Defining a Neuroprotective Pathway for the Treatment of Ataxias

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Dedication

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Abstract

Spinocerebellar Ataxias (SCAs) are a group of genetic diseases characterized by progressive ataxia caused by neurodegeneration of specific cell types, namely Purkinje Cells (PCs) of the cerebellum. Mouse models of SCA Type 1 (SCA1) can be used to study the molecular mechanisms underlying PC degeneration and death. One SCA1 mouse model, ATXN1[30Q]D776, has an initial ataxia but no progressive degeneration or PC death. RNA-seq experiments identified the up-regulation in the cerebellum of the peptide hormone Cholecystokinin (Cck) in these mice. Knocking out Cck or the Cck1 receptor (Cck1R) in ATXN1[30Q]D776 mice confers a progressive disease where PC death occurs by thirty-six weeks of age. Weighted Gene Co-expression Network Analysis (WGCNA) performed on cerebellar RNA-seq data from ATXN1[30Q]D776;Cck^−/− mice identified a disease progression-related gene set named the Pink Module that is influenced by Cck. A Cck1R agonist, A71623, was administered via osmotic minipump to ATXN1[30Q]D776;Cck^−/− mice and AXTN1[82Q] mice, which are a more faithful representation of human SCA1 PC degeneration. In both mouse models, A71623 protected against progressive ataxia and PC degeneration. These results suggest that manipulation of the Cck-Cck1R pathway may be a therapeutic target for treatment of diseases involving PC degeneration.
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Chapter 1: Introduction
1.1 Spinocerebellar Ataxias (SCA) and Purkinje Cell Susceptibility

SCAs are a group of dominantly inherited, progressive diseases that share many clinical and neuropathological features. There are currently 40 SCA types identified to date, and 29 of these have identified genetic causes, though the affected gene, mechanism of action, and type of mutation varies (Sun et al., 2016). Despite the heterogeneity in causes of SCAs, they each affect cells in the central and peripheral nervous systems in similar fashions. Purkinje Cells (PCs) of the cerebellum represent the main target of the SCAs. Degeneration of these neurons results in the clinical manifestation of SCAs includes unsteady gait, uncoordinated limb movements, and slurred speech, while individual SCA types may have variable involvement of extracerebellar areas.

Because many different mutations in many different genes are able to cause degeneration of this specific cell type, one possible approach for treatment of SCAs is to attempt to identify a convergent mechanism of disease (Bushart et al., 2016). In vitro, in vivo, and human studies have identified several convergent mechanisms of disease that point to unique susceptibilities of PCs to changes in signaling at multiple levels. Altered protein homeostasis occurs in several of the SCAs where aberrant protein folding and aggregation appears to contribute significantly to pathogenesis. Several studies suggest specific neuronal types, including PCs, are particularly sensitive to these misfolding events (Williams et al., 2008; Zoghbi and Orr, 2000). Several SCAs also involve altered transcriptional regulation, largely through disruption of transcriptional complexes (Bushart et al., 2016). PCs are additionally sensitive to changes in intracellular calcium handling. Cytosolic calcium levels are tightly regulated in neurons through a number of signaling pathways, calcium buffers, and transporters. Changes in components of these calcium handling pathways have been noted in several mouse models of different SCAs,
and several SCAs are caused by mutations in calcium transporters or channels (Bushart et al., 2016). Similarly, PCs are especially susceptible to altered membrane excitability (Chopra et al., 2014) and synaptic function (Serra et al., 2004; Liu et al., 2009).

These findings in multiple SCAs with multiple genetic causes hint at features of PCs themselves that make them particularly susceptible to certain types of assault. One possibility when pursuing therapeutic strategies is to identify an upstream mechanism that may impact overall PC health by modulating one or more of these mechanisms. In this thesis work, a potential PC protective pathway is identified in a mouse model of Spinocerebellar Ataxia Type 1 (SCA1). It is important to note that, while the rest of this thesis focuses on SCA1, this therapeutic pathway may be protective in other cases of PC dysfunction and death.

1.2 Triplet Repeat expansions

In 1991, a new mutational mechanism of disease was discovered: expansion of unstable trinucleotide repeats (Fu et al. 1991, LaSpada et al. 1994). Since then, 15 neurological disorders have been identified that consist of trinucleotide repeat expansions. Of these, eight are neurodegenerative diseases caused by the expansion of a CAG triplet repeat encoding a polyglutamine (polyQ) tract in the respective protein. These diseases include Huntington disease, spinobulbar muscular atrophy, and some of the spinocerebellar ataxias (SCAs).

Of these, SCA types 1, 2, 3, 6, 7, and 17 have a CAG trinucleotide repeat expansion, falling into the category of polyQ diseases. This expansion caused increased neuronal dysfunction and eventual neuronal loss, typically 10-20 years after the onset of symptoms. Early in the disease patients experience ataxia, including loss of limb and trunk coordination, unstable gait, dysarthric speech, and nystagmus. In the mid- and late-
stages of these diseases, patients may have other symptoms, such as cognitive impairment, and motor and sensory impairments (Orr, 2000; Koeppen, 1998).

1.3 Spinocerebellar Ataxia Type 1 (SCA1)

SCA1 is a fatal, autosomal dominantly inherited neurodegenerative disease caused by a CAG repeat expansion in the gene ATXN1 that encodes a polyglutamine (polyQ) expansion in the ATXN1 protein (Orr, 2000). In SCA1, Purkinje Cells (PCs) of the cerebellum are particularly vulnerable and the loss of these cells is a hallmark pathological finding. Neurons in the cerebellar dentate nuclei and brainstem nuclei are also lost (Zoghbi and Orr, 1995). Unaffected alleles of ATXN1 contain between 6-42 CAG repeats, with larger repeat tracts containing 1-3 CAT (histidine) interruptions. Disease-causing expansions range from approximately 40-80 pure polyQ residues (Chung et al. 1993). Onset of SCA1 is typically in the third or fourth decade (Zoghbi and Orr 1995), though it is dependent on the length of the pathogenic polyQ tract: longer polyQ expansions correspond to younger ages of onset (Haines et al., 1984, Schut, 1950). SCA1 is a progressive, degenerative disease and there is currently no disease-altering treatment or cure.

1.4 SCA1 mouse models

In order to study endogenous and pathogenic ATXN1 function, our lab and others have generated several genetic mouse models. The first SCA1 mouse model generated was the Pcp2-ATXN1[82Q] mouse (Figure 1A). A major disadvantage of using mice to model a 10-20 year progressive neurodegenerative disease is that the short lifespan of mice could prevent clinical symptoms from appearing. When generating the ATXN1[82Q] mouse model, Burright et al. (1995) took several approaches to overcome this complication. First, the transgene is over-expressed in these mice, leading to much
higher levels of expression than endogenous ATXN1. Additionally, a large polyQ expansion only found in one juvenile form of SCA1 was used (82Q) to confer a severe form of the disease. Finally, the transgene was expressed solely in the PCs of the cerebellum under the Pcp2/L7 PC-specific promoter (Oberdick et al., 1990, Smeyene et al., 1991; Vandaele et al., 1991). While pathogenic ATXN1 targets multiple cell types in the human disease, the PCs of the cerebellum exhibit early dysfunction and particular sensitivity to mutant ATXN1. The PCs also are large cells with well-defined connectivity and distinct morphology, making studies of both their pathology and physiology comparatively straightforward. Therefore expressing the \textit{ATXN1[82Q]} transgene solely in the PCs is a strategy to simplify the disease model in order to uncover both pathogenic and endogenous ATXN1 function. The drawback to this approach is that SCA1 is a complex disease affecting many cell types, and therefore these models cannot faithfully recapitulate every aspect of the disease. However, these strategies resulted in \textit{ATXN1[82Q]} mice that have a progressive, PC degenerative disease that occurs over a well-defined period of time. As a control for the \textit{ATXN1[82Q]} mice, the \textit{Pcp2-ATXN1[30Q]} line was created over-expressing a non-pathogenic form of human ATXN1 that contains a wild-type repeat number of 30Q with two His (CAT) interruptions. At one year of age, the \textit{ATXN1[30Q]} mice have a normal phenotype, which suggests no detrimental consequences of the transgene on PC function and health.
Figure 1. SCA1 mouse models. A) ATXN1[82Q] mice express human ATXN1 under the PC-specific Pcp2/L7 promoter. 1 year old ATXN1[82Q] mouse cerebellum. B) ATXN1[30Q]D776 mice express human ATXN1 with a wt repeat expansion of 30Q under the Pcp2/L7 promoter. There is a serine to aspartic acid amino acid change at the phosphorylation site S776 that is potentially phosphor-mimicking. 1 year old ATXN1[30Q]D776 mouse cerebellum. C) Molecular layer (ML) thickness changes from 6 to 12 weeks. ATXN1[82Q] ML thickness significantly decreases from 6 to 12 weeks, but ATXN1[30Q]D776 does not change with time. N>6 per group Two-way ANOVA, tukey post-hoc test. *p<0.05 **p<0.01.
ATXN1[82Q] mice have a well-defined progressive disease pattern. As early as postnatal day 25, pathology is visible in the form of clear cytoplasmic vacuoles in the cerebellar PCs (Clark and Orr, 2000). By 12 weeks of age, the mice have clear ataxic cage behavior and the PC dendritic tree has a distinct atrophied morphology. This is reflected in one measure of PC atrophy, the thickness of the molecular layer (ML) in the cerebellar cortex (Figure 1A, quantified in 1C). There is a sharp decrease in ML thickness from six to twelve weeks of age in ATXN1[82Q] mice (Figure 1C), while ATXN1[30Q] mice have no change in ML thickness out to one year of age (data not shown). By 12 weeks frequent heterotopic PCs are prevalent throughout the cerebellar ML, and there is diminished Calbindin (Calb) immunoreactivity in the surviving PCs. By 36 weeks, ATXN1[82Q] mice have PC loss compared to wt mice (Figure 6C).

It is important to note that the ataxic behavioral phenotype in ATXN1[82Q] mice precedes PC loss. Indeed, by 6 weeks ATXN1[82Q] mice show altered cage behavior, deficits on a rotating-rod (RotaRod) apparatus, and gait abnormalities by footprint pattern analysis (Clark and Orr, 2000). This suggests that PC dysfunction occurs before severe PC atrophy and death in the ATXN1[82Q] mice. This concept was supported by experiments showing decreased functional connectivity in PC-parallel fiber stimulation protocols by 6 weeks of age (Barnes et al., 2011). Additionally, parallel neurochemical changes in both human SCA1 patients and ATXN1[82Q] mice have been observed using proton magnetic resonance spectroscopy (1H MRS). Specifically, both SCA1 patients and ATXN1[82Q] mice have decreased levels of cerebellar N-acetylaspartate and glutamate, and increased levels of myo-inositol, which correlates with ataxia rating score (Öz et al., 2010). These physiological and neurochemical changes support the idea of PC dysfunction preceding overt degeneration and PC death in mice.

While comparing the ATXN1[82Q] and ATXN1[30Q] mice highlighted the importance of the polyQ tract in disease progression, our lab and others have identified
regions outside the polyQ tract that may be important in pathogenic ATXN1 function. Several conserved sequence motifs in the ATXN1 protein, as well as cellular molecules that interact with ATXN1, indicate that ATXN1 functions in the nucleus as a regulator of transcription and RNA-processing. One such motif is the ATXN1/HBP1 (AXH) domain, residues 567-689 that folds into an OB-fold, forming putative RNA-binding and protein-protein interaction surfaces (Yue et al., 2001; de Chiara et al., 2003; Chen et al., 2004; Kim et al., 2013). Several transcription regulators including SMRT (Tsai et al., 2004), Gfi-1 (Tsuda et al., 2005), Capicua (Lam et al., 2006; Kim et al., 2013), and the Rorα/Tip60 complex (Serra et al., 2006; Gehrking et al., 2011) interact with ATXN1 via its AXH domain. Importantly, polyQ expanded ATXN1 lacking the AXH domain is no longer pathogenic (Tsuda et al., 2005).

Towards the C-terminus from the AXH domain is another stretch of highly conserved overlapping sequence motifs in ATXN1. Among these is a nuclear localization signal (NLS) at amino acids 771-774, the function of which is required for pathogenesis (Klement et al., 1998). There are additionally several ATXN1 phosphorylation sites that were identified from the amino acid sequence of ATXN1 (Banfi et al., 1994). In 2003, Emamiam et al. performed mass spectrometry analysis and identified that one serine at position 776 serves as the main site of ATXN1 phosphorylation. Interestingly, phosphorylation at this site was able to modulate the stability of the ATXN1 protein (Jorgensen et al., 2009). Several lines of inquiry suggest that accumulation of ATXN1 is a major contributor to pathogenesis. ATXN1 knockout mice do not have a cerebellar phenotype (Matilla et al., 1998), and studies conditionally decreasing pathogenic ATXN1 are able to restore normal function (Zu et al., 2004). Therefore, the stability of the ATXN1 protein may contribute to its pathogenesis, making the S776 phosphorylation site interesting.
To explore the influence of phosphorylation at the S776 site on pathogenicity of ATXN1, a mouse model mimicking phosphorylation by substituting an aspartic acid for the serine was generated using the original ATXN1[30Q] transgene (Duvick et al., 2014). Whereas the ATXN1[30Q] mice had little to no cerebellar pathogenesis, the ATXN1[30Q]D776 mice have early ataxia around 6 weeks by Rotarod analysis.

Similarly, ATXN1[82Q] mice with a serine to alanine substitution that rendered the 776 position unable to be phosphorylated no longer had any disease pathogenesis, despite the long repeat tract. These two mouse models revealed the importance of the S776 phosphorylation site to pathogenic ATXN1 function.

ATXN1[30Q]D776 mice have both a motor phenotype and cerebellar pathology as substantial as the ATXN1[82Q] mice at 6 weeks (Figure 1B, C). Unlike ATXN1[82Q] mice, however, in ATXN1[30Q]D776 mice pathology does not progress. At 1 year, there is no significant decrease in ML thickness or PC counts, indicating that ATXN1[30Q]D776 mice do not have progressive PC degeneration or death. A reasonable hypothesis is that some protective mechanism has been activated in these mice to prevent disease progression.

