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THE CELLULAR NUCLEIC ACID BINDING PROTEIN REGULATES THE ALZHEIMER'S DISEASE β-SECRETASE PROTEIN BACE1

Christopher J. Holler
University of Kentucky, cjholl4@gmail.com

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Christopher J. Holler, Student

Dr. M. Paul Murphy, Major Professor

Dr. Michael Mendenhall, Director of Graduate Studies

THE CELLULAR NUCLEIC ACID BINDING PROTEIN REGULATES THE ALZHEIMER'S DISEASE β-SECRETASE PROTEIN BACE1

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

Christopher John Holler

Lexington, Kentucky

Director: Dr. Michael Paul Murphy, Professor of Molecular and Cellular Biochemistry

Lexington, Kentucky

Abstract of Dissertation

THE CELLULAR NUCLEIC ACID BINDING PROTEIN REGULATES
THE ALZHEIMER'S DISEASE β-SECRETASE PROTEIN BACE1

Alzheimer's disease (AD) is the most common neurodegenerative disease affecting the elderly population and is believed to be caused by the overproduction and accumulation of the toxic amyloid beta (Aβ) peptide in the brain. Aβ is produced by two separate enzymatic cleavage events of the larger membrane bound amyloid precursor protein, APP. The first, and rate-limiting, cleavage event is made by beta-secretase, or BACE1, and is thus an attractive therapeutic target. Our lab, as well as many others, has shown that BACE1 protein and activity are increased in late-stage sporadic AD. We have extended these findings to show that BACE1 is increased in the earliest stages of AD before the onset of significant Aβ accumulation, indicating a potential causal role in the disease. Interestingly, BACE1 mRNA levels are unchanged in AD, leading to reason that a post-transcriptional method of BACE1 regulation is altered in disease. To date, the mechanism for this aberrant post-transcriptional regulation has not been elucidated. This study has implicated the cellular nucleic acid binding protein (CNBP), a highly conserved RNA binding protein, as a positive regulator of BACE1 translation, with implications for the etiology of sporadic AD. CNBP overexpression in cultured cells or spiked into a cell-free *in vitro* translation system increased BACE1 protein expression without affecting BACE1 mRNA levels. Knockdown of CNBP reduced BACE1 protein and mRNA slightly. Furthermore, CNBP associated with BACE1 mRNA in cell lysates and bound directly to the BACE1 5' UTR *in vitro*, which confers most of the regulatory activity. Importantly, CNBP was increased in the progression of AD and correlated with BACE1 expression. Cellular stressors (such as glucose deprivation and oxidative stress) that occur in the AD brain increase BACE1 translation and we have found that these stressors increased CNBP expression as well. Early experimental evidence suggests that CNBP may enhance BACE1 translation through a cap-independent mechanism, which is an alternative translational pathway activated by cell stress. These studies indicate that the RNA binding protein CNBP is a novel *trans*-acting factor important for the regulation of BACE1 protein production and may be a viable therapeutic target for AD.

Keywords: Alzheimer's Disease, BACE1, CNBP, RNA Binding Protein, Translation

Christopher J. Holler
Student Signature

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Date

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By

Christopher J. Holler

Dr. Michael Mendenhall
Director of Graduate Studies

Dr. M. Paul Murphy
Director of Dissertation

This thesis is dedicated to my wife, Emily, whose unconditional love and support allows me to achieve anything I can dream…

…and to my Grandpa Holler, whose battle with Alzheimer's made me want to fight back.

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CHAPTER ONE: Introduction

Alzheimer's Disease

Alzheimer's disease (AD) is a progressive neurodegenerative disease and the most common type of dementia affecting the elderly population. Current reports estimate that over 5 million Americans and ~35 million persons worldwide have AD, and without effective therapeutic interventions those numbers could rise to over 15 million and 100 million respectively by 2050 ([1], Alzheimer's Disease International World Alzheimer Report, 2011). Such a drastic increase would bring with it an exponential rise in healthcare costs and additional hardships on families caring for those with AD. While there are currently no effective treatments for the prevention or cure of AD, significant advancements in understanding the underlying mechanisms of the disease have been made over the past 30 years that will potentially yield promising therapeutic breakthroughs in the near future.

Clinically, AD is characterized by a progressive loss in memory, impairments in language and intellectual capacity, behavioral changes and physical deterioration that ultimately leads to death [2, 3]. There are two major pathological lesions in the AD brain: (1) intracellular neurofibrillary tangles composed primarily of the hyperphosphorylated tau protein and (2) extracellular neuritic plaques composed largely of the amyloid-beta peptide (Aβ). These two hallmarks of the AD brain are believed to be connected through the amyloid cascade hypothesis [4, 5], in which age-dependent accumulations of Aβ drive the disease process and trigger a number of downstream events including the formation of tangle pathology, resulting in substantial neuronal loss and cognitive impairment. As AD is sometimes difficult to distinguish from other dementias or even normal aging, a

definitive diagnosis can only be made by analyzing the post-mortem brain for the key pathological lesions. Familial, early-onset AD (FAD) cases are rare (<0.5% of all cases) [6] and result from mutations either in the precursor protein from which Aβ is derived (amyoid-β precursor protein; APP) [7] or the presenilins (PS1 or PS2) [8-10] which, as part of the γ-secretase complex, catalyze the final cleavage step of APP to generate Aβ [11]. The remaining AD cases are sporadic, with the same etiology as FAD; however, the causative agents of this form are varied and not well understood. Age is the most significant risk factor for sporadic AD, and aside from apolipoprotein E (*APOE*) genotype, there are few genetic clues available to assess additional risk for development of the disease [12]. Nevertheless, studies from FAD cases and various transgenic mouse models of AD (reviewed in [13]) have helped strengthen the case that Aβ is the causative agent of the disease and therapeutics aimed at reducing Aβ generation or accumulation should prove beneficial for the prevention or treatment of AD.

APP Processing and Aβ Generation

The Aβ peptide is excised from the large type I membrane protein APP by a series of membrane-bound endoprotease cleavage events [14]. APP processing occurs in two main pathways: non-amyloidogenic and amyloidogenic (**Fig 1.1**). Both of these pathways and the products they produce occur under normal physiological conditions; however, it is the increased shift towards the amyloidogenic pathway that leads to AD. The non-amyloidogenic pathway begins with the cleavage of APP by an α-secretase (various metalloproteases such as ADAM9/10/17, TACE; reviewed in [15]) within the Aβ peptide region (Lys16-Leu17) and precludes formation of Aβ [14], instead producing a soluble APP fragment (sAPPα) and a membrane bound C-terminal fragment (CTFα) of 83 amino

acids (C83). C83 is then rapidly cleaved by the γ-secretase complex within the membrane to release a p3 peptide of uncertain function and the APP intracellular domain (AICD), which likely plays a role in signal transduction pathways [16-20]. Approximately 90% of APP is processed via the non-amyloidogenic pathway. However, the remaining APP may be cleaved first by a β-secretase, which generates sAPPβ and CTFβ (C99); C99 is then cleaved by γ-secretase to generate the mature 4 kDa Aβ peptide and AICD. Recent studies have shown that sAPPβ can be further cleaved by an unknown protease to form a 35kDa N-terminal fragment that is a ligand for the death receptor DR6; activation of DR6 triggers caspase-6 mediated axonal pruning and may play a role in AD pathogenesis [21].

The amyloidogenic pathway yields several Aβ species ranging in size from 37-43 amino acids in length, due to the flexibility of γ-secretase cleavage within the membrane. The most abundant form produced is $A\beta_{40}$ (~80-90%), and the most pathogenic is $A\beta_{42}$ (~5-10%) [4]. Aggregation of the more hydrophobic and fibrillogenic $A\beta_{42}$ peptide results in deposition of insoluble, extracellular senile plaques in the brain ([22], reviewed by [23]). Recent advancements in the field have implicated various soluble Aβ oligomers as the most toxic Aβ species and are key targets for therapeutic intervention [24-26]. Although the normal cellular functions of APP and its various cleavage products remain elusive, growing evidence points to critical roles in the developing central nervous system (CNS) [27-29]. The role of APP in the adult brain, which remains highly expressed, warrants further study.

Most emerging treatments for AD aim at minimizing Aβ production or enhancing Aβ clearance from the brain. As such, both β- and γ-secretase are considered prime therapeutic targets to combat AD [23, 30].

Discovery of the Brain β-secretase: BACE1

β-secretase, most commonly referred to as BACE1 (**b**eta-site **A**PP **c**leaving **e**nzyme 1), provides the rate-limiting enzymatic activity needed to generate Aβ and was originally cloned by five different laboratories in 1999 using distinct isolation methods [31-35]. BACE1, located on chromosome 11, is an atypical, type I membrane-bound aspartic protease of the pepsin family (cathepsin D and E, pepsin A and C, and renin) [36]. Early experiments showed that BACE1 had all the characteristics of the major brain β-secretase. Enzymatic activity of BACE1 is highest at low pH (~4.5), consistent with localization to acidic intracellular compartments including endosomes and the trans-Golgi network (TGN) and known β-secretase activity [32, 37]. Overexpression of BACE1 in cultured cells or transgenic animals results in a dramatic increase in Aβ production and APP cleavage products (sAPPβ or C99) and targeted antisense oligonucleotide knockdown of BACE1 reduces these products [32, 38]. In addition, recombinant BACE1 displays a preference for cleaving the FAD-causing Swedish mutation APP sequence (APPswe; β-site mutation KM → NL) over wild-type APP, consistent with β-secretase activity [32, 36].

The most compelling piece of evidence proving that BACE1 is the major β-secretase in the brain comes from BACE1 knock-out animals, generated by several groups [39-41]. $BACE1^{-/-}$ mice are devoid of any amyloid production or deposition in the brain, and are viable and fertile [40, 41]. $BACE1^{-/-}$ mice crossed with various APP transgenic mice have abolished amyloid pathology and improved cognitive capabilities [40, 42-44]. While at first $BACE1^{-/-}$ mice seemed normal and healthy, extensive

behavioral studies revealed subtle, although concerning, phenotypic abnormalities including increased anxiety, timidness, and mild deficits in spatial learning and memory, indicating that therapeutic-targeted BACE1 inhibition would not be without consequences [45-48]. Whether these unwanted side-effects would be reproduced in humans when BACE1 is inhibited remains an important question.

A short time after BACE1 was discovered, the related enzyme BACE2, which maps to the obligate Down syndrome critical region of chromosome 21, was cloned [49-51]. BACE1 and BACE2 are >65% homologous, indicating that BACE2 may be an alternative β-secretase [51, 52]. Indeed, BACE2 is able to cleave APP at the β-site (Asp+1) *in vitro* [53], but prefers to cleave APP within the Aβ peptide region at Phe+19 and Phe+20 [53-55]. Additionally, while BACE1 is highly expressed in neurons [56-58], BACE2 is expressed at much lower levels in the brain and appeared mostly astrocytic [52, 56, 59]. BACE2/APP overexpressing mice do not have increased Aβ levels [60] and combined BACE1 and BACE2 knock-out animals have slightly enhanced morbidity over $BACE1^{-/-}$ mice [46], further indicating that BACE2 is likely a poor β-secretase candidate but has some essential, yet undefined roles in the CNS that may overlap with BACE1. While BACE2 may play a minimal, if any, role in sporadic AD, this enzyme may be of particular importance to individuals with Down syndrome, who harbor an extra copy of chromosome 21 containing both the APP and BACE2 genes [49, 51], and who invariably develop AD-like pathology by their early 40s [61, 62]. Altogether, extensive studies on BACE1 and BACE2 up to this point indicate that BACE1 is the major β-secretase responsible for Aβ production in the brain.

Synthesis, Maturation, and Trafficking of BACE1

BACE1 is synthesized in the endoplasmic reticulum (ER) as a 501 amino acid pre-pro-enzyme with an N-terminal 21 residue ER-signal peptide followed by a prodomain from amino acids 22-45. The prodomain is cleaved by a furin-like convertase in the Golgi apparatus [52, 63, 64]. While studies have shown that immature BACE1 is able to cleave substrates [65], including APP, removal of the prodomain significantly increases its activity. This feature is distinct from traditional aspartic proteases in which the propeptide completely masks the active site of the enzyme, rendering it inactive until cleaved. The BACE1 catalytic domain consists of two conserved active sites (93-96 D*SGT and 289-292 D*TGS; where * = catalytic aspartate residue) and is followed by a single membrane spanning region and a short C-terminal domain. The catalytic site of BACE1 faces the luminal side of the cellular compartments while the carboxyl terminus is cytoplasmic and is involved in trafficking of the enzyme. The structure of the catalytic active site of BACE1 has been solved [66] and there are numerous additional structures of BACE1 in complex with various inhibitors. A structure of the entire immature enzyme does not exist at this point. BACE1 likely functions as a dimer in the membrane *in vivo*, similar to its APP substrate [67-69]. Because BACE1 displays many features distinct from other aspartic protease family members, there is significant hope for designing fairly specific inhibitors against it.

Mature, unglycosylated BACE1 has an estimated molecular weight of 65 kDa, while extensive modifications result in a BACE1 protein of about 75 kDa in its fully mature form. During maturation, BACE1 undergoes extensive post-translational modifications including *N*-glycosylation, acetylation, phosphorylation, and *S*-

palmitoylation which are involved in subcellular localization and trafficking [36, 63, 64, 70-73]. The palmitoylation of BACE1 enhances BACE1 localization to lipid rafts in the bilayer, which increases Aβ generation [74-76]; however subsequent studies showed that palmitoylated-deficient BACE1 processes APP as efficiently as raft-associated palmitoylated BACE1 [77].

BACE1 is present in small quantities on the cell surface [70, 77] and is rapidly internalized and trafficked to endosomal compartments and the TGN, where it has highest activity at low pH (~4.5). BACE1 has a long half-life (8-16 hrs) [36, 70] compared to APP (3 hours) and may undergo numerous rounds of trafficking to the cell surface and back before ultimately being targeted to the lysosome for degradation [70]. Alternatively, BACE1 may be degraded by the proteosomal degradation pathway [78]. Several Golgi-localized γ-ear containing ARF (GGA) binding proteins recognize a dileucine (D^{496}ISLL) motif on the carboxy terminus of BACE1 through a VHS domain [79]. GGA members sort various proteins, including BACE1, from the endosomes to the TGN and also transport ubiquitinated BACE1 to lysosomes for degradation [80-82]. Phosphorylation of Serine498 on BACE1 enhances the affinity of GGA proteins to BACE1 [83]. If the dileucine motif of BACE1 is deleted or mutated to alanines, the Ser498 mutated, or dominant negative GGA proteins overexpressed, BACE1 accumulates in endosomes and results in increased Aβ generation [81, 84, 85]. In addition, GGA proteins (GGA1 and GGA3) are decreased in AD causing decreased BACE1 lysosomal degradation and increased co-localization of BACE1 with APP in endosomes leading to enhanced Aβ production [82, 85, 86]. GGA3 may be degraded in AD due to increased neuronal apoptosis, as it is a substrate for caspase-3 [82].

Another set of proteins, the Nogo/reticulon (RTN) family, is also involved in BACE1 trafficking, in this case to the endoplasmic reticulum (ER) [87, 88]. When RTN3 is overexpressed in cells, the altered subcellular localization of BACE1 to the more neutral pH of the ER results in decreased BACE1 activity and Aβ production [89]. Monomeric RTN3 is reportedly decreased in the AD brain, reducing BACE1 ER localization and possibly contributing to AD pathogenesis [90]. Finally, BACE1 can be shed from the membrane surface through cleavage by a "sheddase" such as ADAM10 or TACE, which may result in decreased Aβ production since soluble BACE1 is unable to closely associate with the membrane-bound APP substrate [71, 91, 92]. However, *in vitro*, overexpression of soluble BACE1 can still cleave APP to produce Aβ [71], although the role and extent of BACE1 shedding *in vivo* remains unclear. Nevertheless, increased α-secretase activity (such as ADAM10) may reduce Aβ generation through cleavage of both APP and BACE1 [93].

BACE1 Substrates

The physiological role of BACE1 is still an area of intense research and is critical for understanding the toxicity-based side effects that may arise by inhibiting BACE1 for therapeutic purposes. While APP is the most commonly studied substrate of BACE1 due to its role in AD, the fact that APP is a rather weak substrate for BACE1 (BACE1 prefers to cleave bulky residues such as Leu at the P-1 position found in the APPswe mutation) indicated additional BACE1 substrates likely existed. By studying similar amino acid sequences to that of APPswe and the phenotypes of $BACE1^{-/-}$ mice, many of these substrates are now known, which suggests an increasingly broad physiological role for

BACE1. These substrates include APLP1/2 (APP family members) [94-96], α-2,6 sialytransferase (ST6Gal I) [97, 98], the P-selectin glycoprotein ligand-I (PSGL-I) [99], the interleukin-1 receptor type-II [100], the β-subunits of voltage-gated sodium channels ($Na_v\beta_2$) [101], the low-density lipoprotein receptor related protein (LRP-1) [102], and neuregulin-I/III (NRG-1, NRG-3) [103-105]. While a consensus cleavage sequence between known BACE1 substrates does not exist, there is a clear preference for single membrane-spanning substrates with a Leu or Phe at the P-1 position and a charged residue at the P+1 position [37, 66].

BACE1 cleaves APP at the β site (Asp+1) and β' site (Glu+11) [32], both of which result in production of amyloid-beta peptide after cleavage by γ-secretase. Cleavage at the β site is most common and leads to the typical Aβ peptides of 40-42 amino acids while cleavage at the β' site is most commonly used in rodents (and also seen *in vitro* when BACE1 is overexpressed) and may also contribute to a significant, yet largely unstudied pathogenic Aβ population [106, 107]. The related enzyme BACE2 can also cleave APP at the β site [53, 108] but prefers to cleave within the Aβ sequence at Phe+20 and Ala+21, precluding pathogenic Aβ generation [53, 55, 91, 108, 109]. Overexpression of BACE2 can reduce Aβ [110] and since BACE1 and BACE2 can compete for substrate *in vitro*, it has been postulated that BACE2 acts as an alternative α-secretase [54, 55, 91]. However, because BACE1 largely resides in neurons and BACE2 in glia, it is unclear what role, if any, BACE2 has on the pathogenesis of AD *in vivo*. APP homologs APLP1/2 also undergo α, β, and γ-secretase cleavage but do not produce Aβ or related peptides, and the *in vivo* function of these products are not fully elucidated [94-96, 111]. Cognitive and emotional deficits reported in various $BACE1^{-/-}$ mice appear to be

largely attributed to impaired APP processing, indicating key physiological roles for β-site derived APP products [42].

