Murine Models of Spinocerebellar Ataxia Type 5

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ABSTRACT

Spinocerebellar ataxia type 5 (SCA5) is a slowly progressive neurodegenerative disease of the cerebellum caused by mutations in the *SPTBN2* gene, which encodes the protein β-III spectrin. To characterize how β-III spectrin with the American SCA5 mutation causes Purkinje cell degeneration and cerebellar dysfunction, I developed the first transgenic murine models of SCA5 and identified brain proteins that potentially interact with the region of β-III spectrin where the American SCA5 mutation occurs. Behavioral studies with a conditional model that drives expression of untagged β-III spectrin and a second 3xFLAG-tagged SCA5 model show that overexpressing mutant β-III spectrin in murine cerebellar Purkinje cells causes cerebellar dysfunction. Further studies with the conditional tet-regulated mice show that untagged mutant β-III spectrin alters the localization of the glutamate transporter EAAT4 and the metabotropic glutamate receptor mGluR1α and produces a concomitant deficit in mGluR1 function. Histologic analysis of the 3xFLAG-tagged SCA5 murine model shows that the American SCA5 mutation also alters the Purkinje cell distribution of the mutant β-III spectrin protein itself. Additionally, I identified a number of brain proteins that are novel β-III spectrin interaction candidates, including the dynactin subunit $p150^{Glued}$. I show that the American and French SCA5 mutations alter the interaction strength of β-III spectrin with $p150^{Glued}$ and α-II spectrin respectively.

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ABBREVIATIONS

aa	amino acid
ARP1	actin related protein 1
BLAST	Basic Local Alignment Search Tool
bp, kb	basepairs, kilobases
°C	temperature in degrees Celsius
CH	calponin homology
CPRG	chlorophenol red-β-D-galactopyranoside
DDO	double dropout
DMEM	dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
EAAT4	excitatory amino acid transporter 4
HEK 293, HEK 293T	human embryonic kidney cell lines
het, HZ	heterozygous, homozygous
IgG	immunoglobulin G
IHC	immunohistochemistry
IP	immunoprecipitation
LTP	long term potentiation
mGluR1α	metabotropic glutamate receptor 1 alpha
MRI	magnetic resonance imaging
µg, mg, g	micrograms, milligrams, grams

µl, mL, L	microliters, milliliters, liters
µM, mM, M	micromolar, millimolar, molar
NCBI	National Center for Biotechnology Information
PBS	phosphate buffered saline
Pcp2	Purkinje cell protein 2
PCR	polymerase chain reaction
PH	pleckstrin homology
QDO	quadruple dropout
RIPA	radioimmunoprecipitation buffer
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SCA	spinocerebellar ataxia
SCA5	spinocerebellar ataxia type 5
S.E.M.	standard error of the mean
SPΔ15	β-III spectrin with the French SCA5 deletion
SPΔ39	β-III spectrin with the American SCA5 deletion
SPTBN2	*spectrin, beta, non-erythrocytic 2*
TIRF	total internal reflection fluorescence
TRE	tet-response element
tTA	tetracycline transactivator
X-α-Gal	5-Bromo-4-Chloro-3-indolyl-α-D-galactopyranoside

CHAPTER 1

INTRODUCTION TO SCA5

I. Introduction to spinocerebellar ataxia

The autosomal dominant spinocerebellar ataxias (SCAs) are a group of progressive neurodegenerative diseases predominantly affecting the cerebellum, a hindbrain structure critical for motor control and balance. Common symptoms and signs of SCA include gait imbalance, limb incoordination, slurred speech, and swallowing difficulties (Rosenberg 2001). In some types of SCA, neurodegenerative changes and associated clinical features are also found in the basal ganglia, brainstem, spinal cord, peripheral nerves, optic nerves, and/or retina. The first classification system for the ataxias, based on clinical features, was proposed by Harding (Harding 1982, Harding 1983). This system divides the autosomal dominant cerebellar ataxias into categories based on the presence or absence of extracerebellar disease. However, the clinical variability of these diseases combined with advances in genetic studies resulted in the development of a genetic classification that has largely replaced the clinical classification (Rosenberg 2001). To date 28 genetic loci have been described for these disorders, and the pathogenic mutation has been identified for 16 of these diseases (Storey et al. 2009).

II. Features of SCA5

SCA5 was initially described in a large American kindred descended from President Abraham Lincoln's paternal grandparents and mapped to the centromeric

region of chromosome 11 (Ranum et al. 1994a). Two additional SCA5 families, which map to the same genetic locus on chromosome 11q13, were subsequently reported from France and Germany (Stevanin et al. 1999, Burk et al. 2004). Both of these families have similar clinical and imaging findings to the American SCA5 family.

Clinical examination of affected members of the American SCA5 family shows that SCA5 is a slowly progressive cerebellar disease with a mean age of onset of 33.0 ± 13.1 years (Table 1) (Liquori et al. 2002). The disease is characterized by a disabling and progressive gait ataxia, with a typical progression from initial mild unsteadiness to increased gait instability requiring walking aids and occasionally resulting in wheelchair dependence. Typical features of SCA5 also include upper and lower limb ataxia. Although upper limb ataxia is often subtle at onset, in most patients it progresses to impair fine dexterity and affects basic activities such as handwriting. Many SCA5 patients have dysarthria, but only 1 of the 58 patients examined had articulation problems severe enough to prevent verbal communication. Overall, clinical features of SCA5 are primarily cerebellar, although occasionally individuals also show signs of mild bulbar or pyramidal tract involvement. In contrast to a number of other forms of ataxia, SCA5 patients typically do not develop the coughing and swallowing problems associated with bulbar dysfunction and in general SCA5 does not reduce life span.

Brain magnetic resonance imaging (MRI) of affected SCA5 individuals supports the clinical picture of isolated cerebellar dysfunction by showing extensive atrophy of the cerebellar cortex with minimal extracerebellar findings (Figure 1) (Liquori et al. 2002). Within the cerebellum, there is preferential atrophy of the superior hemispheres and

anterior vermis and relative sparing of the inferior vermis and tonsils. In later stages of disease there may be minimal brainstem atrophy, and no atrophy is seen in the cerebrum or basal ganglia.

Examination of SCA5 autopsy tissue is consistent with the clinical and MRI findings. Gross pathological examination shows a shrunken cerebellum with atrophy prominently affecting the anterior-superior hemispheres (Liquori et al. 2002). Additional findings show a striking loss of Purkinje cells, resulting in molecular layer thinning (Figure 2) (Ikeda et al. 2006). Purkinje cells are responsible for transmitting all output from the cerebellar cortex to the vestibular and cerebellar nuclei (Jörntell and Hansel, 2006) and thus are critical for cerebellar function.

Mutations in *spectrin, beta, nonerythrocytic 2* (*SPTBN2*), the gene that encodes the protein β-III spectrin, cause SCA5 (Ikeda et al. 2006). Although many of the known mutations for other forms of SCA are microsatellite repeat expansions (Rosenberg 2001), SCA5 is caused by missense or small in-frame deletion mutations in the β-III spectrin gene (*SPTBN2*). Figure 3 depicts the locations of the three originally reported mutations found in *SPTBN2* (Ranum et al. 1994a, Stevanin et al. 1999, Burk et al. 2004): the American family has a 39-bp deletion causing an in-frame deletion at the beginning of the third spectrin repeat (E532_M544del), the French family has a 15-bp deletion causing an in-frame deletion with the insertion of a tryptophan at the end of the third spectrin repeat (L629_R634delinsW), and the German family has a transition mutation causing a leucine to proline change in the calponin homology domain (L253P).

III. Introduction to β-III spectrin

The unicellular organisms *Acanthamoeba* and *Dictyostelium* both possess genes encoding α-spectrin-like proteins, but heteromeric spectrin containing two α-spectrin chains and two β-spectrin chains appears to be characteristic of multicellular animals (Moorthy et al. 2000). The *Caenorhabditis elegans* and the *Drosophila melanogaster* genomes both contain one α-spectrin gene, one canonical β-spectrin gene, and one heavy β-spectrin gene. The rat, mouse, and human genomes contain two α-spectrin genes, four canonical β-spectrin genes, and one heavy β-spectrin gene.

The basic structure of β-III spectrin is that of a canonical β-spectrin (Bennett and Baines 2001), with an N-terminal filamentous actin-binding region containing two calponin homology (CH) domains, a central region containing 17 spectrin repeats, and a C-terminal region containing a pleckstrin homology (PH) domain (Figure 3). Although the triple helical spectrin repeat domains have a high degree of structural similarity, the repeats are not strictly interchangeable since different spectrin repeats bind different ligands (Djinovic-Carugo et al. 2002). Binding sites in the β-III spectrin protein predicted by homology include protein 4.1 and actin binding sites in the N-terminal region, an ankyrin-binding site in the 15th spectrin repeat, and a self-association site in the 17th spectrin repeat (Stankewich et al. 1998).

Three groups independently reported the discovery of β-III spectrin (Ohara et al. 1998, Sakaguchi et al. 1998, Stankewich et al. 1998). Although one of the groups characterized β-III spectrin as the widely expressed Golgi-associated spectrin (Stankewich et al. 1998) which was previously reported by Beck and others (1994), the

spectrin antisera used to identify Golgi-associated spectrin was later shown to cross-react with the Golgi-associated protein syne-1 (Bennett and Healy 2008, Gough 2003). In addition, the broad expression pattern of β-III spectrin reported by Stankewich and colleagues (1998) was only substantiated by RNA analysis and was contradicted by the protein analyses reported by the two other groups (Ohara et al. 1998, Sakaguchi et al. 1998). These other groups show a highly restricted distribution pattern of β-III spectrin protein in rat tissue, with the majority of β-III spectrin located in brain and the most prominent immunohistochemical staining occuring in Purkinje cell dendrites (Ohara et al. 1998). Consistent with this description, immunohistochemical analysis of human brain tissue shows prominent β-III spectrin staining in cerebellar Purkinje cell soma and dendrites (Figure 2) (Ikeda et al. 2006). The relatively high levels of β-III spectrin protein found in cerebellar Purkinje cells are consistent with the cerebellar pathology of SCA5, which causes dramatic Purkinje cell loss and cerebellar degeneration with little or no effect on other parts of the brain.

IV. Potential role of β-III spectrin in membrane protein stabilization

Initial work on spectrin focused on its role in the red blood cell, where erythrocytic spectrin heterotetramers – each containing two α-I spectrin peptides and two β-I spectrin peptides – form a submembranous scaffold that physically supports the cell membrane and links transmembrane proteins to the actin cytoskeleton (Tse and Lux 1999). Mutations in erythrocytic spectrin cause hereditary spherocytosis and hereditary elliptocytosis. These hematologic disorders, which are characterized by red blood cell

membrane fragility, underscore the importance of spectrin for proper erythrocyte cell membrane function.

Other spectrins also play important roles in membrane function; in particular, there appears to be a general scaffolding role for β-spectrins in anchoring proteins at specific membrane domains. In *Drosophila* lacking β spectrin the Na,K ATPase does not properly accumulate at the basolateral plasma membrane in epithelial cells (Dubreuil et al. 2000). β-II spectrin is required for E-cadherin localization at the lateral plasma membrane in human bronchial epithelial cells (Kizhatil et al. 2007). Voltage-gated sodium channel clustering at the nodes of Ranvier and axon initial segments, which is necessary for effective action potential propagation along the axon, is deficient in *β-IV spectrin*–null mice (Komada and Soriano 2002).

Similarly, β-III spectrin may be required for proper localization of the transmembrane excitatory amino acid transporter 4 (EAAT4), a glutamate transporter predominantly expressed in cerebellar Purkinje cells (Gincel et al. 2007, Yamada et al. 1997, Furuta et al. 1997). β-III spectrin physically interacts with EAAT4, and functional studies show that β-III spectrin facilitates glutamate transport by EAAT4 and stabilizes EAAT4 at the plasma membrane (Jackson et al. 2001). Dr. Dan Gincel, a former post-doctoral researcher with Dr. Jeffrey Rothstein at Johns Hopkins University, used total internal reflection fluorescence (TIRF) microscopy to quantitate the effects of the control and mutant β-III spectrin clones that I developed on EAAT4 trafficking. These results show that β-III spectrin with the American SCA5 mutation fails to stabilize EAAT4 at the membrane in HEK 293 cells (Table 2) (Ikeda et al. 2006). Subcellular fractionation

analysis of cerebellar autopsy tissue from an American SCA5 patient reveals relatively less EAAT4 in the enriched synaptosomal fraction compared to control tissue (Ikeda et al. 2006); this alteration in EAAT4 fractionation suggests that less EAAT4 is located near the synapse in SCA5. Together, these results support the hypothesis that wildtype β-III spectrin stabilizes EAAT4 at specific plasma membrane locations and that by preventing this stabilization mutant β-III spectrin may lead to functional changes in glutamatergic signaling.

V. Potential role of β-III spectrin in intracellular transport

Additionally, several studies suggest that spectrin plays a role in the intracellular transport mediated by the microtubule-based motor protein dynein. Functional studies comparing transport in squid axoplasm cytosol to transport in a biochemically reconstituted system show that axonal spectrin, dynactin, and dynein all are required for microtubule-dependent transport of protein-free liposomes (Muresan et al. 2001). Co-localization and co-immunoprecipitation studies implicate spectrin in melanosome transport in *Xenopus laevis* melanophores (Aspengren and Wallin 2004). In addition, expression of spectrin's PH domain – shown to bind phosphatidylinositol 4,5-bisphosphate and thought to mediate spectrin's association with membranes – appears to disrupt retrograde axonal transport of mitochondria in neuronal cell culture by a dominant-negative mechanism (De Vos et al. 2003). Dynactin is a multi-protein complex required for coordinating intracellular bidirectional microtubule-based motility (Haghnia et al. 2007), and co-immunoprecipitation and affinity chromotography studies show that

the ARP1 subunit of dynactin associates with spectrin (Holleran et al. 1996). Additional biochemical experiments specifically implicate β-III spectrin by showing that ARP1 binds directly to β-III spectrin (Holleran et al. 2001). Together, these studies show that spectrin can interact with both membranes and dynactin and functionally implicate spectrin in intracellular transport.

Recently, Damaris Lorenzo, a graduate student in Dr. Laura Ranum's laboratory, has developed a *Drosophila* model of SCA5 in collaboration with Dr. Thomas Hays' laboratory. This model shows that overexpression of spectrins containing the American or German SCA5 mutations leads to axonal transport deficits, impaired motor coordination, and abnormal synaptogenesis in transgenic flies (Lorenzo et al. 2008). These phenotypes, which are characteristic of other mutations known to affect the transport machinery, strongly suggest that both the German and the American SCA5 mutations cause transport deficits. Additional support for this hypothesis is that partial loss of function mutations in dynein or a dominant-negative mutation in the $p150^{Glued}$ component of dynactin genetically enhance these phenotypes. Taken together, these data provide several lines of evidence that SCA5 mutations affect intracellular transport in the context of the *Drosophila* model system.

VI. Conclusions

Clinical, MRI, and pathological findings indicate that SCA5 is a slowly progressive neurodegenerative disease that predominantly affects the cerebellum. The mutations causing SCA5 occur in the *SPTBN2* gene, which is highly expressed in

cerebellar Purkinje cells and encodes the protein β-III spectrin. Putative functions for β-III spectrin include facilitating intracellular transport and stabilizing proteins at specific membrane domains. Characterization of SCA5 mutations will improve understanding of both normal β-III spectrin function and the specific disruptions of β-III spectrin function that lead to ataxia.

VII. Overall Aim

The overall aim of my thesis is to determine how the American SCA5 mutation, a small in-frame deletion in the third spectrin repeat of the large structural β-III spectrin protein, causes Purkinje cell degeneration and cerebellar dysfunction. I am pleased to report that I have developed the first murine models of SCA5 which express mutant β-III spectrin in cerebellar Purkinje cells. I have developed two types of mouse models – a conditional overexpression model in which the tet transactivator drives expression of untagged transgenic β-III spectrin protein (Chapter 2) and a simple overexpression model in which transgenic mice express epitope-tagged β-III spectrin (Chapter 3). I have used these models to test the hypotheses that expressing mutant β-III spectrin in Purkinje cells causes neuronal dysfunction, affects membrane protein localization, and affects protein trafficking. I also have identified novel candidate brain proteins that interact with the second and third spectrin repeat region of β-III spectrin, and I show that the introduction of the American and French SCA5 mutations changes the interaction strength of β-III spectrin with two different interacting proteins (Chapter 4).

Clinical Feature	Frequency in SCA5
Limb ataxia	+ + + +
Gait ataxia	+ + + +
Truncal ataxia	+ + +
Muscle weakness	–
Muscle atrophy	–
Deep tendon reflexes	
Hypoactive	+
Hyperactive	+ +
Bulbar abnormalities	+ / –
Abnormal eye movements	+ + +
Sensory	+ +
Dysarthria	+ + +
Abnormal three-cough sequence	+ / –
Babinski sign	–
Abnormal Romberg	+ + +

Notes:
+ + + +, ≥90%; + + +, 50-89%; + +, 25-49%;
+, 10-24%; + / –, 2-9%; – ≤2%.

Table 1. Clinical features of SCA5. This table reviews the disease features found in affected members of the American SCA5 family and was reprinted with the permission of Cambridge University Press from Liquori et al., (2002). Spinocerebellar ataxia type 5. In: M. Manto and M. Pandolfo (Eds.), *The Cerebellum and its Disorders.* Cambridge University Press©, Cambridge, U.K, pp. 445-450.

Construct	Total diffraction spots analyzed	Percentage of diffraction spots not moving
eGFP-EAAT4 + empty vector	685	62.0 ± 8.7
eGFP-EAAT4 + wildtype β-III spectrin	122	94.2 ± 9.7
eGFP-EAAT4 + mutant β-III spectrin	375	67.5 ± 4.4
eGFP-EAAT3 + empty vector	547	61.0 ± 11.2
eGFP-EAAT3 + wildtype β-III spectrin	337	58.7 ± 5.4

Table 2. Mutant β-III spectrin alters lateral trafficking of glutamate transporters. TIRF microscopy of HEK 293 cells was performed and digital movies of the imaged cells were evaluated using Metamorph. Each diffraction spot was analyzed separately. For each condition, three to six different experiments were recorded from different dishes and different days. Results are given as mean ± s.d. Figure and legend are reprinted with permission from Ikeda et al., (2006), *Nature Genetics*, Vol. 38, pp. 184-190, with permission conveyed through the Copyright Clearance Center.

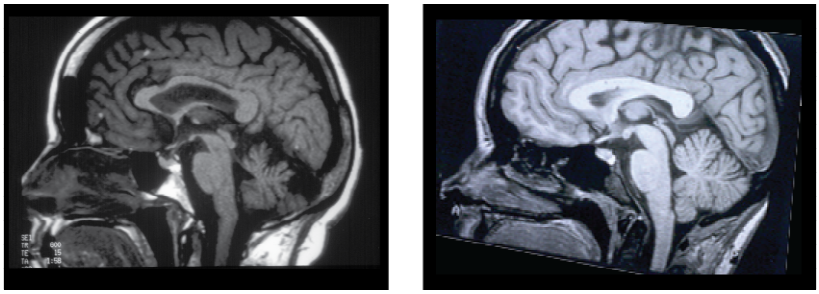


Figure 1. MRI of an American SCA5 patient. Sagittal MRI scan from an affected individual at the age of 64 shows marked cerebellar atrophy, minimal brainstem atrophy, and no evidence of cerebral involvement. Relative preservation of the posterior vermis, posterior hemisphere, and tonsillar cortex is evident. Sagittal MRI scan from an unaffected control individual is shown for comparison. Modified and reprinted with the permission of Cambridge University Press from Liquori et al., (2002). Spinocerebellar ataxia type 5. In: M. Manto and M. Pandolfo (Eds.), *The Cerebellum and its Disorders*. Cambridge University Press©, Cambridge, U.K, pp. 445-450.

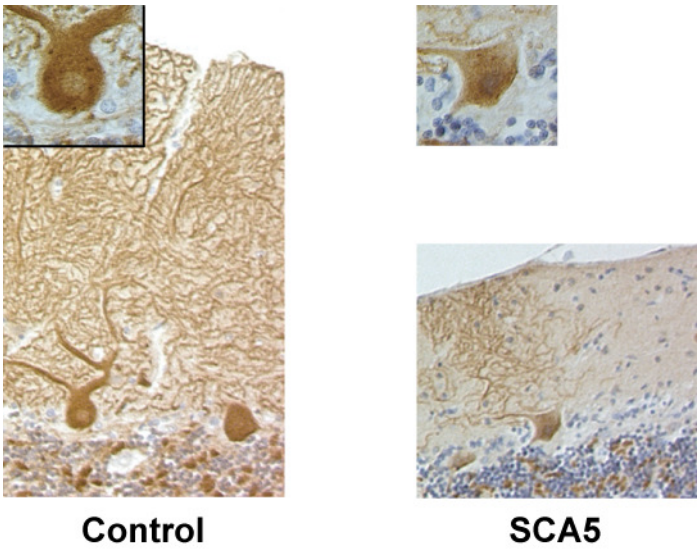


Figure 2. β-III spectrin immunohistochemistry in control and American SCA5 cerebellar tissues. Sections were stained with an antibody raised against the N-terminal portion of the β-III spectrin and visualized at 200X magnification. Enlarged images of the Purkinje cells are also shown (630X). Figure and legend are reprinted with permission from Ikeda et al., (2006), *Nature Genetics*, Vol. 38, pp. 184-190, with permission conveyed through the Copyright Clearance Center.

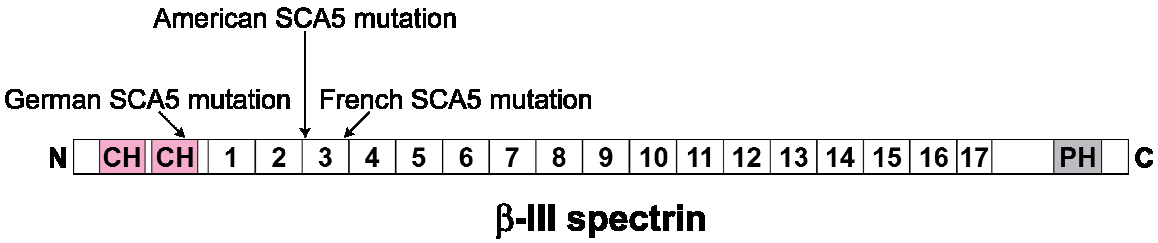


Figure 3. Illustration of SCA5 mutations. Human β-III spectrin is a 2390 aa protein which consists of an N-terminal region containing two calponin homology (CH) domains, a central region containing 17 spectrin repeats, and a C-terminal region containing a pleckstrin homology (PH) domain. The German SCA5 mutation causes a leucine to proline change in the second CH domain (L253P), the American SCA5 mutation causes an in-frame deletion of 13 amino acids at the beginning of the third spectrin repeat (E532_M544del), and the French SCA5 mutation causes an in-frame deletion of 6 amino acids with the insertion of a tryptophan at the end of the third spectrin repeat (L629_R634delinsW).

CHAPTER 2

CHARACTERIZATION OF A CONDITIONAL MURINE MODEL OF SCA5

I. Introduction

The limited availability of relevant human disease tissue, which is often at a late stage in the degenerative process, and the limited scope of the types of investigations that can be done with human subjects underscores the importance of using model organisms to study neurodegenerative diseases such as SCA5. Biomedical research has utilized model organisms ranging in complexity from prokaryotes such as the bacterium *Escherichia coli* to primates such as the Rhesus Macaque; generally, studies using more complex organisms are more time-consuming and expensive but yield results with more relevance to the human condition. The house mouse, *Mus musculus*, has been used extensively for studying genetic neurodegenerative disorders because it has brain cytoarchitecture similar to that of humans, is amenable to genetic manipulation, and has a relatively short generation time.

When designing a mouse model of a genetic disease, the probable genetic mechanism is an important consideration. The autosomal dominant inheritance pattern of SCA5 points toward gain-of-function, dominant-negative, or haploinsufficiency as potential genetic disease mechanisms. All 3 types of mechanisms can be modeled using a knock-in approach, but knock-in models do not always faithfully recapitulate neurodegenerative features of adult-onset human diseases because of the short murine life span. For example, although the reported range of pathogenic human SCA1 alleles is 42-

81 CAG repeats (Ranum et al. 1994b), the first reported SCA1 knock-in model – with 78 CAG repeats inserted into the mouse *Atxn1* gene (Lorenzetti et al. 2000) – had relatively late onset of subtle motor coordination deficits and lacked cerebellar neurodegeneration. A subsequent SCA1 knock-in model did recapitulate the neurodegenerative features of SCA1 (Watase et al. 2002), but the 156 CAG repeats in these mice represent a much larger expansion than any found in human SCA1 patients. The alternative transgene overexpression approach has produced a more representative disease model using a human SCA1 mutation, in which Purkinje cell expression of transgenic ataxin-1 with 82 CAG repeats causes cerebellar degeneration (Burright et al. 1995).

The transgene overexpression approach can be used to model diseases caused by gain-of-function and dominant-negative mutations but not haploinsufficiency. TIRF microscopy studies in HEK 293 cells indicate that β-III spectrin with the American SCA5 mutation acts in a dominant-negative manner to prevent wildtype β-III spectrin from stabilizing the glutamate transporter excitatory amino acid transporter 4 (EAAT4) at the membrane (Dan Gincel, personal communication). Additionally, although mice lacking full-length β-III spectrin develop motor coordination deficits and Purkinje cell loss by 6 months of age, mice with approximately half normal levels of full-length β-III spectrin appear unaffected at least to a year of age (Jackson et al. 2008); these findings argue against β-III spectrin haploinsufficiency as a mechanism for SCA5. Given the EAAT4 evidence for a dominant-negative mechanism and the evidence against haploinsufficiency, I used a transgene overexpression approach to develop a mouse model of SCA5.

Immunohistochemical analysis indicates that within the cerebellum β-III spectrin protein is found predominantly in Purkinje cells (Ohara et al. 1998), so for this transgenic SCA5 model I have used the tet-responsive system to drive expression in murine Purkinje cells of either wildtype human β-III spectrin or human β-III spectrin with the American SCA5 mutation (E532_M544del). BLAST comparison of human (NCBI accession no. NP_008877.1) and mouse (NCBI accession no. NP_067262.1) β-III spectrin shows that the two peptides are 94% identical at the amino acid level, so the transgenic wildtype human β-III spectrin will likely function similarly if not identically to endogenous mouse β-III spectrin.

Characterization of this transgenic murine model shows that expression of mutant β-III spectrin in cerebellar Purkinje cells causes behavioral, histological, and functional changes. The SCA5 mice have significantly reduced latency on the accelerating rotorod, indicating a deficit in motor coordination. Thinning of the molecular layer in SCA5 mice suggests Purkinje cell dendritic atrophy. Immunofluorescence studies reveal changes in localization of the perisynaptic excitatory amino acid transporter 4 (EAAT4) and metabotropic glutamate receptor 1α (mGluR1α) proteins in SCA5 mice. Additionally, *in vivo* flavoprotein optical imaging studies demonstrate a lack of mGluR1-mediated long term potentiation (LTP). These alterations in the SCA5 mice involving the glutamate transporter EAAT4 and the glutamate receptor mGluR1α strongly suggest that aberrant perisynaptic glutamate signaling may contribute to SCA5 pathogenesis.

II. Results

I generated the DNA constructs for this murine model by separately cloning cDNAs encoding wildtype or mutant human β-III spectrin immediately downstream of the tet-response element (TRE) (Figure 4). The resulting wildtype (TRE-SP-WT) and mutant (TRE-SPΔ39) DNA constructs were sent to the University of Minnesota Mouse Genetics Laboratory for pronuclear injection. I identified transgenic animals by Southern blot and PCR (Figure 5). Nine animals were identified with the TRE-SPΔ39 transgene out of 44 potential founders, 4 had transgene-positive offspring, and one of these founders had 2 integration sites (Figure 5), which resulted in a total of 5 TRE-SPΔ39 lines. Nine animals with the TRE-SP-WT transgene were identified by Southern blot and PCR out of 43 potential founders, and 5 had transgene-positive offspring.

