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MOLECULAR PATHOGENESIS OF SPINOCEREBELLAR ATAXIA TYPE 10

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MOLECULAR PATHOGENESIS OF SPINOCEREBELLAR TYPE 10

by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas Medical Branch

December, 2012

Dedication

To my family

Acknowledgements

Without the assistance of numerous individuals, I certainly could not have completed my dissertation. I thank all for contributing to the completion of this dissertation.

Molecular Pathogenesis of Spinocerebellar Ataxia Type 10

Publication No._____

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The University of Texas Medical Branch, 2012

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Spinocerebellar ataxia type 10 (SCA10) is a unique autosomal dominant cerebellar ataxia (ADCA) which habors non-coding ATTCT repeat expansion on the 9th intron of the *ATXN10* gene. The presence of seizure in addition to pancerebellar ataxia is a characteristic clinical manifestation of SCA10. How this enlarged intronic ATTCT repeat leads to degeneration or dysfunction of the nervous system underlying SCA10 phenotype is not well known. The present study focused on elucidating a molecular pathogenesis of this unique inherited disease.

In this study, we demonstrated normally transcribed ATTCT-repeats form a lengthy AUUCU pre-mRNA molecule in SCA10 cells, after being completely spliced out. We identified the specific interaction between this AUUCU RNA repeat and a RNA-binding protein, Nova, which leads to the aberrant splicing of Nova target transcripts, including major inhibitory neurotransmitter receptors, glycine and GABA. Our study also indicated that the sequestration of the RNA-binding proteins by toxic expanded repeats result in complex SCA10 phenotype. We clearly showed that aberrant splicing of $GABA_AR\gamma2$ and GlyR$\alpha2$ causes disruption of inhibitory transmission in Purkinje cells in

the SCA10 cerebellum. We demonstrated that abnormal electrophysiology in SCA10 occurs specifically through modulating GABA neurotransmission but not glycine.

In our current study, we were able to define SCA10 as a toxic RNA disease, and presented one of the possible molecular and electrophysiological mechanisms of SCA10 pathophysiology.

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List of Abbreviations

ADCAs	Autosomal dominant cerebellar ataxias
BEAN	Brain expressed, associated with Nedd4
c-JNK2	c-Jun N-terminal kinase
CNS	Central nervous system
DM1	Myotonic dystrophy type 1
DM2	Myotonic dystrophy type 2
DNA	Deoxyribonucleic acid
DRPLA	Dentatorubral-pallidoluisian atrophy
EMSA	Electrophoretic mobility shift assay
FISH	Fluorescence in situ hybridization
FRDA	Friedreich's ataxia
FXN	Frataxin
GABA	gamma-Aminobutyric acid
GFP	Green fluorescent protein
hnRNPA	Heterogeneous ribonucleoprotein particle A
hnRNPK	Heterogeneous ribonucleoprotein particle K
KH domain	K Homology domain
KO	Knock-out
LOX-15	15-Lipoxygenase
MBNL1	Muscleblind-like
mIPSC	miniature inhibitory postsynaptic current

NMDA	N-Methyl-D-aspartic acid
PAGE	Polyacrylamide gel electrophoresis
PKC	Protein kinase C
Poly-Q	Poly-glutamine
POMA	Paraneoplastic opsoclonus-myoclonus ataxia
RT-PCR	Reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
SBMA	Spinal-bulbar muscular atrophy
siRNA	small interfering RNA
TK2	Thymidine kinase 2
TTX	Tetrodotoxin
UTR	Untranslated region

CHAPTER 1: BACKGROUND

MICROSATELLITE REPEAT INSTABILITY AND HUMAN GENETIC DISORDERS

Microsatellite repeat length polymorphism is a genetic phenomenon that describes the presence of variable numbers of tandemly repeated nucleotide sequences throughout eukaryotic genomes (Jeffreys et al. 1985). These nucleotide repeats consist of varying number of repeat units (mono-, di-, tri-, tetra-, penta- and hexa-nucleotide repeats), and are present at specific loci in all human chromosomes. Importantly, the integrity and lengths of these repeats in alleles are strictly maintained in the genome, and remain unaltered throughout the life-span of a healthy individual. However, the functional significance of the presence of these repeats in specific loci in human genomes remains unclear. Recent studies have established that the microsatellite repeats become highly unstable at certain genomic DNA loci, and repeat numbers often expand, resulting in different human genetic disorders. Specific microsatellite repeat sequences become highly unstable in one of the alleles, and invariably expand in these diseases. A large number of clinical and experimental studies support the idea that expansion of such repeated DNA sequences is the primary cause of this novel class of genetic disorders. The molecular basis of repeat instability, the mechanism by which these repeats expand in human genome, and specifically how expansion of repeats leads to disease phenotypes is a subject of intense investigation. Furthermore, analyses of patient genomic DNA have revealed that the repeat number in the mutant allele expands in small increments during each mitotic cell division in all somatic tissues, and greatly expand during germ line transmission from one generation to the next, in many of repeat expansion disorders. In this novel class of microsatellite repeat expansion-associated genetic disorders, extreme

instability of the microsatellite repeats often results in massive expansions of these repeats, and such expansions contribute to the pathogenic mechanism of this unique and heterogeneous group of genetic disorders.

Since 1991, when instability and expansion of CAG tri-nucleotide repeat in the androgen receptor gene was first found in spinal bulbar muscular atrophy (SBMA) (La Spada et al. 1991), a large number of human diseases have been identified that are characterized by expanded microsatellite repeat sequences in the disease-associated genes. These repeats typically consist of tri-, tetra-, penta-, or even hexa-nucleotide repeats, within the transcription unit of the respective disease gene. At present, there are over 40 human genetic diseases that are recognized as being caused by the instability of microsatellite repeat sequences. Unstable tri-nucleotide repeat sequences are known to be the most common microsatellite repeats as the primary cause of these disorders. Other nucleotide sequences consisting of tetra- and penta-nucleotide repeats have also been found to expand at various genomic DNA loci, leading to diseases such as myotonic dystrophy type 2 (DM2) (Christina et al. 2001), and spinocerebellar ataxia type 10 (SCA10) (Matsuura et al. 2002), respectively.

Each microsatellite repeat expansion-associated disease has distinctive clinical features, but neurological defects are the most typical findings noted in most of the known conditions. In addition, the majority of these diseases follow the general rule of anticipation (La Spada et al. 1994). Importantly, these diseases do not follow the classical Mendelian pattern of genetic inheritance, because the severity and age of onset of the disease increases from one generation to the next. The general consequence of this phenomenon is that the more severe symptoms as well as the earlier onset of disease occur in the next generation.

Paulson et al. (1996) first suggested a classification method for these diseases based on the repeat location in the respective genes (Figure 1-1), where they were divided into 2 groups: type 1 and type 2 (Paulson et al. 1996).

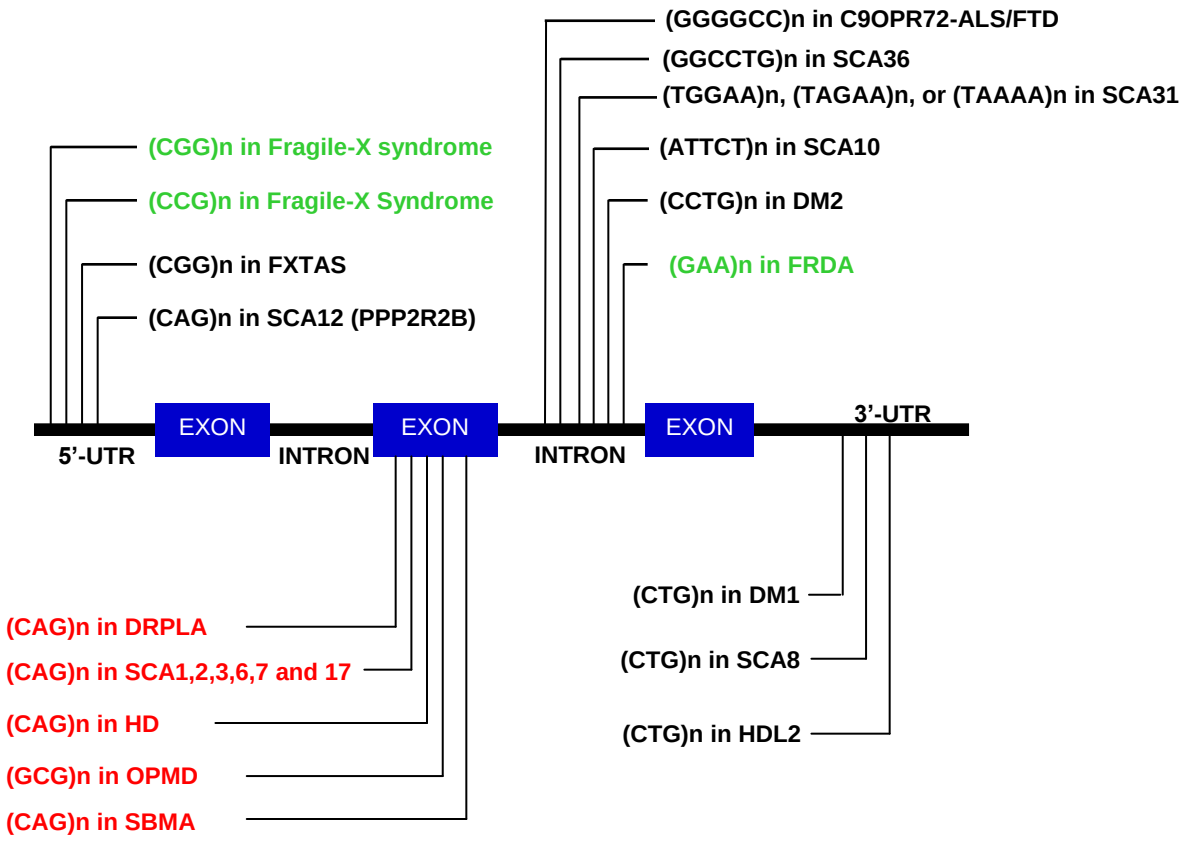


Figure 1-1. Repeat Expansion Diseases.
In 1996, Paulson et al. suggested a classification method for these diseases based on the repeat location in the respective genes. Initially seven trinucleotide repeat diseases were divided into 2 groups, type 1 and 2. There have been significant growing in numbers of repeat expansion disease afterward which includes tetra-, penta-, and hexa- nucleotide repeats.

Currently repeat expansion diseases are classified into type 1, 2 and 3. Diseases with expanded repeats within the coding region (exons) are classified as type 1. These disorders are further sub-categorized into type 1a, caused by an extended CAG repeat

sequence, encoding a stretch of poly-glutamine (poly-Q) tract; and type 1b, caused by an expanded GCN or GCG sequence, encoding poly-alanine sequences in the mutant protein. Diseases where there are expanded repeats either in the 5' or 3' untranslated regions (UTRs) of the disease genes are classified as type 2. Fragile X syndromes and spinocerebellar ataxia type 12 (SCA12) have expanded repeats within the 5' UTR of the mutant genes. Myotonic dystrophy type 1 (DM1) and spinocerebellar ataxia type 8 (SCA8) are typical examples of diseases with CTG repeat expansions occurring within the 3' UTR of the disease genes. Diseases wherein repeat expansions occur within the intronic sequences of the mutant genes are categorized as type 3, and myotonic dystrophy type 2 (DM2), Friedreich ataxia (FRDA) and spinocerebellar ataxia 10 (SCA10) are examples of type 3 disease.

Several possible mechanisms by which expansion of microsatellite repeats cause extensive cellular toxicity and complex disease phenotypes have been proposed. Accumulating evidence suggests that either the gain- or loss-of-function of the mutant genes, associated with the repeat expansions, contribute to the pathogenic mechanisms (Cummings et al. 2000; Todd et al. 2010). Whether abnormal expansion occurs within the coding or non-coding region of the disease genes was initially thought to determine the pattern of these diseases. For instance, in a recessively inherited, non-coding expansion disorder such as Friedreich ataxia (FRDA), the loss of-function mutation plays a dominant role in the pathogenic mechanism of the disease. In FRDA, expansion of the GAA triplet repeats occurs in the first intron of the *frataxin* (*FXN*) gene (Campuzano et al. 1996; Lodi et al. 2006; Kumari et al. 2012). The expanded GAA-TTC repeat sequences have been shown to adopt unusual or non-B-DNA structures, e.g., either sticky DNA (Sakamoto et al. 1999; Clark et al. 2004) or purine-purine-pyrimidine or pyrimidine-purine-pyrimidine type of triple helix DNA structures *in vitro* (Marappan et al. 1999). Various studies support the hypothesis that the expanded GAA·TTC repeat

sequences and/or DNA secondary structures formed by these repeats seriously affect the movement of the RNA polymerase over the template DNA, eventually resulting in marked suppression of the *FXN* gene transcription. In fact, these pathogenic non-B-DNA structures have been shown to inhibit progression of RNA polymerase or transcription factors over the template DNA in vivo (Bidichandani et al. 1998). Furthermore, these extended sequences and/or structures have also been shown to interact with splicing factors e.g., hnRNPA1 or hnRNPA2, and disturb splicing of the *FXN* gene, resulting in mis-splicing and rapid degradation of the *FXN* mRNA (Baralle et al. 2008). Importantly, recent studies also indicate that massive expansion of the GAA sequences results in significant modification of chromatin composition- histone modification, and marked increase in DNA methylation leading to silencing of the mutant gene (Al-Mahdawi et al. 2008; Soragni et al. 2008; Castaldo et al. 2008; Kim et al. 2011). From these key findings, FRDA has been categorized as a non-coding repeat disease with loss-of-function mutation.

In contrast, repeat expansions in the coding region, commonly dominantly-inherited, follows a different path of destiny. The expanded repeat sequences encode lengthy stretches of amino acids, which lead to various pathogenic consequences. For instance, the expanded CAG repeats in the poly-Q diseases (Table 1-1) encode a lengthy tract of glutamines in a product of the respective gene, producing a mutant protein (Zoghbi et al. 2000). The mutant protein encoding elongated poly-Q tracts tends to misfold, causes cellular toxicity and invariably forms insoluble aggregates or inclusion bodies. The mutant poly-Q proteins misfold and also induce misfolding of other proteins that are found in the ubiquitin-positive inclusions (Chen et al. 2002). The production of toxic oligomers (Nagai et al. 2007; Takahashi et al. 2008) or interactions with other cellular proteins might contribute to the pathology of the Poly-Q diseases (Lim et al. 2008). Accumulating evidence suggests that aberrant folding and intermolecular protein-

protein interaction ultimately results in the gain-of-function of the mutant proteins, and loss or diminished function of the proteins that interact with the mutant Poly-Q proteins. The molecular mechanisms of the poly-Q diseases are still being widely investigated, but the altered biophysical properties of the mutant proteins containing the expanded poly-Q stretch underlie the central pathogenesis of the gain-of-function mutation in poly-Q diseases.

Table 1-1. Poly-glutamine (Poly-Q) Diseases.

Disease	Protein Name	Poly-Q positions and Disease Repeat Range	Protein function
SBMA	Androgen Receptor	40-62 Normal=9-36	Testosterone-activated steroid receptor
HD	Huntingtin	36-121 Normal=10-35	Scaffolding protein linked to diverse Cellular pathways
DRPLA	Atrophin-1	49-88 Normal=6-35	Possible transcription co-repressor
SCA1	Ataxin-1	39-91 Normal=6-35	Transcriptional co-repressor involved In cell specification & synaptic activity
SCA2	Ataxin-2	32-200 Normal=14-32	RNA processing & translational regulation
SCA3	Ataxin-3	52-86 Normal=12-40	Deubiquitinating enzyme involved In protein quality control
SCA6	Calcium Channel Subunit 1A	Normal=4-19 20-33	Voltage-sensitive Calcium Channel subunit
SCA7	Ataxin-7	34-300 Normal=7-17	Component of HAT complex (TFTC/STAGA) & transcriptional regulation
SCA17	TATA-Box-binding protein	47-63 Normal=25-42	Component of core transcriptional complex TFIID

A large number of recent studies suggest that the pathogenic mechanism of non-coding repeat disorders e.g., type 1 or 2 myotonic dystrophies do not follow either simple loss- or gain-of-function mutation of the disease gene (Ranum et al. 2006; Osborne et al. 2006; O'Rourke et al. 2009; Cooper et al. 2009). The expanded CTG and CCTG repeat

sequences in myotonic dystrophy type 1 and 2 are located in the 3' UTR and intronic sequences of DMPK and ZNF9 genes, respectively. A large number of recent studies have established that these non-coding repeats in DM1 or DM2 contribute to the development of some overlapping and also distinct and unique pathology in the manifestation of the disease phenotypes. The extended repeat tracts are known to confer high toxicity to the respective mutant mRNAs, and this phenomenon is now commonly referred to as 'toxic RNAs gain-of-function.' A large number of studies support the hypothesis that the mutant CUG and CCUG RNAs in DM1 and DM2 contribute, at least in part, to the development of complex and multi-systemic phenotypes in DM1 and DM2 (Mastroyiannopoulos et al. 2010) (Please refer to RNA-mediated pathogenic mechanisms in DM1/DM2 in Chapter 2). However, the mechanism by which the expanded CUG RNA inflicts degenerative and dystrophic defects in DM1 remains unknown and definitely needs further investigation. It is also not clear why DM2 with its massive CCTG repeat expansions shows milder and less severe phenotypes compared to DM1, presenting relatively shorter CTG repeat expansions.

As described above, SCA10 belongs to a microsatellite repeat-expansion-associated disorder wherein the expansion of a penta-nucleotide repeat expansion occurs within the intron of *ATXN*10 gene. The mechanism by which intronic repeat expansion causes SCA10 pathology is largely unknown.

AUTOSOMAL DOMINANT CEREBELLAR ATAXIA

From the clinical perspective, SCA10 belongs to a heterogeneous group of disorders, which are collectively known as autosomal dominant cerebellar ataxias (ADCAs) or spinocerebellar ataxias (SCAs) (Koeppen et al, 2005) (Table 1-2). The ADCAs are typically difficult to distinguish from each other based on the clinical

findings themselves. The prevalence of these types of inherited ataxias is reported as 3.5 per 100,000 to 8.5 per 100,000, according to several studies (Jin et al. 1999; Tang et al. 2000).