Two other BACE1 substrates that are of particular interest are the neuregulins (NRG-1 and NRG-3) and the β-subunits of voltage-gated sodium channels ($Na_v\beta_2$). Neuregulins are ligand members of the Erbβ family of receptor-tyrosine kinases that have numerous roles in the CNS, including synaptic formation and maintenance, myelination of axons, and neurotransmitter expression and function [103-105]. The voltage-gated sodium channels regulate membrane excitability and play crucial roles in propagating action potentials in the CNS and peripheral nervous system (PNS). Close examination of the subtle phenotype of $BACE1^{-/-}$ mice revealed deficiencies in myelination of neurons in the PNS and CNS [103, 104] and alterations in the inactivation of voltage-gated sodium channels [46]. These events help explain some of the phenotypic abnormalities, i.e. hyperactivity, schizophrenic-like behavior, and epileptic symptoms of the BACE1 null mice [112]. Increased BACE1 expression (as in AD) or inhibiting BACE1 (as a therapeutic strategy) [113] both result in neurological deficits, a result of the increasingly broad physiological role of BACE1. Therefore, optimal BACE1 therapeutic treatments must focus on reducing rather than abolishing BACE1 activity *in vivo*, a task that has been challenging to this point.

BACE1 Expression and Regulation in Normal and AD Brain

BACE1 is found only in vertebrates, which was a first clue to its possible role in complex systems such as the brain and immune system. BACE1 is expressed in most tissue types, with highest levels in the CNS and lower levels in peripheral tissues [32,

33]. Interestingly, BACE1 is also expressed at high levels in the pancreas, where several largely inactive isoforms exist [114, 115]. In the brain, BACE1 is expressed primarily in neurons, consistent with the major Aβ-producing cell type [39], although BACE1 is upregulated in and around reactive astrocytes as well [116, 117]. Since astrocytes outnumber neurons by an order of magnitude in the brain, even a small increase in BACE1 in these cells could play a role in AD pathogenesis.

BACE1 undergoes complex regulation at both the levels of transcription and translation (**Fig 1.2**). The upstream promoter region of BACE1 has been characterized [58, 118-120] and contains several binding sites for common transcription factors such as AP-1, AP-2, HSF-1, NF-κβ, PPARγ, YY1, MZF1, HNF-3β, HIF1α, STAT1/3 and Sp1 (reviewed in [121]). Overexpression of Sp1 increases BACE1 transcription and knockdown of Sp1 reduces, but does not abolish BACE1 transcription, indicating dependence on additional transcription factors [118]. For example, NF-κβ signaling can repress and upregulate BACE1 transcription [122] and increasing evidence points to a role for PPARγ, which is involved in inflammatory response, in BACE1 regulation. Inflammation is an early event in the development of AD and has been a target for numerous AD therapeutics (reviewed in [123]). The use of certain non-steroidal anti-inflammatory drugs (NSAIDs) potentially reduced the risk of AD in epidemiological studies [124] and altered amyloid metabolism *in vitro* [125]. NSAIDs, such as ibuprofen, can target PPARγ directly to suppress BACE1 gene expression in cell culture [126]. A selective agonist of PPARγ, pioglitazone, reduced both BACE1 mRNA and protein in APP transgenic mice [127]. PPARγ agonists such as pioglitazone and rosiglitazone have been approved for treatment of type II diabetes and produce few negative side effects,

indicating they may be useful in treating AD as well. The JNK/c-jun signaling pathway has also been implicated in BACE1 transcriptional regulation during oxidative stress conditions and may involve a positive feedback mechanism dependent on γ-secretase activity and Aβ42 production [128-130].

A hallmark of AD is impaired glucose metabolism [131] and BACE1 displays many characteristics of a stress response protein. Treatment of cells with drugs that impair energy metabolism led to increased levels of BACE1 and Aβ in the brain by approximately 2-fold [132]. In addition, BACE1 is upregulated in response to numerous stressor events such as hypoxia [133], ischemia [134], oxidative stress [135, 136], traumatic brain injury [137], cholesterol content [138, 139], viral infection [140], and altered calcium homeostasis [141]. A small increase in BACE1 activity is also seen in normal aging in rodents, monkeys, and humans [142], which may be related to these events. In the future, it will be important to determine which specific pathways of BACE1 regulation are affected in AD compared to other stress events, and how this influences the overall etiology of the disease process.

The human BACE1 5' untranslated region (UTR) is unusually large (~446 nt), has a high guanine-cytosine (GC) content (77%), and contains three upstream open reading frames (uORFs) similar to other stress response proteins - all indicators of a protein under tight translational regulation [143-145]. It is also highly conserved with other vertebrates, suggesting a critical role in regulation (**Fig 1.3**). The BACE1 5' UTR inhibits BACE1 translation [146], likely owing to the presence of the uORFs and a high degree of secondary structure which may impede efficient ribosome initiation and/or scanning mechanisms [143-145, 147]. Although translation initiation may occur at three

of the four uAUGs [143-145, 148], it is unknown whether any functional peptides are synthesized. The 3' UTR of BACE1 is also extremely long and is of variable length; it likely plays a role in stabilizing the mRNA and controlling expression through several microRNA binding sites [149-151]. MicroRNAs are short noncoding RNA species that target the 3' UTRs of specific genes and either inhibit translation or lead to mRNA degradation [152]. The loss of a specific microRNA cluster led to increased BACE1 expression, but only in a subset of AD patients [150]. In addition, a non-coding BACE1-antisense RNA that stabilizes BACE1 mRNA was increased in AD patients [153]. BACE1 activity in AD may be enhanced by increased direct association with the lipophilic mediator molecule sphingosine-1-phosphate [154] while BACE1 half-life, and subsequent Aβ generation, was reported to be increased by the lipid second messenger ceramide, which is elevated in the brain of AD patients [155]. Alternative splicing events also affect BACE1 expression levels. Several BACE1 variants that display little or no activity exist due to alternative splicing [156]. Recently, it was reported that a G-rich element in exon 3 of BACE1 regulates splice site selection through formation of a G-quadruplex (G4) structure mediated by heterogeneous nuclear ribonucleoprotein H (hnRNP H); knockdown of hnRNP H resulted in decreased full-length BACE1 mRNA [157]. These studies indicate that several post-transcriptional events likely contribute to the sustained increase of BACE1 in AD.

Many groups have looked at the expression and activity of BACE1 in the AD brain. Compared to healthy age-matched controls, BACE1 protein and activity are increased in brain regions affected by AD [158-162], although others have reported no change [163], or decreased levels [164]. One report indicated that only ~30% of sporadic

AD cases had increased BACE1 levels [150], possibly owing to the fact that most measurements are made from end-stage AD postmortem tissue, which could differ from early-stage AD BACE1 levels. The increase of BACE1 in the AD brain may contribute to enhanced Aβ production and advancement of the disease, especially if it occurs early in the disease process. For example, BACE1 is also increased in the cerebrospinal fluid (CSF) in patients with mild-cognitive impairment, a precursor to AD [165]. BACE1 elevation seems to occur mostly in the neurons surrounding plaque deposits and in reactive astrocytes [166, 167].

Interestingly, BACE1 mRNA levels appear largely unchanged in AD, indicating that a post-transcriptional control process may go awry in disease, accounting for the increased BACE1 protein levels [57, 159, 168-170]. BACE1 is highly regulated at both the transcriptional and translational levels, and in particular the 5' UTR of BACE1 appears be the major *cis*-element for translational regulation. While the BACE1 5' UTR keeps translation relatively low in healthy individuals, translation occurs much more efficiently in AD through unresolved mechanisms. This observation leads to the speculation that certain *trans* factors, such as RNA binding proteins and/or translational machinery components, may be altered in AD, resulting in increased BACE1 translation.

Recently, the phosphorylation of the eukaryotic initiation factor (eIF2α-P) has been implicated in the increased expression of BACE1 at the translational level [170, 171]. eIF2 is a GTP-binding protein that mediates the delivery of the initiator methionine-tRNA (Met-tRNA$_i$) to the ribosome to initiate protein synthesis [172]. The activity of eIF2 is regulated by reversible phosphorylation of its α subunit by one of four different stress-induced kinases [173]. When eIF2α is phosphorylated during increased cellular

stress, the eIF2-GDP generated after each round of initiation becomes an inhibitor of the guanine nucleotide exchange factor, eIF2B, and the result is a reduction in exchange of eIF2-bound GDP for GTP. Since eIF2-GTP is needed to bind Met-tRNA$_i$, phosphorylation of eIF2α effectively inhibits recycling of eIF2 for new rounds of initiation; the end result is reduced global translation. Interestingly, specific mRNAs that encode proteins responsible for critical stress-mediated cellular functions are preferentially translated upon eIF2α phosphorylation [173], and BACE1 appears to be one of these proteins.

In one study, energy deprivation in cultured cells and primary neurons lead to increased levels of BACE1 which was shown to be mediated by increased eIF2α-P [170]. Treatment of APP transgenic mice with energy metabolism inhibitors increased phosphorylated eIF2α, BACE1, Aβ, and Aβ deposition [170]. In addition, an analysis of postmortem human brain samples found increased levels of phosphorylated eIF2α that correlated with increased BACE1 and amyloid levels [170]. The increase in BACE1 translation due to eIF2α-P was hypothesized to occur through enhanced ribosomal scan-through of the BACE1 uORFs, allowing for more initiation events to occur at the authentic BACE1 start codon; however, this mechanism has not been demonstrated experimentally [170]. Besides glucose deprivation, hydrogen peroxide (H_2O_2) has also been shown to increase BACE1 protein post-transcriptionally in a phosphorylated eIF2α-dependent manner [171]. Oxidative stress, implemented by H_2O_2 and other reactive oxygen species (ROS), increases in the AD brain due to mitochondrial damage and is thought to play a significant role in the etiology of the disease [174-176]. A recent study indicated that initial cell stressor events may increase eIF2α phosphorylation and BACE1

expression, but once a certain level of amyloid pathology is obtained (and tangle pathology is initiated), the events are self-sustaining and cannot be reversed with partial BACE1 reduction [177]. This indicates that targeting of BACE1 and/or regulating factors must occur early in the disease process to have the most therapeutic value. Determining how BACE1 translation is regulated during times of cellular stress when eIF2α is phosphorylated may lead to novel therapeutic treatments.

CNBP: Role in Disease and Development

The cellular nucleic acid binding protein, or CNBP, is the protein product of the gene commonly known as *ZNF9*, but more recently referred to as "CCHC-type zinc finger, nucleic acid binding protein" by the Human Genome Organization Gene Nomenclature Committee. Even though CNBP was first identified more than 25 years ago, very little is known about how this protein functions. CNBP is strikingly conserved in vertebrates and mounting evidence suggests that it is a multifunctional single-stranded nucleic acid binding protein involved in basic biological functions. So far, CNBP has been implicated in two separate biological processes: the human disease myotonic dystrophy type 2 (DM2) and the embryogenesis of craniofacial/forebrain structures.

Expansion of a CCTG tetranucleotide repeat in the first intron of the human CNBP gene causes DM2 [178]. DM2 is an autosomal dominant disorder that presents clinically with a broad-range multi-systemic phenotype including myotonia, muscle weakness and wasting, cardiac defects, cataracts, hypersomnia, some neurological deficits, and endocrinological problems [179]. It is believed that DM2 is primarily caused by a toxic gain-of-function in which long, untranslated CCUG repeats accumulate and

form RNA foci in the nucleus [180]. These nuclear foci form ribonucleoprotein (RNP) inclusions by sequestering several RNA binding proteins involved in various RNA processing events including splicing, stability, transcription, and translation [181]. However, a growing line of evidence suggests that CNBP loss-of-function may play a role in the pathogenesis of DM2 as well. For example, $CNBP^{+/-}$ mice haploinsufficient for CNBP have a phenotype similar to myotonic dystrophy with multisystemic abnormalities that can be reversed by crossing them with CNBP overexpressing mice [182]. Additionally, several groups have reported that cytoplasmic levels of CNBP are reduced in skeletal muscle satellite cells, myoblasts, and muscle biopsies from DM2 patients [183, 184]. A reduction in CNBP coincided with a decrease in the translation of a number of 5' terminal oligopyrimidine (5' TOP) containing mRNAs that are direct targets of CNBP [184]. These targets are members of the translational machinery apparatus and include ribosomal protein S17 (RPS17), poly-A binding protein (PABP), and the eukaryotic elongation factors eEF1A and EF2. The overall effect was a reduction in global protein synthesis that could be reversed by reintroducing CNBP ectopically to cultured DM2 myoblasts [184, 185]. Finally, recent evidence suggests that CCUG repeats themselves can cause decreased CNBP expression and this is mediated through a post-transcriptional mechanism, likely decreased protein translation or stability [186, 187]. While the exact mechanism for DM2 pathogenesis remains clouded, these studies have shed some light on the basic cellular roles of CNBP.

In addition to DM2, CNBP plays a prominent role in embryonic development, and this area of research has provided important insights into the molecular actions of CNBP. CNBP is ubiquitously expressed in vertebrates with high levels occurring in the testis,

ovary, and brain [188, 189]. Analysis of CNBP during mouse embryonic development showed high mRNA expression in the forebrain, midbrain, craniofacial structures, and limb buds; in general, the highest levels of CNBP were seen where the highest rates of cellular proliferation occurred [189, 190]. Studies in chicken [191] and fish embryos [188, 192] showed similar expression profiles and indicated a conserved mechanism. Complete knockout of CNBP expression in mice is embryonic lethal due to incomplete forebrain development and craniofacial abnormalities [189]. About 40% of the newborn heterozygous mice had similar phenotypes, including growth retardation, smaller mandible, and lack of eyes, and died shortly after birth. This suggests that a certain threshold level of CNBP is necessary for proper development.

CNBP appears to influence embryonic development primarily through transcriptional regulation of a number of genes. The evidence that CNBP binds to the CT-promoter element of *c-Myc* and causes its upregulation [193] and that $CNBP^{-/-}$ mice lack detectable levels of *c-Myc* [189] suggest that CNBP plays a role in stimulating cell proliferation and differentiation through *c-Myc* induction. In support of this, overexpression of a dominant negative form of CNBP downregulated *c-myc* expression in a species of toad (*Xenopus laevis*) [194]. In the chicken, CNBP knockdown also downregulated other genes involved in cell proliferation which led to forebrain truncation [191]. Experiments using Morpholinos that specifically knockdown CNBP in zebrafish embryos showed that the phenotype arising from CNBP deficiency is a result of abnormal gene expression and reduced cell proliferation in the neural crest [192], from which most craniofacial cartilage is derived [195]. Additionally, increased apoptosis and decreased cell proliferation was seen in these CNBP Morphants where CNBP was

knocked down [192]. The essential role of CNBP in craniofacial development has outlined a basic role in embryonic development; however, CNBP remains highly expressed in adult tissue, such as the brain, where its cellular functions are still unknown.

Nucleic Acid and Protein Interactions of CNBP

Human CNBP was originally identified as a zinc finger protein that bound to the sterol regulatory element from the hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase gene and negatively regulated its expression [196], although follow-up studies could not confirm this. Subsequent studies implicated CNBP as a negative transcriptional regulator of the early promoter-enhancer of the JC virus [197] and the beta-myosin heavy chain gene [198], as well as a positive transcriptional regulator of the macrophage colony-stimulating factor gene in fibroblasts [199], and the CT promoter element of the *C-MYC* proto-oncogene [193]. CNBP has also been implicated as a translational regulator. CNBP binds a G-rich sequence downstream of 5' TOP structures in several mRNAs that are part of the translational machinery apparatus, although there is still some discrepancy as to whether CNBP acts as a positive [184, 200] or negative [201-203] regulator of their translation. Several studies have now shown that CNBP and its yeast homolog gis2p directly interact with translating polysomes, indicating a crucial role in translation [204-207]. Interestingly, CNBP also binds directly to the poliovirus internal ribosomal entry site (IRES) to stimulate cap-independent translation [200], an alternative method of translation involving internal initiation that occurs when canonical cap-dependent translation is inhibited by various cellular stressors [208]. CNBP has been identified as an IRES *trans*-acting factor (ITAF) as part of a potential complex involved

in the cap-independent translation of the mouse [204] and human [205] ornithine decarboxylase (ODC1) genes. Since CNBP binds directly to the ODC1 IRES and also directly associates with translating ribosomes, it may function as a bridge between specific mRNAs and the translational apparatus.

Although CNBP binds single-stranded nucleic acids, it also appears to participate in a number of protein-protein interactions. A large-scale proteomics screen in human cells identified numerous candidates for CNBP-interacting proteins through mass spectrometry [209]. The most abundant targets found were ribosomal proteins both of the large and small subunits as well as several heterogenous ribonuclear proteins (hnRNPs) and a number of other RNA binding proteins. A separate study that used tandem affinity chromatography followed by mass spectrometry identified CNBP as well as many other RNA binding proteins and ITAFs that bound the human ODC1 IRES sequence [204]. These studies corroborate with previous findings that CNBP associates with the translation machinery and that CNBP may be a member of an RNP complex with other ITAFs [204], although the exact functional consequences of these protein-protein interactions are unknown.

CNBP Gene Structure and Functional Domains

The human CNBP gene is located on the long arm of chromosome 3 and encodes a protein of 177 amino acids and has a predicted molecular weight of 19 kDa [210]. The amino acid sequence of CNBP is strikingly conserved in human, mouse, rat, chicken, and toad, indicating a basic biological function (**Fig. 1.4**). CNBP also has orthologs in insects, fish, lizard, shrimp [211], and yeast [212, 213], but not in plants or bacteria. Structurally,

CNBP consists of seven tandem CCHC-type zinc finger motifs of 14 amino acids each (C-φ-X-C-G-X_3-H-X_4-C, where φ is an aromatic amino acid and X is any amino acid) [214] connected by predicted intrinsically disordered linker regions [215]. Each zinc finger is formed through the coordinated interaction with a single divalent cation, with Zn^{2+} providing the most stability [193]; this interaction may help maintain the overall structure and stability of the protein. A glycine/arginine-rich region (RGG box) is located between the first and second zinc fingers, which is a common feature of many RNA-binding proteins, such as the Fragile X mental retardation protein (FMRP) [216]. An alternative splice site in this region results in a slightly smaller β-isoform of 170 amino acids that has been reported in mammals [214] and *X. laevis* [217, 218], but whose role is unclear. In addition, CNBP contains a putative nuclear localization signal, P(KR/KK/RK)EREQ, and a putative proteolytic PEST (Pro, Glu, Ser or Thr-enriched) cleavage site. CNBP is localized to the nucleus and cytoplasm in mammalian, amphibian, chicken, and fish cells [189, 190, 211, 219-221], while the PEST sequence allows for an N-terminal truncated product that has been identified in amphibians [202, 220, 222]. The truncated form of CNBP lacks the first zinc finger and RGG box and may possibly function as a dominant negative mutant with important regulatory consequences [194]. A single, putative cAMP-dependent protein kinase (PKA) phosphorylation site of unknown function exists at the C-terminus of CNBP [223]. A second putative phosphorylation site for casein kinase II that is missing in the CNBP β-isoform has been suggested, but not confirmed [218].