These mouse models granted an understanding of both normal and mutant ATXN1 function. Levels of expression of mutant ATXN1, length of the polyQ tract,
One important function of ATXN1 likely to play a role in pathogenesis is via regulation of gene transcription. Mice lacking the ATXN1 nuclear localization sequence do not have ATXN1 that can enter the nucleus, and they do not get disease (Klement et al., 1998). Additionally, previous studies have identified the up- and down-regulation of specific PC genes using a PCR-based cDNA subtractive-hybridization assay on ATXN1[82Q] cerebellar RNA (Lin et al., 2000). The interesting finding here was that PC-specific genes were not globally affected by mutant ATXN1, but rather specific gene targets. It appears that mutant ATXN1 specifically influences
This thesis work describes the identification and characterization of the Cck-Cck1R pathway that is activated and protects against disease progression in \textit{ATXN1[30Q]D776} mice. Experiments discussed in Chapter 2 identified and characterized the overexpression of the gene \textit{Cck} in \textit{ATXN1[30Q]D776} mice. In Chapter 3, upregulated \textit{Cck} is shown to be neuroprotective against behavioral and pathological disease progression in \textit{ATXN1[30Q]D776} mice and that it acts through the canonical Cck1R pathway. In Chapter 4, RNA sequencing (RNAseq) is used to identify pathways that overlap between two progressive mouse models, and describe the role of Cck in modulating those pathways. Finally, in Chapter 5, a Cck1R agonist, A71623, is used to protect against disease progression in two progressive SCA1 mouse models.
Chapter 2: ATXN1[30Q]D776 mice have increased expression of Cck mRNA
2.1 Identification of Cck as a gene of interest in ATXN1[30Q]D776 mice

RNA-sequencing (RNAseq) was performed on whole cerebellar mRNA samples from wt/FVB, ATXN1[82Q], and ATXN1[30Q]D776 mice at 5, 12, and 28 wks of age (Ingram and Wozniak et al., 2016). The goal of this study was to identify genes and gene pathways regulated by pathogenic ATXN1. Because the ATXN1[30Q]D776 mice do not have disease progression, it was hypothesized that a protective mechanism was activated in these mice to prevent progressive PC degeneration. To identify potential candidates, the gene expression changes between wt/FVB, ATXN1[82Q], and ATXN1[30Q]D776 mice at 12 wks was compared and 130 genes were identified that were changed among all three genotypes (Figure 2A). It was reasoned that a protective mechanism might be differentially expressed in mice with progressive disease compared to mice with non-progressive disease, that list was then filtered to genes with opposite expression patterns between ATXN1[82Q] and ATXN1[30Q]D776 mice (32 genes, Figure 2B). Those genes identified were further filtered for a high FPKM (>3) and for PC-expressed genes in an attempt to identify highly expressed genes that may be directly impacted by PC-expressed pathogenic ATXN1. This method resulted in the identification of two genes, Cholecystokinin (Cck) and Col18a1. The gene Cck was expressed 12-fold higher in ATXN1[30Q]D776 cerebella than wt/FVB cerebella and slightly decreased in ATXN1[82Q] mice (Figure 2C). Because of the remarkably high expression of Cck in ATXN1[30Q]D776 mice, it was selected as an initial candidate for exerting these protective effects.
Figure 2. Expression of cholecystokinin (Cck) is elevated in ATXN1[30Q]D776 cerebella. (A) Venn diagram depicting the total number of transcripts changed at 12 weeks of age. (B) Breakdown of the expression changes for the 130 genes with significant changes in common between ATXN1[82Q] and ATXN1[30Q]D776 cerebella. Fifty-nine genes were upregulated in both lines compared to wt (FVB/NJ) and 66 genes downregulated. Thirty-two genes changed in opposite directions in ATXN1[82Q] and ATXN1[30Q]D776 cerebella. C) Of these 32 genes, two genes (Cck and Col18a1) had expression levels of FPKM ≥ 3.0 and enhanced translation in Purkinje cells.
2.2 Cck mRNA expression pattern supports it as a candidate for the protection observed in ATXN1[30Q]D776 mice

To determine whether Cck may be a gene of interest in pursuing as a potentially protective mechanism in ATXN1[30Q]D776 mice, its cerebellar expression patterns were first established. In situ hybridization from the Allen Brain Atlas for Cck mRNA reveals a PC-specific pattern of expression in the mouse cerebellum (Figure 3A). The Cck1R, but not the Cck2R, also is enriched in the PCs. When the in situ hybridization for Cck mRNA was performed in ATXN1[82Q] and ATXN1[30Q]D776 mice, Cck mRNA was found to be enriched in PCs (Figure 3B) and is expressed higher in the ATXN1[30Q]D776 mice. This suggests that Cck and its receptors are indeed localized to the region affected by pathogenic ATXN1 in ATXN1[30Q]D776 mice.
Figure 3. Cck cerebellar localization and expression. A) In situ hybridization (ISH) heatmap expression from the Allen Brain atlas. B) ISH for Cck mRNA in SCA1 mouse models. C) qRT-PCR for Cck and ATXN1 mRNA reveals corresponding timing of expression. N>3 per group. D) qRT-PCR for Cck mRNA in several mouse models at 12 weeks of age. One-Way ANOVA with tukey post-hoc test, *p<0.05, **p<0.01.
If the ATXN1[30Q]D776 protein is leading to the elevation of Cck mRNA, then the timing of expression for ATXN1 and Cck should coincide. qRT-PCR was used to determine the expression pattern of Cck in ATXN1[30Q]D776 mice (Figure 3C). Indeed, the Cck gene in these mice is turned on around p11, coinciding with the increase in ATXN1 expression around that age (Figure 3C).

All ATXN1[30Q]D776 mouse lines (L2, L3, and L6) lack disease progression. While the rest of this thesis work utilizes ATXN1[30Q]D776-L2, Cck mRNA was also found to be elevated in these other non-progressive mouse lines (Figure 3D). Cck mRNA expression was measured in ATXN1[82Q] mice and the conditional ATXN1[30Q]D776 mice, which express the ATXN1[30Q]D776 transgene with a tet-ON inducible system. Conditional ATXN1[30Q]D776 mice have progressive disease similar to that seen in ATXN1[82Q] mice, a puzzling observation. It was interesting to note, then, that Cck mRNA is elevated in the three ATXN1[30Q]D776 lines and not in either progressive line tested (data not shown), perhaps explaining the observation of progressive pathology in the conditional-ATXN1[30Q]D776 mice.

The timing of expression, localization, and specificity to non-progressive mice supported this elevation of Cck as a mechanism of interest to pursue as a potential candidate for preventing disease progression in ATXN1[30Q]D776 mice.
2.3 Discussion

In this chapter, experiments identifying highly upregulated Cck in ATXN1[30Q]D776 mice, and slightly downregulated Cck mRNA in ATXN1[82Q] mice is discussed. While little is known about the role for Cck in the cerebellum, it has been widely studied in many different brain areas.

CCK is a neuropeptide hormone expressed in a cell-specific manner throughout the mammalian central nervous system. Though Cck is well-known for its actions in regulating food intake, it is also implicated in neuronal activation (Meis et al., 2007), depolarization (Rogers et al., 2008), axonal migration (Giacobini et al., 2004), synaptic plasticity (Wyeth et al., 2012), and synaptic transmission (Deng et al., 2010). Cck is first transcribed as a full-length protein, procholecystokinin (proCCK), and is cleaved into several different active forms in a tissue- and cell-specific manner (Rehfeld et al., 2003). Among these, the eight-amino acid peptide sulfated Cck8 (Cck8S) is highly expressed in the CNS (Dockray et al., 1980; Rehfeld et al., 2003), especially in the cerebellum of mice, humans, and non-human primates (Kagami et al., 2001; Matsui et al., 1993; Yamashita et al., 1990; Uhlen et al., 2015).

The expression profile of Cck in the cerebellum changes dramatically throughout development: In wt mice Cck mRNA levels have a bimodal expression pattern, with high levels in the CNS before birth that decrease around P7, then increase significantly to reach adult levels by P21 (Sato et al., 2008). In adult mice, Cck is one of the 1000 most highly expressed PC-specific genes (Heiman et al., 2014 and Figure 3A).

There are two identified CCK receptor subtypes, CCK-1 receptor (Cck1R, also known as CckAR) and CCK-2 receptor (Cck2R, also known as CckBR). Both belong to the family I of G-protein coupled receptors (GPCRs), but there are marked differences in their cell-type specificity and binding affinities for different forms of Cck. The Cck1R and
Cck2R share 50% sequence homology, have seven transmembrane domains, an
E/DRY motif, and a NPXXY motif necessary for initiating intracellular signaling cascades
(Dufresne et al., 2006). The Cck1R has the highest affinity for the sulfated forms of Cck
peptides, particularly Cck8S, but also readily is bound by Cck33, Cck39, and Cck58
(Reeve et al., 2002; Solomon et al., 1984). Binding to the Cck2R does not appear to be
heavily influenced by sulfation state, and the Cck2R, unlike the Cck1R, binds the
peripheral peptide gastrin as readily as Cck8 (Dufresne et al., 1996).

Both receptor subtypes are expressed in a cell-specific manner throughout the
brain, but may have sexually dimorphic expression and function in certain areas, like the
hypothalamus and hippocampus (Zu et al., 2012; Sui et al., 2013). Cerebellar Cck
receptor expression has proven difficult to determine, largely because of species
differences. Autoradiography studies have shown Cck binding sites highly expressed in
the cerebellum of several mammalian species (Dietl et al., 1989; Sekiguchi et al., 1986),
though these studies did not determine which receptor was responsible. Both Cck1R
and Cck2R appear to be expressed in the cerebellum during development in several
rodent species (Kagami et al., 2001). While there is no cerebellar Cck receptor
expression in adult rats, in mice the Cck1R is enriched in PCs, and low levels of
expression have been found for both the Cck1R and the Cck2R in the brainstem
(Kagami et al., 2001; Lein et al., 2007). In adult humans, the Cck1R is highly expressed
in PCs and cells in the molecular layer of the cerebellar cortex, while the Cck2R is not
detected in the cerebellum (Uhlen et al., 2015).

The mechanism of action of Cck is well-studied in both the gut and other areas of
the CNS. Administering Cck in vitro was shown to directly activate neurons in several
brain areas, including the vagal afferents to the nucleus of the solitary tract. Rogers and
Hermann (2008) utilized Ca$^{2+}$ imaging and various pharmacological blockades to show
that Cck1R activation in this area can lead to increases in intracellular Ca$^{2+}$ via parallel
activation of PKA and PLC to activate L-type Ca\(^{2+}\) channels and endoplasmic reticulum Ca\(^{2+}\) release, respectively. In this study, the authors additionally suggest that Cck, via the Cck1R, can indirectly lead to PKC activation, which causes transient K\(^{+}\) current blockage and leads to action potential-like discharges, Ca\(^{2+}\) spikes, and vesicle release. Activation of Cck2Rs leads to an increase in currents from a nonselective cation channel, likely TRP channels (Chung et al., 2009). Importantly, Cck-Cck1R activation can lead to long-term changes in cellular function by manipulating mRNA expression in target cells (Heldsinger et al., 2012). This is thought to underlie Cck’s enhancement of long-term potentiation in brain areas like the hippocampus.

A role for Cck in the cerebellum is less clear. In 1996, Bishop measured PC inward currents induced by exogenous synthetic Cck8S administration and showed Cck8S was capable of modulating both spontaneous activity and excitatory amino acid-induced activity in the opossum cerebellum. They found that Cck administration largely increased spontaneous PC firing, and influenced PC responses to excitatory amino acids. The expression of Cck receptors in the cerebellum suggests that the Cck1R may be more important for mediating cerebellar effects (Uhlen et al., 2015). This is also supported by functional evidence in humans. It has long been known that Cck influences the brain-gut axis, which controls food intake, and that the cerebellum is an important regulator of this system (Zhu and Wang, 2008). A recent study using MRI in humans showed that circulating glucose decreases the cerebellar BOLD signal and that this decrease is enhanced when the subjects are given a Cck1R antagonist (Little et al., 2014). Another study found that ingested lipids increased cerebellar activation, and this increase does not occur when the subjects are also given a Cck1R antagonist (Lassman et al., 2010). While these studies point to a complex role for cerebellar Cck in monitoring and responding to the body’s energy stores, they do reveal functional consequences of Cck-Cck1R activation in the cerebellum.
Cck upregulation has been postulated to constitute an endogenous neuronal protective response, though the mechanism by which it acts is not yet understood. There are many models of neuronal dysfunction and degeneration that have reported increased Cck expression, and several that suggest it is protective. For example, despite the observation that activation of either Cck receptor generally leads to an increase in excitability, Cck has surprisingly been suggested to function as an endogenous anti-convulsant in epilepsy patients. Iadorola and Sherwin (1991) showed elevation of Cck mRNA and pro-Cck protein in actively epileptic patient human temporal cortical foci. Systemic Cck injections have additionally been shown to attenuate or delay convulsions induced in rodents by a variety of mechanisms, including picrotoxin, electroshock, and other convulsant drugs (Kadar et al., 1984; Kadar et al., 1985; Zetler, 1980; Iadarola et al., 1986). Cck8S also protects against NMDA-receptor mediated glutamate excitotoxicity in cultured cerebral cortical neurons, potentially by blocking the formation of nitric oxide (Tamura et al., 1992). While the mechanism by which Cck is able to protect against seizure activity remains unclear, it suggests that upregulation of Cck may constitute an endogenous mechanism by which cells in the CNS attempt to offset neuronal dysfunction.