Table 1-2. Spinocerebellar Ataxia (SCAs) or Autosomal Dominant Cerebellar Ataxia (ADCAs) Caused by Microsatellite

Disorder	Location	Gene	Mutation		Clinical Features
SCA1	6p23	SCA1	CAG	Poly-Q	EPS, Neuropathy
SCA2	12p24	SCA2	CAG	Poly-Q	EPS, Neuropathy
SCA3	14q32.1	SCA3	CAG	Poly-Q	EPS
SCA6	19p13	CACNA 1A	CAG	Poly-Q	Prominent cerebellar findings
SCA7	3p14	SCA7	CAG	Poly-Q	Retina degeneration, Hearing loss
SCA8	13q21	Unknown	CTG	3' UTR	Sensory neuropathy
SCA10	22q13	SCA10	ATTCT	Intronic	Seizure
SCA12	5q31	PPP2R2B	CAG	5' UTR	EPS, Neuropathy
SCA17	6q27	TBP	CAG	Poly-Q	EPS, Psychosis, MR, seizure
SCA31	16q22.1	BEAN, TK2	TGGAA	Intronic	Prominent cerebellar findings
SCA36	20p13	NOP56	GGCCTG	Intronic	Motor neuron signs
DRPLA	12p13.31	DRPLA	CAG	Poly-Q	Myoclonic epilepsy, chorea

EPS indicates extrapyramidal syndrome.
MR indicates mental retardation.

In addition to thorough clinical characterization, genetic analyses such as linkage and mutation analyses have been used extensively to distinctly categorize the subtypes of the SCAs. For example, SCA types 1, 2, 3, 6, 7, and 17 have expanded CAG tri-nucleotide repeats (Globas et al. 2008). SCA type 8 and SCA12 have been identified as type 2 tri-nucleotide repeat disorders containing non-coding tri-nucleotide repeats within the transcription unit of the disease genes (Koob et al. 1999; Holemes et al. 1999). Additionally, several SCAs show some characteristic clinical features, such as retinopathy in SCA7 (Lindbald et al. 1996), Parkinsonism in SCA2, 3, and 17 ((Lu et al. 2004; Klockgether 2007), and seizure in SCA 10, 17, and DRPLA (Grewal et al. 2002; Pfeiffer et al. 1990). Focal epilepsy (Tan et al. 2004) and retinitis pigmentosa (Rufa et al. 2002) were also reported in patients with SCA 2.

The spinocerebellar ataxias are pathologically characterized by degeneration of specific neurons within the cerebellum, with or without its connecting structures (Koeppen et al. 2005). In several of the SCAs, the pathologic changes involve the basal ganglia or long tracts such as corticospinal tract or posterior column. Clinically, patients with ADCA present with progressive ataxia involving gait, articulation and eye movements, variable degree of brainstem dysfunction such as bulbar palsy or parkinsonian symptoms.

Historically, prior to deciphering the genetic etiology of ADCAs, the classification of these conditions was confounding. Harding et al. in 1988 initially developed an authoritative classification according to clinical findings and pathology for the diseases (Harding 1988). Autosomal dominant cerebellar ataxia characterized by cerebellar-plus-brainstem syndrome is classified as type 1, which for example includes SCA 1 to 4, 8, and 17. Autosomal dominant cerebellar ataxia with a progressive cerebellar-plus-brainstem-plus-retinopathy is grouped into type 2, which corresponds to

SCA 7. ADCA with a "pure cerebellar" syndrome is defined as type 3, which for example includes SCA 5, 6, and 10. Currently, genetic categorization takes the place of Harding's classification.

INTRONIC MICROSATELLITE REPEAT EXPANSIONS, AUTOSOMAL DOMINANT CEREBELLAR ATAXIAS (ADCAS) AND SPINOCEREBELLAR ATAXIA TYPE 10

As described above, SCA10 belongs to a class of neurodegenerative disorders which are categorized as type 3 microsatellite repeat expansion disorders, and also as type 3 ADCAs. It is a unique type of neurodegenerative disease both clinically and genetically, due to the fact that the patient with SCA10 presents with seizure in addition to pancerebellar ataxia, and it has a large intronic penta-nucleotide expansion of ATTCT repeats in the *ATXN10* gene on chromosome 22q13.1 (Matsuura et al. 2000). In addition to the ATTCT repeat expansions in SCA10, another novel penta-nucleotide repeat expansion has recently been reported to be the disease causing mutation in spinocerebellar ataxia type 31 (SCA 31) (Sato et al, 2009; Sakai et al. 2010). The underlying mutation in SCA31 was found to be a fairly large expansion (varies in the range of 2.5 to 3.8 kb) of complex penta-nucleotide repeats containing (TGGAA)n, (TAGAA)n, and (TAAAA)n in the intronic region shared by 2 different genes, *BEAN* (brain expressed, associated with Nedd4) and *TK2* (thymidine kinase 2), on chromosome 16q22.1 (Sato et al. 2009). Among the 3 different penta-nucleotide repeats, the TGGAA was the only repeat in which large repeats segregated with the phenotype, suggesting the importance of expanded TGGAA repeats in the SCA31 pathogenic mechanism. Gel electrophoretic mobility-shift assay (EMSA) showed that essential splicing factors, serine/arginine-rich splicing factors SFRS1 and SFRS9, bind to expanded (UGGAA)n sequences in vitro. However, whether these splicing factors are sequestered by the mutant UGGAA repeats *in vivo*, and such aberrant RNA-protein interactions result in

inappropriate splicing and whether aberrant splicing contributes to the disease phenotypes is yet to be established. Also it is not clear whether expanded repeats interact with additional RNA-binding proteins and contribute to the pathogenic mechanism of SCA31. Development of an appropriate fly and/or animal model should provide key insight into the pathophysiology of SCA31.

Importantly, a recent discovery has shown that large expansions of a novel GGCCTG hexa-nucleotide repeats within the intronic region of the *NOP56* gene is the disease causing mutation in hereditary spinocerebellar ataxia type 36 (SCA 36) (Kobayashi et al. 2011). Analyses of lymphoblast cells from patients with SCA36 showed the presence of GGCCUG RNA aggregates or foci similar to the RNA foci observed in DM1 or DM2 cells (Kobayashi et al. 2011). Presence of GGCCUG RNA foci in SCA36 patients' cells suggests that this disease might also be one of the RNA-gain-of-function diseases. Furthermore, presence of RNA processing/splicing factors such as SRSF2 in the GGCCUG foci in patients cells strongly suggest that protein sequestration by the mutant RNA and associated splicing abnormalities probably contribute to the pathogenic mechanism in SCA36. Moreover, identification of the full spectra of RNA-binding proteins that interact with the expanded GGCCUG RNA in vivo and are sequestered into the GGCCUG aggregates or foci will be essential and of paramount importance to obtain important insight into the pathophysiology of SCA36. Development of a fly or mouse model will be very critical to delineate the mechanistic insight into the pathophysiology of SCA36.

These recently identified cerebellar ataxias e.g., SCA31 and SCA36 are now considered to be in the class of a growing number of toxic RNA diseases that exhibit RNA-mediated gain-of-function mechanisms such as myotonic dystrophies type 1 and 2, SCA8, SCA10, and fragile X–tremor ataxia syndromes.

CLINICAL DESCRIPTION OF SCA10

In 1998, Grewal et al. first described a distinct form of SCA in several Mexican families. They reported a novel autosomal dominant cerebellar ataxia which was characterized by dysarthria, gait and limb ataxia, and nystagmus without any major brainstem or long-tract abnormalities (Grewal et al. 1998). They excluded any pre-existing diagnoses of SCA1, 2, 3, 4, 5, 6, and 7 in these patients by both mutation and genetic linkage analysis.

In 1999, Matsuura et al. analyzed four generations of Mexican family members, who were clinically characterized by pure pancerebellar ataxia, and for the first time reported the existence of a previously unknown form of ataxia in the affected individuals (Matsuura et al. 1999). Interestingly, presence of seizure was also described as part of the clinical presentation in this form of SCA with two affected individuals. Careful genetic analyses excluded the possibility of the existence of other forms of established ataxias in these patients. These two key findings by Grewal et al. (1998) and Matsuura et al. (1999) were seminal in delineating the inheritance pattern in this novel type of motor coordination disease and was designated as spinocerebellar ataxia type 10 (SCA10) (Matsuura et al. 2000).

In 1999, Zu et al. and Matsuura et al, independentely localized the candidate gene of this new form of SCA in chromosome 22, and designated it as *ATXN10* gene (Zu et al. 1999; Matsuura et al. 2000). The genomic DNA analysis indicated that *ATXN10* is comprised of 12 exons spanning a 172.8 kb region, and is translated into a 745-amino acid protein which was designated as ataxin-10. *ATXN10* is known to be expressed in various tissues, but the expression is the highest in the brain, heart, and muscle.

In a landmark discovery in early 2000, Matsuura et al. used genetic analyses to show the occurrence of expanded microsatellite repeats in the *ATXN10* gene (Matsuura et al. 2000). Matsuura et al. investigated the possibility of the presence of microsatellite repeats in *ATXN10* gene since numerous previous reports had revealed the presence of expanded CAG repeat sequences in various SCA subtypes such as SCA 1, 2, 3, 6 and 7 and these disorders showed a similar inheritance pattern to SCA10 (Matsuura et al. 2000).

EXPANSION OF INTRONIC ATTCT MICROSATELLITE REPEATS IN SCA10

In a ground breaking discovery, a unique and fairly large ATTCT penta-nucleotide repeat expansion was identified in intron 9 of the *ATXN10* gene in all patients with SCA10 from five Mexican families (Matsuura et al. 2000). Moreover, the pathological range of the ATTCT repeats was determined by subsequent sequence analysis, which revealed the presence of 10 to 32 ATTCT repeats in the normal individuals, and >800 ATTCT repeats in the affected individuals with SCA10 (Matsuura et al. 2006).

In the following year, analysis of additional 18 patients with SCA10 from four Mexican families by Rasmussen et al. (2001) not only confirmed the initial clinical and genetic findings by Matsuura et al. (2000), but also indicated that the pathogenic numbers of ATTCT repeats in SCA10 patients are large and range from 920 to 4140. (Rasmussen et al. 2001). This study also determined the mean age of onset of clinical signs, generally occurring in the mid-twenties. Notably in this study, generalized or complex partial seizures were reported in 13 individuals out of 18, where they failed to describe any evidence of extracerebellar abnormalities on clinical evaluation and imaging studies that can account for seizure. Importantly, seizures have been known to contribute

significantly to the morbidity and mortality of this disease, but the mechanism by which the ATTCT repeat expansion in SCA10 leads to the development of seizure remains largely unknown.

ATAXIN-10

Human *ATXN10* gene consists of 12 exons and is 172.8 kb in length, which encodes the protein of ataxin-10. The normal physiological function of ataxin-10 is still unknown, and requires further investigation. Ataxin-10 belongs to the family of armadillo repeat proteins and in solution it tends to form homotrimeric complexes, which associate via a tip-to-tip association in a horseshoe-shaped contact with the concave sides of the molecules facing each other. The predominant location of ataxin-10 within cell is known to be cytoplasmic and predominantly expressed in the brain, heart and skeletal muscles. A previous study by Marz et al. revealed that targeted inactivation of ATXN10 in neuronal cells (by treating neuronal cells with ATXN10 siRNA) resulted in increased apoptotic death of the primary cerebellar neurons (Marz et al. 2004). The reason why down-regulation of ATXN10 resulted in neuronal death is not yet understood and definitely needs further investigation. Another study by Waragai et al. (2006) demonstrated that over-expression of the ATXN10 gene facilitated neurite extension in the PC12 cells (Waragai et al. 2006). However, the detailed molecular mechanism by which ectopic expression of ATXN10 facilitated neurite extension in cultured neuronal cells is not clear. Together, these studies did not clearly establish whether altered or diminished function of Ataxin-10, due to ATTCT repeat expansion, contributes to SCA10 pathogenic mechanism.

THE PATHOGENIC MECHANISMS OF SCA10

TRANSCRIPTION OF THE MUTANT *ATXN10* GENE IN SCA10

The genetic mutations in several microsatellite repeats expansion disorders are the expansion of CAG tri-nucleotide repeat sequences within the coding region of the mutant genes. The extended CAG sequences in the processed transcripts are translated into poly glutamine (poly-Q) tract proteins. The mutant proteins containing extended poly-Q sequences invariably misfold, induce misfolding of other proteins, and form toxic protein-protein aggregates. Collectively, these disorders are known as Poly-Q diseases and the extended poly-Q sequences are the primary toxic entity in these disorders, and contribute to the pathogenic mechanism. Because the expanded ATTCT repeat tract in SCA10 was found to be located in the non-coding region (intron 9) of the *ATXN10* gene, the possibility that the repeat is translated to produce a mutant toxic protein was ruled out. In the case of DM1, the expanded CTG repeats have been shown to form highly condensed chromatin structures, and cause transcriptional silencing of the neighboring genes. To address this possibility in the SCA10 pathogenic mechanism, Wakamiya et al. (2006) investigated the effect of the ATTCT repeat expansion on the transcription and processing of the *ATXN10* and genes that flank the *ATXN10* locus (Wakamiya et al. 2006). Analyses of the steady state transcript levels of *ATXN10* and neighboring genes indicated that expression of *ATXN10* and its neighboring genes remain unaltered in SCA10. The analyses revealed that the mutant *ATXN10* allele is transcribed efficiently and the pre-mRNA containing an expanded AUUCU repeat is spliced normally. These findings ruled out the possibility that massive expansions of ATTCT repeats in the *ATXN10* locus causes transcriptional silencing of the neighboring genes and contributes to the disease phenotypes in SCA10.

HAPLOINSUFFICIENCY OF *ATNX10*

The early hypothesis was that massive expansion of ATTCT repeats resulted in the silencing of the *ATXN10* mutant allele and haploinsufficiency of *ATXN10* contributes to SCA10 pathogenic mechanism. To test this possibility, and to establish whether loss of *ATXN10* function contributes to SCA10 pathogenesis, the *ATNX10* mutant mouse line was generated by Wakamiya et al. (2006). As the homozygous deletion of *ATXN10* caused embryonic lethality, it was not possible to examine the extent to which complete loss of *ATXN10* function contributes to SCA10 pathology in the adult mouse. Heterozygous deletion of *ATXN10* produced viable mice. Importantly, the heterozygous *ATXN10* adult mice failed to recapitulate any detectable SCA10-like motor phenotypes. This study supported the hypothesis that the haploinsufficiency of *ATXN10* gene due to massive expansion of ATTCT repeats might not be the underlying mechanism involved in SCA10 pathogenesis (Wakamiya et al. 2006).

EFFECT OF MASSIVE ATTCT REPEAT EXPANSION ON THE TRANSCRIPTION OF THE NEIGHBORING GENES THAT FLANK THE MUTANT *ATXN10* LOCUS

To exclude the possibility that the expanded ATTCT repeat in SCA10 affects the transcription of genes that are present on either side of the mutant *ATXN10* gene locus, the levels of expression of the genes flanking the *ATXN10* locus were determined in cells from SCA10 patients. The real time RT-PCR analyses of the total RNA from SCA10 cells showed that there was no consistent change in the level of the neighboring gene transcripts in SCA10 compared to normal samples. This finding indicated that massive ATTCT repeat expansion does not affect the expression of the neighboring genes that flank the *ATXN10* locus (Wakamiya et al. 2006). These analyses ruled out the possibility that massive expansion of ATTCT repeats in the 9th intron of *ATXN10* gene causes transcriptional silencing of neighboring genes and contributes to SCA10

pathology. Both RT-PCR and Western blot analyses supported the idea that massive ATTCT repeat expansion did not alter the steady state level of *ATXN10* mRNA levels in SCA10.

As we have discussed above, the studies described thus far have failed to establish how the clinical features of this dominantly inherited ataxia could be caused by the massive expansion of intronic penta-nucleotide AUUCU repeat sequences. In the present study, in conjunction with our previous report in 2010 (White et al. 2010), which will be briefly presented in Chapter 2, we investigated the possible mechanisms by which a highly expanded non-coding ATTCT repeat can cause neurodegeneration, and give rise to the unique phenotype of SCA10.

CHAPTER 2: HYPOTHESIS AND SPECIFIC AIMS

Spinocerebellar ataxia type 10 (SCA10) is a non-coding repeat expansion genetic disorder of unknown molecular pathophysiology. SCA10 is known to be the second most common autosomal dominant cerebellar ataxia in Mexico and Brazil. The patients with SCA10 present with a gait abnormality, which is usually followed by pancerebellar ataxia and rapidly progressed to complete disability (Ashizawa 2006; Rasmussen et al. 2001; Teive et al. 2004). About 60% of Mexican patients with SCA10 develop epilepsy, particularly complex partial or generalized seizures, which may potentially lead to life-threatening status epilepticus (Grewal et al. 2002; Rasmussen et al. 2001). The genetic mutation which is known to cause SCA10 is the massive expansion of an ATTCT pentanucleotide repeat sequence. It is present within the 9th intron of the *Ataxin-10* (*ATXN10*) gene on chromosome 22q13.31 (Matsuura et al. 2000). The ATTCT repeat is polymorphic in length, with the number of repeats in normal individuals ranging from 10 to 29, and the repeat lengths in the two alleles might be the same or different. In contrast, the repeat length in one of the *ATXN10* alleles in patients with SCA10 is expanded up to 4,500 repeats, whereas the intermediate allele sizes vary in the range of 280-850 repeats (Matsuura et al. 2006; Raskin et al. 2007). Thus the SCA10 repeat expansion represents one of the largest microsatellite expansions known to occur within the human genome. The mechanisms by which the non-coding ATTCT repeat expansion leads to degeneration and dysfunction of the nervous system in SCA10 needs to be more clearly understood in order to develop a rational molecular approach to therapy. In recent studies, several neurodegenerative diseases have been found to have non-coding expanded repeats as the genetic etiology, similar to SCA10.

RNA-MEDIATED PATHOGENIC MECHANISMS IN MYOTONIC DYSTROPHY TYPE 1 AND 2

The underlying pathogenic mechanism(s) of the non-coding microsatellite repeat expansion-associated genetic diseases e.g., Myotonic dystrophy types 1 and type 2 (DM1 and DM2) are being extensively studied (Mastroyiannopoulos et al. 2010; Ashizawa 2012). The disease causing mutation in DM1 is the expansion of polymorphic CTG tri-nucleotide repeats in the 3' untranslated region (UTR) of the protein kinase gene *DMPK* (Mahadevan et al. 1992). In contrast, the mutation in DM2 is the massive expansion of CCTG tetra-nucleotide repeats within the 1st intron of the *ZNF9* gene (Christina et al. 2001).

