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THE CELLULAR NUCLEIC ACID BINDING PROTEIN IN AGING AND DISEASE

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Dr. Michael Mendenhall, Director of Graduate Studies
THE CELLULAR NUCLEIC ACID BINDING PROTEIN IN AGING AND DISEASE

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Molecular and Cellular Biochemistry at the University of Kentucky

By

Robin L. Webb

Lexington, Kentucky

Director: Dr. M. Paul Murphy, Molecular and Cellular Biochemistry

Lexington, Kentucky

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Abstract of Dissertation

THE CELLULAR NUCLEIC ACID BINDING PROTEIN IN AGING AND DISEASE

The ZNF9 gene on chromosome 3 encodes the cellular nucleic acid binding protein (CNBP), a ubiquitously expressed, 177 amino acid (~19.5kDa) protein that is highly conserved among vertebrates. The function of the protein is largely unknown, however an expansion in the first intron of the protein results in myotonic dystrophy type 2 (DM2), a multisystemic disease featuring cardiac arrhythmia, muscle wasting, cataracts, and a range of neuropathologies. Remarkably, we recently discovered that CNBP is involved in regulating the activity of β-secretase, the enzyme that produces the first cleavage event in the generation of the amyloid-β peptide (Aβ). The progressive fibrillization and deposition of Aβ is widely believed to be the primary causal factor in the development of Alzheimer’s disease (AD), and AD-like pathology in individuals with Down syndrome (DS). DS provides a unique model for evaluating how these factors change in the aged brain as compared to young brain, and how such changes affect the proportion of DS patients with AD. In the AD brain, both BACE1 and BACE2 increased from an early stage of disease; in DS brains, BACE1 significantly decreased (p<0.04) with age, whereas BACE2 was unchanged, even though the gene for BACE2 is located within the DS obligate region of chromosome 21. BACE1 and BACE2 activity levels were highly correlated in this series (r² = 0.95), indicating that there may be a higher degree of shared regulation than previously believed. This implicates regulators of BACE as potentially critical for the development of AD, and our data suggests that CNBP may be one such regulator. In AD, CNBP increases early in the disease process, a change that does not occur in the normal aging process or in DS. CNBP and BACE protein levels were correlated in these cases (p<0.001), while there was no relationship between CNBP and age, or CNBP and Aβ, in either the human or mouse brain, indicating that CNBP does not increase as a consequence of normal aging. Thirty day overexpression of CNBP
following adeno-associated viral delivery in murine gastrocnemius muscle resulted in an increase in BACE1 protein (p<0.01) and a consequential increase in Aβ production (p<0.01). Other experiments indicated that CNBP overexpression did not affect the half-life of BACE1 mRNA or protein, but resulted in an increase in BACE1 translation. These data indicate that CNBP is an important regulator of β-secretase, and may play an important role in the onset and progression of AD.

Keywords: Alzheimer’s disease, Cellular Nucleic Acid Binding Protein, Translational Regulation, Down syndrome, RNA binding protein

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THE CELLULAR NUCLEIC ACID BINDING PROTEIN
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DEDICATION

This thesis is dedicated to Josh and Colton, my loves. Thank you for believing in me.
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I had no idea what I was getting myself into when I wandered into the Murphy lab in the winter of 2006. What I thought was going to be a short term job turned into so much more. I have really enjoyed seeing the lab evolve from those early days, when the lab consisted of just three people through today. I will never forget those early days when Tina was teaching me to be a functional person in the lab. The Murphy lab has a unique camaraderie, and is an awesome environment to work in; I couldn’t have joined a better lab. To the lab members past and present, Tina, Chris, Valerie, Thom, Dana, and Kat, thank you for everything, you have been great co-workers and will be life-long friends. Paul, I can’t thank you enough for your mentorship over the years, I would not have gone through graduate school without your encouragement. As much as I’ve learned about science, I’ve learned many other things that equally as important, most of which involve pop culture, board games, or politics. How would I have ever known about "All your base are belong to us", or literal videos for 80s songs, or a million other equally crazy things?! I took my first flight as part of a lab trip, learned that not everyone owns a rototiller, and I will never say, “I reckon” anything, ever, ever again! I will miss my lab family!

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Chapter 1: Background and Significance

CNBP Introduction and Discovery

The cellular nucleic acid binding protein (CNBP) was discovered in 1989 by a group using an oligonucleotide probe that bound the sterol regulatory element, a promoter sequence required for repression of several elements involved in the cholesterol biosynthetic pathway (Rajavashisth, Taylor et al. 1989). In recent years, accumulating evidence has identified the cellular nucleic acid binding protein (CNBP) as a vital regulator of cellular metabolism. We now know that CNBP is involved in several diverse cellular functions, interacting with key regulatory proteins in cellular pathways involved in every stage of the mammalian lifespan, from development through changes associated with the aged cellular milieu.

CNBP is a ubiquitously expressed, 177 amino acid (≈19.5kDa) protein that is highly conserved among vertebrates (Fig. 1.1A) (Warden, Krisans et al. 1994). The single-stranded nucleotide binding protein has 7 zinc finger motifs that have remarkable homology with retroviral nucleocapsid genes (McGrath, Buckman et al. 2003), and an RGG box located between the first two zinc fingers thought to be critical for RNA interaction (Armas, Cabada et al. 2001). The protein has one cAMP dependent protein kinase (PKA) phosphorylation site (Lombardo, Armas et al. 2007). CNBP was first isolated as a potential regulator of the sterol regulatory element of HMG-CoA Reductase (Rajavashisth, Taylor et al. 1989). However, work from several laboratories indicates that
it is unlikely that CNBP plays a role in cholesterol regulation. While biochemical systems indicate that CNBP can bind either ssDNA or RNA (Armas, Aguero et al. 2008), the protein exhibits a many fold preference for RNA, is greatly enriched in the cytoplasm and ER, and has no consensus sequence for nuclear localization. CNBP is found in the nucleus only during early development (Armas, Cabada et al. 2001). CNBP plays a critical role in development, as CNBP knockout severely hinders forebrain development in mice and is embryonic lethal at day E10 (Chen, Liang et al. 2003, Armas, Aguero et al. 2008).

CNBP is implicated in human disease, with a large tetranucleotide (CCTG) expansion in the first intron of ZNF9 (the gene encoding CNBP) resulting in Myotonic Dystrophy type 2 (DM2) (Liquori, Ricker et al. 2001). DM2 presents as a less severe, but similar phenotype to Myotonic Dystrophy type 1 (DM1) (Finsterer 2002). Cardiac arrhythmia, muscle wasting, and cataracts are hallmarks of DM1. DM2, which has a similar, albeit milder clinical course, has recently been shown to share a basic mechanism with DM1 (Finsterer 2002). Both are characterized by large expansions in noncoding regions of RNA: the 3’ UTR of a protein kinase (DMPK) in DM1, and the first intron of ZNF9 in DM2 (Cho and Tapscott 2006). These proteins are highly dissimilar in nature, a protein kinase (DMPK) and a nucleic acid binding protein (CNBP). A parsimonious explanation is that the abnormal buildup of mRNA in the nucleus causes both diseases. Enlarged RNA transcripts accumulate within nuclear foci, and less CNBP is synthesized in myoblasts of DM2 patients (Huichalaf, Schoser et al. 2009, Salisbury, Schoser et al. 2009).
RNA Binding Proteins

With advances in technology, the ability to study not only the proteome but also the transcriptome are becoming more prevalent. The accessibility of these techniques has resulted in an increase of information and a growing appreciation of the transcript, its volatile existence, and its journey to produce a functional protein. There are many things that make it difficult to study the path from RNA to protein, among these, the fact that RNA transcripts are more heterogenous and of lower abundance than was previously appreciated (Keene 2010). In addition to characteristics inherent to the transcript itself, it is likely that localized translational regulation is governed by non-coding regulatory RNA species, as well as RNA binding proteins. These modulators of cellular response likely act to both temporally and spatially fine-tune the translational response to various stimuli, and may act either synergistically or competitively depending on the scenario (Keene 2010).

Within recent years, the concept has emerged that translational control is an important means of spatially regulating protein expression. This is first appreciated during development, where localized translation plays an important role in the process of body patterning and organization of the developing embryo (Pasnoor, He et al. 2012). However, examples of localized translation are important in other cellular contexts as well. For example, internal ribosome entry sites (Spires and Hyman 2005) are functional elements that can substitute for the mRNA 5’ cap structures and recruit translational components. IRES were first identified in viral mRNAs, and while it is not known how widely used these elements are, recent experiments indicate that as many as 3% of the cellular pool of mRNAs may contain IRES (Macdonald 2001). Another example of
localized transcription in adult organisms comes from the recent finding that in neurons, translation events localized to specific dendrites following stimulation results in synthesis of proteins that have long-term effects on synaptic strength (Obeid, Dimachkie et al. 2010). In rats, within two hours of long term potentiation induction, there was a fifty-fold increase in two miRNAs implicated in memory formation in the dentate gyrus (Bredy, Lin et al. 2011). Also, Dicer, Argonaute, and Fragile-X mental retardation proteins are found within the RNA granulues of dendrites. Epigenetic modifications on histone lysine residues that can have drastic effects on transcription and translation vary by hundreds of thousands of positions in the genome at different developmental stages, and affect brain function (reviewed in (Bredy, Lin et al. 2011).

Ultimately, the final decisions regarding gene expression are made in the cytoplasm, largely thought to be based on characteristics of the transcript itself, with signals such as the exon-junction complex and signal peptides governing the fate of the transcript (Mittal, Scherrer et al. 2011). While eukaryotic segregation of transcription and translation explains the lack of operons in higher organisms per se, we are now beginning to appreciate that subpopulations of transcripts are coordinately processed by RNA protein (RNP) complexes. These RNP complexes allow for formation of gene expression modules, in which functionally related transcripts are spliced, transported, stabilized/degraded, or translated in a highly coordinated manner (Keene 2010). These events depend on a class of proteins known as RNA binding proteins (RBPs). This class of proteins indirectly couples post-transcriptional processing with functional outcomes in the cell by interacting directly with transcripts. RBPs are a universal feature of living cells, with 650 known RBPs in yeast, and more than 2500 occurring in mammals. In fact,
RNA processing factors, including RBPs, outnumber transcription factors by 2-fold (Keene 2010). In general, RBPs represent a highly conserved and diverse class of proteins. At the transcript level, RBPs are more rapidly turned over (less stable) than most mRNAs, but are tightly controlled at the protein level with expression varying little between various cell types (Cho and Tapscott 2007).

Besides the ribosome and spliceosome, thousands of RNP complexes exist, including processing bodies and stress granules, and other smaller complexes are dispersed throughout the cytoplasm of the cell. Existence of these RBPs is predicted based on the presence of protein motifs that encode known RNA interaction domains (Cho and Tapscott 2007, Dimachkie and Barohn 2013). The most common of these include the RNA-recognition motif (RRM), K homology (KH) domain, serine/arginine (SR) domain, Zinc fingers, or Pumilio / KBF (PUF) domains (reviewed in Mittal, Scherrer et al. 2011).

Even though these domains are present within these proteins, the specificity of targets that each RBP interacts with varies widely, and is poorly understood. For example, Nop13, an RBP involved in pre-18s RNA processing, has 2 known targets, while Npl13, an mRNA export protein, has 1266 targets. These vary based on spatial and temporal expression of their targets. Our understanding of RBPs is rapidly expanding, and this level of regulation is being mapped into post-transcriptional regulatory networks (reviewed in (Cho and Tapscott 2007)). As more data for RBPs is included, it is becoming apparent that this level of regulation is a multidimensional network, with several RBPs acting as central hubs for cellular processes, and regulating increasingly specific nodes in response to various stimuli (Keene 2010). For example, in yeast
approximately 70% of genes have associations with at least one RBP, but on average interact with 3 RBPs before being degraded (Beckett, Webb et al. 2012). CNBP is thought to directly interact with mRNAs containing a 5’ terminal oligopyrimidine tract (Pellizzoni, Lotti et al. 1997), a feature that may represent as much as 20% of the total mRNA pool in the cell (Cho and Tapscott 2007). Of note, all components of the translational apparatus, as well as several initiation and elongation factors fall into this category (Levy, Avni et al. 1991, Avni, Shama et al. 1994).

**Structure, Localization, and Conservation**

Even though CNBP is ubiquitously expressed in many cell types and throughout the mammalian lifespan, the post-development role of the protein is poorly understood. Structural clues reveal a relatively small protein, consisting of 177 amino acids (≈19.5kDa), that is transcribed from a gene locus on the long arm of chromosome 3 organized into five exons and four introns (Flink and Morkin 1995). The first exon contains the vast majority of the 5’ UTR, which is interrupted by the first, and by far the largest intron (Fig. 1.1B). The UTR extends into exon 2, which also encodes the first zinc-finger. The 2\(^{nd}\) zinc-finger is encoded in its entirety in exon 3, while zinc-fingers 3-5 and the N-terminal region of the 6\(^{th}\) zinc-finger are all encoded on exon 4. The 6\(^{th}\) zinc-finger domain is the only one interrupted by an intron. Its c-terminal region, and zinc-finger 7, as well as the 3’ UTR all reside within the 5\(^{th}\) exon (Flink and Morkin 1995). The most striking structural features of the protein are these seven zinc finger domains that have remarkable homology with retroviral nucleocapsid genes (McGrath, Buckman et al. 2003). Between the first two zinc-finger domains is an RGG box, a domain critical
for RNA interaction (Armas, Aguero et al. 2008). The protein also contains a C-terminal cAMP-dependent protein kinase (PKA) phosphorylation site (Lombardo, Armas et al. 2007). Use of an alternative splice site in the protein’s second exon results in an isoform lacking a 6-16 amino acid region located between the RGG box and the second zinc-finger domain, CNBPβ. The consequences of expression of this smaller protein are unknown (Flink and Morkin 1995).

Interestingly, the zinc-finger domain is one of the most highly conserved elements of orthoretroviruses, and there is absolute homology between the metal ion coordinating cysteine and histidine residues of the nucleocapsid and CNBP domains (McGrath, Buckman et al. 2003). Of CNBP’s seven zinc-fingers, six are capable of supporting both replication and infectivity when substituted for the nucleocapsid of HIV type 1. Only the 5th CNBP zinc-finger was unable to support RNA production and infectivity in the virus. The authors suspect that unique features, like one fewer basic residue gives a net negative 2 charge and the presence of a serine residue following an aromatic residue limits the structural conformations it can adopt, thereby rendering it ineffective as a substitute for the nucleocapsid while the other six can effectively replace the nucleocapsid (McGrath, Buckman et al. 2003).

Post-development, CNBP is largely localized to the cytoplasm and ER in a wide variety of cell types. While the protein is ascribed broad spectrum functions, what those are, and if the protein is essential for cellular functioning after forebrain formation is not known. CNBP is a very highly conserved protein, and displays a similar anterior expression pattern vital for central nervous system development in mouse, chick, zebrafish, and xenopus embryos (Warden, Krisans et al. 1994, De Dominicis, Lotti et al.
CNBP is found in the nucleus during development, and while the molecular events dependent on its presence are vital for CNS development, exactly what those events are is not known (Chen, Liang et al. 2003).

**Role in Forebrain Development**

Although little is known about the function of CNBP in the adult organism, a role for CNBP in development is well established. In the mouse embryo, CNBP is very important in anterior patterning that results in formation of the forebrain (Armas, Aguero et al. 2008), and is later detected in areas of dense cell proliferation, including the limb buds and tail regions (Chen, Liang et al. 2003). Chen and colleagues isolated CNBP in a retroviral insertion screen for insertions that resulted in craniofacial abnormalities. Indeed, hemizygous mouse pups had an increased mortality rate, with fewer pups surviving the gestation period. Those that were born alive displayed various craniofacial abnormalities, including an absent or smaller mandible and the absence of eyes. While some of these animals did survive to adulthood, the authors noted growth retardation as well as eye and skeletal defects (Chen, Liang et al. 2003).

CNBP knockout embryos do not survive past embryonic day 10.5 (Chen, Liang et al. 2003). The authors note that while the node and notochord form, there is an extreme decrease in cellular proliferation in the anterior region preceding gastrulation and neural fold development. However, the trunk and tail of these mutant embryos forms relatively normally, although they are significantly smaller than either their hemizygous or wild-type counterparts (Chen, Liang et al. 2003). These findings indicate that decreases in
CNBP below an unknown threshold have severe developmental consequences, and CNBP is absolutely required for development of the central nervous system.

**CNBP in Human Disease**

In the last 20 years we have learned that many human diseases are caused by expansions in DNA repeats. Certain structural features seem to predispose regions of these genes to expansion by disrupting cellular machineries involved in replication and DNA repair. To date, close to 30 such disorders have been described (reviewed in Mirkin 2007). These repeats consist of 3-12 nucleotides in length, and may be located in coding regions, untranslated regions (including introns), or even in promoter regions of genes. Disease is thought to occur after the number of repeats passes a poorly defined threshold, or after loss of a stabilizing interruption in the growing repeat. After crossing this threshold further expansion becomes more likely, resulting in earlier penetrance with successive generations, and worsening of the disease phenotype, a phenomenon known as genetic anticipation (Ranum and Cooper 2006).

The current model by which this expansion is thought to occur is the strand slippage model, in which extrahelical structures (the most likely secondary structure is an imperfect hairpin) form during the replication process, or perhaps DNA repair or recombination, resulting in changes in repeat length. Strand slippage is generally unfavorable, but the unique ability of these regions of DNA to form secondary structures probably makes conditions more favorable for these repetitive sequences. Consequences of the polymerase encountering a highly repetitive sequence is thought to result in stalling forward movement of the polymerase, causing a looping out of new repeats on
the leading strand the result is an expansion, while it would result in genomic contraction on the lagging strand (reviewed in Mirkin 2007). At some threshold, that varies for different expansions, the length of repeats become pathogenic. One contributing factor in the transition to disease is the loss of stabilizing interruptions in long-normal alleles. This is thought to occur when the stabilizing region slips out of the template strand and is lost during replication. Misalignment on the leading strand creates mismatches in both the hairpin and the duplex part of the slipped strand during replication, and those may be repaired by the co-excision repair pathway, but if they are lost then it results in the 3’ region being expanded.

The unique ability of these RNA sequences to form secondary structures is central to their ability to cause disease (Prange 2011). Virtually all tri-nucleotide repeats have been shown to form imperfect hairpins, as well as the CCTG repeat in DM2 (which have twice as many mismatches). Some of the longer repeats have been hypothesized to form more complex structures. In fact, for DMPK, stability of the hairpin results in increased length of the repeat. These hairpins can sequester proteins, like muscle blind and CELF family proteins in DM1 and DM2, most of which are splicing factors (Fardaei, Rogers et al. 2002, Jones, Jin et al. 2011). With time, it is becoming increasingly clear that alternative splicing during development contributes significantly to the myotonic dystrophy phenotype (Lindemann 2011). Another possible toxic gain of RNA function is that the cell regards them as essentially unprocessed microRNAs. Processing by the RNAi pathway could lead to silencing of genes that contain short complementary sequences in their transcripts. There is evidence that these hairpins are ‘digested’ by Dicer, and some of these are now known to be targets of Dicer in vivo as well. An idea
that surfaced more recently is that transcription of these expanded repeats can proceed in both directions, and both the sense and anti-sense strands lead to the formation of a 21 nucleotide duplex RNA. This leads to recruitment of heterochromatin protein 1, and likely contributes to local formation of heterochromatin, which would likely result in gene silencing of neighboring genes. This silencing may be a contributing factor resulting in some of the more variable secondary phenotypes seen in many of these diseases, including the highly variable phenotypes noted in cases of DM2 (reviewed in (Mirkin 2007)).

For the myotonic dystrophies, DNA expansions occur in non-coding regions of RNA: the 5’ UTR of a protein kinase (DMPK) in DM1 (Brook, Zemelman et al. 1992), and the first intron of ZNF9 (CNBP) in DM2 (Liquori, Ricker et al. 2001, Finsterer 2002, Cho and Tapscott 2006). Disease causing expansions in DM1 range from 80 to more than 2,000 CTG repeats, while the range of repeats in DM2 ranges from 75-11,000 CCTG repeats. The resultant proteins are highly dissimilar in nature, a protein kinase (DMPK) and a nucleic acid binding protein (CNBP). Although the genes involved are very different, the clinical phenotype shares many hallmark features. Myotonic dystrophy is a multisystem disorder characterized by muscular dystrophy, myotonia, cataracts, endocrine abnormalities, and cardiac defects, including conduction defects and cardiac hypertrophy, and generally results in progressive degeneration (Marian and Willerson 2007). DM2 is described as a clinically heterogenous multisystem disorder (Meola 2000), with some patients remarkably clinically similar to DM1, although other patients present with predominantly proximal skeletal muscle involvement, and there is not a congenital form of DM2 (Lindemann 2011). A parsimonious explanation for these similarities
between DM1 and DM2 is that the abnormal buildup of mRNA in the nucleus causes both diseases. Enlarged RNA transcripts accumulate within nuclear foci, and less CNBP is synthesized in myoblasts of DM2 patients (Jones, Jin et al. 2011). This leads to decreased levels of proteins of the translational apparatus, and possibly other targets of CNBP that have not yet been evaluated (Huichalaf, Schoser et al. 2009).

**Translational Regulation, Aging, and Disease**

Within recent years, accumulating evidence has identified the cellular nucleic acid binding protein (CNBP) as a vital regulator of cellular metabolism. We now know that CNBP is involved in several diverse cellular functions, from interacting with key regulatory proteins in cellular pathways involved in every stage of the mammalian lifespan, from development through changes associated with the aged cellular milieu. In fact, CNBP is thought to directly interact with mRNAs containing a 5’ terminal oligopyrimidinetract (Crosio, Boyl et al. 2000), a feature that may represent as much as 20% of the total mRNA pool in the cell (Cypser, Tedesco et al. 2006, Cho and Tapscott 2007). Of note, all components of the translational apparatus, as well as several initiation and elongation factors fall into the category (Li, Tian et al. 2011).

These findings are striking given that changes in both protein synthesis and degradation are thought to contribute to detrimental aspects of the aging phenotype (Tavernarakis 2008). With aging the cellular protein pool tends to accumulate various irreversible protein modifications. These include the increased presence of reactive oxygen species, leading to oxidation of amino acid side chains and peptide cleavage resulting in an increase in carbonyl derivatives in the cell. Presence of these carbonyl
derivatives contributes to the pool of damaged proteins that increase with aging or various pathological states. Also, glyoxal and/or methylglyoxal buildup causes formation of advanced glycation end products that disrupt protein function. Normal physiological processes also lead to amino acid racemization, isomerization and deamination, causing mild to devastating effects on protein function (Tavernarakis 2008). The protein pool is balanced by the rates of protein synthesis and protein degradation. The quality of the protein pool is also influenced by the cellular maintenance and repair pathways. Several components of these pathways are affected by aging, with both protein synthesis and degradation declining during aging (Arumugam, Gleichmann et al. 2006).