Cck also protects against several types of neuronal assault, including central lesions, axotomies, and degenerative conditions in the spinal cord. Peripheral administration of Cck8S protects central cholinergic neurons against fimbria-fornix lesions in mice (Tirassa et al., 1999) and basal forebrain lesions in rats (Sugaya et al., 1992). These effects are thought to be mediated by Cck-induced increases in nerve growth factor, NGF (Manni and Lundeberg, 2003; Tirassa et al., 1999). Indeed, Cck8S can induce NGF expression throughout the central and peripheral nervous system via the Cck1R in the hypothalamus and pituitary, and via the Cck2R in the hippocampus (Tirassa et al., 1998). In the periphery, systemic Cck8 administration leads to localized
increases of NGF in areas injected with the neurotoxin capsaicin and subsequent neuronal protection (Manni and Lundeberg, 2003).

In the spinal cord, Cck upregulation occurs around the time of neuronal assault. The wobbler mouse has a spontaneous mutation in a vesicle trafficking protein that leads to degeneration of the motor neurons in the dorsal spinal cord, and has led to discoveries of how degeneration may occur in infant spinomuscular atrophy and amyotrophic lateral sclerosis. Wobbler mice were found to have increased numbers of Cck8S-immunoreactive fibers in the dorsal horn of the spinal cord in the early stages of disease (Zhang and Vacca-Galloway, 1992). In wild-type mice, Cck transcription also increases in the spinal cord in response to sciatic nerve axotomy (Verge et al., 1993; Verge et al., 1995), possibly as a mechanism to enhance axonal regeneration via subsequent upregulation of NGF.

These studies support the idea that Cck is endogenously upregulated during neuronal assault and can protect against neuronal dysfunction and degeneration in many different cell types throughout the CNS and PNS. While the role for Cck in the cerebellum is undefined, the upregulation of Cck occurs very early in at least three mouse models of ataxia: Rolling Mouse Nagoya, weaver, and Purkinje Cell Degeneration mice (Matsui et al., 1993). However, the cell type source, mechanism of action, or effect on disease of Cck in these mice was never studied beyond the initial observation.
Chapter 3: Increased $Cck$ expression prevents disease progression in $ATXN1[30Q]D776$ mice through the Cck1 receptor
3.1 Cck\(^{-}\) mice have a mild cerebellar deficit

The elevation, timing of expression, and cellular localization of Cck in ATXN1[30Q]D776 mice suggests that it could be a candidate for the protection against disease progression previously observed in these mice. To address whether Cck was protective against disease progression, Cck\(^{-}\) mice were obtained from Jackson labs.

The Cck\(^{-}\) mice were generated by Lacourse et al. (1999). They have a 168bp deletion of the first coding exon in the Cck gene, exon 2. At this site, a lacZ reporter gene and a neomycin selection cassette were inserted. Confirmation of the knockout was performed by peptide radioimmunoassay (Lacourse et al., 1999). The presence of \(\beta\)-galactosidase staining also confirmed the presence of the lacZ reporter gene (Lacourse et al., 1999). Cck knockout mice have normal body weight, fat absorption, and body mass. They eat more food during the light period, and less during the dark, suggesting changes in their circadian components of the brain-gut axis. They are slightly more anxious than wt controls, and may have some subtle memory and cognitive deficits that could arise from decreased hippocampal neurogenesis (Lo et al., 2008).

Cck\(^{-}\) mice have some gastrointestinal deficits, including gallbladder hypomotility (Wang et al., 2010) and excess pancreatic enzyme production. These phenotypic traits do not affect fertility or viability.

Because Cck is highly expressed in both the developing and adult cerebellum, it was first determined whether Cck\(^{-}\) mice had a cerebellar phenotype. To test whether Cck\(^{-}\) mice had a motor phenotype, the balance beam test for motor coordination was used. This test consists of two measures: time to cross and the number of times their hindpaws slip off of the beam. The Cck\(^{-}\) mice performed significantly worse on the balance beam than wt mice (Figure 4A, B). Similarly, Cck\(^{-}\) mice performed worse on the rotarod test for motor coordination than wt mice (Figure 4C).
Figure 4. Cck\(^{-/-}\) mice have mild cerebellar dysfunction at 36 weeks of age. A) Number of footslips recorded on the smallest (10mm round) beam. Numbers in the bars represent the N’s for each group. B) Average length of time it takes to cross the 10mm round beam. C) Latency to fall from the rotarod test of motor coordination. Students t-test, *p<0.05, **p<0.01, ***p<0.001. D) ML thickness change from 20 weeks to 1 year. Two-Way ANOVA, tukey post-hoc test. *p<0.05. E) Number of PCs per 250 um of primary fissure. F) qRT-PCR for Calb mRNA, expressed as a fold change over wt/FVB. Student’s t-test, *p<0.05.
The behavioral analysis suggested a cerebellar phenotype, so their cerebellar morphology was examined. There was no difference in molecular layer (ML) thickness, a measure of PC dendritic tree degeneration, between Cck\(^{-}\) and wt mice at 20 weeks or 1 year (Figure 4D). However, there was a significant decrease in Cck\(^{-}\) ML thickness from 20 weeks to 1 year, while wt/FVB do not have a significant change. There was also no difference in PC number (Figure 4E), indicating that there is no PC loss compared to wt mice.

It has previously been reported that changes in PC signaling can elicit behavioral deficits like those I observed in Cck\(^{-}\) mice in absence of pathology. To test this, expression of the PC marker Calbindin 1 (Calb1) was examined, which has previously been suggested to reflect PC health. By qRT-PCR, Calb1 mRNA was significantly decreased in Cck\(^{-}\) mice. This suggests that, in the absence of Cck, there is some mild PC dysfunction that does not lead to any severe pathological phenotype or PC degeneration. This supports the idea that decreased levels of cerebellar Cck, such as those found in ATXN1\([82Q]\) mice, may be detrimental to cerebellar function and health. The cerebellar phenotype of these mice lends some support to the hypothesis that an upregulation of Cck may serve to ameliorate some of the PC dysfunction observed in SCA1 mice.

3.2 ATXN1\([30Q]\)D776 mice lacking Cck have severe ataxia and progressive PC degeneration

To test whether elevated Cck in ATXN1\([30Q]\)D776 mice is able to protect against disease progression, Cck\(^{-}\) mice were crossed to ATXN1\([30Q]\)D776 mice to produce ATXN1\([30Q]\)D776;Cck\(^{-}\) mice. At 36 wks old, ATXN1\([30Q]\)D776;Cck\(^{-}\) mice performed significantly worse on both the rotarod (Figure 5A), and the balance beam (Figure 5B, C) than wt, Cck\(^{-}\), and ATXN1\([30Q]\)D776 mice.
Figure 5. Motor behavior tests on 1 year old SCA1 mouse models. A) Time to cross the 10mm balance beam. B) Number of footslips on the 10mm round balance beam. C) Latency to fall off the rotarod test. One-Way ANOVA with Tukey post-hoc test. *p<0.05, ***p<0.001. #p<0.05 compared to wt.
To test whether the progressive motor behavior deficit was reflected by changes in cerebellar pathology, ML thickness was measured at 6 and 52 weeks (Figure 6B). As was previously reported, at 6 weeks both ATXN1[82Q] and ATXN1[30Q]D776 mice had significantly thinner ML than wt mice (Two-Way ANOVA with Tukey post-hoc test, p=0.014 and p=0.0118, respectively). ATXN1[30Q]D776;Cck−/− mouse ML thickness at 6 weeks was not different than ATXN1[30Q]D776 or ATXN1[82Q] ML thickness (p>0.999 for both comparisons). As previously reported, ATXN1[82Q] ML thickness decreased sharply between 6 and 52 weeks (p<0.0001), while wt and ATXN1[30Q]D776 ML thickness did not change with time (p=0.3319 and p=9.449, respectively). In contrast, ATXN1[30Q]D776;Cck−/− ML thickness did significantly decreased with time (p=0.0066). This suggests that the elevation of Cck in ATXN1[30Q]D776 mice may protect against PC degeneration.
Figure 6. Pathology on SCA1 mouse models. A) representative images of Calbindin-stained PCs in the primary fissure of the mouse cerebellum. B) Quantification of ML thickness at 6 weeks and 1 year. Two-Way ANOVA with tukey post-hoc test, ***p<0.001 compared to 6 week data. #p<0.05 compared to wt. C) 1 year old mice. Number of PCs per 250 um of the primary fissure. One-Way ANOVA, tukey post-hoc test. *p<0.05 compared to ATXN1[30Q]D776, #p<0.05 compared to wt.
Another measure of cerebellar pathology is the loss of PCs. It has previously been established that $ATXN1[82Q]$ mice have PC death later than 28 weeks of age, while $ATXN1[30Q]D776$ mice never have PC death. At 36 weeks of age, $ATXN1[30Q]D776;Cck^{-/-}$ mice have significant PC loss compared to wt/FVB mice (One Way ANOVA with Tukey post-hoc test, $p=0.0097$). The PC counts are not significantly different than $ATXN1[82Q]$ mice ($p=0.9942$), suggesting that their PC loss is similar at this age.

These data suggest that the absence of overexpressed Cck in the $ATXN1[30Q]D776$ mice confers a progressive disease similar to that seen in $ATXN1[82Q]$ mice.

3.3 $ATXN1[30Q]D776$ mice lacking the Cck1R have progressive PC degeneration

$ATXN1[30Q]D776;Cck^{-/-}$ mice have a progressive disease similar to that seen in $ATXN1[82Q]$ mice, albeit on a longer time scale (Figure 1C). This supports the idea that upregulated Cck in $ATXN1[30Q]D776$ mice is protective against progressive PC degeneration and loss. In order for the Cck pathway to be used as a potential therapeutic, it was important to next determine the mechanism by which it is acting. Cck is translated first a pro-hormone, then cleaved into its active peptides in a tissue- and cell-specific manner in the cytoplasm (Pratt et al., 2004). Upon release from the cell, either at the synapse or from the cell body in some instances, it is able to bind to two identified Cck receptors. One possibility for Cck’s activity in the $ATXN1[30Q]D776$ mice was that it was acting via a non-canonical mechanism yet to be discovered. More likely, Cck is being cleaved into an active peptide, then binding to one of the two receptors. As described in the discussion of Chapter 2, the Cck1R is enriched in the PCs of the cerebellum, while the Cck2R is not expressed in the cerebellum. However, the Cck2R is
expressed in some cells of the brainstem and deep cerebellar nuclei that potentially could mediate these effects. It was therefore important to test both receptors.

This experiment was designed with two goals: 1) determine whether upregulated Cck mRNA is acting through the canonical Cck-receptor pathway and 2) identify the Cck receptor involved. If the Cck receptors are involved, then it can be reasoned that the upregulated Cck is being cleaved into its active peptide form. To answer this question, I used a genetic approach similar to that in Chapter 3.2. I obtained Cck1R−/− and Cck2R−/− mice from Jackson Labs. Cck1R−/− mice were generated by replacing a portion of exon 3 encoding the third transmembrane domain and the second intracellular loop including the “ERY” motif with the neomycin resistance gene (Kopin et al., 1999). A competition binding study using 125I-Cck8 showed high affinity binding in wt mouse pancreatic samples, whereas there was no displaceable binding of 125I-Cck8 in Cck1R−/− mice, affirming that these mice have no functional Cck1R. Cck1R−/− mice are viable and fertile, and while they are more prone to gallstones (Schmitz et al., 1996), they have no overt differences in appearance or general behavior from wt mice (Kopin et al., 1999).

Cck2R−/− mice were generated by deleting a portion of exon 3, all of exon 4, and a portion of exon 5 and replaced with a neomycin resistance gene (Langhans et al., 1997). These mice have an abnormal gastric function and morphology (Langhans et al., 1997; Rindi et al., 1998). However, they are viable, fertile, and any differences from wt in behavioral tasks are subtle and usually depend on external stimuli, such as administration of amphetamine (Runkorg et al., 2006).

No previous studies have examined cerebellar function or motor behavior in Cck1R−/− or Cck2R−/− mice. Cck1R−/− mice have a trend towards a cerebellar deficit similar to Cck−/− mice (Figure 5), but no overt cerebellar pathology (Figure 6). Cck2R−/− mice do not appear to have any pronounced motor deficit or cerebellar pathology.
Cck1R<sup>−/−</sup> and Cck2R<sup>−/−</sup> mice were crossed to ATXN1[30Q]D776 mice to generate ATXN1[30Q]D776;Cck1R<sup>−/−</sup>, ATXN1[30Q]D776;Cck2R<sup>−/−</sup>, and ATXN1[30Q]D776;Cck1R<sup>−/−</sup>;Cck2R<sup>−/−</sup> mice. As shown in Figure 5, at 36 weeks of age, ATXN1[30Q]D776 and ATXN1[30Q]D776;Cck2R<sup>−/−</sup> mice had balance beam and rotarod deficits compared to wt/FVB mice. Interestingly, ATXN1[30Q]D776;Cck<sup>−/−</sup> and ATXN1[30Q]D776;Cck1R<sup>−/−</sup> both performed significantly worse than ATXN1[30Q]D776 mice at 36 weeks, indicating that removing either Cck or the Cck1R confers a more severe motor deficit by three different measures of motor behavior (Figure 5). Supporting this observation, the ATXN1[30Q]D776;Cck1R<sup>−/−</sup> mice also had similar pathology to ATXN1[30Q]D776;Cck<sup>−/−</sup> mice. ATXN1[30Q]D776;Cck1R<sup>−/−</sup> mice had thinner ML thickness at 1 year (Two-Way ANOVA with Tukey post-hoc test, p=0.0481) and loss of PCs at 36 weeks (One-Way ANOVA with tukey post-hoc test, p=0.0329) compared to ATXN1[30Q]D776 mice.

Interestingly, the cerebellar phenotype of both ATXN1[30Q]D776;Cck<sup>−/−</sup> and ATXN1[30Q]D776;Cck1R<sup>−/−</sup> mice is similar to that seen in ATXN1[82Q] mice (Figure 6), while the ATXN1[30Q]D776;Cck2R<sup>−/−</sup> mice are not significantly different from ATXN1[30Q]D776 mice.