A large number of recent studies support the hypothesis that the expanded CTG and CCTG sequences are transcribed and the expanded CUG and CCUG sequences, encoded in the mutant *DMPK* and *ZNF9* transcripts in DM1 and DM2 respectively, are deposited as insoluble aggregates or foci in the affected tissues (Taneja et al. 1995; Davis et al. 1997). Subsequent studies have shown that the insoluble aggregates, are, in fact, the RNA-protein complexes formed by the elongated CUG and CCUG RNA with various RNA-binding proteins. The "RNA gain of function" model for DM1 and DM2 suggests that the mutant transcripts encoding the extended repeat sequences are the primary toxic molecules in the pathogenic mechanisms. According to this novel hypothesis, these mutant transcripts elicit pathogenicity by binding and sequestering specific RNA-processing factors, and either loss or diminished function of the proteins that complex with the mutant repeat-containing RNA eventually lead to the manifestation of the DM1/DM2 phenotypes.

In our recent study, we have shown that the expanded ATTCT-repeats, encoded in the mutant *ATXN10* allele, are efficiently transcribed and spliced out. The spliced out

introns containing extended AUUCU repeats remain unprocessed, and form the RNA-protein complexes. These complexes are deposited as insoluble aggregates in the cytosol and nuclei in SCA10 cells (White et al. 2010). From the perspective that the expanded CUG/CCUG repeats are highly toxic and play crucial toxic roles in the pathophysiology of DM1/DM2 (Mastroyiannopoulos et al. 2010), we postulated that the mutant AUUCU sequences present in the spliced out introns might be toxic and contribute to the pathogenic mechanism. Therefore we investigated the possible trans-dominant toxic effect of the expanded AUUCU RNA repeats which might also form complexes with specific RNA-binding proteins, and the loss-of-function of the sequestered proteins contribute to SCA10 pathogenesis.

We performed RNA FISH analyses of the SCA10 fibroblasts with an antisense RNA probe against the expanded AUUCU repeats and demonstrated that the AUUCU-containing nuclear/intracellular RNA foci are indeed deposited in the SCA10 fibroblasts. Based on this finding, we hypothesized that the expanded AUUCU RNA repeats play an important role in the pathogenic mechanism of SCA10.

THE ROLE OF THE MUTANT AUUCU RNA IN THE PATHOGENIC MECHANISM OF SCA10

Expanded AUUCU repeats form discrete aggregates or foci in SCA10 cells

To determine whether expanded AUUCU RNA form aggregates or foci in SCA10 cells, we performed RNA FISH analysis using a Cy-3-labelled (AGAAU)5 antisense RNA probe. We first stained SCA10 fibroblasts with the Cy-3-labeled antisense probe and found widespread occurrence of AUUCU-containing nuclear/intracellular RNA foci in SCA10 cells (Figure 2-1). With this finding, we hypothesized that AUUCU-containing

RNA foci might harbor specific RNA-binding proteins as has been observed in cases of other microsatellite-expansion-related diseases e.g., DM1 and DM2.

Identification of the proteins that complex with the expanded AUUCU RNA repeats

Previously, in order to identify the RNA-binding proteins that might be sequestered into the AUUCU RNA aggregates or foci, we performed RNA pull down assay and analyzed the pulled-down proteins by mass spectrometry. In this procedure, we first synthesized a biotin-labeled AUUUCU RNA (~500 repeats) using a T7 RNA polymerase in vitro RNA synthesis kit (Roche) as we described previously (White et al. 2010). We next incubated the biotin-labeled (AUUCU)5 RNA oligomer with total protein extract from mouse brain and then pulled down the AUUCU-RNA-protein complexes with magnetic bead-coated streptavidin. The proteins that were pulled down along with the biotin-labeled AUUCU RNA, but not with control RNA, were analyzed by mass spectrometry. Using this methodology, we identified a known RNA-binding protein,

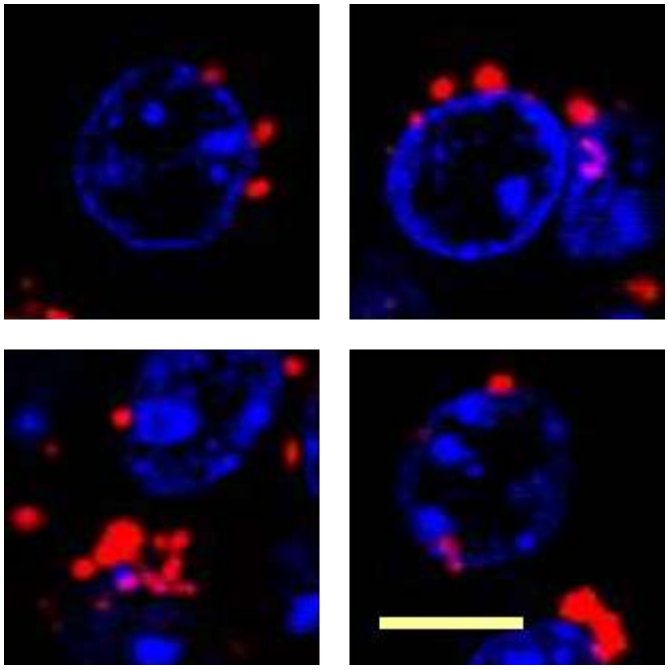


Figure 2-1. RNA foci in SCA10 cells.
RNA fluorescence *in situ* hybridization (FISH) shows nuclear/intracellular AUUCU foci (red) along with nuclei (blue) in fibroblasts from patients with spinocerebellar ataxia type 10 (SCA10). RNA foci are detected, using Cy-3-labelled $(AGAAU)_5$ antisense RNA probe, and nuclei are stained with 4,6 diamino-2-phenylindole dihydrochloride (DAPI). Scale bar represents 10 µm.

hnRNPK which was repeatedly and reproducibly precipitated with the AUUCU RNA but not with the control RNA (White et al. 2010). Subsequently we showed that hnRNPK is present within the AUUCU aggregates in the SCA10 transgenic mouse brain and in SH-SY5Y neuroblastoma cells ectopically expressing ~500 AUUCU repeats (White et al. 2010). Our results showed that the mutant AUUCU-RNA has a highly specific and strong binding affinity for hnRNPK. Furthermore, we demonstrated that sequestration of hnRNPK into the AUUCU aggregates leads to apoptotic neuronal death via intracellular translocation of PKC delta into mitochondria (White et al. 2010).

Sequestration of AUUCU RNA-binding protein in SCA10 brain

In the previous study, however, we could not exclude the possibility that in addition to hnRNPK, additional RNA-binding proteins having high sequence or structural homology with hnRNPK, might also complex with the mutant AUUCU RNA and thus might be sequestered into the aggregates in SCA10 brain. We postulated that identification of additional AUUCU-RNA-binding proteins might provide important insight into the pathogenic mechanism of SCA10. The most critical sequence/structural motifs that are responsible for the RNA-binding property of hnRNPK are the K-homology (KH)-domains. In the present study, we explored the possibility whether expanded AUUCU RNA sequences complex with and sequester neuronal proteins encoding the homologous RNA-binding KH domains in SCA10 brain and explored whether this aberrant RNA-protein interaction might contribute to complex SCA10 pathology.

To achieve this goal, we used in vitro cell culture SCA10 models (human neuroblastoma SH-SY5Y cells transiently expressing various lengths of expanded AUUCU repeats), a SCA10 transgenic mouse line expressing ~500 AUUCU repeats in

brain and a SCA10 patient's postmortem brain tissue to explore the aberrant RNA-protein interactions and delineate the underlying pathophysiology of SCA10. We believe that identifying novel neuronal proteins that complex with mutant AUUCU RNA and thus are sequestered into the AUUCU RNA aggregates not only should provide important insight into the molecular mechanism by which aberrant interaction of AUUCU-RNA with neuronal proteins contribute to neuronal death and neurological deficiencies in SCA10 but also will allow us to develop potential therapeutic approaches aimed at reversing the SCA10 phenotype.

HYPOTHESES

We demonstrated that the neuron-specific RNA-binding protein Nova, which encodes three hnRNPK-like RNA-binding K-homology (KH) domains, shows strong and significant affinity for the expanded AUUCU RNA *in vitro*. The goal of this study was to establish whether Nova interacts with mutant AUUCU RNA *in vivo* and thus was sequestered into the AUUCU RNA aggregates or foci in the SCA10 patient's and SCA10 transgenic mouse brains. Our central hypothesis was as following:

The expanded AUUCU sequences encoded in the mutant ATXN10 transcripts sequester Nova, which leads to altered or diminished function of Nova. Inactivation or diminished function of Nova, due to its sequestration into the AUUCU RNA foci, results in aberrant splicing of the Nova target genes in SCA10 brain. These splicing defects of the Nova target genes regulating electrophysiological properties contribute to the neuronal dysfunction and development of complex neurological phenotype in SCA10 (Figure 2-2).

We used several biochemical, biophysical and molecular biological techniques to analyze the neuronal SH-SY5Y cells ectopically expressing ~500 AUUCU repeats, the brain tissue from the SCA10 transgenic mice and SCA10 patient in order to test the hypothesis that sequestration of Nova into the AUUCU RNA aggregates or foci is a major pathogenic mechanism in SCA10 and this aberrant AUUCU-RNA-Nova interaction contributes to neuronal loss and/or development of complex neurological deficiencies in SCA10.

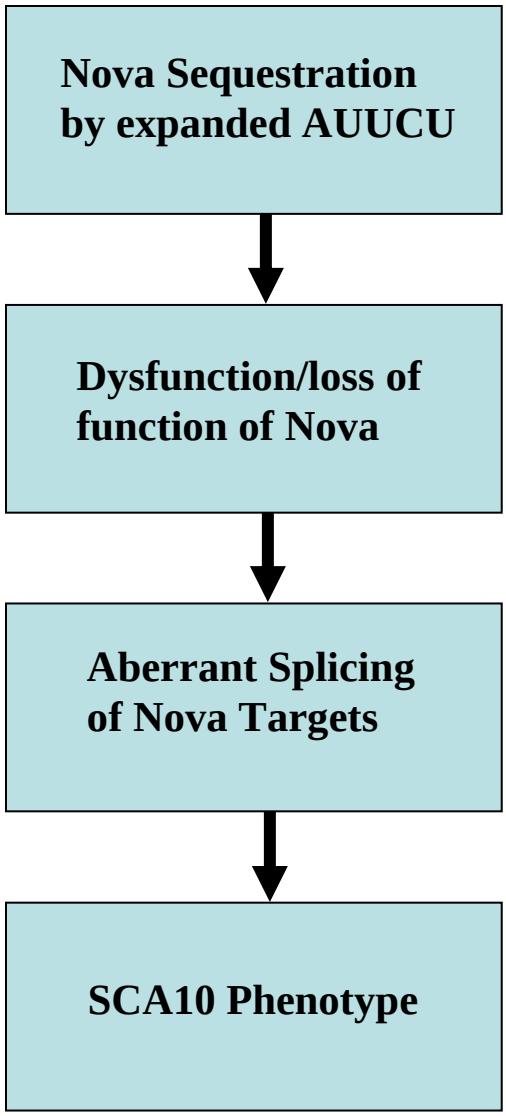


Figure 2-2. Central Hypothesis.
The goal of this study is to understand the mechanism by which expanded AUUCU repeat causes ataxia and seizure in SCA10.

SPECIFIC AIMS

We tested our hypothesis by pursuing the following two specific aims (Figure 2-3).

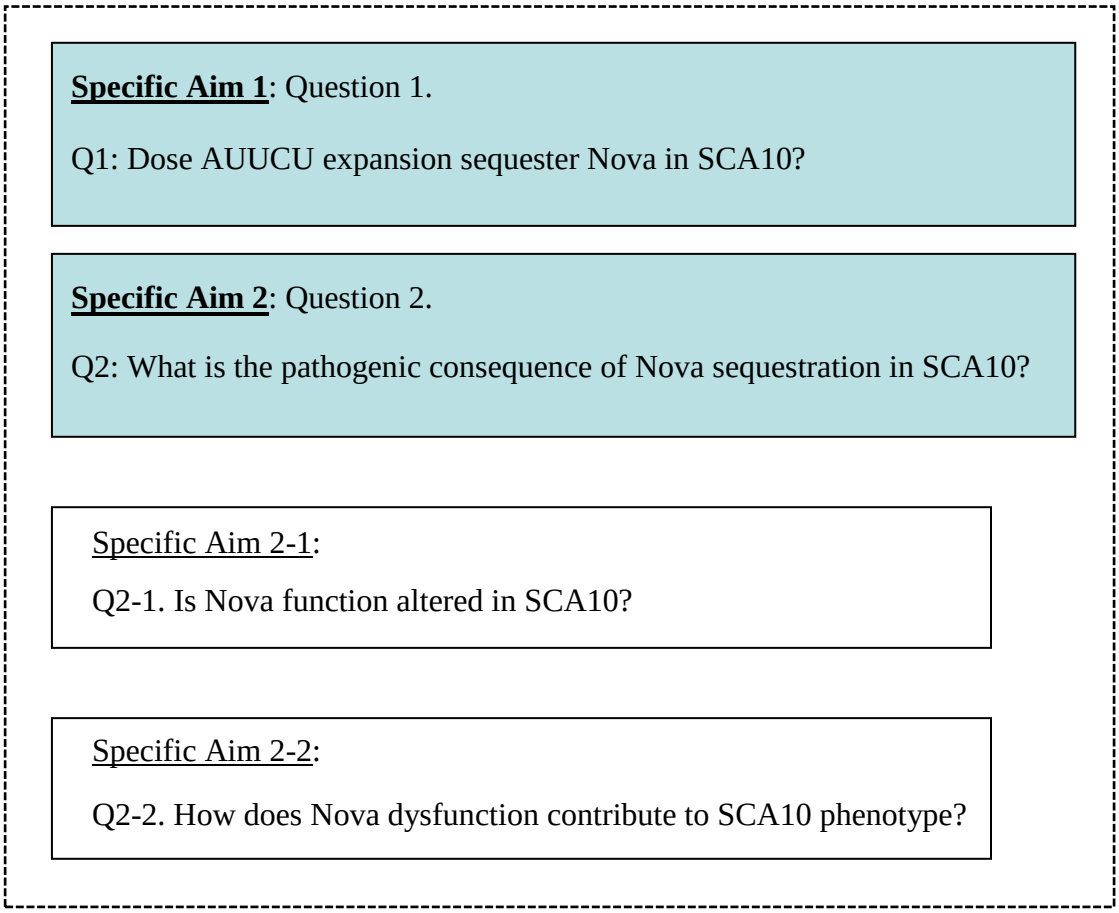


Figure 2-3. Questions in the Specific Aims.
In this study, we investigated the above questions.

Specific Aim 1: Determined that Nova was sequestered into the aggregates or foci formed by the expanded AUUCU RNA repeats in the SCA10 patient's brain, and in SCA10 transgenic mouse brain ectopically expressing ~500 intronic AUUCU repeats

In this specific aim, we used various biochemical and biophysical techniques to determine the binding specificities of Nova with the pathological lengths of AUUCU RNA repeats. We determined whether Nova is present in the AUUCU-RNA-protein aggregates in the SCA10 transgenic mouse brain as well as in SCA10 patients' brain.

Specific Aim 2: Demonstrated Nova dysfunction and its pathogenic consequences.

Specific Aim 2-1: Determined that sequestration of Nova into the AUUCU RNA foci resulted in aberrant splicing of the Nova target genes in SCA10 brain

Nova is known to regulate alternative splicing of several genes that play critical roles in regulating neuronal function, electrophysiological properties and motor coordination. In this aim, we analyzed alternative splicing patterns of several of the known Nova target genes in SH-SY5Y cells transiently expressing various lengths of expanded AUUCU RNA repeats, SCA10 transgenic mouse brain ectopically expressing ~500 AUUCU RNA repeats and in SCA10 patient's brain.

Specific Aim 2-2: Determined that aberrant splicing of Nova targets led to the development of SCA10-like clinical phenotype

To determine whether aberrant splicing of the Nova target genes leads to the clinical phenotype of SCA10, we tested the neurophysiologic effect(s) as a functional consequence of the expanded AUUCU RNA repeats in the SCA10 transgenic mice.

Clinical manifestations of SCA10 are characterized by pancerebellar ataxia and seizure. We have developed a novel transgenic mouse line expressing a pathogenic length of AUUCU repeats (~500 AUUCU repeats) in brain to evaluate the extent to which expression of expanded AUUCU repeats contributes to the development of SCA10-like neurological defects. In this aim, we characterized the neurophysiologic defects and further explored the molecular pathophysiology of SCA10 to identify a functional neurophysiological consequence of the AUUCU RNA repeat in the SCA10 transgenic mice.

CHAPTER 3: SPECIFIC AIM 1. ABERRANT INTERACTION OF NEURONAL PROTEIN NOVA WITH MUTANT AUUCU RNA REPEATS

INTRODUCTION

Our recent study showed that the pathogenic lengths of AUUCU RNA sequences complex with a widely-studied RNA-binding protein, hnRNPK and the RNA-protein complexes formed by hnRNPK and AUUCU repeats are deposited as insoluble aggregates in the SCA10 cells (White et al. 2010). Furthermore, our studies provided strong evidence that either loss or diminished function of hnRNPK in SCA10 results in neuronal cell death. Our study suggested that alteration/inactivation of hnRNPK activity via its aberrant interaction with the mutant AUUCU RNA, might contribute to neuronal loss and progressive cerebellar atrophy, one of the important SCA10 phenotypes. However, the mechanism by which the expression of extended AUUCU RNA results in characteristic SCA10 phenotypes e.g., seizure and ataxia needs to be further investigated. In the present study, we focused on identifying additional neuronal proteins that could possibly bind with the mutant AUUCU RNA repeats and contribute to SCA10 pathology. In addition, we investigated whether the newly identified RNA-binding proteins are indeed sequestered into the AUUCU RNA foci in SH-SY5Y cells ectopically expressing ~500 AUUCU repeats, in the SCA10 patient's brain and SCA10 transgenic mouse brain.

The nucleic acids-binding motifs in hnRNPK: The K-Homology (KH) nucleic acid-binding domains

The RNA-binding sequence/structural motifs in hnRNPK are known as the hnRNPK homology domain or KH-domain, which was first described in hnRNPK in 1994 (Siomi et al. 1994). There are three distinct KH domains present in hnRNPK and these domains have been recognized as the single-stranded nucleic acid binding motifs. Numerous recent studies show that hnRNPK preferentially binds with the pyrimidine-rich motifs present both in RNA and DNA and is involved in the regulation of a wide range of cellular functions.