Although global decline in protein translation is associated with aging, this is not universally true of all proteins. Changes in the translation of specific proteins may result in (or as a consequence of) detrimental aspects of the aging process or various pathological states. CNBP likely represents one of the central ‘hub’ RBPs, capable of binding mRNAs with little sequence specificity. It may be involved in eliciting stress-induced responses in cellular micro-environments, as an activator of cap-independent translation, by binding IRES sequences in the 5’ UTR of its targets (Sonneborn 2005). It is also implicated as a regulator of global translation rates and proliferation control (Calcaterra, Armas et al. 2010), a finding supported by reduced rates of translation in patients with DM2 (Huichalaf, Schoser et al. 2009). It is possible this effect is elicited by CNBPs interactions with mRNAs containing a 5’ terminal oligopyrimidine (5’ TOP) tract (Crosio, Boyl et al. 2000). These findings suggest a potential role for CNBP in the aging process and/or various pathological states in which aberrant translational control is a
contributing factor. CNBP is a ubiquitously expressed, highly conserved protein of unknown function in the mature organism. An expansion in the first intron of the protein causes DM2 (Liquori, Ricker et al. 2001). However, haploinsufficiency of the protein recapitulates several aspects of the DM2 phenotype, including cataracts, myotonia, and muscle pathology, which are rescued with reintroduction of CNBP (Chen, Wang et al. 2007), highlighting our need to separate effects of the expansion from haploinsufficiency of the protein. Herein we examine changes in CNBP that occur with aging in mice and humans both in skeletal muscle and brain tissue. Targets are evaluated that may have relevance to pathology of the skeletal muscle, including DM2 and inclusion body myositis, and also previously unreported targets whose translational control is involved in Alzheimer’s disease and Down syndrome. These findings indicate that these multiple pathological states may share an underlying defect in translational regulation. This work describes efforts to test the hypothesis that CNBP is involved in translational regulation, and that changes in this protein with aging or in various pathological states may have repercussions for degenerative disease in both brain and muscle.
Figure 1.1 CNBP is Highly Conserved
**Figure 1.1 CNBP is Highly Conserved.**  A) CNBP orthologs from 6 different species (>95% identical). The CNBP protein is highly conserved over its entire 177 amino acid length. Human and rat CNBP are completely identical; mouse CNBP has a single glutamic acid insertion after residue 73. CNBP contains seven zinc knuckle domains, and an RGG box domain that likely confers RNA binding preference over DNA. A conserved PKA site close to the C-terminus is the major (and perhaps only) site of phosphorylation.  

**B) Organization of the ZNF9 gene locus.** Exons are shown in blue, coding sequence by blue boxes.
Chapter 2: General Materials and Methods

Human Subjects

We selected two series of cases to study two overlapping questions. The first case series was chosen to examine the role of β-secretase in age-related neurodegenerative disease. These samples were obtained from the Alzheimer’s Disease Center (ADC) tissue repository at the University of Kentucky, Sanders-Brown Center on Aging (Dimachkie and Barohn 2012, Dimachkie and Barohn 2012, Dobrev, Barhon et al. 2012). Controls (n = 9) were age-matched to disease cases (preclinical AD [PCAD]: n = 10; amnestic MCI: n = 7; AD: n = 10). Preclinical AD cases (or high pathology controls) were defined as those that met the NIA-Reagan neuropathology criteria for likely AD, but exhibited no clinical signs of dementia (Mittal, Barohn et al. 2011, Josh Yeh, Dimachkie et al. 2012). Amnestic MCI was defined as per the criteria of Petersen et. al.(Petersen, Smith et al. 1999). We included six cases of frontotemporal dementia (FTD) as an Aβ-unrelated neurodegenerative disease and specificity control (Ibrahim, Dimachkie et al. 2010). The details of the recruitment, inclusion criteria, and mental status test battery for our normal control group have been described previously (Hejazi, Lavenbarg et al. 2011). Details of our tissue collection procedures and consensus diagnosis have also been described (Markesbery, Schmitt et al. 2006, Dimachkie and Barohn 2012). The second case series was selected to better elucidate the role of Aβ deposition as a feedback mechanism for changes in β-secretase. Individuals with DS develop Aβ deposition and other AD-like pathology with age. DS cases and controls were obtained from the University of California at Irvine ADC brain tissue repository, and the NICHD Brain and Tissue Bank
for Developmental Disorders (University of Maryland). Control brains had no history of ante-mortem dementia, and post-mortem intervals (PMI) were short (averaging < 3 hrs).

**Animal Subjects**

All animals were housed and maintained in accordance with the University of Kentucky IACUC committee’s standards and regulations. Details on the specific animal models used are discussed later for each set of experiments. Animals used for viral transduction experiments in vivo were moved to BSL2 housing prior to viral exposure. Adult animals were used for virus injection into skeletal muscle. Animals were anesthetized with isoflurane or ketamine / xylazine cocktail (50-100 µg / kilo). 25 µl of virus in sterile PBS was administered into the gastrocnemius using disposable tuberculin syringes (BD Falcon; Franklin Lakes, NJ). Generally, the left leg of the animal was injected with the test virus, while the right leg was injected with the appropriate empty vector virus, providing a within subject control. Animals were monitored until they recovered from the anesthetic. The animals were monitored on a daily basis for a minimum of three days following injection, with special attention given to each animal’s weight, body condition score, and gait. Monitoring was reduced to once per week thereafter given no concerns were identified during the initial monitoring period. After one month, mice were sacrificed by either barbiturate overdose (Beuthanasia) or by CO2 asphyxiation, and gastrocnemius muscles removed, or either fresh frozen or drop fixed in 10 % formalin solution.

For viral expression in brain P0-P2 mouse pups were cryoanesthetized, and injected bilaterally with AAV (≈ 1 x e10 viral genomes / µl). Litters were injected with
CMV-CNBP AAV2, CMV-GFP AAV2, or empty vector AAV2 virus. Pups were warmed under a heat lamp before being returned to their mother. The animals were then aged out 1 week to 3 months before euthanasia by barbiturate overdose, or CO2 asphyxiation. Brains were removed, the cerebellum and frontal lobes collected for potential BACE enzymatic activity assay after verification of viral expression. The remainder of the brain was cut into three separate sections, one was drop fixed in 10 % formal for immunohistochemistry. The other two were frozen at -80°C, one for RNA extraction (TRIzol®; Invitrogen, Carlsbad, CA ), and the other for protein (2 % SDS) extraction. Tail-snips were also collected for post-mortem genotyping of each animal.

**Cell Culture**

Human embryonic kidney 293 cells (HEK293T or HEK 293A), and neuroblastoma cells (SH-SY5Y) were grown in Dubelco’s modification of Eagle’s media (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (HyClone). Neuroglioma cells (H4) were grown in Opti-Mem (Invitrogen; Carlsbad, CA) also supplemented with 10 % fetal bovine serum and 1 % penicillin / streptomycin. All cell lines were cultured at 37°C under 5% CO2.

For virus harvesting, HEK 293A cells were transfected with 180 µg adeno-helper plasmid, 90 µg rep / cap plasmid (AAV2 or AAV1), and 90 µg pZac2.1 plasmid (either empty vector or CMV-CNBP) (University of Pennsylvania Viral Vector Core), in 7.5 mM PEI (Polysciences, Warrington, PA). For transduction with purified virus, viral particles in PBS were added directly to the cell medium and incubated overnight in standard conditions at 37°C in 5% CO2.
Tissue Collection and Processing

Human tissue samples were resuspended in five volumes (wet weight / volume) of tissue lysis buffer (TLB: 10 mM sodium acetate [NaOAc], 3.0 mM NaCl, 0.1% Triton® X-100, 0.32 M sucrose, pH = 5.0). The buffer was supplemented with a complete protease inhibitor cocktail with EDTA (PIC; Amresco; Solon, OH), with 100 nM pepstatin A added (Sigma-Aldrich; St. Louis, MO). Whole tissue homogenate was centrifuged at ~2,000 x g for 15 min to pellet insoluble material, followed by an additional spin at 20,000 x g for 30 min. Pelleted material was sequentially extracted in an equal volume of RIPA buffer (0.1% SDS, 0.5% deoxycholate, 1.0% Triton® X-100, 50 mM Tris-Base, 150 mM NaCl, pH= 8.0, with PIC) or 2% (w/v) SDS for the determination of detergent-soluble Aβ, followed by 70% (v/v) formic acid (FA) for the determination of insoluble Aβ. In each case the pellet was extracted by brief sonication (10 x 0.5 s microtip pulses @ 20% power; Fisher Sonic Dismembrator, Model 500, Fisher Scientific, Pittsburgh, PA) followed by centrifugation to pellet insoluble material (detergent soluble fraction: 20,000 x g for 30 min, at 14°C; FA fraction: 20,000 x g for 1 h, at 4°C). Protein content was determined by bicinchoninic acid (BCA) assay (Pierce Biotechnology; Rockford, IL).

Frozen animal tissue samples were homogenized using a PowerMax AHS200 (VWR, Radnor, PA) in five volumes (wet weight / volume) of 2 % SDS buffer supplemented with complete protease inhibitor cocktail (PIC; Amresco; Solon, OH). Homogenates were centrifuged at 20,000 x g for 30 min to pellet insoluble material. Protein content was determined by bicinchoninic acid (BCA) assay (Pierce Biotechnology; Rockford, IL).
**SDS-PAGE and Western Blot Analysis**

Cells were lysed in ice cold 1% Triton X-100 lysis buffer (50 mM Tris/150 mM NaCl + Roche Complete Protease Inhibitor Cocktail; pH= 8.0), and the insoluble material pelleted for 5 minutes at 20,000 x g. Animal and human tissue samples were homogenized in 2 % SDS (w/v), and the total amount of protein was determined by BCA assay (Pierce Biotechnology, Rockford, IL). Following separation by SDS-PAGE (BioRad, Hercules, CA), proteins were transferred to either 0.45 μm PVDF or nitrocellulose membranes and blocked with 5% nonfat dried milk. BACE1 was detected using commercially available antibodies, mouse monoclonal MAB931 (R&D Systems, Minneapolis, MN), or rabbit monoclonal EPR3956 (Epitomics, Burlingame, CA). CNBP was detected using a rabbit antibody raised against the C-terminal 20 amino acids of human and mouse CNBP (Niedowicz, Beckett et al. 2010). HRP conjugated secondary antibodies and detection reagents were obtained from Pierce Biotechnology.

**ELISA**

ELISAs for Aβ from transfected cell, human brain, or mouse skeletal muscle and brain were performed as described in Beckett et al. (Beckett, Niedowicz et al. 2010). Detergent and FA-soluble pools of Aβ were measured by antigen capture using monoclonal antibody Ab9 (against the amino-terminus of Aβ), and detection was performed using biotinylated 4G8, against Aβ17-24 (Covance; Denver, PA) followed by horseradish peroxidase (HRP) conjugated Neutravidin™ (Pierce Biotechnology, Rockford, IL); Aβ40 was detected with Ab13.1.1 and Aβ42 was detected with 12F4 (Covance; Denver, PA) (Das, Howard et al. 2003, McGowan, Pickford et al. 2005), or
captured using Ab2.1.3 and detected with Ab9; alternative antibody combinations gave essentially identical results. For the measurement of APP or APP C-terminal fragments (CTF) by ELISA, we used a similar procedure. Full length APP was captured using antibody 22C11 (Millipore; Billerica, MA), which was then detected using biotinylated 6E10 against Aβ1-16 (Covance; Denver, PA). The cleared sample was then transferred to a second plate, coated with affinity purified antibody CT20, raised against the last 20 amino acids of APP (Murphy, Uljon et al. 2000). Antibody 6E10 (for CTFβ) or 4G8 (total CTFs) was used to captured CTFs were detected using either antibody 6E10 or. Oligomeric Aβ was measured using the single-site 4G8/4G8 sandwich ELISA method (LeVine 2004, Beckett, Niedowicz et al. 2010). β-Secretase activity was measured using a commercial kit (Sigma-Aldrich; St. Louis, MO), and BACE1 protein was assessed by ELISA (Ahmed, Holler et al. 2010).

**Immunohistochemistry**

Sections were rehydrated and then blocked for 30 min. in 5 % v/v serum from organism the secondary antibody was prepared in. Peroxidase activity was quenched by 10 min. treatment with 3 % H2O2 in 10 % methanol. Antigen retrieval was performed by boiling the slides in citrate buffer for approximately 5 min. After several brief PBS washes, primary antibody was applied to the sections overnight. This was then washed away, and secondary antibody applied for 30 min. For antigen detection using fluorescent secondary antibodies, slides were treated with autofluorescence eliminator reagent and coverslipped with vectashield mounting media. Otherwise, sections were treated with

**Real-Time PCR**

For RNA isolation, 100 mg of frozen tissue was homogenized using TRIzol® reagent (Invitrogen; Carlsbad, CA). Tissue culture cells were lysed directly in TRIzol® reagent. Extraction was followed by phenol / chloroform extraction and ethanol precipitation, as per the manufacturer’s instructions. RNeasy cleanup columns (Qiagen; Valencia, CA) were run for each sample. The reverse transcriptase reaction (iScript Select cDNA Synthesis Kit; Bio-Rad Laboratories, Hercules, CA) used 1 µg of RNA, purified RNase H, MMLV reverse transcriptase, and a mixture of random hexamers and oligo dT primer, according to the manufacturer’s instructions. Standardization was performed to the geometric mean of a minimum of two housekeeping genes (see chapter specific methods for primer details). Quantitative real-time PCR reactions contained ~20ng of sample cDNA together with PerfeCTa SYBR green SuperMix (Quanta Biosciences; Gaithersburg, MD).

**Adeno-associated Viral Preparation**

The CNBP cDNA was cloned into the XhoI / XbaI restriction sites in the pZac2.1 plasmid (U Penn Viral Vector Core). The CNBP pZac2.1 plasmid, Adeno-helper plasmid, and rep / cap (AAV1 or AAV2) plasmids were then co-transfected using the PEI (Polysciences, Warrington, PA) transfection method into HEK293A cells. Cells were harvested 72 hours later, by centrifugation at 3,300 x g for 5 minutes. DMEM was
decanted and cells were resuspended in a PBS wash buffer. 10 % w/v sodium deoxycholate (Fisher Scientific; Pittsburg PA) was added to a final concentration of 0.5 \% w/v, and benzonase (Sigma-Aldrich; St. Louis, MO) added to a final concentration of 50 IU / ml. The viral suspension was then incubated in a 37°C water bath for 30 min, and then at 50°C for 30 min. Resuspended cells were lysed by three freeze-thaw cycles (-80°C for 30 min and thawed at 50°C for 30 min), and left at -80°C overnight. Viral lysates were thawed and debris pelleted by centrifugation at 18,500 x g for 10 min. Viral particles were harvested using a commercially available filter kit as per the manufacturer’s instructions, and concentrated using 100,000 MWCO filter (Amicon) to a final volume of ≈ 100 µl, or by iodixanol gradient. Aliquots of individual viral preparations were tested for ability to transduce human neuroglioma cells (H4), and preparations that resulted in CNBP overexpression as detected by western blot were all combined, producing a ≈ 1 mL viral stock (enough to perform all planned animal experiments from this single batch). This stock viral solution was aliquoted into 100 µl aliquots to prevent freeze-thaw and stored at -80°C.

Data Analysis

Data were analyzed using SPSS®. Simple group comparisons were made using either Student’s t-test, or the Mann Whitney U-test, where appropriate. Group data were analyzed by a general linear model ANOVA, co-varying for age, gender, PMI, and loading controls when necessary, and post-hoc comparisons performed using Dunnett’s test. Correlations were determined using either Pearson’s r or Spearman’s ρ.
Chapter 3: Changes in CNBP in Neurodegenerative Disease

Portions of this chapter are reprinted from:


INTRODUCTION

Alzheimer’s disease

Alzheimer’s disease is a devastating disorder and a growing public health concern as our population ages: AD now ranks as the 6th leading cause of death in the United States. Rates of mortality from AD continue to increase as the age of our population creeps upward, unlike other disease states whose mortality rates are on the decline, and also unique among top causes of death in its inability to be prevented (http://www.alz.org). The most common form of dementia among the elderly, AD is already taking a toll on our health care system, and many families struggle to provide necessary care for loved ones as the disease progresses unimpeded by current therapeutics. AD manifests as a progressive cognitive decline, including memory loss, speech dysfunction, and impaired spatial orientation, as well as a host of other symptoms (McKhann, Drachman et al. 1984) that begin inconspicuously many years prior to development of symptomology. In the general population, AD manifests in two forms: an
autosomal dominant early onset form of the disease, familial AD (FAD), that accounts for less than 5% of disease cases, and the more common sporadic form of late-onset AD. Age of onset distinguishes the two groups but clinical presentation and neuropathology are essentially identical (Johnson, Williams et al. 2003). Thus, studying FAD gene mutations has provided insight into the molecular mechanisms that lead to neuropathology (Hardy and Higgins 1992, Haass, Hung et al. 1994, Haass, Lemere et al. 1995, Walsh, Hartley et al. 2001), even though the process may begin as much as 20 years before the patient begins to present clinically with symptoms (Braak and Braak 1991, Bateman, Xiong et al. 2012).

The Molecular Neurobiology and Histopathology of AD

AD is characterized by the presence of two neuropathological lesions, extracellular plaques composed largely of a 40-42 amino-acid peptide called β-amyloid (Aβ), and intracellular tangles and striated neuropil threads composed of a hyperphosphorylated form of the cytoskeletal protein tau (Lassmann, Bancher et al. 1995, Dimachkie, Ohanian et al. 2000, Verma, Gushiken et al. 2001). Synapse loss in areas of the brain vital for learning and memory correlates with a patient’s performance on cognitive tests even in cases of mild AD, and precedes neuronal loss, which becomes prevalent in mild-AD. (Patel and Dimachkie 2000, Verma, Forman et al. 2000). This neuronal loss eventually encompasses most of the brain, which ultimately becomes atrophied, with enlarged ventricles and significantly less overall brain weight than a comparatively aged healthy brain.
Characterization of genomic mutations present in early onset FAD led to development of the amyloid cascade hypothesis (Taylor, Hardy et al. 2002). The amyloid precursor protein (APP) is a ubiquitously expressed type 1 transmembrane protein similar in structure to a receptor (Kang, Lemaire et al. 1987), but after years of intense study no universally accepted ligands have been identified (reviewed in (Pasnoor, He et al. 2012)). The processing of the protein is now known in considerable detail (Selkoe 1996, Dimachkie, Justiz et al. 2000, Huse and Doms 2000) (Fig. 3.1A). Non-amyloidogenic APP processing by α-secretase on the cell surface results in cleavage within the Aβ peptide fragment thereby abrogating Aβ peptide formation and resulting in secretion of a large fragment, sAPPα. The remaining transmembrane c-terminal fragment (CTFα) is a substrate for γ-secretase processing, and results in secretion of a peptide fragment much smaller than Aβ, called p3. Cleavage of APP by a transmembrane aspartyl protease, β-site APP site cleaving enzyme (BACE), occurs in the endocytic pathway (Fig. 3.1B) and results in the transmembrane fragment CTFβ. Subsequent cleavage in the transmembrane domain of CTFβ by γ-secretase generates secreted Aβ peptide fragments 38-43 residues in length. Cleavage of either CTFα or CTFβ by γ-secretase also results in the generation of a small, cytosolic fragment (AICD) of poorly understood function. FAD-linked mutations in APP generally result in an increase in Aβ42 production (Suzuki, Cheung et al. 1994, Maruyama, Tomita et al. 1996); this is thought to be the most toxic peptide species generated by this non-canonical APP processing pathway and leads to aggregation and formation of higher order structures including oligomers (reviewed in (Glabe 2008)) that damage neurons and induce pathogenesis. This slightly longer peptide fragment is more hydrophobic and is thought to seed neuritic plaque deposition by
causing aggregation of other species that are more abundant and soluble, such as Aβ40 (Cummings, Head et al. 1996, Lemere, Blusztajn et al. 1996).

BACE2 is an aspartyl protease with ~65% sequence homology to BACE1, the major form of β-secretase in the brain. BACE1 was originally discovered by multiple groups as the primary β-secretase responsible for Aβ generation in the brain (Hussain, Powell et al. 1999, Sinha, Anderson et al. 1999, Vassar, Bennett et al. 1999, Yan, Bienkowski et al. 1999, Lin, Koelsch et al. 2000), and the homologue BACE2 was discovered shortly thereafter (Ishii, Tamaoka et al. 1997, Solans, Estivill et al. 2000). The β-secretases belong to the pepsin family of aspartyl proteases, and are the only transmembrane domain containing members. The BACE1 gene is found on chromosome 11 and encodes a 501 amino acid protein, while the BACE2 protein is found on chromosome 21 and encodes a 518 amino acid protein (reviewed in (Stockley and O'Neill 2007)). Like other aspartyl proteases, both BACE1 and BACE2 have an N-terminal prodomain that is cleaved by a furin-like protease or through autoproteolytic cleavage (Hussain, Christie et al. 2001) to generate the mature enzyme. One of the primary differences between the enzymes occurs within the C-terminal portion of the proteins, with the BACE1 active-site containing 3 disulfide bonds, while BACE2 has 2 (Chou 2004).

**β-secretases and Neuropathology**

Since its discovery little more than a decade ago, a vast body of work has amassed supporting the role of BACE1 in AD. BACE1 activity has been established as the rate-limiting step in formation of the Aβ-peptide. BACE1 levels increase slightly during the
normal aging process (Austin, Pappolla et al. 1995, Fukumoto, Rosene et al. 2004), but it is well established that both BACE1 protein and enzymatic activity are further increased in the AD brain (Fukumoto, Cheung et al. 2002, Yang, Lindholm et al. 2003, Ahmed, Holler et al. 2010). In the Swedish familial form of AD, an APP mutation immediately N-terminal to the β-site makes the protein a more efficient substrate for BACE, resulting in early onset dementia and a more rapid disease progression (Mullan, Crawford et al. 1992). Importantly, BACE1 knockout prevents formation of the Aβ peptide in vivo, a finding that solidly supports BACE1 as the major β-secretase in the brain, and a prime therapeutic target for AD (Roberds, Anderson et al. 2001). Although phenotypic changes in BACE1 knockout mice are subtle, it is likely that BACE1 has multiple substrates and is involved in myelination (Willem, Garratt et al. 2006, Ursu, Alekov et al. 2012), and is important during development and following traumatic brain injury (Turner and Hilton-Jones 2010, Ursu, Alekov et al. 2012).