To generate mice expressing wild-type or mutant β-III spectrin in cerebellar Purkinje cells I bred the previously characterized *Pcp2-tTA* transgenic mice, in which the *Pcp2* promoter expresses the tetracycline transactivator (tTA) specifically in the Purkinje cells of the cerebellum (Zu et al. 2004), to *TRE-SP-WT* and *TRE-SPΔ39* transgenic mice. I performed relative quantification of *TRE-SP* RNA expression by quantitative RT-PCR analysis on cerebellar tissue from doubly transgenic *Pcp2-tTA*$^{+/-}$ / *TRE-SP*$^{+/-}$ mice at 3 weeks of age. The highest transgene expression level was found in control line 225, with SCA5 lines 184-1, 184-2, 643, and 645 also expressing the transgene at the RNA level (Figure 6); however, transgene expression in mice from SCA5 line 690, control line 246, and control line 266 was barely detectable.

I tested doubly transgenic animals from lines robustly expressing the transgene for deficits in motor coordination by assessing performance on the accelerating rotorod at 26 weeks of age. *Pcp2-tTA / TRE-SPΔ39* animals from lines 184-2 and 645 had significantly reduced average latencies on the accelerating rotorod compared to age-matched *Pcp2-tTA* animals and to *Pcp2-tTA / TRE-SP-WT* mice from line 225 (Figure 7). *Pcp2-tTA / TRE-SPΔ39* animals from lines 643 and 184-1 had reduced average latencies compared to line 225 *Pcp2-tTA / TRE-SP-WT* mice, but the difference was not statistically significant. I carried out further characterization using the SCA5 lines with significant differences in rotorod performance (lines 184-2 and 645) and control line 225.

First I performed histological studies of the cerebella, which show thinning of the molecular layer in SCA5 line 184-2 mice at 37 weeks of age compared to age-matched *Pcp2-tTA* mice (p-value < 0.02) and control line 225 mice (p-value < 0.02) (Figure 8). While SCA5 line 645 mice did not have reduced thickness of the molecular layer at 37 weeks of age compared to controls (data not shown), at 80 weeks of age the average molecular layer thickness in the SCA5 line 645 mice as well as the SCA5 line 184-2 mice was reduced compared to age-matched control line 225 mice (p-value of control line 225 vs. SCA5 line 184-2 < 0.01; p-value of control line 225 vs. SCA5 line 645 < 0.01) (Figure 8).

Next I used the SCA5 mice and human SCA5 autopsy tissue to examine the effect of mutant β-III spectrin on the glutamate transporter EAAT4. Immunoblot analysis shows that total cerebellar levels of EAAT4 protein are unchanged in SCA5 samples compared to controls after normalizing for Purkinje cell abundance with calbindin in both human

autopsy tissue (Ikeda et al. 2006) and the conditional SCA5 murine model (Figure 9). This finding is verified by immunohistochemical analysis of SCA5 autopsy tissue (Figure 10) (Ikeda et al. 2006), which shows abundant EAAT4 protein in the remaining Purkinje cells. Although EAAT4 is not lost in SCA5 Purkinje cells, immunohistochemical analysis of an initial human SCA5 autopsy specimen suggested altered localization of EAAT4, with more intense EAAT4 staining of the Purkinje soma compared to control tissue (Ikeda et al. 2006). I show here that immunohistochemical analysis of a second, better preserved American SCA5 autopsy specimen verifies this initial finding (Figure 10), although conclusive analysis of EAAT4 localization in human autopsy tissue is complicated by extensive Purkinje cell atrophy and loss. The conditional SCA5 mice show a similar change in EAAT4 distribution pattern, with relatively more EAAT4 located in the soma than in dendrites compared to controls (Figure 11). Additionally, higher magnification images show that EAAT4 dendritic distribution in controls is more punctate, with clustering of EAAT4 at dendritic spines; whereas EAAT4 is more diffusely located throughout the dendritic membrane of SCA5 mice (Figure 12).

I also examined the effect of mutant β-III spectrin on the metabotropic glutamate receptor 1α (mGluR1α), a Purkinje cell receptor with a perisynaptic dendritic distribution similar to that of EAAT4 (López-Bendito et al. 2001, Dehnes et al. 1998). Biochemical analysis of non-transgenic murine cerebellar extracts shows that a small amount of mGluR1α co-immunoprecipitates with β-III spectrin and a small amount of β-III spectrin co-immunoprecipitates with mGluR1α (Figure 13); these data show that β-III spectrin and mGluR1α interact in cerebellar extracts. As with EAAT4, total cerebellar levels of

mGluR1α protein are similar in SCA5 and control mice (Figure 14), but cerebellar mGluR1α localization is different: mGluR1α is clustered at Purkinje dendritic spines in control mice but appears more diffusely distributed within the Purkinje dendrites of SCA5 mice (Figure 15A). I quantified this distribution difference by determining the degree to which mGluR1α was concentrated in clusters, using an approach similar to that developed by Das and Banker (2006) to analyze receptor clustering in cultured hippocampal neurons. I defined mGluR1α clusters to be regions of fluorescence intensity at least double that of the average fluorescence intensity of the entire image. After setting the threshold for each image at twice its average fluorescence intensity (Figure 15B), I used the particle analysis function in ImageJ (Rasband 1997-2009) to automatically analyze the mGluR1α clusters. I show that both average mGluR1α cluster size and average mGluR1α cluster coverage – defined as the percentage of the image area covered by clusters – are significantly less in SCA5 mice compared to controls (Figure 16).

This change in mGluR1α localization prompted examination of mGluR1 function. Dr. Xinming Wang, a post-doctoral researcher in Dr. Timothy Ebner's laboratory, used *in vivo* optical imaging techniques (reviewed in Reinert et al. 2007) to examine mGluR1-mediated long term potentiation (LTP) at the parallel fiber – Purkinje cell synapse (Wang et al. 2009) in the SCA5 mice (Figure 17). High intensity, burst stimulation of parallel fibers elicited a subsequent increase in the normalized response in control mice, with response amplitude significantly above baseline up to 60 minutes after conditioning; however, this potentiation was absent in SCA5 mice. The response of the

SCA5 mice is reminiscent of the lack of potentiation seen in non-transgenic mice treated with the mGluR1 antagonist LY36785 (Wang et al. 2009).

III. Discussion

This transgenic SCA5 murine model is the first mammalian model to show that overexpression of mutant but not wildtype β-III spectrin in cerebellar Purkinje cells causes a motor coordination phenotype and degenerative cerebellar changes. These cerebellar phenotypes validate the hypothesis that mutant β-III spectrin causes ataxia through a dominant gain-of-function or dominant-negative effect and provide evidence against a haploinsufficiency mechanism. This work, which addresses the nature of the mutation, is important for understanding the fundamental disease mechanisms that underlie SCA5 and therefore may be important for future rational therapeutic development.

Two of the four SCA5 lines that express the mutant β-III spectrin transgene have a significant deficit in rotorod latency. I only compared expression at a single time point, so although the two SCA5 lines without a significant rotorod deficit are not the lowest expressing lines by my analysis, it is possible that timing of transgene expression varies so that some lines do express the transgene at 3 weeks of age but not at time points important for developing disease. Also, my expression comparison is indirect since I measured transgenic RNA levels. The cross-reactivity of all available antibodies that recognize transgenic human β-III spectrin protein with endogenous mouse β-III spectrin prevented me from directly comparing transgenic protein levels. Additionally, although

the rotorod can be a sensitive test for motor coordination, it is a behavioral test and the high amount of animal-to-animal variability can mask minor changes in motor coordination.

Analysis of this conditional SCA5 murine model corroborates other studies showing dysregulation but not loss of the glutamate transporter EAAT4 in SCA5. Expression of β-III spectrin with the American SCA5 mutation does not affect EAAT4 protein levels in Purkinje cells of these conditional SCA5 mice or in human SCA5 autopsy tissue (Ikeda et al. 2006). However, mutant β-III spectrin expression does affect EAAT4 localization in two American SCA5 patients and in this murine model of SCA5.

The EAAT4 findings present a fundamental difference between this conditional SCA5 murine model and mice lacking full-length β-III spectrin. These β-III spectrin deficient mice have been proposed as a potential SCA5 disease model (Jackson et al. 2008), though these mice do not have an SCA5 mutation and sequencing the *SPTBN2* gene in patients with unknown forms of ataxia has not revealed any predicted null mutations to date (Laura Ranum, personal communication). Loss of EAAT4 occurs early in the β-III spectrin deficient mice and produces functional electrophysiological changes in 3-week-old mice (Jackson et al. 2008), but since EAAT4 loss is not a feature of SCA5, the β-III spectrin deficient mice do not accurately model functional changes related to EAAT4 in SCA5. Comparing features of the β-III spectrin deficient mice with the conditional SCA5 mice and the human SCA5 autopsy specimens will establish which normal functions of β-III spectrin are affected in SCA5, although the analysis is complicated to some extent because the β-III spectrin deficient mice express a small

amount of β-III spectrin protein containing all but the N-terminal amino acids encoded by exons 2-6.

Similar to EAAT4, dysregulation but not loss of a second perisynaptic protein, the glutamate receptor mGluR1α, also occurs in this conditional SCA5 murine model. The clustering of mGluR1α at Purkinje dendritic spines is reduced in the SCA5 mice, and optical imaging studies show deficiencies in an mGluR1-mediated function in the SCA5 mice. This disruption of mGluR1α is intriguing since mGluR1 knockout mice develop ataxia (Aiba et al. 1994), and restoring mGluR1α to cerebellar Purkinje cells of the mGluR1 knockout mice restores motor coordination (Nakao et al. 2007). Additionally, mGluR1 plays a critical role in cerebellar synaptic plasticity: mGluR1 is required for both long term depression and long term potentiation at parallel fiber – Purkinje cell synapses (Aiba et al. 1994, Wang et al. 2009).

My results from Chapter 3 and previously reported findings suggest a potential model for the interaction I observed between β-III spectrin and mGluR1α. β-III spectrin interacts with α-II spectrin in cerebellar lysates (Chapter 3). Böckers and colleagues (2001) describe a potential link between α-II spectrin and the complex associated with mGluR1α by showing that α-II spectrin binds to the synaptic scaffolding protein Shank. Within the mGluR1α complex, Homer is an adaptor between Shank and mGluR1α (Tu et al. 1999, Brakeman et al. 1997). Binding experiments with different combinations of these proteins would determine whether each of these proteins is necessary and sufficient for the interaction between β-III spectrin and mGluR1α.

In summary, analysis of this conditional SCA5 murine model shows that expression of mutant β-III spectrin in murine Purkinje cells causes changes in cerebellar function. Unlike β-III spectrin deficient mice but like human SCA5 autopsy specimens, the conditional SCA5 mice have no significant change in cerebellar EAAT4 protein levels after controlling for Purkinje cell abundance. Characterization of these conditional SCA5 mice shows altered EAAT4 localization, altered mGluR1α localization, and deficient mGluR1-mediated LTP compared to controls. Taken together, these results indicate that aberrant glutamate signaling in perisynaptic regions of cerebellar Purkinje cells may contribute to SCA5 pathogenesis.

A potential future direction using this conditional SCA5 murine model is to address whether expressing mutant β-III spectrin causes EAAT4 loss-of-function effects. For instance, glutamate clearance at the climbing fiber – Purkinje cell synapse is deficient in the EAAT4 knock-out mice (Huang et al. 2004), so examining climbing fiber – Purkinje cell synaptic function would be one way to evaluate EAAT4 function in the conditional SCA5 mice. Additionally, the EAAT4 knock-out mice have increased parallel fiber evoked activation of perisynaptic metabotropic glutamate receptors (mGluRs) such as mGluR1, with increased amplitude and faster rising kinetics of mGluR-mediated excitatory postsynaptic currents (mGluR-EPSCs) (Nikkuni et al. 2007). This finding corresponds to previous work showing that glutamate transport of postsynaptic neuronal EAATs limits the activity of mGluRs (Brasnjo and Otis 2001, Otis et al. 2004, Wadiche and Jahr 2005). Impaired mGluR1α function in the conditional SCA5 mice may preclude the importance of any potential EAAT4 functional change; conversely, EAAT4

mislocalization may contribute to the mGluR1α dysfunction seen in the conditional SCA5 mice. Comparing mGluR-EPSCs in conditional SCA5 mice deficient in EAAT4 to conditional SCA5 mice with normal levels of EAAT4 would address this hypothesis and provide a more complete understanding of EAAT4 and its relationship to mGluR1α in SCA5.

Additionally, the disruption of mGluR1α, a protein involved in cerebellar plasticity, is particularly intriguing given that SCA5 patients show a different type of dysdiadochokinesis compared to patients with other forms of SCA (Laura Ranum, personal observation). Rapid alternating movements, a common clinical task to evaluate motor coordination, requires the patient to tap his hand on the same flat surface area while rapidly alternating his hand between pronation and supination (Gelb 2009). Patients with other types of SCA typically perform this task slowly and arrhythmically, but accurately. Many SCA5 patients have problems not only with speed and rhythm but also with learning how to accurately perform this task: instead of flipping their hand and touching the same surface area during pronation and supination, they hinge the edge of their hand against the flat surface. This consistent and unusual finding even after repeated correction suggests that SCA5 may affect motor learning as well as motor coordination. More extensive analysis of motor learning in the SCA5 mice and SCA5 patients and comparisons of motor learning to other SCAs with similar disease onset such as SCA8 (Koob et al. 1999, Moseley et al. 2006) would address whether motor learning is disproportionately affected in SCA5.

Many neurodegenerative diseases such as SCA5 progress from cell dysfunction to cell dysmorphism, finally culminating in cell death. This conditional SCA5 murine model may provide insight into the early stages of disease pathogenesis, but even by 80 weeks of age the mice do not develop end-stage SCA5 disease characteristics such as dramatic Purkinje cell loss. Modifying the murine model to increase transgene expression levels may recapitulate more neurodegenerative features of SCA5 at an earlier time point. Various ways to try to increase transgene expression in the existing conditional SCA5 model include homozygosing the TRE transgene, using a line with higher tTA expression in Purkinje cells, and reinjecting the TRE transgene and selecting for lines with significantly higher expression. Alternatively, increasing transgene expression may require an entirely different transgenic approach.

Another potential approach for developing an SCA5 murine model with an earlier, more severe disease phenotype is to generate and characterize mice with the other reported SCA5 mutations. The varying disease severity and age of onset in murine models of amyotrophic lateral sclerosis with different *superoxide dismutase 1* point mutations (Shibata 2001) provides rationale for this strategy. Also, this approach would allow researchers to compare the effects of different SCA5 mutations on the cerebellar Purkinje cell. The German SCA5 mutation is located in an entirely different domain of β-III spectrin than the other SCA5 mutations and causes an aberrant interaction with actin (Krueger 2008) not seen with the American or French SCA5 mutations (K. Krueger and K. Armbrust, personal observations). Quantitative interaction studies show that the American and French SCA5 mutations have dissimilar effects on the strength of protein-

protein interactions (Chapter 4). These demonstrated differences between the mutations suggest the possibility that all three reported SCA5 mutations may disrupt different spectrin-protein interactions. Still, each of these mutations leads to similar neurodegenerative changes in humans, and comparing mouse models with different SCA5 mutations is one approach to identify similarities in Purkinje cell dysregulation.

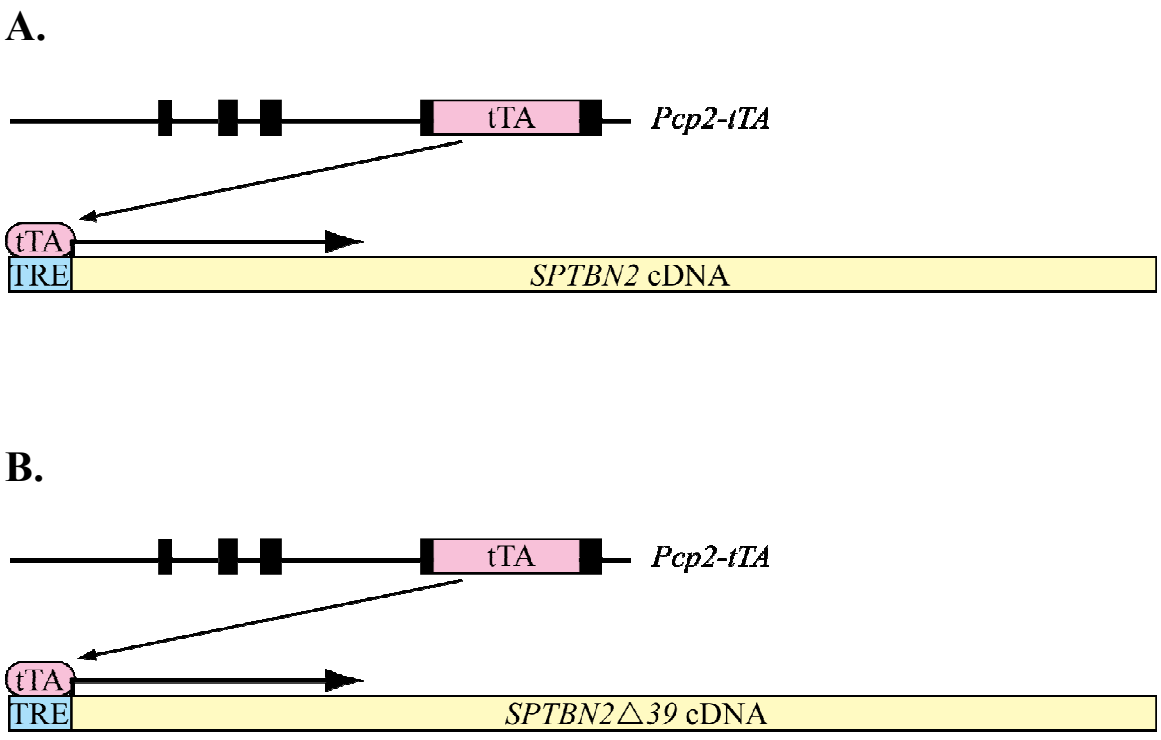


Figure 4. Conditional SCA5 murine model design. (A) Schematic of control *Pcp2-tTA* / *TRE-SP-WT* mice. The *Pcp2-tTA* transgene produces tTA protein in cerebellar Purkinje cells. The tTA protein binds to the TRE and drives expression of wildtype β-III spectrin. (B) Schematic of SCA5 *Pcp2-tTA* / *TRE-SPΔ39* mice. The *Pcp2-tTA* transgene produces tTA protein in cerebellar Purkinje cells. The tTA protein binds to the TRE and drives expression of β-III spectrin with the 39bp American SCA5 deletion.

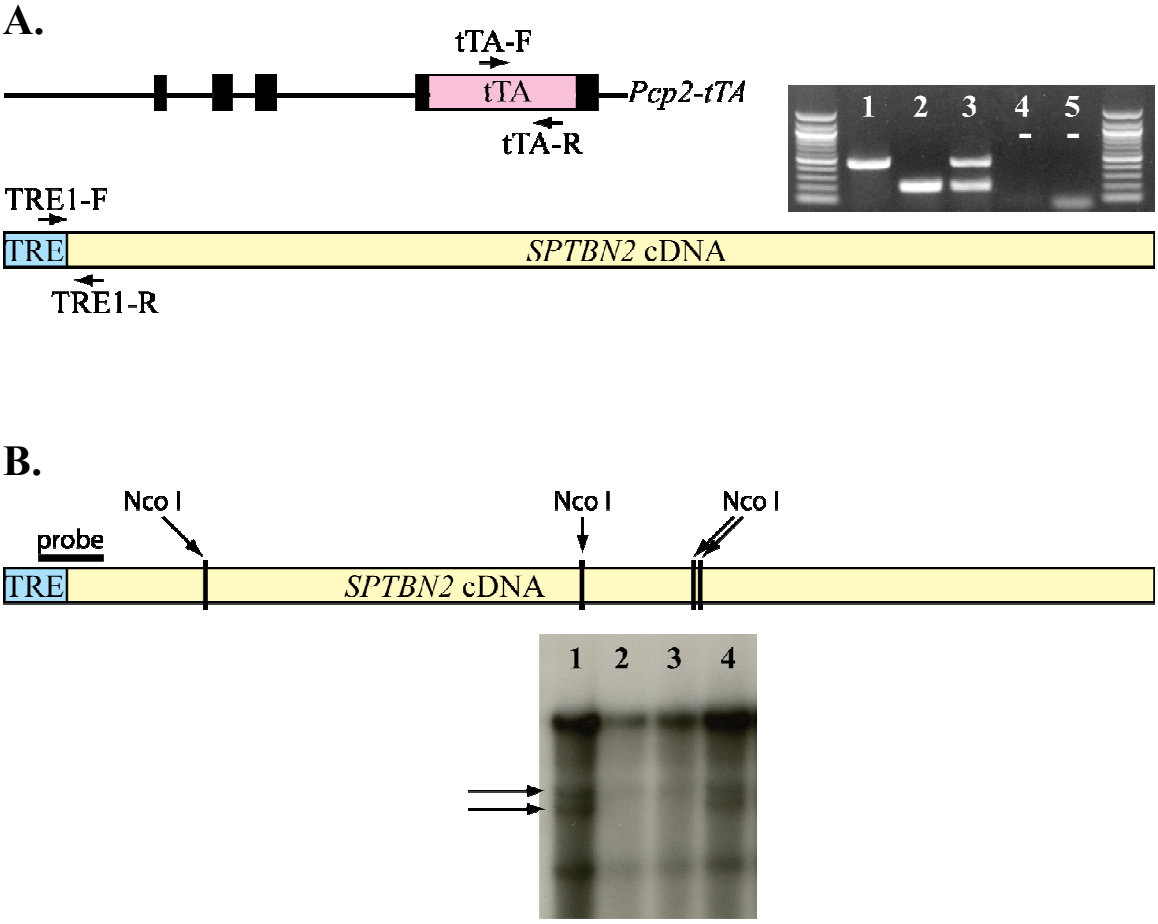


Figure 5. Genotyping conditional SCA5 mice. (A) PCR assay for identifying *Pcp2-tTA* and *TRE-SP* transgenes. Primers tTA-F and tTA-R anneal within the tTA sequence. Primer TRE1-F anneals within the TRE sequence, and primer TRE1-R anneals within the *SPTBN2* cDNA. A 469 bp product is generated by genotyping an animal with the *Pcp2-tTA* transgene (lane 1), a 212 bp product is generated by genotyping an animal with the *TRE-SP-WT* transgene (lane 2), and both products are generated by genotyping a doubly trangenic *Pcp2-tTA* / *TRE-SP-WT* animal (lane 3). DNA from a known non-transgenic animal (lane 4) and no DNA template (lane 5) are negative controls. (B) Southern blot assay to identify distinct *TRE-SP* transgene integration sites. Locations of Nco I restriction sites and the Southern probe sequence are shown on the *TRE-SP* transgene. Distinct integration site patterns identified by Nco I digestions of genomic DNA from transgene-positive offspring of founder 184 are shown in Lanes 1-4: the arrows point out 2 integration site-specific bands in Lanes 1 and 4 and only 1 integration site-specific band in Lanes 2 and 3.

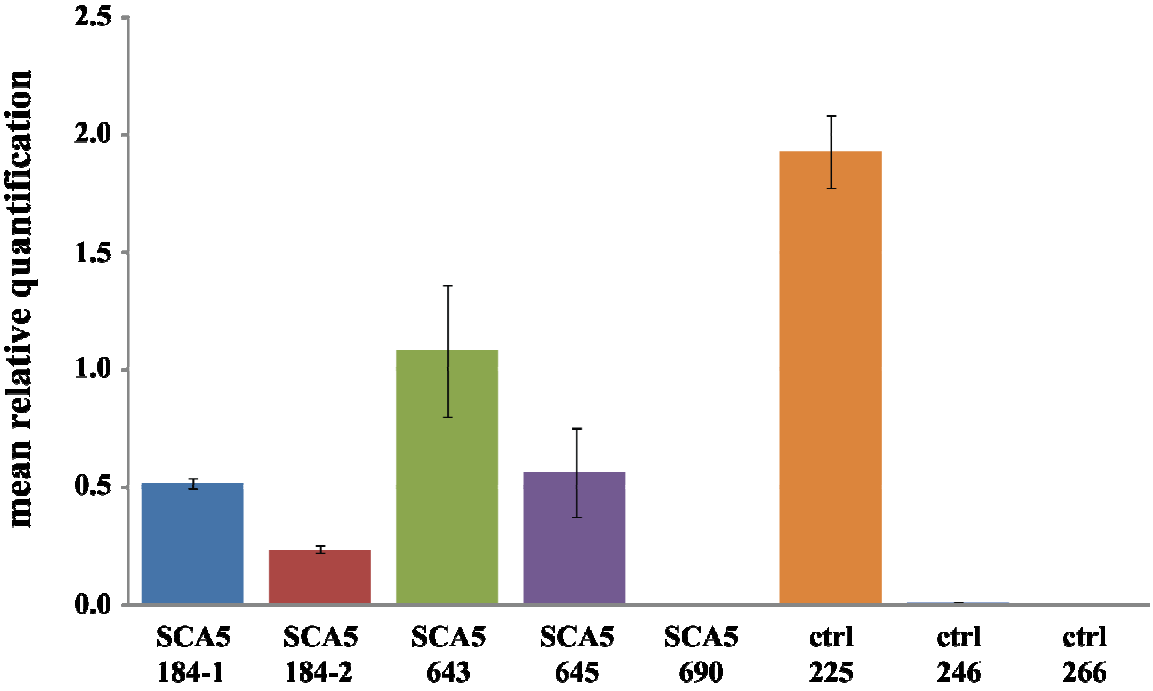
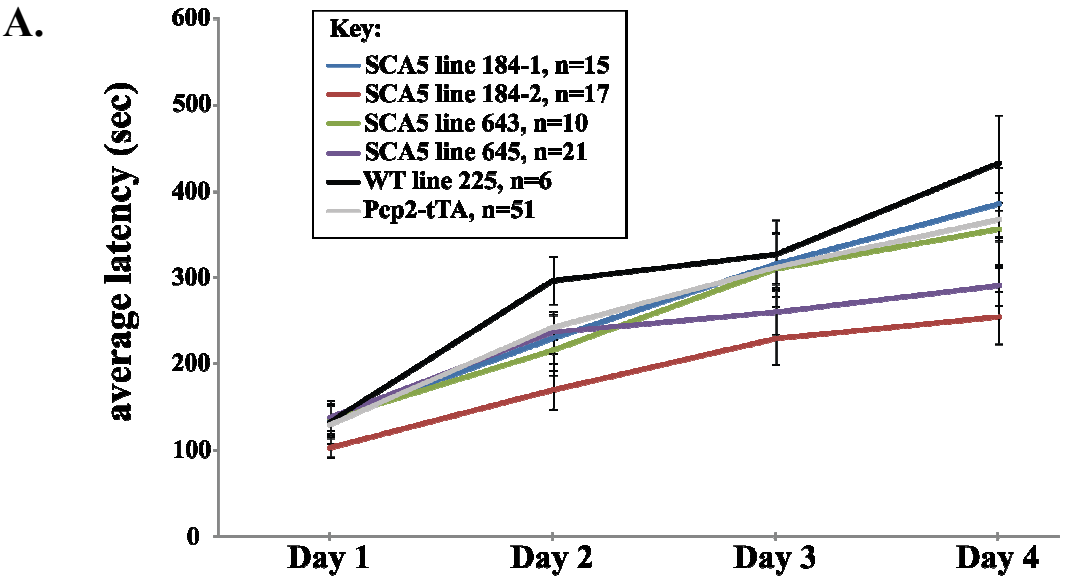
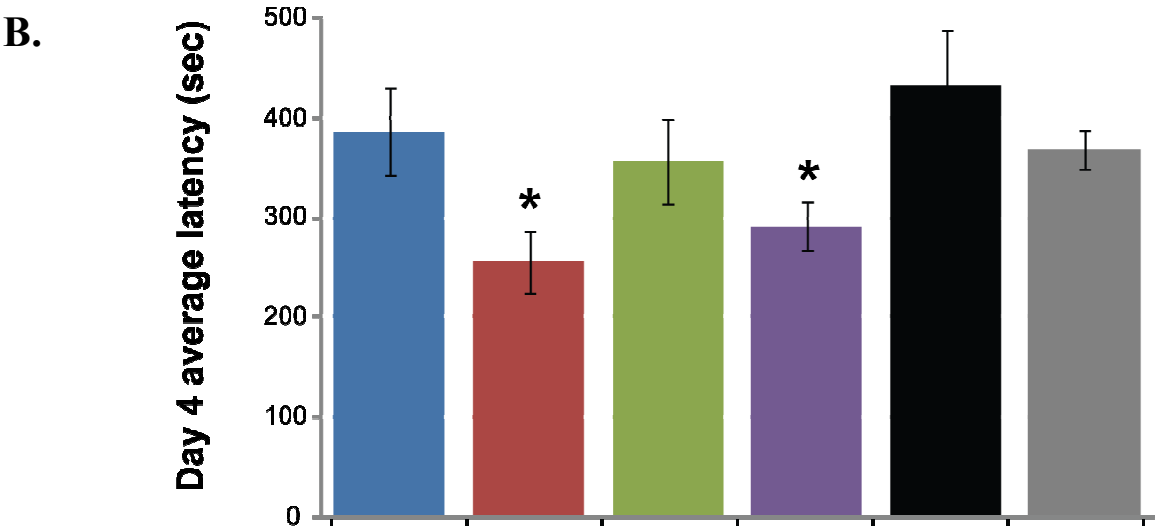


Figure 6. Transgene expression levels. Mean relative quantification values of transgenic *TRE-SP* RNA in cerebella from doubly transgenic *Pcp2-tTA* / *TRE-SP* mice at 3 weeks of age. Relative quantification was estimated for each mouse by normalizing the threshold cycle (C_T) of the *TRE-SP* transcript to the calbindin cDNA C_T. SCA5 lines 184-1, 184-2, 643, and 645 as well as control line 225 express the spectrin transgene. Error bars indicate S.E.M.



