, 800), so we should also consider dual drug therapies for TSC and ASD behaviors. This strategy may result in more complete rescue of symptoms as well as reduce the dose of rapamycin needed (and consequently reduce the resulting side effects). These studies, and the potential for future studies will further our knowledge and ability to treat both TSC and ASD.

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Vita

Rachel Michelle Reith was born in San Antonio, TX on June 28, 1985. Her loving parents are Marv and Glenda Reith. Moving around a lot as a child, she has lived in Texas, Louisiana, Arkansas, and Oklahoma. However, after graduating in 2003 from Jenks High School, she returned to Texas to study at Biochemistry and Cell Biology at Rice University. She graduated from Rice in 2007 and only made it down the street to The University of Texas Graduate School of Biomedical Sciences at Houston, where she matriculated in August of 2007. Her doctoral work in Human and Molecular Genetics was performed in the lab of Dr. Michael Gambello in the department of Pediatrics/Medical genetics at The University of Texas Medical School. She will receive her doctoral degree in August 2012.