**APP and Aβ**

There is much debate about which characteristics confer toxicity to the Aβ peptide. The N-terminal end of the peptide, formed by β-secretase cleavage, is fairly heterogeneous and subject to various modifications. The C-terminus, produced by intramembrane processing of the CTF by the γ-secretase, yields a peptide 39-43 amino acids long, with Aβ40 and Aβ42 being the most abundant species. The peptide likely exists as a dynamic pool of forms ranging from soluble dimers through higher order oligomers that become increasingly insoluble with size and result in plaque deposition. While many of the events regarding this process are poorly understood, it is likely driven biochemically
by sequestration of hydrophobic regions from the aqueous environment (Watson, Castano et al. 2005). It is widely accepted that the 42 amino acid peptide is more hydrophobic and aggregation prone, and is proposed to seed plaque formation in the brain. Aβ42 is the first peptide species to form extracellular deposits in the DS brain, and these deposits are abundant in brains from young individuals with DS by 12 years of age, approximately 20 years before significant Aβ40 and tau histopathology can be found (Mori, Spooner et al. 2002).

The Aβ peptide is a fragment of APP, a transmembrane protein of unknown function. Recently, it was proposed that APP stimulates neuroprogenitor cells to develop into various glial cell lineages, and could be a possible contributor to the decreased neurogenesis and delayed development seen in DS (Lu, Esposito et al. 2011). A role in vasodilation has also been suggested, and represents a potential mechanism for APP mediated cerebral amyloid angiopathy, a process that could contribute to early neuropathology in AD (Han, Zhou et al. 2008).

This thesis project originated from an initial finding using insertional gene-trap mutagenesis to identify products that alter Aβ production, as determined by Aβ ELISA. From ~3500 cell lines screened, one cell line was identified that produced less Aβ, and the resultant DNA fragment was sequenced, yielding a match to the ZNF9 gene, that encodes CNBP. Replacement of CNBP in the hemizygous cell line normalized Aβ levels, indicating that disruption of this protein did induce the noted decrease in Aβ (MPM, unpublished data). Analysis of the ZNF9 disrupted cell line indicated a decrease in BACE1 (MPM, unpublished data). Cleavage of APP by BACE1 is the rate-limiting step in formation of the Aβ-peptide, and this enzyme is an important therapeutic target for
treat AD, as it is rate-limiting step in formation of the pathogenic Aβ peptide. For these reasons, we wanted to determine if changes in CNBP accompany changes in BACE1 in post-mortem human brain tissue. We also wanted to characterize BACE1 and BACE2 pathology in the brain. AAV2 was used to overexpress CNBP in tissue culture cells, so that we could begin to determine how CNBP is mechanistically regulating BACE. We then used this tool in vivo to determine if overexpressing CNBP would cause a concomitant increase in BACE1 that would exacerbate Aβ-related pathology in the brain of a mouse model of AD.
Figure 3.1 APP Processing and Imbalance in Age-Related Neurodegeneration.
Figure 3.1 APP Processing and Imbalance in Age-Related Neurodegeneration. A) The amyloid precursor protein is processed either by an amyloidogenic pathway (Klunk, Lopresti et al. 2005) or a canonical pathway (right). Canonical processing by α-secretase results in secretion of a large extracellular fragment, sAPPα. Importantly, this cleavage occurs within the Aβ peptide fragment (light blue), preventing its formation. A membrane bound C-terminal fragment, CTFα, then becomes a substrate for γ-secretase. This cleavage occurs within the membrane, releasing a short extracellular p3 peptide, and the APP intracellular domain (AICD,dark blue). Amyloidogenic processing occurs as APP interacts with β-secretase, or BACE, in the endocytic pathway. This generates the secreted sAPPβ, and a longer C-terminal fragment, CTFβ; γ-secretase cleavage of this fragment generates Aβ and AICD. B) In mature endosomes, BACE1 (an enzyme that is most active at acidic pH) then cleaves APP resulting in increased amounts of CTFβ and Aβ peptide (light blue) being secreted outside the cell. Increased extracellular accumulation of toxic Aβ species, particularly Aβ42, results in the formation of Aβ oligomers. These oligomers then overwhelm the brain’s capacity for clearance and degradation and form extracellular plaques, ultimately leading to neurodegeneration and severe brain atrophy. C) Normally, most APP is cleaved by the α-secretase, releasing sAPPα. CTFα is endocytosed and then processed by γ-secretase, resulting in formation of the p3 peptide, which is secreted, and releasing the AICD into the cytosol. BACE processing of APP may occur, resulting in formation of Aβ (Eissner, Iacobelli et al. 2004), but these are largely degraded and cleared. While few small plaques may accumulate with aging, they are much smaller and fewer in number than those associated with disease. Reproduced from: (Webb and Murphy 2012).
METHODS

Aβ and APP Immunoblots

For the examination of Aβ and APP by Western blot, 10 μL of the FA fractions were dried under vacuum (Labconco Centrifugal Concentrator), then reconstituted in standard loading buffer. These samples, as well as 10 μl of the PBS and SDS extracts, were separated on 12% Bis-Tris SDS-PAGE with MES XT running buffer (BioRad; Hercules, CA). The proteins were then transferred to 0.45 μm nitrocellulose (Biorad; Hercules, CA). After transfer, the membranes were boiled in PBS for 5 minutes and blocked overnight in 1% BSA and 2% BlockAce (Serotec) in PBS. The membranes were probed with antibody 6E10 (Covance; 2 μg/mL in PBS with 5% nonfat dry milk), followed by rabbit anti-mouse IgG (Rockland). AβPP was detected using AbCT20, as described (Dimachkie and Barohn 2013). Reactive bands were visualized with Super Signal West Dura HRP Substrate (Pierce Biotechnology, Rockford, IL) and exposed to film overnight.

APP C-terminal fragment (CTF) ELISA

For the measurement of APP or APP C-terminal fragments (CTF) by ELISA, we used a similar procedure to the standard ELISA procedure described in the general methods section. Antibody 22C11 (Millipore; Billerica, MA) was used to capture full length APP from the sample (N-terminal capture), which was then detected using biotinylated 6E10 against Aβ1-16 (Covance; Denver PA). The cleared sample was then transferred to a second plate, coated with affinity purified antibody CT20, raised against
the last 20 amino acids of APP (Murphy, Uljon et al. 2000)(Fig. 3.1). Captured CTFs were detected using either antibody 6E10 (for the β-secretase derived fragment, CTFβ) or 4G8 (total CTFs). Captured CTFs were detected using either antibody 6E10 (for the β-secretase derived fragment, CTFβ) or 4G8 (total CTFs).

**BACE Enzymatic Assays**

We recently described and validated our assays for BACE1 and BACE2 for human tissue in considerable detail, including reagent specificity (Ahmed, Holler et al. 2010). This method is similar to that of Fukumoto et al. (Fukumoto, Cheung et al. 2002, Fukumoto, Rosene et al. 2004). Briefly, NaOAc extracted (10 mM sodium acetate [NaOAc], 3.0 mM NaCl, 0.1% Triton® X-100, 0.32 M sucrose, pH = 5.0 buffer supplemented with PIC) samples were loaded at least in duplicate onto blocked (Protein Free; Pierce Biotechnology, Rockford, IL) 384-well plates containing 0.5 μg of antibody / well (BACE1: MAB931, raised against the BACE1 ectodomain, R&D Systems, Minneapolis, MN; BACE2: Ab1, raised against amino acids 496-511 of BACE2; EMD Biosciences). A second set of samples, loaded in the absence of antibody, served as controls for background activity. In some cases, further validation experiments were performed by using two different antibodies, directed against the opposite ends of BACE1 (C-terminus, EPR3956; Epitomics) or BACE2 (N-terminus, rabbit polyclonal Ab2; EMD Biosciences). Plates were washed 5 times (with: 10 mM NaOAc, 1.5 mM NaCl, 1.0 % Triton® X-100, 0.32 M sucrose, pH = 5.0). To determine enzymatic activity, we used an octameric peptide substrate (1 μM) corresponding to the P4’ to P4 amino acids flanking the β-secretase site in the human APP sequence, and containing the human
Swedish APPΔNL mutation (Peptides International, Louisville, KY; EDANS/DABCYL conjugate, excitation/emission: 350 nm/490 nm). The Swedish familial mutation in APP (ΔNL), which results in early-onset, autosomal dominant AD, enhances β-secretase cleavage (Citron, Oltersdorf et al. 1992, Mullan, Crawford et al. 1992, Cai, Golde et al. 1993); the APPΔNL sequence is a preferred substrate for both BACE1 and BACE2 (Andrau, Dumanchin-Njock et al. 2003). The assay was performed in a buffer similar to the wash buffer, except with a higher salt concentration (10 mM NaCl). Net fluorescence was measured after 2 h at 37°C using a BioTek plate reader.

**BACE Transcription**

While we hypothesized that CNBP controls BACE translation, our preliminary data did not rule out the possibility that CNBP acts at the transcription level. In order to determine if CNBP affects BACE transcription, either directly or indirectly we inhibited transcription and monitored the effect on BACE mRNA levels, monitored by quantitative real-time PCR. 6 x 35mm AAV infected cultures were evaluated for each of 5 time points plus one plate of uninfected cells per construct (CNBP, anti-CNBP shRNA, or eGFP). Cultures were treated with 10µg/ml actinomycin D to block new transcription, and the amount of target mRNA measured by qRT-PCR. For positive and negative controls we used mRNAs reported to be transcriptionally repressed or activated by CNBP. As a negative control, we monitored RPS16 mRNA. As a positive control we monitored levels of c-myc mRNA. Changes in transcription were defined as an increase or decrease in steady state mRNA, in the absence of a change in mRNA half-life. This set of experiments was performed in triplicate.
BACE Translation

15 cm plates of either SY5Y or H4 cells were transduced with CNBP AAV2 (≈50 genome copies / cell) overnight. The following morning, cells were plated into 6-well dishes and left in a 37°C incubator for 8 hours (to allow reattachment). Medium was removed, and replaced with Methionine / Cysteine free media (+10mM HEPES) containing $^{35}$S radiolabeled methionine and cysteine (≈100 μCi / 35mm well) and incubated overnight to allow a steady-state label incorporation. The radioactive medium was removed by aspiration; cells were washed with pre-warmed chase media, and incubated in the non-radioactive media until collected. Triplicate wells were collected for time zero, 1 hr, 2 hrs, 4 hrs, 8 hrs, and 24 hrs. Cells were washed once more, then lysed in ice-cold RIPA buffer containing complete protease inhibitor cocktail. Samples were spun at 14,000 x g for 2 min to pellet insoluble debris before being transferred to a fresh 1.5 ml tube. For immunoprecipitation of BACE 2 μl BACE1 monoclonal antibody (Epitomics, Burlingame, CA) diluted 1:1 in glycerol was added to each tube, along with 5 ul protein G and 25 ul protein A conjugated Sepharose beads. Samples were incubated with rotation at 4°C overnight. Immunoprecipitated protein was collected by spinning at 14,000 x g for 2 min, and aspirating medium for a total of 3 washes. BME and loading buffer was added to samples, which were heated to 65°C for 15 min, and transferred to a bis-Tris gel for separation by SDS-PAGE. The gel was dried and transferred to a phosphoscreen for one week, before being developed on a Typhoon phosphoimager.
5XFAD Mouse Model

In order to test the hypothesis that increasing levels of CNBP have a causative role in increased levels of BACE1 in vivo, we chose to overexpress the protein in the brains of a mouse model of Alzheimer’s disease. While animal models of disease recapitulate several aspects of the AD-phenotype, age-related anomalies are often unapparent until very late in the animal’s lifespan. The 5XFAD model was advantageous for this study because the model develops pathology very early, with soluble Aβ detectable by 21 days of age (Oakley, Cole et al. 2006). Generally, autosomal dominant human mutations that result in familial Alzheimer’s disease tend to cluster near the β and γ-secretase cleavage sites of APP, or in the presenilin subunits of the γ-secretase complex. These mutations result in increased production of Aβ42, the more fibrillogenic peptide species, resulting in early onset disease in humans. Because previous reports indicated that these mutations could have an additive affect, Vassar and colleagues made an accelerated model of amyloid deposition by generating a transgenic with three familial mutations into APP (K670N / M671L (Swedish), I716V (Florida), and V171L (London), and two PS1 mutations (M146L, and L286V)(Oakley, Cole et al. 2006). Importantly, BACE was not manipulated in the making of this model, making it an ideal model-system for evaluating the effect of overexpressing CNBP on the BACE, and Aβ-pathology. If CNBP is sufficient to drive increases in BACE, then this should result in increased downstream Aβ-production, and earlier deposition into amyloid plaques.
**CNBP Overexpression in vivo**

Proven male transgenic animals were bred with wild-type C57BL/6 SJL females. P0 or P1 pups were cryo-anesthetized and 2 µl of CNBP (~2.5 x 10^{10} viral particles) virus was transferred bilaterally into each hemisphere using a Hamilton syringe and beveled needle inserted approximately 2μm into the cortex. Pups were warmed under a heat-lamp and returned to the dam until aged to pre-selected time points, 3 months, 1 month, 14 days, and 7 days. Animals were euthanized by barbiturate overdose and brains collected and dissected as detailed in the general methods section. All animals were genotyped postmortem.
Figure 3.2 CT20 Antibody Specificity
**Figure 3.2 CT20 Antibody Specificity.** CT20-18 is specific for full length βAPP (APP) and βAPP C-terminal fragments (CTFs). A) Samples were lysed in TBS + 1% Triton® X-100, and approximately 90 μg of protein was loaded / lane of a Criterion XT 4-12% gel. After blocking, three separate membranes were probed with either the pre-immune serum, unpurified anti-serum, or anti-serum absorbed with the peptide immunogen. APP(+) = Human H4 neuroglioma cells, overexpressing human APPΔNL695; APP(-) = Nontransgenic mouse brain. B) CTFs were immunoprecipitated from APP(+) cell lysate using CT20-18, and then separated and immunoblotted as in A), above.
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Table 3.1. Human Case-Series (continued)

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|      | 1105 | 84 | 3/3 | Female | 1035 | 2.50 | 4 | 30 | 0.51 | Congestive Heart Failure | N/A |
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|      | 1119 | 87 | 3/3 | Female | 1160 | 2.25 | 4 | 30 | 0.27 | Aortic Aneurysm | N/A |
|      | 1141 | 84 | 3/3 | Female | 1300 | 3.25 | 4 | 29 | 1.18 | Lung Cancer | N/A |
|      | 1150 | 90 | 3/3 | Female | 1110 | 3.50 | 3 | 29 | 1.20 | Stroke | N/A |
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|      | 1187 | 77 | 3/4 | Male | 1340 | 2.75 | 4 | 30 | 1.00 | Esophageal Cancer | N/A |
|      | 1179 | 87 | 3/3 | Female | 1220 | 2.25 | 5 | 30 | 0.67 | N/A | N/A |
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| s.d. | 3.7 | 110.1 | 0.5 | 0.6 | 0.7 | 0.46 |
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Table 3.1 Human Case-Series (continued)

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Table 3.1 Human Case Details. Samples from the Alzheimer’s disease Center (ADC) tissue repository at the University of Kentucky, Sanders-Brown Center on Aging. Controls (n = 9) were age-matched to disease cases (preclinical AD [PCAD]: n = 10; amnestic MCI: n = 7; AD: n = 10). Preclinical AD cases (or high pathology controls) were defined as those that met the NIA-Reagan neuropathology criteria for likely AD, but exhibited no clinical signs of dementia. Amnestic MCI was defined as per the criteria of Petersen et. al. (Petersen, Smith et al. 1999). We included six cases of frontotemporal dementia (FTD) as an Aβ-unrelated neurodegenerative disease and specificity control. Tissue processing details provided for various methodologies provided in the General Methods Chapter. Abbreviations: FTD = Frontotemporal Dementia; MCI = Mild Cognitive Impairment; AD = Alzheimer’s Disease; ApoE = Apolipoprotein E genotype; PMI = Post-Mortem Interval; MMSE = Mini-Mental State Exam; C.O.D. = Cause of Death; N/A = Not Applicable or Unavailable.
RESULTS

The Cellular Nucleic Acid Binding Protein (CNBP) Regulates β-Secretase (BACE1)

In an effort to identify gene products that alter Aβ production, our lab transduced a functionally hemizygous cell line, chinese hamster ovary (CHO) cells, with a retroviral gene trap vector; random viral integration can generate a knock-out phenotype, and the investigator can screen mutant cell lines for the desired phenotype, in this case by ELISA for Aβ (~3500 cell lines screened). Plasmid rescue techniques were used to excise DNA from one cell line (FF1) that produced less Aβ, and the resultant DNA fragment was sequenced and searched for in a non-redundant database, yielding a match to the ZNF9 gene, that encodes CNBP. Replacement of CNBP in the FF1 cell line normalized Aβ production (MPM, data not shown). Two sequential enzymatic activities produce the Aβ peptide. First, β-site APP Cleaving Enzyme (BACE1) cleaves the Amyloid Precursor Protein (APP) into a secreted APP fragment (sAPPβ) and leaves behind a 99 residue C-terminal fragment (CTFβ), which is cleaved by γ-secretase to Aβ and the APP intracellular domain (AICD).

CNBP and BACE1 are Highly Correlated in Human Brain and Change in Alzheimer’s Disease.

After validating that CNBP was the interrupted gene, we wanted to determine if the amount of protein was altered in the brain, and if these changes could account for changes in BACE1 that occur in AD. We examined a disease affected (the superior and middle temporal gyri; SMTG) and unaffected (the cerebellum; CB) region from a cohort
of AD cases and age-matched controls (N = 41; Figure 3.3 A, B). In both brain regions, the amount of CNBP and BACE1 were positively correlated (Spearman’s ρ: SMTG = 0.423, p<0.01; CB = 0.316, p<0.05). We next performed an analysis of CNBP and BACE1 levels using age, post-mortem interval, and GAPDH levels (the loading control) as covariates, and including gender as a variable (Figure 3.3 C). In the SMTG, both CNBP [F(1,34) = 9.29, p<0.005] and BACE1 [F(1,34) = 11.43, p<0.005] were increased in the AD cases. In contrast, in the CB, both CNBP [F(1,34) = 4.30, p<0.05] and BACE1 [F(1,34) = 8.03, p<0.01] were decreased. Neither CNBP nor BACE1 mRNA changed significantly in the disease.

**BACE1 and BACE2 Proteins and Activities Increase in Neurodegenerative Disease**

In an earlier study (Ahmed, Holler et al. 2010), we noted that BACE2 was nearly as abundant in human brain as BACE1. This was also true in this larger series of cases (Fig. 3.4.). Overall, BACE1 (F[4,32] = 2.89, p<0.04) and BACE2 (F[4,32] = 2.87, p<0.04) activities were both increased in neurodegenerative disease (Fig. 3.4. A, B). The increase occurred in a disease-affected region (the SMTG; superior and middle temporal gyri, areas 21 and 22) but not in a brain region unaffected by disease (the CB; cerebellum). Even though enzymatic activity was not increased in the CB with disease, BACE1 (R² = 0.25, p<0.001) and BACE2 (R² = 0.08, p<0.04) activities in the CB were still significantly correlated with activities in the SMTG. The same increases in a disease-affected region (SMTG) but not in an unaffected region (CB) were seen for BACE1 (F[4,32] = 5.87, p<0.001) and BACE2 (F[4,32] = 15.03, p<0.0001) proteins on Western blot, (Fig. 3.4 C, D) when examined by densitometry. The results were unchanged when
the data were not standardized to β-Actin as a loading control. The results were also the same if MCI/PCAD cases were treated as a single, combined group (the amount of pathology in these cases is nearly identical); (Table 3.1). As expected from earlier assay validation studies, BACE1 and BACE2 activity were correlated with their respective protein bands detected by immunoblot (BACE1: $R^2 = 0.17$, $p<0.006$; BACE2: $R^2 = 0.21$, $p<0.002$). Also, consistent with a general increase in β-secretase activity, the APP CTFβ:α ratio was higher in AD in the SMTG ($F[1,38] = 12.00$, $p<0.001$), but not in the CB. In SMTG from AD cases, the CTFβ:α ratio was nearly twice that of the other cases ($118 \pm 14$ vs. $60 \pm 8$; units are arbitrary). The CTFβ:α ratio was also correlated with both BACE1 ($R^2 = 0.09$, $p<0.03$) and BACE2 ($R^2 = 0.07$, $p<0.05$) activity. The outcome was similar when the SMTG ratio was standardized to the CTFβ:α ratio in the CB, although in this case the overall disease effect was also significant ($F[4,34]= 4.97$, $p<0.01$). These data indicate that an increase in β-secretase activity and protein in the brain, both BACE1 and BACE2, likely occurs at an early stage in the disease process, before cognitive impairment is detectable. Further, this increase may not be entirely specific to AD, since both BACE1 and BACE2 were similarly elevated in FTD cases.

We observed a striking correlation between BACE1 and BACE2 in human brain, a phenomenon observed with multiple antibody combinations and assay conditions. Using our standard capture assays for determining BACE1 (MAB931) and BACE2 (Ab1) activities, we found strong correlations between the two enzymatic activities in both the SMTG ($R^2 = 0.9$, $p<0.0001$) and CB ($R^2 = 0.7$, $p<0.001$), indicating that this correlation was unrelated to disease. To confirm this finding, we repeated the assay using a different method. In the validation test, we reversed the orientation of the assay and used different
antibodies, capturing BACE1 at the C-terminus (using EPR3956) and BACE2 at the N-terminus (using Ab2). BACE1 and BACE2 activity remained highly correlated in both the SMTG (R² = 0.64, p<0.001) and CB (R² = 0.47, p<0.001) (Fig. 3.3, E, F). We observed similar disease-related increases in BACE1 and BACE2 using this alternate method (data not shown). Finally, we examined the BACE1 (using MAB931) and BACE2 (using Ab5670) relationship by immunoblot and detected a similarly strong correlation (R² = 0.32, p<0.001). The correlation was significant whether or not the data were standardized to the β-actin loading control. Hence, we reproducibly detected exceptionally strong correlations between BACE1 and BACE2 proteins and activities using different methodologies.

We next wanted to determine whether changes in the β-secretase enzymes occurred only at the level of protein and activity, or extended down to the mRNA level. The total amount of BACE1 or BACE2 mRNA did not change with disease state (data not shown). The amount of BACE1 and BACE2 mRNA did correlate with each other in both the SMTG (R² = 0.48, p<0.001) and CB (R² = 0.72, p<0.001), but in neither case did the amount of total mRNA correlate with the amount of BACE1 or BACE2 activity or protein.