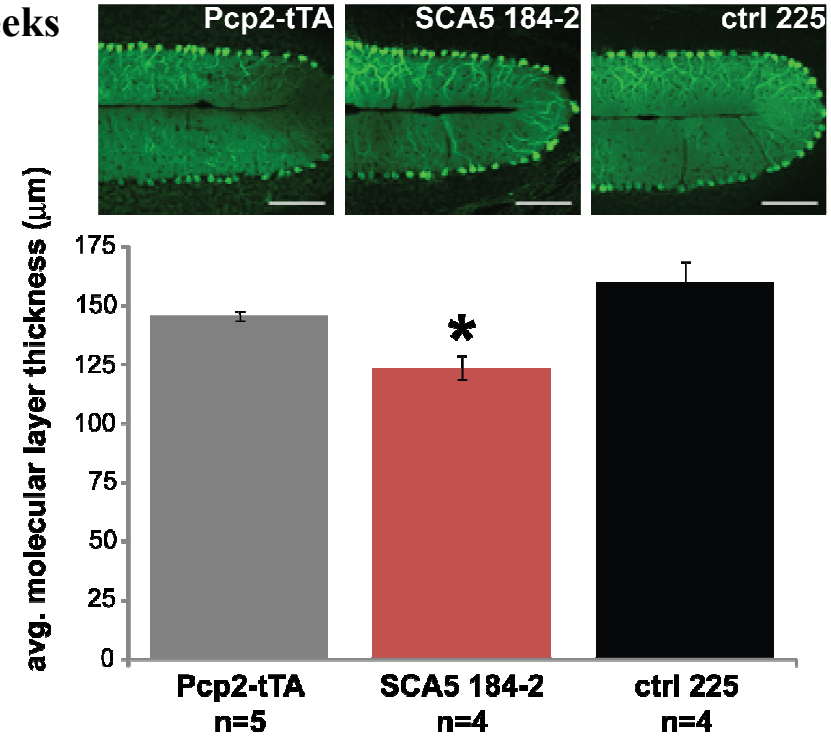


	SCA5 184-1	SCA5 184-2	SCA5 643	SCA5 645	WT 225	PCP2-tTA
Day 4 average	386	255	356	291	433	368
n	15	17	10	21	6	51
S.E.M.	43	31	43	24	55	20
p-value vs PCP2-tTA	0.706	0.004	0.809	0.017	0.303	
p-value vs WT line 225	0.512	0.021	0.292	0.050		0.303

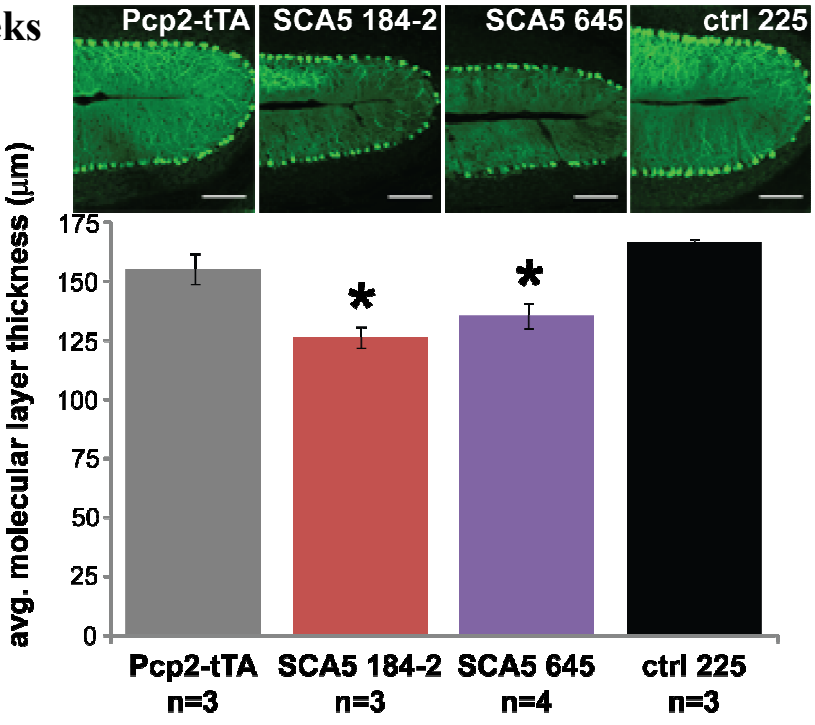
Figure 7. Rotorod analysis of SCA5 mice. (A) Mice were tested for latency on the accelerating rotorod for 4 consecutive days at 26 weeks of age. (B) Histogram of average latencies on Day 4. * indicates p-value ≤ 0.05 compared to *Pcp2-tTA* and line 225 *Pcp2-tTA/TRE-SP-WT* controls. All error bars indicate S.E.M.

Figure 8. Molecular layer thickness of SCA5 mice. Primary fissure of cerebellar cortex visualized by calbindin immunofluorescence from 37 week (A) and 80 week (B) mice with quantitation of molecular layer thickness. Error bars indicate S.E.M. Scale bars = 100 μm. * indicates p-value < 0.05 compared to control line 225.

A. 37 weeks



B. 80 weeks



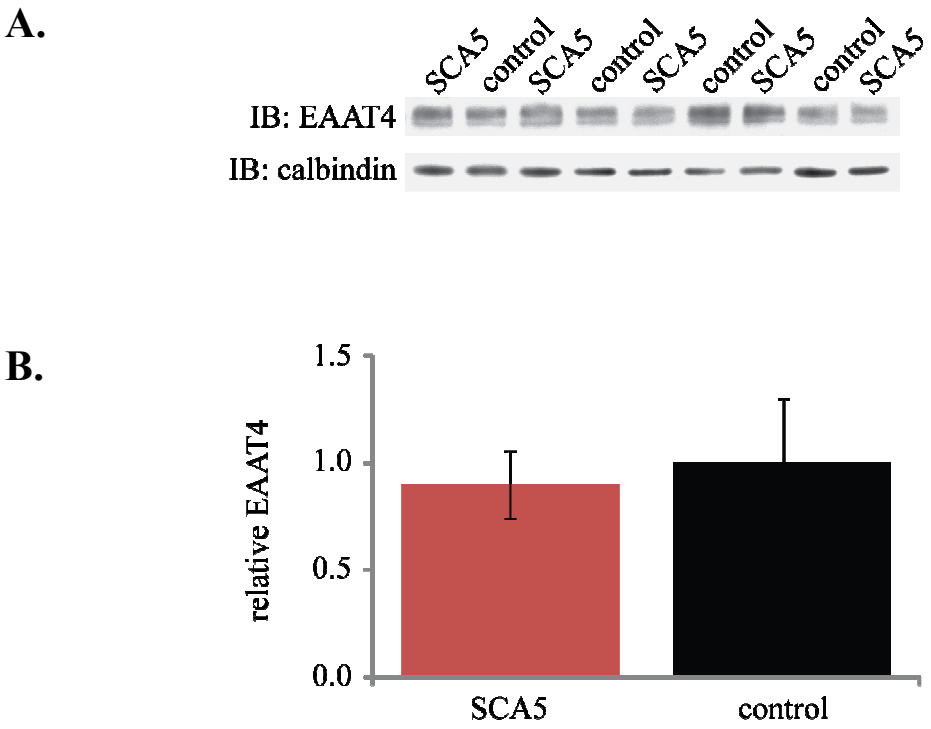


Figure 9. EAAT4 protein levels are preserved in SCA5 mice. (A) Immunoblot analysis of cerebellar lysates from SCA5 (*Pcp2-tTA/TRE-SPΔ39* line 184-2, n=5) and control (*Pcp2-tTA/TRE-SP-WT* line 225, n=4) mice aged 9-11 months. (B) I calculated the ratio of EAAT4 to calbindin for each sample from densitometric analysis of immunoblots. The average ratio for the SCA5 samples was not significantly different from that of control samples (p-value = 0.78). Error bars indicate S.E.M.

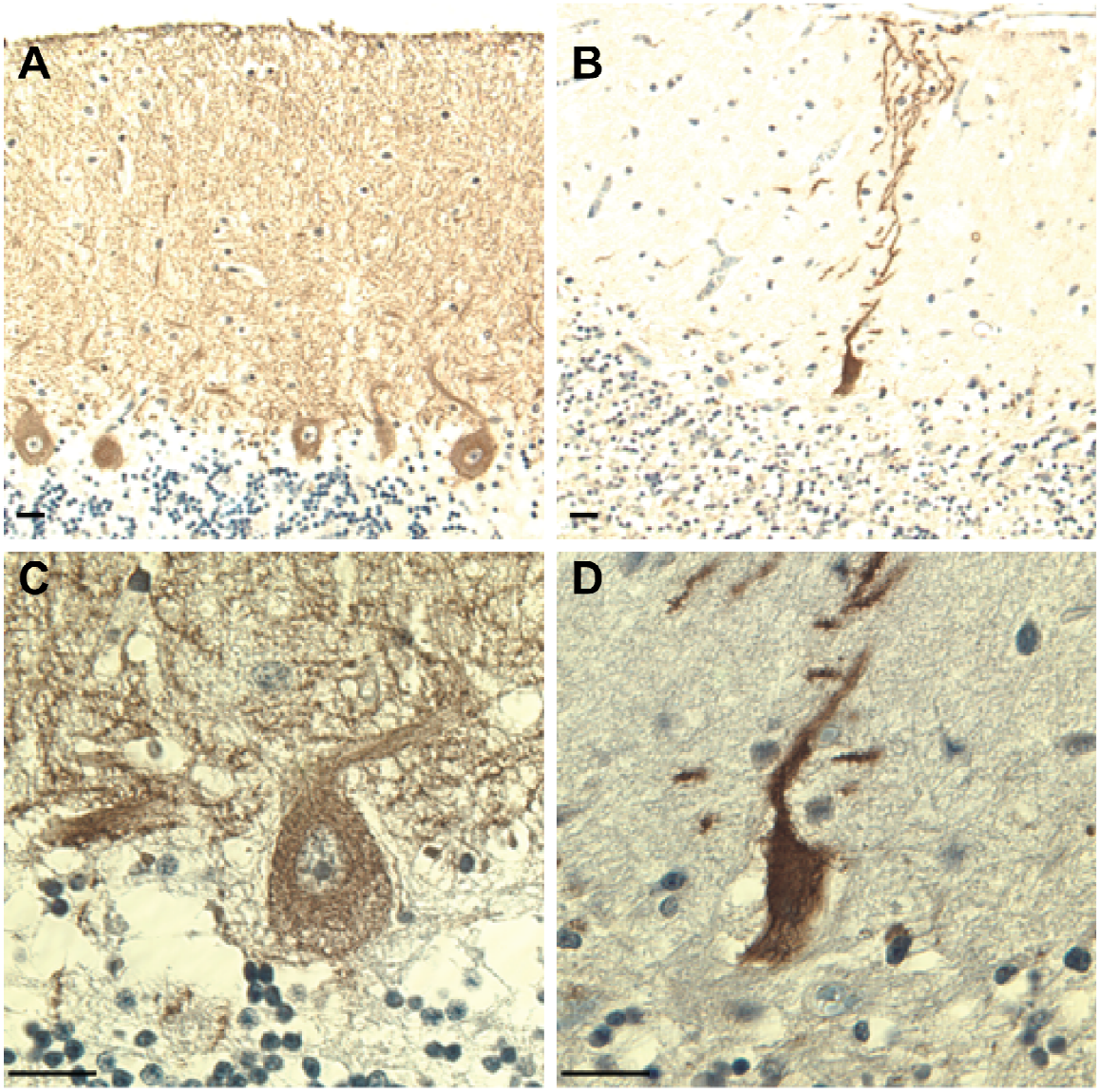


Figure 10. EAAT4 immunohistochemistry in SCA5 autopsy tissue. I stained cerebellar autopsy tissue from control (A,C) and American SCA5 (B,D) individuals with an EAAT4 antibody and used a hematoxylin counterstain. Images are at 200X magnification (A,B) and 630X magnification (C,D). Scale bars = 20 µm.

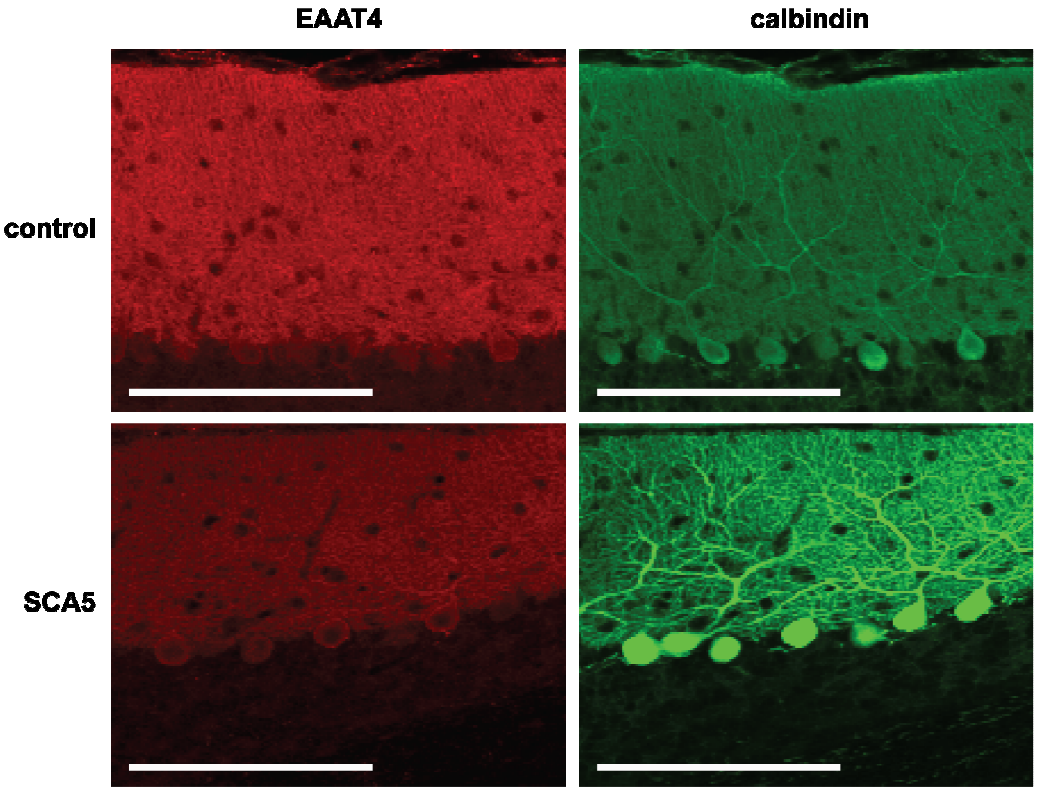


Figure 11. EAAT4 immunofluorescence in SCA5 mice. I immunostained cerebella from 8-10 month SCA5 line 184-2 mice and age-matched control line 225 mice with calbindin and EAAT4 antibodies and visualized Purkinje cells adjacent to the primary fissure by confocal microscopy. The dendrites in the SCA5 tissue stain less intensely with the EAAT4 antibody than the dendrites in the control tissue, but the intensity of EAAT4 staining is higher in Purkinje soma in SCA5 compared to control. The calbindin staining in the right panels shows the Purkinje cells. Calbindin staining intensity varied from section to section, but the calbindin intensity of SCA5 mice was not reproducibly higher than controls, as shown above, or vice versa. Scale bars = 100 µm.

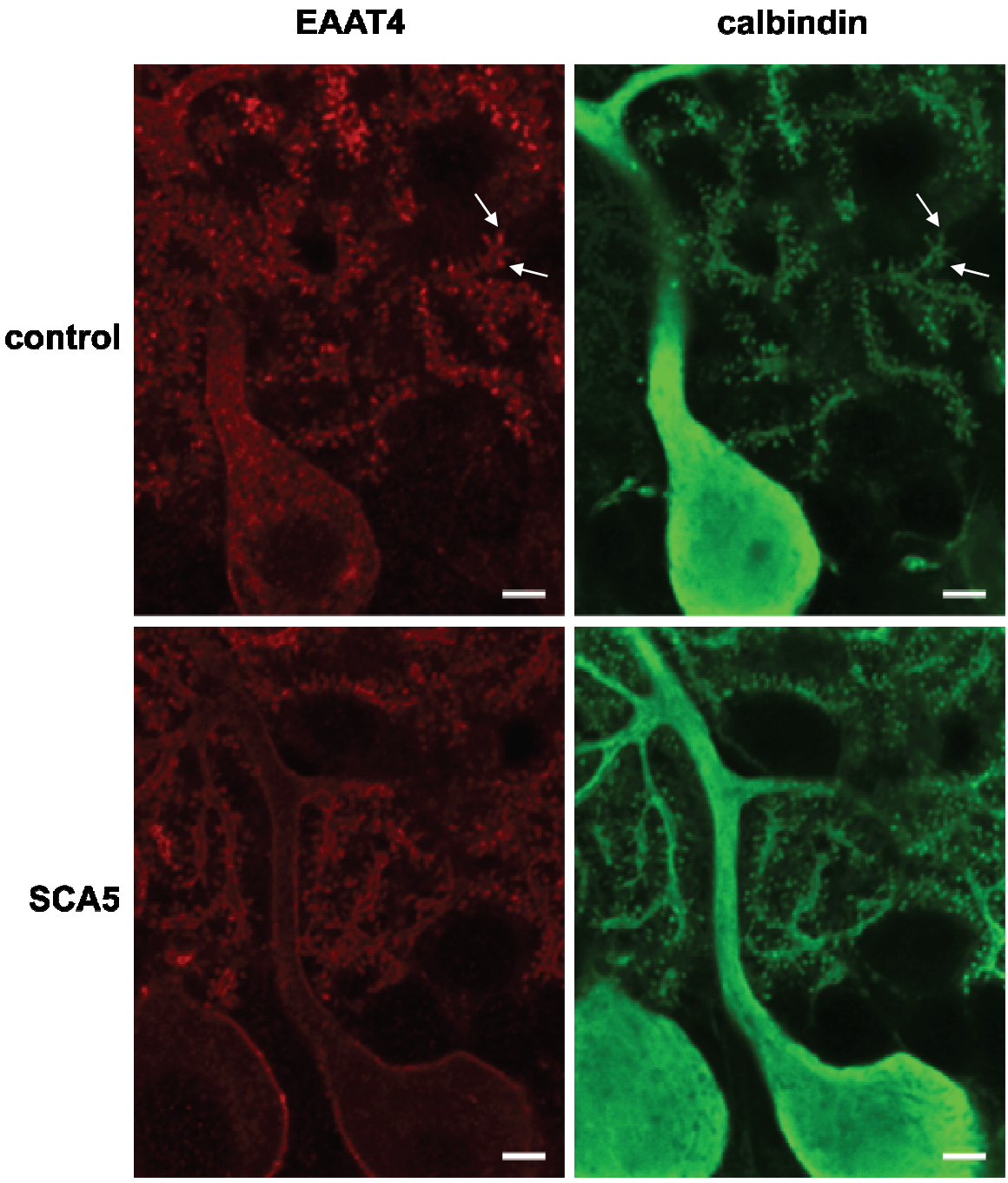


Figure 12. Higher magnification EAAT4 immunofluorescence in SCA5 mice. I immunostained cerebella from SCA5 line 184-2 mice and age-matched control line 225 mice with calbindin and EAAT4 antibodies. High magnification images show punctate EAAT4 staining corresponding to spines (arrows) in control Purkinje dendrites. EAAT4 staining in SCA5 Purkinje cells is localized to the cell membrane in soma and dendrites, without a particular preference for dendritic spines. Scale bars = 50 μm.

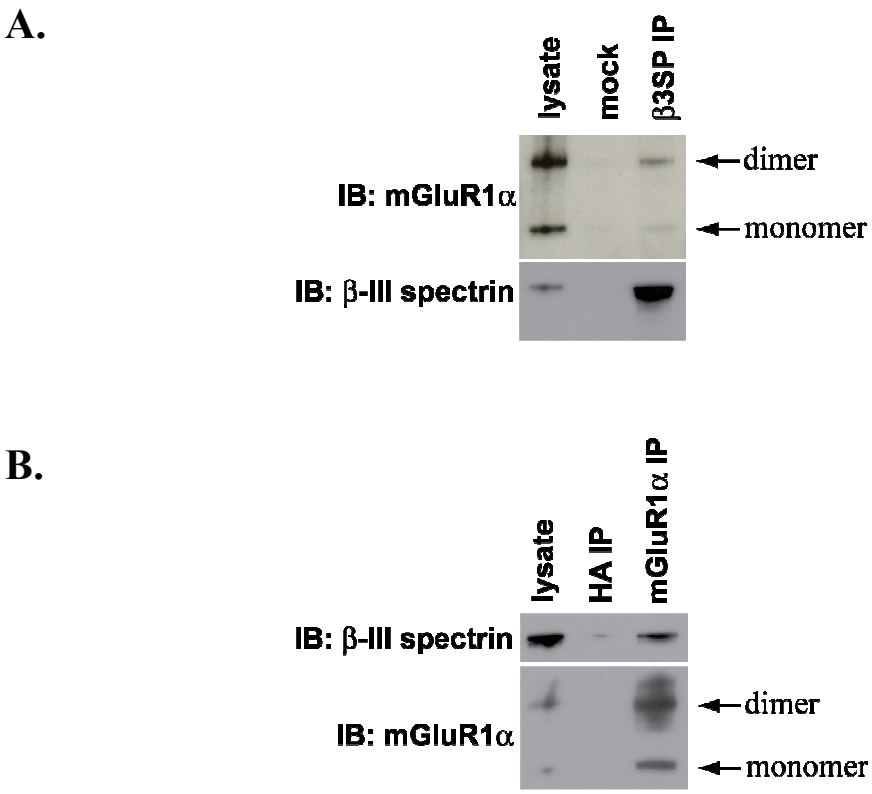


Figure 13. β-III spectrin associates with mGluR1α. I performed immunoprecipitations using cerebellar extracts from non-transgenic FVB mice. (A) I used the goat β-III spectrin antibody (β3Sp IP) or no antibody (mock) for immunoprecipitation, then immunoblotted with rabbit mGluR1α antibody and reprobed with goat β-III spectrin antibody. The right lane shows that mGluR1α co-precipitates with β-III spectrin. (B) I used the rabbit mGluR1α antibody (mGluR1α IP) or the irrelevant rabbit hemagglutinin antibody (HA IP) for immunoprecipitation, then immunoblotted with goat β-III spectrin antibody and reprobed with rabbit mGluR1α antibody. The right lane shows that β-III spectrin co-precipitates with mGluR1α.