These data suggest that the elevated Cck that was protective in the ATXN1[30Q]D776 mice is acting through the Cck1R, but not the Cck2R. This is especially interesting given the expression of both Cck and Cck1R on PCs of the cerebellum (Figure 3A). It is possible that, in the ATXN1[30Q]D776 mice, Cck is being released from the PCs, and also acting on the PCs in a cell-autonomous manner.
3.4 Discussion

A consequence of enhanced expression of Cck in ATXN1[30Q]D776 PCs in relation to their lack of progressive disease was disclosed by crossing ATXN1[30Q]D776 mice with mice lacking either Cck or Cck1R. Absence of either Cck or Cck1R in ATXN1[30Q]D776 mice resulted in a PC disease in which pathology now progressed to cell death.

Importantly, Cck<sup>−/−</sup> and Cck1R<sup>−/−</sup> mice also have a motor deficit, but only subtle molecular changes in PC health and no overt cerebellar pathology. One possibility is that the significant motor deficits in Cck<sup>−/−</sup> mice is largely due to contributions from extra-cerebellar areas requiring Cck activity. The interpretation of rodent behavioral tests for motor coordination is inherently complicated. Other factors mediated by extra-cerebellar brain regions undoubtedly contribute to motor performance, including anxiety, stress, body weight, and motivation. Anxiety is unlikely to contribute to the motor deficit, as two tests of anxiety, the elevated plus maze, and stress-induced hyperthermia (a non-motor test of anxiety), showed no difference in behavior at 36 weeks of age between wt and Cck<sup>−/−</sup> mice (Appendix Figure 1). Additionally, the Cck1R<sup>−/−</sup> mice, but not the Cck2R<sup>−/−</sup> mice have motor deficits. Given the widespread expression of the Cck2R in the central nervous system, and the limited expression of brain Cck1R, it seems likely that any non-cerebellar contributions to a deficit in motor behavior would be impacted as much by the Cck2R. Finally, a significant decrease in Calb1 mRNA, and a slight but significant decrease in ML thickness with age in Cck<sup>−/−</sup> mice does suggest mild PC dysfunction. Indeed, a decrease in Calb1 is correlated with poor PC health (Clark and Orr, 2000).

Taken together, these data suggest a role for cerebellar Cck in maintaining PC function in the adult mouse.
The natural ligand with the highest affinity for the Cck1R is Cck-8, an octapeptide cleaved from the C-terminal portion of Cck (Dufresne et al., 2006). While the precise localization of Cck1R protein in the cerebellum has not been convincingly shown, in situ hybridization data (Figure 3A) from the Allen Brain Atlas suggests that it is expression primarily in the PCs. Thus, these results strongly support a model of where Cck is elevated, cleaved into an active peptide, and binds to PC Cck1R, potentially in an autocrine manner. This model may underlie the lack of progressive pathology in \textit{ATXN1[30Q]D776} mice, and may thus constitute an attractive therapeutic mechanism of disease for a number of reasons. First, the body of literature covered in the discussion in Chapter 2 highlights the findings that \textit{Cck} is upregulated in specific cell types under specific forms of neuronal assault, and may be protective in those instances. Second, a similar neuroprotective role for another neuropeptide, NPY, was found in mouse models of SCA3/MJD was recently reported by Duarte-Neves et al. 2015. Finally, there are considerable \textit{in vivo} data, including in humans, on the use of Cck1R agonists, peptides, peptoids, and small molecules, as satiety agents for the treatment of obesity disease with minor side effects, depending on the drug and dosage (Asin et al., 1992; Cannon et al., 1996; Jordan et al., 2008; Wang et al., 2011). Taken together, these data suggest that manipulation of the Cck-Cck1R pathway may be of interest as a therapeutic against neuronal death in the cerebellum.
Chapter 4: RNA-seq reveals potential downstream pathways of Cck-Cck1R activity
4.1 RNA-seq identifies Cck-specific gene changes in ATXN1[30Q]D776 mice

In Ingram and Wozniak et al. (2016), upregulation of Cck was identified in ATXN1[30Q]D776 mice, while it was downregulated in ATXN1[82Q] mice. In Chapter 3, the findings that this upregulation protects against disease progression in these mice was discussed. In order to understand downstream targets of Cck, RNA-seq was performed on whole cerebellar RNA samples from ATXN1[30Q]D776;Cck⁻/⁻ and Cck⁻/⁻ mice at 5, 12, and 28 weeks of age. To maintain consistency with the earlier RNA-seq runs of wt, ATXN1[82Q] and ATXN1[30Q]D776 mice, the samples from ATXN1[30Q]D776;Cck⁻/⁻ and Cck⁻/⁻ mice were processed and run in the same manner (see Methods). In brief, samples were sent to the University of Minnesota Biomedical Genomics Center for library creation and Illumina GAIIx sequencing. For quality control, each sample was required to have an RNA integrity number greater than 8. Using three biological replicates per genotype and per age, a total of 1.17 billion paired-end reads were generated for this experiment, with a minimum of 42.7 million reads per replicate (Table 1).
Table 1. Whole cerebellar samples used in RNA-seq experiments.

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<th># Reads Mapped</th>
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Initial read quality was determined using FastQC (Andrews). Reads were processed to remove adapter sequences and trimmed. Following data quality control and prepping, the samples were mapped to the UCSC mm9 mouse annotated genome (iGenomes, Illumina). Expression analysis was completed using the Tophat, CuffDiff pipeline (Langmead et al., 2010; Trapnell et al., 2009, 2010) on the Galaxy interface (Blankenberg et al., 2010; Giardine et al., 2005; Goecks et al., 2010). Alignment was completed using default parameters except for using a Std. Dev of Distance between
Mate Pairs of 60 and a gene annotation model only looking for supplied junctions. Greater than 70% read pairs correctly mapped following these parameters. Differential gene expression was determined using CuffDiff with default parameters.

Differential gene expression was completed in pairwise comparisons: wt vs. \textit{ATXN1[82Q]}, \textit{ATXN1[30Q]D776}, \textit{ATXN1[30Q]D776;Cck}^- or \textit{Cck}^- . Changes across time for each genotype was also examined. As shown in Figure 6, \textit{ATXN1[30Q]D776;Cck}^- mice have progressive pathology over a longer timescale than \textit{ATXN1[82Q]} mice. Therefore it wasn’t surprising to see that there are comparatively fewer gene changes in \textit{ATXN1[30Q]D776;Cck}^- mice from 5 to 12 weeks than from 5 to 28 weeks (Figure 7). Interestingly, the majority of those genes changed from 5 to 28 weeks are downregulated, similar to that seen in \textit{ATXN1[82Q]} mice (Ingram and Wozniak et al., 2016).

\textbf{Figure 7. Gene changes detected by RNA-seq.} Comparison of CuffDiff gene changes in \textit{ATXN1[30Q]D776;Cck}^- samples over time. Also includes the number of up- or down-regulated genes changed between \textit{ATXN1[30Q]D776} and \textit{ATXN1[30Q]D776;Cck}^- samples at 28 weeks.
Comparing \textit{ATXN1[30Q]D776} and \textit{ATXN1[30Q]D776;Cck\textsuperscript{−/−}} samples identified genes changed due to upregulated Cck, directly or indirectly. At 28 weeks, about 50\% of genes differentially expressed between \textit{ATXN1[30Q]D776} and \textit{ATXN1[30Q]D776;Cck\textsuperscript{−/−}} are upregulated, and 50\% downregulated (Figure 7). These upregulated and downregulated genes sets were run through the Ingenuity Pathway Analysis software (IPA, Quiagen) to look for common pathways that may be influenced by upregulated Cck at 28 weeks. Pathways significantly overlapping with the upregulated genes were involved in two canonical immune response pathways: Dendritic Cell Maturation (B-H Multiple testing correction, \(p=1.01\times10^{-9}\)) and the Complement System (\(p=2.7\times10^{-8}\)). Not surprisingly based on the body of available research, the most significant disease pathways influenced by genes upregulated in the 28 week \textit{ATXN1[30Q]D776} vs. \textit{ATXN1[30Q]D776;Cck\textsuperscript{−/−}} dataset were Cancer (\(p=6.08\times10^{-44}\)) and Gastrointestinal Disease (\(p=1.38\times10^{-32}\)).

The downregulated genes fell into canonical pathways heavily influenced by the decades of literature surrounding Cck’s role in neural development (Axonal Guidance Signaling, \(p=2.28\times10^{-6}\)), and in the gut (Epithelial Adherens Junction Signaling, \(p=2.14\times10^{-5}\)). The top Disease pathway influenced by these downregulated genes, however, was Neurological Disease, specifically Movement Disorders (\(p=4.81\times10^{-23}\)). The IPA software predicted an “increased activation” for the movement disorder pathway (Z-score=4.859), indicating that, according to the literature, the downregulation of these genes has been implicated in pathogenesis of movement disorders. A full list of the movement disorder-related genes and their differential expression can be found in Appendix 1.
4.2 Weighted Gene Coexpression Network Analysis (WGCNA) reveals mechanistic similarities in disease-related gene expression between \textit{ATXN1[82Q]} and \textit{ATXN1[30Q]D776;Cck\textsuperscript{-/-}} mice.

In Ingram and Wozniak et al., (2016), a Weighted Gene Coexpression Network Analysis (WGCNA) was applied to the RNA-seq data from \textit{ATXN1[82Q]}, \textit{ATXN1[30Q]D776} and wt mice at 5, 12, and 28 weeks (Presson et al., 2008; Langfelder and Horvath, 2008) in order to gain insight into gene expression networks. This allowed identification of gene sets whose expression significantly correlated with one another across the three ages. In this paper, nineteen modules were detected. In order to determine which of these modules correlated with disease, \textit{ATXN1[82Q]} and \textit{ATXN1[30Q]D776} genotypes at all ages were classified as “ataxic” and wt animals at all ages were classified as “healthy”. Correlation of the modules with the “ataxic” classification was performed with a t-test (Bonferroni corrected for a p-value <1e-5). Two modules significantly correlated with the ataxic phenotype, Magenta and Lt Yellow. Further analysis determined that the genes in the Magenta Module represented primary downstream targets of pathogenic ATXN1 in PCs, while the Lt Yellow Module represented secondary gene targets, likely in other cell types.
Table 2. Depiction of the samples included in three separate WGCNAs that were performed on the RNA-seq data. Green indicates samples included in each test.

<table>
<thead>
<tr>
<th>WGCNA</th>
<th>Wt</th>
<th>ATXN1[82Q]</th>
<th>ATXN1[30Q]D776</th>
<th>ATXN1[30Q]D776;Cck/−</th>
<th>Cck/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingram and Wozniak et al., 2016</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>WGCNA including ATXN1[82Q]</td>
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<tr>
<td>WGCNA excluding ATXN1[82Q]</td>
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</table>

To identify downstream targets of upregulated Cck in the ATXN1[30Q]D776 mice, the WGCNA was repeated using two strategies (Table 2). The first strategy analyzed gene expression changes from all genotypes at all ages, including Cck/−, and ATXN1[30Q]D776;Cck/− mice at 5, 12, and 28 weeks. Twenty-two modules were identified. As in Ingram and Wozniak et al., correlation of all identified modules with disease was performed using an “ataxic” or “healthy” disease classification, this time including all ATXN1[82Q], ATXN1[30Q]D776, and ATXN1[30Q]D776;Cck/− samples at all ages as “ataxic” and all wt and Cck/− samples as “healthy”. Unlike in Ingram and Wozniak et al., no modules were identified (Figure 8). This was potentially due to stark differences in timing of disease progression between ATXN1[82Q] and ATXN1[30Q]D776;Cck/− mice (Figure 6). ATXN1[82Q] mice have significant pathological changes by 12 weeks, but ATXN1[30Q]D776;Cck/− mice don’t have changes in pathology until after 28 wks. Therefore, the “ataxic” or “healthy” classification would not take into account disease severity, or these differences in progressive timeline between these two progressive mouse models.
Figure 8. WGCNA correlation with disease. WGCNA including all cerebellar samples does not correspond with a classification of “ataxic” or “healthy. Fischer’s Exact test.

Therefore, ML thickness for each genotype at each age was used as a more accurate representation of disease state. With this new analysis, one module significantly correlated with ML thickness, the Pink Module (Fisher’s exact test, p=9e-18, 373 genes).

While this new WGCNA analysis identified a gene module significantly correlated with a measure of cerebellar disease, there was a chance that the ATXN1[82Q] samples were heavily weighting the data towards identification of these genes, and that the contribution from ATXN1[30Q]D776;Cck−/− mice was minimal. The second strategy, therefore, was to repeat this WGCNA, now excluding all ATXN1[82Q] samples at all ages from the analysis. Thirty-six modules were detected with one, again named the Pink Module, significantly correlating with disease (Figure 9A, Fisher’s exact test, p=7e-13, 366 genes).
Figure 9. A) WGCNA including all samples reveals one gene module (Pink) that correlates significantly with ML thickness across all ages. B) Two WGCNA tests run: including all samples, or excluding only ATXN1[82Q] samples reveals similar gene module groupings. Importantly, the two pink modules correlating with disease significantly overlap with 229 genes. Fischer’s Exact test.
To test the extent to which inclusion or exclusion of the ATXN1[82Q] samples influenced the module compositions, the two module sets were examined for overlap. A total of 8,242 genes overlapped between the two WGCNA sets (Figure 9B). The two identified Pink Modules significantly overlapped (Fischer’s exact test, p=2.9e-244), with 229 genes. The similarity of these two modules obtained both in the presence and absence of ATXN1[82Q] indicates that the Pink Module indeed represents disease-related gene changes that occur in ATXN1[30Q]D776;Cck⁻ mice, and was not solely representing changes in ATXN1[82Q] mice.