**BACE1 and BACE2 localization**

Our initial findings revealed that BACE2 was nearly as abundant in the human brain as BACE1. Using well characterized antibodies (Ahmed, Holler et al. 2010), we first performed an examination of the localization patterns of both BACE1 and BACE2 in the human brain. Although some areas overlap, we observed noticeable differences (Fig.
BACE1 positive cells (left panels) were abundant throughout the neocortex, and were often of distinct pyramidal cell morphology. BACE1 immunoreactivity was mainly present as punctate perinuclear and cytoplasmic staining, consistent with a primary localization to the endosomal/lysosomal compartment. BACE2 positive pyramidal neurons were also abundant. However, we also observed numerous BACE2 positive cells that had the signature stellate appearance of astrocytes, particularly close to the brain surface. These areas frequently contained few BACE1 positive cells. BACE2 immunoreactivity within cells of astrocytic morphology was more widely dispersed, and the processes were frequently labeled extensively. We did not observe a clear relationship between BACE1 or BACE2 positive cells and deposits of the Aβ peptide. However, BACE2 positive processes resembling dystrophic neurites were found closely associated with some plaques.

To examine the distribution of BACE1 and BACE2 in more detail, we performed a double label immunofluorescence study (Fig. 3.6). Both forms of β-secretase were found in neurons. However, even in areas where astrocytes were present in large numbers, we did not observe any that were clearly BACE1 positive. Although we did find examples of BACE2 positive astrocytes, these were surprisingly less common in areas of extensive gliosis and neurodegeneration. BACE2 positive astrocytes in these areas usually had more extensive cytoplasmic labeling and less distinctly labeled processes. We observed BACE2 positive astrocytic processes in association with blood vessels. We did not observe significant immunostaining for either BACE1 or BACE2 in microglia (data not shown). Hence, although there were some differences in the distribution pattern of
BACE1 and BACE2 in the human brain (notably the absence of BACE1 in astrocytes), both were found in neurons.

**Changes in the BACE Product Aβ in Neurodegenerative Disease**

The full length APP levels did not change noticeably between disease and non-disease groups, even though there is a modest relationship between CTFβ and β-secretase activity in these cases (Dimachkie and Barohn 2013). There were several notable differences between the distribution of the Aβ peptide among the different soluble fractions. First, there was substantially less Aβ in the cerebellum compared to the SMTG in both the PBS and SDS fractions (Fig 3.7A. left and center panels, respectively). In contrast, there was a strong, positive Aβ signature in the FA fraction in the cerebellum in the AD cases that was comparable to that of the SMTG (Fig 3.7A. right panels). Second, there is only a faint monomeric Aβ band detectable in the PBS fraction in the SMTG, whereas there are multiple higher molecular weight bands in the SDS fraction, and a wide range of dissociated fragments in the FA fraction. These most likely correspond to both oligomeric and fibrillar Aβ species, and were clearly elevated in the AD cases. Within the SDS fraction, there were multiple distinct bands of oligomeric Aβ species that could also be seen in the MCI and PCAD cases. We did not detect a distinct overabundance of any single prominent band, such as Aβ*56 (Lesne, Koh et al. 2006), although there were certainly bands within this size range. There was little, if any, Aβ detected in either the control or FTD cases.
Overexpression of CNBP leads to Increased BACE1 Protein In Tissue Culture

The β-secretase cleavage is the rate-limiting step in formation of the pathogenic Aβ peptide, and represents a major potential therapeutic target for AD. BACE1 knockout in mice abolishes formation of sAPPβ, as well as CTFβ and Aβ, supporting a causal role for BACE enzymatic activity in AD pathogenesis. Metabolic labeling and RT-PCR experiments indicated that the effect of CNBP on Aβ was caused by changes in BACE1 expression, at either the mRNA or protein level, or both.

We began by generating an adeno-associated viral vector (AAV) to overexpress the CNBP protein for use in various in vitro and in vivo systems. CNBP was cloned into the pZac.1 viral system, containing the CMV promoter (U Penn) and transfected into HEK 293T cells along with the AAV helper plasmid and AAV2 capsid plasmid. Virions were harvested, purified, and reconstituted in sterile PBS. This virus was then used to determine which immortal and primary cell lines were amenable to viral transduction and what multiplicities of infection (MOIs) were necessary to induce overexpression without reaching toxicity. The CMV-CNBP AAV2 virus was able to transduce all cell lines tested, including SY5Y and C2C12 cells (Figure. 3.8) as well as H4s, and HEK 293T cells. Primary neurons were also efficiently transduced by overnight incubation with the virus. In all cell lines examined, BACE1 protein levels increased with CNBP overexpression. At MOIs of 50 genome copies / cell and over, even the empty vector showed an increase in BACE1, possibly as a consequence of cellular stress.

We tested the efficacy of several shRNAs to knockdown CNBP protein in cell culture using a commercially available system (OriGene HuSH). In several cell lines
shRNA 30 resulted in the most dramatic decrease in protein, and was chosen for further analysis (Fig. 3.8 B). Transfection with the shRNA also resulted in decreased BACE1 protein (Fig. 3.8 C).

**CNBP Overexpression Leads to Increased BACE1 Protein Without Changes in mRNA or Protein Half-life.**

While biochemical systems indicate that CNBP can bind either ssDNA or RNA (Armas, Nasif et al. 2008), in a cellular context the protein exhibits a many-fold preference for RNA, is greatly enriched in the cytoplasm and ER, and has no consensus sequence for nuclear localization. This, and the fact that other reports indicate that CNBP interacts with the 5’ UTR of mRNAs (Pellizzoni, Lotti et al. 1997), and the presence of a potential binding site with weak sequence homology within the 5’ UTR of BACE1 led us to consider that CNBP regulates BACE at the translational level. While we hypothesized that CNBP controls BACE translation, our preliminary data did not rule out the possibility that CNBP acts at the transcriptional level. In order to determine whether CNBP affects BACE transcription, either directly or indirectly, we overexpressed CNBP (by viral transduction) then inhibited transcription using Actinomycin D (forms a non-specific complex with DNA that inhibits RNA synthesis) and monitored the effect on BACE mRNA levels, by quantitative real-time PCR. For positive and negative controls we used mRNAs reported to be transcriptionally repressed or activated by CNBP. Changes in transcription were defined as an increase or decrease in steady state mRNA, in the absence of a change in half-life. CNBP overexpression had no effect on BACE1 mRNA half-life (Figure 3.9) (hours ± 95% C.I.: CNBP: 7.3 ± 0.3, Vector: 7.4 ± 0.7
hours); the housekeeping gene TPT1 mRNA was similarly unaffected (hours ± 95% C.I.: CNBP: 5.1 ± 0.5 hours, Vector: 4.5 ± 0.7 hours) (2 experiments, 3 replicates each / time point). This is consistent with our data, and the data of several other groups, showing that the BACE1 increase in the AD brain is not accompanied by an increase in BACE1 mRNA, further supporting a role for CNBP in post-transcriptional BACE1 regulation.

The increase in BACE expression could result from either increased translation, or a reduced rate of protein turnover. In order to determine if the half-life of the BACE1 protein was affected by CNBP overexpression, cells were treated with radiolabelled $^{35}$S Methionine / Cysteine overnight, to steady-state label all proteins being translated. BACE1 was immunoprecipitated at timepoints out to 24 hours. (Fig. 3.10 A, B) Steady state metabolic labeling experiments (in H4 cells) showed no obvious effect of CNBP overexpression on BACE1 protein half-life (2 experiments, 2-3 replicates each / time point); as expected, CNBP overexpression did result in slightly higher starting amounts of BACE1. Specificity is shown for BACE1 IP (antibody EPR3956; Epitomics) using mock (rabbit IgG + protein A/G beads), blank (beads alone) or after absorbing the antibody with an excess of BACE1 antigen (CNBP overexpression is also shown). Follow up experiments indicated that CNBP was able to bind BACE1 mRNA directly, but it was not able to bind BACE2 mRNA (CJH, submitted).

**CNBP is Tightly Regulated *in vivo***

In order to test the hypothesis that increasing levels of CNBP have a causative role in increased levels of BACE1 *in vivo*, we chose to overexpress CNBP in mouse skeletal muscle. Viral transfer was efficacious in vivo, and resulted in a significant
increase in BACE1 protein and downstream Aβ production, supporting a role for CNBP in regulating BACE1 in vivo (data discussed in detail in section 3.3). The realization that CNBP affected BACE1 translation in several in vitro and in vivo systems prompted us to ask whether this could be exploited to either accelerate or decelerate pathology formation in the brain of an animal model of AD. We chose the 5XFAD mouse model, which has 3 human familial AD mutations in APP, as well as 2 mutations in presenilin1, part of the enzymatic subunit of the γ-secretase complex. These mutations have an additive effect on pathology, resulting in a highly accelerated model of Aβ pathology, with soluble forms of the peptide detectable by 21 days of age (Oakley, Cole et al. 2006). Importantly, BACE was not manipulated in the making of this model, making it an ideal model-system for evaluating the effect of overexpressing CNBP on the BACE, and Aβ-pathology. If CNBP is sufficient to drive increases in BACE, then this should result in increased downstream Aβ-production, and earlier deposition into amyloid plaques. The viral delivery strategy was advantageous in this case for several reasons. First, using a viral delivery strategy in specific target tissues allowed us to study effects of changes in CNBP while circumventing systemic effects of gene manipulation throughout the organism, as it is known that CNBP knockout is lethal at embryonic day 10 (Chen, Liang et al. 2003). Also, this technique was advantageous because it was more time and cost effective than generating and characterizing several lines of genetically modified mice. Entire litters of P0 or P1 pups (≈50% transgenic) were cryo-anesthetized and 2 µl of CNBP (≈1 x e10 GC / ml) virus was transferred bilaterally into each hemisphere using a Hamilton syringe. Brain tissue was examined at various time points from 1 week through 3 months of age, well past the age when amyloid begins to deposit into amyloid plaques in the 5XFAD
model. We have used AAV successfully in the brain. Expression of AAV2/1-hSyn1-WPRE (also from the U Penn Viral Core) vector containing eGFP demonstrated stable neocortical expression out to 2-3 months (Fig 3.11 A). Expression of the hSyn1 promoter drives primarily neuronal expression, in this case large pyramidal neurons in neocortical layers 4 and 5 (20x) (Fig 3.11 B). We have been able to obtain stable brain overexpression (~3x or better, for ~3 months) of two other proteins (mLepR and Pin1; not shown) using this approach, but were not able to overexpress CNBP (CNBP, N = 35; pZac2.1, N = 21; eGFP, N = 7; Uninjected controls, N = 10; not shown). Because CNBP-AAV2 transduction was not efficacious (Fig 3.11 D), the virus was remade with the AAV1 capsid, but this was also not effective in the brain at 21 days (Fig 3.11 C) (CNBP, N = 16; Uninjected controls, N = 9), 14 days, (CNBP, N = 8; Uninjected controls, N = 4; not shown) or 7 days (CNBP, N = 9; Uninjected controls, N = 4; not shown). Finally, an alternate animal strain, the APP/PS1 knock-in model was tested also without significant increase in CNBP (not shown). Inability to overexpress the protein in brain, even though the virus did transduce primary neurons in culture, likely reflects a redundant mechanism for strictly regulating levels of the protein, as overexpression may be toxic, and may be restricted within a very narrow range by a developmentally regulated miRNA or similar mechanism. To test this, we performed stereotactic injection into the hippocampus of adult APP / PS1 animals (CNBP, N =6; pZac2.1 Vector, N = 6). Modest overexpression was achieved with this method (p<0.05) (Fig 3.12 A, B).
Figure 3.3. CNBP and BACE1 are Highly Correlated in Human Brain and Change in Alzheimer’s Disease
Figure 3.3. CNBP and BACE1 are Highly Correlated in Human Brain and Change in Alzheimer’s Disease. A) Representative Western blots for BACE1 and CNBP protein in AD-affected (the SMTG) and unaffected (the CB) regions. B) CNBP and BACE1 levels are significantly and positively correlated in both the SMTG (p<0.01) and the CB (p<0.05). C) In AD, CNBP and BACE1 are significantly higher in the SMTG, and significantly lower in the CB; the mRNA for CNBP was unchanged (p > 0.4), although data were only available for a subset of the cases (10 AD, 9 control; data were standardized to the amount of TPT1 mRNA; no effect was observed for alternative methods of standardization). * = p < 0.05, ** = p < 0.01. CNBP and BACE1 are Highly Correlated in Human Brain and Change in Alzheimer’s Disease. *Reprinted from (Holler, Webb et al. 2012), with permission from Elsevier.*
Figure 3.4. BACE1 and BACE2 are increased in neurodegenerative disease.
Figure 3.4. BACE1 and BACE2 are increased in neurodegenerative disease. A) BACE1 activity (as determined by the MAB931 capture assay) is higher in PCAD, AD, and FTD. B) BACE2 activity (as determined by the Ab1 capture assay) is higher in FTD and PCAD and strongly trends towards an increase in AD cases (p<0.07). C) BACE1 protein is higher on Western blot in both FTD and AD. D) BACE2 protein is higher on Western blot in MCI, PCAD, AD and FTD. Immunoblot results were essentially unchanged when not standardized to βActin, indicating that outlier cases with very low βActin levels had minimal influence on the analysis. E, F) BACE1 and BACE2 activities are highly correlated, a phenomenon observed with multiple antibody combinations and assay conditions (shown: BACE1 EPR3956 capture assay, BACE2 Ab2 capture assay). Dunnett’s Test, * = p<0.05; ** = p<0.01. BFU = Base Fluorescence Units; 1000 BFU / mg = 2.3 nmol · min⁻¹ · mg⁻¹ total protein. Reprinted from (Holler, Webb et al. 2012), with permission from Elsevier.
Figure 3.5. BACE1 and BACE2 Distinct Patterns of Cellular Immunoreactivity in the Human Brain.
Figure 3.5. BACE1 (left panels) and BACE2 (right panels) Distinct Patterns of Cellular Immunoreactivity in the Human Brain. A,C) Strongly BACE1 positive cells (brown) of distinct neuronal morphology are found throughout the neocortex, and are not clearly associated with Aβ deposits. B,D) BACE2 immunoreactivity (brown) was more commonly found in cellular processes, occasionally intertwined with plaques. E,G) Many BACE2 positive cells are clustered in the outer cortical layer near the surface of the brain, in areas largely devoid of BACE1 positive cells. F,H) Although some BACE1 is found in cellular processes, most BACE1 immunoreactivity is localized in a punctate perinuclear pattern; in contrast, BACE2 has a broader intracellular distribution (counter stain: cresyl violet). Antibodies: BACE1, mouse monoclonal MAB931; BACE2, rabbit polyclonal Ab2; Aβ, mouse monoclonal 4G8. Substrates: 3, 3’-Diaminobenzidine (DAB, brown), or Vector® SG. Reprinted from (Holler, Webb et al. 2012), with permission from Elsevier.
Figure 3.6. BACE2 was Observed in Both Neurons and Astrocytes.
**Figure 3.6. BACE2 was Observed in both Neurons and Astrocytes.** A) BACE1 was not seen in astrocytes, even in areas where substantial numbers of astrocytes were clearly labeled or in areas of extensive gliosis. B) BACE2 positive astrocytes were rarely found ([arrowhead](#)) in areas of extensive neurodegeneration and gliosis. C,D) Both BACE1 and BACE2 were present in neocortical pyramidal neurons. E,F) BACE1 and BACE2 negative astrocytes in close proximity to positive labeled cells, some of which are distinctively neuronal in morphology. G) A BACE2 positive astrocyte ([yellow arrow](#)) showing strong cytoplasmic labeling. H,I) BACE2 positive astrocytic processes ([white arrow](#)) are often found in association with blood vessels. **Antibodies:** BACE1, rabbit monoclonal EPR3956 (red); BACE2, rabbit polyclonal Ab2 (red); GFAP, mouse monoclonal MAB360 (green). **Counter stain:** DAPI. Scale bars = 25 μm. **Reprinted from** (Holler, Webb et al. 2012), *with permission from Elsevier.*
Figure 3.7. Aβ and APP in a Disease Affected (SMTG) and Unaffected (Cerebellum) Region.

A.

B.
Figure 3.7. Aβ and APP in a Disease Affected (SMTG) and Unaffected (Cerebellum) Region. A) The Aβ peptide can be detected at various levels on immunoblot. A synthetic Aβ42 standard was run on the first lane of each gel; full length APP can be seen in both the PBS and SDS fractions, between the 188 and 62 kDa markers. A faint band corresponding to monomeric Aβ can be seen in a few cases in the SMTG, but not in the cerebellum. A substantially stronger signal especially for monomeric Aβ, can be seen in the SDS fraction, along with a variety of higher molecular weight, oligomeric forms of the peptide. SDS soluble Aβ can be seen in the AD, MCI and PCAD cases, and is mostly absent from the control and FTD cases. As with the PBS fraction, very little SDS soluble Aβ can be detected in the cerebellum. There was a large amount of FA soluble Aβ in both the SMTG and cerebellum in AD cases; a large portion of this signal appears as a smear, representing a wide range of Aβ species that have been dissociated from relatively insoluble fibrillar material. B) The expression of full length AβPP is not remarkably different between control and disease cases. Lysate from H4 cells overexpressing human AβPP<sub>695ΔNL</sub> was run in as a marker in the first lane. All immunoblots were probed and developed for the same amount of time. Reproduced from (Beckett, Webb et al. 2012).
Figure 3.8. Changes in BACE1 Parallel CNBP in Culture

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### B.

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Figure 3.8. Changes in BACE1 Parallel CNBP in Culture. A) A test of AAV2-CNBP virus and control over a range of multiplicities of infection (MOI) in human SH-SY5Y neuroblastoma cells (BACE1 antibody: MAB931). Interestingly, the highest MOI of the control AAV2 virus also caused a slight increase in both CNBP and BACE1. It is possible that this is a nonspecific effect of the cells being stressed at this higher concentration, and would be broadly consistent with the effects observed of more direct cellular stressors. B) Several commercially available shRNAs were tested for ability to knockdown CNBP protein in multiple cell lines. OriGene HuSH shRNA 30 was most consistent (H4 cells shown). C) Reduction of CNBP via shRNA treatment also resulted in decreased BACE1 protein.
Figure 3.9. CNBP Overexpression does not Affect BACE1 mRNA Half-life
Figure 3.9. CNBP Overexpression does not Affect BACE1 mRNA Half-life. H4 WT cells transfected with ≈ 50 GC/cell CMV CNBP AAV2 or CMV pZac2.1 Empty Vector overnight had essentially the same rate of mRNA decay (hours ± 95% confidence interval. CNBP: 7.3 ± 0.3, Vector: 7.4 ± 0.7 hours); the housekeeping gene TPT1 was similarly unaffected (hours ± 95% confidence interval. CNBP ± 5.1 ± 0.5 hours, Vector: 4.5 ± 0.7 hours) (2 experiments, 3 replicates per time point).
Figure 3.10. CNBP does not Change BACE1 Protein Half-life

A. CNBP and Vector expression of BACE1 over time.

B. Bar graph showing total BACE1 (AU) over time.

C. Western blot for BACE1 expression at different time points.
Figure 3.10. CNBP does not Change BACE1 Protein Half-life. A, B) CNBP overexpression had no effect on BACE1 half-life. C) Similarly, steady state metabolic labeling experiments (in H4 cells) showed no obvious effect of CNBP overexpression on BACE1 protein half-life (2 experiments, 2-3 replicates each / time point); as expected, CNBP overexpression did result in slightly higher starting amounts of BACE1. Specificity is shown for BACE1 IP (antibody EPR3956; Epitomics) using mock (rabbit IgG + protein A/G beads), blank (beads alone) or after absorbing the antibody with an excess of BACE1 antigen (CNBP overexpression is also shown).
Figure 3.11. CNBP transduction is ineffective in brain
Figure 3.11. CNBP transduction is ineffective in brain.  A) Tests of an AAV2/1-hSyn1-WPRE (also from the U Penn Viral Core) vector containing eGFP demonstrated stable neocortical expression out to 2-3 months (shown: 1 month, 10x). B) The hSyn1 promoter drives primarily neuronal expression, in this case large pyramidal neurons in neocortical layers 4 and 5 (20x). We have been able to obtain stable brain overexpression (~3x or better, for ~3 months) of two other proteins (mLepR and Pin1; not shown) using this approach, but were not able to overexpress CNBP (CNBP, n = 12; controls, n = 10; not shown). C) Aβ values were stable in the absence of changes in CNBP and BACE1 (data shown are for 5xFAD mice, ~3-4 months of age, but similar data were collected in other experiments). Neither AAV1 (D; CNBP, n = 33; controls, n = 17; shown: 21 days) nor AAV2 (E; CNBP, n = 34; controls, n = 50; shown: 3-4 months) using a generic CMV promoter were able to drive CNBP overexpression in the mouse brain (post-injection time: 7 – 120 days). The same batch of AAV2 (Fig. 3.7) was able to effectively transduce multiple cell lines (including rat primary neurons; Figure 3.7D) and was able to drive stable overexpression in mouse skeletal muscle tissue in vivo (Figure 5.1; see main text); these data may indicate that the brain exerts tight restriction on CNBP expression in vivo.
Figure 3.12 Modest CNBP Overexpression in Transduced Hippocampus

A.

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B.

![Graph showing protein expression levels]

Protein Expression (AU)

- CNBP AAV2
- pZac2.1 AAV2
- Uninjected

* Statistical significance
Figure 3.12 Modest CNBP Overexpression in Transduced Hippocampus. Stereotactic injection into the hippocampus of adult APP / PS1 animals. Overexpression was analyzed two weeks post-injection (CNBP, N = 6; pZac2.1 Vector, N = 6). Modest overexpression was achieved with this method (p<0.05).
DISCUSSION

During development BACE1 is involved in myelination of the peripheral and central nervous system through regulation of neuregulin (Hu, Hicks et al. 2006). This may be representative of the function that BACE1 plays following traumatic brain injury, or even early in the AD process. However, both increased enzymatic activity and protein levels play a role in the development of AD, as β-secretase cleavage is the rate-limiting step in formation of the pathogenic Aβ-peptide. Increased production of Aβ, along with potential decreases in mechanisms that clear Aβ from the brain, like neprilysin and / or insulin degrading enzyme all conspire in the AD process(Qiu, Walsh et al. 1998, Russo, Borgh et al. 2005).