CNBP consists of 5 exons, with exon 1 containing the 5’ UTR and exon 5 containing the majority of the 3’ UTR [214]. Exons 2-5 are highly conserved in all

species whereas the introns are more variable, with intron 1 being the longest amongst invertebrates [224]. The 3' UTR of CNBP is also highly conserved indicating a likely regulatory role [219]. A putative promoter region was identified in mouse CNBP within a 3 kb proximal region upstream of the transcriptional start site that contains potential recognition sequences for numerous common transcription factors including Sp1, AP-1, GATA-1/2/3, STAT, and C/EBP among others [190]. Additionally, putative promoter and regulatory elements were identified in intron 1 in zebrafish [225]. Interestingly, the canonical sequences for a TATA- or CAAT-box may be located very far from the transcription start site [190, 214].

CNBP Biochemical Activities and Molecular Mechanism of Action

CNBP binds single-stranded DNA (ssDNA) and RNA, but not double stranded DNA [194, 215, 226], and has been implicated in the transcriptional and translational regulation of various genes. Evidence of CNBP localization to the cytoplasm, ER, and nucleus support this claim [220-222]. In the absence of crystallographic structures of CNBP alone or bound to nucleic acid molecules, the different structural domains of CNBP have been studied experimentally in an attempt to dissect their molecular function. The CCHC-type zinc fingers are structurally similar to those found in retroviral nucleocapsid (NC) proteins and, in fact, substitution of the human CNBP zinc fingers in place of the two human immunodeficiency virus 1 (HIV1) NC zinc fingers preserved viral structure and replication ability [227]. The HIV1 NC protein primarily functions in the assembly of virions and packaging of the genomic RNA [228]. The zinc fingers also interact with ssDNA and each of the seven zinc fingers were predicted to interact with a

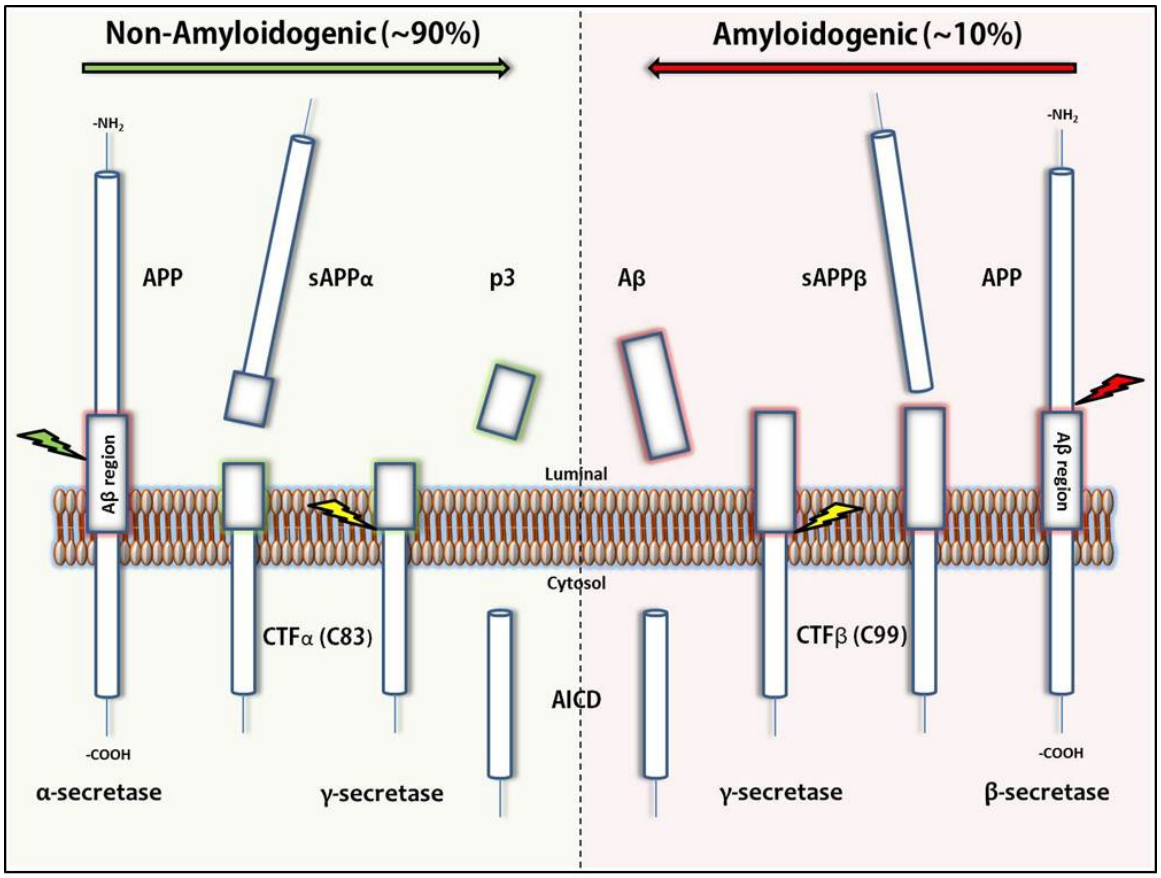
single G nucleotide of an octonucleotide probe *in silico* [229]. Studies in *X. laevis* indicated that the presence of the zinc fingers is required but not individually necessary for achieving maximal binding activity to nucleic acids [194]. However, the RGG box was essential for RNA-protein binding while removal of the RGG box preserved a weak ssDNA binding activity. Microinjection of *X. laevis* embryos with CNBP that lacked the N-terminal zinc finger and RGG region, mimicking the proteolytic form, functioned as a dominant negative *in vivo*.

CNBP can bind ssDNA as a monomer or dimer; however, CNBP binds to RNA as a preformed dimer [215, 230], leading to speculation that non-interconvertable pools of CNBP monomers and dimers exist; exactly how CNBP dimerization occurs is unknown. Analysis into the biochemical and molecular features of CNBP revealed that its preferred targets contain unpaired guanosine-rich stretches constrained by an organized sequence environment [215]. Additionally, it was shown using nucleic acid melting and annealing assays that CNBP acts as a nucleic acid chaperone through ATP-independent binding, remodeling, and stabilization of nucleic acid secondary structures [215]. As an example, a recent study revealed that CNBP enhanced *C-MYC* transcriptional activity by promoting parallel G-quadruplex (G4) formation; however, CNBP does not actually bind the G4 structures [231]. The chaperone activity appears to depend on the N-terminus of the protein where the first zinc finger and RGG box reside [215]. Since G4 structures are also present in RNA, CNBP may influence translation through remodeling G-rich specific sequences.

Rationale for Study

BACE1 is the rate-limiting enzymatic activity needed to generate the toxic Aβ peptide that is believed to be the causal agent of Alzheimer's disease. Studies in our lab as well as others have shown that BACE1 protein and activity are increased in AD, which may underlie the etiology of the disease. Increased BACE1 in AD occurs in the absence of increased mRNA levels, indicating a change in post-transcriptional regulation. This raises an intriguing question about the regulatory mechanisms that govern BACE1 translation and how they may be altered in disease states. We do not yet fully understand how BACE1 is translated under normal conditions, let alone during cellular stress or disease processes.

Recently, our lab identified CNBP as a potential regulator of BACE1 (MPM, unpublished data). The purpose of this study was to determine if CNBP is a *bona fide* regulator of BACE1 expression and if so, to determine how CNBP might carry out this function. This study progresses the field of knowledge of both BACE1 regulation and CNBP molecular function, and has important implications for both Alzheimer's disease and myotonic dystrophy type 2.


Non-Amyloidogenic (~90%)
Amyloidogenic (~10%)
-NH2
APP
sAPPα
p3
Aβ
sAPPβ
APP
-NH2
Aβ region
Luminal
Cytosol
CTFα (C83)
AICD
CTFβ (C99)
Aβ region
-COOH
-COOH
α-secretase
γ-secretase
γ-secretase
β-secretase

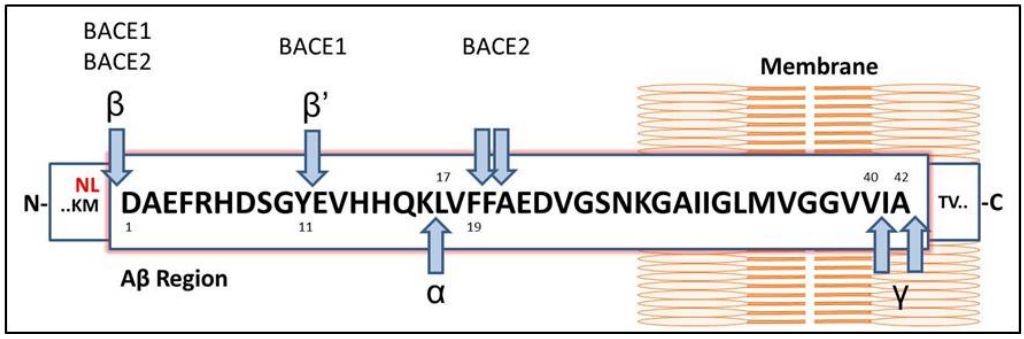

BACE1
BACE2
BACE1
BACE2
Membrane
β
β'
NL
..KM
N-
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
TV..
-C
1
11
17
19
40
42
Aβ Region
α
γ

Figure 1.1 APP Processing Pathways and Aβ Generation. *Top*, Constitutive processing of APP by α-secretase (left) results in a secreted, soluble APP fragment (sAPPα) and a membrane-bound C-terminal fragment (C83), which is rapidly cleaved by the γ-secretase complex within the membrane to liberate the p3 peptide. Approximately 90% of APP is processed by this pathway which precludes Aβ generation. However, 10% of APP may be first cleaved by β-secretase (right) producing sAPPβ and C99. C99 in turn is cleaved by γ-secretase to produce the Aβ peptide. Both pathways produce the APP intracellular domain (AICD) after γ-secretase cleavage. *Bottom*, A schematic of the Aβ peptide region (1-42) shows the locations of various proteolytic cleavage events at the β, β', α, and γ sites. The locations where BACE1 and BACE2 cut are labeled, along with the Swedish mutation (NL) that enhances β-secretase cleavage in familial AD cases.

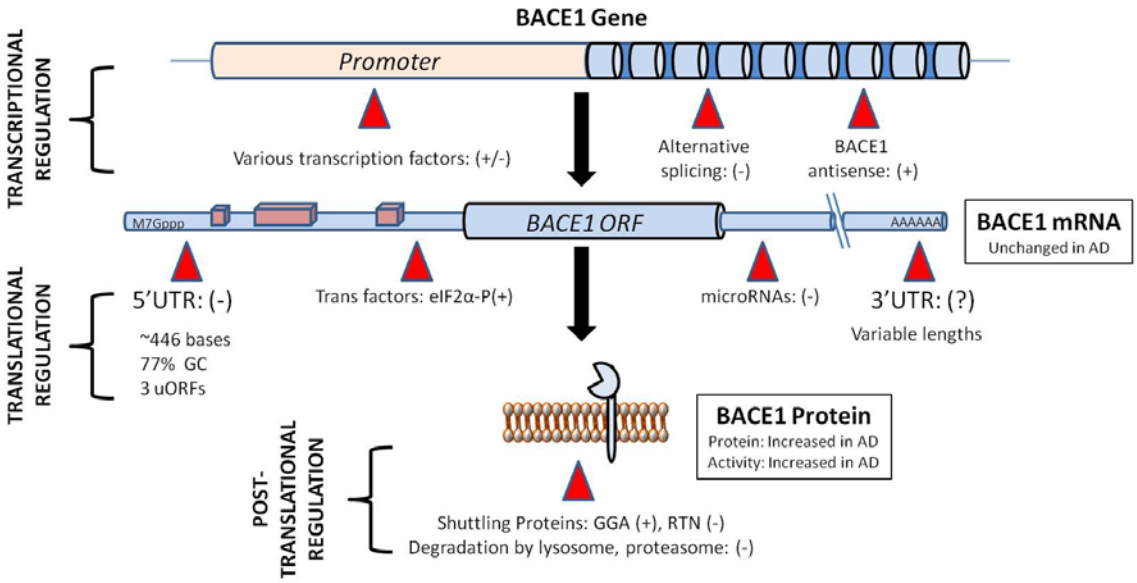

BACE1 Gene
Promoter
TRANSCRIPTIONAL REGULATION
Various transcription factors: (+/-)
Alternative splicing: (-)
BACE1 antisense: (+)
M7Gppp
BACE1 ORF
AAAAAA
BACE1 mRNA
Unchanged in AD
TRANSLATIONAL REGULATION
5'UTR: (-)
~446 bases
77% GC
3 uORFs
Trans factors: eIF2α-P(+)
microRNAs: (-)
3'UTR: (?)
Variable lengths
BACE1 Protein
Protein: Increased in AD
Activity: Increased in AD
POST-TRANSLATIONAL REGULATION
Shuttling Proteins: GGA (+), RTN (-)
Degradation by lysosome, proteasome: (-)

Figure 1.2 BACE1 is Regulated at Various Stages of Expression. BACE1 transcription is variably regulated by its promoter region which contains binding sites for many transcription factors such as NF-κβ, Sp1, and PPARγ among others. BACE1 transcription can also produce alternative splice forms which lack enzymatic activity or a stabilizing antisense transcript. Translation of BACE1 is highly regulated by numerous mechanisms including its own 5' UTR which is extremely long, highly structured, and contains three upstream open reading frames (red boxes); all of which likely contribute to an inhibitory effect. The BACE1 3' UTR may be variable in length and its role in regulating translation is largely unknown. Several microRNAs can bind to the 3' UTR which results in decreased transcript stability. Under conditions of energy deprivation, increased phosphorylation of translation initiation factor eIF2α leads to enhanced BACE1 translation. BACE1 protein levels may be regulated by altered subcellular localization through interactions with shuttling proteins or through degradation pathways. In AD, BACE1 protein and activity are increased without noticeable changes in mRNA levels, indicating that post-transcriptional regulation is altered in disease. See text for additional details.

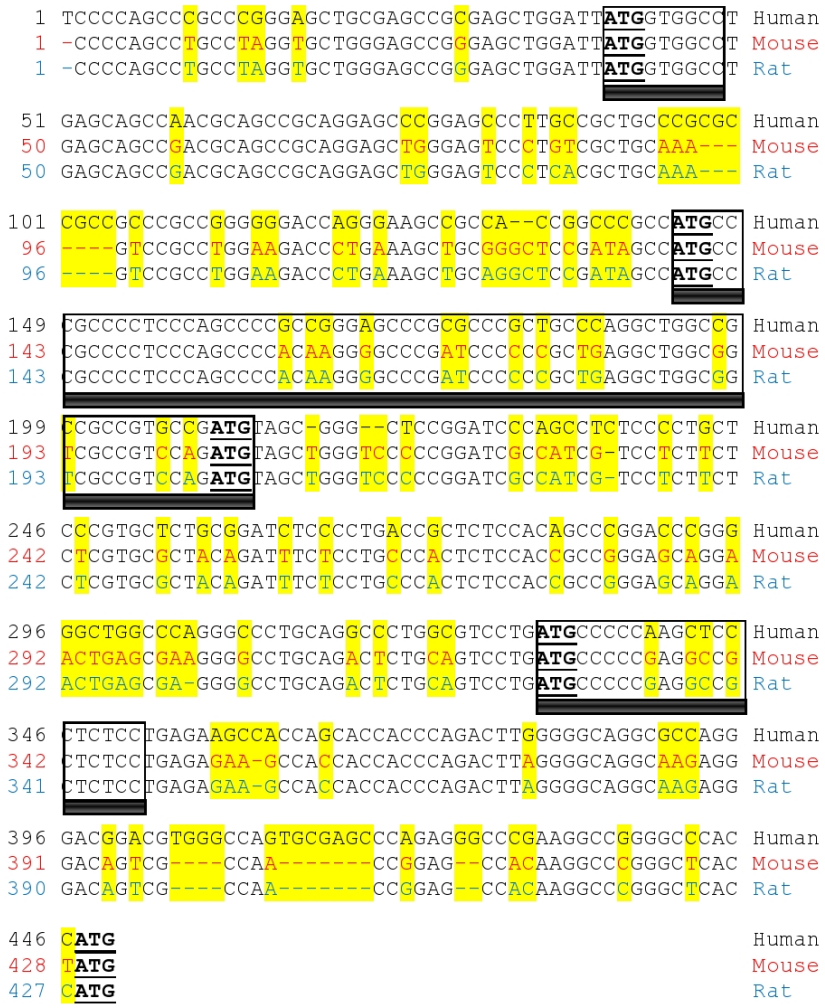

1 TCCCCAGCCCGCCCGGGAGCTGCGAGCCGCGAGCTGGATTATGGTGGCCT Human
1 -CCCCAGCCTGCCTAGGTGCTGGGAGCCGGGAGCTGGATTATGGTGGCCT Mouse
1 -CCCCAGCCTGCCTAGGTGCTGGGAGCCGGGAGCTGGATTATGGTGGCCT Rat
51 GAGCAGCCAACGCAGCCGCAGGAGCCGGGAGCCCTTGCCGCTGCCCGCGC Human
50 GAGCAGCCGACGCAGCCGCAGGAGCTGGGAGTCCCTGTCGCTGCAAA--- Mouse
50 GAGCAGCCGACGCAGCCGCAGGAGCTGGGAGTCCCTCACGCTGCAAA--- Rat
101 CGCCGCCCCGCCGGGGGGACCAGGGAAGCCGCCA--CCGGCCCGCCATGCC Human
96 ----GTCCGCCTGGAAGACCCTGAAAGCTGCGGGCTCCGATAGCCATGCC Mouse
96 ----GTCCGCCTGGAAGACCCTGAAAGCTGCAGGCTCCGATAGCCATGCC Rat
149 CGCCCCTCCCAGCCCCGCCGGGAGCCCGCGCCCGCTGCCCAGGCTGGCCG Human
143 CGCCCCTCCCAGCCCCACAAGGGGCCCGATCCCCCCGCTGAGGCTGGCGG Mouse
143 CGCCCCTCCCAGCCCCACAAGGGGCCCGATCCCCCCGCTGAGGCTGGCGG Rat
199 CCGCCGTGCCGATGTAGC-GGG--CTCCGGATCCCAGCCTCTCCCCTGCT Human
193 TCGCCGTCCAGATGTAGCTGGGTCCCCCGGATCGCCATCG-TCCTCTTCT Mouse
193 TCGCCGTCCAGATGTAGCTGGGTCCCCCGGATCGCCATCG-TCCTCTTCT Rat
246 CCCGTGCTCTGCGGATCTCCCCTGACCGCTCTCCCACAGCCCGGACCCGGG Human
242 CTCGTGCGCTACAGATTTCTCCTGCCCACTCTCCCACCGCCGGGAGCAGGA Mouse
242 CTCGTGCGCTACAGATTTCTCCTGCCCACTCTCCACCGCCGGGAGCAGGA Rat
296 GGCTGGCCCCAGGGCCCTGCAGGCCCTGGCGTCCTGATGCCCCCCAAGCTCC Human
292 ACTGAGCGAAGGGGCCTGCAGACTCTGCAGTCCTGATGCCCCCGAGGCCG Mouse
292 ACTGAGCGA-GGGGCCTGCAGACTCTGCAGTCCTGATGCCCCCGAGGCCG Rat
346 CTCTCCTGAGAAGCCACCAGCACCACCCAGACTTGGGGGCAGGCGCCAGG Human
342 CTCTCCTGAGAGAA-GCCACCACCACCCAGACTTAGGGGCAGGCAAGAGG Mouse
341 CTCTCCTGAGAGAA-GCCACCACCACCCAGACTTAGGGGCAGGCAAGAGG Rat
396 GACGGACGTGGGCCAGTGCGAGCCCAGAGGGCCCGAAGGCCGGGGGCCCAC Human
391 GACAGTCG----CCAA-------CCGGAG--CCACAAGGCCCGGGGCTCAC Mouse
390 GACAGTCG----CCAA-------CCGGAG--CCACAAGGCCCGGGGCTCAC Rat
446 CATG Human
428 TATG Mouse
427 CATG Rat

Figure 1.3 The BACE1 5' UTR is Conserved in Vertebrates. The BACE1 5' UTR in human, mouse, and rat is long (446-nt, 428-nt, and 427-nt, respectively) with a high GC-content (~77%, 68%, and 67%, respectively). The sequence identity between human and mouse or rat is 72.1 and 72.7%, respectively. Sequences highlighted in yellow indicated non-conserved regions between human and mouse/rat. The boxed sequences correspond to the three conserved uORFs, which may or may not be translated in various cell types. Start codons (ATG) are shown underlined in bold. There are four uATGs present; however, the third uATG is immediately followed by a stop codon (TAG). The last ATG indicate the authentic BACE1 start codon.