To determine the contribution of the ATXN1[30Q]D776;Cck⁻ samples to disease-related gene changes, both the new WGCNA excluding the ATXN1[82Q] and the WGCNA including ATXN1[82Q] were compared to the Ingram and Wozniak et al. WGCNA (Figures 10 and 11, respectively). Figure 10 shows the overlap between the WGCNA excluding ATXN1[82Q], however both this and the analysis including the ATXN1[82Q] samples (Figure 11) produced similar results. In both new WGCNAs, the two disease-related modules, Pink and Magenta, significantly overlapped. 122 genes overlapped in the WGCNA excluding ATXN1[82Q], and 166 overlapped in the WGCNA including ATXN1[82Q] (Appendix Table 2; Fischer’s exact test, p=1.4e-103).
Figure 10. WGCNA excluding \textit{ATXN1[82Q]} samples. Correspondence of WGCNA modules from Ingram and Wozniak et al., (2016) and the WGCNA modules excluding \textit{ATXN1[82Q]} samples. The two disease models- Pink and Magenta (Ingram and Wozniak et al., 2016), significantly overlap with 122 genes.
Figure 11. **WGCNA including all samples.** Correspondence of WGCNA modules from Ingram and Wozniak et al., (2016) and the WGCNA modules including all samples. The two disease modules significantly overlap with 166 genes.

Figure 12A shows the Eigengene expression for the Pink Module, including \textit{ATXN1[82Q]} samples. Wt/FVB and \textit{Cck}⁻ gene expression remains the same from 5 to 28 weeks. While \textit{ATXN1[30Q]D776} expression starts out lower than wt expression, it also doesn’t change from 5 to 28 weeks. \textit{ATXN1[82Q]} gene expression of the Pink Module decreases substantially from 5 to 28 weeks. These findings replicate what was found in Ingram and Wozniak et al., for the Magenta Module, with a majority of the \textit{ATXN1[82Q]} genes decreasing in expression from 5 to 28 weeks. Interestingly, the gene expression for \textit{ATXN1[30Q]D776;Cck}⁻ mice starts out low at 5 and 12 weeks similar to \textit{ATXN1[30Q]D776}, however by 28 weeks the expression drops to the level of \textit{ATXN1[82Q]} samples. This change in pattern of expression with time supports the idea
that these genes represent genetic changes correlated with progressive disease pathways. Figure 12B shows that the majority of genes in the Pink Module are downregulated in both ATXN1[82Q] mice and ATXN1[30Q]D776;Cck<sup>−/−</sup> mice.

**Figure 12. Expression of the genes included in the Pink Module.** A) Eigengene expression reveals similar genes expression changes in ATXN1[82Q] and ATXN1[30Q]D776;Cck<sup>−/−</sup> samples across time. B) Percent of Pink Module genes up- or down-regulated in ATXN1[82Q] or ATXN1[30Q]D776;Cck<sup>−/−</sup> samples.
4.3 Discussion

In Chapter 3, the finding that \textit{ATXN1[30Q]D776;Cck}\textsuperscript{−/−} mice had disease progression similar to that seen in \textit{ATXN1[82Q]} mice was discussed. In this Chapter, RNA-seq was used to examine the gene expression changes that occur in these two progressive disease mouse models. Analysis of the gene changes in \textit{ATXN1[30Q]D776;Cck}\textsuperscript{−/−} mice revealed the greatest number of changes between 12 and 28 weeks of age. Differential expression of genes between \textit{ATXN1[30Q]D776} and \textit{ATXN1[30Q]D776;Cck}\textsuperscript{−/−} mice at 28 weeks revealed upregulated genes involved with inflammatory signaling, and downregulated genes implicated in movement disorders. WGCNA was used as an unbiased, statistically rigorous test to determine which gene changes correlated with one another. Two WGCNAs were run: one including every sample, and one excluding all \textit{ATXN1[82Q]} samples (Table 2). Both analyses identified the Pink Module, which significantly correlates with disease and significantly overlaps with the disease-related Magenta Module (Ingram and Wozniak et al., 2016).

One strategy for disseminating information from gene lists is to perform pathway analysis. This is a crude way to determine whether genes in a specific set correlate with predetermined gene lists created and curated by combing the literature for relationships between different molecules. Any pathways identified from a particular gene list are heavily influenced by pre-existing literature, and so it is not surprising that, when genes from \textit{Cck}\textsuperscript{−/−} or \textit{ATXN1[30Q]D776;Cck}\textsuperscript{−/−} gene sets are run through pathway analysis software, the primary pathways identified are those involved in neuronal development, gastrointestinal function, and cancer. There is a large body of literature surrounding the role of Cck and Cck receptors in these areas of study, and so the pathway analysis results were not surprising.
As an attempt at identifying pathways relevant to this study, only the genes upregulated or the genes downregulated between ATXN1[30Q]D776 and ATXN1[30Q]D776;Cck−/− were run separately through pathway analysis. Interestingly, the upregulated genes fell into pathways involved in inflammatory signaling and immune response. It is possible that this signaling is a secondary effect of PC degeneration that is occurring at this age in ATXN1[30Q]D776;Cck−/−, but it is also possible that the upregulated Cck is actively suppressing a potentially harmful immune response in the ATXN1[30Q]D776 mice. A limited number of studies have suggested that the Cck-Cck1R pathway can inhibit inflammatory signaling both peripherally and centrally (Miyamoto et al., 2012; Zhang et al., 2011; Luyer et al., 2005; Cong et al., 2003). Indeed, changes in inflammatory signaling and glia in PC dysfunction and death has been observed in SCA1 mice (Cvetanovic et al., 2015).

The genes that were downregulated in ATXN1[30Q]D776;Cck−/− mice compared to ATXN1[30Q]D776 mice belonged to predictable canonical pathways involved in gastrointestinal function and neuronal development. The identification of these categories is likely heavily influenced by the large body of previously existing literature for Cck function in the gastrointestinal and developing systems. Interestingly, the most significant Disease category was Neurological Disease, and more specifically Movement Disorders. This category includes ataxia (mouse or human)-related genes (Cntnap1: Bhat et al., 2001; ATP1A3: Ikeda et al., 2013), previously identified human SCA-related genes (ITPR1, Foskett, 2010), and genes implicated in other neurodegenerative disorders with a movement component, including Parkinson’s Disease (PLA2G6: Shinzawa et al., 2008) and Huntington’s Disease (Calb1: Hodges et al., 2006). This indicates that at least a subset of the genes differentially expressed in ATXN1[30Q]D776 mice in the presence or absence of upregulated Cck have been previously implicated in neurodegenerative disorders.
Findings discussed in Chapter 3 reveal pathological and behavioral similarities between $ATXN1[82Q]$ and $ATXN1[30Q]D776;Cck^{-/-}$ mice. This RNA-seq experiment was used to identify disease progression-related genes in these two mouse models, and to use comparisons between $ATXN1[30Q]D776$ and $ATXN1[30Q]D776;Cck^{-/-}$ mice to identify Cck’s role in eliciting these changes. While the pathway analysis potentially identified a subset of genes regulated by Cck involved in neurodegenerative disease, this is an extremely biased method of analyzing genetic data. To remove the bias, WGCNA was used to identify sets of disease progression-related genes, and, more specifically, determine whether the protective effects of upregulated Cck discussed in Chapter 3 were caused by changes in downstream targets.

In order to remove any bias that could occur by inclusion of the $ATXN1[82Q]$ samples, the WGCNA was run twice: both with and without $ATXN1[82Q]$. This did change the composition and the number of modules detected. For the purposes of this study, the disease-related modules were identified, however the further categorization of the rest of the modules and the influence of $ATXN1[82Q]$ may be a topic of future inquiry.

While the first WGCNA in Ingram and Wozniak et al. used a bimodal disease classification of “ataxic” or “healthy” and accurately identified a module of disease-related genes, this approach was limited in that it does not take into account severity of disease or disease progression. This is especially important in this study, as there are two disease-progressive mouse models that differ in their timing of disease progression. In this analysis, ML thickness was chosen because it is a pathological measure that has been shown to reliably reflect disease state across the three chosen ages, and reflects subtle changes in disease progression better than motor behavior. This resulted in a robust correlation of a single disease-related module, whose eigengene expression suggested progressive downregulation of its included genes in $ATXN1[82Q]$ and
ATXN1[30Q]D776;Cck−/− mice. Importantly, the Pink Module was correlated with disease in both WGCNAs, indicating that this is a robust finding that is not heavily weighted by changed in ATXN1[82Q] samples.

It is interesting to note that no significant canonical pathways emerge from running a pathway analysis on either the Magenta or the Pink gene clusters. This could be due to a lack of literature surrounding the identified genes that could be used to connect them. Alternatively, this could be an indication that this disease-related set of genes doesn’t fall under any single pathway, and instead represents many different pathways that are critical to PC health.

The observation that a core set of disease-related genes consistently emerges as the Pink Module both with and without inclusion of the ATXN1[82Q] samples suggests that these genes are related to progression in ATXN1[30Q]D776;Cck−/− mice. Taking together the observation that the ATXN1[30Q]D776 mice do not have a progressive change in the expression of the genes in the Pink Module and that Chapter 3 showed involvement of the Cck1R and not the Cck2R, it is reasonable to conclude that the elevation of Cck, acting through the Cck1R, is influencing the levels of these important disease-related genes in order to exert its protective effects. As discussed in Chapter 2, the ultimate downstream consequence of Cck1R activation is changes in gene expression. The results discussed here suggest that modulation of the Cck-Cck1R pathway may upregulate these core disease-related genes, and thereby could protect against disease progression.
Chapter 5: Activation of the Cck1R protects against disease progression in two SCA1 mouse models
5.1 The Cck1R agonist A71623 protects against disease progression in 
\textit{ATXN1[30Q]D776;Cck}\textsuperscript{-/-} mice.

The RNA-seq results suggest that upregulated \textit{Cck} in \textit{ATXN1[30Q]D776} mice is 
activating a subset of genes whose decreased expression contributes to progressive PC 
pathology in both \textit{ATXN1[82Q]} and \textit{ATXN1[30Q]D776;Cck}\textsuperscript{-/-} mice. In Chapter 3, it was 
revealed that the upregulated Cck is acting through the Cck1R. Therefore, a 
commercially available Cck1R agonist, A71623 (Tocris), was chosen to test whether 
modulation of the Cck1R is protective against progressive disease. A71623 is a modified 
version of the Cck tetrapeptide, and has a 1,200-fold selectivity for the Cck1R over the 
Cck2R (Asin et al., 1992; Lin et al., 1990). A71623 can be delivered intraperitoneally 
(i.p.) and cross the blood brain barrier to elicit behavioral changes in rodents and pigs 
(Asin et al., 1992).

This is the first study to administer A71623, or any Cck1R agonist, continuously 
over a long-term experiment, and few studies have suggested, but not directly tested, its 
ability to cross the blood brain barrier. Therefore, a preliminary experiment was first 
performed to determine whether peripheral administration of A71623 could elicit 
changes in the cerebellum. A known downstream target of Cck1R activation is increased 
phosphorylation of ERK1/2 (P-ERK1/2, or MAPK1/2) in the central nervous system 
(Campos et al., 2012; Sutton et al., 2004). While peripheral administration of A71623 
has previously been shown to influence centrally-mediated behaviors (Asin et al., 1992), 
cerebellar effects of A71623 administration or changes in cerebellar P-ERK1/2 had not 
been determined. A bolus injection of either a low (0.02 mg/kg) or a high (1 mg/kg) dose 
of A71623 was administered i.p. The 0.02 mg/kg (low) dose of A71623 was able to 
increase the ratio of P-ERK1 to total ERK1 as early as 6 hours (data not shown) and up 
to 24 hours (Fig 13A, B; One-Way ANOVA with Tukey post-hoc, p=0.0454 compared to
vehicle). Similarly, the 1 mg/kg (high) dose was able to increase the ratio of P-ERK1 to total ERK1 (p=0.0046 compared to untreated) to a greater extent than the lower dose, although the difference between the high and low dose was not significant (p=0.0615). This initial experiment showed that A71623, even in a low dose, was able to cross the blood brain barrier to elicit changes in the cerebellum in a dose-dependent manner. In similar experiments, 0.02 mg/kg of A71623 was able to elicit behavioral changes in rats and mice, specifically inhibition of food intake (Asin et al., 1992). For the following experiments, 0.02 mg/kg (31 nmol/kg) of A71623 is used to determine whether activation of the Cck1R can protect against disease.

![Figure 13. Cerebellar Phospho-ERK1/2 is increased 24 hours after bolus injection of Cck1R agonist A71623. A) Western blot image of phosphor-ERK1/2 (P-ERK) and total ERK1/2. Two doses were administered i.p. and the cerebellum was collected 24 hours post-injection for analysis. B) Quantification of (A). N=2 per group. One-Way ANOVA, tukey post-hoc test, *p<0.05.](image-url)
First, ATXN1[30Q]D776;Cck−/− mice were treated with A71623 (0.02 mg/kg/day). In designing this experiment, it was first important to consider the timeline of progressive pathology that occurs in these mice. Figure 14A shows that a significant decrease in ML thickness in ATXN1[30Q]D776;Cck−/− mice doesn’t occur until after 28 weeks of age. The RNA-seq findings discussed in Chapter 4 support the idea that major disease-related genetic changes in these mice occurs between 12 and 28 weeks. In order to reliably measure changes in pathology, these mice needed to be aged until after measurable changes occur. Thus, the treatment paradigm described in Figure 14B was used.
Figure 14. Changes in ML thickness over time. To identify appropriate treatment paradigms, changes in cerebellar pathology over time were quantified using ML thickness. \textit{ATXN1[82Q]} ML thickness decreases dramatically from 6 to 12 weeks, whereas \textit{ATXN1[30Q]D776;Cck\textsuperscript{-/-}} ML thickness decreases from 28 to 36 weeks.

In brief, mice underwent baseline motor behavior testing, which takes one week per test. Baseline balance beam test was performed at 5 weeks of age, then baseline rotarod behavior was performed at 6 weeks of age. At the conclusion of the rotarod test, an osmotic minipump (Alzet, model 1004), was implanted i.p. to continuously administer either A71623 at 0.02 mg/kg/day or vehicle (20mM PBS). Tests of motor behavior were
repeated at 11 to 12, 17 to 18, and 35 to 36 weeks of age. Pumps were replaced three
times during the lifespan of the mice, and the mice were sacrificed for pathology at the
completion 36 week rotarod test.