Although much attention has focused on the role of BACE1 as a therapeutic target for AD, there are actually two atypical aspartyl proteases found in the brain, BACE1 and BACE2. The enzymes share ≈65% sequence homology, and are the only transmembrane domain containing members of the pepsin family of aspartyl proteases. Previous reports indicated that BACE1 represents the majority of β-secretase activity in the brain, based on the fact that knockout of BACE1 halts Aβ production in the brains of rodents, and also because there was thought to be substantially less BACE2 in the brain. However, we recently reported that the two enzymes are actually expressed at comparable levels in the human brain (Holler, Webb et al. 2012). Further, the two are likely subject to the same regulatory mechanisms, as they are correlated in both disease affected and unaffected regions in the brain, and disease-related increases in BACE1 are accompanied by comparable increases in BACE2, and importantly, both occur early in the disease
process. While BACE1 is only expressed in neurons, BACE2 is found in both astrocytes and neurons. BACE2 can either cleave APP at the β-site, or within the peptide, actually abrogating Aβ formation, and acting as an alternative α-secretase. For these reasons it was important to re-examine the role of these enzymes in the AD brain as BACE2 could also contribute to the disease process in a way not yet appreciated. While the BACE1 acts as the rate-limiting step in formation of the Aβ-peptide, a role for BACE2 in disease is not as clear but could contribute to neuropathology seen in AD (Holler, Webb et al. 2012).

Recently, we discovered that the β-secretase may be regulated by a small RNA-binding protein, CNBP. While this protein is important in forebrain patterning and head development in several organisms (Austin, Pappolla et al. 1995, Chen, Liang et al. 2003, Dimachkie and Barohn 2012), little is known about the role of this protein in the adult organism, even though it is very abundant, and occurs in every tissue examined for expression. An expansion in the first intron of the ZNF9 gene, that encodes CNBP, causes myotonic dystrophy type II, and this will be discussed at length in Chapter 5. While several transcription factors have been implicated in β-secretase regulation, CNBP is unique in that it represents a new layer of regulation that, along with small non-coding RNAs, could act to regulate its targets at the post-transcriptional level in response to stimuli in a temporal and spatial manner that may be important for neurons specifically. This could be important for regulating protein translation at the synapse, and could therefore have a role in memory formation, structural integrity of the synapse, or contributing to pathology in other ways. Post-transcriptional mechanisms regulating BACE1 are known to change in AD (Wong 2008). When this translational control goes
awry, with aging or pathological conditions, global rates of translation could become slower or abberant (Kaeberlein and Kennedy 2008).

In cell culture models and primary neuronal cultures manipulating CNBP resulted in corresponding changes in BACE1 in the same direction with both overexpression and knockdown. Follow up experiments indicated that CNBP was able to bind BACE1 mRNA directly, but it was not able to bind BACE2 mRNA (CJH, submitted). Given this difference, it seems likely that although the two seem to share regulatory mechanisms at the post-transcriptional level, it is probably not through CNBP. However, both BACE1 and BACE2 are expressed peripherally, where CNBP regulation may be important, as will be discussed in the following chapters.

Interestingly, it was not easy to assess the relationship between CNBP and BACE1 in the brain of mouse pups. Efforts to overexpress CNBP using a validated AAV virus were thwarted by the brain, even though the same virus was able to transduce primary neurons. We know that CNBP levels are required above a certain unknown threshold in order for rostral head development to proceed properly, with even haploinsufficiency resulting in severe problems. However, while there was some concern that drastic overexpression of CNBP may be toxic (based on unpublished results indicating higher levels of cell death in CNBP AAV transduced cell lines), we did not anticipate the inability to overexpress the protein in vivo. In the initial experiment CNBP AAV2 was transferred bilaterally into the brains of litters of mouse pups estimated to be ≈50% transgenic 5XFAD mice. These mice were aged out for 3 months, a time-point chosen based on the earliest plaque deposition becoming apparent in the brain. By evaluating pathology at this early stage we hoped to be able to discern differences in rates
of pathology formation between the CNBP injected groups and empty vector or eGFP injected groups, and thus either support or undermine a role for the small RNA-binding protein in regulating the β-secretase and driving neuropathology in vivo. When we found that CNBP was not overexpressed after 3 months, we decided that the viral capsid protein type may not be optimal for transduction, even though other reports indicated AAV2 was able to transduce brain tissue, or perhaps the virus was silenced relatively quickly, and so shorter time points were evaluated. The virus was made with the packaging protein AAV1, and the procedure was repeated at 21 day, 14 day, and 7 day time points, but in no instance was there evidence of protein overexpression. In an effort to determine if there was perhaps a small increase in CNBP not detectable by western blot analysis, all samples were evaluated for changes in Aβ production by ELISA, but again, there was no difference between the groups (not shown). Finally, in an effort to see if this inability to overexpress the protein was a mouse strain-specific anomaly, litters of APP / PS1 (APP<sub>NLh</sub>/APP<sub>NLh</sub>×PS-1<sub>P246L</sub>/PS-1<sub>P246L</sub>) human double knock-in animals were injected with the virus, but again overexpression was not achieved.

The fact that it was not possible to overexpress the protein using the same virus that worked in multiple other model systems may indicate that levels of the CNBP are tightly regulated in vivo. Perhaps the local abundance of the protein is controlled by production of an mRNA that would necessarily have to target the coding sequence of the protein (as the viral insert was cDNA only). This seems possible given that increased levels of the protein in cell culture did result in higher rates of cell death, and also because development of the brain continues after birth, until the animal reaches maturity. Given the decreased expression levels of CNBP after birth, it may be possible to
manipulate levels of the protein in the adult organism without severe repercussions that would result from an increase during the developmental stage. To test this, we performed stereotactic injections into the hippocampus of a small group of animals, and indeed a modest amount of overexpression was achieved \textit{in vivo}, supporting the hypothesis outlined above.
Chapter 4: CNBP in Aging and Down syndrome

Portions of this chapter are reprinted from:


INTRODUCTION

In the previous chapter a strong correlation between CNBP and BACE1 in AD was described in post-mortem brain tissue. From this we wanted to know if aging drives CNBP expression, as age is the greatest risk factor for AD. Because CNBP is implicated in both neurodegenerative and neuromuscular disease, both brain and muscle tissues were examined for changes in protein levels. Also, we wanted to determine if the homologous enzyme BACE2 was connected, or similarly subject to regulation by CNBP.

CNBP in Aging and Down syndrome

There are several theories as to why the aging process occurs. When considered from an evolutionary perspective, for example, lifespan likely represents the internal struggle that favors reproduction over self-preservation, and therefore dictates that cellular resources are shunted toward processes that ensure fecundity over somatic DNA
repair and cellular maintenance (Jin 2010). There are also several overlapping theories as to how the aging process occurs, and these are thought to contribute to lifespan determination in a species-specific manner. Epigenetic changes, telomere attrition, changes in mitochondria and the redox states of proteins, and inflammatory processes are all thought to contribute to the aging process (reviewed in (Stadtman 1988, Wisniewski and Frangione 1996)). However, which changes are causal and which are consequential are the matter of much debate, and indeed aging likely represents the culmination of many of these factors over time. This is a very complex process, as illustrated by the concept of hormesis, or the idea that being subjected to mild versions of these same cellular stressors acts as a pre-conditioning mechanism that actually improves longevity. It is thought that these low level stressors actually extend lifespan by stimulating cellular maintenance and repair pathways (Li, Tian et al. 2011).

Until very recently, factors that determine lifespan were considered a ‘fixed’ species-specific trait that was variable only within a fairly small range. However, this has been challenged by the discovery in C.elegans and Drosophila that interfering with individual genes can have profound (positive or negative) effects on longevity of the organism (Jazwinski 2000). With increasing complexity of the model organism, the percent change lifespan seems less substantial, but nonetheless, aging is indeed a plastic process that can be manipulated. While there are several species specific factors that must be considered when examining mutations that affect longevity, it seems across species, three cellular signaling pathways are central to the aging process. The insulin growth factor, the kinase target of rapamycin, and p38 mitogen-activated protein kinase pathways seem to be universally affected by the aging process.
On the cellular level, aging is thought to occur as the result of slowing rates of both new protein translation, and degradation pathways, resulting in a protein pool that is subject to a barrage of insults, resulting in increased damage over time (Kaeberlein and Kennedy 2008). While the rate of translation of new proteins decreases with aging, the fidelity of the process does not seem to decrease. Protein degradation pathways, including proteasomal degradation, autophagy, and lysosomal degradation all become less efficient with aging, and damaged proteins may further inhibit efficient degradation, exacerbating altered protein homeostasis. All components of the translational apparatus are likely subject to regulation by presence of a 5’ TOP sequence in their mRNA (Levy, Avni et al. 1991). Because CNBP is known to interact with 5’TOP sequences, changes in levels of the protein with aging could negatively impact basal levels of components of the translational apparatus (Pellizzoni, Lotti et al. 1997). This could have consequences for the aging process as well as various pathological states by causing or contributing to the decrease in global translation rates that occur with aging.

**Introduction to Down syndrome**

According to the CDC, 1 in 700 infants born have Down syndrome (DS) – approximately 400,000 people in the US and 6 million people world-wide. DS is caused by an extra copy of chromosome 21 that arises during gametogenesis. In 95% of cases, this occurs as the result of chromosomal nondisjunction (Sherman, Allen et al. 2007). This is usually due to improper segregation of chromosomes into daughter cells during meiosis I, although nondisjunction in meiosis II also occurs (Fig 4.1). This results in gametes that have two copies of chromosome 21 (HSA 21), and upon fusion with another
gamete, results in trisomy 21. Although HSA 21 is the smallest human autosome, the chromosome encodes more than 400 known genes (Gardiner and Costa 2006), a number that may increase with further study. Less frequently, DS occurs due to somatic mosaicism or translocations (Sherman, Allen et al. 2007). DS presents with an easily recognizable phenotype, including a characteristic set of facial features, delayed development and varying levels of intellectual disability, shortened stature, muscle hypotonia, joint laxity, AD-like neuropathology, and a heterogeneous range of other traits.

Advances in health care have led to improved longevity for individuals with DS, with the expected lifespan now approaching 60 years. While advanced maternal age is the only well documented risk factor for DS (Allen, Freeman et al. 2009), many socioeconomic and environmental factors that are difficult to evaluate may affect prevalence and survivability. With aging, the DS population faces an entirely different set of challenges. By the late 1800s, it was documented that individuals with DS develop plaque and tangle neuropathology that is similar to the one described in 1906 by Alois Alzheimer and is now known as Alzheimer’s disease (AD) pathology (reviewed in (Mann 2006)). AD is a disease that has progressed in our social consciousness from a peculiar rarity less than half a century ago to one of the greatest public health concerns of our generation (Abbott 2011). We now know that essentially all individuals with DS develop AD-like pathology by the fourth decade of life. Interestingly, this realization predated the finding that an extra copy of chromosome 21 causes DS by almost 50 years (Lejeune 1959). Clues as to how this predisposes individuals with DS to AD-like pathology
became more clear with the finding that HSA 21 harbors the genes for the amyloid precursor protein (APP) and BACE2, two genes directly implicated in AD pathogenesis.

The 400 known genes on HSA 21 represent many protein families and diverse functions, including the transmembrane phosphatase with tensin homology (TPTE) and superoxide dismutase (SOD1). HSA 21 harbors at least two genes implicated in the development of AD-like pathology. The first is APP, the substrate from which the pathogenic Aβ peptide is derived. The second is BACE2, an aspartyl protease with ~65% sequence homology to BACE1, the major form of β-secretase in the brain. BACE1 was originally discovered by multiple groups as the primary β-secretase responsible for Aβ generation in the brain (Hussain, Powell et al. 1999, Sinha and Lieberburg 1999, Vassar, Bennett et al. 1999, Yan, Bienkowski et al. 1999, Lin, Koelsch et al. 2000), and the homologue BACE2 was discovered shortly thereafter (Ishii, Li et al. 1997, Solans, Estivill et al. 2000).

Because BACE2 is located on chromosome 21 and initial reports indicated an ability to generate the Aβ peptide from APP (Farzan, Schnitzler et al. 2000), it seemed plausible that this enzymatic activity might contribute to AD pathology in DS (Solans, Estivill et al. 2000). Recent evidence indicates that BACE1 and BACE2 activities and expression are highly correlated in the brain, including in individuals with DS. However, significant effort from multiple groups has uncovered little evidence to support a role for BACE2 in driving the disease process. While BACE2 mRNA is increased in DS (Barbiero, Benussi et al. 2003), post-transcriptional regulatory mechanisms either prevent an increase in translation, or affect flux of the protein by increasing the rate of degradation. Many groups have reported that levels of BACE2 protein in the DS brain are
comparable to control brains in various brain regions (Barbiero, Benussi et al. 2003, Cheon, Dierssen et al. 2008, Holler, Webb et al. 2012). Even though structural studies indicate that the active sites of both BACE1 and BACE2 are very similar (Chou 2004), overexpression studies of BACE2 in both primary and immortalized cell culture models generally result in decreased Aβ production (Sun, He et al. 2006). Other studies indicate that BACE2 has a higher propensity to cleave APP downstream from the BACE1 protease site, actually abrogating Aβ formation (Fluhrer, Capell et al. 2002, Sun, He et al. 2006, Stockley and O'Neill 2007). In vivo studies using transgenic mice that overexpress BACE2 alone (Azkona, Amador-Arjona et al. 2010), or co-overexpress both BACE2 and APP (Azkona, Amador-Arjona et al. 2010) do not show a resultant increase in Aβ peptide in the brain. These findings taken together indicate that BACE2 is probably not responsible for AD pathology in the DS brain, and indeed, may be have a protective function in this instance.

The APP gene is found in the DS obligate region, and the protein is overexpressed in the adult DS brain (Cheon, Dierssen et al. 2008, Cheon, Dierssen et al. 2008, Dimachkie and Barohn 2013). Overexpression of APP leads to dysfunction of the endocytic system, resulting in increased turnover from the cellular surface, thereby increasing the likelihood that APP will encounter β-secretase and be processed via the amyloidogenic pathway (Chou and Howe 2002). This will result in more intracellular APP carboxyl-terminal fragment(s) cleaved at β-site(s) (CTFβ), and in turn more Aβ will be generated in the DS brain.

In order to determine if an age-related increase of CNBP occurs in humans, we decided to examine the protein, as well as BACE in a very unique set of autopsy
specimens, a series of DS and age-matched control (non-DS) subjects ranging from < 6 months to 67 years old. This case series would allow us to answer several questions. Do levels of CNBP change with aging in humans, or only in pathological states? Also, individuals with DS have life-long overexpression of both APP and BACE2 as a consequence of carrying an extra copy of HSA21. Do BACE1 activity and protein levels increase as a result of this gene-dosage affect, and do increases in CNBP precede these changes as they do in AD? Mouse tissue was used to examine CNBP and BACE1 expression in neonates, as well as throughout the murine lifespan in both brain and skeletal muscle.

METHODS

**CNBP determination across the murine lifespan**

Hemibrains and gastrocnemius muscles from mice of various strains were processed as described in the general methods section. Briefly, tissues were homogenized in five volumes (wet weight / volume) RIPA buffer supplemented with complete protease inhibitor cocktail (PIC; Amresco; Solon, OH). Homogenates were centrifuged at 20,000 x g for 30 min to pellet insoluble material. Protein content was determined by bicinchoninic acid (BCA) assay (Pierce Biotechnology; Rockford, IL). 20 µg total proteins were transferred to 0.45µm nitrocellulose membrane via spot blot apparatus. Membranes were probed for CNBP, then stripped via 1M glycine pH=8.0, and re-probed for β-actin. A subset of the youngest and oldest groups was separated by SDS-PAGE and transferred to 0.45 µm nitrocellulose at 1amp for 1 hour. This membrane was probed for CNBP as described previously.
Mice were sorted into three age categories for analysis. A young group consisting of animals less than 6 months (n = 45), a middle-aged group, 7-15 months: (n = 16), and an old group, 16 months and older (n = 23).

**CNBP determination in Down syndrome**

Down syndrome cases and controls were obtained from the University of California at Irvine ADC brain tissue repository, and the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland. Cause of death exhibited no distinct pattern in any group. Control brains had no history of ante-mortem dementia, and post-mortem intervals (PMI) were short (averaging < 14 hrs) (Table 4.1). Human tissue collection and handling followed guidelines issued by the PHS and the University of Kentucky IRB. Tissues were handled as described in the general methods section.
Table 4.1. Down syndrome Case Details

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Table 4.1. Down syndrome Case Details. DS cases and controls were obtained from the University of California at Irvine ADC brain tissue repository, and the NICHD Brain and Tissue Bank for Developmental Disorders (University of Maryland). Control brains had no history of ante-mortem dementia, and post-mortem intervals (PMI) were short (averaging < 15 hrs?). Abbreviations: PMI = Post-Mortem Interval; MMSE = Mini-Mental State Exam; C.O.D. = Cause of Death; N/A = Not Applicable or Unavailable.
RESULTS

Changes in CNBP with aging and Down syndrome

BACE enzymatic activity and protein levels both increase early in the disease process that ultimately results in AD and a similar pathogenic process may occur in DS. While the mechanisms that drive this increase are largely unknown, our data indicate that CNBP is a key regulator of BACE, with consequences for Aβ production. While genetic predispositions account for a small proportion of AD cases, the greatest risk factor for AD in the general population is increasing age. Because much of our knowledge of CNBP function is limited to developmental processes, several questions remain regarding the role of the protein in the adult organism, as well as during the aging process. Even in the absence of pathology, global decreases in protein translation occur either causally or consequentially with aging. Given CNBP’s role in governing translation of 5’ TOP mRNAs, changes in the protein could contribute to detrimental aspects of the aged phenotype by influencing the basal rate of protein translation.

Both CNBP and BACE1 proteins are highly expressed in the developing embryo and the neonate, reflecting their importance during development. BACE1 is involved in the myelination of axons through cleavage of neuregulin, while CNBP is likely important for proliferation control and early signaling events important for establishing the anterior / posterior axis of the embryo and initiating forebrain development. After birth, levels of both decrease dramatically (Fig 4.1), although both are widely expressed in the mature
organism. The normal physiological function of both proteins in the adult organism is poorly understood.

Given that CNBP is involved in disease processes affecting both the brain and skeletal muscle, and both tissues are negatively affected by the aging process, we next decided to examine CNBP levels broadly across the murine lifespan, dividing animals into three age groups, a young group less than 6 months (n = 45), a middle-aged group, 7-15 months: (n = 16), and an old group, 16 months and older (n = 23). There was a small overall increase in CNBP with age (Fig 4.2) (F [2, 58] = 4.62, p<0.02). This was significant in both brain (F[2,59]=3.61, p<0.04) and skeletal muscle (quad) (F[2,80]=3.39, p<0.04). Mann-Whitney U-test (vs. young): * = p<0.05, ** = p<0.01.

In order to determine if an age-related change occurs in humans, we decided to examine CNBP and BACE in a very unique set of autopsy specimens, a series of DS and age-matched control (non-DS) subjects ranging from < 6 months to 67 years old. This case series would allow us to answer several questions. Do levels of CNBP change with aging in humans, or only in pathological states? Also, individuals with DS have life-long overexpression of both APP and BACE2 as a consequence of carrying an extra copy of HSA21 that arises most commonly from chromosomal nondisjunction (Fig 4.3). Do BACE1 activity and protein levels increase as a result of this gene-dosage affect, and do increases in CNBP precede these changes as they do in AD?

mRNA levels of both APP and BACE2 were significantly higher in DS cases, while BACE1 mRNA levels were not significantly different from controls (not shown). DS cases had slightly more APP protein, but no additional BACE2 protein (Fig 4.4 A,C),
supporting the literature finding that even though individuals with DS have an extra copy of BACE2, this does not translate to greater expression of BACE2 protein. BACE1 and BACE2 enzymatic activities were unchanged between DS cases and controls (Fig 4.4B, D). Neither BACE1 (MAB931) nor BACE2 (Ab5670) proteins differed between DS cases and controls (Fig 4.4 C) (Randomized subset of control and DS samples of various ages shown). BACE1 activity showed a slight age-related decrease (Fig 4.4 D). Mann Whitney U-test, * = p<0.05; ** = p<0.01. Localization of BACE1 protein is comparable in AD and DS (Fig 4.5), with expression limited to largely to neurons, (immunostained with Anti-SMI311-green, Anti-BACE1-red) while there was no detectable BACE1 protein expression in astrocytes (immunostained with anti-GFAP-green, and anti-BACE1-red) (anti-GFAP antibody for Glial Fibrillary Acidic Protein; EMD Millipore, Billerica MA, anti-BACE1 antibody 931; R&D Systems, Minneapolis MN, anti-SMI311 antibody for Pan Neuronal Neurofilament marker; EMD Millipore; Billerica, MA).

Samples from the entire case-series of DS and control samples (Table 4.1) in randomized order were separated by SDS-PAGE and immunoblotted for APP and it’s C-terminal fragments (detected with Ab-CT20, MPM, University of KY). While there is a substantial increase in both APP and it’s CTFs in the DS cases there is not an apparent increase in CNBP, although there is variability between subjects (subset shown in Fig 4.6 A.). CNBP was not significantly different across the lifespan of either control or DS cases (Fig 4.6 B; mean densitometry value plotted from triplicates evaluated from each subject).
These data reveal a large increase in APP and its CTFs in the absence of a robust change in CNBP and no concomitant increase in either BACE1 protein levels or activity in this case-series. This differs from the AD cases, that have increased BACE1 protein levels and increased enzymatic activity early in the disease process, so Aβ levels were examined across the age range represented. Overall amounts of Aβ were higher in DS from both RIPA buffer and Formic Acid soluble fractions (p<0.01) (Fig 4.7. A). The amount of Aβ increased with age in both DS cases (p<0.001) and matched controls (p<0.05), with substantially more Aβ being deposited in the DS brain after 40 years of age (Fig 4.7 B). Data shown represent total Aβ extracted in either RIPA buffer (F[1,34] = 14.14, p<0.001) or 70% FA (F[1,34] = 40.09, p<0.0001). Neither BACE1 nor BACE2 (Fig 4.7 C, D) enzymatic activities were related to the amount of either Aβ or CNBP (Fig 4.8) in the brain.
Figure 4.1 BACE1 and CNBP Protein Levels in the Murine Neonate.