The lengths of the KH domains vary from protein to protein and usually consist of 70-100 amino acids that bind to specific pyrimidine-rich nucleotide sequences. The KH domains are primarily found in proteins involved in transcriptional regulation and other intracellular functions. The RNA-binding proteins other than hnRNPK that contain the well-conserved KH domains include vigilin, human FMR1 (Musco et al. 1996), Nova-1 and Nova-2 (Lewis et al. 2000), ribosomal protein 3 (Wimberly et al. 2000), and ERA (Chen et al. 1999).

The functional significance of the KH-domains

The KH domains play an important role in RNA binding, as indicated by the study of Siomi et al (Siomi et al. 1994). Siomi et al. showed that a mutation in the KH domain of the FMR1 gene significantly disturbs the RNA binding capacity, which eventually leads to the development of some of the typical phenotype of fragile X syndrome (Siomi et al. 1994).

The most conserved sequence in the KH domain is designated as GXXXGXXG, which is also referred to as the 'KH domain signature', and resides in the center of the KH domain (Musco et al. 1996). This sequence has a notable evolutionary significance, since it is highly conserved in various organisms such as bacteria, archaea, and eukaryotes. The study of the secondary structures of the KH domain revealed it contains either a maxi-KH domain which presents βααββα in sequence, or a mini-KH domain, with βααββ in sequence. Studies for crystal structure of KH domains reveal the presence of β1-α1-α2-β2-β3-α3 topology (Lewis et al. 1999; Teplova et al. 2011).

Nova

Since our previous study demonstrated a significantly high binding affinity between the AUUCU repeat RNA and hnRNPK in SCA10 (White et al. 2010), we hypothesized that in addition to hnRNPK, additional KH-domain-containing neuronal proteins might complex with the expanded AUUCU RNA repeats, and thus preventing them from their normal cellular function, contributing to the complex SCA10 phenotypes.

The Nova proteins belong to the large class of KH-type family of RNA-binding proteins. Nova was initially described as an antigen in a paraneoplastic syndrome called POMA (paraneoplastic opsoclonus-myoclonus ataxia) (Musunuru et al. 2001). It has a KH domain highly homologous to hnRNPK. The function of Nova is known to regulate several of the brain-specific alternative splicing events including the pre-mRNA of inhibitory neurotransmitter receptors, GABA and Glycine. Nova is known to bind consensus sequences which are primary transcripts containing UCAY motifs.

Interestingly, Nova proteins are known to be functionally related to hnRNPK, where they are involved in cytoplasmic translocation of RNA transcripts from the nucleus (Michael et al. 1997), and regulate LOX-15 (lipoxygenase-15) mRNA translation in red blood cells (Ostareck et al. 1997).

The objective of the present study was to test our central hypothesis that in addition to hnRNPK, additional RNA-binding proteins containing the KH-domain(s) complex with the mutant AUUCU RNA repeats in SCA10 brain, and understanding this unusual RNA-protein interaction should provide important insight into the molecular pathogenic mechanism of SCA10.

RESULTS

Nova contains highly conserved RNA-binding sequences, homologous to the KH-domain of hnRNPK

The hnRNPK and Nova share common structural motifs, the hnRNPK homology (KH) domain, which provides a structural basis for single-stranded mRNA or DNA binding. Evidence suggests that the KH-domains interact directly with single-stranded RNA through its signature sequence, and mutation in the KH domains abrogates its nucleic acid-binding properties.

To determine the alignment of the KH-domain in hnRNPK and Nova proteins, we analyzed and aligned sequences using Vector NTI software (Invitrogen Co.). Amino acid sequence alignment suggests that the KH-domain sequences of hnRNPK and Nova are highly conserved (Figure 3-1).

Figure 3-1. Highly conserved KH domain sequences of hnRNPK, Nova-1 and -2. Amino acid sequence alignment shows that the KH-domain sequences of hnRNPK, Nova-1 and -2 are highly conserved.

The hnRNPK contains three KH-domains that mediate its specific interaction with the target sequences. Interestingly the KH-domains in Nova harbor additional conserved sequences in its N- and C-terminal which are also known to be required for specificity of RNA-binding. The alignment of amino acid sequences of hnRNPK and Nova reveals that the GXXXGXXG sequence motifs present in the KH-domains are highly conserved (Musco et al. 1996).

Nova shows strong affinity for AUUCU repeats and forms RNA-protein complex under normal physiological conditions *in vitro*

Sequence analyses of hnRNPK and Nova revealed that the KH-domains present in hnRNPK and Nova share a significant sequence homology. Nova contains three KH-domains, similar to that observed in hnRNPK. Our previous studies have shown that the expanded AUUCU RNA shows a strong affinity for hnRNPK both *in vivo* and *in vitro* (White et al. 2010). Since Nova contains three highly conserved hnRNPK-like KH domains, we hypothesized that the mutant AUUCU RNA might complex with Nova and this unusual and aberrant RNA-protein interaction *in vivo* might alter/diminish Nova activity, and altered/diminished function of Nova might contribute to complex neurological pathology in SCA10. To investigate whether the expanded AUUCU RNA

binds Nova, we first determined the specificity and strength of bi-molecular interaction of the extended AUUCU repeat RNA and Nova using an RNA solution-binding assay described in White et al. (2010).

To determine whether expanded AUUCU RNA specifically interacts with Nova, we performed the RNA solution binding assay using purified Nova. To achieve this goal, we purified Nova using gel-filtration affinity chromatography as described in White et al. (2010). The purified Nova was then incubated with the single-stranded 5′-biotin-labeled synthetic $(AUUCU)_{15}$ RNA molecules at room temperature overnight to allow the formation of RNA-protein complex under normal physiological conditions. Nova was then serially extracted from the RNA-protein complex with HEPES buffer containing increasing salt (sodium chloride) concentrations. The extracted protein fractions were analyzed using PAGE and the Nova was detected by Western blot analysis using anti-Nova antibody. The Western blot data demonstrates that Nova could be extracted from the AUUCU-RNA-protein complex only with higher salt concentration ($\geq$ 150 Mm) but not with lower physiological salt concentration, suggesting a significantly higher affinity of Nova for the $(AUUCU)_{15}$ RNA. In contrast, Nova was completely dissociated from the control RNA molecules at significantly lower ($\leq$ 100 mM) salt concentrations (Figure 3-2). This finding suggested that Nova binds tightly to the expanded AUUCU RNA repeats.

Nova shows marked co-localization with the AUUCU RNA foci in SH-SY5Y cells ectopically expressing pathogenic lengths of AUUCU repeats

The *in vitro* solution binding assay described above suggested the possibility that Nova might also bind with the mutant AUUCU RNA repeats *in vivo*. To investigate this possibility, we first evaluated the possible co-localization of Nova with the extended

AUUCU RNA foci in SH-SY5Y cells. To achieve this goal, we co-transfected SH-SY5Y cells with two plasmids: one expressing ~500 AUUCU repeats, and the second expressing GFP-tagged human Nova. The *FISH* analysis of the double-transfected SH-SY5Y cells with cy3-labeled antisense $(AGAAU)_5$ RNA probe showed distinct and highly significant co-localization of the red fluorescence from the ~500 AUUCU RNA repeats, and the green fluorescence from the GFP-tagged Nova (Figure 3-3). This finding substantiated our central hypothesis that Nova might complex with the expanded AUUCU RNA repeats *in vivo* and be present as RNA-protein aggregates or complexes in the double-transfected SH-SY5Y cells.

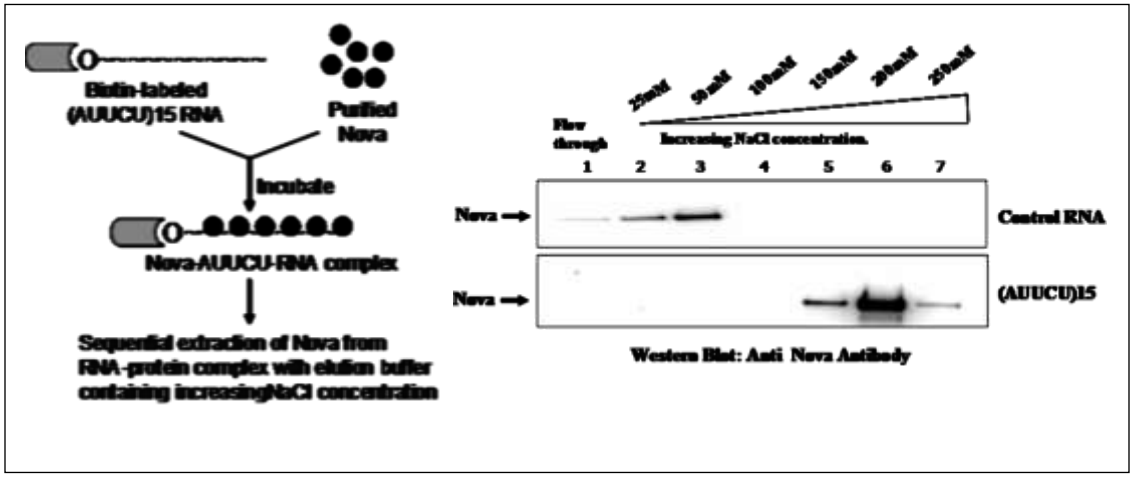


Figure 3-2. RNA Solution Binding Assay.
Purified Nova was incubated with biotin-labeled $(AUUCU)_{15}$ RNA or control RNA, and sequential extraction of Nova from RNA-protein complex with elution buffer containing increasing NaCl concentration was performed. Finally extraction was analyzed through western blot. Top blot is Nova extracted from control RNA-protein complex and the bottom one is Nova extracted from $(AUUCU)_{15}$ RNA-protein complex. It shows Nova protein is extracted from RNA-protein complex only with higher salt concentration ($\geq$ 150 mM), but is completely dissociated from the control RNA at significantly lower salt concentration ($\leq$ 50mM).

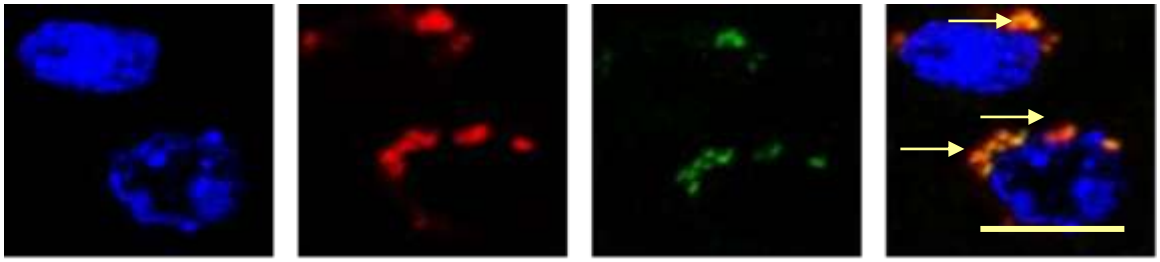


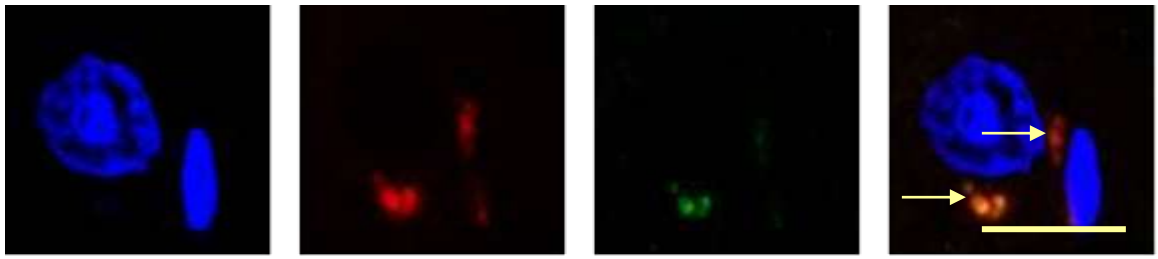
Figure 3-3. Co-localiztion of AUUCU-RNA and Nova in SH-SY5Y cells. SH-SY5Y cells were co-transfected with AUUCU repeat and GFP-tagged human Nova. RNA foci are detected using Cy-3-labelled (AGAAU) antisense RNA probe through RNA fluorescence *in situ* hybridization (FISH). Analysis shows distinct co-localization of the red fluorescence from AUUCU RNA and the green fluorescence from Nova protein. Yellow/Orange fluorescence indicates overlap of red and green fluorescence (arrow). Nuclei are counterstained with 4, 6 diamino-2-phenylindole dihydrochloride (DAPI). Scale bar represent 10µm.

The AUUCU RNA foci show strong co-localization with endogenous Nova in the SCA10 transgenic mouse brain ectopically expressing ~500 AUUCU repeats

To further investigate the possible *in vivo* interaction between the expanded AUUCU repeats and Nova, we examined whether Nova shows any significant co-localization with the aggregates formed by the mutant AUUCU RNA in the SCA10 transgenic mouse brain. We have developed a transgenic plasmid DNA construct which harbors the expanded ATTCT repeats (~500 ATTCT repeats) within the rabbit β-globin intron cloned upstream of the *LacZ* reporter gene and downstream of the human α-enolase promoter genes (White et al. 2010). Using this transgenic construct, we generated a novel transgenic mouse line that expresses ~500 AUUCU repeats in brain. Our study demonstrated that the transcripts containing the ~500 AUUCU repeats formed distinct nuclear as well as cytosolic aggregates (White et al. 2010). We carried out RNA *FISH* analysis on the sagittal section of the SCA10 transgenic mouse brain and found a significant co-localization of hnRNPK with the AUUCU RNA (White et al. 2010). To

further establish whether Nova also interacts with the AUUCU RNA in vivo, the AUUCU repeat-containing aggregates were detected by RNA *FISH* (Cy3 fluorescent-labeled antisense probe), and Nova was visualized with immunofluorescence-tagged anti-Nova antibody in mouse brain. The RNA *FISH* and immunofluorescence analysis of the transgenic mouse brain shows highly significant co-localization of AUUCU RNA foci with endogenous Nova in the sagittal sections of hippocampus CA1 (Figure 3-4A) and cerebellar cortex (Figure 3-4B) from the 6-month-old SCA10 mice (n=6). In contrast, no detectable RNA foci or its co-localization were observed in the control mouse brains (Figure 3-5).

A



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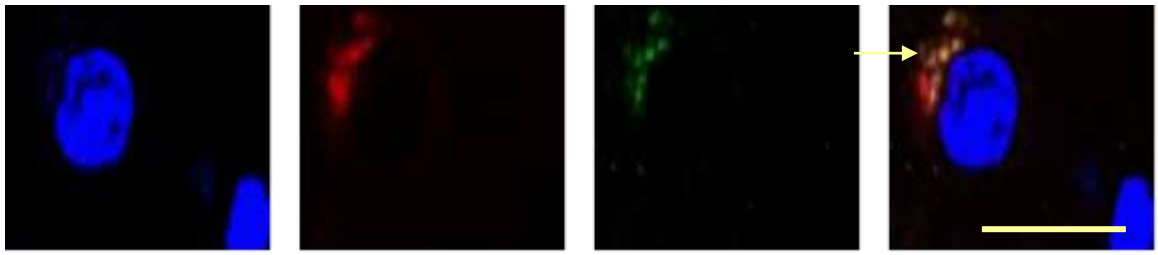


Figure 3-4. Co-localization of AUUCU RNA and Nova protein in transgenic SCA10 mouse expressing intronic ~500 AUUCU repeat.
Expanded AUUCU RNA forms aggregates which were detected by RNA fluorescence *in situ* hybridization (FISH), and Nova was visualized with immunofluorescence-tagged anti-Nova antibody in SCA10 transgenic mouse brain expressing ~500 AUUCU repeat. Analysis shows co-localization of AUUCU repeat (red) with endogenous Nova (green) in sagittal section of hippocampus CA1 (Fig. 3-4A) and cerebellar cortex (Fig. 3-4B) from SCA10 transgenic mouse. Yellow/Orange fluorescence indicates overlap of red and green fluorescence (arrow). Nuclei are counterstained with 4, 6 diamino-2-phenylindole dihydrochloride (DAPI). Scale bar represents 10 µm.

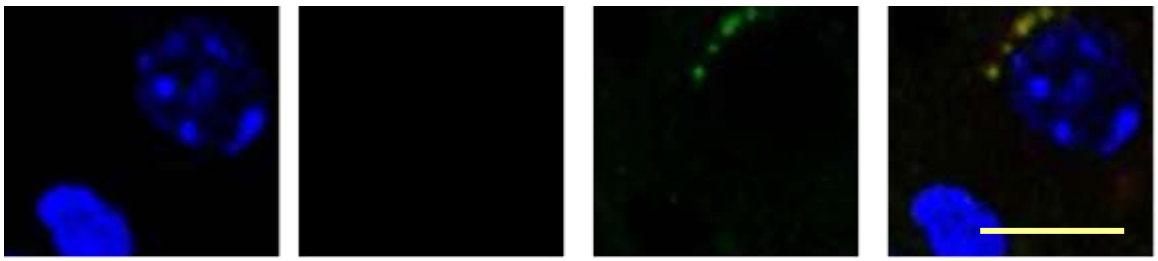


Figure 3-5. RNA foci or its co-localization of AUUCU RNA and Nova protein are not detected in control mouse.
RNA fluorescence *in situ* hybridization (FISH) analysis does not show any distinct RNA foci on the sagittal section of wild type mouse brain. Endogenous Nova was detected with immunofluorescence-tagged anti-Nova antibody (green). There is no detectable co-localization of Nova with RNA in wild type mouse brain. Nuclei are counterstained with 4, 6 diamino-2-phenylindole dihydrochloride (DAPI). Scale bar represents 10 µm.

Nova co-localizes with the AUUCU RNA foci in SCA10 patient's brain.

To further confirm the *in vivo* interaction of the expanded AUUCU RNA repeats and Nova in SCA10 patients' brain, we examined co-localization of endogenous mutant *ATXN10* transcripts with endogenous Nova in the patient's brain. Sagittal sections from SCA10 patient's brain and control brains were analyzed by *FISH*; Nova was detected with anti-Nova antibody and AUUCU RNA aggregates were detected with Cy3-labelled antisense RNA probe as described above. Distinct and marked co-localization of endogenous *ATXN10* transcripts encoding expanded AUUCU repeats with endogenous Nova was observed in the cerebellar cortex of an SCA 10 patient. In contrast, control human brain did not show any detectable foci or co-localization under identical experimental conditions (Figure 3-6).