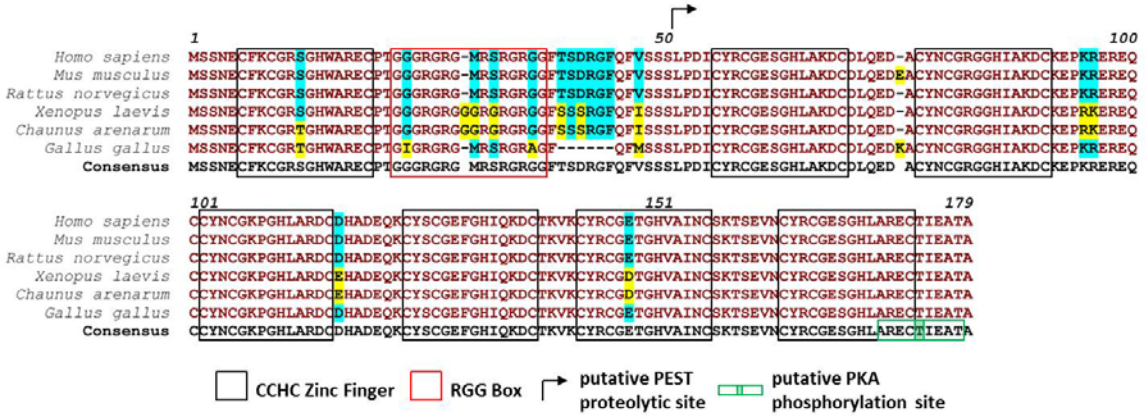


Figure 1.4 CNBP is Strikingly Conserved in Vertebrates. CNBP sequence similarity is >90% conserved in most vertebrates, and human CNBP sequence similarity between mouse and rat is 99% and 100%, respectively. Human and toad (*Chaunus arenarum*) CNBP are 94% conserved. Highlighted sequences indicate non-conserved regions. CNBP contains seven CCHC-type zinc fingers and an RGG region between the first and second zinc fingers; these regions are critical for carrying out CNBP's interactions with ssDNA and RNA molecules. Full-length human CNBP is 177 amino acids in length, but an alternative splice site exists in the RGG region which produces a shorter CNBPβ isoform (170 amino acids) of unknown function. A putative PEST proteolytic cleavage site occurs after serine 50, which produces a truncated dominant negative protein observed in amphibians, but has not been found in human, mouse, or rat. A putative PKA phosphorylation site of unknown function exists at the C-terminus.

CHAPTER TWO: General Materials and Methods

Plasmids and Reagents

The pCMV6-XL5 empty and human CNBP vectors were purchased from Origene Technologies. The HuSH 29-mer short-hairpin vectors (pGFP-V-RS: empty, scrambled, and CNBP [TAGTTTGGTAGAGGTGTTATGTATAATGC]) as well as recombinant full-length human CNBP protein with C-terminus DDK (i.e. Flag) tag were also purchased from Origene. The pCMV6-XL5 human BACE1 construct (Origene) with 5' and 3' UTRs was a kind gift from Dr. Pete Nelson (University of Kentucky; Lexington, KY). The pcDNA6.1-seAP vectors (with human BACE1 5' UTR, 3' UTR, both UTRs, or no UTRs) were kindly provided by Dr. Todd Golde (University of Florida; Gainesville, FL). The *Chaunus arenarum* (toad) wild-type CNBP construct (pGEX-2T GST-$CNBP_{WT}$) and recombinant proteins (GST-$CNBP_{WT}$ and GST-$CNBP_{1\text{-}\Delta RGG}$) were a kind gift from Dr. Nora Calcaterra (National University of Rosario, Argentina). The human pcDNA3.1-hODC1-IRES dual luciferase vector was a kind gift from Dr. Andrew J. Link (Vanderbilt University; Nashville, TN). The rat BACE1 5' UTR and null dual luciferase constructs (designated RP and RPh) were kindly provided by Dr. Vincent Mauro (Scripps University; San Diego, CA).

To make the viral vectors, the CNBP cDNA was cloned into the XhoI / XbaI restriction sites in the pZac2.1 plasmid (CMV promoter driven; U Penn Viral Vector Core). The CNBP pZac2.1 plasmid, Adeno-helper plasmid, and rep / cap (AAV2) plasmids were then co-transfected using the polyethyleneimine (PEI) transfection method into HEK293A cells. Virus was harvested 72 hours later, either by iodixanol gradient or

filter kit, and reconstituted in PBS [232]. Empty vector virus was used as a negative control.

Cell Line Cultures and Transfections

All cell lines were purchased from ATCC. Human embryonic kidney (HEK293T or HEK293A), human SH-SY5Y (neuroblastoma), and mouse N2a (neuroblastoma) cells were cultured in Dulbecco's Modified Eagle Media (DMEM; HyClone). Human H4 (neuroglioma) cells were cultured in Opti-MEM media (Gibco) or DMEM. Media was supplemented with 10% heat-denatured fetal bovine serum (FBS; HyClone) and 1% Penicillin (5,000 Units/ml) / Streptomycin (5,000 µg/ml) (Pen-Strep; Lonza) unless otherwise specified. Cells were grown in a 37°C incubator under humidified conditions with 5% CO_2. Plasmid transfections were performed as described in the text or figure legends with either FuGene 6 liposomal transfection reagent (Roche) or FuGene HD transfection reagent (Promega) at a 3:1 lipid:cDNA ratio. Transfections in SH-SY5Y cells were achieved via electroporation with an Amaxa Nucleofector using Amaxa Cell Line Nucleofector Kit V (Lonza) according to the manufacturer's instructions for SH-SY5Y cells. Pooled stable cells were made by transfecting shRNA vectors (empty, scrambled, or CNBP) into H4 cells and selecting for cells expressing the plasmids with 1µg/mL puromycin (Sigma). Selective pressure was kept on the cells for approximately two weeks before pooling and freezing down plugs of cells. Prior to freezing, analysis of GFP expression by fluorescent microscopy indicated that the majority of the cells remaining expressed the shRNA plasmid for each cell line.

Primary Cell Culture and Viral Transductions

Primary enriched cortical neuronal cultures were prepared from embryonic day 18 Sprague-Dawley rats. Cortical tissue was dissected from the brain, washed three times in Minimum Essential Media (MEM; Invitrogen), and transferred to a new centrifuge tube. After the tissue had settled, the supernatant was removed and the tissue was washed with MEM two more times. Tissue was then dissociated in 0.25% trypsin (Gibco) for 7 min at 37°C followed by repeated pipetting to aid in the process. The cells were then passed through a 0.22-micron nylon mesh (BD Falcon) and pelleted by centrifugation at 272×g for 3 min at 4°C. The supernatant was removed and the cells were resuspended in neurobasal (NB)/MEM (60%/40%) media (1 mL / brain). Cells were plated at the indicated concentrations on PEI-coated 35-mm dishes. After cells were attached (3-4 hours later), cell culture media was replaced with fresh NB/MEM media. Half of the media was exchanged for fresh media every 2-3 days until experiments were started. Experiments began at 6 or 7 days *in vitro* (DIV). Cells were incubated at 37°C and 5% CO_2 throughout. Primary astrocyte cultures were prepared the same as for neurons except after dissociation of cortical tissue with 0.25% trypsin, cells were diluted into 1× MEM (Invitrogen) and transferred to a 75 cm^2 flask. Cells were grown for approximately 2 weeks at 37°C and 5% CO_2 to allow for neurons to die-off and for astrocytes to reach confluency. Afterwards, cells were trypsinized, counted, and grown in 10 cm dishes in MEM until use.

Primary rat cortical neurons were transduced with AAV2 viral vectors according to published methods [233]. Briefly, approximately 1 million neurons were plated onto PEI-coated 35 mm dishes. On DIV 7, virus was diluted into NB/MEM media and applied

directly to each dish in triplicates. The starting virus concentration was estimated to be 5×10^{10} genome copies / mL using QuickTiter AAV Quantitation Kit (Cell Biolabs, Inc.). Since we had observed some toxicity in other cell lines with high CNBP overexpression, we chose to use a low multiplicity of infection (MOI) of 0.1. After 4 days, 0.5 mL fresh NB/MEM media was added to each dish to replenish nutrients. Seven days after transduction, cells were harvested for biochemical analysis.

Protein Extraction, SDS-PAGE, and Western Blotting

Cells were lysed in ice cold radioimmunoprecipitation (RIPA) buffer (50 mM Tris-Base, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100) supplemented with complete protease inhibitor (PIC) cocktail + EDTA (Amresco) followed by brief sonication or freeze-thaw fracturing at -80°C to enhance cell lysis. The lysates were cleared by centrifugation for at least 5 minutes at 20,000×*g* and 4°C to remove insoluble cellular debris. Human samples were homogenized in 1× PBS or 2% SDS (w/v) supplemented with protease inhibitors, unless otherwise indicated. The total amount of protein was determined by bicinchoninic (BCA) assay (Pierce Biotechnology).

SDS-PAGE samples were prepared in Laemmli buffer (2% SDS, 10% glycerol, 62.5 mM Tris-HCl, 0.002% bromophenol blue) with 10% (v/v) β-mercaptoethanol (BME), and heated to 70ºC for 15 minutes prior to loading. Equal protein amounts were loaded per lane on a range of Criterion (Bio-Rad Laboratories) or Nu-PAGE (Invitrogen) precast SDS-PAGE gels in MES, MOPS, or Tris-glycine running buffer and electrically transferred to 0.45 μm nitrocellulose or polyvinylidine fluoride (PVDF) membranes (Bio-Rad Laboratories). For spot blots, protein extracts were diluted in 1× PBS to 20 μg/mL

and 50 µL (1 µg total protein) was loaded into each well of a MINIFOLD I Spot Blot system (Whatman). Proteins were spotted onto a 0.45 µm nitrocellulose membrane via vacuum filtration. Membranes were typically blocked overnight at 4°C with 1% BSA / 2% BlockAce (Serotec) in 1× PBS, pH 7.4 (Fisher Scientific).

Blots were incubated in the indicated primary antibody (usually 1 µg / mL) for 1-2 hours at room temperature (RT), and in secondary antibody (1:5000 – 1:20,000 dilution) for 30 minutes to 1 hour at RT. Primary antibodies were diluted in TBST buffer (1× Tris-Buffered Saline (TBS) + 0.1% Tween-20; Fisher) with 5% non-fat dried milk or 5% bovine serum albumin (BSA; Calbiochem). Horseradish peroxidase (HRP) conjugated secondary antibodies (Pierce Biotechnology) were diluted in TBST + 5% milk. Between antibody applications, blots were washed 3 × 10 minutes in TBST buffer. A final wash in TBS for 5 minutes served to remove the detergent. Membranes were incubated with enhanced chemiluminescent detection substrate (West Dura or West Pico; Pierce Biotechnology) for 5 minutes followed by exposure to autoradiographic film (ISC Bioexpress; Fisher Scientific). Films were developed using a Kodak X-OMAT 2000A processor (Eastman Kodak Company). Densitometric analysis of protein bands was conducted using freely available Scion Image software (http://www.scioncorp.com/).

Primary antibodies used were as follows: BACE1 was detected using mouse monoclonal MAB931 (BACE1 ectodomain, R&D Systems), rabbit monoclonal D10E5 (Cell Signaling), or rabbit monoclonal EPR3956 (Epitomics). BACE2 was detected using rabbit polyclonal Ab5670 (amino acids 441-457; Abcam). CNBP was detected using a rabbit polyclonal antibody raised against the C-terminal 20 amino acids of CNBP, which is conserved in multiple species including human and rodents [234]. This antibody

recognizes one band of approximately 20-24 kDa, depending on the ladder used. β-Actin (AC15, Sigma), β3-Tubulin (Abcam) and GAPDH (Abcam) were detected using common commercial antibodies. Recombinant BACE1 or BACE2 protein (R&D Systems) was sometimes run as a marker for molecular weight comparison.

Reverse Transcription-PCR and Quantitative Real-Time PCR

For RNA isolation from brain, 100 mg of frozen tissue was homogenized in TRIzol reagent (Invitrogen) 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. Total RNA was isolated from cell cultures using AllPrep DNA/RNA/Protein Mini-Prep kits (Qiagen) according to the manufacturer's instructions. The reverse transcriptase (RT) reaction (iScript Select cDNA Synthesis Kit; Bio-Rad Laboratories) used 1 µg of RNA, purified RNase H, MMLV reverse transcriptase, and a mixture of random hexamers and oligo dT primers, according to the manufacturer's instructions. General cycling conditions for quantitative real-time PCR (qPCR; MJ Opticon 4 or MiniOpticon thermal cyclers; Bio-Rad Laboratories) included a 3 minute denaturation step at 95°C followed by 40 cycles of denaturation for 15 seconds at 95°C and annealing/extension for 45 seconds at 60°C. Standardization was performed to the geometric mean of a minimum of two housekeeping genes. qPCR reactions contained ~20 ng of sample cDNA together with PerfeCTA SYBR green SuperMix (Quanta Biosciences) unless otherwise specified. A melting curve was generated following cycling to assess the purity of amplification as well as visual inspection of PCR products on an 8% Tris-Borate EDTA (TBE) polyacrylamide gel (Bio-Rad Laboratories)

followed by SYBR gold staining and fluorescence imaging. All primer sets (Integrated DNA Technologies, Inc.) used in general qPCR experiments can be found in Appendix Two.

Aβ Enzyme Linked Immunosorption Assay (ELISA)

Aβ measurements were carried out using a well-characterized sandwich ELISA procedure, the details of which, including antibodies used, have been published previously [235-237]. Briefly, pellets from the initial sodium acetate (NaOAc) fraction used for β-secretase activity measurements (see Chapter Three) were serially re-extracted by brief sonication in 2% SDS+PIC (w/v) or RIPA buffer + PIC, followed by 70% (v/v) formic acid (FA). Sample extracts were stored frozen at −80°C until time of assay. Formic acid extracted material was initially neutralized by a 1:20 dilution in TP buffer (1 M Tris base, 0.5 M Na_2HPO_4), followed by a further dilution as needed (1:100 to 1:400, final) in AC buffer (0.02 M sodium phosphate buffer, pH 7, 0.4 M NaCl, 2 mM EDTA, 0.4% Block Ace (Serotec), 0.2% BSA, 0.05% CHAPS, and 0.05% NaN_3). RIPA or SDS soluble fractions were diluted (1:5 to 1:100) in AC buffer alone. Immunoassay plates (Immulon 4HBX; Nunc) were coated with 0.5 μg / well of antibody, and blocked with a solution of Synblock (AbD Serotec) per manufacturer's instructions.

Total Aβ, $Aβ_{40}$ and $Aβ_{42}$ measures from human samples were performed using monoclonal antibody Ab9 (against the amino-terminus of Aβ) for capture. Total Aβ was detected with biotinylated 4G8, against $Aβ_{17-24}$ (Covance), $Aβ_{40}$ was detected with biotinylated 13.1.1 (own; QED Bioscience, Inc.), and $Aβ_{42}$ was detected with biotinylated 12F4 (Covance). Oligomeric Aβ was measured using the single-site 4G8/4G8 sandwich

ELISA [235]. Biotinylated antibodies were detected with HRP-conjugated NeutrAvidin (Pierce Biotechnology). A peptide standard curve of recombinant $A\beta_{40}$ or $A\beta_{42}$ (rPeptides) was run on the same plate for comparison, and standards and samples were run at least in duplicate. Plates were developed with TMB reagent (Kirkegaard & Perry Laboratories), stopped with 6% *o*-phosphoric acid, and read at 450 nm using a BioTek multiwell plate reader. Aβ values were determined by interpolation relative to the standard curve.

Rodent Aβ ELISAs were performed essentially as described for the human samples. Briefly, media from primary neuronal cultures were collected and supplemented with 1 mM EDTA, pH 8.0 prior to storage at -80^{o}C until use. Media samples were centrifuged briefly to remove insoluble material and loaded onto 384-well plates neat (100 µL per sample). For rodent Aβ ELISAs, capture was performed using 34.2.1 (against N-terminus; QED Bioscience, Inc.) and detection was performed using biotinylated 4G8. Recombinant rodent $A\beta_{42}$ (rPeptide) was used to generate a standard curve.