To confirm activation of Cck1R in the cerebellum, P-ERK1 expression was
assessed as in the preliminary experiments. As in the bolus injection experiment, the
ratio of cerebellar P-ERK1:total ERK1 in the long-term administration study was elevated
in A71623-treated mice compared to vehicle-treated mice (Figure 15).

Figure 15. P-ERK1 is increased in A71623-treated mice compared to Vehicle-
treated mice. A) Western blot for P-ERK1/2 and total ERK1/2. B) Densitometric analysis
of the western blot in (A). Student’s t-test, ***p=0.00038.

The vehicle-treated ATXN1[30Q]D776;Cck−/− mice performed worse on the motor
behavior tasks than the A71623 mice, beginning at 18 weeks. The balance beam test
involves three days of training for the mice to learn how to cross a medium-sized, square
beam. On test day, the mice cross six different balance beams of increasing difficulty.
Data shown in Figure 16A and B is from the most difficult (10mm diameter, round) beam
on test day. The balance beam has two measures of motor coordination: footslips and
time to cross the beam. An interesting finding is the change in motor ability observed
over time. On this test, the vehicle-treated ATXN1[30Q]D776;Cck−/− mice performed
significantly worse beginning at 11 weeks than they had at 5 weeks on both time to
cross (Two-Way ANOVA with Bonferroni post-hoc, p=0.0246) and footslips (p=0.0060).
The A71623-treated ATXN1[30Q]D776;Cck^ mice, however, did not have a change in motor performance from the baseline behavioral tests (time to cross, p=0.9703; footslips, p=0.9307). Additionally, the A71623-treated mice performed significantly better on both measures of motor behavior from the balance beam test than the vehicle-treated mice, beginning at 18 weeks (time to cross, p=0.0.0105; footslips, p=0.0337), or in other words after 10 weeks of treatment.
Figure 16. Cck1R agonist A71623 treatment in ATXN1[30Q]D776;Cck−/− mice. A) Treatment paradigm. Mice are tested for motor performance using two tests. Then either 0.02mg/kg/Day A71623 or vehicle (20mM PBS) is given until 36 weeks of age, at which time mice are sacrificed for pathology. The osmotic minipumps are replaced every 6 weeks. B) Number of footslips on the smallest balance beam, 10mm round. C) Time to cross the 10mm round balance beam. D) Latency to fall on the Rotarod. Two-Way ANOVA with tukey post-hoc test. #p<0.05 compared to 5 weeks. *p<0.05, **p<0.01, ***p<0.001 compared to vehicle-treated mice.
The Rotarod test is a widely-accepted test of motor coordination where mice are placed on an accelerating rotating rod and their latency to fall off the rod is recorded. Similar to the findings from the balance beam test, the vehicle-treated mice performed significantly worse with time (Figure 16C). The A71623-treated mice did perform significantly worse with age (Two-Way ANOVA with Sidak post-hoc, p<0.0206), however they still performed better than vehicle-treated mice, beginning at 12 weeks (p=0.0012).

The observation that the A71623-treated mice performed better across these two tests of motor behavior than the vehicle-treated mice indicates that the agonist was able to protect against progressive motor deficits in $ATXN1[30Q]D776;Cck^{-/-}$ mice. However, these tests are not conclusive, as tests of motor behavior are influenced by many extra-cerebellar areas. Therefore histology was performed to examine the effect of A71623 on cerebellar cells.
Figure 17. Pathology analysis of A71623 or vehicle-treated ATXN1[30Q]D776;Cck−/− mice. A) ML thickness in 12 week old untreated mice compared to 36 week old untreated and treated mice. B) Number of PCs per 250 um of cerebellar primary fissure in 12 week old untreated mice compared to 36 week old untreated and treated mice. Two-Way ANOVA, tukey post-hoc test, **p<0.01, ***p<0.001 compared to A71623 treatment. #p<0.05 compared to 12 week old measurements.

The morphology of vehicle- or A71623-treated ATXN1[30Q]D776;Cck−/− mouse cerebellum was next examined. Two measures of pathology, ML thickness and PC counts per 250um, are shown in Figure 17A and B, respectively. At 12 weeks of age untreated ATXN1[30Q]D776;Cck−/− mice are not significantly different from ATXN1[30Q] mice. At 36 weeks, there is no change in ML thickness or PC counts for
ATXN1[30Q]D776 mice. Vehicle-treated ATXN1[30Q]D776;Cck<sup>−/−</sup> mice, however, have a significant decrease in ML thickness (Two-Way ANOVA with Bonferonni post-hoc, p<0.001), and significant PC loss (Two-Way ANOVA with Bonferonni post-hoc, p<0.01) by 36 weeks. A71623-treated ATXN1[30Q]D776;Cck<sup>−/−</sup> mice, in contrast, do not have any change in ML thickness by 36 weeks and do not have any PC loss. Indeed, they have a significantly thicker ML than vehicle-treated ATXN1[30Q]D776;Cck<sup>−/−</sup> mice (p<0.001), and significantly more PCs (p<0.01). These data indicate that A71623 treatment was able to protect against progressive neurodegeneration in ATXN1[30Q]D776;Cck<sup>−/−</sup> mice.

Both the behavioral tests and the histopathology in the A71623- treated ATXN1[30Q]D776;Cck<sup>−/−</sup> mice confirms that A71623 was able to protect against progressive cerebellar dysfunction and atrophy. Particularly exciting is the finding that A71623 protects against PC death, indicating that Cck1R agonist may be a viable therapeutic target for PC degeneration.

5.2 A71623 treatment protects against progressive disease in ATXN1[82Q] mice.

While the ATXN1[30Q]D776;Cck<sup>−/−</sup> mice represent one progressive SCA1 mouse model, they are genetically not as representative of SCA1 PC degeneration and death as the ATXN1[82Q] mice. Because the RNA-seq data revealed overlapping genetic changes between ATXN1[30Q]D776;Cck<sup>−/−</sup> and ATXN1[82Q] mice, and because ATXN1[82Q] mice more faithfully represent the PC pathology seen in human SCA1 patients, the ATXN1[82Q] mice were next treated with A71623.

A similar treatment protocol was used to treat ATXN1[82Q] mice with A71623 (Figure 16A). Because the disease progression in these mice occurs on a more rapid timescale than in ATXN1[30Q]D776;Cck<sup>−/−</sup> mice (Figure 14A), the baseline motor behavior tests were performed earlier, at 3 to 4 weeks of age. At 4 weeks of age the
*ATXN1*82Q* mice were too small for pump implantation, so they were given daily i.p. injections (0.02 mg/kg) until 6 weeks of age. The osmotic minipumps were then implanted to continuously administer either A71623 (0.02 mg/kg/day) or vehicle (20mM PBS). At 11-12 weeks the behavior tests were repeated, and the animals sacrificed for pathology.
Figure 18. Cck1R agonist A71623 treatment in ATXN1[82Q] mice. A) Treatment paradigm. Mice are tested for motor performance using two tests. Then either 0.02mg/kg/Day A71623 or vehicle (20mM PBS) is given until 12 weeks of age, at which time mice are sacrificed for pathology. B) Number of footslips on the smallest balance beam, 10mm round. C) Time to cross the 10mm round balance beam. D) Latency to fall on the Rotarod. Two-Way ANOVA with tukey post-hoc test. #p<0.05 compared to 5 weeks. *p<0.05, **p<0.01, ***p<0.001 compared to vehicle-treated mice. E) ML thickness of 6 week old untreated mice compared to 12 week old treated mice. One-Way ANOVA, tukey post-hoc test, ***p<0.001.
At 11 weeks of age, the vehicle-treated \textit{ATXN1[82Q]} mice took longer to cross the balance beam (Figure 18B; Two-Way ANOVA with Tukey post-hoc test, \(p=0.0008\)), and had more footslips than they had at 3 weeks (Figure 18C; \(p=0.0002\)). Similarly, the vehicle-treated mice performed worse on Day 4 of the rotarod at 12 weeks than they had at 3 weeks (Figure 18D; Two-Way ANOVA with Tukey post-hoc, \(p=0.0005\)). In contrast, mice treated with A71623 had no significant change in time to cross (\(p=0.8875\)) or number of foot slips (\(p=0.702\)) on the balance beam, and no significant change in rotarod performance on Day 4 from 4 to 12 weeks of age (\(p=0.9984\)). The A71623-treated mice additionally performed better at 11-12 weeks than the vehicle-treated mice on all motor behavior measures, including time to cross (\(p=0.0003\)) and number of footslips on the balance beam (\(p=0.0013\)), and latency to fall on Day 4 of the Rotarod test (\(p=0.0263\)). These behavioral outcomes indicate that A71623 treatment is able to protect against progressive motor behavior deficits.

ML thickness and PC counts were measured. As has been previously reported, there were no differences in PC counts in \textit{ATXN1[82Q]} mice at 6 or 12 weeks of age, regardless of treatment (data not shown). The vehicle-treated mice at 12 weeks of age had significantly smaller ML thicknesses than 6 week old untreated \textit{ATXN1[82Q]} mice (Figure 18E; Two-way ANOVA with Tukey post-hoc, \(p=0.0001\)), whereas A71623-treated mice had no change in ML thickness by 12 weeks (\(p=0.2114\)). A71623-treated mice at 12 weeks of age have significantly thicker ML than vehicle-treated mice (\(p=0.002\)). These pathological findings indicate that a low dose of A71623 is able to prevent progressive PC degeneration.
5.3 Discussion

These data indicated that the Cck1R agonist A71623 was able to prevent progressive motor behavior deficits and progressive PC atrophy in ATXN1[30Q]D776;Cck<sup>−/−</sup> and ATXN1[82Q] mice. ATXN1[30Q]D776;Cck<sup>−/−</sup> mice have a slow progressive timeline compared to ATXN1[82Q] mice. Therefore the ATXN1[30Q]D776;Cck<sup>−/−</sup> mice were treated with either A71623 (0.02 mg/kg/Day) or vehicle (20mM PBS) from 6 to 36 weeks of age. ATXN1[82Q] mice have a faster disease progression, and the most significant change in PC pathology is seen as early as 12 weeks. Therefore, these mice were given A71623 or vehicle from 4 to 12 weeks of age. In both progressive mouse models, A71623 was able to significantly prevent disease progression, as evidenced by no worsening of motor behavior performance, and no progressive PC degeneration.

Careful evaluations of A71623 pharmacodynamics have not been performed. Therefore, choosing a dose that both crosses the blood brain barrier and elicits changes in the brain was challenging. The 0.0264mg/kg/Day dosage was chosen as the low end of potentially efficacious dosages for two reasons. The first is that this is the lowest dose that has been shown to inhibit food intake after a single, bolus i.p. injection (Asin et al., 1992). Inhibition of food intake is the most well-studied behavioral consequence of Cck1R activation that is known to be, at least in part, centrally-mediated (Bellissimo and Anderson et al., 2003; Reidelberger et al., 2003; Lo et al., 2014). The second, more practical reason, is that it is 5-fold less than the maximum solubility of A71623 (1mg/kg in 20nM PBS). Currently in progress is a treatment study using the dose of 1mg/kg/Day to determine whether the protective effects of A71623 will be even more pronounced with a much higher dose. Most interesting will be to determine if a higher dose, or perhaps a more direct route of administration such as intracerebroventricular injection, will restore motor behavior and PC pathology rather than just preventing progression.
It was interesting that activation of the Cck1R with A71623 appears to protect against the progressive aspects of the disease, but doesn’t improve motor behavior or pathological measures to wt levels. As noted in Chapter 2, $ATXN1[30Q]D776;Cck^{-/-}$ mice have a progressive, degenerative disease compared to $ATXN1[30Q]D776$ mice. However, the $ATXN1[30Q]D776$ mice have a smaller ML thickness, and impaired motor behavior compared to wt mice, beginning from 6 weeks. It is therefore worth noting that elevated $Cck$ in these mice does not appear to restore cerebellar health to the level of wt mice, and does not protect against some degeneration. One possibility is that this is due to timing of expression of the Cck peptide. While $Cck$ mRNA is elevated at the same time as $ATXN1$ in the $AXTN1[30Q]D776$ (Figure 3C), the Cck prohormone still needs to be cleaved into its active forms capable of binding the Cck1R. It is possible that, while $Cck$ and the Cck1R is elevated at the correct time to immediately exert its protective effects, the molecules needed to process Cck in the cerebellum may not turn on until later, thus allowing mutant $ATXN1$ to cause damage to PCs at an early age. Another possibility is that the elevated Cck in the $ATXN1[30Q]D776$ mice is not at a level high enough to fully activate available Cck1Rs, and therefore these mice do not return to wt levels because there is still not enough Cck at the Cck1R to restore PC health. If this is the case, perhaps the experiments using a 1mg/kg/Day dose rather than the low 0.02mg/kg/Day dose will provide restoration of motor behavior performance and PC health.

In $ATXN1[30Q]D776;Cck^{-/-}$ mice, A71623 was able to protect against progressive PC death. However, in $ATXN1[82Q]$ mice, no PC death was observed, even in vehicle-treated mice. This is likely because PC death does not occur by 12 weeks of age in $ATXN1[82Q]$ mice. Future studies may try treating $ATXN1[82Q]$ mice with A71623 for a longer period of time in order to discern whether it also protects against PC death in these mice.
Chapter 6: Overall Discussion
6.1 Major Findings

SCAs are a group of heritable diseases that cause progressive neurodegeneration of specific cell types, namely PCs of the cerebellum. Here are described a series of experiments identifying and characterizing the potentially PC-protective Cck-Cck1R pathway.

Upregulated Cck mRNA in the non-progressive SCA1 mouse model ATXN1[30Q]D776 was first identified in Ingram and Wozniak et al., which used RNA-seq on whole cerebellar tissue from progressive, ATXN1[82Q], and non-progressive, ATXN1[30Q]D776, mouse cerebellar extracts. Further analysis using qRT-PCR confirmed that Cck is solely upregulated in non-progressive mouse models, and downregulated in progressive mouse models. The temporal regulation and localization of both Cck and Cck1R suggests that, in the normal adult cerebellum, Cck is both released from, and activates PCs in a cell-autonomous manner. This finding, as well as a body of literature supporting a potential role for the endogenous upregulation of Cck in various types of neuronal assault, suggested that Cck may be of interest as a potential endogenous neuroprotective mechanism in ATXN1[30Q]D776 mice.