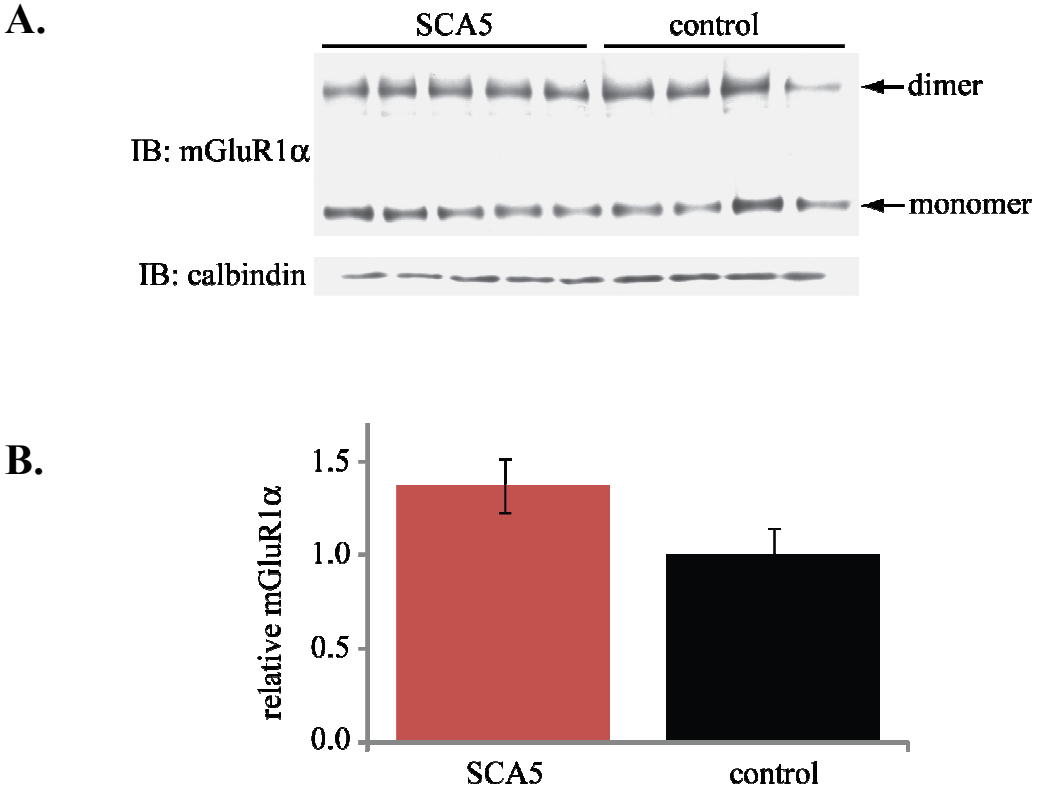
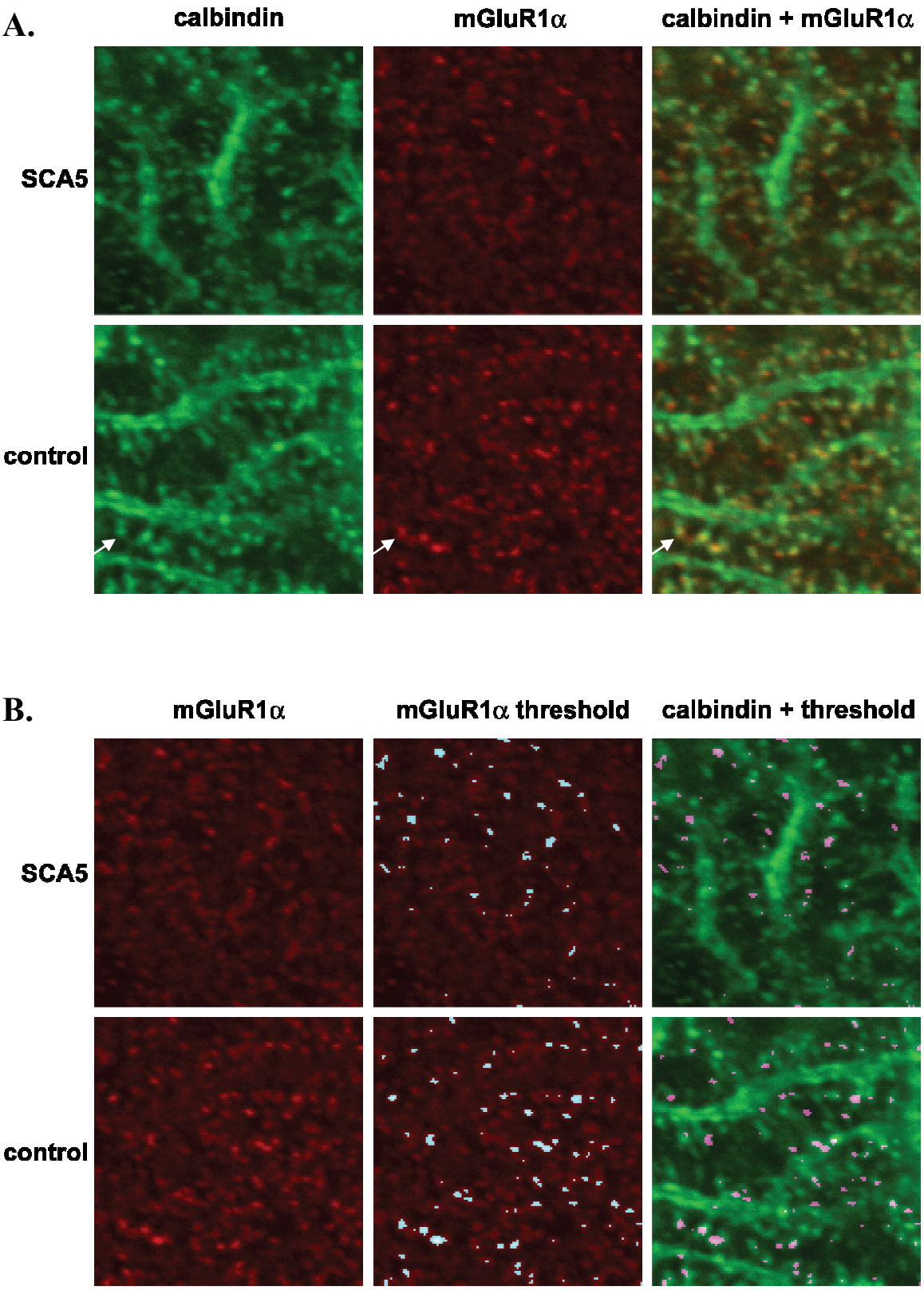


Figure 14. mGluR1α protein levels are preserved in SCA5 mice. (A) Immunoblot analysis of cerebellar lysates from SCA5 (*Pcp2-tTA/TRE-SPΔ39* line 184-2, n=5) and control (*Pcp2-tTA/TRE-SP-WT* line 225, n=4) mice aged 9-11 months. (B) I calculated the ratio of mGluR1α (monomer + dimer) to calbindin for each sample from densitometric analysis of immunoblots. The average ratio for SCA5 samples was not significantly different from that of control samples ($p = 0.11$). Error bars indicate S.E.M.

Figure 15. Loss of mGluR1α dendritic spine clustering in SCA5 mice. I immunostained cerebella from 8-10 month SCA5 line 184-2 mice and age-matched control line 225 mice with calbindin and mGluR1α and visualized dendrites by confocal microscopy. (A) Cropped images display 1/16th of the full original image. The calbindin staining in the left panels shows Purkinje cells, the middle panels show mGluR1α staining, and the right panels show a merged image. In the control image obvious mGluR1α clustering appears at the dendritic spines (arrow). The mGluR1α staining pattern is more diffuse in the SCA5 mouse. (B) The left panels show the original mGluR1α image, the middle panel shows the original mGluR1α image merged with the thresholded mGluR1α image, and the right panel shows the calbindin image merged with the thresholded mGluR1α image. For each image, I calculated the average mGluR1α fluorescence intensity using ImageJ software (Rasband 1997-2009), then set the threshold for each image at twice its average fluorescence intensity, as previously described (Das and Banker 2006).


A.
calbindin
mGluR1α
calbindin + mGluR1α
SCA5
control
B.
mGluR1α
mGluR1α threshold
calbindin + threshold
SCA5
control

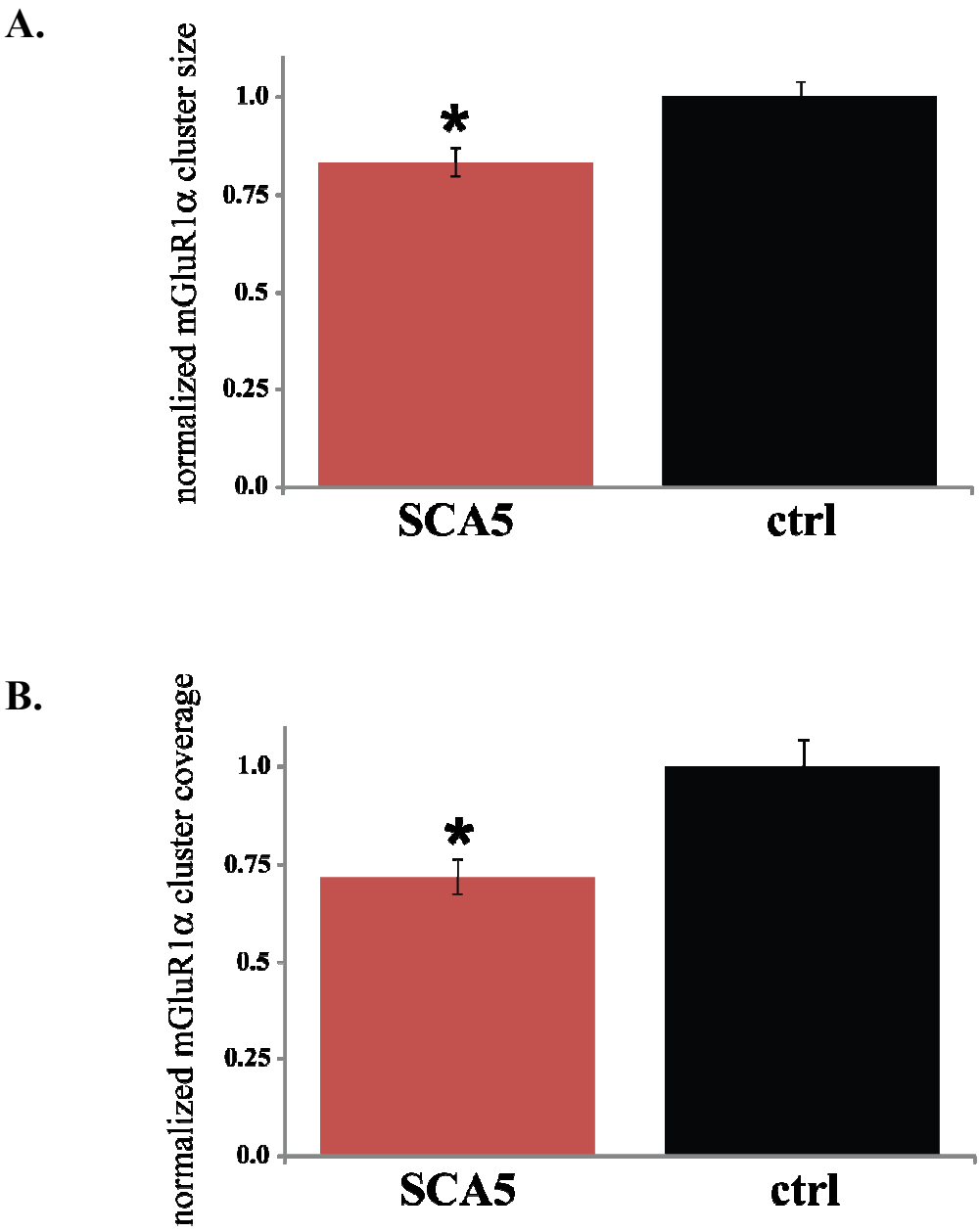


Figure 16. Quantification of mGluR1α clustering in SCA5 mice. I used ImageJ software (Rasband 1997-2009) to set the image threshold and perform subsequent mGluR1α cluster quantification. I analyzed animals from SCA5 line 184-2 and control line 225, n=5 per genotype. (A) Normalized average size of mGluR1α clusters. (B) Normalized average percentage of image area covered by mGluR1α clusters. Error bars indicate S.E.M. * indicates p-value < 0.02 compared to control.

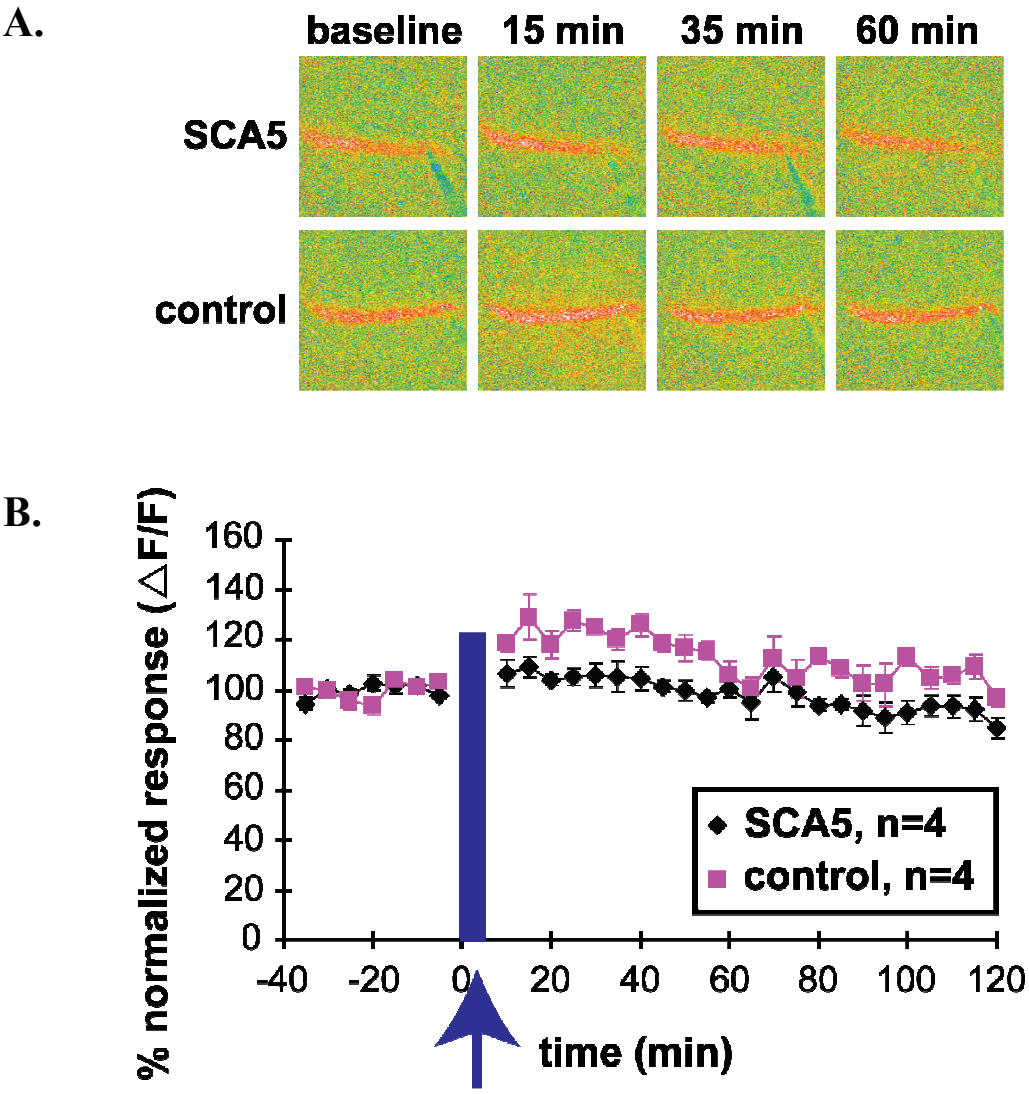


Figure 17. Response to high frequency, burst stimulation in SCA5 mice. (A) Pseudocolored images (with increases shown in yellow to red and decreases shown in green to blue) show a representative recording from a line 184-2 SCA5 mouse and a line 225 control mouse. At baseline, surface stimulation of parallel fibers elicits a beam of increased fluorescence caused by excitation of Purkinje cells and interneurons. At 15 and 35 minutes after a conditioning stimulation of high frequency, burst stimulation (15 pulses at 100 Hz) repeated every 3 sec for 5 min the control mouse shows an increased response over baseline but the SCA5 mouse does not. (B) Graph showing the average response over baseline for SCA5 and control animals, with the conditioning stimulation starting at time = 0 min and lasting 5 min (duration of conditioning stimulation is blocked with box indicated by arrow).

CHAPTER 3

CHARACTERIZATION OF A 3xFLAG-TAGGED MURINE MODEL OF SCA5

I. Introduction

The American SCA5 mutation potentially affects β-III spectrin localization within cerebellar Purkinje cells or its interaction with known binding partners. To address these hypotheses I generated and characterized a mouse model of SCA5 that allows unambiguous identification of the transgenic protein for histological and biochemical studies.

In this model the *Pcp2* promoter drives expression of the β-III spectrin transgenes in murine cerebellar Purkinje cells. Several versions of the *Pcp2* promoter have been used in other models to drive transgene expression; here I have used the L7ΔAUG expression construct (Smeyne et al. 1995), which consists of a mouse genomic DNA fragment containing approximately 1 kb of the *Pcp2* promoter, the *Pcp2* gene with all potential translational start sites mutated, and approximately 200 bp of downstream sequence. Successful Purkinje cell-specific expression of several different transgenes inserted into the BamH I site of this construct has been reported (Smeyne et al. 1995, Zhang et al. 2001, Zu et al. 2004, Jorgensen 2007).

A key feature of this SCA5 mouse model is an epitope tag for unambiguous identification of the transgenic protein. Because an amino terminal myc tag on my previously developed β-III spectrin clones did not prevent wildtype β-III spectrin from stabilizing EAAT4 (Ikeda et al. 2006), I selected an amino terminal position for the tag

for this mouse model. However, I decided to use a 3xFLAG tag instead of a myc tag to increase detection sensitivity (Hernan et al. 2000) and to avoid potential cross-reactivity with endogenous murine c-myc protein. Additionally, Nathan Jorgensen (2007) has shown that driving expression of a 3xFLAG-tagged transgene with the L7ΔAUG expression construct successfully produces detectable transgenic protein in murine cerebellar Purkinje cells. The SCA5 murine model described here, with the L7ΔAUG construct driving expression of wildtype and mutant 3xFLAG-tagged β-III spectrin, is a straightforward system to biochemically and histologically compare wildtype β-III spectrin with β-III spectrin containing the American SCA5 mutation.

II. Results

To generate mice expressing epitope-tagged wildtype and mutant β-III spectrin in cerebellar Purkinje cells, I separately cloned cDNAs encoding wildtype and mutant 3xFLAG-tagged human β-III spectrin into the L7ΔAUG expression construct (Smeyne et al. 1995) (Figure 18). The resulting control (Pcp2-3xFLAG-SP-WT) and SCA5 (Pcp2-3xFLAG-SPΔ39) DNA constructs were sent to the University of Minnesota Mouse Genetics Laboratory for pronuclear injection. I identified transgenic animals by PCR and Southern blot analysis (Figure 19). Four animals were identified with the Pcp2-3xFLAG-SPΔ39 transgene out of 15 potential founders; one of these founders had 2 integration sites, which after breeding and transgene segregation resulted in a total of five Pcp2-3xFLAG-SPΔ39 lines. Ten animals with the Pcp2-3xFLAG-SP-WT transgene were

identified out of 42 potential founders, with multiple integration sites identified in the *Pcp2-3xFLAG-SP-WT* founder 792 (Figure 19).

Immunoblot analysis of cerebellar extracts showed that of the SCA5 *Pcp2-3xFLAG-SPΔ39* mice, line 414 had the highest level of transgenic protein, followed by lines 415, 509-1, 509-2, and 417 (Figure 20). The two highest expressing SCA5 lines – lines 414 and 415 – were bred to homozygosity to maximize transgene expression levels for further characterization. Homozygous *Pcp2-3xFLAG-SPΔ39* mice from lines 414 and 415 had significantly lower levels of transgenic protein than heterozygous *Pcp2-3xFLAG-SP-WT* mice from line 782, and homozygous *Pcp2-3xFLAG-SPΔ39* mice from line 415 had similar transgenic protein levels to heterozygous *Pcp2-3xFLAG-SP-WT* line 792-1 mice. Additionally, as expected for this L7ΔAUG promoter-driven transgene, immunoblot analysis with the flag antibody showed that the transgenic protein in *Pcp2-3xFLAG-SPΔ39* line 414 mice was found specifically in cerebellar tissue: no transgene-specific flag immunoreactivity was detected even after prolonged exposure in extracts from other tissues important for movement and coordination such as skeletal muscle, spinal cord, and frontal cerebral cortex (data not shown).

At 26 weeks of age homozygous *Pcp2-3xFLAG-SPΔ39* mice from lines 414 and 415 had significantly reduced latency on the accelerating rotorod compared to age-matched line 782 *Pcp2-3xFLAG-SP-WT* heterozygous controls (Figure 21). Additionally, the performance of line 782 *Pcp2-3xFLAG-SP-WT* heterozygous mice was not significantly different from that of age-matched non-transgenic mice. Since the heterozygous line 782 mice have higher levels of transgenic protein than homozygous

mice from either 414 or 415 mutant lines, these rotorod results indicate that overexpression of mutant but not control β-III spectrin in Purkinje cells causes a deficit in motor coordination. Additionally, the homozygous mutant mice develop an unsteadiness of gait that is readily discernable on elevated beam crossing (data not shown).

I used immunohistochemical and immunofluorescent techniques to compare the localization patterns of mutant and wildtype β-III spectrin proteins within cerebellar Purkinje cells. Immunohistochemical staining with the flag antibody in the SCA5 *Pcp2-3xFLAG-SPΔ39* cerebella shows intense staining in the Purkinje cell soma and much lighter staining in the molecular layer containing the Purkinje cell dendrites (Figure 22); whereas the staining levels of Purkinje cell soma and dendrites are more comparable in *Pcp2-3xFLAG-SP-WT* control mice. I used indirect immunofluorescence to verify this difference in transgenic protein localization in *Pcp2-3xFLAG-SPΔ39* compared to *Pcp2-3xFLAG-SP-WT* mice, and high magnification images show small cytoplasmic puncta that are immunopositive with both flag and β-III spectrin antibodies in Purkinje cell soma of SCA5 *Pcp2-3xFLAG-SPΔ39* but not control *Pcp2-3xFLAG-SP-WT* mice (Figure 23).

This change in β-III spectrin localization within the Purkinje cell was difficult to verify in human SCA5 autopsy tissue. Although I have analyzed a second SCA5 autopsy specimen that is better preserved than the initially described SCA5 autopsy sample (Liquori et al. 2002, Ikeda et al. 2006), the disease has progressed to a late stage for both specimens at autopsy. Very few Purkinje cells remain in SCA5 autopsy tissue, and the dramatic dendritic atrophy and occasional ectopic somal localization of the remaining Purkinje cells (Figure 24) complicates comparison: it is difficult to determine whether the

intensity of β-III spectrin staining represents true β-III spectrin protein distribution or if it is more reflective of changes in overall protein levels as the Purkinje cells degenerate. Some of the SCA5 Purkinje cell bodies stain very intensely compared to control, but overall the intensity of β-III spectrin staining in human SCA5 Purkinje cells is variable with both intensely stained and lightly stained Purkinje cell soma.

To test whether the American SCA5 mutation affects the interaction of β-III spectrin with its known binding partners α-II spectrin and EAAT4, I performed co-immunoprecipitations from cerebellar extracts of *Pcp2-3xFLAG-SP* mice. For these experiments I compared age-matched control *Pcp2-3xFLAG-SP-WT* mice from line 792-1 to SCA5 *Pcp2-3xFLAG-SPΔ39* mice from line 415 since these lines have similar transgenic protein levels (Figure 20). Both mutant and wildtype 3xFLAG-β-III spectrin co-immunoprecipitate with α-II spectrin, and α-II spectrin co-immunoprecipitates with both mutant and wildtype 3xFLAG-β-III spectrin (Figure 25); these findings indicate that both wildtype and mutant β-III spectrin interact with α-II spectrin. Similarly, both mutant and wildtype 3xFLAG-β-III spectrin co-immunoprecipitate with EAAT4, and EAAT4 co-immunoprecipitates with both mutant and wildtype 3xFLAG-β-III spectrin (Figure 26), which indicates that the American SCA5 mutation does not prevent β-III spectrin from interacting with EAAT4.

III. Discussion

I have generated a second transgenic murine model in which overexpression of β-III spectrin with the American SCA5 mutation in cerebellar Purkinje cells causes a motor coordination phenotype not found in mice overexpressing higher levels of wildtype β-III spectrin. Like the conditional SCA5 murine model described in Chapter 2, the cerebellar phenotype of these 3xFLAG-tagged SCA5 mice validates the hypothesis that mutant β-III spectrin expression in cerebellar Purkinje cells leads to ataxia through a dominant-negative or dominant gain-of-function mechanism and provides evidence against the hypothesis that the American SCA5 mutation causes disease by haploinsufficiency. Also, because the rotorod latency phenotype is more pronounced in the 3xFLAG-tagged SCA5 model than in the conditional SCA5 model, this 3xFLAG-tagged model may be more useful for future studies examining the effects of other genes, drugs, or therapies on cerebellar phenotype modulation. Additionally, the enhanced rotorod phenotype suggests that future characterization of aged 3xFLAG-tagged SCA5 mice might provide insight into features that typically occur later in disease progression.

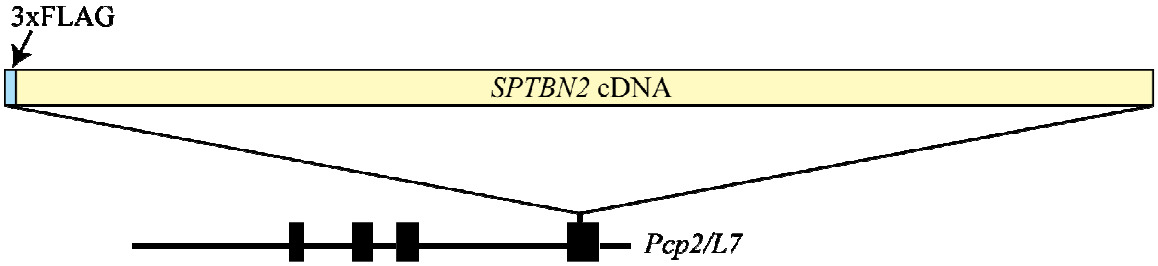
Studies using the 3xFLAG epitope tag, which is the distinguishing feature of this model and allows unambiguous identification of transgenic protein, show that the American SCA5 mutation does not prevent the interaction of β-III spectrin with its known binding partners EAAT4 and α-II spectrin. The EAAT4 result is not unexpected since EAAT4 interacts with a C-terminal region of β-III spectrin far from the American SCA5 mutation (Jackson et al. 2001). In contrast, α-II spectrin is a better candidate for an aberrant interaction with mutant β-III spectrin. The proximity of the American SCA5

mutation at the beginning of the third spectrin repeat to the α-spectrin / β-spectrin dimer nucleation site consisting of the first 2 spectrin repeats in β-spectrin (Begg et al. 2000) raises the possibility that the American SCA5 mutation is detrimental to the interaction between α-spectrin and β-III spectrin. However, my co-immunoprecipitation data refute the hypotheses that impaired interactions between mutant β-III spectrin and EAAT4 or mutant β-III spectrin and α-II spectrin cause the lack of EAAT4 stabilization by mutant β-III spectrin in HEK 293 cells (Ikeda et al. 2006) or the changes in EAAT4 localization in the conditional SCA5 mice (Chapter 2).

On the other hand, my data showing that the American SCA5 mutation alters the cellular localization of β-III spectrin provide an explanation for these changes in EAAT4 stabilization and localization. Anchoring integral membrane proteins at specific membrane domains is a fundamental role for spectrin (reviewed in Bennett and Healy 2008), and my finding that relatively less mutant β-III spectrin is located in the Purkinje dendrites in this mouse model predicts that mutant β-III spectrin is inadequate at stabilizing dendritic proteins such as EAAT4, and possibly mGluR1α, within the Purkinje cell. The small puncta of mutant β-III spectrin that I observed specifically in the soma of SCA5 mice are consistent in size with transport vesicles, indicating that the American SCA5 mutation may impair β-III spectrin trafficking to the dendrites. This change in β-III spectrin trafficking may exert a dominant effect on membrane protein distribution by directly affecting target protein trafficking or by sequestering additional cytoskeletal proteins required for anchoring membrane complexes.

Future studies to identify components associated with these puncta may provide insight into the mechanism underlying the change in mutant β-III spectrin localization as well as downstream effects. Additionally, future experiments with these mice that would take advantage of the epitope tag include flag affinity purification followed by mass spectrometry to compare protein complexes that interact with wildtype and mutant β-III spectrin as well as immunogold electron microscopy to compare wildtype and mutant β-III spectrin localization at higher resolution and with additional structural information.

A. Pcp2-3xFLAG-SP-WT transgene



B. Pcp2-3xFLAG-SPΔ39 transgene

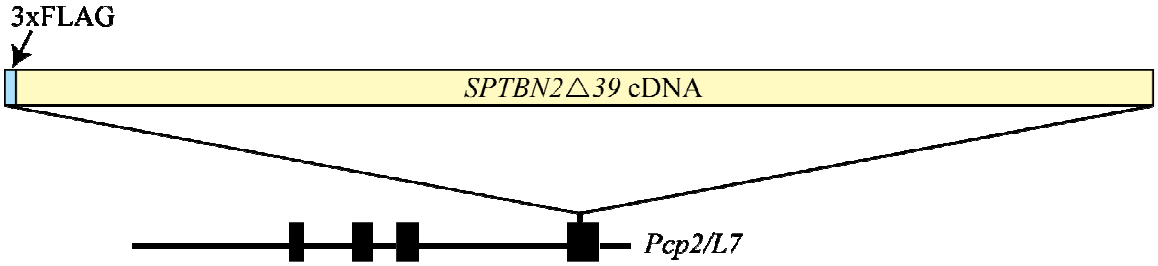


Figure 18. Diagrams of Pcp2-3xFLAG-SP transgenes. The Purkinje cell regulatory region *Pcp2/L7* drives expression of human β-III spectrin protein with an amino-terminal 3xFLAG epitope tag. Two transgene constructs were developed: (A) Pcp2-3xFLAG-SP-WT to express wildtype human β-III spectrin, and (B) Pcp2-3xFLAG-SPΔ39 to express human β-III spectrin with the American SCA5 mutation.