RNA Experiments

All RNA experiments were done using RNase-free reagents and equipment including buffers, tips, tubes, gloves, glassware, etc. when possible to minimize any spurious contamination or degradation.

Statistical Analysis

Data were analyzed using SPSS software (SPSS Inc., Chicago, IL). 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 where appropriate. Specific information regarding experimental replicates and statistical methods used are indicated in the figure legends.

CHAPTER THREE: β-Secretase Expression is Increased in Early-Stage AD

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INTRODUCTION

The amyloid cascade hypothesis remains the most definitive theory outlining the etiology of Alzheimer's disease, stating that the abnormal production and deposition of amyloid beta in the brain drives the disease process [4, 5]. Mutations that cause familial AD result in mismetabolism of APP and increased levels of the more fibrillogenic $A\beta_{42}$ species. The more common sporadic AD cases also show increased Aβ production, but the reason why is less clear. Numerous therapeutic strategies have targeted decreasing production and/or enhancing clearance of Aβ from the brain; however, these interventions need to occur very early in the disease process to have the most benefit (reviewed in [239, 240]).

Since its discovery in 1999, BACE1 has been a prime therapeutic target for the prevention of AD (reviewed in [241]). As BACE1 is needed for the initial cleavage event to generate Aβ from APP, blocking this crucial step should theoretically prevent the disease if implemented before a threshold level of Aβ can accumulate. In support of this, a rare mutation in APP (A673T) was recently discovered in an Icelandic population that prevents cleavage by BACE1 and ultimately protects these individuals against developing AD [242]. The increase in Aβ production and deposition in sporadic AD cases is likely due to a combination of factors, but several studies have now shown that BACE1 protein and activity are increased in late-stage AD brain tissue compared with age-matched controls [158-162]. This suggests that increased BACE1 expression may be at least partially responsible for the development of sporadic AD. Because BACE1 is also

increased by various cell stresses that occur in the AD brain, an important question arises: is the BACE1 increase in late-stage AD a cause of the disease or a consequence that helps potentiate it? A more definitive conclusion could be reached by studying so-called “early-stage” AD cases. Considerably less is known about the homologous enzyme BACE2. Even though BACE2 can cleave APP at the β site, it does not appear to play a significant role in Aβ production in AD [55, 60, 108, 110]. Nevertheless, we have previously observed relatively high levels of BACE2 in the brain [162], and the role of BACE2 in disease and its relationship to BACE1 is unresolved.

As a progressive neurodegenerative disease, the pathophysiology of AD likely begins years, if not decades, before the diagnosis of dementia [243]. As such, a clinical continuum of AD has been used to characterize groups of individuals that can be considered to be in the earliest stages of AD. Mild cognitive impairment (MCI) represents an intermediate stage between normal cognitive decline due to healthy aging and the more rapid decline typical of various dementias, including, but not limited to AD [244]. These individuals show small but significant declines on cognition-based questionnaires designed to screen for signs of dementia, such as the Mini-Mental State Exam (MMSE), and have increased risk for developing sporadic AD with approximately 10-15% of MCI patients progressing to AD each year. MCI is the earliest stage of clinical dementia and most patients have significant neuropathological changes associated with AD upon autopsy as well. Even though MCI represents an early stage in the progression of AD, many experts believe that therapeutic intervention at this step may already be too late due to an extensive accumulation of pathology that results in irreversible cognitive decline.

In light of this, researchers have begun to look for an even earlier time point in the disease and have outlined a broad category for a stage termed pre-clinical AD (PCAD) [243]. PCAD encompasses asymptomatic individuals who display the pathophysiological changes associated with AD, but who are indistinguishable from cognitively and clinically normal age-matched individuals. Normally, a diagnosis of PCAD can only be obtained by evaluating the brain pathology upon autopsy, but recent advancements in neuroimaging, cerebrospinal fluid (CSF) analysis, and cellular biomarker discovery have begun to detect these changes *in vivo*. PCAD cases may display slight cognitive deviations from their own baseline measures, but do not yet meet criteria for MCI. Since these two stages represent the earliest events in the progression towards AD, determining what pathological changes occur in the brain during these times could shed light onto the underlying mechanisms of the disease. Finding biomarkers that can accurately predict a future diagnosis of AD could allow for early intervention therapies to prevent or delay the progression towards dementia.

Little information is available on how specific the changes to BACE1 in AD are compared with other neurologic diseases [245]. It is conceivable that BACE1 increases in other neurodegenerative diseases due to cellular stress; however it is unknown if this may play a role in the disease process since Aβ accumulation is not typical of other dementias. Comparing BACE1 expression between various neurodegenerative diseases may provide new information on how BACE1 is regulated and its role in disease processes.

In this chapter, we have used various biochemical assays to analyze a large set of post-mortem brain tissue from control (cognitively normal), PCAD, MCI, and late-stage-AD cases, as well as several cases of frontotemporal dementia (FTD), a

neurodegenerative disease distinct from AD. We used tissue from two brain regions – the superior and middle temporal gyri (SMTG), which is heavily affected in AD; and the cerebellum (CB), which is largely unaffected in AD, to study changes in BACE1 and BACE2 expression and how they relate to each other and to Aβ. We showed that BACE1 is increased in the progression of AD in the SMTG tissue only, which indicates a possible causal role in the development of AD. Furthermore, we described evidence for the support that increased BACE1 expression is not limited to AD, but may occur in other neurodegenerative diseases as well. Interestingly, BACE2 expression mirrored that of BACE1 in all disease states, and BACE1 and BACE2 protein and activity were strikingly correlated, indicating a possible shared mechanism of regulation. Finally, we confirmed what numerous other groups have shown, that increased BACE1 protein levels are not accompanied by a corresponding increase in mRNA levels, emphasizing a change in BACE1post-transcriptional regulation in AD.

METHODS

Subjects

Human tissue samples (**Table 3.1**) were obtained from the Alzheimer's Disease Center tissue repository at the University of Kentucky, Sanders-Brown Center on Aging. Controls (n = 9) were age-matched to disease cases (preclinical AD, or PCAD: n = 10; amnestic mild cognitive impairment, or MCI: n = 7; AD: n = 10). Preclinical AD cases (sometimes also called high pathology controls) were defined as those that met the NIA-Reagan neuropathology criteria for likely AD, but showed no clinical signs of dementia ([246]; NIA, 1997). Amnestic MCI was defined according to the criteria of Petersen *et al.*

[244]. We included six cases of frontotemporal dementia (FTD) as an additional neurodegenerative disease, and as a specificity control [247]. Aβ is not considered to play a significant role in FTD. The details of the recruitment, inclusion criteria, and mental status test battery for our normal control group have been described previously [248]. Human tissue collection and handling followed guidelines issued by the PHS and the University of Kentucky IRB.

Human Tissue Collection and Processing

Details of our collection procedures and consensus diagnosis have been described elsewhere [249]. Briefly, brain weights were determined and a gross neuropathologic evaluation carried out at the time of autopsy. Tissue samples were dissected and immediately frozen or fixed in 4% paraformaldehyde. For histology (**Fig 3.1**), paraffin embedded specimens were cut at 8-μm, stained with hematoxylin-eosin, and the modified Bielschowsky method. Braak staging [250] was performed using Gallyas stained sections from ventromedial temporal lobe structures and Bielschowsky-stained neocortical sections. Neurofibrillary tangles (NFTs), diffuse plaques (DPs), and neuritic plaques (NPs) were counted using Bielschowsky stained sections. Plaques were counted using a 10× objective (field size: 2.35 mm^2) in the 5 most involved fields in each section. The most involved fields were determined by studying the whole section and marking it. Plaques were classified as DPs (plaques without neurites) and NPs (plaques with neurites) in each region. Neurofibrillary tangles were counted with a 20× objective (field size: 0.586 mm^2), also in the 5 most involved fields.

Frozen samples were homogenized using a PowerMax AHS200 in five volumes (w/v) of sodium acetate (NaOAc) buffer (10 mM NaOAc, 1.5 mM NaCl, 0.1% Triton X-

100, 0.32 M sucrose, pH 5.0). The buffer was supplemented with PIC+EDTA and 100 nM pepstatin A to inhibit aspartyl proteases; BACE1 and BACE2 are not inhibited by pepstatin A. Whole tissue homogenate was centrifuged at 20,800×*g* for 30 minutes at 4°C to pellet insoluble material. Pelleted material was sequentially extracted in an equal volume (0.7 mL) of RIPA buffer with PIC+EDTA or 2% SDS with PIC+EDTA followed by 70% formic acid (FA) for the determination of detergent soluble and insoluble Aβ, respectively. In each case the pellet was extracted by brief sonication (10 × 0.5 s microtip pulses at 100 W; Fisher Sonic Dismembrator, Model 500; Fisher Scientific) followed by centrifugation to pellet insoluble material (RIPA fraction: 20,800×*g* for 30 minutes; FA fraction: 20,800×*g* for 1 hr; both spins were performed at 4°C). Protein content was determined by BCA assay relative to bovine serum albumin (BSA) standards prepared in the same buffer.

β-Secretase Activity Assays

We recently described and validated the assays for BACE1 and BACE2 for human tissue in considerable detail, including reagent specificity [162]. This method is similar to that of Fukumoto et al. [142, 158]. Briefly, BACE1 (MAB931, raised against the BACE1 ectodomain; R&D Systems) or BACE2 (Ab1, raised against amino acids 496–511 of BACE2; EMD Biosciences) antibodies were loaded onto a 384-well black opaque plate (Nunc) at 0.5 μg antibody / well (in 50 μL PBS) and incubated overnight at 4ºC. The following day, the plate was blocked (Protein Free; Pierce) for 1 hr at RT. After blocking, the samples extracted in NaOAc buffer (100 μL) were loaded at least in duplicate onto the plate. A second set of samples, loaded in absence of antibody, served as controls for background activity. Further validation experiments were performed on a

subset of samples by using a different antibody, directed against the opposite end of BACE1 (C-terminus, EPR3956; Epitomics) or BACE2 (N-terminus, rabbit polyclonal Ab2; EMD Biosciences). The plate was washed extensively (at least 5 times) with NaOAc buffer, and then loaded with 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, Lousiville, KY; EDANS/DABCYL conjugate, excitation/emission: 350 nm/490 nm). Net fluorescence was measured after 2 hours at 37ºC using a BioTek plate reader.

RESULTS

BACE1 and BACE2 Protein and Activity Increase in Neurodegenerative Disease

In an earlier study, we noted that BACE2 was nearly as abundant in human brain as was BACE1 in a small number of cases [162]. This was also true in this larger series of cases (**Fig 3.2;** compare band intensities to 100 ng recombinant standards for both BACE1 and BACE2). Overall, BACE1 ($F_{4,32} = 2.89$, $p<0.04$) and BACE2 ($F_{4,32} = 2.87$, $p<0.04$) activities were increased in neurodegenerative disease (**Fig 3.3A,B**). The increase occurred in a disease-affected region (the SMTG) but not in a brain region unaffected by disease (the CB). The same increases in the SMTG but not in the CB were seen for BACE1 ($F_{4,32} = 5.87$, $p<0.001$) and BACE2 ($F_{4,32} = 15.03$, $p<0.0001$) proteins (NaOAc fraction) using Western blot analysis (**Fig 3.3C,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 when MCI and PCAD cases were treated as a single, combined group (the amount of pathologic disease in these cases

is nearly identical; see **Table 3.1**). As expected from earlier assay validation studies, BACE1 and BACE2 activities were correlated with their respective protein bands detected by immunoblot analysis (BACE1: R^2 = 0.17, p<0.006; BACE2: R^2 = 0.21, p<0.002). These data indicate that an increase in BACE1 and BACE2 activities and protein levels likely occur at an early disease stage.

We observed a striking correlation between BACE1 and BACE2 in human brain, a phenomenon observed with multiple antibody combinations and assay conditions. Using a standard assay for BACE1 (MAB931) and BACE2 (Ab1) activities, we found strong correlations between the two enzymatic activities in the SMTG (R^2 = 0.9, p<0.0001) and the CB (R^2 = 0.7, p<0.001), indicating that this 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 activities remained highly correlated in the SMTG (R^2 = 0.64, p<0.001) and the CB (R^2 = 0.47, p<0.001) (**Fig 3.3E,F**). We observed similar disease-related increases in BACE1 and BACE2 using this alternate method (**Fig 3.4**). Finally, we examined the BACE1 (using MAB931) and BACE2 (using Ab5670) relationship by immunoblot analysis and detected a similarly strong correlation (R^2 = 0.32, p<0.001). The correlation was significant regardless of whether the data were standardized to β-Actin. Hence, the strong correlations between BACE1 and BACE2 proteins and activities were detected using different methods. Interestingly, we also detected a significant increase in both BACE1 and BACE2 activity and protein in frontotemporal dementia

cases (**Fig 3.3A-D**) compared to controls in the SMTG, but not the CB, indicating that increased β-secretase expression is not specific to AD.

BACE1 and BACE2 mRNA Levels are Unchanged in Disease

Since we observed increases in BACE1 and BACE2 in disease, 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 (**Fig 3.5**). The amounts of BACE1 and BACE2 mRNA correlated with each other in the SMTG ($R^2 = 0.48$, $p<0.001$) and the CB ($R^2 = 0.72$, $p<0.001$), but in neither case did the amount of total mRNA correlate with the amount of BACE1 or BACE2 activity or protein. These data indicate that, in disease, BACE1 and BACE2 protein levels are increased through a change in post-transcriptional regulation.

BACE1 Activity is Related to Soluble but Not Insoluble Aβ in the Brain

We next decided to explore the relationship between BACE1 and Aβ in greater detail by studying human cases in which we had access to detailed neuropathology data. We detected a significant increase in total Aβ only in late-stage AD cases in both the SMTG and CB ($p<0.01$) (**Fig 3.6**). A trend of increased total Aβ in PCAD and MCI cases in the SMTG was observed, but was not statistically significant. There was no accumulation of Aβ in FTD cases, as expected. Neither BACE1 nor BACE2 activity could predict the number of diffuse plaques or neuritic plaques. BACE1 ($R^2 = 0.12$, $p<0.03$) and BACE2 ($R^2 = 0.13$, $p<0.03$) activities were significantly positively related to the amount of Aβ in the NaOAc-soluble fraction isolated from the SMTG in this case

series (**Fig 3.7A,B**). Although these relationships were modest, we found similar results in the CB in these same cases (*data not shown*). The NaOAc fraction is the first in the extraction series, contains the most soluble pool of Aβ peptides, and is also where BACE1 activity is localized [162]. There was no relationship between either BACE1 or BACE2 enzymatic activities and any form of Aβ (total Aβ, $A\beta_{40}$, $A\beta_{42}$, or oligomeric Aβ) extracted in the RIPA, SDS, or FA fractions (**Fig 3.7C,D**; FA fraction shown). Finally, the amount of BACE1 protein showed a slight positive correlation with the amount of SDS-extractable Aβ (R^2 = 0.10, p=0.05). Taken together, these data suggest that BACE1 (or BACE2) activity and protein are not particularly strong predictors of the concentrations of various Aβ forms in the brain.

DISCUSSION

Increased BACE1 expression in late-stage AD has been reported by multiple groups, including our own. However, it has remained unclear whether this increase is a cause or consequence of the disease process. In this chapter, we have shown that BACE1 is increased in early-stage AD cases, compared with healthy age-matched controls, which indicates that it is likely a driving factor in the progression of disease. The increase in BACE1 protein could not be explained by increased mRNA levels, confirming previous studies [57, 159, 168-170], and emphasizes an alteration in post-transcriptional regulation in disease. Similar expression profiles were found for the related enzyme, BACE2. BACE1 and BACE2 protein and enzymatic activities were highly correlated regardless of disease, indicating a possible shared mechanism of regulation. Since BACE2 is not believed to play a major role in Aβ production, further research is needed to determine

the consequences, if any, of increased BACE2 levels in the brain. This will likely require the discovery of novel BACE2 substrates.

Few groups have looked at BACE1 in early-stage AD cases, partly due to a limitation in available samples. Elevated BACE1 activity and total protein levels have been reported in the CSF of two separate cohorts of MCI patients [165, 251] as well as from platelets [252], indicating that BACE1 may be considered an important biomarker for AD [253]. No studies had been reported from PCAD cases or from early-stage AD brain tissue. We did not detect a significant increase in BACE1 activity or protein in MCI cases compared to controls in the SMTG. This may be due to a small sample size of MCI cases (n=7) as we were able to detect significant BACE1 increases in PCAD cases (n=10), which is expected to occur before MCI on the AD clinical spectrum. Furthermore, MCI cases do not always progress to AD, indicating that different pathological processes may be a factor.

We showed that BACE1 protein and activity were increased in the progression of AD in a disease affected region of the brain, the SMTG, but not in the cerebellum, which is largely spared from neuronal loss. Interestingly, we found high levels of BACE1 protein and activity in the cerebellum (compare to SMTG) that were unchanged throughout all disease states. We also found elevated levels of total Aβ in the CB of late-stage AD cases only. Determining why different brain regions show varying susceptibility to neurodegeneration could be critical for understanding the basic mechanisms of AD.

Although we observed overall increases in both BACE1 and Aβ levels in late-stage AD cases, there was not a strong correlation between BACE1 and Aβ in the brain.

BACE1 was weakly correlated with soluble Aβ in the NaOAc fraction, but did not correlate with detergent soluble or formic acid soluble Aβ, which usually correspond to diffuse plaques and neuritic plaques, respectively. Several studies have looked at the relationship between BACE1 and Aβ in the brain with varying results. An association between elevated BACE1 and Aβ deposition in dystrophic neurites has been reported in transgenic mouse models of AD [254]. Likewise, BACE1 enzymatic activity was correlated with Aβ plaques in the temporal cortex of AD brains [161]. Conversely, BACE1 or β-secretase activity has previously been shown not to correlate with Aβ-related pathologic findings in the brain or to be a weak association [142, 158, 255]. Even though it has been reported that BACE1 is elevated in neurons surrounded by amyloid plaques, this may occur only around a subset of plaques in the AD brain [166]. We did note that the amount of CTFβ in the SMTG was related to the amount of Aβ in multiple fractions and was also related to the number of neuritic plaques (*data not shown*; [238]); this stronger relationship is likely due to CTFβ being the immediate precursor to Aβ, whereas BACE1 protein does not often encounter its APP substrate in the cell and thus β-secretase activity is a step further removed [256]. Furthermore, differences in extraction methods and immunoassays factor into these discrepancies as well. We found that a better indicator of insoluble Aβ and plaques in the brain is the activity of Aβ-degrading enzyme neprilysin (NEP) (*data not shown*; [238]) and this corroborates a recent similar finding in frontal cortex [257]. Interestingly, a decrease in NEP activity was observed in AD cases in the disease-affected SMTG, but an increase in NEP activity was found in the cerebellum (*data not shown*; [238]), suggesting a possible reason for differences in brain region disease susceptibility.