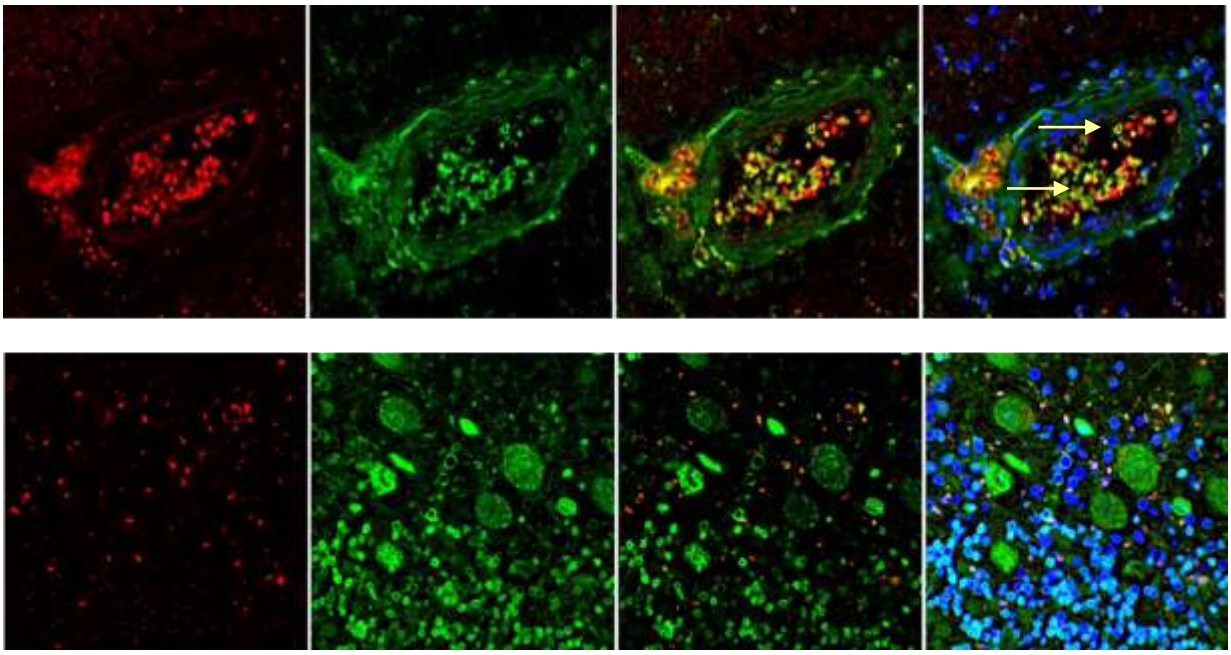


Figure 3-6. Co-localization of AUUCU RNA and Nova in human brain.
RNA fluorescence *in situ* hybridization (FISH) analysis shows the distinct co-localization of AUUCU RNA with endogenous Nova in SCA10 human brain (upper row). RNA foci are detected using Cy-3-labelled (AGAAU) antisense RNA probe (red fluorescence). Nova was visualized with immunofluorescence-tagged anti-Nova antibody (green fluorescence). Yellow/Orange fluorescence indicates overlap of red and green fluorescence (arrow). In contrast, control human brain does not show any distinct co-localization (lower row). Nuclei are counterstained with 4, 6 diamino-2-phenylindole dihydrochloride (DAPI).

SUMMARY/DISCUSSION AND FURTHER STUDY

Several hereditary neurological disorders are found to be caused by expanded microsatellite repeats in non-coding portions of genes (Todd et al. 2010). For instance, DM1 is attributed to the expansion of a polymorphic CTG tri-nucleatide repeats located in the 3' untranslated region (UTR) of the *DMPK* gene. A large number of recent studies suggest that the expanded CTG repeats are efficiently transcribed and the extended CUG sequences, encoded in the mutant *DMPK* transcripts, complex with specific RNA-binding

proteins including muscle blind (MBNL1) protein and CUG-BP1, and aberrant RNA-protein interactions result in inappropriate splicing of multiple target pre-mRNA molecules, leading to multi-organ pathophysiology in DM1.

In the present study, we have provided strong evidence that support our central hypothesis that Nova interacts with the mutant AUUCU RNA repeats *in vitro*, in cell culture, transgenic mouse brain and in SCA10 patient's brain. As described in DM1, ATTCT expansion in SCA10 is transcribed to AUUCU repeats, and this lengthy AUUCU repeat sequences sequester RNA-binding proteins e.g., hnRNPK and Nova. Since Nova plays a crucial role in maintaining neuronal integrity in the nervous system, we hypothesize that this aberrant RNA-protein interaction in patients' brain would result in partial or complete inactivation of Nova, leading to aberrant splicing of the Nova target genes and neurological phenotypes in SCA10. The present consensus is that Nova regulates alternative splicing of several target genes in the central nervous system (CNS) and therefore studying the aberrant splicing of the Nova target genes in SCA10 should provide important insight into the mechanism of disease pathogenesis.

In the next chapter, we used SH-SY5Y cells and SCA10 mouse model to perform experiments focusing on determining the Nova dysfunction in SCA10 pathogenic mechanism to obtain further evidence that supports our central hypothesis that the gain-of-function of AUUCU-RNA repeats leading to either diminished or loss of function of Nova is one of the primary contributors of SCA10 pathogenic mechanism. In addition, we determined that the splicing regulatory function of Nova is altered in SCA10.

CHAPTER 4: SPECIFIC AIM 2. ABBERANT SPLICING OF NOVA TARGET GENES IN SCA10

INTRODUCTION

Alternative splicing

Differential processing of pre-mRNAs, commonly referred to as alternative or alternate splicing, is one of the most important and highly efficient cellular mechanisms to provide cells different sets of proteins from a single RNA precursor, ultimately leading to cellular and functional diversity. Alternative splicing also defines characteristic expression: differential, spatial and temporal, depending on cell type. Distinct functions and structures of cells determined by alternative splicing play a fundamental role in maintaining the integrity and complexity of neuronal function in nervous system.

Since 1982, when Amara et al. first demonstrated alternative splicing in the *CGRP* gene (Amara et al. 1982), various patterns of alternative splicing from many genes have been discovered. Subsequently, the reports for differentially spliced isoforms of major neurotransmitter receptor subtypes including glutamate (Sommer et al. 1990), NMDA (Hollmann et al. 1993), and $GABA_A$ (Whiting et al. 1990) receptors followed. The physiologic or pathologic significance of most of these splice variants has been actively studied and needs to be elucidated further.

Nova

Nova is known to be exclusively expressed in the CNS, and was originally identified as a target antigen in a paraneoplastic neurological condition, POMA (paraneoplastic opsoclonus myoclonus ataxia) (Darnell 1996), a condition wherein patients develop characteristic inhibitory motor defects including opsoclonus, myoclonus, and ataxia. Nova is also well known to harbor three KH-domains, which are involved in interactions with specific nucleotide sequence.

Nova has been found to function as a neuron-specific splicing factor, and regulates alternative splicing of a group of genes in the CNS. There are consensus sequences including HYCAY motifs (H indicates A, C, or U and Y indicates C or U) (Jensen et al. 2000), which are usually located closely in the target exon of pre-mRNAs. Nova binds to these cluster motifs through mediation of its KH-domains, and regulates splicing of the target pre-mRNAs. However, the exact Nova target sequence motifs have not been conclusively established.

Targets of Nova have been widely investigated where they have been found to be closely involved in inhibitory synaptic functions (Ule et al. 2005). Pre-mRNAs of GlyRα2 and $GABA_AR\gamma2$ are two inhibitory neurotransmitter receptor subunits which were identified as Nova target RNAs.

Splicing regulatory function of Nova

Nova has been shown to regulate alternative splicing of GlyRα2 exons E3A and E3B, which are known to encode a crucial component of the extracellular domain of the GlyRα2 receptor. These exons are known to be spliced in or out in a mutually exclusive

fashion in presence/absence of Nova. The intronic sequence $(UCAU)_3$ of the GlyRα2 pre-mRNA transcript, known to be Nova binding motif, is located 80 nucleotides upstream of an exon, E3A. Once Nova binds the target RNA sequence during transcription, the inclusion of E3A is enhanced, while the exclusion of a downstream exon, E3B occurs simultaneously. The alternative splicing of E3A and E3B exons in the GlyRα2 gene by Nova provides a different set of the extracellular domain of GlyRα2, ultimately leading to different properties of the receptor ligand binding (Kuhse et al. 1991).

$GABA_AR\gamma2$ is another important transcript, the alternative splicing of which has been shown to be regulated by Nova (Dredge et al. 2003). As a cassette, exon E9 of $GABA_AR\gamma2$ is alternatively spliced, it generates two mRNA isoforms, E9L (long) and E9S (short). Interestingly, the E9L exon encodes an additional 8 amino acids that are expressed in an intracellular loop of $GABA_AR\gamma2$. These sequences of amino acids are found to harbor a protein kinase C phosphorylation site, which further modulates $GABA_A$ receptor function. Once Nova binds the Nova- intronic motif, (YCAY) which is located 80 nucleotides upstream of exon E10 of $GABA_AR\gamma2$, it causes enhancement of exon E9L inclusion and exon E9S exclusion at the same time.

In a previous study by Jensen et al. using Nova knock-out (KO) mice (Jensen et al. 2000), a characteristic phenotype of inhibitory motor defect as well as characteristic aberrant splicing pattern of GlyRα2 and $GABA_AR\gamma2$ in the absence of Nova was demonstrated. Analysis of splicing indicated a significant decrease in utilization of GlyRα2 E3A and $GABA_AR\gamma2$ E9L in KO mice compared to wild-type. In the present study, we hypothesized that normal function of Nova would be disrupted by aberrant interaction of Nova with the mutant AUUCU RNA in SCA10, leading to aberrant splicing of Nova targets in SCA10 patient's brain. Because Nova protein is sequestered

by lengthy AUUCU RNA repeats in SCA10 which causes depletion of intracellular levels of Nova, decreased level of Nova will eventually cause the splicing defect observed in Nova null mice. In this chapter, to determine whether the RNA-Nova interaction sequesters Nova from its splicing regulatory function, we assessed alternative splicing of Nova target genes, including two important inhibitory neurotransmitter receptors, GlyRα2 and $GABA_AR\gamma2$.

In the previous chapter, we used cell culture model, SCA10 mouse model and SCA10 patient's tissue to demonstrate that Nova binds with expanded AUUCU repeats *in vivo* in a highly specific manner. In this chapter, we investigated whether the function of Nova was altered due to the inappropriate interaction of Nova with the mutant AUUCU RNA repeats in SCA10.

RESULTS

Alternative splicing of GlyRα2 and $GABA_AR\gamma2$ transcripts

Briefly, during transcription, exon 3A of GlyRα2 is included in the presence of Nova, whereas exon 3B is excluded in the absence of Nova (Figure 4-1). In similar fashion, the E9L of $GABA_AR\gamma2$ RNA is predominantly expressed in presence of active Nova in brain, whereas the E9S of $GABA_AR\gamma2$ RNA is predominantly detected in the absence of Nova (Figure 4-2).

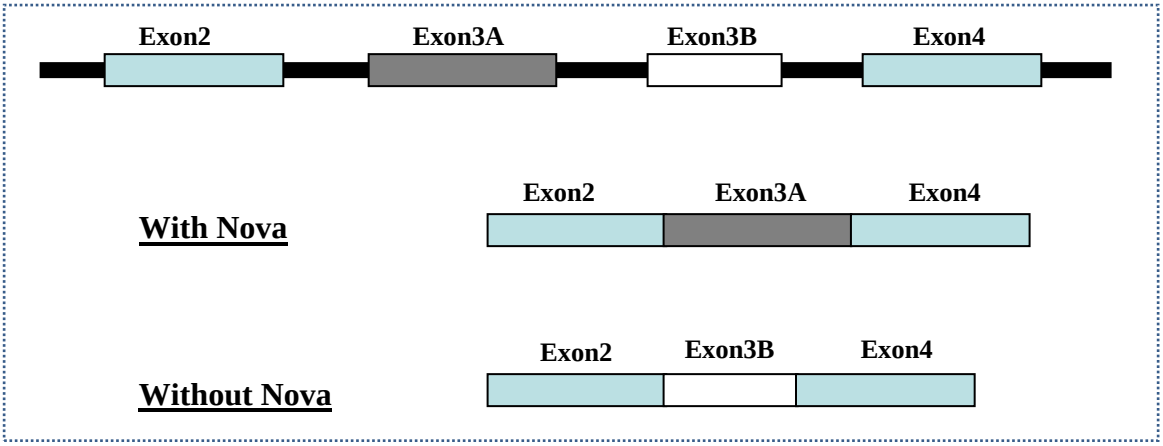


Figure 4-1. Alternative splicing of GlyRα2 and a splicing regulatory factor, Nova.

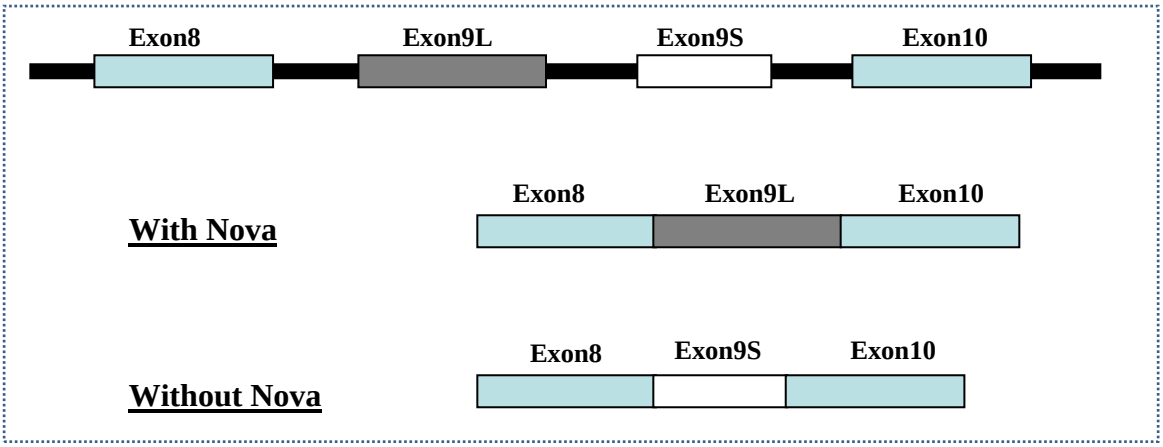


Figure 4-2. Alternative splicing of $GABA_AR\gamma2$ and a splicing regulatory factor, Nova.

Using RT-PCR analyses, we examined whether any changes in splicing isoforms occurred in various SCA10 models. Detection of any shift in splicing isoforms would indicate that the normal splicing regulatory function of Nova is altered by the RNA repeat

in SCA10. Furthermore, the splicing defect may lead to the alteration of the physiologic properties of Nova targets, contributing to SCA pathogenesis.

Aberrant splicing of GlyRα2 and $GABA_AR\gamma2$ transcripts in human neuroblastoma (SH-SY5Y) cells expressing the expanded AUUCU RNA repeats

To identify the alternative splicing pattern of GlyRα2 and $GABA_AR\gamma2$ pre-mRNAs in SH-SY5Y cells, ectopically expressing the mutant AUUCU repeats, we performed RT-PCR analysis of the total RNA isolated from cells expressing ~500 and 25 AUUCU repeats. With the assumption that the proportion of inclusion or exclusion of exons relative to the total number of transcripts alters significantly in ATTCT repeat expressing cells, we designed primers to detect two well-known splicing isoforms of GlyRα2 and $GABA_AR\gamma2$, respectively.

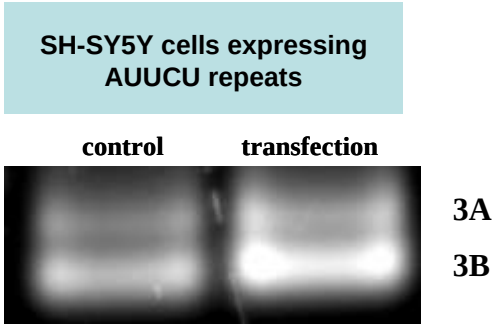


Figure 4-3. Aberrant splicing of GlyRα2 in SH-SY5Y cells expressing mutant AUUCU. RT-PCR products of control and mutant AUUCU expressing SH-SY5Y cells were analyzed and two well-known splicing isoforms of GlyRα2 were detected. The data indicate that both E3A and E3B isoforms were transcribed with E3B being relatively predominant in control SH-SY5Y cells, whereas in mutant AUUCU expressing SH-SY5Y cells, the 3A isoform was decreased, and the 3B isoform was increased significantly.

The RT-PCR analysis of total RNA isolated from SH-SY5Y cells expressing ~500 AUUCU RNA repeats showed two splice isoforms of GlyRα2; one isoform is GlyRα2 E3A ("exon 3A inclusion and exon 3B exclusion" isoform) and the other is GlyRα2 E3B ("exon 3B inclusion and exon 3A exclusion" isoform). We found that in control SH-SY5Y cells, both E3A and E3B isoforms were transcribed with

E3B being relatively predominant, whereas in SH-SY5Y cells expressing the mutant ATTCT repeats, the 3A isoform was decreased, and the 3B isoform was increased significantly (Figure 4-3).

In the same fashion, we performed RT-PCR analysis and determined the two isoforms of $GABA_AR\gamma2$ splicing, E9L ("inclusion of exon 9L and exclusion of exon 9S" isoform), and E9S ("inclusion of exon exon 9S inclusion and exclusion of exon 9L" isoform). The pattern of $GABA_AR\gamma2$ splicing revealed that the E9L isoform was more common than the E9S isoform in the control SH-SY5Y cells (expressing 25 AUUCU repeats) whereas the E9S isoform predominated in SH-SY5Y cells expressing ~500 AUUCU repeats. Significantly, the E9L isoform expressed in the normal condition was largely replaced by the E9S isoform in SH-SY5Y cells expressing the mutant AUUCU repeats (Figure 4-4).

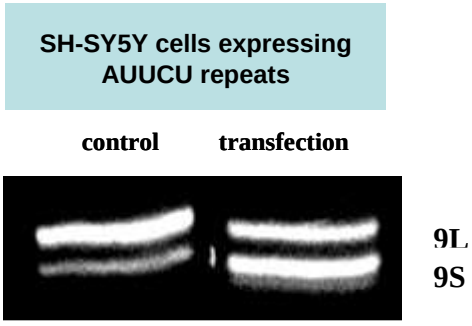


Figure 4-4. Aberrant splicing of $GABA_AR\gamma2$ in SH-SY5Y cells expressing mutant AUUCU. RT-PCR products of control and mutant AUUCU expressing SH-SY5Y cells were analyzed and two well-known splicing isoforms of $GABA_AR\gamma2$ were detected. The data indicate that the E9L isoform was more common than the E9S isoform in the control SH-SY5Y cells, whereas in mutant AUUCU expressing SH-SY5Y cells, the E9S isoform predominated in SH-SY5Y cells expressing ~500 AUUCU repeats.