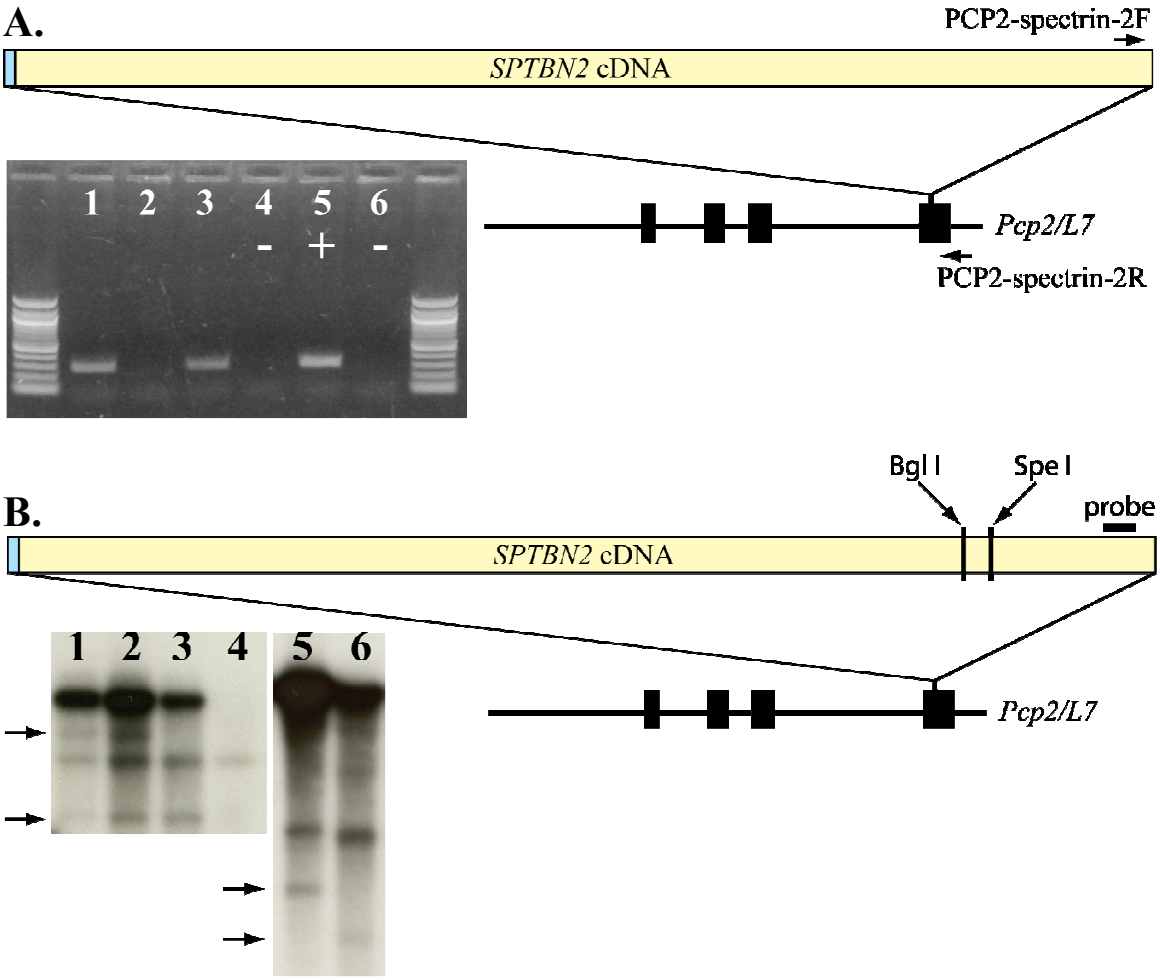
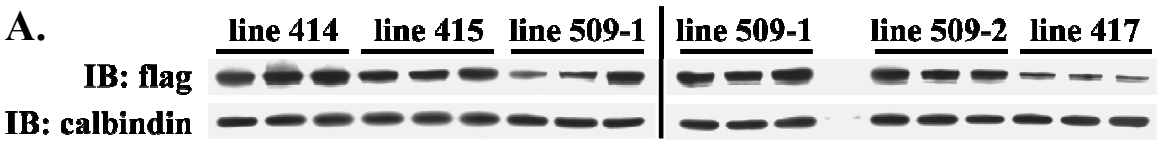
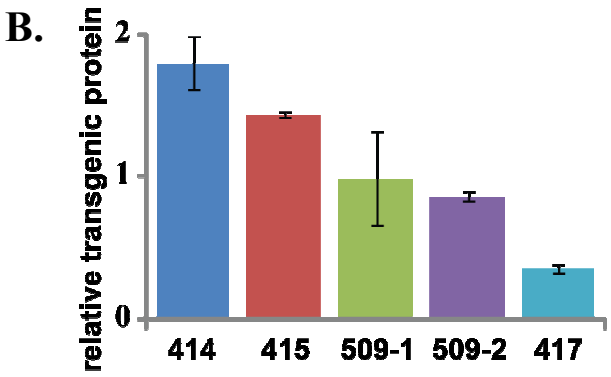
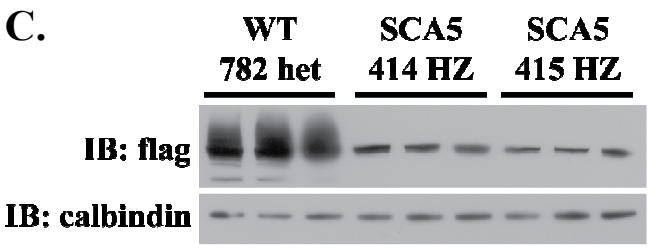


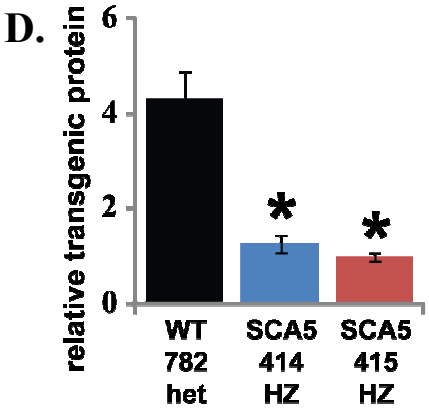
Figure 19. Genotyping of Pcp2-3xFLAG-SP mice. (A) PCR assay for identifying transgenic *Pcp2-3xFLAG-SP* mice. Primer PCP2-spectrin-2F anneals within the *SPTBN2* cDNA, and primer PCP2-spectrin-2R anneals within the *Pcp2/L7* gene. This PCR assay specifically identifies *Pcp2-3xFLAG-SPΔ39* (lane 1) and *Pcp2-3xFLAG-SP-WT* (lane 3) transgenic animals with no amplification product from DNA of a non-transgenic littermate (lane 2). Purified transgene plasmid DNA (lane 5) is a positive control, and DNA from a known non-transgenic animal (lane 4) and no DNA template (late 6) are negative controls. (B) Southern blot assay to identify multiple transgene integration sites. The transgene contains exactly one *Bgl I* restriction site and exactly one *Spe I* restriction site, and the probe sequence corresponds to 205 bp near the 3' end of the *SPTBN2* cDNA. Distinct integration sites (arrows) were identified in 2 lines. Lanes 1-4 show *Bgl I* digestions of genomic DNA from offspring of *Pcp2-3xFLAG-SPΔ39* founder 509, with 2 transgene integration sites apparent in Lanes 1 and 2, 1 transgene integration site apparent in Lane 3, and non-transgenic DNA in Lane 4. Lanes 5 and 6 show *Spe I* digestions of genomic DNA from offspring of *Pcp2-3xFLAG-SP-WT* founder 792 in which 2 transgene integration sites showed independent segregation (arrows).

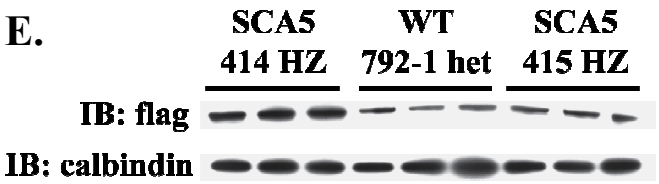
Figure 20. Transgenic protein levels in Pcp2-3xFLAG-SP mice. All experiments compare levels of flag-tagged transgenic protein to the calbindin loading control in cerebellar lysates from 6-week-old aminals. (A) Immunoblots from heterozygous *Pcp2-3xFLAG-SPΔ39* animals. (B) Densitometric analysis comparing transgenic flag-tagged protein levels normalized to calbindin in heterozygous *Pcp2-3xFLAG-SPΔ39* animals. (C) Immunoblots from heterozygous *Pcp2-3xFLAG-SP-WT line 782* and homozygous *Pcp2-3xFLAG-SPΔ39* animals. (D) Densitometric analysis comparing transgenic flag-tagged protein levels normalized to calbindin in heterozygous line 782 WT and homozygous SCA5 animals. * indicates p-value < 0.05 compared to heterozygous line 782 animals. (E) Immunoblots from heterozygous *Pcp2-3xFLAG-SP-WT* line 792-1 and homozygous *Pcp2-3xFLAG-SPΔ39* animals. (F) Densitometric analysis comparing transgenic flag-tagged protein levels normalized to calbindin in heterozygous line 792-1 WT and homozygous SCA5 animals. * indicates p-value < 0.05 compared to heterozygous line 792-1 animals. All error bars indicate S.E.M.

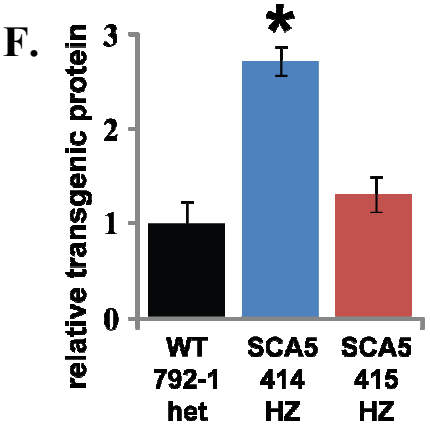

A.
line 414
line 415
line 509-1
line 509-1
line 509-2
line 417
IB: flag
IB: calbindin


B.
relative transgenic protein
2
1
0
414
415
509-1
509-2
417

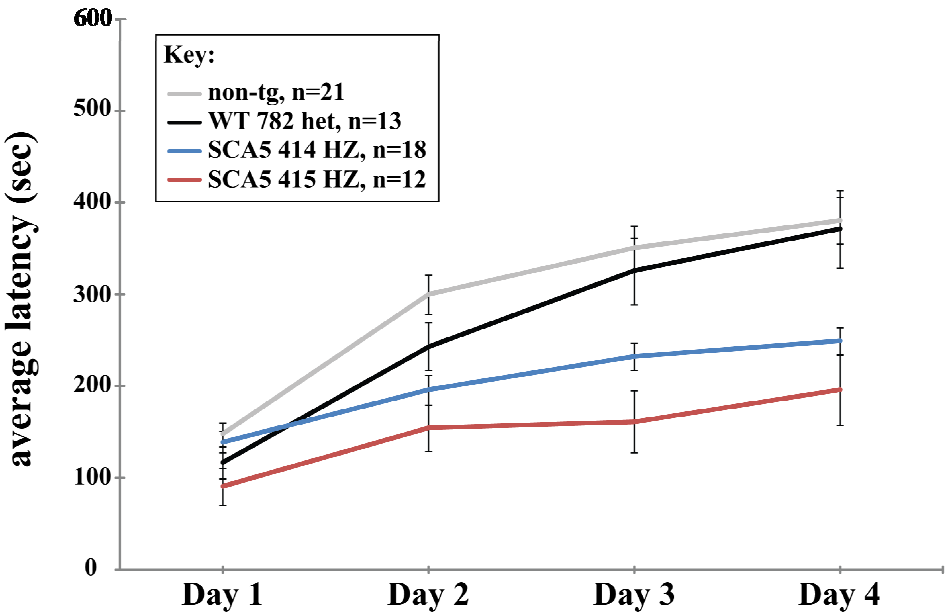

C.
WT
782 het
SCA5
414 HZ
SCA5
415 HZ
IB: flag
IB: calbindin


D.
relative transgenic protein
6
4
2
0
*
*
WT
782
het
SCA5
414
HZ
SCA5
415
HZ

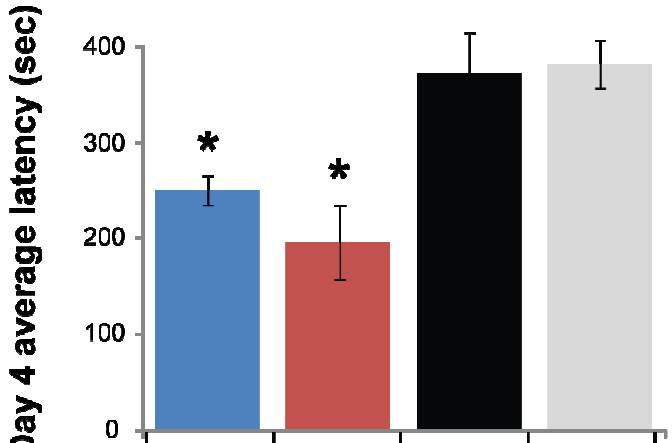

E.
SCA5
414 HZ
WT
792-1 het
SCA5
415 HZ
IB: flag
IB: calbindin


F.
relative transgenic protein
3
2
1
0
*
WT
792-1
het
SCA5
414
HZ
SCA5
415
HZ

A.



B.



	SCA5 414 HZ	SCA5 415 HZ	WT 782 het	non-tg
Day 4 average	250	196	372	381
n	18	12	13	21
S.E.M.	15	38	43	25
p-value vs non-tg	0.0001	0.0006	0.8568	
p-value vs WT 782 het	0.0168	0.0057		0.8568

Figure 21. Rotorod analysis of Pcp2-3xFLAG-SPΔ39 mice. (A) Mice were tested for latency on the accelerating rotorod at 26 weeks of age. Error bars indicate S.E.M. (B) Histogram of average Day 4 latencies. Error bars indicate S.E.M. * indicates p-value ≤ 0.05 compared to heterozygous line 782 animals.

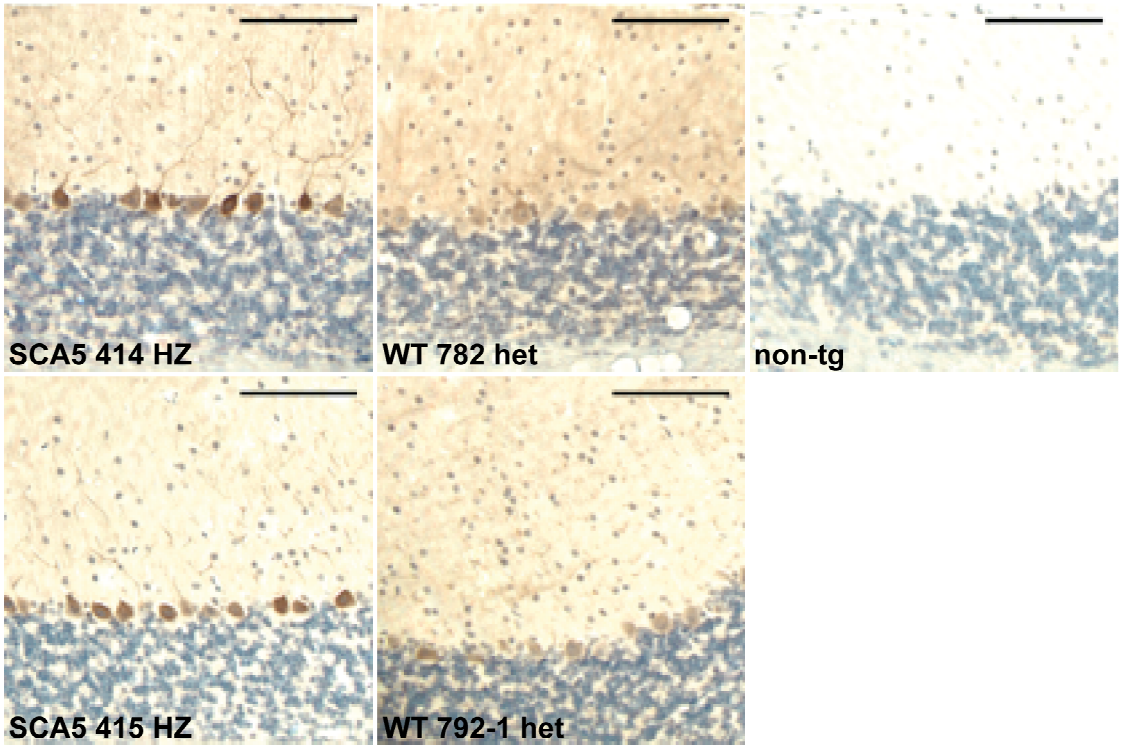


Figure 22. Transgenic protein immunohistochemistry in Pcp2-3xFLAG-SP mice. Cerebellar sections from homozygous (HZ) SCA5 *Pcp2-3xFLAG-SPΔ39* line 414, homozygous SCA5 *Pcp2-3xFLAG-SPΔ39* line 415, heterozygous (het) *Pcp2-3xFLAG-SP-WT* line 782, heterozygous *Pcp2-3xFLAG-SP-WT* line 792-1, and non-transgenic (non-tg) mice at 26 weeks of age were stained with the flag antibody, counterstained with hematoxylin, and visualized at 200X magnification. Scale bars = 100 µm.

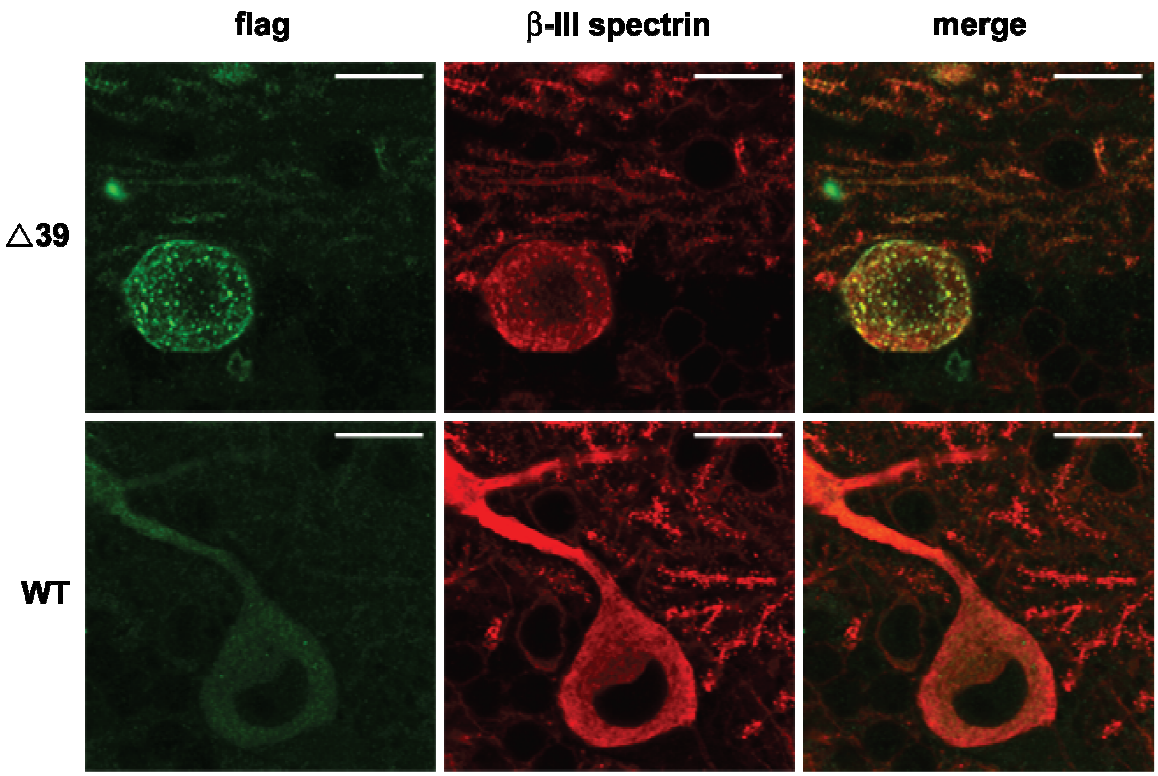


Figure 23. Transgenic protein immunofluorescence in Pcp2-3xFLAG-SP mice. Indirect immunofluorescence of cerebellar sections from homozygous *Pcp2-3xFLAG-SPΔ39* line 414 mice (top) and heterozygous *Pcp2-3xFLAG-SP-WT* line 782 mice (bottom) at 41 weeks of age stained with flag (left) or β-III spectrin (middle) antibodies. Merged images are shown on the right. Scale bars = 10 μm.

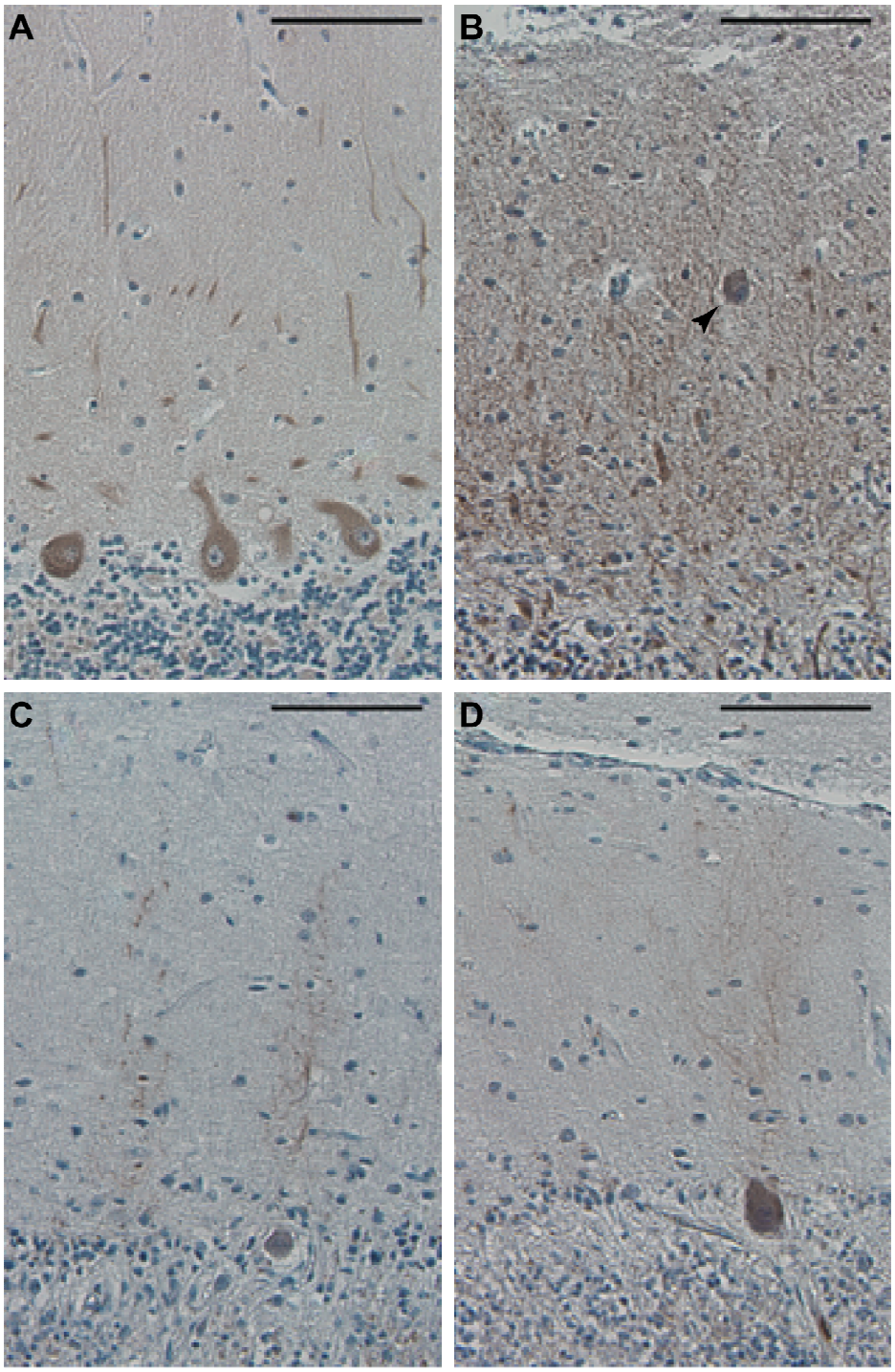


Figure 24. β-III spectrin immunohistochemistry in control and SCA5 cerebella. Cerebellar autopsy tissue from control (A) and American SCA5 (B,C,D) individuals was stained with a β-III spectrin antibody and visualized at 200X magnification. (B) The arrowhead points to an ectopically located Purkinje cell body. (C,D) There is variability in staining intensity of the SCA5 Purkinje cell bodies. Scale bars = 100 μm.

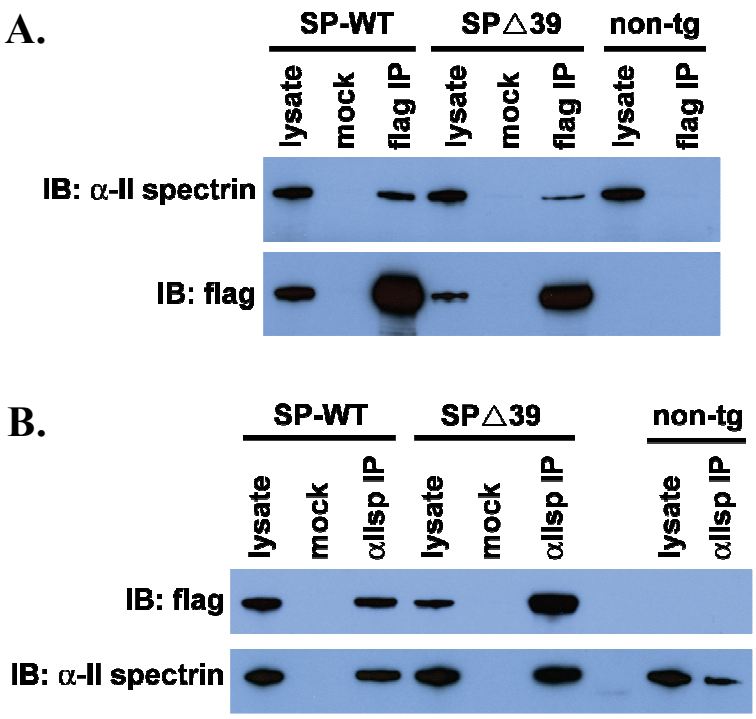


Figure 25. α-II spectrin associates with both mutant and control β-III spectrin. Immunoprecipitations of cerebellar extracts from a *Pcp2-3xFLAG-SP-WT* line 792-1 mouse (SP-WT), a *Pcp2-3xFLAG-SPΔ39* line 415 mouse (SPΔ39), and a non-transgenic mouse (non-tg) were analyzed for all experiments. (A) Immunoprecipitations were performed with no antibody (mock) or the flag antibody (flag IP), followed by immunoblotting (IB) with goat α-II spectrin antibody and flag-HRP antibody. (B) Immunoprecipitations were performed with no antibody (mock) or the mouse α-II spectrin monoclonal antibody (αIIsp IP), followed by immunoblotting (IB) with flag-HRP antibody and goat α-II spectrin antibody.