Supporting this idea, this is the first study to show that Cck−/− mice have modest cerebellar deficits. Cck−/− mice have distinct motor deficits by 1 year of age, as determined by two separate tests of motor behavior: the balance beam and the rotarod. Despite this observation, only mild cerebellar pathology was detected. The motor deficits observed in these mice could be due to input from extra-cerebellar areas, given that the Cck−/− is a global knock-out. Behavior tests are inherently complicated by a number of factors. Both the balance beam test and the rotarod could potentially be influenced by things like mouse weight, heightened anxiety, or changes in motivation. However, the findings here suggest at least some cerebellar contribution to the motor deficits, given
the increased number of footslips on the balance beam test, as well as the decrease in 
*Calb1* mRNA and slight decrease in ML thickness with age. The finding that *Cck*<sup>-/-</sup> mice have cerebellar deficits supports the idea that endogenous Cck is important for PC health, but it is important to understand the influence of highly upregulated *Cck* found in *ATXN1[30Q]D776* mice.

A genetic approach was employed by crossing the non-progressive *ATXN1[30Q]D776* mice to *Cck*<sup>-/-</sup> or *Cck1R*<sup>-/-</sup> mice. *ATXN1[30Q]D776;Cck*<sup>-/-</sup> mice and *ATXN1[30Q]D776;Cck1R*<sup>-/-</sup> mice have a progressive, degenerative disease resulting in severe ataxia and PC death. Therefore the up-regulation of *Cck* does indeed represent a protective pathway in these mice. From a therapeutic perspective, it was interesting to note the necessity of the Cck1R for the protective effects of upregulated *Cck*. In order to determine a therapeutic strategy, it was important to determine whether the upregulated *Cck* mRNA was transcribed, cleaved, and released to act through a canonical Cck-receptor pathway, or if it was acting through a novel mechanism. Because the Cck1R is necessary for protection, it stands to reason that Cck is indeed being cleaved into an active peptide capable of binding to the Cck1R and released from the PCs.
Figure 19 shows one potential model for the mechanism of action in a PC of \textit{ATXN1[30Q]D776} mice. Nuclear ATXN1 influences transcription of target genes, leading to the elevation of \textit{Cck} mRNA. Based on localization of both \textit{Cck} and its cerebellar receptor, \textit{Cck1R}, \textit{Cck} is translated, processed, and released from the PCs to bind to PC \textit{Cck1Rs} in a cell-autonomous manner. \textit{Cck-8S} is the most common peptide form of \textit{Cck} in the brain, and in the cerebellum. It is also the peptide with the highest affinity for \textit{Cck1R}, making it the likely active form in this model.

It must also be noted that, while \textit{ATXN1[30Q]D776} mice have elevated \textit{Cck} and lack disease progression, they do have an initial ataxia, and thinner ML than wt mice. It is possible that this is due to a developmental deficit in these mice. While ATXN1 and
Cck both turn on around p11 in the ATXN1[30Q]D776 mouse cerebellum, there are a host of processing steps necessary to obtain the Cck peptides. Pro-peptide (or prohormone) convertases are expressed in cell-specific manners, and are known to cleave Cck into its active forms, including Cck-8. The Brain Transcriptome Database (Sato et al., 2008) shows expression of two pro-peptide convertases, Pcsk1 and Pcsk2, known to cleave Cck-8 (Tagen and Beinfeld, 2005) increasing in cerebellar expression levels around P21, much later than the elevation of mutant ATXN1. Additionally, cerebellar Cck1R doesn’t reach its peak expression until after P21 (Sato et al., 2008). It is therefore possible that the initial cerebellar dysfunction observed in ATXN1[30Q]D776 mice is due to the influence of mutant ATXN1 before elevated Cck is able to be transcribed, processed, released, and activate the Cck1R.

Activation of the Cck1R has been shown to ultimately elicit changes in gene expression, however no in-depth analysis of Cck1R targets has been performed. In Ingram and Wozniak et al., RNA-seq on cerebellar RNA extracts from wt, ATXN1[82Q], and ATXN1[30Q]D776 mice at 5, 12, and 28 weeks was performed. That study identified a group of genes called the Magenta Module that significantly correlated with disease progression. In this thesis work, RNA-seq on cerebellar RNA extracts from ATXN1[30Q]D776;Cck−/− and Cck−/− mice was added to the data from the Ingram and Wozniak et al. study. This led to the identification of the Pink Module, a set of cerebellar genes associated with disease progression. The Pink Module was a robust finding, given its emergence whether the ATXN1[82Q] samples were included or excluded in the analysis, suggesting that its identification is driven by ATXN1[30Q]D776;Cck−/− samples. The two Pink Modules- with and without ATXN1[82Q]- significantly overlapped with each other, significantly correlated with disease progression, and significantly overlapped with the previously identified Magenta Module. The observation that these genes are PC-specific, related to disease progression in ATXN1[82Q] mice, and differentially
expressed in ATXN1[30Q]D776 and ATXN1[30Q]D776;Cck<sup>-/-</sup> samples suggests that it represents not only genes contributing to disease in these models, but also that these genes are regulated by elevated Cck.

This experiment suggested that Cck upregulation may impact disease-related gene changes, largely by preventing their down-regulation, and led to the hypothesis that activating the Cck1R using a Cck1R agonist, A71623, could be protective. Previous studies using Cck1R agonists identified Phosphorylation of ERK1/2 (P-ERK1/2) as a downstream target of Cck1R activation. Here, both a bolus injection of A71623, and long-term administration led to increases in levels of cerebellar P-ERK1. Others have identified P-ERK1/2 in the pathway upstream of ATXN1 phosphorylation (Park et al., 2013). Unlike in the daoy-cell experiment conducted in the Park et al. (2013) study, this study showed no change in cerebellar ATXN1 expression after an increase in P-ERK1. One explanation for this apparent discrepancy is that the ERK-ATXN1 pathway in Daoy cells functions differently than in PCs, or that the levels of P-ERK1 achieved following A71623 administration aren’t enough to encourage ATXN1 phosphorylation and subsequent accumulation.

In two progressive mouse models- ATXN1[82Q] and ATXN1[30Q]D776;Cck<sup>-/-</sup> mice- A71623 was able to protect against progressive deficits in motor behavior, and against progressive PC degeneration and death. It is interesting that treatment with the drug did not return either mouse model to the level of the wt mice for any measure. This could be due to the aforementioned early developmental expression of mutant ATXN1. Drug administration occurs when both mouse models already have thinner ML and mild ataxia, and the primary effect of A71623 is to prevent further deterioration. It is possible that earlier administration could improve the phenotype. Another explanation could be that a higher dose of A71623 or a more direct route of administration is needed. As discussed below, these experiments are planned.
The studies described here highlight a PC-protective pathway in mouse models of SCA1. Based on these findings, a model of Cck activity in the ATXN1[30Q]D776 mice is postulated in Figure 18.

Mutant ATXN1 leads to the elevation of Cck mRNA. This is cleaved and released as an active peptide. The most likely important peptide is Cck-8S, given the high levels of sulfated Cck-8 (Cck-8S) in the cerebellum and the preference of Cck1R for this form of Cck peptide.

6.2 Future Directions

This thesis work identified, characterized, and completed preliminary proof-of-concept studies examining the Cck-Cck1R pathway as a potential protective mechanism in PCs. While the work described here shows the promise of this pathway as a therapeutic mechanism, there are several studies that must be completed in order to fully grasp the potential of Cck1R activation as a therapy.

Several follow-on studies using the A71623 agonist must be completed. The first set of studies will examine a dose-response for the protective capabilities of A71623. While the results obtained with i.p. administration of 0.02mg/kg/Day A71623 were promising, this dose did not result in restoration of motor behavior or PC pathology to the level of wt mice. One possibility is that the dose was too low. Therefore a 5-fold increase in dose (1mg/kg/Day) would determine whether maximal protection has already been achieved. Another possibility is that, while some of the agonist crosses the blood brain barrier, not enough reaches its targets in the cerebellum. Levels of A71623 in the cerebellum can be determined by Mass Spectrometry. Administration of 0.02mg/kg/Day using an osmotic minipump attached to a catheter implanted i.c.v. would determine whether increasing the amount of agonist in the brain improves its efficacy. Mass Spectrometry analysis of i.c.v. delivered peptide compared to i.p. delivered peptide
would additionally determine how much A71623 is reaching the cerebellum. Indeed, other studies have shown that administering A71623 i.c.v. improves the response of centrally mediated behaviors (Asin et al., 1993).

The agonist study remains incomplete in interpretation because it lacks several control experiments that would give a better idea of mechanism of action for A71623 in the cerebellum. The Pink Module identified a set of genes influenced by Cck and associated with progressive cerebellar disease. It would be of great interest to determine whether administration of A71623 changes the levels of any of these genes. Rather than testing all of them, the most heavily connected genes (i.e. genes whose expression significantly changes with the highest number of other genes in the same Module) can be determined as in Ingram and Wozniak et al. These genes could then be used as markers for A71623 activity in the cerebellum.

Apart from follow-on studies, the Cck1R represents a very interesting target to improve PC health in general. It would be interesting to next determine whether other PC-degenerative diseases, like other SCAs, can be improved using A71623 treatment. For this to feasibly work, these other PC-specific diseases would likely need to be caused, in part, by changes in regulation of the Cck1R-targets genes. In Chapter 4, Cck-regulated genes were found to significantly overlap with a curated “movement disorder” affected gene list. This is encouraging, as it suggests that downstream of the Cck1R in the cerebellum lies multiple gene targets whose regulation is impacted in several movement disorders, including several SCAs. Therefore obtaining and testing A71623 in mouse models of other SCAs may reveal whether the Cck1R represents a global PC protective mechanism applicable to several target diseases.

The ultimate goal of these agonist studies would be to move the treatment eventually to humans. One possibility would be to continue using commercially available A71623. However, a strategy that could be employed to get through to human clinical
studies faster would be to test a Cck1R agonist that has already completed several clinical trials. GlaxoSmithKline created a peptoid Cck1R agonist similar to A71623, GI181771X, has been tested in human clinical trials for obesity (Castillo et al., 2004). While this proprietary drug represents several hurdles in obtaining the drug and running the trial, it is orally available, crosses the blood brain barrier, and has been more thoroughly studies for its pharmacokinetic and pharmacodynamic properties. Therefore obtaining GI181771X may be a faster route to humans than continuing to use A71623.
Chapter 7: Methods
Mice

The Institutional Animal Care and Use Committee approved all animal use protocols. All mice were housed and managed by Research Animal Resources under SPF conditions in an AAALAC-approved facility. For all experiments, equal number of male and female mice were used.

*Cck*⁺⁻, *Cck1R*⁺⁻, and *Cck2R*⁺⁻ mice were obtained from Jackson labs and crossed to *ATXN1[30Q]D776* mice for at least three generations before experimental use. All three mouse lines were on the Sv.129/B6 background, and *ATXN1[30Q]D776* mice were on the FVB/NJ background. To maintain as much background strain consistency as possible, the FVB/NJ wt mice, *ATXN1[30Q]D776* mice, and *ATXN1[82Q]* mice used in all experiments were backcrossed to Sv.129/B6 lines at least three generations. The mice used in the *ATXN1[82Q]* agonist studies were not back-crossed, and were on the FVB/NJ background.

Histology and Pathology

Animals were anesthetized and the cerebella rapidly removed. Half of the cerebellum was post-fixed overnight in 10% formalin and placed in PBS at 4°C before sectioning. The other half was snap-frozen in liquid nitrogen for western blotting experiments. Fixed cerebella were sectioned into 50 μm sagittal sections using a vibratome. Epitopes were exposed using antigen retrieval by boiling sections three times for 15 sec each in 0.01M urea. Sections were blocked overnight in 2% normal donkey serum and 0.3% Triton X-100 in PBS. Subsequent staining was carried out in 2% normal donkey serum and 0.3% Triton X-100 in PBS. Anti-calbindin antibodies used were mouse (Sigma-Aldrich Cat# C9848/RRID:AB-10115846) and rabbit (Sigma-Aldrich Cat# C2724/RRID:AB-258818) at a 1:250 dilution. Sections were incubated for 24 hrs with primary antibodies at 4°C. Following incubation, sections were washed three times in PBS and exposed to secondary antibodies (Alexa Flou 488 antimouse-cat# 715-546/RRID:AB-2340850 and
Alexa Fluor 647 antirabbit-cat# 711-605-152/RRID:AB-2492288, Jackson Immunoresearch Labs, West Grove, PA) for 24 hrs at 4°C. Sections were washed three times in PBS and mounted onto charged slides (Colorfrost Plus, Fisher, Waltham, MA). Fluorescently labeled tissue was imaged using a confocal Olympus 1000 IX inverted microscope.

Molecular layer thickness was measured using the Olympus Fluoview imaging software. 20um-thick z-stack images were taken of the cerebellar primary fissure at 20x. A ruler was drawn from the leading edge of the primary fissure along the visible length. Six measurements- three on each side- were taken of the molecular layer from the base of the Purkinje Cell to the edge of the pial surface. At least 3 cerebellar sections per animal were measured this way, and averaged. Data shown in Figures 1 and 2 represent the averages across treatments.

**Western Blotting**

Frozen cerebella were homogenized in standard tris triton lysis buffer containing protease (Roche) and phosphatase (Sigma-Aldrich) inhibitors, then underwent 3 free/thaw cycles between liquid nitrogen and a 37°C water bath. The samples were spun at 10,000 RPM for 10 min at 4°C, then the pellet was discarded. A Bradford assay was performed to determine protein concentration, and 30 ug of protein per sample was loaded onto a 4-20% gradient gel (Bio-Rad). The gels were run for 1-1.5hrs at 125V and then transferred onto nitrocellulose membranes using the Biorad Tans-blot Turbo transfer system. Membranes were incubated for 10 min at room temperature in blocking solution (1X PBS, 0.01% Tween20, 5% BSA). Membranes were incubated overnight at 4°C with anti-P-ERK1/2 antibody (Cell Signaling Technology, 1:1000) in blocking solution. They were then washed 3X with 1X PBS and 0.01% Tween20.