Targeted inactivation of Nova results in aberrant splicing of the GlyRα2 and $GABA_AR\gamma2$ transcripts in the SH-SY5Y cells

To determine the alternative splicing pattern of GlyRα2 and $GABA_AR\gamma2$ transcripts in the Nova-depleted condition, we performed a Nova inhibition study using small interfering RNA (siRNA). Specific siRNA sequences were used for the targeted down-regulation of Nova in SH-SY5Y cells. The effect of Nova down-regulation on alternative splicing of GlyRα2 and $GABA_AR\gamma2$ transcripts was analyzed by RT-PCR in SH-SY5Y cells.

Successful down-regulation of Nova expression in SH-SY5Y cells was assessed by Western blot analysis, which showed significant decrease in the expression of Nova in the SH-SY5Y cells transfected with Nova siRNA compared with cells treated with control siRNA (Figure 4-5). Using RT-PCR analysis, alternative splicings of GlyRα2 and $GABA_AR\gamma2$ transcripts were assessed. The siRNA-mediated down-regulation of Nova resulted in dramatic shift in alternative splicing of both GlyRα2 and $GABA_AR\gamma2$ RNA, and this aberrant splicing change was similar to the splicing change that was observed in SH-SY5Y cells expressing the mutant AUUCUrepeats (Figure 4-6). In SH-SY5Y cells treated with Nova siRNA, the 3A isoform of the GlyRα2 transcript, and E9L isoform of the $GABA_AR\gamma2$ transcript were drastically decreased.

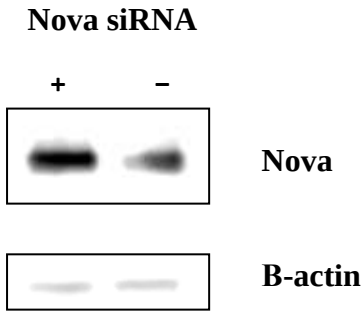


Figure 4-5. Down-regulation of Nova by siRNA. SH-SY5Y cells were transfected with100 pmoles siRNA for Nova. Western blot analysis after 72 hours transfection indicates treatment with Nova siRNA efficiently suppresses Nova expression in SH-SY5Y cells. β-actin control is shown.

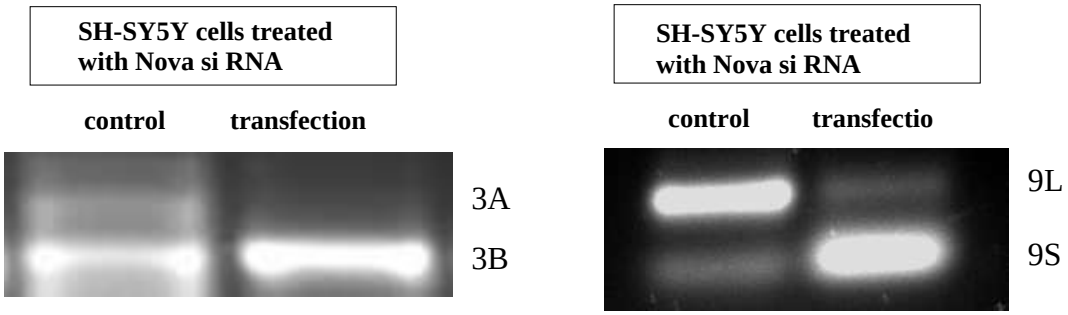


Figure 4-6. Alternative splicing of GlyRα2 and $GABA_AR\gamma2$ in SH-SY5Y cells treated with Nova si RNA.
RT-PCR products of control and Nova siRN- treated SH-SY5Y cells were analyzed and two well-known splicing isoforms of GlyRα2 and $GABA_AR\gamma2$ were detected, respectively. The utilizations of exon 3A in GlyRα2 and exon 9L in $GABA_AR\gamma2$ are significantly decreased in SH-SY5Y cells treated with Nova siRNA.

Splicing defect of Nova target genes in SCA10 transgenic mouse brain

To determine aberrant splicing of the Nova targets *in vivo*, we assayed splicing of GlyRα2 and $GABA_AR\gamma2$ in total RNA isolated from the brain of SCA10 transgenic mice. Using previously described primers for GlyRα2 and $GABA_AR\gamma2$ (Jensen et al. 2000), we performed RT-PCR analysis of total RNA from the SCA10 transgenic brain. Our data indicates that the alternative splicing of exon 3A of the GlyRα2 transcript was significantly disrupted compared to wild-type brain. There was approximately 5-fold increase in the utilization of exon 3B in RNA from transgenic mice, compared to that from wild type brain. At the same time, there was significant change in the utilization of E9S alternatively spliced $GABA_AR\gamma2$ exon as well in transgenic mice relative to wild type brain, consistent with previous observations in SH-SY5Y cells. Significantly, the E9L isoform is completely replaced by the E9S isoform in transgenic mice brain expressing the expanded ATTCT repeat (Figure 4-7).

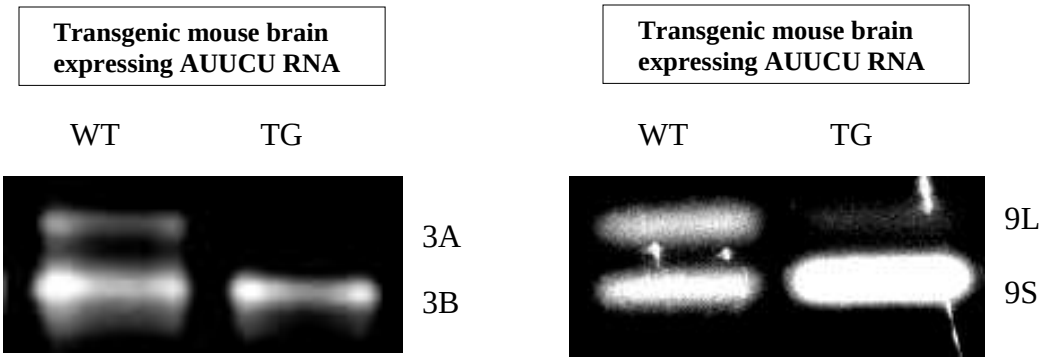


Figure 4-7. Alternative splicing of GlyRα2 and $GABA_AR\gamma2$ in SCA10 transgenic mouse brain.
RT-PCR products of wild type and SCA10 transgenic mouse brain were analyzed and splicing isoforms of GlyRα2 and $GABA_AR\gamma2$ were detected, respectively. The utilizations of exon 3A in GlyRα2 and exon 9L in $GABA_AR\gamma2$ are significantly decreased in SCA10 transgenic mouse brain.

These changes in alternative splicing in SH-SY5Y cells expressing expanded ATTCT repeats and SCA10 transgenic mice are significant, ranging from 3-fold to 10-fold depending on sample types. The bar graph representing splicing percentages of GlyRα2 were calculated as (E3A band)/(All isoform' bands) and (E3B band)/(All isoform' bands); those of $GABA_AR\gamma2$ were calculated as (E9L band)/(All isoform' bands) and (E9S band)/(All isoform' bands), respectively (Figure 4-8). The signal density of the bands was measured and quantified using Multigauge software (Fujifilm). The analysis showed a net 5-fold decrease in exon 3A use of GlyRα2 RNA in SCA10 to wild-type brain: a 10-fold decrease in use of the exon 9L isoform in $GABA_AR\gamma2$ RNA in SCA10 to wild-type brain.

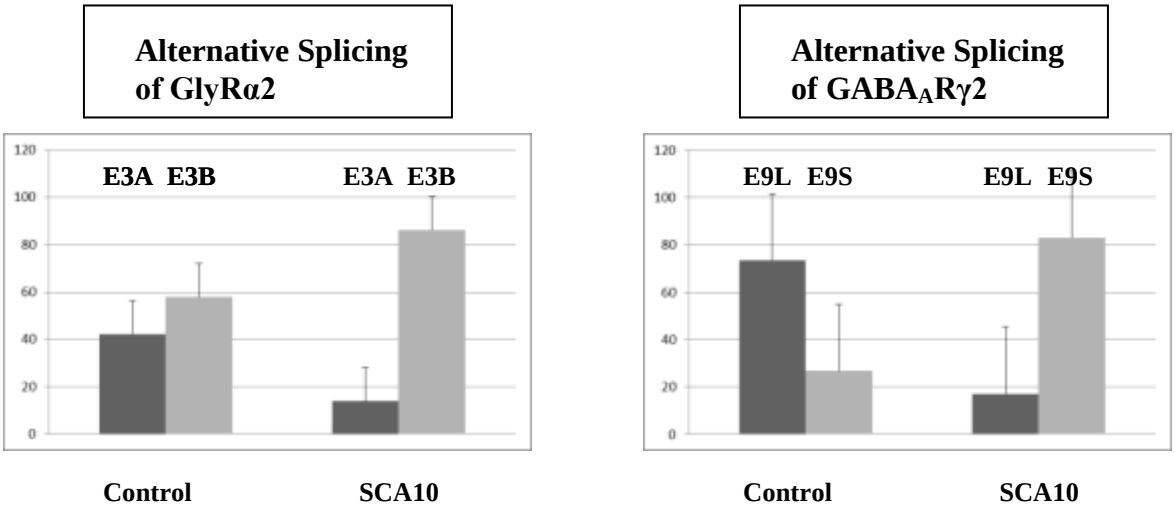


Figure 4-8. Alternative splicing of GlyRα2 and GABA$_A$Rγ2 in SCA10.
The quantitative analysis of signal density through multigauge software (Fujifilm) indicates significant decrease in exon 3A of GlyRα2 and exon 9L of GABA$_A$Rγ2, and increase in exon 3B of GlyRα2 and exon 9S of GABA$_A$Rγ2 in SCA10 transgenic mouse brain compared to wild-type brain.

Splicing defect of other Nova targets in SCA10 models

Other important Nova targets include gephyrin, neogenin, and c-Jun N-terminal kinase 2 (c-JNK2) (Ule et al. 2003), which are also known to be involved in inhibitory synaptic transmission. To determine whether the splicing patterns of these Nova targets are also altered in the SCA10 transgenic brain, we investigated the splicing of transcripts of gephyrin, neogenin, and c-JNK2 in SCA10 transgenic mouse brain. Using RT-PCR analysis and previously described primers (Ule et al. 2003), we determined the alternative splicing of the pre-RNA transcripts of gephyrin, neogenin, and c-JNK2 in the SCA10 mouse brain. Our data suggest that the splicing patterns of these targets are significantly

altered in SCA10 transgenic mice, as previously described in Nova mutant mice (Ule et al. 2003) (Figure 4-9).

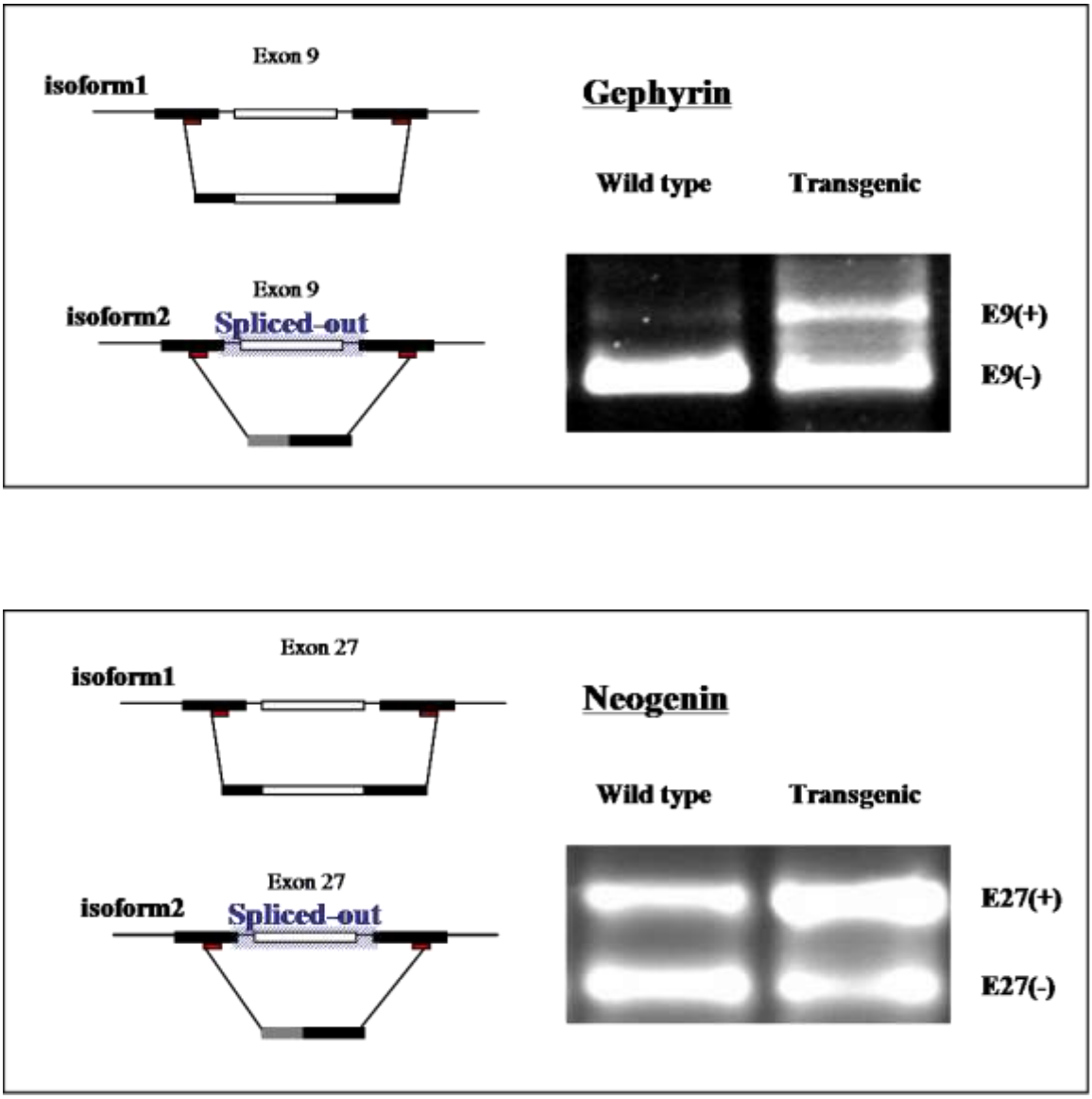


Figure 4-9. (Continued) Aberrant splicing of other Nova targets in SCA10.

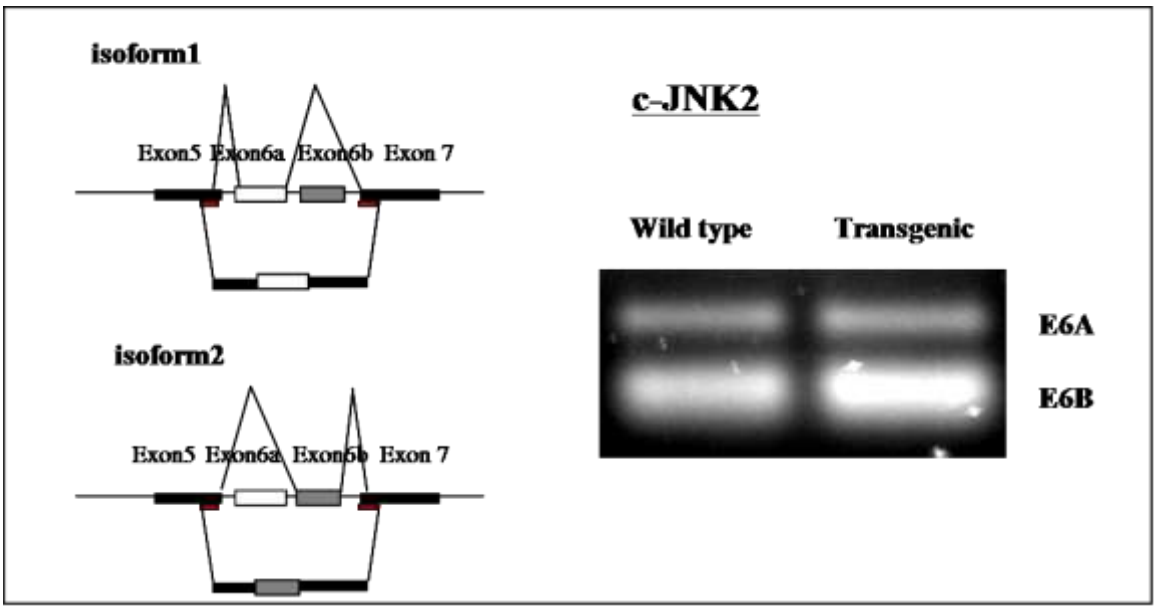


Figure 4-9. (Continued) Aberrant splicing of other Nova targets in SCA10.
RT-PCR products of wild type and SCA10 transgenic mouse brain were analyzed and splicing isoforms of gephyrin, neogenin, and c-JNK2 were detected, respectively, as previously described (Ule et al. 2003). The amount of exon 9 in gephyrin and exon 27 in neogenin, are significantly increased in SCA10 transgenic mouse brain compared to wild-type. The utilization of exon 6B in c-JNK2 is significantly increased in SCA10 transgenic mouse. Alternative splicing of the pre-RNA transcripts of gephyrin, neogenin, and c-JNK2 is significantly altered in SCA10 transgenic mouse brain.

DISCUSSION

Nova is a neuron-specific protein which plays an important role in alternative splicing regulation. Nova was originally identified in an auto-immune-mediated condition known as paraneoplastic opsoclonus-myoclonus ataxia (POMA), which was associated with the production of auto-antibodies against neuron-specific antigens in tumors (Musunuru et al. 2001). Antibodies generated in POMA patients attack targets of these antigens in the CNS, which eventually cause the neurodegeneration and neurological deficits in these patients.