We also observed an increase in BACE1 and BACE2 protein and activity in a limited set of FTD cases when compared to controls. FTD is a neurodegenerative disease distinct from AD, in which the accumulation of Aβ does not occur. FTD encompasses a group of related conditions caused by mutations in several genes that cause shrinking of the temporal and frontal lobes of the brain and results in death 7 years after diagnosis on average [258]. The evidence that BACE1 is increased in FTD likely indicates a consequence of the disease. The reason why FTD cases do not accumulate Aβ as in AD is unclear but may be explained by two factors: (1) cellular processes such as Aβ clearance mechanisms may differ between the two diseases (NEP activity was not decreased in FTD cases in our study) and (2) FTD patients generally die on average 20 years younger than AD patients (**see Table 3.1**), indicating that significant Aβ accumulation and deposition may occur had they lived longer. The evidence remains that increased BACE1 (and BACE2) expression is not an AD-specific event and may play a role in other neurodegenerative diseases as well.

While an increase in BACE1 expression early in the disease process points to a causal role, the evidence that levels remain high in late-stage AD cases also suggest that a positive feedback loop in which Aβ deposition drives β-secretase expression may be important, as has been suggested [130, 160, 161, 166, 170]. While this may be the case later in the disease process, our results indicate that the initial rise in BACE1 protein and activity does not depend on elevated Aβ. We showed that increased BACE1 in early AD cases occurred in the absence of a significant rise in total Aβ, and that FTD cases, which do not accumulate Aβ, have significantly elevated levels of BACE1 as well. These results fit in well with the current amyloid cascade hypothesis and suggest that an initial trigger,

possibly relating to accumulated cellular stress, increases BACE1 expression which in turn leads to an increase in Aβ production and deposition. The increase in Aβ production may cause further stress to the cell, keeping BACE1 levels chronically elevated into late-stage AD. Once this cascade of events reaches a certain threshold of neuropathology to drive advancement of disease, therapeutic interventions may have little impact.

In summary, the data presented in this chapter indicate that sustained elevation of BACE1 may play a causal role in the development and progression of AD and remains a prime therapeutic target for the prevention of AD. In addition, the increase in BACE1 occurs through a post-transcriptional mechanism not specific to AD. How this regulation occurs and what factors may be involved is the primary focus of subsequent chapters.

Table 3.1 Case Details

	Ref #	Age (y)	ApoE	Sex	Brain Weight (g)	PMI (h)	Braak	MMSE	Last MMSE (y)	C.O.D.	Duration (y)
Control	1066	81	3/4	Male	1410	2.00	2	30	0.46	Pulmonary Embolism	N/A
	1069	87	3/3	Male	1230	2.42	2	29	0.52	Prostate Cancer	N/A
	1070	82	3/4	Male	1290	2.10	1	29	1.38	Pneumonia	N/A
	1089	74	3/3	Male	1440	4.00	1	26	0.36	Congestive Heart Failure	N/A
	1127	91	3/4	Female	1230	1.75	2	29	0.76	Myocardial Infarct	N/A
	1134	90	2/3	Male	1310	3.50	0	30	0.18	N/A	N/A
	1159	86	3/3	Female	1200	3.50	0	28	1.03	N/A	N/A
	1163	84	3/3	Female	900	3.00	1	26	0.29	Cardiac Arrest	N/A
	1170	84	3/3	Female	1100	2.50	1	29	1.04	Breast Cancer	N/A
mean		**84.3**			**1234.4**	**2.8**	**1.1**	**28.4**	**0.67**		
s.d.		**5.1**			**163.2**	**0.8**	**0.8**	**1.5**	**0.41**		
s.e.m.		**1.7**			**54.4**	**0.3**	**0.3**	**0.5**	**0.1**		
FTD	1009	68	N/A	Male	900	4.00	0	14	6.34	N/A	N/A
	1078	53	N/A	Female	940	4.75	2	N/A	N/A	Pneumonia	N/A
	1124	48	3/3	Male	900	3.75	0	0	0.34	N/A	6
	1166	59	N/A	Male	1230	6.00	0	17	4.28	N/A	2
	1183	87	N/A	Female	970	4.50	0	N/A	N/A	N/A	6
	5047	51	3/4	Female	705	6.00	0	0	7.28	N/A	9
mean		**61.0**			**940.8**	**4.8**	**0.3**	**7.8**	**4.56**		**5.8**
s.d.		**14.6**			**169.4**	**1.0**	**0.8**	**9.0**	**3.08**		**2.9**
s.e.m.		**5.9**			**69.1**	**0.4**	**0.3**	**4.5**	**1.54**		**1.4**
MCI	1065	87	3/4	Male	1200	3.50	4	24	0.99	Cardiac Arrest	N/A
	1077	87	3/3	Male	1170	2.25	3	21	0.41	Congestive Heart Failure	N/A
	1087	82	3/3	Female	1075	2.90	3	29	0.83	Pulmonary Embolism	N/A
	1130	99	2/3	Female	930	2.00	5	21	0.53	Congestive Heart Failure	N/A
	1152	84	3/4	Male	1350	3.50	4	24	0.97	Congestive Heart Failure	N/A
	1164	88	3/3	Female	1130	3.00	3	28	0.07	N/A	N/A
	1178	96	3/3	Female	970	2.50	4	27	0.59	Colon Cancer	N/A
mean		**89.0**			**1117.9**	**2.8**	**3.7**	**24.8**	**0.63**		
s.d.		**5.8**			**132.3**	**0.5**	**0.7**	**3.1**	**0.31**		
s.e.m.		**2.2**			**50.0**	**0.2**	**0.3**	**1.2**	**0.12**		
PCAD	1082	84	3/3	Female	1020	2.75	4	29	0.60	N/A	N/A
	1097	89	3/3	Female	1210	1.75	4	30	1.76	Stroke	N/A
	1105	84	3/3	Female	1035	2.50	4	30	0.51	Congestive Heart Failure	N/A
	1112	86	2/3	Female	1240	2.41	4	28	0.37	N/A	N/A
	1119	87	3/3	Female	1180	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
	1151	88	2/3	Female	1070	2.25	3	29	0.85	N/A	N/A
	1167	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
mean		**85.6**			**1172.5**	**2.6**	**3.9**	**29.4**	**0.84**		
s.d.		**3.7**			**110.1**	**0.5**	**0.6**	**0.7**	**0.46**		

Table 3.1 continued

s.e.m.		**1.2**			**34.8**	**0.2**	**0.2**	**0.2**	**0.14**		
AD	1084	86	N/A	Female	870	3.25	6	N/A	N/A	Respiratory Infection	N/A
	1086	90	3/3	Female	1090	2.75	6	11	1.91	N/A	8
	1116	84	3/4	Male	1090	2.75	6	18	2.55	Aspiration	8
	1118	83	3/3	Female	1110	3.50	6	5	0.45	N/A	9
	1125	74	4/4	Male	1190	3.00	6	N/A	N/A	Pneumonia	N/A
	1139	86	3/3	Female	1010	2.00	6	9	7.32	Acute Respiratory Fail	15
	1144	85	4/4	Male	1020	2.75	6	15	1.48	Pneumonia	10
	1168	80	3/3	Female	1130	4.00	6	16	2.02	N/A	7
	1173	75	4/4	Male	1120	3.75	6	5	1.32	N/A	8
	1180	91	N/A	Female	1110	3.00	6	1	2.40	N/A	8
mean		**83.4**			**1074.0**	**3.1**	**6.0**	**9.9**	**2.43**		**9.1**
s.d.		**5.7**			**88.5**	**0.6**	**0.0**	**6.0**	**2.08**		**2.5**
s.e.m.		**1.8**			**28.0**	**0.2**	**0.0**	**2.1**	**0.7**		**0.9**

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.

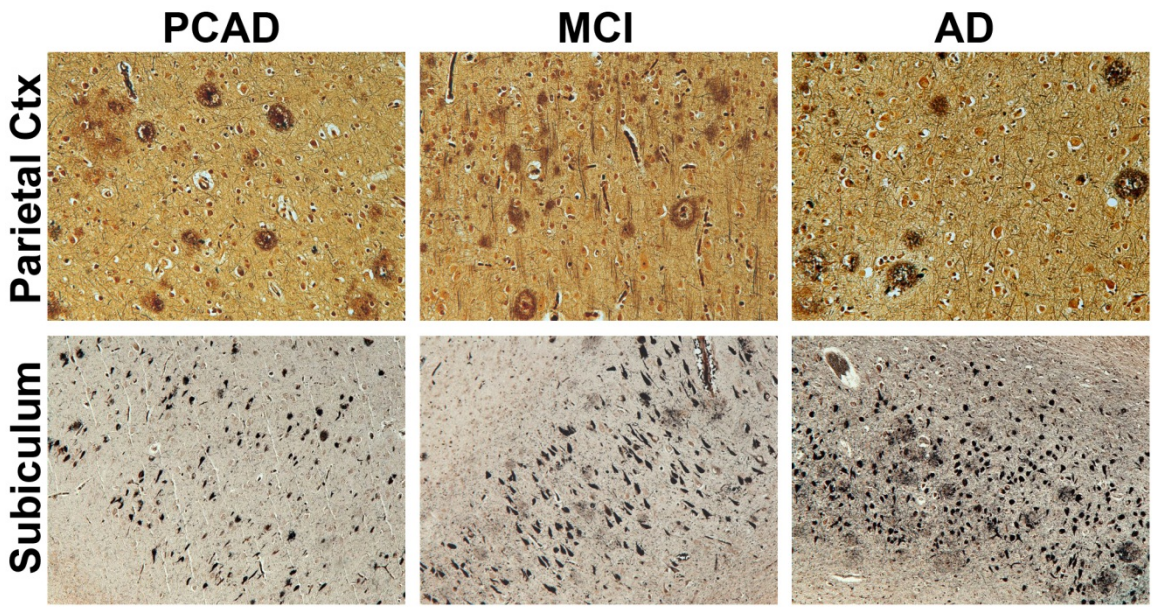


Figure 3.1 Histological Representations of Brain Pathologies. Examples of neuropathology (20× objective) in parietal cortex (Bielschowsky) and subiculum (Gallyas) from representative cases of amnestic mild cognitive impairment (MCI), pre-clinical Alzheimer's Disease (PCAD), and AD. Although the level of cognitive impairment varies from minimal to demented, all three disease states display the same basic elements of neuropathology (plaques and tangles), frequently at similar levels.

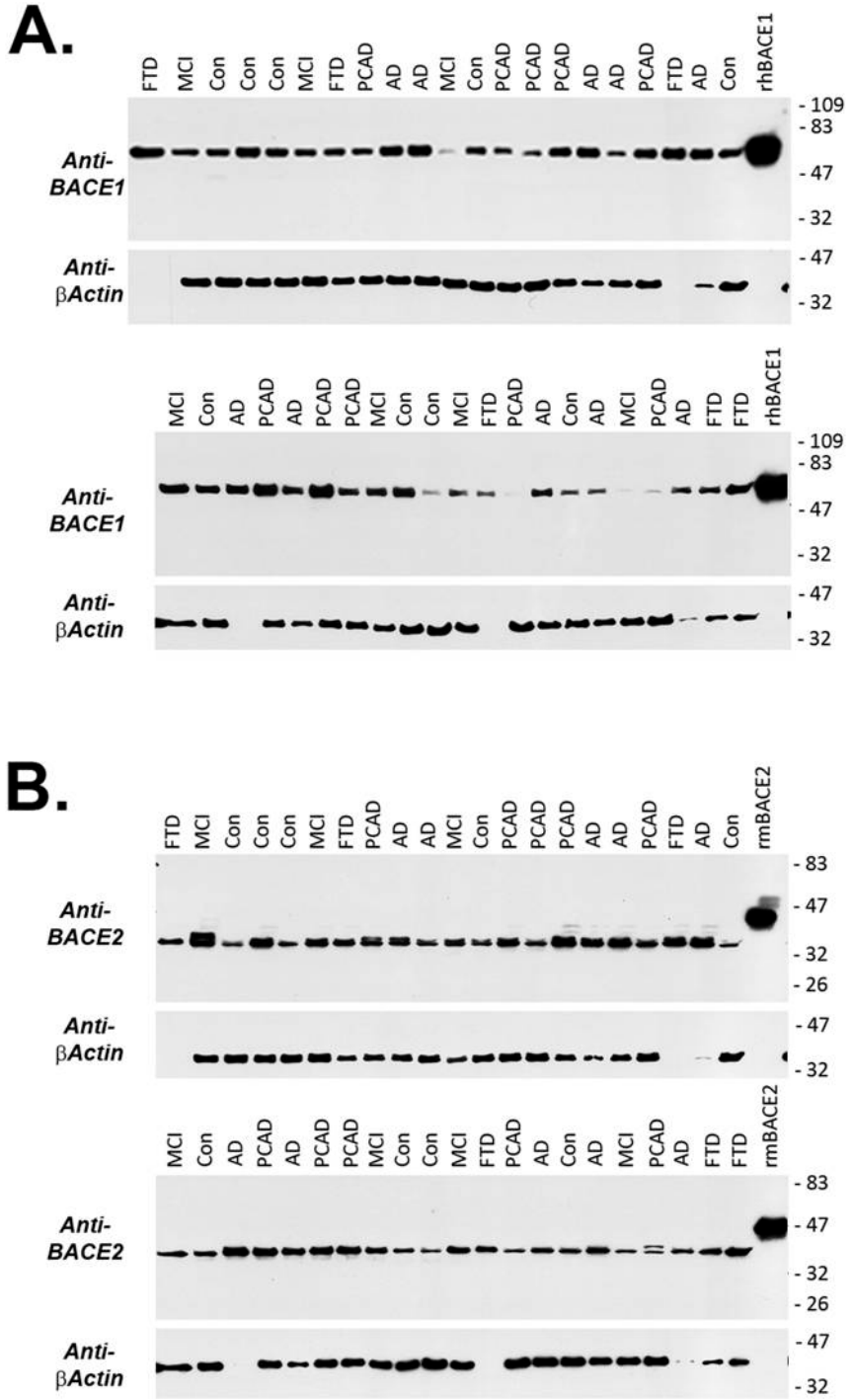

A.
FTD MCI Con Con Con MCI FTD PCAD AD AD MCI Con PCAD PCAD PCAD AD AD PCAD FTD AD Con rhBACE1
Anti-BACE1
Anti-βActin
- 109
- 83
- 47
- 32
MCI Con AD PCAD AD PCAD PCAD MCI Con Con MCI FTD PCAD AD Con AD MCI PCAD AD FTD FTD rhBACE1
B.
FTD MCI Con Con Con MCI FTD PCAD AD AD MCI Con PCAD PCAD PCAD AD AD PCAD FTD AD Con rmBACE2
Anti-BACE2
Anti-βActin
- 83
- 47
- 32
- 26
MCI Con AD PCAD AD PCAD PCAD MCI Con Con MCI FTD PCAD AD Con AD MCI PCAD AD FTD FTD rmBACE2

Figure 3.2 BACE1 and BACE2 Proteins are Abundant in Human Brain. Equal amounts of protein from the SMTG (25 µg) were separated by SDS-PAGE. Human recombinant BACE1 (100 ng; R&D Systems) or mouse recombinant BACE2 (100 ng; R&D Systems) were included as molecular weight comparisons. After probing for (A) BACE1 (MAB931) or (B) BACE2 (Ab5670), blots were stripped and reprobed for β-Actin. Although β-Actin was broadly similar, in some cases the levels were very low. Comparison with recombinant protein standards indicated similar amounts of BACE1 and BACE2 protein in human brain. Although the BACE1 band migrated close to the recombinant protein standard, BACE2 ran at a slightly lower apparent molecular weight. The lower than expected molecular weight of this band and the observed pattern of antibody immunoreactivity together indicate that the active form of BACE2 in the brain is likely BACE2Δ7 (splice form C) as discussed in Holler *et al.* [238]. CON, control.