Chemiluminescent detection was performed by bathing the membrane in Super Signal West Pico Luminol (Thermo Fisher Scientific), then imaged using the ImageQuant LAS
4000 (GE Healthcare, Lifesciences) and densitometry analysis is performed using the ImageQuant Software (GE Healthcare, Life Sciences). Once an image is obtained, the membrane was stripped using Restore Western Blot Stripping Buffer (Thermo Fisher Scientific). It was then washed 3X 10 min in 1X PBS with 0.01% Tween20, and incubated again overnight in anti-total ERK1/2 (Cell Signaling Technology, 1:1000) in blocking buffer. Detection was again performed as described above.

**RNA Isolation**

Total RNA was isolated from dissected cerebella using TRIzol Reagent (Life Technologies, Carlsbad, CA) following the manufacturer’s protocols. Cerebella were homogenized on ice in 500mL TRIzol Reagent using an RNase-free disposable pellet pestles in a motorized chuck. Once tissue was homogenized, 500mL TRIzol Reagent was added for a total of 1mL TRIzol Reagent per cerebella. Samples were then incubated for 5 minutes at room temperature to permit dissociation of nucleoprotein complexes. Phase separation was completed by adding 0.5mL chloroform, shaking vigorously for 15 seconds, and incubating at room temperature for 2-3 minutes. Samples were centrifuged at 12,000xg for 15 minutes at 4ºC, allowing for collection of the clear upper aqueous layer into a new RNase-free tube. RNA was precipitated from the aqueous layer by the addition of 0.5mL 100% isopropanol, incubating at room temperature for at least 10 minutes and centrifugation at 12,000 x g for 10 minutes at 4ºC. The RNA pellet was washed with 75% ethanol, briefly vortexed, and centrifuged at 7,500 x g for 5 minutes at 4ºC. The pellet was allowed to air-dry for 5-10 minutes at room temperature, making sure not to allow the pellet to dry completely. RNA was resuspended in 50-200 uL RNase-free water by passing the solution through a pipette tip several times. RNA was incubated for 10-15 minutes at 60ºC and stored at -80ºC.

For RNA-sequencing RNA was further purified to remove any organic carryover, which can inhibit the enzymatic reactions used in Illumina library preparation and can increase
the risk of failure of library generation. RNA was purified using the RNeasy Mini Kit (Qiagen, Venlo, Limburg), and following the manufacturer's RNA Cleanup protocol. Briefly, buffer RLT and ethanol are added to the sample to promote selective binding of RNA to the RNeasy membrane. The sample is then applied to the RNeasy Mini spin column. Total RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in RNase-free water.

cDNA synthesis

Random-primed cDNA was generated from 1ug RNA using either the SuperScript VILO cDNA Synthesis Kit (Life Technologies) or the iScript Reverse Transcription Supermix (Life Technologies) according to the manufacturer's protocols. For the VILO Kit, each reaction contained 4ul 5X VILO Reaction Mix, 2ul 10X SuperScript Enzyme Mix, 1ug RNA, and DEPC-treated water to 20uL. Tube contents were mixed, placed in a PCR block, and incubated at 25ºC for 10 minutes, followed by 42ºC for 60 minutes, then 85ºC for 5 minutes to terminate the reaction. The cDNA generated was then diluted 1:5 for use in qRT-PCR.

For the iScript Kit, each reaction contained 4ul iScript RT Supermix, 100-1000ug RNA depending on the experiment, and DEPC-treated water to 20uL. Tube contents were mixed, placed in a PCR block, and incubated at 25ºC for 5 minutes, the 46ºC for 20 minutes, then 95ºC for 1 minute to terminate the reaction.

qRT-PCR

qRT-PCR reactions were completed using LightCycler 480 Probes Master Mix and hydrolysis probe (Roche, Penzberg, Germany) following manufacturer’s protocols. Primer sequences and probe combinations were generated using Universal ProbeLibrary for mouse Assay Design Center (Roche). Universal ProbeLibrary Mouse GAPD Gene Assay was used as a control. Human ATXN1 primers were: probe 67, left: AGAGATAAGCAACGACCTGAAGA right: CCAAAACTTCAACGCTGACC. To mouse
Cck: probe 9, left: TGATTCCCCATCCAAAGC right: GCTTCTGCAGGGACTACCG. To mouse Calb1: probe 17, left: ACGGAAGTGGTTACCTGGAA right: CATTCCGGTGATAGCTCCA.

RNA-sequencing

Whole cerebellar RNA from three biological replicates for each genotype was isolated using TRIzol Reagent (Life Technologies) followed by purification with RNeasy Kit according to the manufacturer's protocol (Quiagen) [methods detailed above]. Purified RNA was sent to the BioMedical Genomic Center at the University of Minnesota for Quality Control, including quantification using fluorimetry (RiboGreen assay, Life Technologies) and RNA integrity assessed with capillary electrophoresis (Agilent BioAnalyzer 2100, Agilent Technologies, Inc.) generating an RNA integrity number (RIN, Table 1). All submitted samples had greater than 1ug total mass and RINs greater than 8. Library creation was completed using oligo-dT purification of polyadenylated RNA, which was reverse transcribed to create cDNA. The cDNA was fragmented, blunt-ended, and ligated to barcoded adaptors. The library was size selected to 320bp +/- 5% to produce average inserts of approximately 200bp, and size distribution validated using capillary electrophoresis and quantified using fluorimetry (PicoGreen, Life Technologies) and q-PCR. The libraries were then normalized, pooled, and sequenced.

ATXN1[30Q]D776;Cck^{-/-} and Cck^{-/-} samples were sequenced on an Illumina HiSeq 2500 using a 150nt paired-end read strategy. Data was stored and maintained on University of Minnesota Supercomputing Institute (MSI) servers. Research Informatics Support Systems (RISS) bioinformaticians were available at MSI for data analysis support. Gene expression analysis with the Tuxedo pipeline was done using the Galaxy platform hosted by the MSI. Initial read quality was determined using FastQC (Andrews) to determine the following: basic statistics, per base sequence quality, per sequence quality scores, per base sequence content, per base GC content, per sequence GC
content, per base N content, sequence length distribution, sequence duplication levels, overrepresented sequences, kmer content. Using the FastQC results, reads were trimmed to an acceptable length, e.g. per base sequence quality greater than 28. Contaminating adapter sequences were removed. All paired-end reads were correctly synchronized.

Reads were aligned to the mouse reference genome (Illumina igenomes mm9) with Tophat by using mostly default parameters, except a Std. Dev for Distance between Mate Pairs of 60 and using a gene annotation model only looking for supplied junctions. Differential gene expression was determined with Cuffdiff using default parameters. Splicing analysis was completed using GSNAP and DEXseq by using most default parameters. Genes/introns with a q≤0.05 were considered significant. Genome tracks were visualized with Integrated Genomics Viewer (Broad Institute). Results were graphed with CummeRbund. Pathway and clustering analysis was completed with Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, California) and DAVID Bioinformatics Resource (NIAID, NIH).

WGCNA was conducted using an R package developed by Langfelder and Horvath (2008). Prior to the WGCNA, data were log transformed to reduced heteroscedasticity (unequal variability among samples expressed at different levels), and quantile normalized to generate experiment-appropriate FPKM values. For the WGCNAs described here, an FPKM cutoff value of >10 FPKM was used. WGCNA using an FPKM >3 was also run, and still robustly identified the Pink Module.

**Agonist treatments**

The Cck1 receptor (Cck1R) agonist A71623 (Tocris Biosciences) was resuspended in 20mM PBS according to the manufacturers directions. For the experiment with ATXN1[30Q]D776;Cck−/− mice (Figure 14B), osmotic minipumps (Azlet, 1004) containing
either A71623 (0.02mg/kg/day) or Vehicle (20mM PBS) were implanted intraperitoneally (i.p.) in 6 week old mice. Briefly, 5 week old mice were deeply anesthetized by intramuscular injection of a ketamine/xylazine cocktail (100mg/kg ketamine and 10mg/kg xylazine). Fur on i.p. implantation site (1cm below ribcage) was shaved. Incision sites were scrubbed with povidone-iodine (Betadine) scrub. A 1cm-long incision was made under the ribcage. The peritoneal wall was gently incised beneath the cutaneous incision and the pump cannula was placed into the peritoneal cavity. The musculoperitoneal layer was closed with 4.0 absorbable suture and the skin wound was closed using surgical staples (Alzet). Ten days after surgery, the staples were removed and wounds examined for healing.

For the duration of the experiment, pumps were removed and replaced every 7 weeks. Behavioral data was collected at the timepoints indicated in Figure 14B.

Because of the size of the osmotic minipumps, the mice have to be ~20g or larger for safe implantation into the i.p. space. In ATXN1[82Q] mice, single bolus injections of A71623 (0.02mg/kg) or Vehicle were administered daily beginning at week 5 and continuing until the mice were ~20g (for approximately 7 days, or until the mice were 6 weeks old). At this time the pumps were implanted for the remainder of the experimental timeline.

Behavioral Tests

Rotarod

An accelerating Rotarod was used to assess motor performance and learning. Mice were placed on a rotating rod (3cm diameter) for four trials per day for four consecutive days. Each trial lasted a maximum of 5 min, during which time the rotating rod underwent linear acceleration from 4 to 40 rpm over the entire 5 min. Animals were scored for their latency to fall (in seconds) for each trial. Mice rested a minimum of 10 min in between each trial to avoid fatigue. Data were analyzed using a Two-Way
ANOVA and Tukey post-hoc test for multiple comparisons that factors in day and genotype. Data presented in Figures 1 and 2 represent day 4 of testing for each timepoint.

**Balance Beam/Bar cross test**

The balance beam tests for balance and motor coordination. The apparatus consists of a linear, horizontal beam that is 3 ft in length and hangs 19 inches above the table. At the end of the beam opposite the starting point there is a safety box, which is a dark, enclosed structure measuring 7.5 x 7.5 x 5.5 inches that is accessible from the end of the balance beam. The beam is marked at 4 inches from both the beginning and the end to indicate start and stop lines that are used to determine time it takes to cross the beam. The baseline test occurs over four consecutive days: the first three days are training days and the fourth is a testing day. During the training days, naïve mice were run for four trials per day on the 15mm wide square beam (data not shown). Prior to the first trial on each training day, each mouse was placed in the safety box to acclimate for 30 seconds. Both time to cross (in seconds) and the total number of foot slips were recorded for each trial. Time to cross is defined as the time from when the animal’s hind legs cross the starting line to when their hind legs cross the finish line. Foot slips are defined as any time either of the hind paws unexpectedly slips off the beam. To reduce the impact of learning, only two training days were run for all non-naïve animals following the baseline experiments.

On the test day, animals underwent two trials on each of six beams, ranging in order from easy (large) to difficult (small): 25mm wide square, 27mm diameter round, 15mm wide square (training beam), 17mm diameter round, 8mm wide square, and 10mm diameter round. As during the training trials, latency and the number of foot slips per trial were recorded. Tests were performed between 9 A.M. and 4 P.M.
For each mouse, we found the average time to cross and the average number of foot slips per beam on test day. Test time to cross and foot slips were compared using a Two-Way repeated measures ANOVA and Tukey post-hoc test for multiple comparisons. Data presented in Figures 1 and 2 represent the time to cross and number of foot slips recorded on the smallest beam (10mm round) on test day.

Elevated Plus Maze

The Elevated plus Maze is a widely used animal model of anxiety that is based on conflicting tendencies. The apparatus (Med-Associated, St. Albans, Vermont) consists of two open and two enclosed arms that form a “plus”. The mouse is given the choice of spending time in open, unprotected maze arms or enclosed, protected arms, all elevated from the floor. Mice are placed into the center of the apparatus at the beginning of the session and the number of arm entries and the amount of time spent in the open and closed arms are recorded for 5 min.

Stress-Induced Hyperthermia

Each animal will only be tested in a single behavioral task. For temperature measurements, the mouse is held at a 45° angle with its head upwards. Securing its tail, the probe (RET-3, Rectal probe for mice, Physitemp Instruments Inc.) is dipped into silicon oil and inserted it ~2 cm into the rectum, and held there for ~20 sec until a stable rectal temperature is observed. The probe is withdrawn, temperature is recorded to 0.1°C accuracy, and the mouse is returned to its own cage. Repeat ten minutes later. The probe is thoroughly cleaned with ethanol (70%) between each usage. The stress/anxiety response is determined by the change in temperature from the first measurement to the second. The change in temperature from the first measurement to the second is the hyperthermic stress response.
Bibliography


Appendix
Appendix Figure 1. Anxiety tests. A) Percent of time spent in the open arm of the elevated plus maze (EPM). B) Total distance traveled in the EPM. C) Change in body temperature in response to the stress-induced hyperthermia (SIH) test. D) Initial basal body temperature measured at the beginning of SIH test. Error bars are ±SEM, One-way ANOVA with tukey post-hoc test.
Appendix Table 1. Motor Disease-related genes changed between ATXN1[30Q]D776 and ATXN1[30Q]D776;Cck/- mice at 28 weeks. "Increased" indicates the down-regulation of this gene has been observed to contribute to motor disease, "decreased" indicates the down-regulation of this gene is protective against motor disease. "Affected" indicates the literature identified this gene as related to motor disease, but its influence on disease state remains unclear. Exp Log Ratio is the log-fold change in expression between the two mouse models.
Appendix Table 2. Genes from the Pink Module identified in the WGCNA including all samples.

These 166 genes significantly overlapped with the previously identified Magenta Module (Ingram and Wozniak et al., 2016). Log2(fold change) is the fold change in expression between wt and ATXN1[30Q]D776;Cck/- samples at 28 weeks of age. Q_value is the level of significance, Students t-test with Bonferroni multiple testing correction for p<0.005.

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