POMA patients developed significant motor incoordination; impaired control of inhibitory motor neurons in the cerebellum and brainstem. Significantly, a previous study by Jensen et al. indicates that a phenotype of Nova-1-/- mice is similar to POMA patients; difficulties in controlling motor function due to atrophy of the motor neurons. Subsequently, it was hypothesized that disruption in the alternative splicing of genes involved in the inhibitory synaptic transmission leads to POMA pathogenesis (Jensen et al. 2000). Growing evidence indicates that defects in alternative splicing of the synaptic proteins such as voltage-gated calcium channels can contribute to the disease phenotypes (Faustino et al. 2003; Liao et al. 2009). Notably, Nova-1 mutant animals showed a marked phenotype, which developed severe impairment of motor function postnatally (Jensen et al. 2000).

We demonstrated abnormal splicing of Nova target pre-mRNAs e.g., receptor subunits of two of the important inhibitory neurotransmitters, GlyRα2 and $GABA_AR\gamma2$, in the SCA10 brain. We also showed that exon 3A of GlyRα2 and exon 9L of $GABA_AR\gamma2$ were aberrantly spliced in SH-SY5Y cells ectopically expressing mutant ATTCT repeats and also in SCA10 transgenic mouse brain expressing ~500 AUUCU repeats. From this analysis of alternative splicing of the Nova targets, we hypothesized that the splicing defects would result in changes of receptor properties, which might eventually contribute to the development of SCA10 phenotype.

Does the splicing defect of the Nova targets lead to the SCA10 phenotype?

The GlyRα2 and $GABA_A$ receptor are the two main types of inhibitory synaptic receptors predominantly present in the cerebellum of vertebrates. They belong to the superfamily of Cys-loop-based ligand-gated ion channels (Grenningloh et al. 1987). Two splicing variants of the GlyRα2, GlyRα2A and GlyRα2B, are distinct in the

substitution of two adjacent amino acids, V58 and T59 in GlyRα2A; and, I58 and A59 in GlyRα2B, which are located in the extracellular domain of the N-terminal. The previous study by Miller et al. indicated that the GlyRα2B isoform holds a much higher sensitivity to the agonists, β-alanine, glycine, and taurine, compared to the GlyRα2A variant. Interestingly, this study also demonstrated that substitution of the two amino acid residues in the N-terminal extracellular domain between two GlyRα2 splice variants underlie this functional difference (Miller et al. 2004), suggesting these subtle molecular differences in the molecules can lead to significantly distinct functional consequences.

The study by Jin et al. indicated that the γ2 subunit of the $GABA_A$ receptor plays an essential role in clustering as well as postsynaptic localization of $GABA_A$ receptors (Jin et al. 2004). This subunit is also known to be required for the endocytosis of $GABA_A$ receptors (Essrich et al. 1998; Kittler et al. 2000). More importantly, mutations in the γ2 subunit lead to the dysfunction of $GABA_A$ receptor which eventually results in familial epilepsy syndromes (Baulac et al. 2001; Wallace et al. 2001). Genetically manipulated mice which express only the short form of the $GABA_AR\gamma2$ subunit develop severe anxiety (Homanics et al. 1999), and higher sensitivity to benzodiazepines compared to wild-type mice (Quinlan et al. 2000). The mutant AUUCU RNA repeats abolish and/or diminish the binding of Nova to its targets leading to alteration in Nova-dependent splicing regulation in SCA10. Thus sequestration of Nova by mutant AUUCU RNA results in significant disruption of the regulation of alternative splicing of GlyRα2 and $GABA_AR\gamma2$. This finding provides an important molecular basis for understanding SCA10 pathogenesis, particularly the motor coordination defects due to ATTCT repeat expansions. It also suggests that dysfunction of Nova's splicing regulation underlies the neurologic defects observed in SCA10.

To address the pathogenic implication of the splicing defects of the Nova targets in eliciting SCA10 pathogenesis, we hypothesized that the splicing defect of the Nova targets - GlyRα2 and $GABA_AR\gamma2$ - contributes to the SCA10 phenotype. Because GlyRα2 and $GABA_AR\gamma2$ are two main receptor subunits involved in inhibitory synaptic transmission in cerebellum, we decided to determine postsynaptic inhibitory currents in Purkinje cells in cerebellum of transgenic mice (please refer to additional experiments, in Chapter 5).

CHAPTER 5: DISCUSSION AND ADDITIONAL EXPERIMENTS

Spinocerebellar ataxia type 10 is one of the autosomal dominant cerebellar ataxias (ADCAs) and the mutation in SCA10 is the expansion of a polymorphic ATTCT pentanucleotide repeats located in the 9th intron of the *ATXN10* gene. Although a major part of the pathogenic mechanism of the disease is not clear, previous studies have provided strong evidence suggesting that the expanded intronic ATTCT repeats are effectively transcribed and the mutant *ATXN10* transcripts encoding the extended AUUCU repeats are highly toxic and might play major pathogenic roles in eliciting neurological phenotypes in SCA10.

SCA10 AS A TOXIC RNA GAIN OF FUNCTION DISEASE

The present study showed compelling evidence that supports the notion that SCA10 is a toxic RNA gain-of-function disease. We demonstrated specific and aberrant interaction between the mutant AUUCU RNA repeats and RNA-binding protein, Nova in SCA10. The expanded AUUCU repeat in the 9th intron of the *ATXN10* gene attracts RNA-binding proteins to form intracellular RNA-protein aggregates which ultimately leads to impaired function of the RNA-binding proteins. Moreover, our study showed the altered function of Nova in SCA10, which could be represented by splicing defects of major inhibitory neurotransmitter receptors, Glycine and GABA. As a result of the sequestration of the RNA-binding proteins by a toxic expanded repeat, mis-splicing of several transcripts occurs.

We demonstrated a 'toxic' gain-of-function effect of the AUUCU-repeat RNA in neuronal cells ectopically expressing expanded AUUCU-RNA repeats, SCA10 transgenic mouse model and in human brain tissues. Our study strongly supports the hypothesis that aberrant RNA-protein interaction between the mutant AUUCU repeat RNA with specific RNA-binding proteins is the major pathogenic mechanism of SCA10. In this regard, the pathophysiological consequence of mRNA toxicity must be dissected for better understanding of this non-coding repeat disorder, although its molecular mechanism is still largely unknown.

SCA10 PHENOTYPE

Previously, cerebellar atrophy was demonstrated in magnetic resonance imaging studies in SCA10 patients, which represents neurodegeneration in SCA10. It was also demonstrated that specific loss of cerebellar Purkinje cells mainly consists of decreased cerebellar volume in SCA10 patients. We pointed out 'the depletion of hnRNPK' as its possible underlying mechanism in which the expanded AUUCU-RNA repeat causes neuronal cell death. In our previous study, we postulated sequestration of RNA-binding protein, hnRNPK leads to apoptotic neuronal death through intracellular shift of PKC delta into mitochondria. The other principal clinical features of SCA10 are progressive pancerebellar ataxia and seizure, although other nervous system involvement such as peripheral neuropathy or cognitive decline may be associated. How this interaction between AUUCU RNA repeat and RNA-binding proteins contributes to the SCA10 phenotype is unknown and needs further study.

The present study demonstrated Nova depletion and subsequent splicing defects of its targets in SCA10. Previous study of the phenotypic effects of Nova depletion in

Nova knockout (KO) mice indicates significant dysregulation of the inhibitory motor function in mice. We hypothesize that sequestration of Nova by the extended AUUCU RNA repeats contributes to motor co-ordination phenotypes in SCA10 similar to the phenotypes observed in Nova KO mice. To briefly speak of the transgenic construct, as previously described (White et al. 2010), ~500 ATTCT repeats from SCA10 patient was PCR-amplified, cloned into the second intron of the rabbit β-globin gene. This intron was cloned downstream of the neuron-specific α-enolase promoter and upstream of the LacZ gene to develop the transgenic construct. The transgene containing the α-enolase promoter, β-globin intron with the ATTCT repeats, lacZ and the BGH plyA sequences was microinjected into mouse fertilized eggs, and the injected eggs were implanted into the uterus of a surrogate female. The founder mice were screened by Southern blotting and PCR analysis. Several positive founder mice were bred with wild type mice, and one F1 mouse from each line was analyzed for the expression of the ATTCT repeats. We were thus able to generate several transgenic mouse lines which express expanded pathogenic lengths of ATTCT repeats specifically in the brain. Interestingly, our SCA10 transgenic mice developed significant motor defects and seizure (Figure 5-1) (Quantitative data to be published elsewhere).

Figure 5-1. SCA10 phenotype of transgenic mouse expressing AUUCU RNA repeat in brain.
Transgenic mouse expressing ~500 ATTCT repeats in brain develops significant motor defects and seizure. Captured photos describe the sudden loss of upright posture and generalized tonic clonic seizure activity.

Since Nova KO mice were only able to survive for less than 3 weeks postnatally, it is difficult to postulate how Nova depletion fully affects the phenotype in animals. However, in patients with SCA10, it takes 2-3 decades for clinical symptoms to manifests. We postulate this phenomenon is correlated to the gradual process of sequestration of RNA-binding proteins by repeat, to reach the threshold to cause dysfunction of targets. Because alternative splicing plays crucial roles in neuronal cells to maintain integrity and integrate complicated signals among neuronal networks, we propose that splicing defects contribute to the disease phenotype in SCA10.

THE PATHOGENIC IMPLICATIONS OF ABERRANT SPLICING IN SCA10

The distinct biophysical properties of the functional splice variants may give rise to underlying conditions of the molecular pathogenesis of the SCA10 phenotype. To address the implications of aberrant splicing of inhibitory neurotransmitter receptors, and to better understand genotype-phenotype correlations in SCA10, we explored electrophysiological properties as functional consequences of defective splicing.

THE ELECTROPHYSIOLOGICAL DEFECTS IN SCA10

Cerebellar dysfunction causes ataxia, which arises from dis-inhibition of the motor control in the cerebellar neurons. GABA and glycine, two important inhibitory neurotransmitters, play important roles in eliciting inhibitory synaptic transmission in the cerebellum. Importantly, the Purkinje neurons consist of the sole output in the cerebellum by integrating inhibitory inputs from the primary afferent signals from various cerebellar neurons. Pancerebellar ataxia is one of main neurologic deficits in SCA10.

Our previous study demonstrated sequestration of Nova by the mutant AUUCU RNA in SCA10, and the aberrant splicing of receptor subunits of the major inhibitory neurotransmitters, GABA and glycine in the SCA10 cerebellum. These splicing defects may underlie the pathophysiology of ataxia, one of the cardinal SCA10 manifestations. To determine the functional consequence of the aberrant splicing of Nova targets in SCA10, we hypothesized that the splicing defects would cause disruption in the inhibitory synaptic transmissions in the SCA10 cerebellum. More specifically, the disturbance would be in the inhibitory input to cerebellar Purkinje cells. To further test our central hypothesis, we examined the inhibitory electrophysiological properties in cerebellum of the SCA10 transgenic mice.

RESULTS

To investigate the effects of aberrant splicing on inhibitory transmission to Purkinje neurons in cerebellum in SCA10, we measured miniature inhibitory postsynaptic currents (mIPSCs) in Purkinje cells of the wild type and SCA10 transgenic mice. Patch clamp recordings at Purkinje cell somata in animal brain slices were performed.

mIPSCs of Purkinje cells(PCs) in wild type and SCA10 transgenic mice

Patch clamp recordings revealed significant differences in the morphology of each tracing of mIPSC between wild type and SCA10 transgenic mice (Figure 5-2). Cumulative probability on the amplitude and inter-event interval of mIPSCs was determined. The analysis of the mIPSCs revealed significant differences between wild type and transgenic animals; there is a left shift in the probability of current amplitude,

and downward shift in the probability of the current inter-event interval in SCA10 mice (Figure 5-3).

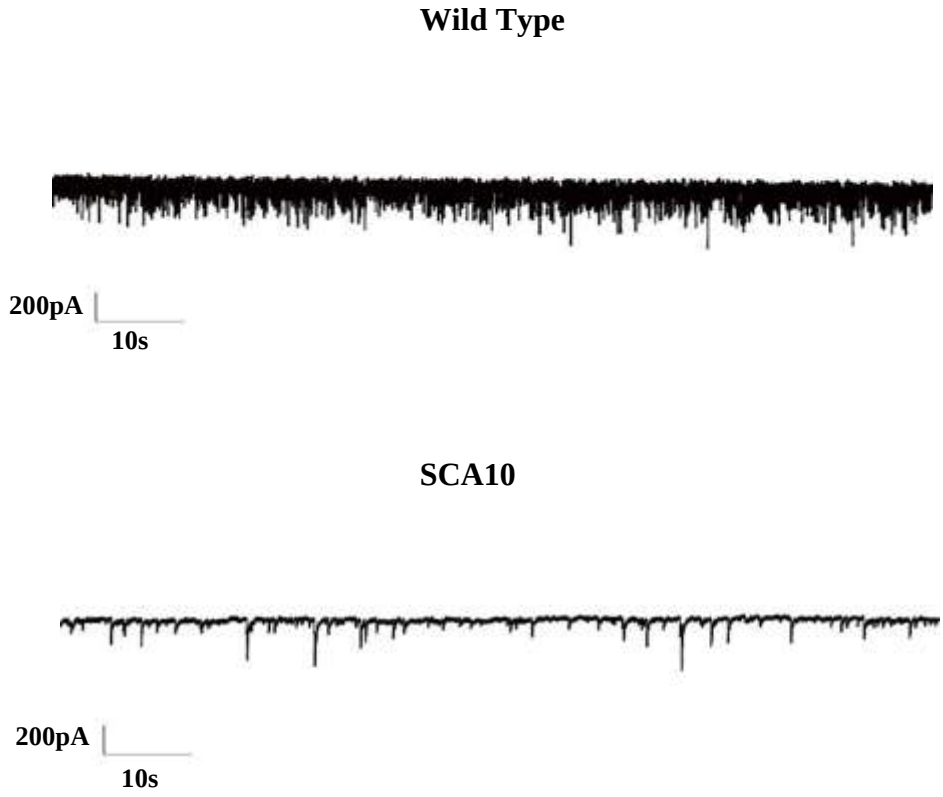


Figure 5-2. mIPSC(miniature inhibitory postsynaptic current) in Purkinje cells of wild type and transgenic mice.
The data represent typical examples of current recording containing mIPSC in Purkinje cells of wild type and SCA10 transgenic mice. The gross morphology of each tracing of mIPSCs in Purkinje cells indicates significant difference between wild type and SCA10 mice.

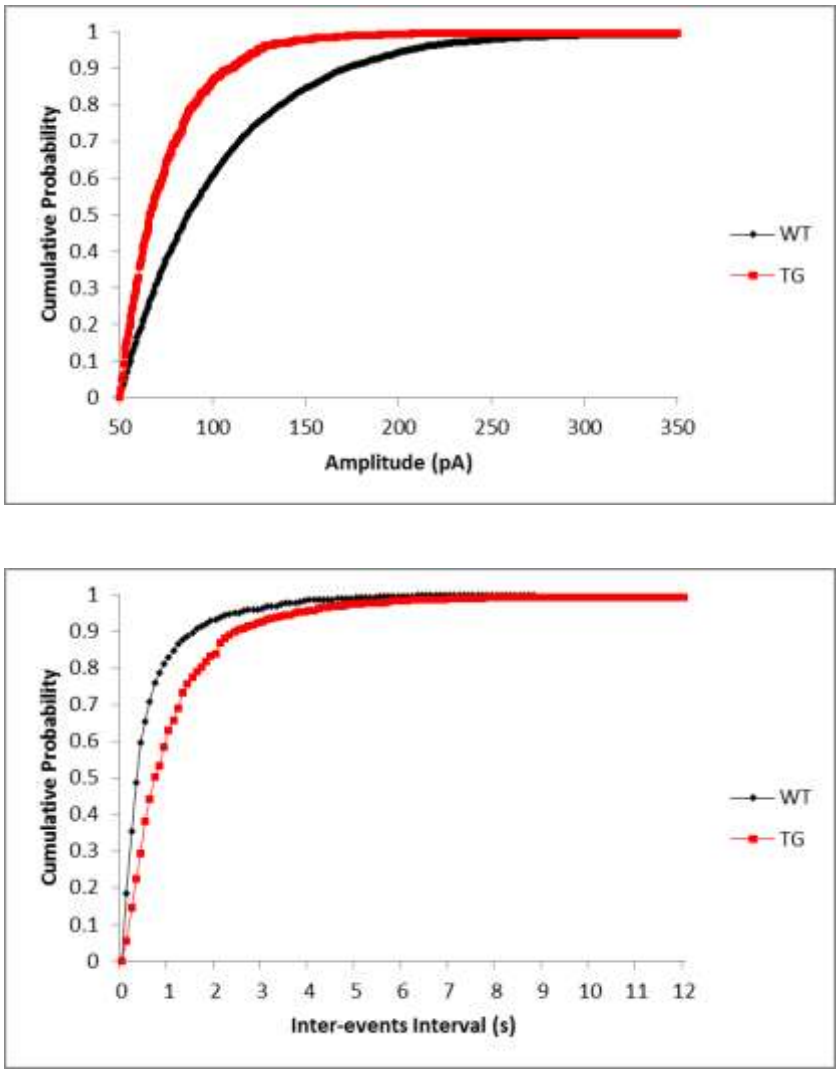
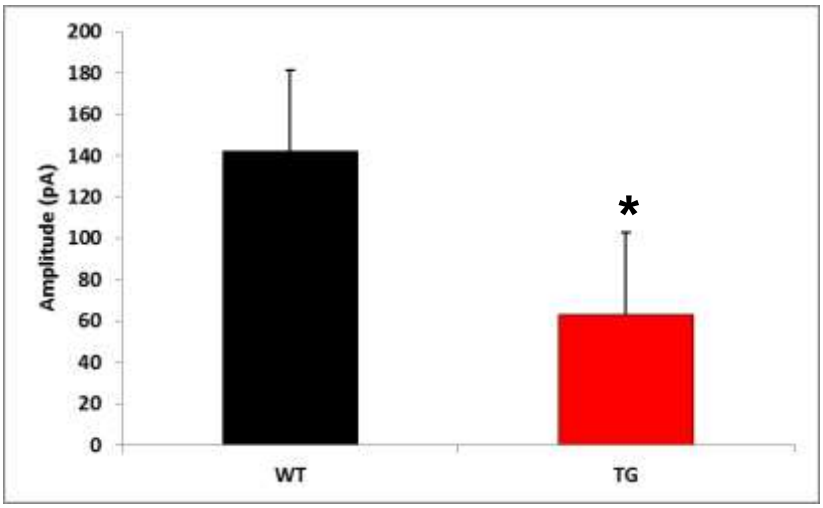


Figure 5-3. Cumulative probability on the amplitude and inter-event interval of mIPSCs in wild type and transgenic mice.
The analysis of cumulative probability indicates significant differences between wild type and transgenic mice. Left shift in the probability of current amplitude and downward shift in the probability of the current inter-event interval are notable in transgenic mouse.