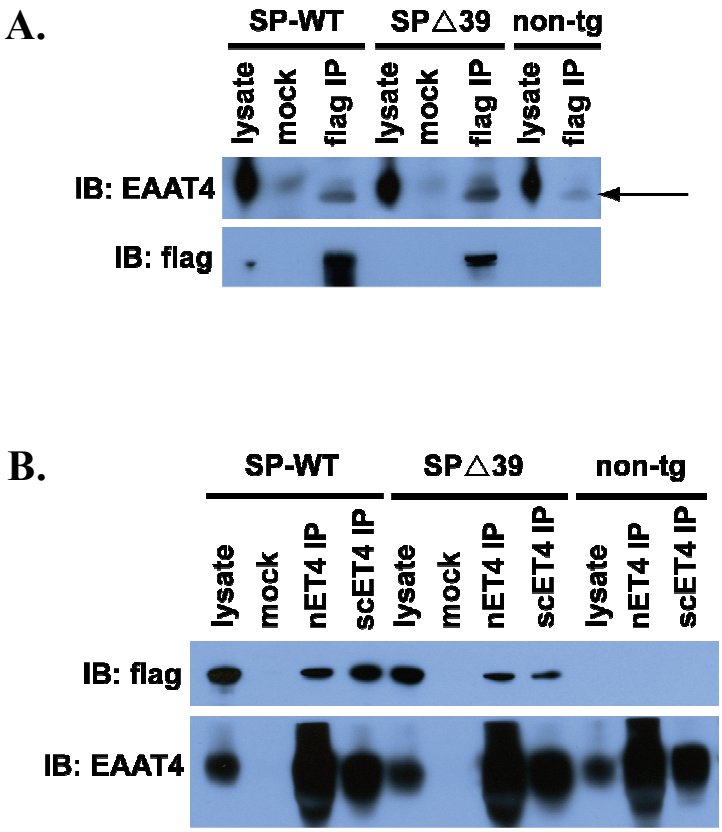


Figure 26. EAAT4 associates with both mutant and control β-III spectrin. Immunoprecipitations of cerebellar extracts from a *Pcp2-3xFLAG-SP-WT* line 792-1 mouse (SP-WT), a *Pcp2-3xFLAG-SPΔ39* line 415 mouse (SPΔ39), and a non-transgenic mouse (non-tg) were analyzed for all experiments. (A) Immunoprecipitations were performed with no antibody (mock) or the flag antibody (flag IP), followed by immunoblotting (IB) with the rabbit cEAAT4 and flag-HRP antibodies. IgG heavy chain, indicated by the arrow, runs at a slightly lower molecular weight than EAAT4 and is visible in all flag IP lanes, while EAAT4 is only visible in the SP-WT and SPΔ39 flag IP lanes. (B) Immunoprecipitations were performed with no antibody (mock), rabbit nEAAT4 antibody (nET4 IP), or goat EAAT4 antibody (scET4 IP), followed by immunoblotting (IB) with the flag and rabbit cEAAT4 antibodies.

CHAPTER 4

PROTEINS INTERACTING WITH SPECTRIN REPEATS 2 AND 3 OF β-III SPECTRIN

I. Introduction

β-III spectrin has a canonical β-spectrin structure consisting of three distinct regions: an amino terminal region containing two calponin homology (CH) domains, a central region of 17 spectrin repeats, and a carboxy terminal region containing a pleckstrin homology (PH) domain. Protein sequence homology between β-III spectrin and the more extensively characterized β-I and β-II spectrins predicts an actin binding site and a protein 4.1 binding site in the amino terminal region as well as an ankyrin binding site in the central region at spectrin repeat 15 (Stankewich et al. 1998). Proteins shown to interact specifically with β-III spectrin include the dynactin subunit actin related protein 1 (ARP1) and the synaptic vesicle release regulator Munc13. ARP1 binds the amino terminal CH-containing region of β-III spectrin (Holleran et al. 2001), and Munc13 binds the region containing spectrin repeats 15, 16, and 17 of β-III spectrin (Sakaguchi et al. 1998).

Two pathogenic, dominantly inherited mutations that cause SCA5 are located in the third spectrin repeat of β-III spectrin (Ikeda et al. 2006): the American SCA5 mutation is a 39 bp deletion at the beginning of the third spectrin repeat (E532_M544del), and the French SCA5 mutation is a 15 bp deletion at the end of the third spectrin repeat (L629_R634delinsW). The fact that these deletions do not disrupt the reading frame is

consistent with the hypothesis that these separate, small, in-frame deletions may act as dominant-negative mutations and disrupt the normal function of β-III spectrin. In addition, screening patients with unknown causes of ataxia for *SPTBN2* mutations has revealed point mutations in this region: an alanine to threonine change in the second spectrin repeat (A486T) and an arginine to tryptophan change in the third spectrin repeat (R634W) (Laura Ranum, personal communication). Although it is not known whether the A486T and R634W mutations are pathogenic, they were found only in ataxia patients and not in over 1000 control chromosomes.

The apparent clustering of pathogenic and potentially pathogenic in-frame mutations at the second and third spectrin repeats of β-III spectrin indicates that the sequence of this particular region may be important for proper function of the protein. A reasonable hypothesis is that functionality of this region involves interaction with other protein(s). To test this hypothesis and to identify proteins that interact with this region of β-III spectrin I performed a yeast two-hybrid screen.

II. Results

I used the Matchmaker GAL4 yeast two-hybrid system (Clontech) to identify putative binding partners of β-III spectrin. Using a fusion construct expressing the second and third spectrin repeats of human wildtype β-III spectrin as bait (Figure 27) and a human adult brain cDNA library (Clontech) as prey I screened 1.57×10^7 clones, then sequenced and identified prey cDNA inserts from 225 positive colonies. Table 3 lists the names of these putative interactors as well as the number of times each was

independently identified in the screen. Among the positives were clones encoding spectrin repeats from α-spectrin, β-spectrin, and α-actinin. Since recombinant spectrin repeat-containing peptides are known to form both homodimers and heterodimers (Harper et al. 2001), identification of clones encoding spectrin repeat-containing prey confirms that the yeast two-hybrid screen is working.

I identified many proteins that potentially interact with β-III spectrin using this yeast two-hybrid screen. For further analysis I selected prey that represent novel interactors of β-III spectrin and have a known function related to maintenance of specialized membrane domains or intracellular transport. Microtubule-associated protein 1A (MAP1A) is a cytoskeletal regulator that links membrane proteins to the cytoskeleton (reviewed in Halpain and Dehmelt 2006), and it may play a role in dendritic remodeling (Szebenyi et al. 2005). Prey of interest with known roles in intracellular transport include the axonal transport regulator MAP-kinase activating death domain (MADD) (Niwa et al. 2008) and $p150^{Glued}$, a critical component of the dynactin complex (reviewed in Schroer 2004).

Next, I performed nutritional selection experiments to determine whether the American SCA5 mutation affects the interaction of the β-III spectrin bait with these prey clones of interest. For each of these three prey clones, I cotransformed yeast with the prey clone isolated in the yeast two-hybrid screen and either an empty bait vector or a bait vector containing the second and third spectrin repeats of human wildtype or mutant β-III spectrin fused to the GAL4 DNA binding domain (SR23-WT; SR23Δ39). I grew the transformed yeast on plates lacking leucine and tryptophan to select for both bait and

prey plasmids, then streaked individual colonies on quadruple dropout (QDO) plates lacking adenine, histidine, leucine, and tryptophan. Growth on QDO plates requires activation of the *ADE2* and *HIS3* reporter genes and thus indicates an interaction between bait and prey. The MAP1A prey clone is viable on the QDO plates when cotransformed with the empty bait vector (Table 4); this MAP1A prey viability on QDO plates even without the spectrin bait indicates that the isolation of MAP1A in the yeast two-hybrid screen is artifactual. The MADD prey clone does require the presence of the spectrin bait plasmid to grow on QDO plates; however, the American SCA5 mutation does not noticeably change the interaction of the MADD prey with the spectrin bait, since the same number of SR23-WT/MADD and SR23Δ39/MADD colonies grew on QDO plates. Intriguingly, all of the SR23-WT/p150^{Glued} colonies grew on QDO plates, whereas only half of the SR23Δ39/p150^{Glued} colonies grew on QDO plates. This result verifies that p150^{Glued} interacts with spectrin repeats 2 and 3 of β-III spectrin in yeast, and it raises the possibility that the American SCA5 mutation affects the strength of the interaction between p150^{Glued} and β-III spectrin.

The p150^{Glued} coding sequence of all five independently isolated clones contain the C terminus of p150^{Glued} and encode a p150^{Glued} peptide fragment that varies in length from 249 to 380 amino acids (Figure 28). I verified the interaction between β-III spectrin and p150^{Glued} in a mammalian system by co-immunoprecipitating lysates from HEK 293T cells cotransfected with constructs encoding flag-tagged full-length p150^{Glued} and myc-tagged spectrin repeats 2, 3, and 4 of β-III spectrin (Figure 29). Although weak, this co-immunoprecipitation was reproducible.

To quantitate the effects of SCA5 mutations on the interaction between $p150^{Glued}$ and β-III spectrin, I performed β-galactosidase assays in yeast cotransformed with $p150^{Glued}$ prey and wildtype or mutant β-III spectrin bait. In this study I used the shortest $p150^{Glued}$ prey clone isolated in the yeast two-hybrid screen in combination with β-III spectrin clones containing spectrin repeats 2, 3, and 4 (SR24). I selected these larger β-III spectrin clones to allow testing of the French SCA5 mutation, which occurs at the end of the third spectrin repeat, as well as the American SCA5 mutation. I found a significant decrease in the β-galactosidase activity of SR24Δ39/$p150^{Glued}$ colonies compared to SR24-WT/$p150^{Glued}$ colonies (p-value = 0.002) (Figure 30). Western blot analysis of protein extracts from these yeast cultures shows that protein levels of bait and prey are relatively comparable, with slightly higher bait protein expression in SR24Δ39/$p150^{Glued}$ colonies. Therefore, the reduced β-galactosidase activity indicates that the American SCA5 mutation decreases the strength of the interaction between β-III spectrin and $p150^{Glued}$. On the other hand, the French SCA5 mutation does not significantly affect the interaction between β-III spectrin and $p150^{Glued}$ (p-value = 0.43).

I also used this method to test the effects of the American and French SCA5 mutations on the interaction between β-III spectrin and its known binding partner α-II spectrin. One of the α-II spectrin clones isolated in the original yeast two-hybrid screen contains 2.5 spectrin repeats (the C terminal half of spectrin repeat 19, spectrin repeat 20, and spectrin repeat 21); I conducted quantitative β-galactosidase assays in yeast cotransformed with this α-II spectrin clone and a clone expressing spectrin repeats 2, 3,

and 4 of wildtype or mutant β-III spectrin (Figure 31). There was no significant difference in β-galactosidase activity between clones cotransformed with α-II spectrin and wildtype β-III spectrin and clones cotransformed with α-II spectrin and β-III spectrin with the American SCA5 mutation (p-value = 0.61), which indicates that the American SCA5 mutation does not change the interaction strength between α-II spectrin and β-III spectrin. However, clones cotransformed with α-II spectrin and β-III spectrin with the French SCA5 mutation had a significant increase in β-galactosidase activity over wildtype β-III spectrin (p-value = 0.02). These data indicate that the French but not the American SCA5 mutation significantly increases the strength of the interaction between α-II spectrin and β-III spectrin.

III. Discussion

Spectrin repeat domains provide mechanical structure, separate distinct domains within large proteins, and act as binding sites for protein-protein interactions (Djinovic-Carugo et al. 2002). One such protein-protein interaction site in the canonical β-spectrins lies within spectrin repeat 15, which interacts with the adaptor protein ankyrin (Kennedy et al. 1991). Spectrin repeat domains in both α-spectrin and β-spectrin also are critical for α-spectrin / β-spectrin dimer formation: α-spectrin spectrin repeats 20 and 21 form an essential nucleation site with β-spectrin spectrin repeats 1 and 2 (Ursitti et al. 1996), and additional spectrin repeats increase overall binding affinity (Begg et al. 2000). Additionally, some spectrin repeats are required for protein-protein interactions that are

specific to certain spectrins. For instance, spectrin repeats 2 and 3 of β-I spectrin bind to the intracellular domain of neural cell adhesion molecule (NCAM) 140 and NCAM180 (Leshchyns'ka et al. 2003); this interaction is involved in assembly of postsynaptic signaling complexes (Sytnyk et al. 2006).

Here I performed a yeast two-hybrid screen to characterize protein-protein interactions involving the second and third spectrin repeats of β-III spectrin, a region that contains the American and French SCA5 mutations. I focused on characterizing putative prey proteins with cytoskeletal or intracellular transport functions, and I show that the American SCA5 mutation significantly decreases the interaction between β-III spectrin and one of these interactors, the dynactin subunit $p150^{Glued}$. Work examining the previously described association between spectrin and dynactin (Holleran et al. 1996) has focused solely on the interaction between the dynactin subunit ARP1 and β-III spectrin (Holleran et al. 2001), so the specific interaction that I identified between β-III spectrin and $p150^{Glued}$ is novel. Parenthetically, this interaction is unlikely to be ARP1-mediated because the β-III spectrin clones that I used in the yeast two-hybrid and co-immunoprecipitation studies do not contain an ARP1 binding site.

Separate work in *Drosophila* SCA5 models shows that both the German and American SCA5 mutations cause intracellular transport deficits (Lorenzo et al. 2008). Since the German SCA5 mutation occurs in the region of β-III spectrin previously shown to bind the dynactin subunit ARP1 (Holleran et al. 2001), a change in ARP1 binding may cause the transport deficits seen with the German mutation. However, it was unclear how the American SCA5 mutation would cause transport deficits. My results suggest a

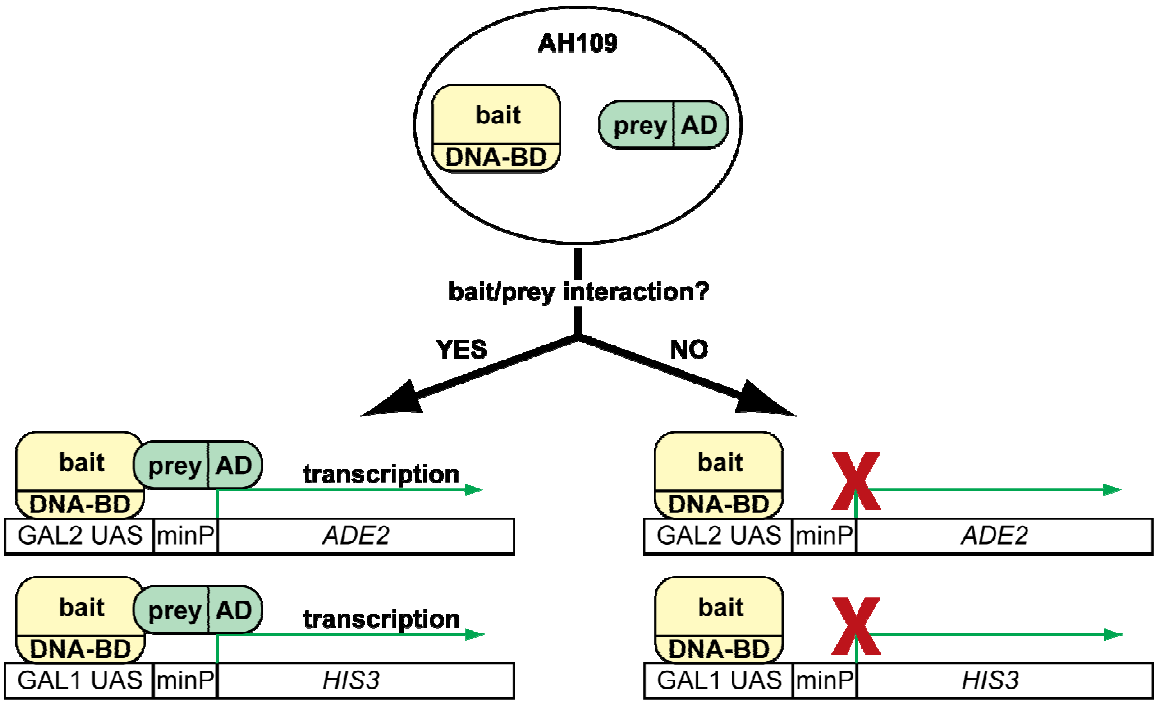
potential mechanism for this finding: the American SCA5 mutation may disrupt intracellular transport by impairing the interaction between β-III spectrin and the dynactin subunit $p150^{Glued}$.

My results also reveal differences between the American and French SCA5 mutations. I found that the interaction between β-III spectrin and α-II spectrin, a positive control prey clone isolated in the yeast two-hybrid screen, was significantly increased by the presence of the French SCA5 mutation but not affected by the American SCA5 mutation. Also in contrast to the American SCA5 mutation, the French SCA5 mutation does not reduce the interaction strength between β-III spectrin and $p150^{Glued}$. These results indicate that the molecular mechanism for pathogenesis of the American and French SCA5 mutations may not be as similar as expected given that both mutations are in-frame deletions in the third spectrin repeat.

	Sequence identity (official full name)	official symbol	no. of clones
1	actinin, alpha 2	ACTN2	1
2	ADP-ribosylation factor-like 2 binding protein	ARL2BP	2
3	ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G1	ATP6V1G1	3
4	coiled-coil domain containing 72	CCDC72	1
5	chaperonin containing TCP1, subunit 7 (eta)	CCT7	1
6	CDK2-associated protein 1	CDK2AP1	1
7	centrosomal protein 63kDa	CEP63	2
8	chondroadherin-like	CHADL	1
9	chromodomain helicase DNA binding protein 4	CHD4	1
10	CAP-GLY domain containing linker protein 3	CLIP3	1
11	component of oligomeric golgi complex 4	COG4	1
12	COP9 constitutive photomorphogenic homolog subunit 4	COPS4	2
13	cytochrome c oxidase I	COX1	1
14	aspartyl-tRNA synthetase 2, mitochondrial	DARS2	2
15	dysbindin domain containing 2	DBNDD2	1
16	dynactin 1 (p150, glued homolog, Drosophila)	DCTN1	5
17	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	DDX5	3
18	2-deoxyribose-5-phosphate aldolase homolog	DERA	1
19	DNA methyltransferase 1 associated protein 1	DMAP1	1
20	DnaJ (Hsp40) homolog, subfamily C, member 19	DNAJC19	4
21	enoyl Coenzyme A hydratase, short chain, 1, mitochondrial	ECHS1	1
22	eukaryotic translation initiation factor 3, subunit C-like	EIF3CL	1
23	enolase 1, (alpha)	ENO1	1
24	fasciculation and elongation protein zeta 1	FEZ1	17
25	fasciculation and elongation protein zeta 2	FEZ2	2
26	fibroblast growth factor receptor 1	FGFR1	1
27	flotillin 1	FLOT1	3
28	G protein pathway suppressor 2	GPS2	1
29	trifunctional protein, alpha subunit	HADHA	1
30	trifunctional protein, beta subunit	HADHB	1
31	isocitrate dehydrogenase 3 (NAD+) beta	IDH3B	1
32	intraflagellar transport 81 homolog (Chlamydomonas)	IFT81	7
33	inhibitor of kappa light peptide gene enhancer in B-cells, kinase complex-associated protein	IKBKAP	19
34	IQ motif and WD repeats 1	IQWD1	1
35	leucine aminopeptidase 3	LAP3	4
36	lactate dehydrogenase B	LDHB	1
37	lin-37 homolog	LIN37	1
38	MAP-kinase activating death domain	MADD	1
39	mannosidase, alpha, class 1A, member 2	MAN1A2	1
40	mannosidase, endo-alpha-like	MANEAL	1
41	microtubule-associated protein 1A	MAP1A	11

42	microtubule-associated protein 1S	MAP1S	7
43	mediator of RNA polymerase II transcription subunit 10	MED10	13
44	microtubule associated monoxygenase, calponin and LIM domain containing 2	MICAL2	1
45	MLX interacting protein-like	MLXIPL	2
46	mortality factor 4 like 1	MORF4L1	1
47	myelin transcription factor 1-like	MYT1L	1
48	no significant homology by BLAST search	none	26
49	TRIM-like	none	12
50	NGFI-A binding protein 2	NAB2	1
51	NADH dehydrogenase (ubiquinone) 1 β subcomplex, 10, 22kDa	NDUFB10	5
52	nucleoporin 62kDa	NUP62	1
53	polycystic kidney disease 1	PKD1	2
54	pleckstrin homology domain containing, family B member 1	PLEKHB1	2
55	plexin A2	PLXNA2	1
56	protein phosphatase 2, regulatory subunit B', epsilon isoform	PPP2R5E	3
57	replication protein A2	RPA2	1
58	salvador homolog 1	SAV1	1
59	succinate dehydrogenase complex, subunit B	SDHB	1
60	septin 7	SEPT7	1
61	SET domain containing 5	SETD5	2
62	spectrin, alpha, non-erythrocytic 1	SPTAN1	2
63	spectrin, beta, non-erythrocytic 1	SPTBN1	3
64	spectrin, beta, non-erythrocytic 4	SPTBN4	1
65	starch binding domain 1	STBD1	1
66	stathmin-like 2	STMN2	1
67	TNF receptor-associated factor 3 interacting protein 1	TRAF3IP1	3
68	thyroid hormone receptor interactor 12	TRIP12	1
69	tumor susceptibility gene 101	TSG101	2
70	thioredoxin 2	TXN2	1
71	ubiquitin specific peptidase 15	USP15	2
72	ubiquitin specific peptidase 4 (proto-oncogene)	USP4	1
73	WW and C2 domain containing 1	WWC1	5
74	zinc finger protein 155	ZNF155	5
75	zinc finger protein 670 (Pan troglodytes)	ZNF670	1

Table 3. β-III spectrin (aa 416-642) yeast two-hybrid screen results. List of prey cDNA inserts identified in positive colonies (blue colonies on SD/-Ade/-His/-Leu/-Trp + X-α-Gal plates) from a yeast two-hybrid assay that used spectrin repeats 2 and 3 of human wildtype β-III spectrin as bait. The no. of clones column lists the number of times I independently isolated each clone in the yeast two-hybrid screen.



Bait vector	Prey Vector	Growth on QDO
empty	MAP1A	8/8
SR23-WT	MAP1A	7/8
SR23Δ39	MAP1A	7/8
empty	MADD	0/3
SR23-WT	MADD	2/3
SR23Δ39	MADD	2/3
empty	p150 clone 1	1/8
SR23-WT	p150 clone 1	8/8
SR23Δ39	p150 clone 1	4/8

Table 4. Growth on quadruple dropout (QDO) plates. I cotransformed selected prey plasmids with empty, wildtype, or mutant β-III spectrin bait plasmids into yeast strain AH109. I isolated individual colonies containing both bait and prey plasmids by selecting for growth on plates lacking leucine and tryptophan, then streaked these colonies on QDO plates (medium lacking adenine, histidine, leucine, and tryptophan) to test for activation of *ADE2* and *HIS3* reporter genes. This table shows the number of viable colonies on QDO plates out of the total number of colonies tested.

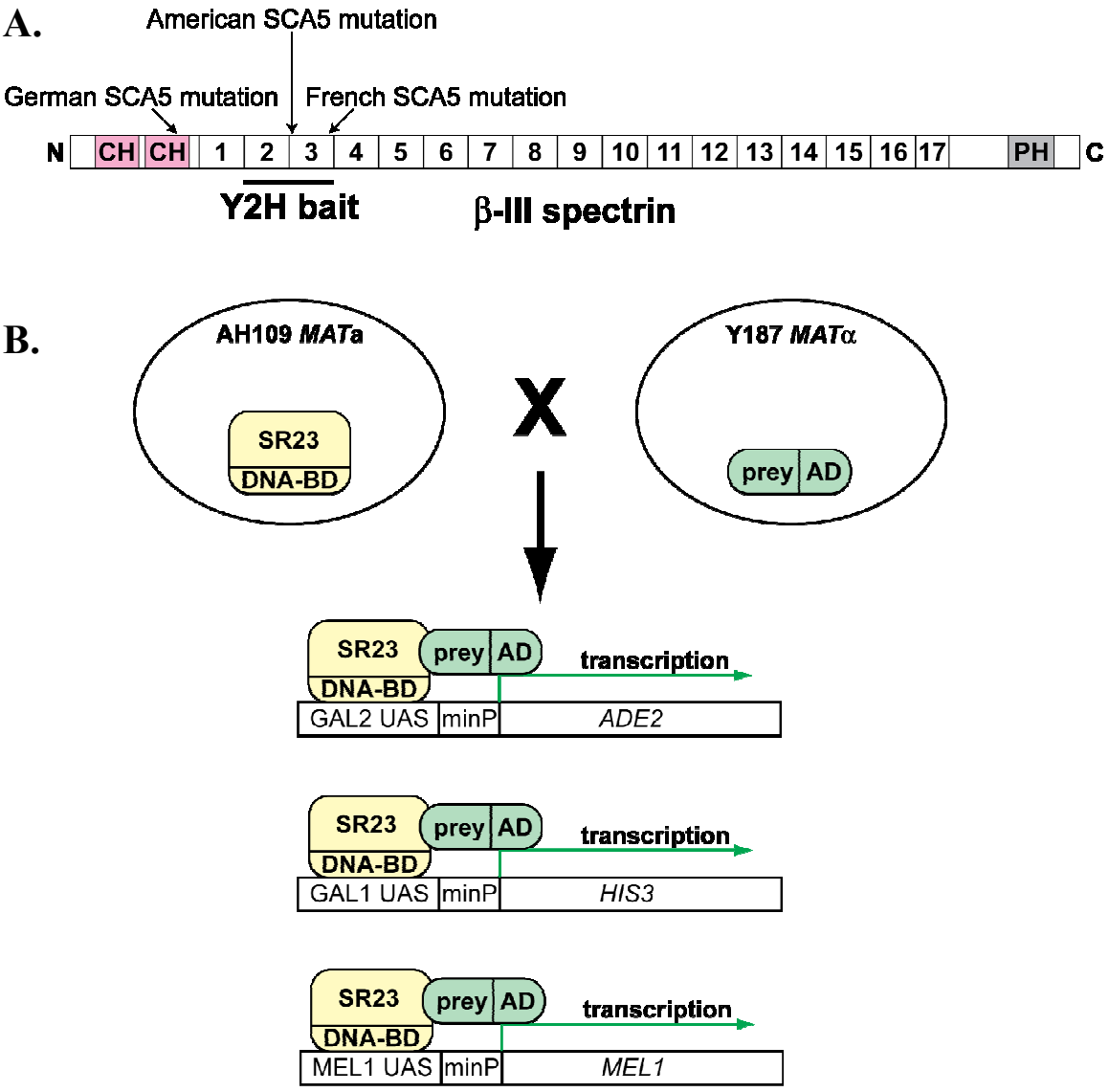


Figure 27. Schematic of yeast two-hybrid screen. (A) Illustration of the β-III spectrin protein showing the region I used as bait for the yeast 2-hybrid screen and the locations of the reported SCA5 mutations. The calponin homology (CH) domains are near the N terminus, the 17 spectrin repeat domains are centrally located, and the pleckstrin homology (PH) domain is near the C terminus. (B) The bait fusion protein SR23/DNA-BD consists of spectrin repeats 2 and 3 from wildtype human β-III spectrin (aa 416-642) fused to the yeast GAL4 DNA-binding domain (DNA-BD). AH109 *MAT*a yeast expressing SR23/DNA-BD were mated with Y187 *MAT*α yeast pretransformed with human brain cDNA prey fused to the yeast GAL4 activation domain (AD). The physical proximity of the DNA-BD and the AD that occurs when bait and prey proteins interact activates transcription of *ADE2*, *HIS3*, and *MEL1* reporter genes, so I selected for an Ade+ His+ Mel1+ phenotype to identify colonies with interacting bait and prey.

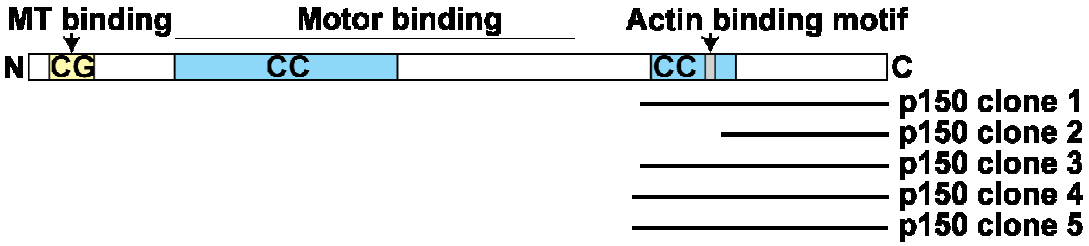


Figure 28. Dynactin p150^{Glued} is a novel binding partner of β-III spectrin. Illustration of the p150^{Glued} protein showing the 5 interacting prey clones found by yeast 2-hybrid. The cytoskeleton-associated protein, glycine-rich (CG) domain near the N terminus binds microtubles (MT), the first coiled-coil (CC) domain is involved in binding microtubule-based motors, and a small conserved actin binding motif within the second coiled-coil domain is thought to bind the dynactin subunit ARP1.