A) Both BACE1 protein and activity are higher in brains isolated from newborn mouse pups (N = 3 – 5 pups / time point; P1 pups were pooled to yield sufficient material for analysis) and decreases rapidly after birth. B) CNBP is similarly highly expressed initially, and decreases rapidly in the neonate.
Figure 4.2. CNBP Increases with Age in Mouse Brain and Skeletal Muscle
Figure 4.2. CNBP Increases with Age in Mouse Brain and Skeletal Muscle. A) CNBP levels in brain are higher in old (>16 months) compared to young (<6 months) mice. B) CNBP levels were also increased with age in skeletal muscle. C) CNBP levels were quantified by densitometry in mice of multiple strains (young, <6 months: n = 45; middle-aged, 7-15 months: n = 16; old, 16+ months: n = 23) by spot blot; β-actin was also quantified in the same samples and used as a covariate in the analysis. There was an overall increase in CNBP with age (F[2,58] = 4.62, p<0.02); this was significant in both brain (F[2,59] = 3.61, p<0.04) and skeletal muscle (F[2,80] = 3.39, p<0.04). Mann-Whitney U-test (vs. young): * = p<0.05, ** = p<0.01.
Figure 4.3 Chromosomal Nondisjunction Results in Down syndrome
Figure 4.3. Chromosomal Nondisjunction Results in Down syndrome. (a) Most often DS occurs as an error in meiosis I (usually in the oocyte). Chromosomal nondisjunction, or improper segregation of chromosome 21 (the smallest autosome; orange), results in one precursor cell having 2 copies (b, upper half) while the other has zero (b, lower half). (c) Meiosis II then proceeds, with the outcome being two gametes that possess an extra copy of chromosome 21 which, after fusion with another gamete, bears 3 copies of chromosome 21, the genetic condition known as DS or trisomy 21. Also produced in this process are two nonviable gametes that possess zero copies of chromosome 21 (bottom).

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Figure 4.4. BACE Enzymatic Activities and Protein are not Increased in DS.
Figure 4.4. BACE Enzymatic Activities and Protein are not Increased in DS. A) As expected, the total mRNA for both BACE2 and APP were significantly higher in DS cases; BACE1 mRNA was unchanged (*not shown*). DS cases had slightly more APP protein, but no additional BACE2 protein. B) BACE1 and BACE2 enzymatic activities were unchanged between DS cases and controls. C) Neither BACE1 (MAB931) nor BACE2 (Ab5670) proteins differed between DS cases and controls. D) BACE1 activity showed a slight age-related decrease. Mann Whitney U-test, * = p<0.05; ** = p<0.01.

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Figure 4.5 BACE1 is Localized to Neurons in DS. BACE1 is not found in astrocytes (immunostained with Anti-GFAP-green and Anti-BACE1 Ab931-red) but is located within neurons (immunostained with Anti-SMI311-green, Anti-BACE1-red).
Figure 4.6. CNBP does not Change in DS

A. 

B. 

APP
CTFs
Actin
CNBP

CNBP (AU)

Age (Years)
Figure 4.6. CNBP does not Change in DS. A) A subset of DS and control samples of various ages in randomized order separated by SDS-PAGE and immunoblotted for APP and it’s C-terminal fragments (detected with Ab-CT20, MPM, University of KY). These illustrate an increase in both APP and CTFs in DS cases, but no pattern of increase in CNBP in these samples. B) CNBP does not change significantly with aging in either control or DS cases (densitometry in triplicate from each case).
Figure 4.7. Aβ Increases with Age in DS but is not Related to β-secretase Activity
**Figure 4.7. Aβ Increases with Age in DS but is not Related to β-secretase Activity.**

(A) The overall amount of Aβ was higher in DS in all fractions (p<0.01). *Shown:* total Aβ extracted in either RIPA buffer (F[1,34] = 14.14, p<0.001) or 70% FA (F[1,34] = 40.09, p<0.0001). (B) The amount of Aβ increased with age in both DS cases (p<0.001) and matched controls (p<0.05). Neither BACE1 (C) nor BACE2 (D) enzymatic activities were related to the amount of either RIPA (*not shown*) or FA-soluble Aβ in the brain. *Scale:* 1 BFU / μg = 2.3 nmol · min⁻¹ · mg⁻¹ total protein. *Reprinted from* (Holler, Webb et al. 2012), *with permission from Elsevier.*
Figure 4.8. BACE Activity is not Related to CNBP in either Aging or Down syndrome
Figure 4.8. BACE Activity is not Related to CNBP in either Aging or Down syndrome. Neither CNBP or BACE protein levels nor enzymatic activity (shown) change significantly in the case series examined with either DS or control subjects between the ages of 6 months and 67 years.
DISCUSSION

Within recent history, we have seen a dramatic extension in the human lifespan, a trend likely to continue for many years to come. This is in part due to increased availability of resources and distribution of life-sustaining nutrients and clean water technology, and also due to decreased morbidity as a result of advances in healthcare (antibiotics) for the elderly. This shift in lifestyle has resulted in a situation where predators and disease pose considerably less risk than was historically true, and exposes humans to an extended period of aging, the most significant risk-factor for a variety of pathological states. This, along with the findings indicating that lifespan in model organisms is a plastic process that can be modified by single-gene mutations, has changed the way we think about aging. However, much remains to be learned about the aging process. For example, technical limitations have resulted in the identification of genes whose impairment affects longevity, but surely there are proteins whose overexpression favor healthy aging. Also, why is there such a disparity in lifespan between genetically similar organisms, like wolves and dogs? What is the Achilles’ heel in such an instance, and can it be negated in a way that influences aging in a healthy way?

Given the role that CNBP has in regulating 5’ TOP mRNAs it seemed plausible that limiting amounts of the protein may negatively affect rates of protein translation that occurs with aging. This does not appear to be the case, as CNBP levels do not seem to change significantly across the human lifespan, although levels of the protein do likely drop significantly after birth due to its role in forebrain development. However, CNBP may be involved in the movement of ribosomal components from an inactive cytoplasmic
fraction to a translationally active polysomal fraction of the cell or have other indirect effects that change global rates of protein synthesis.

We also looked at CNBP in a uniquely available aging series of DS cases, a pathological state where increased Aβ deposition has been implicated as a feedback mechanism for the β-secretase. Our data indicate that unlike the pathological process that occurs in AD, there is no age associated increase in BACE protein levels or enzymatic activity in individuals with DS. Importantly, there is also no change in CNBP, supporting its regulatory role for BACE1. These findings indicate that the increased Aβ deposition that occurs is likely driven by overexpression of the substrate APP, and not a case of an Aβ-driven positive feedback loop.

While there are similar neuropathological changes in people with DS compared to AD, the brains of these populations are quite different. The DS brain is slower to develop and smaller at maturity than the brain of a diploid individual, weighing less than 1250 and often under 1000 grams, several hundred grams less than normal. Anatomically, the DS brain is more rounded with a distinct fore-shortened shape, and smaller frontal lobes, hippocampi, and cerebellum (reviewed in (Mann 2006)). The brain in older individuals with DS is susceptible to cell loss in both cortical and subcortical regions, resulting in dysfunctions in both neurotransmitter systems and neuronal circuitry.

Emerging evidence from both fetal and adult DS tissues and animal models of DS indicates that changes at the molecular level are more wide-spread than previously acknowledged. While there are about 400 hundred known genes on chromosome 21, a meta-analysis of the transcriptome and proteome reveals that many more are affected.
Several – but not all – genes on chromosome 21 were overexpressed, while expression of others was unchanged or even decreased (Vilardell, Rasche et al. 2011). This indicates that the *in vivo* state is the result of a more complex interplay of factors than a simple gene dosage effect. There may be over 300 genes that are significantly changed in DS, the majority of which are not located on chromosome 21, and many of which have known roles in early developmental processes. The role of these various changes in development and the penetrance of many of the typical phenotypes of DS is largely unknown. Recently exon tiling arrays have been used to interrogate the role of various genomic loci in DS features, using rare segmental trisomies (Korbel, Tirosh-Wagner et al. 2009). Importantly, this work highlights that the obligate region of chromosome 21 is more heterogeneous than anticipated, and may not exist at all, as individuals with segmental trisomies can still present with a moderate to severe DS phenotype. One of the patients characterized, a 65-year old without an additional copy of APP, did not have dementia or indication of amyloid accumulation when assessed by brain imaging, supporting a causative role for APP overexpression in neuropathology in DS (Korbel, Tirosh-Wagner et al. 2009).

In the general population, a definitive neuropathological diagnosis of AD requires that the classical hallmarks of AD, namely neuritic plaques and neurofibrillary tangles, be present along with a clinical history of dementia. Although this characteristic AD-like pathology is present by the fourth decade of life, not all individuals with DS develop dementia, even with complete trisomy 21 (Krinsky-McHale, Devenny et al. 2008). Even though changes in cognitive ability and social withdrawal are often reported by caregivers of middle-aged persons with DS, there is some controversy about whether this
represents a clinically defined dementia (Mann 2006). Prevalence rates for dementia in DS vary considerably between studies, but are approximately 15%, slightly higher than that in the general population; however, in DS, the dementia occurs at significantly younger ages (reviewed in (Nieuwenhuis-Mark)). Cognitive testing for DS has proven difficult, which is not surprising given the wide range of intellectual disabilities presented. Also, because there is often little cognitive data for individual patients before their decline, establishing a cognitive baseline is not often possible for individuals. These issues at the individual level make it difficult to elucidate effects in groups, resulting in floor effects plaguing cognitive tests, and difficulty making conclusions regarding population-wide affects in DS (Haxby and Schapiro 1992, Devenny, Krinsky-McHale et al. 2000). A better understanding of the cognitive strengths and weaknesses of individuals with DS (reviewed in (Rasmussen, Whitehead et al. 2008)) and how these change over time represent a huge need for the DS community. Recently, much effort has been put into developing cognitive tests specifically for DS, such as the Arizona Cognitive Test Battery (Edgin, Mason et al. 2010). These testing methods that can be used across a wide range of ages and cultures with little dependence on language skill are an important step forward. In addition, both functional and cognitive abilities are assessed, which are particularly useful for longitudinal studies of basic cognitive ability in persons with DS and discerning if they do indeed develop AD. As a diagnosis of AD requires both neuropathology and dementia, it is important for many reasons that we know the clinical consequences of AD-like pathology in DS versus the non-DS population.

DS is commonly recognized as a model for AD pathology, and is very much proof of principle for the amyloid cascade hypothesis, because the additional copy of
APP in DS results in pathology long before it occurs in the general population. As such, if the progression to dementia is delayed or absent in DS this may help us elucidate a therapeutic strategy that may be applicable to patients with familial or sporadic AD as well. Therapies to treat Alzheimer’s disease in both the DS population and general population are limited. No pharmacological agents have been described that are able to alter disease progression. Symptoms may be improved by a cholinesterase inhibitor (donepezil, rivastigmine, galantamine), or NMDA receptor antagonist (memantine) (reviewed in (Askanas, Engel et al. 1993)). Current goals include determining which biomarkers are indicative of the disease process years before development of pathology, which may lead to therapeutics designed to alter the disease process. Still, many questions remain. Although the pathway driving the degenerative process in DS may be different than the one in familial or sporadic AD, and is likely fueled by substrate (APP) overexpression, the neuropathological hallmarks of the disease are the same. How much do these pathways overlap compared to sporadic AD that occurs in the general population? Are there factors responsible for controlling progress for dementia that are altered in DS, and are these a direct or indirect consequence of an extra copy of HSA 21? Many non-DS individuals who have been followed longitudinally and come to autopsy have sufficient neuritic plaques and neurofibrillary tangles to meet the critiera for a neuropatholical diagnosis of AD, yet there is no evidence to suggest they experienced cognitive impairment or decline, and so are referred to as pre-clinical AD (Price, McKeel Jr et al. 2009). Although it is possible that they would eventually progress to dementia, it is also possible that these individuals exhibit a compensatory mechanism that allows
them to tolerate this neuropathology relatively unscathed. A similar mechanism may be at work in DS.

While there is much to learn, developing and executing longitudinal studies for persons with DS is difficult, and success will depend on an integrated, informed, and motivated network of parents and caregivers of persons with DS, medical professionals that better understand the range of primary and secondary complications that result from DS, and involvement and outreach from the research community. This process has already begun as two goals stemming from the National Institutes of Health’s Research Plan on Down syndrome will be realized within the next year. The first is the development and testing of a national registry for DS, and the second is the establishment of a consortium to bring clinicians and researchers together (Oster-Granite, Parisi et al. 2011). These are exciting steps for the DS community and hopefully just the beginning of many resources that will benefit individuals with DS. However, there are still many challenges and areas where improvements are needed, including identifying socioeconomic factors that impact the early development and increased risk of mortality among certain ethnicities; developing learning tools and programs specifically for intellectual disabilities; educating families and healthcare personnel so individualized health plans and testing for routine and secondary afflictions can be monitored routinely; performing routine functional and cognitive testing prior to decline; and finally, using therapeutics for age-related concerns such as depression and AD.
INTRODUCTION

Experiments from both human and rodent brains indicate that there is little change in CNBP in the aged brain of either species, meaning the normal aging does not drive the increase in protein that results in the BACE increase that occurs in sporadic AD. However, there is an age-related increase of CNBP in the skeletal muscle. This is intriguing, given that BACE activity and Aβ deposits have been implicated as causal factors in a common degenerative disease of the skeletal muscle, sporadic inclusion body myositis (sIBM). Also, we know that an expansion in the 1st intron of ZNF9 (encodes CNBP) causes DM2, a disease with both brain and muscle abnormalities, but it is unknown how BACE1 changes in skeletal muscle with DM2, and how or if this is related to increases that occur in other age-related diseases.

Myotonic Dystrophy

Myotonic dystrophy is the most common of the range of disorders characterized as the muscular dystrophies. The multisystemic disorder known as myotonic dystrophy was described over a hundred years ago. Myotonic dystrophy was thought to be a single disorder until researchers discovered a mutation approximately 20 years ago which indicated there were actually two separate entities, DM1 and DM2 (Patel and Dimachkie 2000). The disease (combined prevalence of DM1 and DM2) affects 1:8,000 people and occurs as the result of two different pathogenic mutations present in repetitive areas of their resident genes. A trinucleotide repeat (CTG) in the 3’ UTR of a kinase (DMPK) results in myotonic dystrophy type 1 (DM1) (Brook, McCurrach et al. 1992), while a
tetranucleotide repeat (CCTG) in the 1st intron of ZNF9 causes myotonic dystrophy type 2 (DM2) (Finsterer 2002). While these two genes encode very different proteins, a kinase and an RNA binding protein, the clinical phenotype that occurs is very similar.

Clinical presentation varies widely, but generally patients with both types of myotonic dystrophy experience progressive muscle weakness and degeneration. Several other hallmark features are also shared between the two diseases, including myotonia, cataracts, endocrine abnormalities (insulin resistance), and cardiac defects, including conduction defects and cardiac hypertrophy (Dimachkie, Justiz et al. 2000, Marian and Willerson 2007). One of the major differences between DM1 and DM2 is which muscle groups are affected first, with proximal muscle groups affected first in DM2 and distal muscle groups affected first in DM1. Genetic anticipation occurs in DM1, and may present as a congenital, childhood onset, or adult onset form of disease. DM2 is described as a clinically heterogenous multisystem disorder, with some patients remarkably clinically similar to DM1, while other patients present with predominantly proximal skeletal muscle involvement (Yang, Ding et al. 2000), and no congenital form of DM2 (Dimachkie, Justiz et al. 2000, Finsterer 2002). From a diagnostic standpoint these diseases present many unique challenges that lead to their underdiagnosis. These include the incredible spectrum of disability which occurs, from barely perceptible myalgia and atrophy that occurs late in life (that is difficult to distinguish from changes which occur with normal aging), through cases of childhood onset disease where mental retardation is the first notable disease symptom. Even when restricted to adult onset forms, presentation ranges from minor muscle weakness and myotonia through severely debilitating respiratory insufficiency and death. Rates of progression vary widely, and it
is not readily apparent that the mutations are 100% penetrant in all cases, further complicating clinical recognition. Other mutations can exacerbate the myotonic dystrophy phenotype, with one of the best known examples being recessive mutations in chloride channels having a profound effect on the DM2 phenotype, leading to conduction defects that can ultimately result in fatal cardiac complications (Ursu, Alekov et al. 2012). Myalgia becomes problematic with disease progression, and while these diseases do not respond to conventional pain treatment, approximately 25% of patients are on continuous pain treatment, as there are no effective treatments for myotonic dystrophy of either type of myotonic dystrophy (reviewed in (Turner and Hilton-Jones 2010)).

At the molecular level, mutations that result in DNA expansion diseases occur in highly repetitive areas of the genome that have a tendency to expand or contract as the result of polymerase slippage (discussed in detail in the introduction) (Mirkin 2007). These new lengths of sequence are repetitive elements that have a unique ability to form secondary structures, with the most likely being imperfect hairpins (O’Rourke and Swanson 2009). Recently, evidence from peripheral blood leukocytes suggests a bias toward expansion, with the occurrence of expansion and contraction events at an unprecedented rate, as often as every 48 hours in dividing leukocytes. Transcription of loci that contain these expansions results in the formation of nuclear foci, and haploinsufficiency of the proteins that should result from the gene loci. In fact, a parsimonious explanation for the considerable phenotypic overlap between DM1 and DM2 is that the abnormal buildup of mRNA in the nucleus causes both diseases.

These transcripts likely contribute to the disease phenotype in several ways. The first is sequestration of splicing factors of the muscleblind (MBNL) family into
ribonuclear foci. Also, the aberrant activation of CUG-binding proteins like CELF1 contribute further to splicing abnormalities. These are likely shared in DM1 and DM2 reviewed in (O'Rourke and Swanson 2009). More variable aspects of the phenotype are likely due to haploinsufficiency of proteins that would normally result from transcription events at the gene locus, DMPK in the case of DM1, and CNBP in the case of DM2 (Chen, Wang et al. 2007). Other more variable aspects of the phenotype likely occur due to changes in local chromatin structure altering the availability of neighboring gene loci to components of the transcriptional apparatus (reviewed in (Cooper, Wan et al. 2009). Less CNBP is found in the myoblasts of patients with DM2. This leads to decreased levels of proteins of the translational apparatus, and possibly other targets of CNBP that have not yet been determined (Huichalaf, Schoser et al. 2009).

**Sporadic Inclusion Body Myositis**

While mutations in CNBP lead to DM2, CNBP is likely also involved in another disease of the muscle, inclusion body myositis (sIBM) (Munshi, Thanvi et al. 2006). Pathology reminiscent of the plaques observed in AD are found in muscle biopsies of patients suffering from sIBM, an age-related degenerative disease of the muscle fibers. Importantly, both AD and sIBM share an increase in BACE protein and enzymatic activity (Dimachkie and Barohn 2009, Pasnoor, Wolfe et al. 2010) that may be regulated by CNBP. It seems likely that the protein is also involved in peripheral regulation of BACE1—actions that may have implications for determining the incidence of sIBM.

Sporadic IBM is the most common idiopathic inflammatory myopathy affecting persons over 50 years of age. sIBM usually manifests as a progressive weakening of
muscle groups, with the quadriceps and finger flexors being most commonly affected, often asymmetrically. There is typically a significant delay between presentation of symptoms and diagnosis of the disease, usually of 5 to 8 years (reviewed in (Dimachkie and Barohn 2012)). While not considered fatal, with disease progression the loss of dexterity and increased propensity to fall becomes a significant risk to the well-being of the patient. Potentially fatal complications from dysphagia accompany mild to moderate facial weakness in approximately half of all advanced cases (reviewed in (Dimachkie 2011).

Biopsy reveals marked inflammatory and degenerative changes in the muscle (Askanas and Engel 2002). Pathologically, sIBM is a heterogenous disorder, with characteristics of both a neurodegenerative disorder, and an autoimmune related myopathy (Askanas, Engel et al. 2012). A subset of sIBM patients (approximately 15 %) has some evidence of an autoimmune disorder, including systemic lupus erytheatosis, Sjogren’s syndrome, and others. Increased prevalence of various cancers, myocarditis, and interstitial lung disease also plague persons with sIBM , (reviewed in (Dimachkie 2011). However, this predisposition to autoimmune disorders is puzzling given that symptoms do not improve with corticosteroid treatment, even though most other idiopathic inflammatory myopathies are responsive to prednisone (Hughes, Bensa et al. 2001). In rare instances of responsiveness, improvement is transient, as the condition becomes progressively resistant to treatment. Histology from the muscle reveals heterogeneously sized fibers resulting in an abnormal architecture of the tissue and multiple individual fibers that bear large vacuoles. The aggregates found within and surrounding these vacuoles contain several proteins implicated in neurodegenerative
disease, including Aβ, hyperphosphorylated microfilament binding protein tau, ubiquitin, and others (Dimachkie and Barohn 2009). It is still a matter of debate as to whether these are causal or consequential in the development of sIBM. Animal models of sIBM that overexpress APP or its C-terminal fragment develop rimmed vacuoles, lymphocytic infiltration of the muscle and Aβ deposition in the skeletal muscle (Jin, Hearn et al. 1998, Sugarman, Yamasaki et al. 2002). Phenotype development in this model is accelerated by introduction of a presenilin (PS1) mutation that favors increased Aβ42 production (the slightly longer more aggregate-prone peptide—considered the most pathogenic peptide species in AD). Importantly, the amount of Aβ-peptide deposited in the muscle is highly correlated with muscle weakness in the animals, lending strong support to the notion that the degenerative process is key to development of sIBM (Gambello, Bai et al. 2006). Also, patients subjected to positron emission tomography had higher median levels of Pittsburgh Compound B (an in vivo marker of Aβ) binding in muscle compared to patients with other types of myopathy, supporting a causal role for Aβ in this particular degenerative process of the muscle (Zhou, Dimachkie et al. 2004).

Importantly, like AD brain, IBM muscle displays an elevated level of total BACE activity. Both BACE1 and BACE2 are expressed peripherally, and both may contribute to this increase (Dimachkie and Barohn 2009). Because CNBP is a regulator of BACE, it may play a causal role in sIBM, as well as DM2. In order to determine if this is true, we used either an AAV2 or lentiviral delivery system to transduce the gastrocnemius muscle group of a mouse model of sIBM, along with normal WT mice, and measured disease related changes in Aβ-peptide and other markers as indicators of pathology.
METHODS

CNBP Overexpression in Skeletal Muscle of a Mouse Model of sIBM

The sIBM mouse model was created by cloning the human APP gene (with the Swedish familial AD mutation, APPΔNL695) into a targeting construct under control of the Muscle Creatine Kinase promoter, restricting expression of the cDNA construct to the skeletal musculature. This construct was used for pronuclear microinjection into C57BL6/SJL embryos following standard practices for generating transgenic embryos. From a transgenic colony maintained at the University of Kentucky (Beckett, Niedowicz et al. 2010), a group of 18 month old hemizygous animals, analogous to a state of moderate disability, and a group of WT control animals were anesthetized with ketamine and xylazine for viral delivery into the gastrocnemius muscle. Twenty five µl CNBP AAV2 (~2.5 x 10^{10} viral particles) (see General Methods pg. 24 for viral preparation) was injected into the left leg, and virus expressing the empty vector was injected into the right leg. Animals were routinely monitored for gait abnormalities, weight loss, or other signs of distress, and were euthanized by barbiturate overdose one month post-injection. Gastrocnemius muscles were removed and stored fresh frozen at -80 °C for protein and RNA analysis.