Furthermore detailed analysis indicated that both the frequency as well as the amplitude of mIPSCs were significantly decreased in SCA10 transgenic mice compared to wild type. In the transgenic mice, the frequency of mIPSCs decreased from 4.69 ± 0.29 Hz to 0.26 ± 0.15 Hz ($P < .05$), and the amplitude of mIPSCs decreased from 142.00 ± 7.93 mA to 63.36 ± 12.33 mA ($P < .05$) (Figure 5-4).



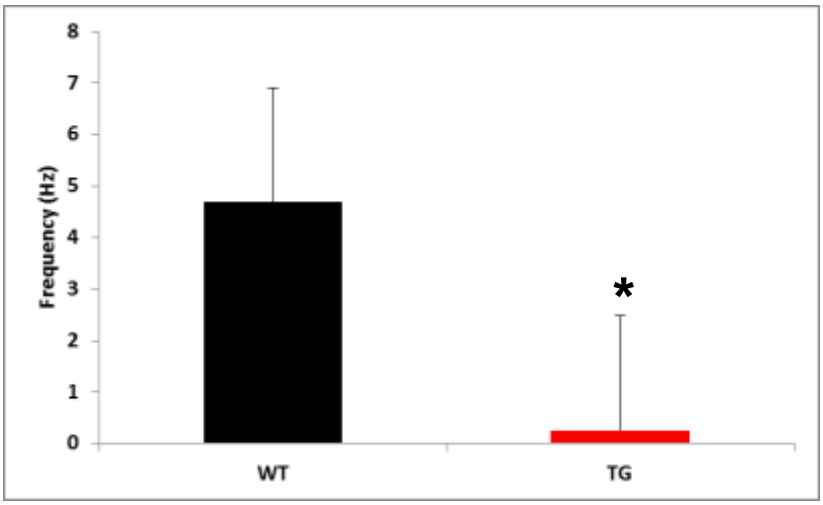


Figure 5-4. The amplitude and frequency of mIPSCs in wild type and transgenic mice. The averaged data indicate significant decrease in the amplitude and frequency of mIPSCs in the transgenic mouse compared to wild-type (*$p < 0.05$, wild type vs. transgenic). Statistical analyses were conducted by one-way analysis of variance (ANOVA).

These findings indicate a disturbed firing pattern of spontaneous inhibitory postsynaptic currents, suggesting that an alteration in inhibitory action is involved in the manifestation of the motor phenotype of SCA10. This aberrant firing pattern in Purkinje neurons indicates that AUUCU-RNA affects the neuronal activity of Purkinje cells, thus impairs the function of the cerebellar neuronal network, leading to the SCA10 phenotype. Furthermore, this altered electrophysiological property may represent the phenotypic consequence of aberrant splicing of the Nova targets in SCA10.

Effects of GABA inhibitor and Glycine inhibitor on mIPSCs of Purkinje cells in the SCA10 transgenic mice

Our data indicated that the inhibitory synaptic transmission was significantly altered in SCA10 transgenic animals. GABA and glycine play important roles as two major inhibitory neurotransmitters in the cerebellum in modulating cerebellar inhibitory transmission. To further characterize the main electrophysiological defects in the SCA10 transgenic mice, we performed a series of electrophysiological measurements to identify the inhibitory neurotransmitter system that was significantly altered in the cerebellum in the SCA10 transgenic mice. To achieve this goal, we analyzed mIPSCs of Purkinje cells after treatment with GABA and glycine inhibitors. To determine which neurotransmitter system mediates abnormal inhibitory transmission in SCA10, GABAA- and glycine-mediated mIPSCs were analyzed in Purkinje cells of SCA10 mice. To identify glycine-mediated mIPSCs (n = 6) or GABAA-mediated mIPSCs (n = 6), either 10 μmol/L bicuculline with 1 μmol/L TTX, or 0.5 μmol/L strychnine with 1 μmol/L TTX, were treated respectively during recordings. The Purkinje cells were perfused with the solution containing either 10 μM bicuculline (GABA inhibitor), or 1 μM strychnine (Glycine inhibitor), then washed out (wash-out).

Our recordings revealed significant morphological changes in the tracings of mIPSCs after bicuculline perfusion (GABA inhibitor), but not after strychnine perfusion (Glycine inhibitor) (Figure 5-5). Further analysis of the data indicated a significant reduction in the frequency of GABAA-receptor–mediated mIPSC, from 1.65 ± 1.19 Hz to 0.05 ± 0.07 Hz, and amplitude of GABAA-receptor–mediated mIPSC from 73.42 ± 21.21 mA to 40.61 ± 15.18 mA (Figure 5-6). There was no significant change in Glycine-receptor-mediated mIPSC frequency and amplitude (Figure 5-7). Therefore, the data suggested that disruption of mIPSCs in the SCA10 occurs predominantly due to aberrant GABA receptor activity. This study demonstrated a significant alteration of inhibitory synaptic transmission in SCA10. Moreover, this study strongly indicates that this defect in inhibitory transmission of Purkinje cells is mediated through GABAA- but not glycine-receptor in cerebellum.

SUMMARY & FURTHER STUDY

Mutant AUUCU RNAs in SCA10 complex with and sequester Nova into RNA foci or aggregates and thus prevent Nova from its normal splicing-regulatory function. This results in aberrant splicing of the Nova target $GABA_AR$ and GlyRα2 leading to the disruption of inhibitory transmission in the Purkinje cells in SCA10 cerebellum. Furthermore, our data suggest that disruption of inhibitory transmission occurs specifically through the modulation of the $GABA_A$- but not via glycine-receptor–mediated neurotransmission. Through our current studies we were able to define SCA10 as a toxic RNA disease, and presented one of the possible molecular and electrophysiological mechanisms of SCA10 pathophysiology. In addition, to validate the functional consequence of Nova targets splicing defects in SCA10 through Nova dysfunction by the AUUCU RNA repeat, we suggest future electrophysiological investigation with Nova KO animals which may provide further insight into the

complexity and functional importance of Nova function in the molecular mechanisms underlying the SCA10 phenotype.

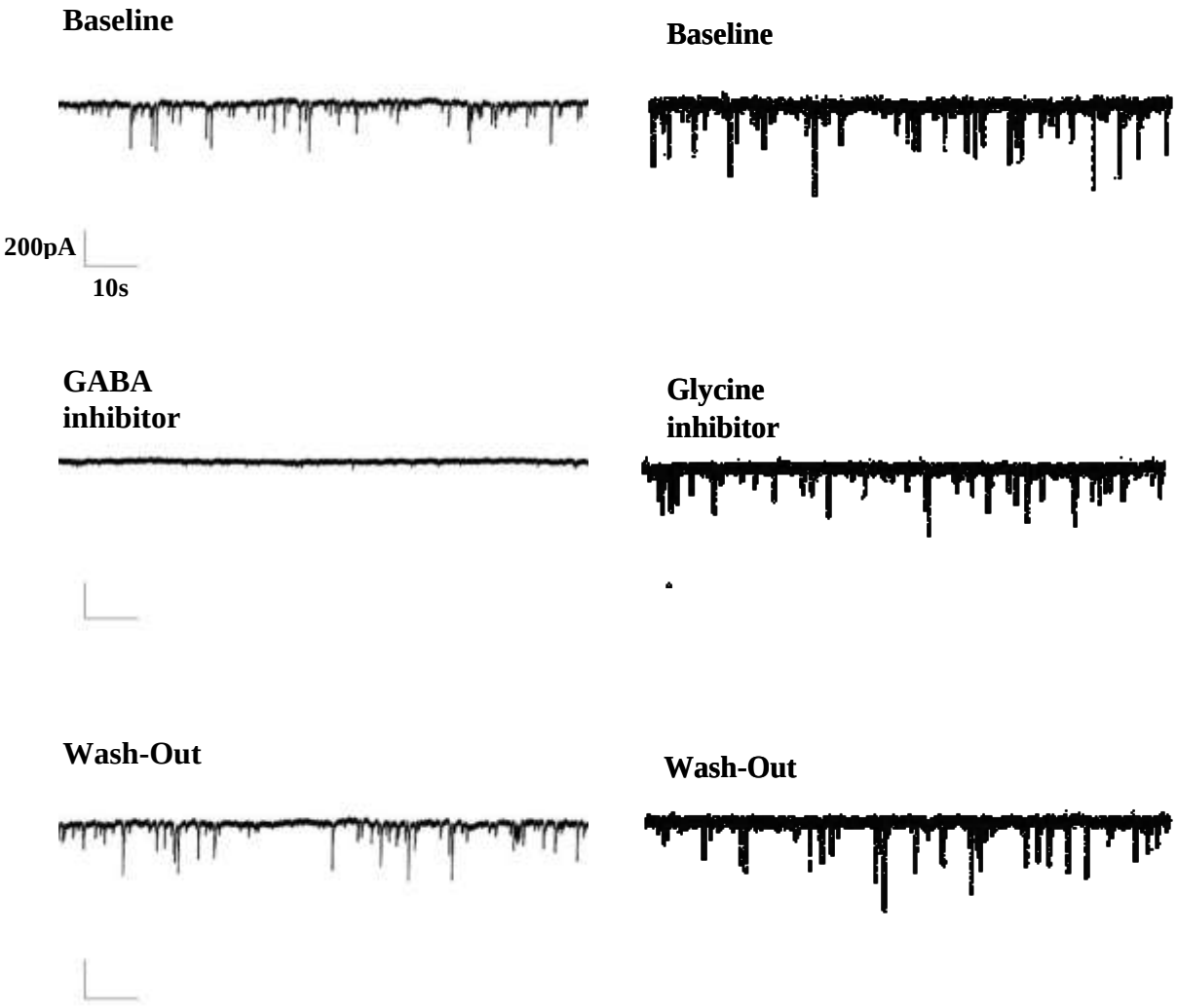


Figure 5-5. mIPSCs after treatment of bicuculline (GABA inhibitor), or strychnine (Glycine inhibitor) in Purkinje cells of SCA10 mice.
The Purkinje cells were sequentially perfused with the solution containing either 10μM bicuculline (GABA inhibitor) or 1 μM strychnine (glycine inhibitor), then washed out (wash-out). The tracing of mIPSCs of Purkinje cells demonstrates significant morphological changes after bicuculline perfusion in transgenic mouse. In contrast, there is no change in mIPSCs after strychnine treatment.

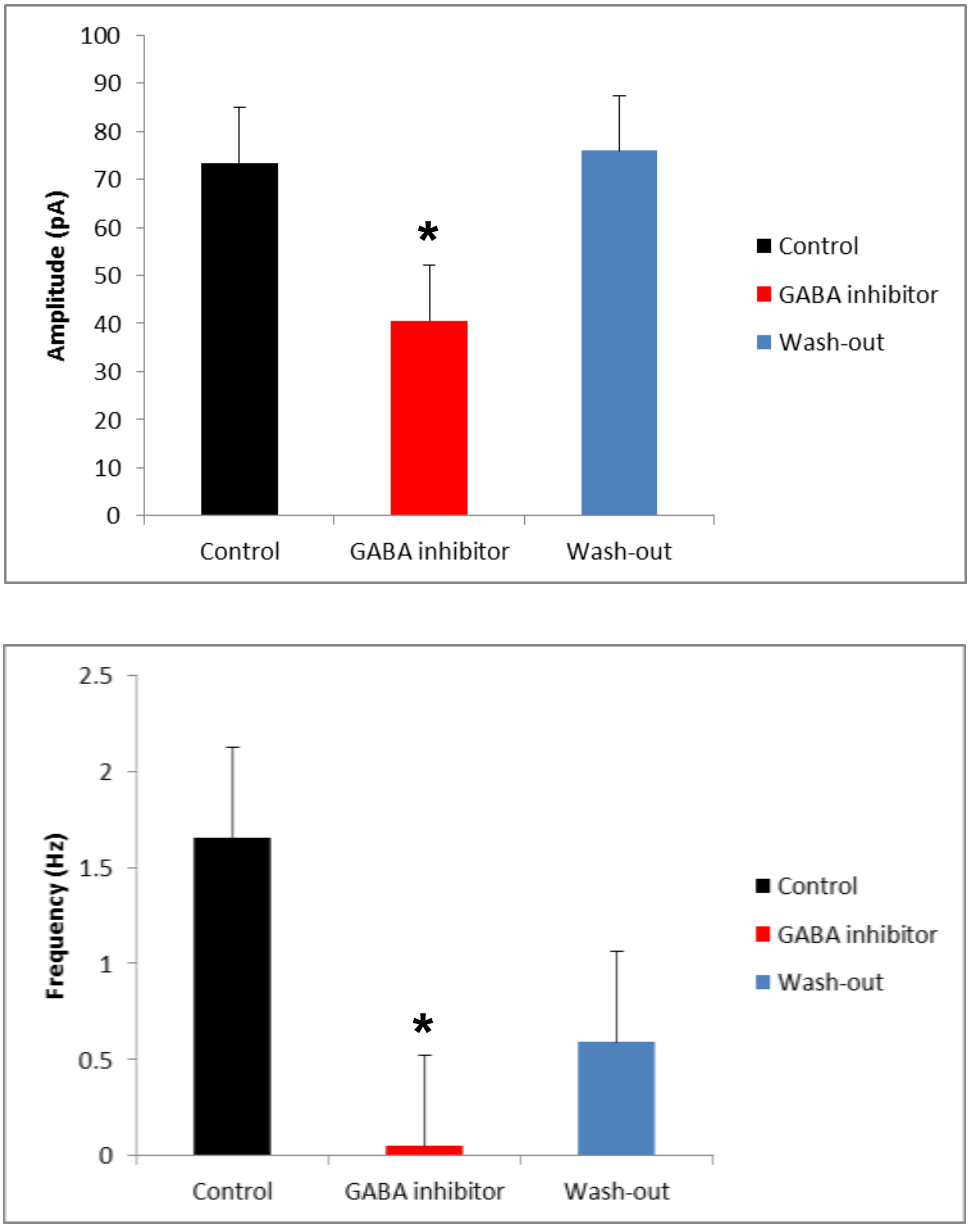
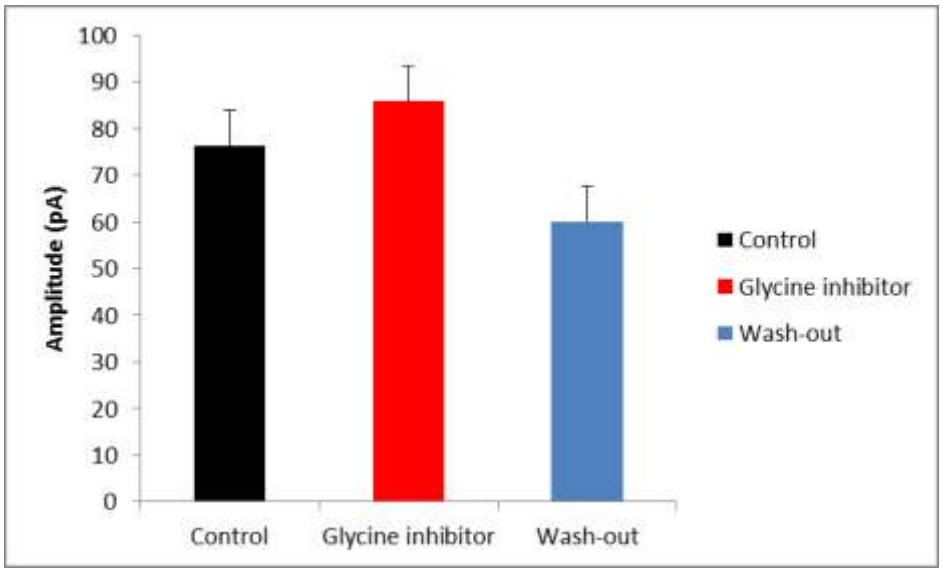


Figure 5-6. The amplitude and frequency of mIPSCs of Purkinje cells after bicuculline (GABA inhibitor) treatment in transgenic mouse.
The averaged data indicate significant decrease in the amplitude and frequency of mIPSCs after bicuculline perfusion (GABA inhibitor) in transgenic mouse (*p< 0.05, GABA inhibitor vs. control and wash-out). Statistical analyses were conducted by one-way analysis of variance (ANOVA).



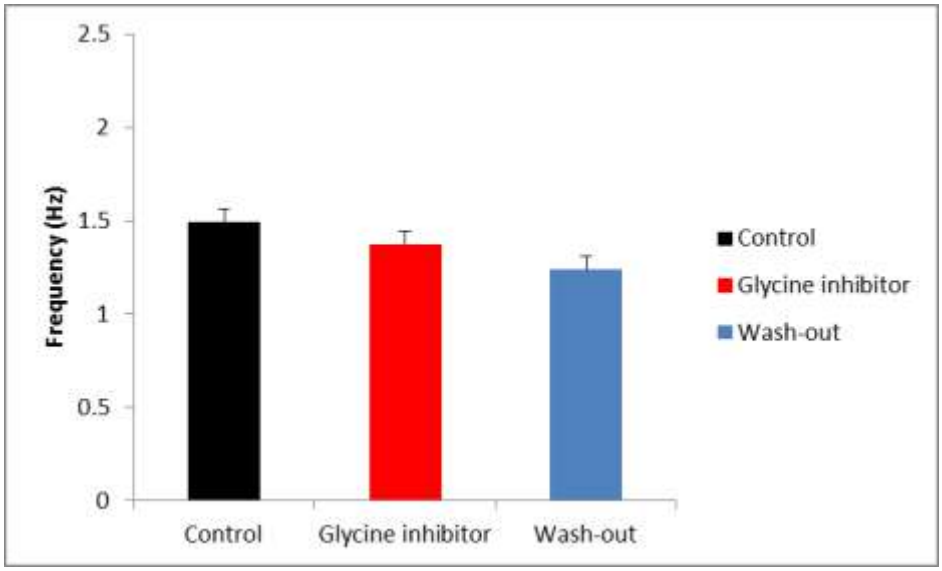


Figure 5-7. The amplitude and frequency of mIPSCs of Purkinje cells after strychnine (glycine inhibitor) treatment in transgenic mouse.
The averaged data indicate no significant change in the amplitude and frequency of mIPSCs in Purkinje cells of transgenic mouse after strychinine perfusion (glycine inhibitor). Statistical analyses were conducted by one-way analysis of variance (ANOVA).

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This dissertation was typed by Jung Gook Lim.

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