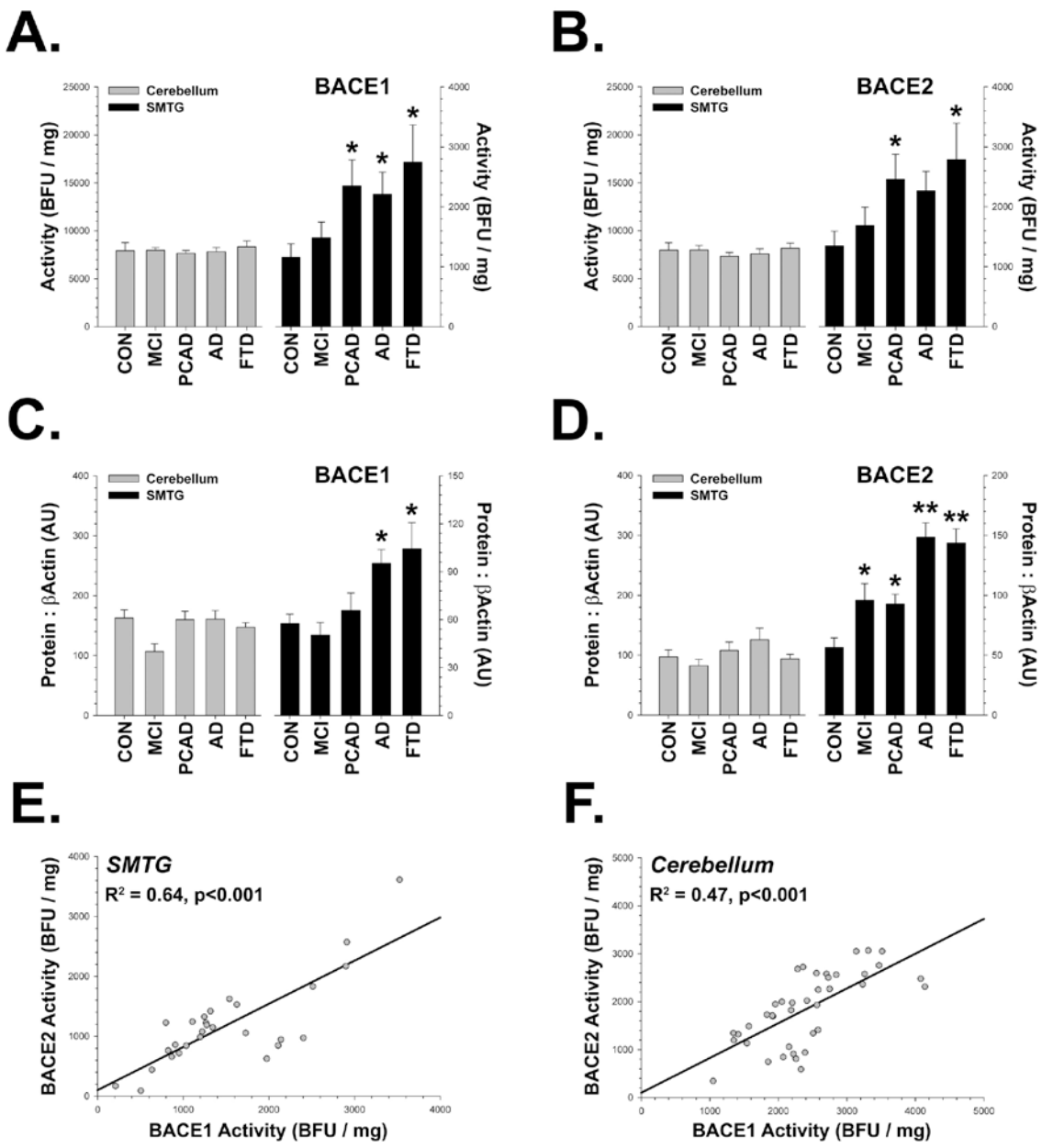

A.
BACE1
Cerebellum
SMTG
Activity (BFU / mg)
Activity (BFU / mg)
CON
MCI
PCAD
AD
FTD
B.
BACE2
C.
BACE1
Protein : βActin (AU)
D.
BACE2
E.
SMTG
R² = 0.64, p<0.001
BACE2 Activity (BFU / mg)
BACE1 Activity (BFU / mg)
F.
Cerebellum
R² = 0.47, p<0.001

Figure 3.3 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 compared to controls. (B) BACE2 activity (as determined by the Ab1 capture assay) is higher in FTD and PCAD and strongly trends toward an increase in AD ($p<0.07$) compared to controls. (C) BACE1 protein is higher using Western blot analysis in FTD and AD (see Fig. 3.2) compared to controls. (D) BACE2 protein is higher using Western blot analysis in MCI, PCAD, AD, and FTD (see Fig. 3.2) compared to controls. Western blot analysis results were essentially unchanged when not standardized to β-Actin, indicating that outlier cases with very low β-Actin levels had minimal effect on the analysis. BACE1 and BACE2 activities are highly correlated in the (E) SMTG and (F) CB, a phenomenon observed with multiple antibodies and assay conditions (shown: BACE1, EPR3956 capture assay; BACE2, Ab2 capture assay). Dunnett's test, * = $p<0.05$, ** = $p<0.01$. Scale: 1000 base fluorescence units (BFU)/mg = 2.3 nmol · min^{-1} · mg^{-1} total protein. Error bars represent standard error of the mean (SEM).

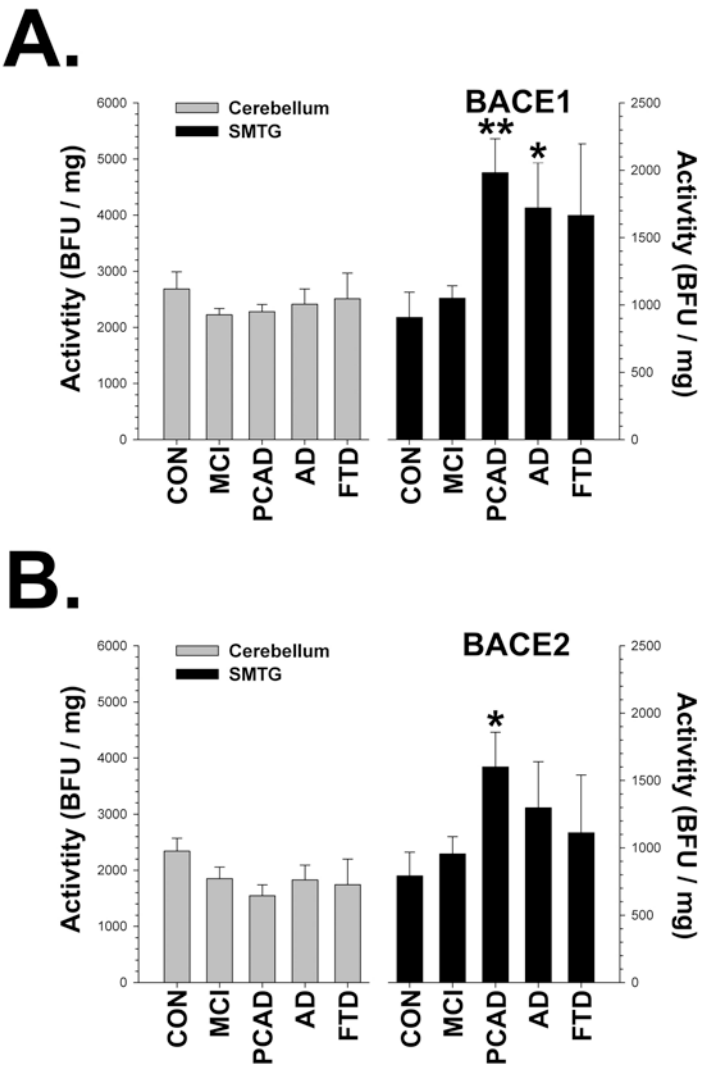

A.
BACE1
Cerebellum
SMTG
Activtity (BFU / mg)
Activtity (BFU / mg)
**
*
CON
MCI
PCAD
AD
FTD
B.
BACE2
Cerebellum
SMTG
Activtity (BFU / mg)
Activtity (BFU / mg)
*
CON
MCI
PCAD
AD
FTD

Figure 3.4 BACE1 and BACE2 Activities are Replicated With Alternative Capture Antibodies. BACE1 (A) and BACE2 (B) enzymatic activity assays were repeated using a different combination of antibodies raised against opposite ends of the proteins. Overall, BACE1 ($p<0.01$) and BACE2 ($p<0.05$) were still higher in the SMTG from disease-affected cases compared with controls. Using this method, the group differences by disease state were essentially unchanged from those of the standard MAB931/Ab1 method (*cf.* Fig. 3.3). The overall significance in this second run was reduced due to slightly lower power because this was a subset of cases. PCAD (BACE1, $t_9 = 3.24$, $p<0.01$; BACE2, $t_9 = 2.40$, $p<0.03$) and AD (BACE1, $t_{11} = 1.86$, $p<0.05$) were the only groups that showed differences versus the control group. We observed marginally lower BACE2 activity ($p<0.05$) in the CB in disease-affected cases with this combination of antibodies; this finding was inconsistent with the first data set and was possibly spurious. * = $p<0.05$, ** = $p<0.01$, Dunnett's test. Error bars represent SEM. Scale is same as in Fig. 3.3.

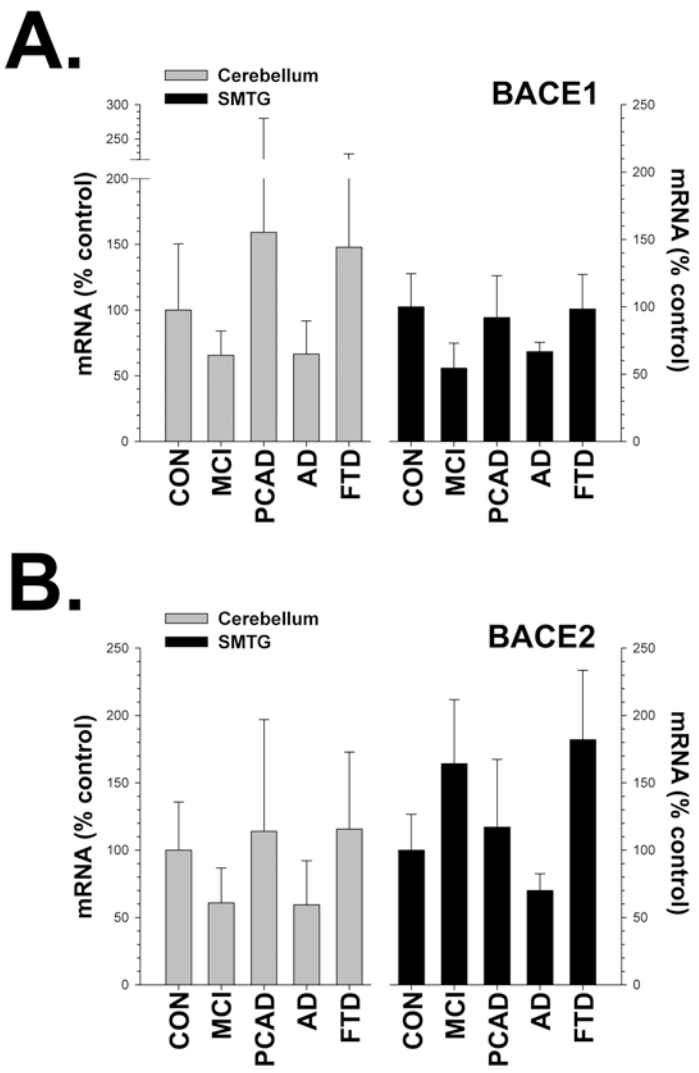


Figure 3.5 BACE1 and BACE2 mRNA Levels Are Unchanged in the Progression of AD. The mean total mRNA levels (*ie.*, all splice forms) for BACE1 (A) and BACE2 (B) did not show consistent alterations across multiple disease states in either the SMTG or CB (all comparisons, $p>0.2$). Values were standardized to the geometric mean of GAPDH and TPT1. Error bars represent SEM.

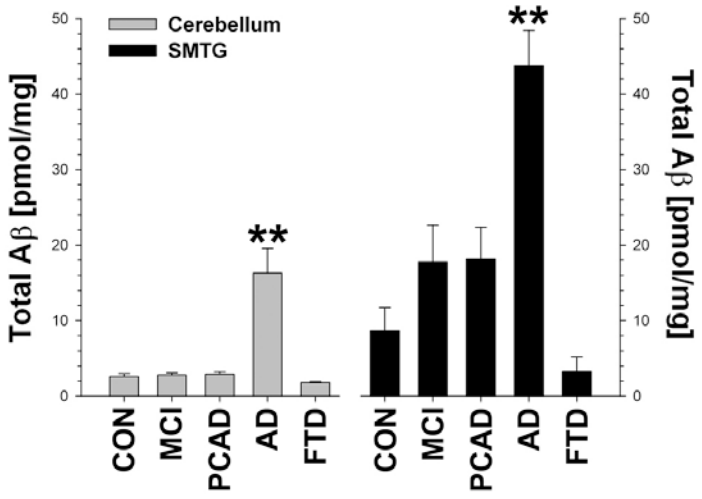


Figure 3.6 Total Aβ Levels are Increased in AD, Similar in MCI and PCAD Cases, and Minimal in FTD Cases. Total Aβ showed a disease-related increase in the SMTG ($F_{4,32} = 16.38$, $p<0.001$) and the CB ($F_{4,29} = 30.14$, $p<0.001$), although the overall amount of Aβ was far lower in the CB (however, two data points in the AD group that were ~20-fold higher than the group mean were dropped from the CB analysis; this did not affect overall significance). Total Aβ in MCI and PCAD cases in the SMTG were similar and trended towards being increased compared to controls. ** = $p<0.01$, Dunnett's test. Error bars represent SEM.

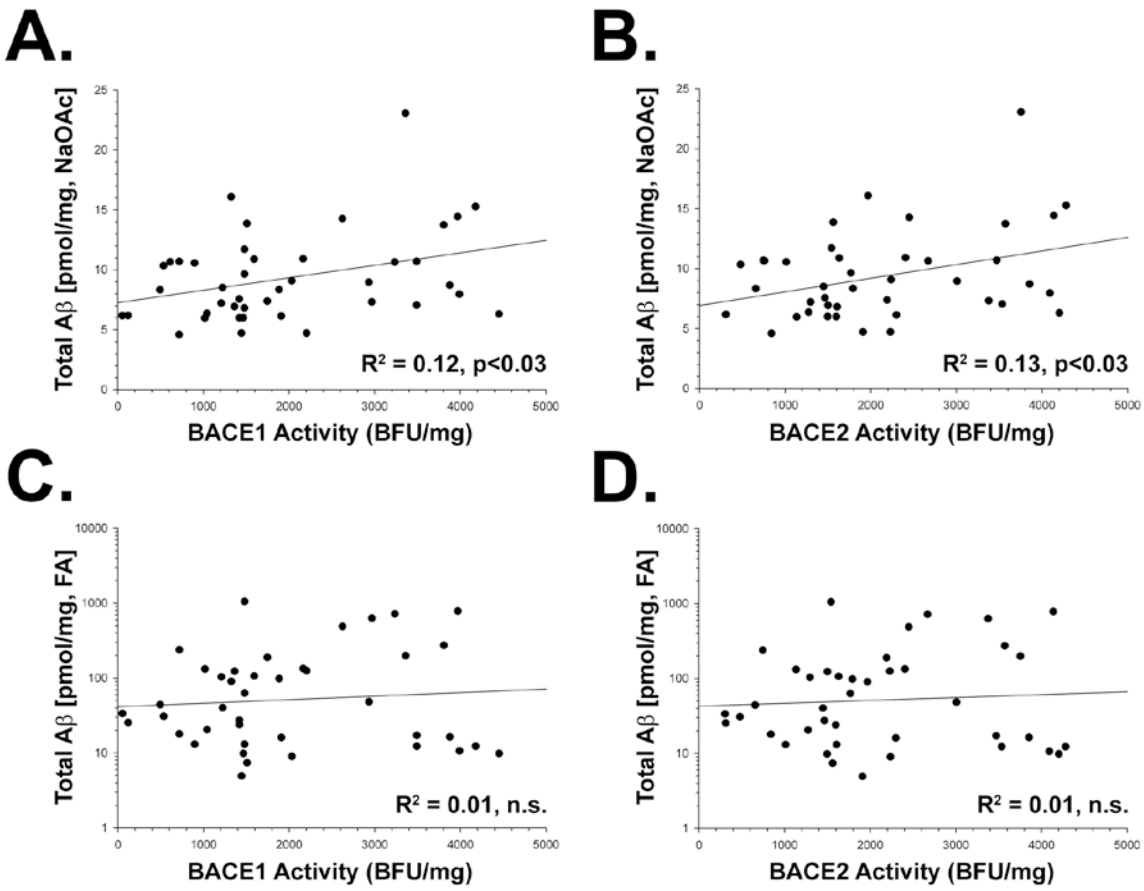


Figure 3.7 BACE1 and BACE2 Enzymatic Activities are Related to Soluble, but Not Insoluble, Aβ in the Brain. BACE1 (A) and BACE2 (B) enzymatic activities were significantly correlated with the amount of Aβ solubilized in the NaOAc fraction, which contains the most soluble pool of peptide. Neither BACE1 (C) or BACE2 (D) enzymatic activities were correlated with less-soluble forms of Aβ, such as the pool extracted with 70% formic acid, FA. Scale: 1000 base fluorescence units (BFU)/mg = 2.3 nmol · min^{-1} · mg^{-1} total protein.

CHAPTER FOUR: CNBP is a Positive Regulator of BACE1 Translation

INTRODUCTION

BACE1 is highly regulated at the level of transcription and translation. However, in the progression of AD, BACE1 protein levels are increased with no concomitant increase in mRNA, indicating that a post-transcriptional mechanism is responsible for this rise. The BACE1 5' UTR appears to be the main *cis*-element responsible for BACE1 translational regulation and has all the characteristics of an mRNA under tight translational control: it is extremely long (~446 nt), highly structured (77% GC content), and contains three uORFs (four uAUGs). Each of these features likely contributes in some way to the repressive nature of the BACE1 5' UTR, although exactly how has been debatable. For example, Lammich *et al.*, concluded that the high GC content and secondary structure of the BACE1 5' UTR was the main factor in repressing BACE1 translation [146]. Other groups have done extensive mutagenesis studies on the 5' UTR and have suggested that the various uAUGs have differing effects on translation through ribosome shunting [144] or leaky scanning and reinitiation events [143, 145]. These conflicting results illustrate that we still do not fully understand how BACE1 is translated under normal conditions. The questions of how and why BACE1 translation is enhanced in the progression of AD remain unanswered, but may hold the key to promising new therapeutic strategies.

BACE1 protein expression is maintained at relatively low levels under physiologically normal conditions and this is reflective of its role in the brain. The list of BACE1 substrates is growing and suggests BACE1 may carry out protective functions (reviewed in [241]). This is evidenced by its role in cleaving members of the neuregulin

family, a process needed for re-myelination after nerve injury (reviewed in [259]). Indeed, increased BACE1 expression has been observed after traumatic brain injury (TBI) [137]. In addition, physiological cleavage of APP (reviewed in [260]) and the related APLP1 and APLP2 proteins [94, 95] appears to be important for cell-cell signaling, synaptic function, and memory. Furthermore, BACE1 is increased in response to various cell stresses such as glucose deprivation [170] and oxidative stress [171]. While brief, transient increases in BACE1 may be neuroprotective, it is the sustained elevation seen in the progression of sporadic AD that underlies its pathogenic role. Determining what factors contribute to sustained BACE1 elevation is an important question in the AD field that has yet to be elucidated.

A number of RNA binding proteins have been implicated in the control of complex brain functions and have been linked to several neurodegenerative diseases (reviewed in [261]). These RNA binding proteins usually have broad cellular functions including RNA processing, transport, stability, and translational regulation. For example, APP translation was shown to be modulated by the competing interactions of the RNA binding proteins FMRP and heterogeneous nuclear ribonucleoprotein C (hnRNP C) [262]. These proteins exhibited negative or positive regulation, respectively, and affected the subcellular localization of APP. Additionally, mutations in RNA binding proteins such as FMRP, FUS (fused in sarcoma), and TDP-43 (TAR DNA-binding protein 43) lead to neurodegenerative diseases, highlighting their importance in cellular functions (reviewed in [263]). To date, no *trans*-acting RNA binding proteins have been implemented in BACE1 translational regulation.

Previously, members of our lab conducted a targeted gene knockout study in Chinese hamster ovary (CHO) cells stably overexpressing human APP to look for disrupted genes that caused alterations in Aβ metabolism (MPM, unpublished data). One cell line displayed a down-regulation of BACE1 expression and a decrease in Aβ production that could not be attributed to changes in APP or the γ-secretase complex. The knocked-out gene linked to the down-regulation of BACE1 was identified as *ZNF9*, which encodes the cellular nucleic acid binding protein, or CNBP. Initial experiments identified CNBP as a positive regulator of BACE1 expression, but this had not been confirmed. Nor was it known whether CNBP affected BACE1 expression at the level of transcription or translation. Although not much is known about CNBP's cellular function, it has been implicated as an RNA binding protein and translational regulator of several seemingly unrelated mRNAs [184, 202, 204].