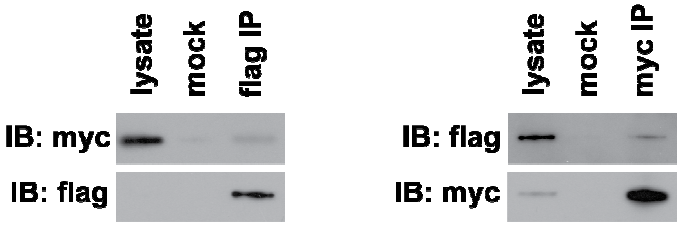


Figure 29. p150^{Glued} associates with spectrin repeats 2-4 of β-III spectrin. Flag-tagged full-length p150^{Glued} and myc-tagged spectrin repeats 2-4 of β-III spectrin were cotransfected into HEK 293T cells. Immunoprecipitations of cell lysates were performed with no antibody (mock), flag antibody (flag IP), or myc antibody (myc IP), followed by immunoblotting with the myc antibody and flag-HRP antibody.

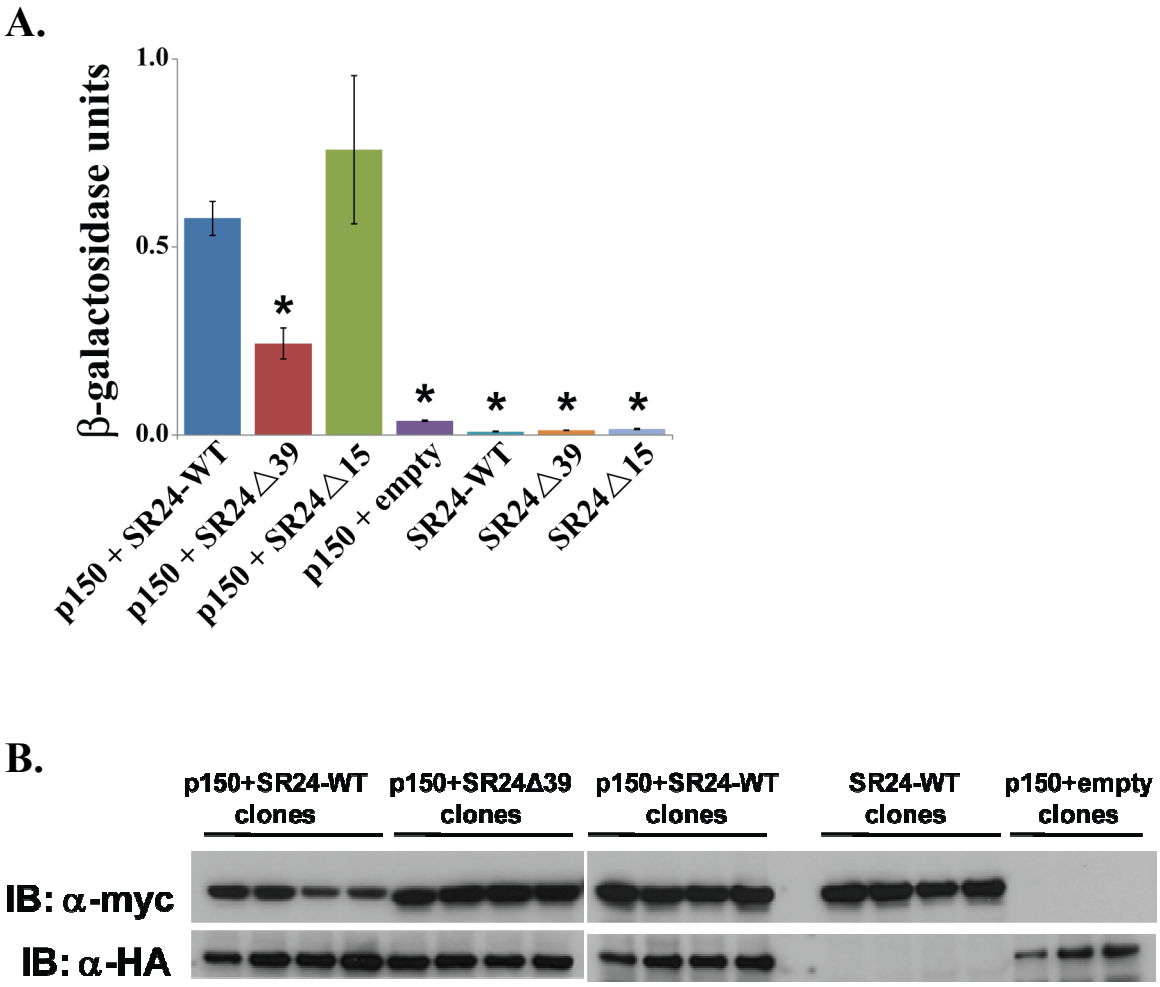
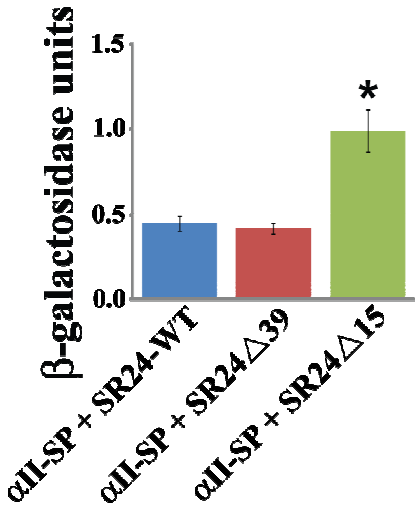


Figure 30. The American mutant β-III spectrin shows reduced interaction with p150^{Glued}. (A) Quantitation of interaction strength between a p150^{Glued} C terminal peptide and spectrin repeats 2-4 of β-III spectrin in a yeast two-hybrid assay, with CPRG as the β-galactosidase substrate. Error bars depict S.E.M.; * indicates p-value < 0.05 compared to p150 + SR24-WT. (B) Western blot showing myc-tagged β-III spectrin bait and HA-tagged p150^{Glued} prey protein levels.

A.



B.

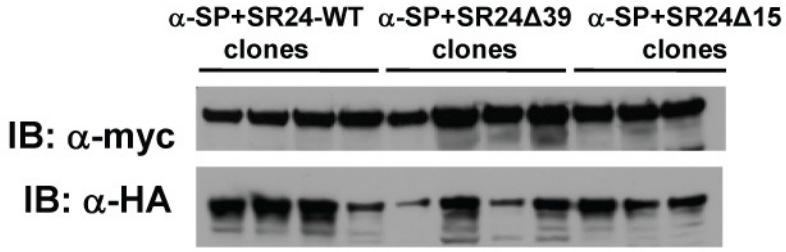


Figure 31. The French mutant β-III spectrin shows increased interaction with α-II spectrin. (A) Quantitation of interaction strength between spectrin repeats 19-21 of α-II spectrin and spectrin repeats 2-4 of β-III spectrin in a yeast two-hybrid assay, with CPRG as the β-galactosidase substrate. Error bars depict S.E.M. * indicates p-value < 0.05 compared to αII-SP + SR24-WT. (B) Western blot showing myc-tagged β-III spectrin bait and HA-tagged α-II spectrin prey protein levels.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Cumulatively, my results suggest a model for SCA5 pathogenesis (Figure 32). I show that the American SCA5 mutation reduces the interaction strength between β-III spectrin and its novel interactor $p150^{Glued}$ (Chapter 4); this altered interaction with the dynactin complex may affect β-III spectrin trafficking, thus providing an explaination for the change in mutant β-III spectrin localization that I show in the 3xFLAG-tagged SCA5 mice (Chapter 3). Altered β-III spectrin trafficking may have a dominant effect on distribution of target membrane proteins by directly affecting target protein trafficking or by sequestering cytoskeletal proteins required for anchoring membrane complexes. Either of these mechanisms could cause the altered distribution of EAAT4 and mGluR1α in the conditional SCA5 mice (Chapter 2). Improper localization of EAAT4 and mGluR1α, and potentially other target proteins, would affect target protein function and likely contributes to Purkinje cell dysfunction.

An important future research objective is to understand how the mechanisms of the different SCA5 mutations converge to cause a similar neurodegenerative effect. Although my work has for the most part focused on characterizing the American SCA5 mutation, I show that the French SCA5 mutation alters the interaction strength of β-III spectrin with α-II spectrin (Chapter 4). Previous work showing that α-II spectrin interacts with Shank (Böckers et al. 2001), a scaffolding protein involved in stabilizing the

mGluR1α membrane complex (Tu et al. 1999, Brakeman et al. 1997), suggests the possibility that the French SCA5 mutation may affect scaffolding protein interactions to affect mGluR1α localization (Figure 32). Since mice expressing β-III spectrin with the American SCA5 mutation have aberrant mGluR1α localization (Chapter 2), one possible model of convergence for the American and French SCA5 mutations is that both may affect stabilization of the metabotropic glutamate receptor mGluR1α – and potentially other target proteins – at specific membrane domains. Binding experiments to determine whether the French SCA5 mutation alters the interaction between full-length β-III spectrin and full-length α-II spectrin as well as further characterization of the role of β-III spectrin in mGluR1α stabilization would address this hypothesis.

Another potential model of convergence is that the three reported SCA5 mutations all may affect intracellular transport. I show that the American SCA5 mutation reduces the interaction of β-III spectrin with the dynactin subunit $p150^{Glued}$ (Chapter 4), and the German SCA5 mutation occurs in the region of β-III spectrin shown to bind the dynactin subunit ARP1 (Holleran et al. 2001). Additionally, both the American and German SCA5 mutations impair intracellular transport in the context of the *Drosophila* system (Lorenzo et al. 2008). These biochemical and functional results support the idea that the American and German SCA5 mutations affect intracellular transport. So far, there is no evidence to support the hypothesis that the French SCA5 mutation affects intracellular transport, but it is possible that the increased strength of interaction with α-II spectrin causes dysregulation of the interaction with dynactin (Figure 32). One way to

test this model of convergence is to determine whether *Drosophila* expressing spectrin with the French SCA5 mutation have intracellular transport deficits.

Refining this model of SCA5 pathogenesis also will involve determining the relative contributions of transport and scaffolding to target membrane protein mislocalization in the SCA5 mice. Biotinylation and trafficking studies in cultured Purkinje neurons from SCA5 and control mice would test the hypothesis that mutant β-III spectrin has a direct effect on EAAT4 and mGluR1α trafficking. Biotinylation studies would determine whether mutant β-III spectrin affects target protein trafficking to the plasma membrane, and transfecting neurons with fluorescently-tagged target proteins would allow calculation of target protein transport velocity in dendrites. Additionally, histological analysis of 3xFLAG-tagged SCA5 mice to determine whether scaffolding proteins such as α-II spectrin colocalize with the flag puncta would test the hypothesis that mutant β-III spectrin sequesters cytoskeletal factors.

At this point, a connection between intracellular transport deficits and the Purkinje cell dysfunction caused by mutant β-III spectrin is only correlative. I am performing an ongoing study to test for the possible contribution of dynactin-dependent intracellular transport to the cerebellar phenotype in mice expressing mutant β-III spectrin in Purkinje cells. In the *Drosophila* model system, $p150^{Glued}$ mutations genetically enhance the neurodegenerative phenotype obtained by expressing mutant β-III spectrin (Lorenzo et al. 2008). I have set up a similarly designed study (Figure 33) by crossing mice heterozygous for a null $p150^{Glued}$ mutation ($p150^{Glued+/-}$)(Lai et al. 2007) to SCA5 line 414 mice heterozygous for the Pcp2-3xFLAG-SPΔ39 transgene (*Pcp2-*

3xFLAG-SPΔ39$^{+/-}$) (Chapter 3). Anastasia Rupp-Moody, a technician in Laura Ranum's laboratory, and I will evaluate motor coordination and analyze cerebellar histology of littermates comprising all 4 resulting genotypes. Finding that the *p150*$^{Glued+/-}$; *Pcp2-3xFLAG-SPΔ39*$^{+/-}$ mice have a more severe phenotype than the predicted additive phenotype of the p150$^{Glued+/-}$ null allele and the Pcp2-3xFLAG-SPΔ39$^{+/-}$ transgene would demonstrate genetic enhancement, which then would provide evidence for the hypothesis that intracellular transport plays a role in the deficits caused by mutant β-III spectrin in murine Purkinje cells. Additionally, I will assess localization of endogenous wildtype β-III spectrin in *p150*$^{Glued+/-}$ mice to determine whether reducing p150^{Glued} levels by half alters wildtype β-III spectrin localization in the Purkinje cell. A potential problem with these studies is that negative results are uninformative. An alternative approach in which a negative result would be informative is to analyze the effect of knocking down p150^{Glued} in cultured Purkinje cells on β-III spectrin localization.

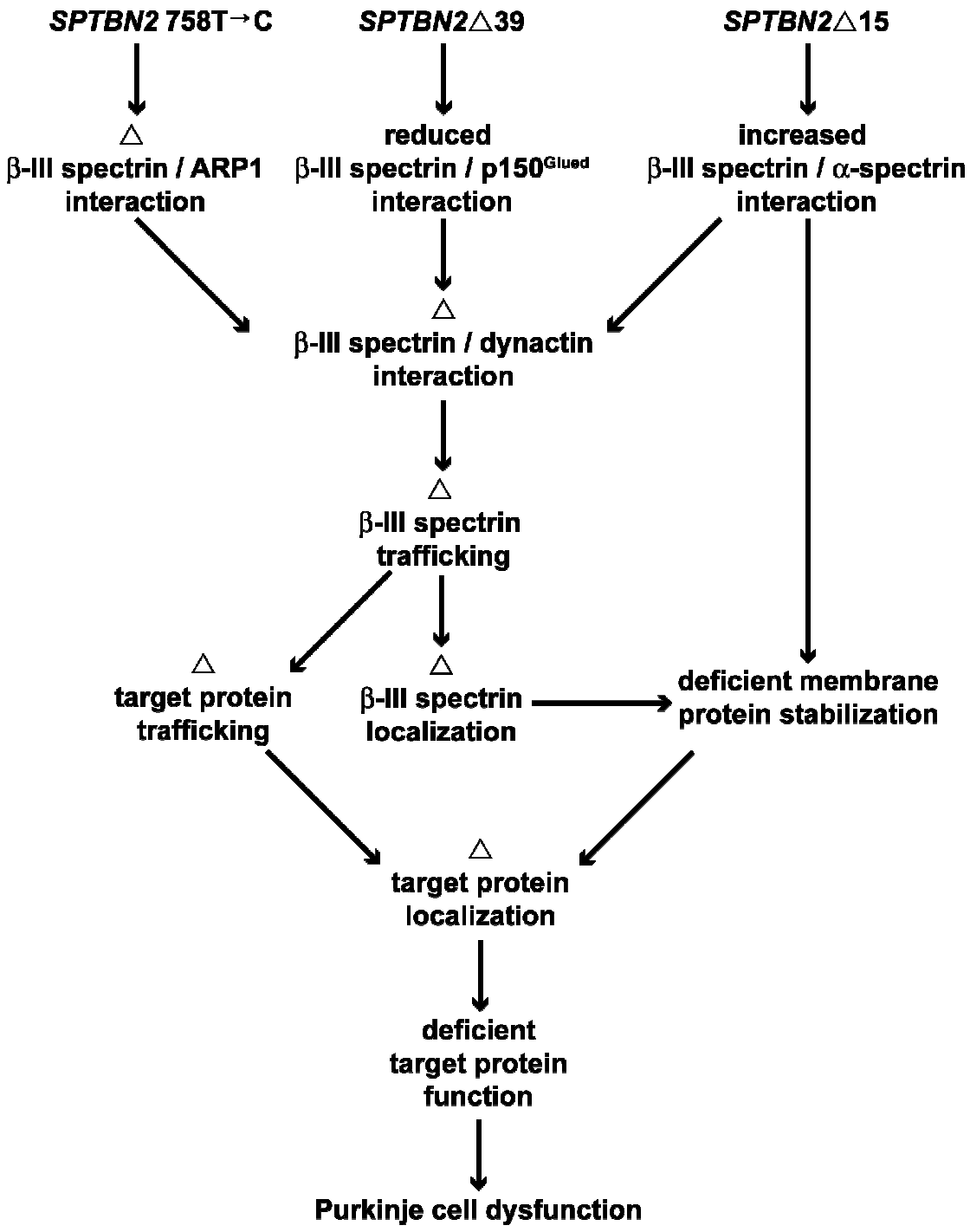


Figure 32. Model of SCA5 pathogenesis.

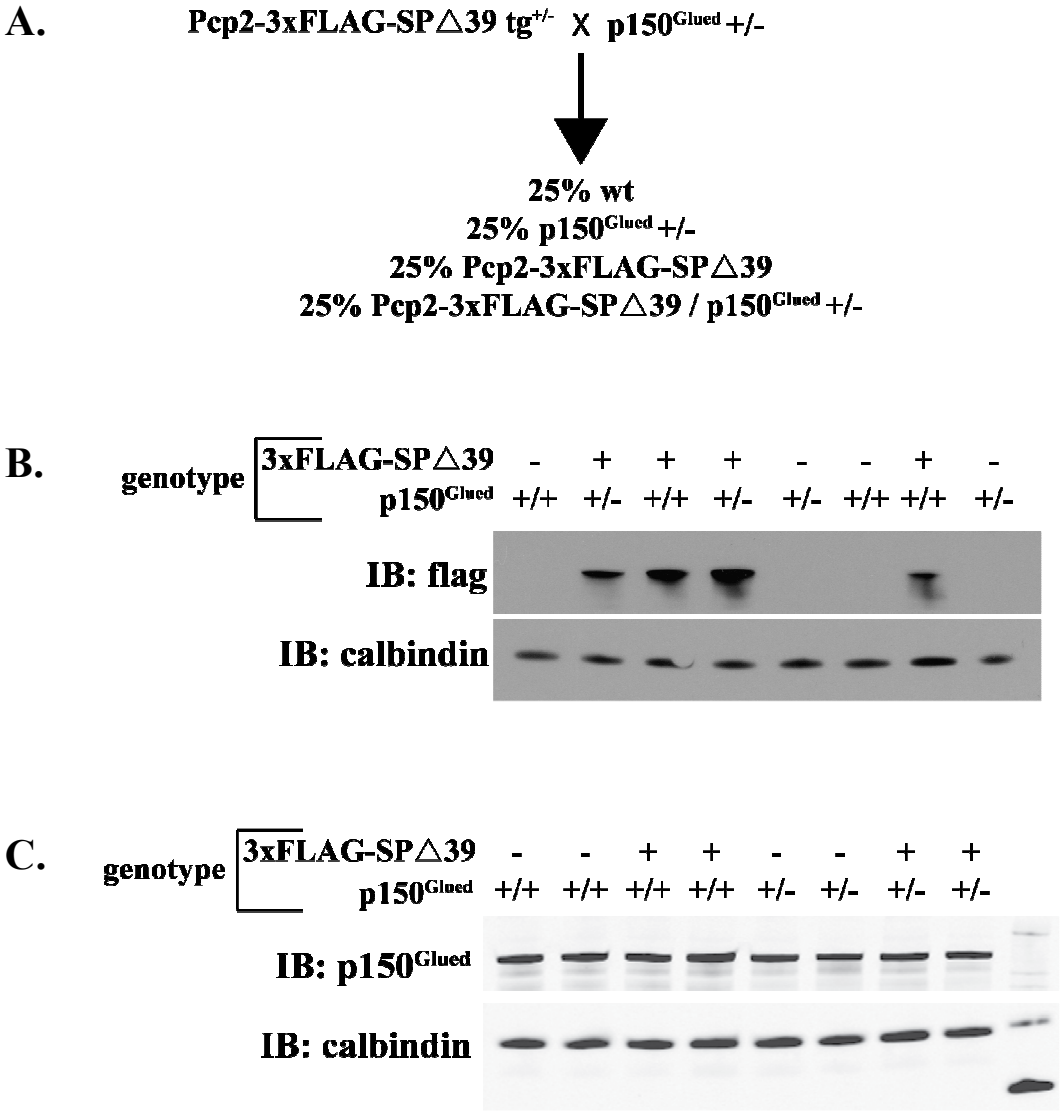


Figure 33. Preliminary data for murine genetic enhancement study. (A) Breeding scheme to generate *Pcp2-3xFLAG-SPΔ39*$^{+/-}$ / *p150*$^{Glued+/-}$ mice. (B) Immunoblot analysis showing cerebellar 3xFLAG-tagged β-III spectrin transgenic protein in the 4 genotypes, with calbindin as a loading control. The presence of the null p150^{Glued} allele does not noticeably change transgenic protein levels. (C) LiCor imaging of cerebellar p150^{Glued} protein levels in the 4 genotypes, with calbindin as a loading control, verifies lower p150^{Glued} protein levels in cerebella of mice heterozygous for the p150^{Glued} null mutation (p150^{Glued} +/-).

CHAPTER 6

MATERIALS AND METHODS

I. Chapter 2 methods

Constructs:

(1) A full-length *SPTBN2* pBluescript cDNA clone (KIAA0302, Kazusa DNA Research Institute) was cloned into the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA) by Kpn I and Not I digestion. Sequencing was performed to verify the integrity of the cDNA and two coding errors were fixed using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

(2) The American SCA5 mutation was generated by PCR using overlapping primer sets (set1: SPΔ39-1f and SPΔ39-1r) and (set 2: SPΔ39-2f and SPΔ39-2r). The PCR products from SPΔ39 primer sets 1 and 2 were used as an overlapping template in a third PCR with primers SPΔ39-1f and SPΔ39-2r to generate a fragment containing the 39bp American SCA5 deletion mutation. This resulting fragment was then subcloned into the *SPTBN2* cDNA by BsmB I and Age I digestion to generate a full-length *SPTBN2* cDNA with the American SCA5 mutation (SPΔ39).

(3) A fragment containing the Tet-response element (TRE) and minimal CMV promoter from the plasmid pTRE2 (Clontech) was cloned upstream of SPTBN2 cDNA in the plasmid pcDNA3.1. See Page 102 for the full-length TRE-SP-WT sequence and Page 105 for the full-length TRE-SPΔ39 sequence. See Table 5 for all primer sequences.

Generation and maintenance of transgenic mouse lines: The transgenes TRE-SP-WT and TRE-SPΔ39 were each cut from the vector background by digestion with Mfe I and Xba I, gel-purified, and dialyzed against injection buffer (5 mM Tris, 0.25 mM EDTA, pH 7.4). Prior to microinjection, the full-length cut constructs were verified by sequencing and transgenic protein expression was confirmed by transfection into HEK 293T cells. 2-4 μg/ml transgene DNA in injection buffer was microinjected into FVB/N zygotes at the University of Minnesota Transgenic Mouse Facility. Transgene-positive founders for both constructs were initially identified using Southern blotting of TaqI-cut tail DNA using the probe generated by amplifying a 323 bp product from the TRE-SP-WT construct template with primers TaqISouthernF and 2TaqISouthernR. For identification of independently segregating integration sites, tail DNA from multiple offspring of a single founder was separately digested with *Nco I*, *Pst I*, and *Xmn I* and subsequently probed with the 212 bp PCR product amplified with primers TRE1-F and TRE1-R from a transgenic tail DNA template. After line establishment, animals were genotyped by multiplex PCR with a primer pair to amplify TRE-SP (TRE1-F and TRE1-R) and a primer pair to amplify the tTA (tTA-F and tTA-R). PCR cycling conditions consisted of an initial 3 minute denaturation step at 94ºC; then 35 cycles of 94ºC for 45 sec, 57ºC for 45 sec, and 72ºC for 1 minute; followed by a final 6 minute elongation step at 72ºC. Mice were housed in a specific-pathogen-free facility, and animal care adhered to the guidelines established by the Institutional Animal Care and Use Committee at the University of Minnesota.

Transgene expression analysis: Total RNA was extracted from frozen cerebella using Trizol® reagent (Invitrogen, Carlsbad, CA). 2 μg total RNA from each animal was treated with amplification grade DNase (Invitrogen, Carlsbad, CA). Half of each DNase reaction was reverse transcribed using the Invitrogen SuperScript™ First-Strand Synthesis System for RT-PCR kit (Invitrogen, Carlsbad, CA) with random primers, and the other half was processed in the same way except that the reaction lacked reverse transciptase (-RT). Absence of transgene transcript PCR product from –RT samples to confirm lack of DNA contamination was verified before proceeding to qRT-PCR. Two-stage PCR was performed using 10% of each RT reaction and Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen, Carlsbad, CA) for 40 cycles (95ºC for 15 s, 60ºC for 1 min) on a Mastercycler® ep *realplex* (Eppendorf, Westbury, NY). I used TRE1-F and TRE1-R primers to amplify the transgene *TRE-SP* transcript and calbindin1-F and calbindin1-R primers to amplify the housekeeping gene *calbindin* transcript. Each reaction was performed in duplicate. The threshold cycle (C_T) of the transgene transcript normalized to the C_T of the calbindin transcript was used to estimate the relative quantitation compared with line 643 *PCP2-tTA/TRE-SPΔ39* animals. Expression levels were determined from 3 animals per line. PCR product purity following quantitative PCR was confirmed by dissociation curve analysis and ethidium bromide gel analysis. Graphs show relative quantification values normalized to line 643 *PCP2-tTA/TRE-SPΔ39* animals.

Rotorod analysis: Rotorod latency was assessed for 26-week mice using an accelerating rotorod apparatus (Ugo Basille, Collegeville, PA) as previously described (Clark et al. 1997). Mice were tested for four trials per day for four consecutive days, with at least 10 min rest between trials. For each trial the rod accelerated from 4 to 40 rpm over 5 min and then remained at 40 rpm for 5 additional min. Latency was scored as time until the mouse fell from the rod or clasped rod without moving paws for greater than two consecutive rotations, up to a maximum possible time of 10 min. Group averages were compared using the *t* test function in Microsoft Excel, assuming two-tailed distribution and two-sample unequal variance.

Immunoblot analysis: Frozen cerebella from mice aged 9-11 months were homogenized in ice-cold 0.25 M Tris pH 7.5 with 1X protease inhibitors (Complete, Roche, Indianapolis, IN). After 3 freeze-thaw cycles and repeated passage through a 27-gauge needle, lysates were clarified twice by centrifugation at 700 X *g* for 5 min at 4ºC. Clarified lysates were incubated on ice for 1 hour with 20 mM dithiothreitol. 5 μg total protein was separated by SDS-PAGE, transferred to a nitrocellulose membrane, and blocked in PBS containing 5% skim milk and 0.1% Triton X-100. The membrane then was incubated with mGluR1α (1:2000, EMD Chemicals, Gibbstown, NJ), EAAT4 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), or calbindin D28K (1:50,000, Sigma, St. Louis, MO) antibodies in PBS containing 1% skim milk and 0.1% Triton X-100. After incubation with the appropriate HRP-conjugated antibody, bands were visualized with enhanced chemiluminescence reagent (GE Healthcare, Buckinghamshire, England).

HRP-conjugated anti-goat IgG was from Santa Cruz Biotechnology (Santa Cruz, CA), and HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were from GE Healthcare (Buckinghamshire, England). Quantitation was performed using the Molecular Analyst Software after scanning with the Model GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA). Graphs show the average amount of mGluR1α (sum of monomer and dimer values) or EAAT4 relative to the amount of calbindin as a percentage of the average value for control *Pcp2-tTA*$^{+/-}$*/TRE-SP-WT*$^{+/-}$ line 225 mice.