Lentiviral Preparation

Lentivirus harboring shRNAs to CNBP was generated from Sigma-Aldrich’s (St. Louis, MO) MISSION® pLKO.1-puro validated shRNA system. HEK293LTV cells were grown in 10 cm dishes with Dulbecco’s modification of Eagle’s media (DMEM)
supplemented with 10% fetal bovine serum and 500 µg/ml geneticin (Invitrogen, Carlsbad CA). Cells were co-transfected with plasmids by the PEI method (≈7.5mM in ddH₂O with 150 mM NaCl) in antibiotic-free DMEM with 10% FBS using either the 2 helper (PsPAX2 and pMD2.g packaging vectors) or 3 helper (pCMV-VSV-G, pRSV-Rev, pMDL-gp-RRE packaging vectors) plasmid system in addition to the donor plasmid. PE- containing medium was removed 4-6 hours later, and replaced with DMEM containing 10% FBS and 1% penicillin/streptomycin (HyClone, Logan UT). Medium containing viral particles was harvested at 36, 48, and 60 hours post-transfection. Pooled samples were centrifuged at 3880 rcf for 5 minutes at 4°C, and the supernatant transferred to a fresh tube for concentration by addition of polyethylene glycol diluted to ≈ 2% of total volume of the combined suspension, and incubated at 4°C for 24-72 hours. Viral particles were collected by centrifugation at 1500 rcf (30 min. at 4°C) and resuspended in 200 µl of PBS.

**CNBP Knockdown in the Gastrocnemius of WT C57BL/6 Animals**

Wild type C57BL/6 mice (N = 15) were anesthetized with ketamine and xylazine (50-100 mg / kg and 10 mg / kg, respectively), and 25 ul of CNBP shRNA (2.45 x 10⁸ IFU / ml) injected into the gastrocnemius of the left leg, and 25 ul scrambled control shRNA vector (1.3 x 10⁸ IFU / ml) injected into the gastrocnemius of the right leg. Animals were euthanized by either CO₂ asphyxiation or barbiturate overdose 2 weeks later, muscles removed and fresh frozen. Samples were then extracted in RIPA buffer and subjected to western blot analysis as described in the general methods section.
**CNBP Knockdown in the Soleus Muscle of CD-1/129 Mice**

Similar to other animal injection experiments, wild type CD-1/129 mice (6 ½ months old) were anesthetized with isoflourane for viral transfer. A single incision was made over the gastrocnemius, and this group of muscles were moved laterally, thereby exposing the underlying soleus muscle. The soleus was injected with 2 µl of either anti-CNBP shRNA virus (2.45 x 10⁸ IFU / ml), or a scrambled shRNA control virus (1.3 x 10⁸ IFU / ml) at distal ends of the muscle (4 µl of virus total), allowing more efficient transduction throughout the muscle. Mice were euthanized 6-7 weeks post treatment for contractile function experiments.

**Contractile Function of the Intact Soleus Muscle**

shRNA injected animals were anesthetized with isoflourane prior to euthanasia by cervical dislocation. Soleus muscles were dissected from a mouse and were mounted for *in vitro* manipulations. Briefly, the distal tendon was tied to a glass rod and the proximal tendon was attached to a force transducer (BG Series, 100 g, Kulite, Leonia, NJ) using silk suture (4-0). The fiber bundle was placed in a water-jacketed organ bath containing Krebs buffer, continuously gassed with 95% O₂–5% CO₂. Prior to the experimental treatment, we positioned the fiber bundle at the length that elicited the highest twitch force (Lo). Soleus muscles were stimulated to contract using electrical field stimulation (supramaximal voltage, 0.25–0.30 ms pulse, and 250–300 ms train duration) via platinum electrodes at frequencies of 1–300 Hz. We measured force using a digital oscilloscope and computed cross-sectional area using muscle weight and Lo (14) to calculate specific
force (in kN/m$^2$). The contractile protocol was conducted at 37°C. All solutions used for measurement of muscle force contained 25 μM D-tubocurarine.

RESULTS

CNBP Regulates BACE1 in vivo

A group of 12 mice (6 hemizygous sIBM, and 6 control C57BL6/SJL animals) were mildly anesthetized with ketamine and xylazine (50 mg / kg and 10 mg / kg, respectively), and 25 μl CNBP AAV2 virus (~2.5 x 10$^{10}$ viral particles) was injected into the gastrocnemius muscle group. The contralateral muscle was injected with empty pZac2.1 vector, providing a within-subject control. Animals were monitored daily for gross changes in weight, behavior, or gait. One month post-injection, mice were euthanized by barbiturate overdose and muscles harvested and fresh frozen. Analysis revealed that while there was variation between the animals, overexpression of CNBP was successful (p<0.05) (Fig 5.1 A), and it was accompanied by a comparable increase in BACE1 protein (p<0.01) (Fig 5.1 B), changes that were not different between genotypes. Importantly, there was also an increase in Aβ (p<0.05), a product of BACE1 activity (Fig 5.1 C). These data suggest that an age-related increase in CNBP may predispose individuals to Aβ pathology of the muscle, and SIBM. Interestingly, CNBP overexpression induced an increase in β-actin in the muscle, a change that seems to be muscle specific, as we did not see this from either tissue culture or brain experiments.
CNBP Reduction does not Recapitulate Aspects of the DM2 Phenotype \textit{in vivo}

A lentivirus harboring either an shRNA validated to knockdown CNBP, (Sigma-Aldrich) or a scrambled shRNA control were injected into the gastrocnemius muscles of C57BL/6 WT (N = 15) animals using a procedure similar to the one described for AAV transduction. The anti-CNBP shRNA virus was injected directly into the gastrocnemius muscle group of the left leg, and the scrambled control virus injected into the right leg, providing a within-subject control. CNBP levels in the gastrocnemii were analyzed by western blot, and densitometry performed using ScionImager. This analysis revealed an approximate 20% knockdown of CNBP \textit{in vivo} (p<0.05 by matched t-test) \textbf{(Fig 5.2 A, B)}. Levels of Aβ were the same in both CNBP shRNA and scrambled shRNA injected muscles \textbf{(Fig 5.2C)}.

Having validated that viral transduction resulted in modest CNBP reduction \textit{in vivo} allowed us to answer several questions. There is some evidence in animal models that haploinsufficiency of CNBP itself recapitulates certain aspects of the DM2 phenotype (Chen, Wang et al. 2007). With this in mind, we wanted to know if reduced CNBP impaired the ability of the muscle to produce force. Also, did less CNBP result in a myotonic phenotype, where the muscle could contract normally, but not release as efficiently as unimpaired muscle? After validating efficacy of the virus to transduce a large and heterogeneous skeletal muscle group, the anti-CNBP shRNA lentivirus was used to transduce the soleus muscle of a group of CD-1/129 mice. The soleus was chosen because it is a small muscle composed largely of slow oxidative muscle fibers, a trait that reduces variability and makes it ideal for force transduction measurements. The muscle was transduced with 2 μl of anti-CNBP shRNA lentivirus, or scrambled control at two
points (4 μl injected / soleus). Analysis of force revealed no difference between the anti-CNBP shRNA and scrambled control in either absolute force (Fig 5.3 A), or average force (Fig 5.3 B) generated by the muscle. There was a small difference in specific force, or force normalized to cross-sectional area of the muscle, but the scrambled control measurements were very erratic, and actually generated less force than the muscle transduced with the anti-CNBP shRNA. Even though this scrambled control is not supposed to target known mammalian transcripts, it is possible that off-target affects are responsible for this variability. The anti-CNBP shRNA injected muscles produced very stable twitches, and generated a sigmoidal curve comparable to what one would expect from an uninjected muscle (data not shown). Half time to relaxation was also not significantly different between the anti-CNBP shRNA injected muscles versus controls (Fig 5.3 C), indicating this small reduction in CNBP did not negatively affect the ability of the muscle to relax, a prominent feature of DM2. One would also suspect that an impaired muscle would fatigue faster, but indeed, we did not see this in the anti-CNBP shRNA injected muscle (Fig 5.3 D).
Figure 5.1. CNBP Regulates BACE1 in Mouse Skeletal Muscle
**Figure 5.1. CNBP Regulates BACE1 in Mouse Skeletal Muscle.** CNBP AAV2 was used to overexpress CNBP in a group of sIBM mice or WT animals (N = 6 vs. 6). The gastrocnemius muscle was transduced, while the contralateral muscle was injected with empty vector serving as the control. **A)** Western blots from a subset of animals (N = 6, shown) reveals that while there was variation among the group, there was a notable increase in CNBP, and a corresponding increase in BACE1 in the CNBP AAV2 injected muscle vs. the empty vector control. Genotype had no effect on CNBP overexpression. **B)** Densitometry from the entire group, N = 12, shows that the increase in BACE1 protein was comparable to levels of CNBP overexpression, and **C)** a significant increase in BACE1 product, the Aβ-peptide, as detected by ELISA. **D)** Interestingly, β-actin also increases with CNBP overexpression. * = p<0.05, ** = p<0.01 (by matched samples t-test)
Figure 5.2. CNBP is Reduced ≈20% Using anti-CNBP shRNA Lentivirus
Figure 5.2. CNBP is Reduced ≈20% Using anti-CNBP shRNA Lentivirus. A commercially available validated anti-CNBP shRNA lentivirus was used to knockdown CNBP in the gastrocnemius muscles of C57BL/6 WT (N = 15) animals using a procedure similar to the one described for AAV transduction. The left leg was injected with virus harboring the anti-CNBP shRNA, and the contralateral muscle was injected with a scrambled non-target shRNA control. A) Analysis by western blot indicated a decrease in CNBP protein, B) approximated at a 20% reduction in CNBP (p<0.05 by matched t-test) (as detected by densitometry using freely available ScionImage). C) Levels of Aβ-peptide were the same in both muscle groups.
Figure 5.3. Reduced CNBP Does Not Recapitulate Aspects of DM2 in vivo
Figure 5.3. Reduced CNBP Does Not Recapitulate Aspects of DM2 in vivo. The soleus muscle of CD-1/129 mice (N = 15) was transduced with 2 μl of anti-CNBP shRNA lentivirus, or scrambled control shRNA at two points (4 μl injected / soleus). Analysis of force revealed no difference between the anti-CNBP shRNA and scrambled control in either absolute force A), or average force B) generated by the muscle. Half time to relaxation was also not significantly different between the anti-CNBP shRNA injected muscles versus control C), indicating this small reduction in CNBP did not negatively affect the ability of the muscle to relax. No difference was noted in rates of fatigue between the anti-CNBP shRNA injected muscle and scrambled control injected muscles D).
DISCUSSION

While most of the DM2 phenotype can be attributed to transdominant effects on the cellular splicing machinery, there are also reduced global levels of protein translation in persons with DM2 (Huichalaf, Schoser et al. 2009). Interestingly, defects in protein translation and splicing dysfunction are both thought to contribute to AD, and indeed the studies discussed here may indicate a previously unknown synergy between DM2 and AD, as well as with sIBM. Splicing abnormalities in DM1 and DM2 result in substantial brain pathology due to adverse effects on the splicing of targets such as tau and Bin1 (Verma, Gushiken et al. 2001, Johnson, Williams et al. 2003), and likely other unknown targets affected by splicing anomalies or haploinsufficiency of CNBP itself. Indeed, the RNA binding protein TDP-43 has recently been implicated in the pathology of both Amyotrophic lateral sclerosis (ALS, a neuromuscular disorder characterized by motor neuron loss in the brain and spinal cord) and FTD (which impacts mainly the brain) (Dimachkie, Ohanian et al. 2000, Verma, Forman et al. 2000), indicating that anomalies that affect both the brain and skeletal muscle may be more prevalent than is immediately obvious.

Our data indicate that it is possible to decrease CNBP levels without adverse effects on skeletal muscle. Data shown in the previous chapter also indicate that CNBP increases with aging in skeletal muscle, which may drive the BACE1 expression important for formation of Aβ inclusions found in skeletal muscle of sIBM. In the animals, modest overexpression of CNBP resulted in a small but significant increase in Aβ in only one month. Chronically elevated levels of CNBP with aging could contribute to the progressive nature of sIBM, exacerbating pathology by longterm deposition of increasing amounts of Aβ. Given these data, it is conceivable that decreasing CNBP may
have therapeutic potential for persons with sIBM, the most common degenerative idiopathic inflammatory myopathy in people over 50 years of age. While gene therapy approaches are technically very challenging, and are likely far from overcoming the obstacles keeping these approaches from the clinic, in theory, an approach like this could be optimized to reduce CNBP levels within a therapeutic range while doing no harm by targeting the ZNF9 locus.

Taken together, these data indicate that it may be possible to improve Aβ pathology in the skeletal muscle of persons with sporadic inclusion body myositis by using a gene-therapy approach to reduce expression of CNBP in skeletal muscle. Our data also indicate that reducing CNBP slightly below basal levels does not result in detrimental changes in the muscle, so while gene-targeting strategies may result in variable retroviral expression, it is encouraging to know that the threshold of CNBP required for basic cellular functions may be lower than anticipated. CNBP may be involved in diseases beyond its known role as a disrupted gene in type 2 myotonic dystrophy. Understanding what CNBP does, and how it functions in brain and other tissues, may shed light on aspects of pathogenic disease mechanisms that have thus far proven elusive.
CHAPTER 6: Summary and Future Directions

Summary

This dissertation project stems from an initial experiment using insertional gene-trap mutagenesis to identify products that alter Aβ production, as determined by Aβ ELISA. From ~3500 cell lines screened, one cell line was identified that produced less Aβ, and the resultant DNA fragment was sequenced, yielding a match to the ZNF9 gene, that encodes CNBP. Replacement of CNBP in the hemizygous cell line normalized Aβ levels, indicating that disruption of this protein did induce the noted decrease in Aβ (MPM, unpublished data).

Two sequential enzymatic activities produce the Aβ peptide. First, β-site APP Cleaving Enzyme (BACE1) cleaves the Amyloid Precursor Protein (APP) into a secreted APP fragment (sAPPβ) and leaves behind a 99 residue C-terminal fragment (CTFβ), which is cleaved by γ-secretase to Aβ and the APP intracellular domain (AICD) (Fig 3.1). Analysis of the ZNF9 disrupted cell line indicated a decrease in BACE1. Cleavage of APP by BACE1 is the rate-limiting step in formation of the Aβ-peptide, and this enzyme is an important therapeutic target for treating AD. For these reasons, we began in Chapter 3 by analyzing CNBP and BACE1 in the human brain. We examined a disease affected region of the brain (the superior and middle temporal gyri; SMTG) and a region largely unaffected (the cerebellum; CB) from a cohort of AD cases and age-matched controls.

We found that CNBP and BACE1 are highly correlated in both the SMTG and CB; both proteins increase in the SMTG of the AD cases, in the absence of a change in
their mRNA levels (Fig 3.2 C, D). A detailed pathological analysis of BACE revealed that BACE1 and a homologous enzyme, BACE2 are highly correlated in the AD brain (Fig 3.3 E, F), and their activities increase early in the disease process (Fig 3.3 A, B). These data supported the idea that CNBP was a positive regulator of BACE1, and given that BACE1 protein levels were increased in the absence of a change in mRNA levels, we hypothesized that CNBP regulated translation of BACE1. We used an AAV system to overexpress CNBP in various culture systems including H4 human neuroglioma cells, C2C12s, SY5Ys, and HEKS, (all immortalized cell lines) as well as primary neurons. In all instances, overexpressing CNBP induced an increase in BACE1 protein (Fig 3.7). In order to determine if CNBP affects BACE transcription, either directly or indirectly, we overexpressed CNBP (by viral transduction), then inhibited transcription using Actinomycin D (forms a non-specific complex with DNA that inhibits RNA synthesis), and monitored the effect on BACE mRNA levels by quantitative real-time PCR. For positive and negative controls we used mRNAs reported to be transcriptionally repressed or activated by CNBP. Changes in transcription were defined as an increase or decrease in steady state mRNA in the absence of a change in half-life.

CNBP overexpression had no effect on BACE1 mRNA half-life (Fig 3.8). This supports our data, and the data of several other groups, showing that the BACE1 increase in the AD brain is not accompanied by an increase in BACE1 mRNA, and further supporting a role for CNBP in post-transcriptional BACE1 regulation. However, we realized that the increase in BACE expression could result from either increased translation, or a reduced rate of protein turnover. In order to determine if the half-life of the BACE1 protein was affected by CNBP overexpression, cells were treated with
radiolabelled $^{35}$S Methionine / Cysteine overnight, allowing a steady-state label of all proteins being translated. These experiments showed no obvious effect of CNBP overexpression on BACE1 protein half-life; as expected, CNBP overexpression did result in slightly higher starting amounts of BACE1 (Fig 3.9). This and other data from our lab, indicating that CNBP can bind directly to the 5’ UTR of BACE1 (CJH, submitted) supports the hypothesis that CNBP is a positive translational regulator of BACE1.

The realization that CNBP affected BACE1 translation in several in vitro systems prompted us to ask whether this could be exploited to either accelerate or decelerate pathology formation in the brain of an animal model of AD. We chose the 5XFAD mouse model, an accelerated model of Aβ pathology with soluble forms of the peptide detectable in brain extracts by 21 days of age (Oakley, Cole et al. 2006). In the initial experiment CNBP AAV2 was transferred bilaterally into the brains of litters of mouse pups (≈50% transgenic 5XFAD mice). These mice were aged out for 3 months, a time-point chosen based on the earliest plaque deposition becoming apparent in the brain. Considerable effort went into making this experiment work. After finding that CNBP was not overexpressed initially, we changed the capsid protein (from AAV2 to AAV1), and evaluated brain extracts at a range of shorter time-points (21, 14, and 7 days) in an effort to determine if the virus was being silenced quickly, but in no instance was there evidence of protein overexpression. In an effort to determine if there was, perhaps, a small increase in CNBP not detectable by western blot analysis but substantial enough to affect BACE1 levels, all samples were evaluated for changes in Aβ production by ELISA. There was no difference between the groups (data not shown). Finally, in an effort to see if this inability to overexpress the protein was a strain-specific anomaly,
litters of APP / PS1 knockin animals were injected with the virus, but again overexpression was not achieved. While there was some concern that drastic overexpression of CNBP may be toxic (based on unpublished results by myself and other lab members indicating higher levels of cell death in CNBP AAV transduced cell lines), we did not anticipate the inability to overexpress the protein in vivo, especially after transducing primary rat neurons. From this exhaustive experiment, we conclude that CNBP is tightly regulated in vivo. It is tempting to speculate that the overexpression induced by the virus was silenced by a developmentally regulated shRNA, one that would necessarily target the coding sequence of CNBP, as only the cDNA is packaged in the virus. To test this, we performed stereotactic injection into the hippocampus of adult APP / PS1 animals, resulting in modest overexpression of CNBP (Fig 3.12), indicating tight developmental control of CNBP.

Our data indicate that CNBP may be involved in regulating translation of BACE1, the rate-limiting enzymatic activity in formation of the pathogenic Aβ-peptide, which is thought to play a causal role in AD. Because the most significant risk factor for AD in the general population is age, we wanted to determine if CNBP changes with age in either brain or skeletal muscle. We speculated that an age-related increase in CNBP would result in a concomitant increase in BACE1 (and possibly BACE2 as well) and a worsening AD-phenotype with time. We already know BACE2 enzymatic activities and protein levels increase in the aged brain and could also be involved in AD-pathology but did these BACE2 processes change with aging?

Our studies from neonatal mice indicate that both CNBP and BACE are highly expressed early in the brain, and their expression decreases rapidly after birth (Fig 4.2).
This is not surprising, given that both play important roles in CNS development. BACE1 is involved in the myelination of axons, while CNBP is likely important for proliferation control and early signaling events important for establishing the anterior / posterior axis of the embryo and initiating forebrain development. The normal physiological function of both proteins in the adult organism is poorly understood, although both are ubiquitously expressed at moderate levels. Given that CNBP is involved in disease processes affecting both the brain and skeletal muscle, and both tissues are negatively affected by the aging process, we next decided to examine CNBP levels broadly across the murine lifespan (Fig 4.3). We noticed a modest increase in CNBP in muscle with aging, and a barely perceptible increase in CNBP in the rodent brain.

In order to determine if an age-related change occurs in humans, we decided to examine CNBP and BACE in a very unique set of autopsy specimens, a series of DS and age-matched control (non-DS) subjects ranging from < 6 months to 67 years old. This case series would allow us to answer several questions. Do levels of CNBP change as a consequence of aging in humans, or only in pathological states? Also, individuals with DS have life-long overexpression of both APP and BACE2 as a consequence of carrying an extra copy of HSA21. Do BACE2 activity and protein levels increase as a result of this gene-dosage affect, and do increases in CNBP precede these changes as they do in AD? Our findings indicate a substantial increase in Aβ deposition in the DS brain at 40 years of age, a finding that supports the literature regarding Aβ deposition in persons with DS. This is not accompanied by increases in BACE1 protein or enzymatic activity, suggesting that overexpression of APP, the substrate that results in Aβ formation, drives
the pathology in these cases. Interestingly, in the aged human, CNBP levels do not change substantially, but actually may decrease slightly with age (Fig 4.8).

Interestingly, there is a neuromuscular disease that shares several key features with APP processing in common with the AD brain, another disease whose most significant risk factor is age. sIBM is the most common idiopathic inflammatory myopathy affecting persons over 50 years of age. Proteinaceous components of plaques reminiscent of AD pathology are found within the muscle fibers in sIBM. Because CNBP increases with age in the muscle, it is possible that it is responsible for upregulating BACE1, and plays a causal role in development of pathology. In Chapter 5, we used CNBP AAV2 to overexpress the protein in murine muscle. Within 1 month, this resulted in a significant increase in both BACE1 and Aβ-peptide in the muscle. This is an important proof-of-concept experiment that supports our reasoning that CNBP may play a causal role in sIBM pathology. While there in accumulating evidence that sIBM and AD have a shared etiology, there are few reports of sIBM in persons with AD (Roos, Vesterberg et al. 2011), however changes that occur in skeletal muscle have not been routinely examined in patients with AD.

In order to determine how much CNBP expression would decrease in vivo, we used an anti-CNBP shRNA lentivirus to knockdown expression of the protein in mouse muscle. Treatment resulted in approximately 20% reduction in CNBP protein levels, but did not change Aβ levels in the muscle in 2 weeks, as detected by ELISA. We next wanted to evaluate if this reduction in CNBP resulted in adverse effects on muscle force, or contractility. Measurements were performed 6-7 weeks post-transduction in mouse soleus muscle. This small reduction in CNBP did not change the absolute force (N)
generated by the muscle, the rate of fatigue, or the length of time required for the muscle to relax. Taken together, these data indicate that it is possible to decrease CNBP slightly without adverse effects on the muscle that recapitulate aspects of the DM2 phenotype. This means that reducing CNBP by using a gene therapy approach to target the ZNF9 gene locus could conceivably have therapeutic implications for treating people who suffer from sIBM, without deleterious effects on skeletal muscle.