Immunoprecipitation: Whole cerebella from non-transgenic FVB/N mice 2-3 months of age were homogenized in ice-cold IP buffer (10 mM sodium phosphate pH 7.4, 130 mM NaCl, 2 mM EDTA, 0.1% Triton X-100) with 1X protease inhibitors (Complete, Roche, Indianapolis, IN). Lysates were clarified twice by centrifugation at 10,000 X *g* for 5 min at 4ºC. Lysates were precleared twice for 20 minutes with Protein G Sepharose or Protein A Sepharose beads (Amersham Biosciences, Uppsala, Sweden), then immunoprecipitated at 4ºC with an mGluR1α antibody (EMD Chemicals, Gibbstown, NJ), an HA antibody (Sigma, St. Louis, MO), a β-III spectrin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or no antibody (mock). Protein G Sepharose or Protein A Sepharose beads (the same type used for preclearing) were added to each tube and mixture was incubated at 4ºC for 1 hour. Beads were washed 6 times in 1 ml ice-cold IP buffer, then resuspended in loading buffer. Samples were vortexed, boiled for 5 minutes, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. After the membrane was blocked in 5% skim milk in PBS-T, it was incubated with β-III spectrin antibody antibody (1:500 Santa

Cruz Biotechnology, Santa Cruz, CA) or mGluR1α antibody (1:2000 EMD Chemicals, Gibbstown, NJ) in 1% skim milk in PBS-T. After incubation with the appropriate HRP-conjugated antibody, bands were visualized with enhanced chemiluminescence reagent (GE Healthcare, Buckinghamshire, England). HRP-conjugated anti-goat secondary antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), and HRP-conjugated anti-rabbit secondary antibody was from GE Healthcare (Buckinghamshire, England).

Immunofluorescent staining: Mice were anesthetized and transcardially perfused with phosphate-buffered saline followed by 10% phosphate-buffered formalin. Brains were dissected out of skull, post-fixed overnight in 10% phosphate-buffered formalin, and stored at 4ºC in phosphate-buffered saline. 50-µm-thick vibratome sections were boiled three times in 0.01 M urea to expose epitopes. After blocking in PBS with 0.3% Triton X-100 and 2% normal serum, slices were incubated for 48 hours at 4ºC with antibodies against calbindin (1:1000, Sigma, St. Louis, MO), EAAT4 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), or mGluR1α (1:500, EMD Chemicals, Gibbstown, NJ) diluted in blocking solution. Slices were washed 4 times for 20 minutes in PBS. After incubation for 48 hours at 4ºC with the appropriate fluorescent-conjugated antibody diluted 1:500 in blocking solution, slices were washed 4 times for 20 minutes in PBS. Secondary antibodies used for these experiments included Alexa Fluor® 488 conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA), Cy3 conjugated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA), and Cy3 conjugated anti-goat IgG (Jackson

ImmunoResearch, West Grove, PA). Sections were mounted in Gel / Mount^{TM} (Biomedia, Foster City, CA) and stored in the dark at -20ºC.

Quantification of molecular layer thickness: Sections were imaged by confocal microscopy with an Olympus FluoView FV1000 inverted confocal microscope at 200X magnification. Molecular layer thickness for each mouse was calculated by measuring from the edge of a Purkinje cell body to the fissure. I recorded 5 independent measurements on each side of the primary cerebellar fissure for a total of 10 measurements per animal. Group averages were compared using the *t* test function in Microsoft Excel, assuming two-tailed distribution and two-sample unequal variance.

Quantification of mGluR1α clustering: Sections were imaged by confocal microscopy with an Olympus FluoView FV1000 inverted confocal microscope at 2400X magnification. Analysis was modeled after techniques described by Das and Banker (2006) and quantification was performed using ImageJ software (Rasband 1997-2009). In this analysis, I defined clusters as regions having twice the average intensity of the entire image. The threshold for each image was manually set to twice the average intensity of the entire image. Normalized cluster size was defined as the average size of particles selected by this threshold, normalized to the average control cluster size calculated for images taken with the same microscope settings on the same day. Normalized cluster area was defined as the percentage of image area selected by this threshold, normalized to the average control cluster area calculated for images taken with the same microscope

settings on the same day. Group averages were compared using the *t* test function in Microsoft Excel, assuming two-tailed distribution and two-sample unequal variance.

Optical imaging: Optical imaging experiments were performed by Dr. Xinming Wang in Dr. Timothy Ebner's laboratory as previously described (Wang et al. 2009). SCA5 and age-matched control mice were anesthetized with urethane and supplemented as needed. The animals were artificially respirated. Core temperature was maintained with a feedback-controlled heating pad. For each experiment, the animal was placed in a stereotaxic frame, Crus I and II of the cerebellar cortex were exposed, and the dura removed. The mouse and stereotaxic frame were placed on a large stage beneath the optics from a modified Nikon epifluorescence microscope. Illumination was provided by a 100 W xenon-mercury lamp powered by a stabilized D.C. power source. For flavoprotein imaging, excitation light was passed through a band-pass filter (455±35 nm) and the emitted epifluorescence was passed through a long-pass filter (>515 nm) using a 500 nm dichroic mirror.

To stimulate parallel fibers a parylene-coated microelectrode was placed on the surface of the cerebellar cortex. The basic imaging paradigm involved collecting a time series of images resulting from parallel fiber test stimulation (10 pulses at 100 Hz (175 μA, 150 μs duration)) at 5 minute intervals before, during and after application of the conditioning stimulus. The conditioning stimulation consisted of a high frequency, burst stimulation of 15 pulses (175 μA, 150 μs duration) at 100 Hz repeated every 3 sec for 5 min.

Quantification of the optical signal was performed as previously described (Wang et al. 2009). Briefly, quantification of the optical signal for each experimental image was based on the net fluorescence change in the region of interest after subtracting the average of the background images. Here, the beam of increased fluorescence evoked by parallel fiber stimulation was the region of interest. ANOVA was used to test for statistical differences between the SCA5 and control mice. The optical responses were displayed by pseudocoloring increases in fluorescence using yellow to red and decreases in fluorescence using green to blue.

II. Chapter 3 methods

Constructs: Wildtype and American SCA5 mutant full-length *SPTBN2* cDNA clones in the vector pcDNA3.1 were ligated into the *Pcp2*/L7 expression construct provided by Tao Zu (Zu et al. 2004). See Page 108 for the full-length Pcp2-3xFLAG-SP-WT sequence and Page 112 for the full-length annotated Pcp2-3xFLAG-SPΔ39 sequence.

Generation and maintenance of transgenic mouse lines: The transgenes Pcp2-3xFLAG-SP-WT and Pcp2-3xFLAG-SPΔ39 were each cut from the vector background by digestion with Pme I, gel-purified, and dialyzed against injection buffer (5 mM Tris, 0.25 mM EDTA, pH 7.4). Prior to microinjection, the full-length cut constructs were verified by sequencing and transgenic protein expression was confirmed by transfection into HEK

293T cells. 2-4 µg/ml solution was microinjected into FVB/N zygotes at the University of Minnesota Transgenic Mouse Facility. Transgene-positive founders for both constructs were initially identified using Southern blotting of TaqI-cut tail DNA using the probe generated by amplifying a 323 bp product from the TRE-SP-WT construct template with primers TaqISouthernF and 2TaqISouthernR. For identification of independently segregating integration sites, tail DNA from multiple offspring of a single founder was separately digested with *Bgl I* and *Spe I* and subsequently probed with the 205 bp PCR product amplified with primers PCP2intSouthern-1F and PCP2intSouthern-1R from a transgenic tail DNA template. After line establishment, animals were genotyped by PCR with the primers PCP2-spectrin-2F and PCP2-spectrin-2R. PCR cycling conditions consisted of an initial 3 minute denaturation step at 94ºC; then 35 cycles of 94ºC for 45 sec, 60ºC for 45 sec, and 72ºC for 1 minute; followed by a final 6 minute elongation step at 72ºC. Mice were housed in a specific-pathogen-free facility, and animal care adhered to the guidelines established by the Institutional Animal Care and Use Committee at the University of Minnesota.

Generating homozygous mice: Tail DNA of all transgene-positive offspring from a breeding pair of heterozygotes was examined using a quantitative PCR strategy similar to that described by Alexander et al. (2004). Two-stage PCR was performed using 10 ng tail DNA and Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen, Carlsbad, CA) for 40 cycles (95ºC for 15 s, 60ºC for 1 min) on a Mastercycler® ep *realplex* (Eppendorf, Westbury, NY). I used primers PCP2-spectrin-2F and PCP2-

spectrin-2R to amplify the transgene DNA and primers IMR042 and IMR043 to amplify the murine housekeeping gene *interleukin-2*. Each reaction was performed in duplicate. The threshold cycle (C_T) of the transgene normalized to the C_T for *interleukin-2* was used to estimate the relative transgene copy number. Homozygosity was verified by breeding to non-transgenic animals.

Immunoblot analysis: Half cerebella were homogenized with a Polytron homogenizer in 400 µl ice-cold RIPA lysis buffer (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 µg/ml PMSF, 50 KIU/ml aprotinin, 1 mM sodium orthovanadate). Lysates were clarified by centrifugation, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was blocked in 5% skim milk in PBS-T, and then incubated with flag (1:5000 anti-flag-HRP, Sigma, St. Louis, MO) or calbindin (1:20,000 mouse anti-calbindin D28K, Sigma, St. Louis, MO) antibodies in 1% skim milk in PBS-T. After calbindin antibody incubation the membrane was incubated with an anti-mouse-IgG secondary antibody conjugated to HRP (Amersham Biosciences, Uppsala, Sweden). Bands were visualized with enhanced chemiluminescence (Amersham Biosciences, Uppsala, Sweden). Quantitation was performed using the Molecular Analyst Software after scanning with the Model GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA). Graphs show the amount of flag relative to the amount of calbindin as a percentage of control *Pcp2-3xFLAG-SP-WT* mice.

Rotorod analysis: Rotorod latency was assessed for 26-week mice using an accelerating rotorod apparatus (Ugo Basille) as previously described (Clark et al. 1997). Mice were tested for four trials per day for four consecutive days, with at least 10 min rest between trials. For each trial the rod accelerated from 4 to 40 rpm over 5 min and then remained at 40 rpm for 5 additional min. Latency was scored as time until the mouse fell from the rod or clasped rod without moving paws for greater than two consecutive rotations, up to a maximum possible time of 10 min. Group averages were compared using the *t* test function in Microsoft Excel, assuming two-tailed distribution and two-sample unequal variance.

Immunohistochemistry: Cerebellar tissue from transgenic mice, cerebellar autopsy tissue from an American SCA5 family member, and cerebellar autopsy tissue from a control individual without neurological disease were embedded in paraffin. 8 μm sections were incubated in 0.3% H_2O_2 for 30 min to bleach endogenous peroxidase activity, and then steamed for 30 min in 10 mM citrate buffer pH 6.0. For flag staining, sections were blocked in PBS with 10% skim milk and 1% BSA, then incubated at 4˚C overnight with flag antibody (F1804, Sigma, St. Louis, MO) diluted at 1:500 in PBS with 0.2% BSA. For β-III spectrin staining, sections were blocked in 5% normal horse serum in PBS, then incubated at 4˚C overnight with β-III spectrin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted at 1:500 in blocking solution. Positive staining was visualized by the avidin-biotin-peroxidase complex method (Vector, Burlingame, CA) with

diaminobenzidine as the chromogen and counterstained with hemotoxylin. Images were obtained using a Zeiss Axiovert 2 upright microscope.

Immunofluorescent staining of mouse cerebellum: Mice were anesthetized and transcardially perfused with phosphate-buffered saline followed by 10% phosphate-buffered formalin. Brains were dissected out of skull, post-fixed overnight in 10% phosphate-buffered formalin, and stored at 4ºC in phosphate-buffered saline. 50-µm-thick vibratome sections were boiled three times in 0.01 M urea to expose epitopes. After blocking in PBS with 0.3% Triton X-100 and 2% normal donkey serum, slices were incubated for 48 hours at 4ºC with flag antibody (F1804, Sigma, St. Louis, MO) and β-III spectrin antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) in blocking solution. Slices were washed 4 times for 20 minutes in PBS. After incubation for 48 hours at 4ºC with Alexa Fluor® 488 conjugated donkey anti-mouse IgG (Invitrogen, Carlsbad, CA) and Cy3 conjugated donkey anti-goat IgG (Jackson ImmunoResearch, West Grove, PA), slices were washed 4 times for 20 minutes in PBS. Sections were mounted in Gel / Mount^{TM} (Biomedia, Foster City, CA) and stored in the dark at -20ºC. Sections were imaged with an Olympus FluoView FV1000 inverted confocal microscope at 2400X magnification.

Immunoprecipitation: Whole cerebella were homogenized in ice-cold IP buffer (10 mM sodium phosphate pH 7.4, 130 mM NaCl, 2 mM EDTA, 0.5% Triton X-100) with 1X protease inhibitors (Complete, Roche, Indianapolis, IN). Lysates were clarified twice by

centrifugation at 10,000 X *g* for 5 min at 4ºC. Lysates were precleared twice for 20 minutes with Protein G Sepharose beads (Amersham Biosciences, Uppsala, Sweden), then immunoprecipitated at 4ºC with flag antibody (F1804, Sigma, St. Louis, MO), goat EAAT4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit nEAAT4 antibody (generous gift from Jeff Rothstein), α-II spectrin antibody (MAB1622, Millipore, Billerica, MA) or no antibody (mock). Protein G Sepharose was added to each tube and mixture was incubated at 4ºC for 1 hour. Beads were washed 6 times in 1 ml ice-cold IP buffer, then resuspended in loading buffer. Samples were vortexed, boiled for 5 minutes, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. After the membrane was blocked in 5% skim milk in PBS-T, it was incubated with flag (1:2000 anti-flag-HRP, Sigma, St. Louis, MO), cEAAT4 (1:200, generous gift from Jeff Rothstein), or α-II spectrin (1:500 C11, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies in 1% skim milk in PBS-T. After incubation with the appropriate HRP-conjugated antibody, bands were visualized with enhanced chemiluminescence reagent (GE Healthcare, Buckinghamshire, England). HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were from GE Healthcare (Buckinghamshire, England).

III. Chapters 4 and 5 methods

Generation of β-III spectrin bait constructs: Standard cloning techniques were used to generate constructs, and the integrity of all clones was confirmed by sequencing.

Restriction sites were introduced by PCR or by the QuikChange XL site-directed mutagenesis kit (Stratagene, Cedar Creek, TX). To construct pGBKT7-SR23-WT, the bait plasmid used for the yeast 2-hybrid screen, a human wild-type *SPTBN2* cDNA fragment containing spectrin repeats 2 and 3 (aa 416-642) was cloned in frame with the GAL4 DNA binding domain and the myc tag in vector pGBKT7 (Clontech). To construct pGBKT7-SR23Δ39 the American SCA5 mutation was introduced into pGBKT7-SR23-WT. To construct pGBKT7-SR24-WT a human wild-type *SPTBN2* cDNA fragment containing spectrin repeats 2, 3, and 4 (aa 416-745) was cloned in frame with the GAL4 DNA binding domain and the myc tag in vector pGBKT7 (Clontech). To construct pGBKT7-SR24Δ39 the American SCA5 mutation was introduced into pGBKT7-SR24-WT. To construct pGBKT7-SR24Δ15 the French SCA5 mutation was introduced into pGBKT7-SR24-WT.

Yeast 2-hybrid screen: Yeast 2-hybrid screening was performed using the Matchmaker GAL4 Two-Hybrid System 3 (Clontech, Mountain View, CA) following the manufacturer's instructions detailed in the Clontech Yeast Protocols Handbook. The bait plasmid pGBKT7-SR23-WT was introduced into yeast strain AH109 (*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4*Δ, *gal80*Δ, *LYS2 : : GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*HIS3, MEL1 GAL2*$_{UAS}$-*GAL2*$_{TATA}$-*ADE2, URA3::MEL1*$_{UAS}$-*MEL1*$_{TATA}$-*lacZ*) by lithium acetate-mediated transformation, and yeast expressing the bait protein were mated to yeast strain Y187 (*MATα, ura3-52*, *his*3-*200, ade 2-101*, *trp1-901, leu2-3*, *112*, *gal4*Δ, *met*$^{-}$, *gal80*Δ, *URA3 : : GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*lacZ, MEL1*) pretransformed with a human adult brain

cDNA library (Clontech, Mountain View, CA). I screened 1.57 x 10^7 clones and identified 335 positive colonies. Positive colonies were identified by growth on dropout medium lacking histidine, leucine, and tryptophan supplemented with 3 mM 3-amino-1,2,4-triazole. Colonies were replica plated on dropout medium lacking adenine, histidine, leucine, and tryptophan supplemented with 4 mg/ml X-α-Gal for more strigent selection requiring activation of the 3 reporter genes *ADE2*, *HIS3*, and *MEL1* to reduce false positives. Total yeast DNA was extracted from positive blue colonies on these quadruple dropout plates, then prey cDNA inserts were amplified by PCR with primers AD-library-F2 and AD-library-R and sequenced with primer AD-library-F2. Results were BLASTED against the NCBI human database (URL: http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the corresponding official full gene name and official symbol as designated by the HUGO Gene Nomenclature Committee (URL: http://www.genenames.org/index.html) were recorded for each clone.

Quantitative β-galactosidase assay: Bait and prey vectors were transformed into the yeast strain SFY526α (*MATα, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, can^r, gal4-542, gal80-538, URA3 : : GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*lacZ*) and grown on agar plates lacking the appropriate amino acids to maintain selective pressure on the transformed plasmids. 4 independent colonies were selected from each transformation and grown in the appropriate selective liquid medium. β-galactosidase assays were performed in triplicate on each liquid culture using the substrate chlorophenol red-β-D-galactopyranoside (CPRG), essentially as described in the Clontech Yeast Protocols

Handbook. Yeast protein was extracted from the same liquid cultures that were used for the CPRG assay to compare expression levels of myc-tagged bait and HA-tagged prey. CPRG was obtained from Roche Applied Science (Indianapolis, IN), and the SFY526α yeast strain was a generous gift from Dr. Judy Berman (University of Minnesota).

Immunoblot analysis of yeast cells: Yeast cells were resuspended in a cracking buffer of 8 M urea, 5% SDS, 40 mM Tris-HCl pH 6.8, 0.1 M EDTA, 0.4 mg/ml bromophenol blue, 1% β-mercaptoethanol, 1X protease inhibitors (Complete Mini, Roche Applied Science, (Indianapolis, IN)) prewarmed to 60ºC. Acid-washed 425-600 µm glass beads were added to cell mixtures and tubes were were vortexed at 4ºC, boiled at 100ºC for 5 min, vortexed at 4ºC, and centrifuged at 18,000 x g for 5 min at 4ºC. Supernatants were boiled at 100°C for 2-3 min, separated by SDS-PAGE on NuPage 4-12% Bis-Tris gels (Invitrogen), and transferred to a nitrocellulose membrane (GE Healthcare). Blots were incubated in 5% non-fat milk in PBS-T (1 X PBS, 0.1% Triton X-100) for 1 hour to block non-specific binding. The membranes were incubated at 4˚C overnight or at RT for 1 hour with the primary antibody in 1% non-fat milk/PBS-T. Primary antibodies used in these analyses included: 1) c-*myc* mouse monoclonal antibody (Sigma-Aldrich, Saint Louis, MO) (1:5,000 dilution); and 2) HA-HRP mouse monoclonal antibody (Sigma-Aldrich) (1:5,000 dilution). Blots were washed 2 times with PBS-T. Membranes were incubated with sheep anti-mouse-IgG-HRP (Sigma Aldrich) (1:5,000 dilution) secondary antibodies in 1% non-fat milk in PBS-T for 1 hr at room temperature, followed by 4 PBS-T washes. Bands were visualized with enhanced chemiluminescence (GE Healthcare).

Cell culture and co-immunoprecipitation: HEK 293T cells were cultured in DMEM (Invitrogen) and 10% fetal bovine serum (Invitrogen) at 37°C and 10% CO_2. Cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Cells were lysed in 25 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 1X protease inhibitors (Complete Mini, Roche Applied Science, (Indianapolis, IN) 48 hours post-transfection. Lysates were precleared twice for 20 minutes with Protein G Sepharose beads (Amersham Biosciences, Uppsala, Sweden), then immunoprecipitated with flag, c-myc, or no (mock) antibodies at 4ºC. Protein G Sepharose beads were added to each tube and mixture was incubated at 4ºC for 1 hour. Beads were washed 6 times in 50 mM Tris-Cl pH 7.5. Samples were vortexed, boiled for 5 minutes, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. After the membrane was blocked in 5% skim milk in PBS-T, it was incubated with flag antibody (1:5000 anti-flag-HRP, Sigma) or c-myc antibody (1:5000 mouse anti-c-myc 9E10, Sigma) in 1% skim milk in PBS-T followed by incubation with an anti-mouse-IgG secondary antibody conjugated to HRP (Amersham Biosciences, Uppsala, Sweden). Bands were visualized with enhanced chemiluminescence (Amersham Biosciences, Uppsala, Sweden).

Immunoblot analysis of mouse tissue: Cerebella were homogenized in ice-cold RIPA lysis buffer (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 50 KIU/ml aprotinin, 1 mM sodium orthovanadate). Lysates were clarified by centrifugation, separated by SDS-PAGE, and transferred to a nitrocellulose membrane.

(1) For flag transgenic protein analysis the membrane was blocked in 5% skim milk in PBS-T, and then incubated with flag antibody (1:5000 anti-flag-HRP, Sigma) or calbindin antibody (1:50,000 mouse anti-calbindin D28K, Sigma) in 1% skim milk in PBS-T followed by incubation with an anti-mouse-IgG secondary antibody conjugated to HRP (Amersham Biosciences, Uppsala, Sweden). Bands were visualized with enhanced chemiluminescence (Amersham Biosciences, Uppsala, Sweden).

(2) For $p150^{Glued}$ protein analysis, the membrane was incubated with $p150^{Glued}$ (1:500, BD Biosciences) or calbindin (1:50,000 mouse anti-calbindin D28K, Sigma) antibodies in 1% skim milk in PBS-T, followed by incubation with IRDye 800CW-conjugated anti-mouse (LI-COR, Lincoln, Nebraska) secondary antibodies. Imaging was performed using the Odyssey Infrared Imaging System (LI-COR, Lincoln, Nebraska).

IV. TRE-SP-WT transgene sequence

Notation: the tet-responsive promoter sequence is italicized; the translational start codon is underlined.

aattgcatgaagaatctgcttagggttaggcgttttgcgctgcttcgcgatgtacgggccagatatacgcgttgacattgattattgactagtgccctttc
gtctcgag*tttaccactccctatcagtgatagagaaaagtgaaagtcgagtttaccactccctatcagtgatagagaaaagtgaaagtcgagttt*
accactccctatcagtgatagagaaaagtgaaagtcgagtttaccactccctatcagtgatagagaaaagtgaaagtcgagtttaccactccct
atcagtgatagagaaaagtgaaagtcgagtttaccactccctatcagtgatagagaaaagtgaaagtcgagtttaccactccctatcagtgata
gagaaaagtgaaagtcgagctcggtacccgggtcgagtaggcgtgtacggtgggaggcctatataagcagagctcgtttagtgaaccgtcag
*atcgcctggagacgccatccacgctgttttgacctccatagaagacaccgggaccgatccagcct*ccgcggccccgaattcgagctcggtacc
cggggatcctctagtcagctgacgcgtgctagcgcggccgcatcgataagcttgtcgacgatatctctagcttaagcttggtaccgggccccccctc
gaggtcgacccacgcgtgggttccactgagcagccaaccgcagcctctggccacaaggagagcggagcacaggagcaggaagccgcctacc
acc<u>atg</u>agcagcacgctgtcacccacagactttgacagcttggaaatccagggccagtacagtgacatcaacaaccgctgggaccttcctgactcg
gactgggacaatgacagcagctcggcccgcctctttgagaggtctcgcattaaggctctggcagatgaacgagaagctgtgcagaagaaaacctt
caccaagtgggtaaactcgcacctggcccgggtcacgtgccgggtgggggacctgtacagcgacctccgggacggacgcaacctgctgaggct
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V. TRE-SPΔ39 transgene sequence

Notation: the tet-responsive promoter sequence is italicized; highlighted "g" is an insertion mutation that does not affect coding sequence or expression; the translational start codon is underlined.

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ccatgttcccaagggcagcccttctcttctgctgtttaattccagactggtggtgggacccaggtaacccccctctcccacccccgccgacttctcccctt
tccccagcctcgtgcctctgtccctcaccacggtgtggacagtgccgcaccctcaacataggccatgtggggagtggctgcccctgcctcagggtc
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cagaagtgccagatgacagtcagaggcgcaggagccctccctccccacccccacccctgtaactccagctgccactccatctccagctgctctcaat
ggcttccaggtgtgttgttcggggacagccaccgccttgagtctggccaaggaggtgattaaacagctcagcttctcaaaaaaaaaaaaaaaaaggg
cggccgctcgagtctag

VI. Pcp2-3xFLAG-SP-WT transgene sequence

Notation: sequence from the L7ΔAUG expression construct is italicized; the translational start codon is underlined; the 3xFLAG sequence is capitalized.

taaacgggccctctagactcgac*aagcttctcagagcatggtcagaaagccacagctcatcaatgaatggtcagggacttcctgtcctgctccat*
gcataaatgaaagacgaagacaactcaaattggcatttgaggggcagataaacaggagcatccggtagtttcacaggtggtcgggtagcag
gagccgggttggttggttggtctgtggagagtgcagggattaagggaagaggcctggaccccaacttcttccttggctacccccctgaaaatgt
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TAAAGATCATGACATCGATTACAAGGATGACGATGACAAGagcagcacgctgtcacccacagactttgaca
gcttggaaatccagggccagtacagtgacatcaacaaccgctgggaccttcctgactcggactgggacaatgacagcagctcggcccgcctctttg
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*tccggatcttctgcttaaattcttctagctcctggcgctggaatggggaaaggggtgtgatgggaaggccaagctt*aagttta

VII. Pcp2-3xFLAG-SPΔ39 transgene sequence

Notation: sequence from the L7ΔAUG expression construct is italicized; the translational start codon is underlined; the 3xFLAG sequence is capitalized.

taaacgggccctctagactcgac*aagcttctcagagcatggtcagaaagccacagctcatcaatgaatggtcagggacttcctgtcctgctccat*
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*ggggaaaggggtgtgatgggaaggccaagctt*aagttta

Primer name	Primer sequence
SPΔ39-1f	5’- GTGTCCCAGGACAACTTTGG -3’
SPΔ39-1r	5’- ATCCAGTCCAGGTTGAGGAGGAGCCGCTCC -3’
SPΔ39-2f	5’- CTCCTCAACCTGGACTGGATGGAAGAGATG -3’
SPΔ39-2r	5’- CTCCAGGGTGAGCTTCAGG -3’
TaqISouthernF	5’- CCTGCTCAACAAGCACACAG -3’
2TaqISouthernR	5’- CTAGAGCCTGCGTGGAGAAC -3’
tTA-F	5’- CGCTGTGGGGCATTTTACTTTAG -3’
tTA-R	5’- CATGTCCAGATCGAAATCGTC -3’
TRE1-F	5’- ATCCACGCTGTTTTGACCTC -3’
TRE1-R	5’- GTTGGCTGCTCAGTGGAAC -3’
calbindin1-F	5’- AGGCTGGATTGGAGCTATCA -3’
calbindin1-R	5’- TTCCTCGCAGGACTTCAGTT -3’
PCP2-spectrin-2F	5’- CTCTGGAGAGCCTGAAGAGC -3’
PCP2-spectrin-2R	5’- TATTGTTTTCAGGGGCCAGT -3’
PCP2intSouthern-1F	5’- AGGGCAGCGTCGCCTTT -3’
PCP2intSouthern-1R	5’- GTCATGCCCCGGGTGGT -3’
IMR042	5’- CTAGGCCACAGAATTGAAAGATCT -3’
IMR043	5’- GTAGGTGGAAATTCTAGCATCATCC -3’
AD-library-F2	5’- GCCATGGAGTACCCATACGA -3’
AD-library-R	5’- GCACGATGCACAGTTGAAGT -3’

Table 5. Primer sequences. This table lists the nucleotide sequences of all primers mentioned in this methods chapter.

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