Conclusions and Future Directions

Manipulating CNBP to elucidate its role in degenerative disease

A question that still remains from this work is, can increased expression of CNBP drive increases in BACE1 activity and Aβ-pathology in the brain? While our analysis of both rodent and human brain indicates that age-related increases in the protein are only marginally significant, perhaps over many years, this small increase can contribute to disease. One of the largest animal experiments described in this work details an exhaustive effort to overexpress the CNBP protein in the mouse brain (Figs 3.11 and 3.12). After two different capsid proteins for viral packaging, two different mouse strains, and many time points were evaluated, we have concluded that CNBP is tightly regulated in vivo at P0, probably due to its importance in forebrain development. However, with stereotactic injection into the adult hippocampus, it is possible to overexpress the protein, although the increase we have seen is small (~20%). Given the success of overexpression in the adult hippocampus, it remains possible that CNBP can be overexpressed to determine if this has adverse effects on pathology long-term in a
mouse model of AD. The APP / PS1 (homozygous APPNLh-PS1P264L) double knock-in model would probably be a better choice for this experiment than the 5XFAD strain we evaluated for most of the experiments in neonatal brain. The combination of mutations in the 5XFAD strain result in an early, rapid, and aggressive plaque deposition compared to other AD-models. While these were the very reasons we chose the 5XFAD model, it would be a poor choice for going forward with this experiment for several reasons. First, the animal would be developing pathology long before viral transduction would be effective. Also, and perhaps of most concern, the altered amounts of APP relative to the amount of BACE present may overwhelm any CNBP effect that may occur. The APP / PS1 mice would be a better choice because they exhibit Aβ pathology similar to that observed in AD (Flood, Reaume et al. 2002), including neuritic plaque development with age in the absence of APP overexpression. Because this model develops pathology rather slowly, it would allow for a detailed analysis of CNBP overexpression, which will likely result in upregulation of BACE1, changes that may induce cognitive disability as detected by behavioral analysis of the animals. Data presented in Chapter 5 support the hypothesis that overexpressing CNBP will result in a worsened phenotype.

Elucidating the Molecular Targets of CNBP

CNBP is a ubiquitously expressed, highly conserved protein of unknown function in the mature organism. CNBP likely represents one of the central RBPs, capable of binding mRNAs with little sequence specificity, as data from our lab and others indicate that it prefers to bind unpaired guanine-rich regions of unknown secondary structure (CJH, unpublished, (Van Horn, Arnett et al. 1996). This information does little to exclude
potential targets of the protein. CNBP is implicated as a regulator of global translation rates and proliferation control (Calcaterra, Armas et al. 2010), a finding supported by reduced rates of translation in patients with DM2 (Huichalaf, Schoser et al. 2009). It is possible this effect is elicited by CNBP’s interactions with mRNAs containing a 5’ terminal oligopyrimidine (5’ TOP) tract (Crosio, Boyl et al. 2000), a feature found in most components of the translational apparatus. CNBP may also be involved in eliciting stress-induced responses in cellular micro-environments, as an activator of cap-independent translation by binding IRES sequences in the 5’ UTR of its targets (Sammons, Antons et al. 2010). Data from our lab supports this, as CNBP likely regulates BACE1 translation in a cap-independent manner (CJH, unpublished). These findings suggest a potential role for CNBP in the aging process and/or various pathological states in which aberrant translational control is a contributing factor which includes multiple neurodegenerative and neuromuscular diseases.

One potential way to determine exactly what the molecular targets of CNBP actually are is to immunoprecipitate mRNA from brain extract using our CNBP antibody, and then sequence the RNA to determine what these interacting RNAs are, as well as their abundance relative to one another (reviewed in (Dimachkie, Austin et al. 1995). This approach has been used recently for other RNA binding proteins, most famously TDP-43 (Dimachkie 2011). This was novel in that it was the first time post-mortem brain tissue had been used for such a study, and it allowed the investigators to identify and characterize the RNAs that TDP-43 binds, and determine how these change by reduction of the protein by comparing healthy and familial FTD brain tissue. Similar information for CNBP would be very useful for determining how CNBP elicits functions in such a
wide variety of cellular processes and how these functions change post-development. For example, during development CNBP is involved in regulation of the cell-cycle and control of cellular proliferation, but is implicated as both a positive and negative regulator of different reported translational targets (Rajavashisth, Taylor et al. 1989, Michelotti, Tomonaga et al. 1995).

A more indirect approach to verify CNBP targets *in vivo* could come from optimization of CNBP knockdown in skeletal muscle, which could be used as a source of RNA for comparison to basal levels of CNBP. We were able to obtain ~20% reduction in CNBP with the first test of the virus and in a single injection. Perhaps this procedure could be improved by using a more concentrated viral stock, or by performing dual injections into the muscle would more efficiently reduce CNBP levels as was done in the soleus experiments described (Fig 5.3). Use of microarray and RNA-sequencing technology is becoming more mainstream and less cost-prohibitive; therefore, it would be possible to compare targets elucidated from both scenarios and verify bona fide targets of CNBP.

**Sorting Effects of the DNA Expansion from Effects of CNBP Haploinsufficiency in DM2**

Myotonic dystrophy is frequently described in the literature as a multisystemic heterogeneous disorder, referring to the highly variable spectrum of phenotypes caused by the disorder. In DM2, for example, it is nearly impossible to determine what effects are caused by dysfunction of the splicing machinery versus haploinsufficiency of CNBP itself, and which secondary effects are caused by altered chromatin structure affecting
neighboring genes. Haploinsufficiency of CNBP was reported to result in a phenotype that recapitulates some key aspects of the DM2 phenotype, inducing changes in muscle morphology including presence of centrically located and multi-nucleated fibers, and variable fiber size. Other traits noted were cataracts, gait abnormalities, cardiac hypertrophy and fibrosis, and vastus muscle myotonia (Obeid, Dimachkie et al. 2010). In our experience, reducing CNBP by ≅20% did not result in any notable phenotype in the skeletal muscle.

In order to separate effects of the expansion from those caused by haploinsufficiency of the protein, a first step would be to produce a platform for studying haploinsufficiency in the absence of an expansion alone, and then in combination with the expansion, which could be achieved in a variety of ways. Because CNBP knockout (CNBP -/-) is embryonic lethal, a reasonable next step in pursuing this project would be to produce a conditional knockout animal in which CNBP is selectively reduced post-development in a tissue specific manner. The ES cell line to make the animal is available from the mouse knockout consortium. The ES cells would be injected into the blastocoel of a mouse blastocyst, several of which would be transferred into a pseudopregnant dam. Resultant chimeras would then be screened for germline transmission and resultant offspring would be bred to a flippase recombinase mouse, a recombination event that would remove the neomycin resistance cassette that allowed for selection of the ES cells. The offspring from this cross could finally be bred to tissue specific Cre mice to generate models where CNBP expression could be drastically reduced in either skeletal muscle or brain. For example, the skeletal muscle specific Cre mouse (Golas, Parhi et al. 2010) would be an excellent candidate, resulting in a mouse model where tamoxifen treatment
would result in much less CNBP in the skeletal muscle (although there will still be some, as the system is slightly leaky). There are several good candidates for brain specific expression, a hippocampal Cre mouse (Li, Tian et al. 2011) may be a good choice.

While costly, and time-consuming, useful tools would be generated by this approach. The chimeric animals that resulted from this process would be heterozygous for CNBP (CNBP +/-) due to one allele being interrupted by the neomycin resistance cassette. It would be useful to see if these animals develop a DM2-like phenotype. Would this reduction in CNBP be sufficient to induce muscle weakness or would myotonia develop with age? One could then restore CNBP levels or introduce an expanded construct into either the skeletal muscle or brain by viral transduction. Behavioral and phenotypic analysis of the animals would be fairly straightforward, and length of treatment (with tamoxifen, to induce knockout) to occurrence of pathophysiology would be useful in elucidating the physiological role of CNBP.

Another way to do a similar experiment would be to use the treated conditional knockout mouse and then deliver viral constructs that express various sizes of expansion in-cis with CNBP (Fig 6.1). Constructs could include mini-genes encoding CNBP with either (i) no intronic expansion, (ii) a minimal intronic expansion (100 repeats) to reach the approximate disease threshold, or (iii) a larger intronic expansion (500 repeats) which clearly crosses the disease threshold (Fig 6.1B). The advantage of this method is that, in theory, it should more exactly model DM2. The disadvantage is the constructs described must contain an artificial intron due to packaging size limitations of both AAV and lentivirus. This intron will have to be spliced out correctly for CNBP to be expressed, meaning that all of the parts needed to interact with the splicing machinery will have to
be in the intron, and they will have to function efficiently in order for this modeling method to be effective.

Finally, the conditional knockout animal could be used, and the individual parts supplied *in-trans*. The knockout itself should suffice for a model of haploinsufficiency post-development and ‘rescuing’ CNBP expression by reintroduction using viral transduction should also abrogate any negative consequences of CNBP’s absence. A separate group that expresses an expanded, supra-threshold construct (Fig 6.1B) would provide haploinsufficiency and would more completely model DM2.

Long-term, such a model would provide useful for determining if it is possible to remove the expansion, and allow normal development to continue, unimpeded by toxic effects of the RNA expansion. Removal of the intronic expansion would probably have to occur early, as each division would result in more affected cells, but if possible it would have a huge impact on families affected by diseases in which genetic anticipation. A good example of the utility of such therapeutics would be DM1, in which the first affected generation has barely perceptible symptoms and the next generation experiences onset of muscle weakness in their 30s, often after having children who suffer from an extreme congenital form of the disease. While strictly hypothetical at this point, there are many ethical considerations that must be considered when contemplating altering the genome of an embryo.

**Gene Therapy Approach for Treating Neuromuscular Disease**

The most direct therapeutic implication from this work is for treating people who suffer from sIBM, the most common idiopathic inflammatory myopathy affecting
persons over 50 years of age. Proteinaceous components of plaques reminiscent of AD pathology are found within the muscle fibers in sIBM (Askanas and Engel 1998). In Chapter 5, we show data indicating that overexpression of CNBP in the skeletal muscle results in upregulation of BACE1 with a concomitant increase in Aβ, the peptide produced from BACE cleavage of the APP in the sIBM mouse model. This model expresses human APP (ΔNL) under control of the creatine kinase promoter, restricting its expression to the skeletal muscle (Bidichandani, Garcia et al. 2000). These data paired with our finding that CNBP increases substantially in the muscle with age (Fig 4.2) leads me to speculate that this age-related increase may drive formation of Aβ pathology in the muscle. We also show an approximate 20% decrease in CNBP in the muscle of C57BL/6 animals using an anti-CNBP shRNA lentivirus. This tool would allow us to reduce CNBP from the increased levels that occur with aging, back to near basal levels, and this would likely have a positive effect on muscle pathology in the sIBM mouse model. We did not see a reduction in Aβ with lentiviral knockdown in the animals, but the endpoint for the study was only two weeks post-transduction, so a longer time point may be necessary. It is possible that in order to reduce Aβ would require a more substantial reduction in CNBP below basal levels, but this would need to be tested. If decreasing CNBP does result in lowered BACE activity and, therefore, less Aβ-pathology over time; using a retroviral gene therapy approach to lower CNBP may have therapeutic benefits for persons with sIBM. There are many challenges that limit the use of such approaches in the clinic, including lack of a reasonable way to estimate individual variation in transduction efficiency or spread of the virus from the injection site, as not to lower protein levels into a harmful range.
In our experience, we were able to reduce CNBP levels by ~20% from basal levels without adverse effects on muscle function. The ability to reduce CNBP from an elevated level in aged muscle to a level consistent with those found in mature muscles may result in less Aβ inclusion pathology in the muscle, which could prevent inflammatory changes. This would be a huge advancement because there is no treatment for it sIBM, leaving those afflicted with a rather dismal prognosis of progressive myopathy that is not responsive to pain treatment.

**Conclusions and Implications**

CNBP is an RNA-binding protein typically ascribed ‘broad-spectrum’ functions in the literature (Calcaterra, Armas et al. 2010). Knockout of the protein is embryonic lethal very early, with development terminating likely due to extreme truncation of the forebrain, but other defects including skeleton defects and the absence of eyes in heterozygous animals (Chen, Liang et al. 2003) indicate that the protein plays a fundamental role in development. It is plausible that the normal physiological role of CNBP in development is activating a cellular program that regulates cellular proliferation (Calcaterra, Armas et al. 2010), but is also likely important for setting in motion a cellular process resulting in secretion of stereotactic factor that govern recruitment of progenitor cells to their proper location for development of the embryo. While expression of CNBP decreases rapidly after birth, all cell types and primary / immortalized tissue culture cell lines we have evaluated express robust amounts of the protein, but what role does it play in the adult and / or aged brain?
Data from this dissertation project support a role for CNBP as a translational regulator of BACE1 in several in vitro tissue culture models and in murine skeletal muscle. Importantly, the BACE1 mRNA can be immunoprecipitated from brain tissue with CNBP, indicating a direct role for CNBP in the translational process of the β-secretase. This is likely accomplished through a cap-independent process (CJH, unpublished). While there is little evidence that CNBP increases as a result of the aging process in the brain, data collected by other members of the lab indicate that cellular stressors like glucose deprivation or hydrogen peroxide exposure do cause an increase in CNBP expression (CJH, unpublished). In both primary neurons and skeletal muscle, these increases resulted in increased Aβ; therefore, one of the interesting therapeutic applications of this work would be to determine if blocking this increase in CNBP is sufficient to abrogate downstream BACE1 and Aβ pathology. However, these events suggest that under physiological conditions, an upstream stressor is responsible for driving the cellular regime that results in both increased CNBP and BACE1. Perhaps this indicates that while it is not likely that CNBP drives BACE1 pathology in sporadic AD, the protein could drive pathology after brain injury, and have a causal role in increased AD following chronic head trauma, as occurs in professional football players and boxers (Forstl, Haass et al. 2010, Vanacore, Lehman et al. 2013). Interestingly, I did not see an increase in either CNBP or BACE1 protein or activity in Down syndrome, indicating that a gene-dosage effect of APP likely drives Aβ-pathology in persons with DS. There was no increase in BACE2 protein in post-mortem DS tissue, indicating that although the obligate region of HSA 21 contains an extra copy of BACE2 and more mRNA is made from this locus, it is not translated. Our data from other disease states do indicate that
BACE1 and BACE2 proteins and activities are highly correlated, and likely subject to the same regulatory mechanisms (Holler, Webb et al. 2012). However, unlike BACE1, BACE2 mRNA does not coimmunoprecipitate with CNBP (CJH, unpublished), indicating it’s translation is likely not directly affected by CNBP. This raises the intriguing question; can BACE1 and BACE2 be regulated independently? BACE2 has been implicated as an alternative α-secretase, capable of cleaving APP within the Aβ-region (Farzan, Schnitzler et al. 2000, Sun, He et al. 2006). While our data indicate that expression of BACE1 is primarily neuronal, while BACE2 is found in both neurons and astrocytes, perhaps exploitation of BACE2 could prevent Aβ-pathogenesis if it were expressed in Aβ-generating neurons.

The most direct therapeutic implication from this work is for treating people who suffer from sIBM, the most common idiopathic inflammatory myopathy affecting persons over 50 years of age. Proteinaceous components of plaques reminiscent of AD pathology are found within the muscle fibers in sIBM (Askanas and Engel 1998). In Chapter 5, we show data indicating that overexpression of CNBP in the skeletal muscle results in upregulation of BACE1 with a concomitant increase in Aβ, the peptide produced from BACE cleavage of the APP in the sIBM mouse model. These data paired with our finding that CNBP increases substantially in the muscle with age, (Fig 4.2) leads me to speculate that this age-related increase may drive formation of Aβ pathology in the muscle. We also show an approximate 20% decrease in CNBP in the muscle of C57BL/6 animals using an anti-CNBP shRNA lentivirus. This tool would allow us to reduce CNBP from the increased levels that occur with aging, back to near basal levels, and this would likely have a positive effect on muscle pathology in the sIBM mouse model. If decreasing
CNBP does result in lowered BACE activity and therefore, less Aβ-pathology over time, using a retroviral gene therapy approach to lower CNBP may have therapeutic benefits for persons with sIBM. There are many challenges that limit the use of such approaches in the clinic, including lack of a reasonable way to estimate individual variation in transduction efficiency or spread of the virus from the injection site, so as not to lower protein levels into a harmful range.

The ability to reduce CNBP from an elevated level in aged muscle to a level consistent with those found in mature muscles may result in less Aβ inclusion pathology in the muscle, which could prevent inflammatory changes. This would be a huge advancement there are no treatments for sIBM, leaving those afflicted with a rather dismal prognosis of progressive myopathy that is not responsive to pain treatment.

While we are still far removed from manipulating CNBP in a clinically applicable way, accumulating evidence from our lab and others indicate that CNBP plays a vital role as a global regulator of translation, and there are intriguing insights into various diseases that share Aβ-related pathology from this work. There are many avenues for future research regarding CNBP and its role in both neurodegenerative and neuromuscular disease. Our understanding of RNA-binding proteins like CNBP is rapidly expanding and understanding the role these highly conserved proteins play is of utmost importance, as accumulating evidence suggests that aberrant RNA metabolism is shared among many disease states.
Figure 6.1 Constructs to Introduce CNBP and Expansions in-cis
Figure 6.1 Constructs to Introduce CNBP and Expansions in-cis. (A) The normal mouse ZNF9 gene has 5 exons, including a large first intron. The protein coding sequence (annotated CDS2, 3, 4 and 5) begins within exon 2. Our validated shRNA sequence is against a region in the 3’ UTR. (B) To design the experimental constructs, we will delete the large intron 1, and replace it with the much smaller intron 2. This is purely a practical decision, since the very large size of intron 1 would make the constructs too large to package in the virus. Intron 2 thus serves simply as a carrier element for the pathologic expansions. The modified intron will either contain no expansion, or a small (~100 CCTG) or larger (~500 CCTG) repeating element; the other introns will not be used. The end of the coding sequence will contain a tag (FLAG or similar) to allow CNBP generated from the foreign gene to be distinguished from the endogenous protein (our lab and others have made various tagged versions of CNBP, and a small C-terminal tag does not appear to interfere with its function). The heterologous 3’ UTR (normally contained within the pZac2.1 plasmid) will prevent knockdown by the shRNA targeting the endogenous mouse CNBP mRNA.
## Appendix 1: List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid Precursor Protein</td>
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<tr>
<td>Aβ</td>
<td>Amyloid-beta</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BIN1</td>
<td>Bridging integrator 1</td>
</tr>
<tr>
<td>BME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CB</td>
<td>Cerebellum</td>
</tr>
<tr>
<td>CNBP</td>
<td>Cellular Nucleic Acid Binding Protein</td>
</tr>
<tr>
<td>DM1</td>
<td>Myotonic Dystrophy type 1</td>
</tr>
<tr>
<td>DM2</td>
<td>Myotonic Dystrophy type 2</td>
</tr>
<tr>
<td>DMPK</td>
<td>Protein Kinase</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DS</td>
<td>Down syndrome</td>
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<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<tr>
<td>KH</td>
<td>K-homology domain</td>
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<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PCAD</td>
<td>Preclinical AD</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
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<tr>
<td>PEST</td>
<td>Pro, Glu, Ser or Thr-enriched</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
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<tr>
<td>PMI</td>
<td>Postmortem interval</td>
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<td>PUF</td>
<td>Pumilio domain</td>
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<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time PCR</td>
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<tr>
<td>RBP</td>
<td>RNA Binding Protein</td>
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<tr>
<td>RGG box</td>
<td>Glycine/arginine rich region</td>
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<tr>
<td>RIP</td>
<td>RNA immunoprecipitation</td>
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<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNA-Seq</td>
<td>RNA-high throughput sequencing</td>
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<tr>
<td>RNP</td>
<td>RNA Protein Complex</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>Rpl32</td>
<td>Rodent large ribosomal subunit 32</td>
</tr>
<tr>
<td>RPS17</td>
<td>Human small ribosomal subunit 17</td>
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<tr>
<td>RRM</td>
<td>RNA Recognition Motif</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription PCR</td>
</tr>
<tr>
<td>sAPP(α/β)</td>
<td>Soluble APP (a/b)</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SMTG</td>
<td>Superior middle temporal gyri</td>
</tr>
<tr>
<td>SR</td>
<td>Serine/Arginine</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single-stranded DNA</td>
</tr>
<tr>
<td>TBS(T)</td>
<td>Tris-buffered saline (+ Tween)</td>
</tr>
<tr>
<td>TDP-43</td>
<td>TAR-DNA binding protein 43</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>TPT1</td>
<td>Tumor protein translationally controlled 1</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>ZNF9</td>
<td>Zinc-finger protein 9</td>
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</tbody>
</table>

**Symbols**

- Alpha: $\alpha$
- Beta: $\beta$
- Gamma: $\gamma$
- Mu: $\mu$
## Appendix 2: PCR Primers

### RT-PCR

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<thead>
<tr>
<th>Primer Identifier</th>
<th>Sequence (5' to 3')</th>
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<tbody>
<tr>
<td>BACE1 Forward</td>
<td>TATCATGGAGGGCTTCTACGTG</td>
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<tr>
<td>BACE1 Reverse</td>
<td>GTCCCTGAACCTCATCGTGACAT</td>
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<tr>
<td>CNBP Forward</td>
<td>TCCTTCATGCGAGGTTCGTTCAGT</td>
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<tr>
<td>CNBP Reverse</td>
<td>GACAAGGTGGAAATGTGCACAGCA</td>
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<tr>
<td>TPT1 Forward</td>
<td>GATCGCAGGAACGGTTG</td>
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<tr>
<td>TPT1 Reverse</td>
<td>TTCAGCGGAGGCATTTC</td>
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### Animal Genotyping

<table>
<thead>
<tr>
<th>Primer Identifier</th>
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<tbody>
<tr>
<td>APP Forward</td>
<td>AGAGTACCAACTATGACTACG</td>
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<td>APP Reverse</td>
<td>ATGCTGGATAGCTGGTTTATC</td>
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<tr>
<td>PS1 Forward</td>
<td>ATGACAGAGTTACCTGCACCTTGTG</td>
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<tr>
<td>PS1 Reverse</td>
<td>CTGACTTAATGGTAGCAGCACGA</td>
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</table>
References


of amyloid precursor protein are specific for the increased secretion of A beta 42(43)." Biochem Biophys Res Commun 227(3): 730-735.


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