In this chapter, we have used both cell culture and cell-free systems to show that CNBP acts as a positive regulator of BACE1 expression and this occurs at the level of translation. Furthermore, we show that CNBP interacts with BACE1 mRNA in cell lysates and likely regulates BACE1 translation through direct interaction with its 5' UTR. Finally, we present evidence that CNBP is increased in AD-affected brain tissue, as well as early-stage AD cases and that CNBP expression is highly correlated with BACE1 expression in the brain. Taken together, these results implicate CNBP as an important *trans*-acting factor in the regulation of BACE1 translation.

METHODS

Human Tissue

Human autopsy samples were obtained from the University of Kentucky Alzheimer's Disease Research Center. Frozen tissue from a disease affected (SMTG) or unaffected (CB) region was homogenized in 2% SDS or PBS (both with PIC+EDTA), as described in Chapter Two. Controls (n = 19, 6M/13F; 86.0 ± 1.4 years) were age-matched to AD cases (n = 22, 6M/16F; 83.0 ± 1.3 years). A second set of controls (n = 19, 6M/13F; 85.0 ± 4.3 years, MMSE score = 28.9 ± 1.2) included 10 cases that met neuropathologic criteria of likely AD [246] but showed no clinical signs of dementia (MMSE score = 29.4 ± 0.7); this group was compared to 7 cases of amnestic mild-cognitive impairment (MCI; 89.0 ± 6.2 years; MMSE score = 24.8 ± 3.1), the earliest recognized clinical precursor of AD [244]. Post-mortem intervals (PMI) were short for both set one (2.8 ± 0.2 hours) and two (2.7 ± 0.6 hours). Controls had no history of dementia. Obtaining of informed consent, human tissue collection and handling followed guidelines issued by the PHS and was approved by the University of Kentucky Institutional Review Board (IRB).

Subcellular Fractionation

Primary rat cells (neurons, DIV=7 and astrocytes, DIV=28) were lysed and separated into nuclear and non-nuclear (referred to as cytoplasmic) fractions using the NE-PER kit (Pierce) according to the manufacturer's instructions. Tissue from aged rat brain (4 animals; 20 months old) was harvested and separated into cytoplasmic and nuclear fractions as described previously [264]. Total protein was measured by BCA assay.

Recombinant CNBP Purification

Recombinant CNBP was purified from *E. coli* in order to generate enough protein to use in these studies. First, the pGEX-2T plasmid vector containing wild-type *Chaunus arenarum* CNBP with an N-terminal GST fusion tag (GST-$CNBP_{WT}$) was transformed into One Shot Max Efficiency DH5α-T1R (Invitrogen) chemically competent cells according to the manufacturer's instructions and plated on LB agar supplemented with 50 µg/L carbenicillin (Sigma). Transformed colonies were selected and plasmid was purified using Qiagen mini-prep kit. The purified plasmids were digested with BamH1 and EcoR1 (New England Biolabs) restriction enzymes to verify the presence of the 541-nt CNBP insert. Fresh cultures (100 mL) were induced with 0.5 mM IPTG (Fisher) overnight. Bacterial cells were harvested by centrifugation for 10 min at 2150×g and 4ºC in 50 mL tubes (BD Falcon). The pellet was washed with 50 mL of 25 mM Tris-HCl, pH 8.0, centrifuged to remove wash buffer, and stored at -20ºC until use.

The GST-$CNBP_{WT}$ batch purification protocol was provided by Dr. Nora Calcaterra (National University of Rosario, Argentina) and is based on previous published reports for purification of GST-fusion proteins [265]. Briefly, GST-agarose (Genscript) was equilibrated in Buffer TEN (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 200 mM NaCl). Bacterial pellets in 50 mL conical tubes were resuspended in 3 mL Lysis Buffer (Buffer TEN + 5 mM DTT, 0.5% Triton X-100, PIC+EDTA). Cell lysis was enhanced by brief sonication (10 pulses of 0.5 sec each at 100 W). 1 mL aliquots of bacterial lysates were transferred to 1.5 mL eppendorf microfuge tubes and centrifuged for 30 min at 4^{o}C and 20,800×g and the supernatants were transferred to new tubes. Next, 175 µL of pre-equilibrated GST-agarose slurry (about 100 µL beads) was added to each 1 mL of cleared lysate and the tubes were incubated with end-over-end rotation for 1 hr at

4°C to allow GST-CNBP$_{WT}$ to bind to the resin. After incubation, the beads were pelleted by brief centrifugation and washed several times with 1 mL Wash Buffer (Buffer TEN, 5 mM ATP). Proteins were eluted from the beads in 100 µL Elution Buffer (Buffer TEN, 15mM glutathione, pH 7.4) by incubation at 4ºC for 1 hr with gentle agitation. After elution, the beads were pelleted by centrifugation at 100×g at 4ºC for 10 min. The supernatants were collected and pooled to get a final volume of about 3.5 mL which was then dialyzed into CNBP EMSA binding buffer (final concentration of 50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 50 mM KCl, 0.5 mM DTT, 5% glycerol). This fraction was then concentrated using centrifugal concentrators (Pierce; MWCO = 20 kDa) and total concentrated protein was measured by BCA assay. Aliquots of protein were run on SDS-PAGE and purity was judged to be greater than 90% by Coomassie stain while Western blot confirmed the presence of mostly GST-CNBP$_{WT}$ monomers (~45.7 kDa) and some SDS-stable multimers (~100, 150, 200 kDa) of the expected size. Aliquots of the recombinant GST-CNBP$_{WT}$ protein were stored at -80°C until use.

RNA Immunoprecipitation (RIP)

RIP experiments were based on a previously published protocol [266] and Millipore's MagnaRIP kit instructions. Approximately 18×10^6 H4 cells or 100 mg mouse brain (from postnatal day 2-4 pups) tissue was lysed in polysome lysis buffer (PLB; 10 mM HEPES, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 0.5% NP40) supplemented with PIC (without EDTA; Calbiochem) and 200 U/mL RNase inhibitor (RNasin Plus RNase Inhibitor, Promega). Cells were pipetted repeatedly and mouse brain tissue was homogenized with a Dounce homogenizer to aid extraction. Raw extracts were frozen at -80ºC until use to complete the lysis process. Thawed extracts were centrifuged at

20,800×*g* and 4ºC for 15 minutes to pellet insoluble material. Supernatants were saved for RIP experiments.

Equal amounts of Protein A and Protein G agarose beads (Pierce) were equilibrated in NT2 buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 0.05% NP40). Approximately 20-50 µL of beads resuspended in NT2 buffer were used for each RIP sample. To the beads, antibody was added either against a specific protein of interest or as an isotype specific control. Antibodies used were rabbit polyclonal antisera (CNBP; C-terminus last 20 aa; own), pre-CNBP rabbit serum, rabbit polyclonal anti-PABP (Abcam), mouse monoclonal anti-PABP 10E10 (Abcam), rabbit IgG (Millipore), and mouse IgG (Millipore). Equal amounts of antibody were added to sample and control tubes (10 µg for CNBP and pre-immune sera, 5 µg for anti-PABP and IgG) and the antibody-bead mixture was incubated overnight (~16 hours) at 4ºC with end-over-end rotation. After incubation with antibody, the samples were centrifuged at 2,000×*g* to pellet the beads and the supernatant removed. The beads were then washed 4× with ice-cold NT2 buffer and resuspended in NT2 buffer with 200 U/mL RNase inhibitor and 20 mM EDTA (to disrupt polyribosomes) followed by addition of 100 µL sample extract. A 10 µL aliquot of sample extract was saved for total RNA extraction. Extracts were incubated with the antibody-bead complexes for 2-4 hours at 4ºC with end-over-end rotation. Samples were then pelleted by centrifugation at 2,000×*g* and the supernatant was removed. The beads were then washed at least five times (5 minutes each at 4ºC) with either ice-cold NT2 buffer (H4) or 1× RIPA (mouse brain, for a more stringent wash). An aliquot of the final washed beads was saved for IP / Western blot analysis to confirm pull-down of CNBP. The beads were pelleted by centrifugation and resuspended

in NT2 buffer with 10% SDS and 30 µg of proteinase K. Samples were heated at 55ºC for 30 minutes with agitation to digest protein complexes.

RNA was extracted with phenol/chloroform/isoamyl alcohol (125:24:1, pH 4.3, Fisher) followed by ethanol precipitation overnight at -80ºC. Glycogen (20 µg; Roche) was added to each sample to enhance RNA precipitation. Precipitated samples were centrifuged at 20,800×g for 30 min at 4ºC and the pelleted RNA was resuspended in nuclease-free water. Equal volumes of RNA (usually 2 µL) were reverse transcribed using Verso cDNA synthesis kit (Fisher) according to the manufacturer's protocol. The RNA samples were heated to 70ºC for 5 min prior to the RT reaction to remove secondary structure. cDNA samples (2 µL) were then analyzed by quantitative real-time PCR on a Bio-Rad Mini-Opticon using iScript reagent (Bio-Rad) and gene-specific primers designed against intron-exon splice site junctions (Integrated DNA Technologies, Inc.). Cycling parameters used were: Heat activation 90ºC for 30 sec, then 40-45 cycles of 95ºC for 3 sec, 60ºC for 30 sec, 72ºC for 30 sec, followed by a final extension step of 72ºC for 5 min. The C(t) values for each sample were used to calculate the fold enrichment of the target of interest. After amplification, an aliquot of the product was run on a 2% agarose gel and visualized with SybrGreen stain to ensure correct product size.

The procedure for crosslinked RIP experiments was similar and based on a previously published protocol [267]. One 15-cm dish of H4 cells (~16 million cells) were trypsinized, washed once with 10 mL Opti-MEM media (with 10% FBS), washed twice with 10 mL sterile PBS, and then resuspended in 10 mL sterile PBS. Formaldehyde (AR grade, from a 37% HCHO/10% Methanol stock; Fisher) was added to the cells at a final concentration of 1% and cells were incubated at room temperature for 10 minutes with

slow mixing to induce crosslinking. The crosslinking reaction was quenched by the addition of 2.5 M glycine (pH 7.0) to a final concentration of 0.25 M followed by a five minute incubation at room temperature. The cells were then centrifuged at ~800×*g* for four minutes followed by two washes with ice-cold PBS. The fixed cell pellet was resuspended in 2 mL of 1× RIPA buffer containing PIC without EDTA and 200 U/mL RNase inhibitor. The cells were then sonicated for three rounds of 20 sec each at 100 W. Samples were incubated on ice for 2 minutes between each round of sonication. The lysates were then centrifuged at 20,800×*g* for 10 min at 4ºC to remove insoluble material. The supernatant was saved for RIP experiments. Antibodies were incubated with Protein A and G beads for 2 hours at 4ºC, and lysates were incubated with antibody-bead complexes for 1 hour at 4ºC, both with end-over-end rotation. RIPA buffer was used in place of PLB buffer and a high stringency RIPA buffer (50 mM Tris-HCl, pH 7.5, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 M NaCl, 1 M urea) was used for washing. Protein-antibody-bead complexes were resuspended in 100 µL of resuspension buffer (50 mM Tris-HCl, pH 7.0, 5 mM EDTA, 10 mM DTT, 1% SDS) and heated at 70ºC for 45 minutes to reverse crosslinks. RNA was extracted using TRIzol followed by phenol/chloroform and ethanol/glycogen precipitation. RNA pellets were resuspended in sterile water. RT and qPCR steps were done as described above. UV-crosslinking experiments were performed the same as for formaldehyde crosslinking except H4 cells grown in 10 cm dishes were irradiated once with a Stratalinker 1800 (Stratagene) at 120 mJ/cm^2 at 254 nm in 1× PBS prior to lysis. No heating step was performed prior to RNA purification.

All primer sets (Integrated DNA Technologies, Inc.) used in RIP qPCR experiments can be found in Appendix Two.

In Vitro Transcription / Translation (IVTT) Assay

IVTT assays were carried out using the TnT Coupled Reticulocye Lysate System (Promega) according to the manufacturer's protocol. This system does not require capped mRNA. Briefly, reactions were assembled in nuclease free 1.5 mL microfuge tubes with 0.5 µg of vector (pCMV6-XL5 BACE1 or pcDNA6 seAP vectors; all with T7 promoters) and indicated amounts of recombinant GST-$CNBP_{WT}$ in the presence of 2 µL ^{35}S-cystein/methionine (1175 Ci/mmol; Perkin Elmer) in a final reaction volume of 25 µL. Samples were incubated at 30ºC for 1.5 h. After incubation, a 5 µL aliquot was mixed with SDS loading buffer and heat-denatured for 15 min at 70ºC. Proteins were separated by SDS-PAGE on a Criterion polyacrylamide gel. After electrophoresis, the gel was dried and exposed on a PhosphorImager. Bands were quantified by densitometry using Scion Image.

Electrophoretic Mobility Shift Assays (EMSA)

Given the length and structural complexity of the BACE1 5' UTR, we first attempted binding using short 5'-biotin labeled RNA probes (Integrated DNA Technologies, Inc.) for L4 (5'-GUGGCCGCUGUGGAGAAGCAGCGAGGAGAUG-3') and BACE1 (5'-GCCGCCGCCGUGCCGAUGUAGCGGGCUCCGG-3'). The L4 probe contained most of the 5' UTR of the ribosomal protein L4 which is a direct target of CNBP [202, 230]. This region of the BACE1 5' UTR (210 – 240) was identified by alignment in Vector NTI as the most likely match with L4 (58% sequence identity).

Probes were resuspended in nuclease free water and stored at -80ºC until use. Biotinylated 31-nt probes were detected with the Pierce Lightshift chemiluminescent EMSA kit according to the manufacturer's instructions.

To generate the BACE1 5' UTR probe used in electrophoretic mobility shift assays, the pCMV6-XL5 BACE1 vector was first linearized with EcoN1 (New England Biolabs) which cuts the vector only once at 370-nt inside the BACE1 coding region. The choice of restriction enzymes were limited to ones that only cut the vector once, that would avoid leaving a 3' overhang, and that would generate the entire 5' UTR of BACE1, thus leaving EcoN1 as the best option. Following digestion, one-twentieth volume 0.5 M EDTA was added to stop the reaction, followed by sodium acetate and ethanol precipitation. The digested DNA pellet was resuspended in Tris-EDTA (pH 7.4) buffer to a concentration of 0.5 µg/uL. Ambion MEGAscript kit (Applied Biosystems) was used to generate unlabeled or ^{35}S-UTP (120 µCi/sample, Perkin Elmer) labeled nucleotide probes according to the manufacturer's protocol. Ambion MEGAclear kit (Applied Biosystems) was used to purify, concentrate, and recover the generated RNA probes. Following purification, the probes were checked for concentration and purity using a UV-Vis spectrophotometer (260/280 nm ratio) and by agarose gel electrophoresis to confirm estimated product size of approximately 835-nt. Radiolabeled and unlabeled probes were stored at -80ºC until use.

EMSA reactions were carried out in 1.5 mL nuclease free microcentrifuge tubes. Briefly, RNA probes were incubated in the absence or presence of varying amounts of recombinant CNBP in EMSA binding buffer (50 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 50 mM KCl, 0.5 mM DTT, 5% glycerol + 10U RNase Inhibitor) for 30 minutes at room

temperature in a final volume of 20 µL, unless otherwise specified. Some samples were subjected to UV-crosslinking using a Stratalinker 1800 at 254 nm (5-cm below bulb) for 5 minutes on ice after binding. CNBP concentrations ranged from 0.25 to 10 µM, and the ^{35}S-UTP labeled BACE1 5' UTR was used at 10-100 nM. The reactions were then separated on a Criterion 5% TBE gel (Bio-Rad Technologies) in 0.5× TBE running buffer (Bio-Rad). Gels were dried and exposed on a PhosphorImager. Bands were quantified by densitometry using Scion Image.

RESULTS

CNBP is Mostly Cytoplasmic and Found in Neurons and Astrocytes in the Brain

CNBP localizes to the cytoplasm, ER, and nucleus in a variety of tissues and species during development [220-222]. However, the subcellular localization of CNBP in the adult brain has not yet been reported. It was also unknown if CNBP expression is largely neuronal or extends to other cell types. To study this, we first lysed embryonic rat cortical neurons or astrocytes and looked at CNBP expression via Western blot. We found that both neurons and astrocytes express CNBP at comparable levels (**Fig 4.1A**). It is interesting to note that BACE1 expression is absent in rat primary astrocytes even though CNBP is highly expressed. It has been reported recently that a translational block of BACE1, but not BACE2, occurs in these cells [268]. We next looked at the subcellular localization of CNBP in primary rat neurons. CNBP was found at high levels in both the nuclear and cytoplasmic fractions (**Fig 4.1A**). This was not unexpected, as CNBP has been reported to localize to the nucleus of various cell types in embryonic development, where it plays a critical role in transcriptional processes [190, 220].

To see if CNBP localization in the brain changed with age, we separated nuclear and cytoplasmic fractions from aged rat brain tissue followed by Western blot for CNBP. We found that CNBP was located exclusively to the cytoplasm in four different aged rat brains (**Fig 4.1B**), the same compartment where BACE1 was located. Overall, these results indicate a primary role for CNBP in the cytoplasm in adult brain tissue, consistent with a role in translation.

CNBP Positively Regulates BACE1 Protein Levels

Preliminary findings from our lab in CHO cells indicated that CNBP may be a positive regulator of BACE1 expression (MPM, unpublished data). In order to confirm this, we transfected several human cell lines with either a full-length untagged human CNBP expression vector or an empty vector and then analyzed CNBP and BACE1 protein levels via Western blot. BACE1 protein was significantly increased when HEK293T cells were transiently transfected with CNBP, as detected by immunoblot (**Fig 4.2A**). Increasing the amount of CNBP cDNA used in the transfection increased the amount of both BACE1 and CNBP (**Fig 4.2A**), and BACE1 and CNBP expression were positively correlated ($\rho = 0.31$, $p<0.05$). To confirm our findings, we overexpressed CNBP in DIV=7 rat primary cortical neurons using adeno-associated virus (AAV2) vectors. CNBP overexpression caused an increase in BACE1 protein and in the amount of secreted Aβ peptide (**Fig 4.2B**). We observed no effects of CNBP overexpression on β-Actin or β3-Tubulin protein levels, and adjusting band densities to either of these as internal loading controls did not alter the results.

Finally, we evaluated the effect of CNBP on BACE1 expression in a cell-free *in vitro* transcription / translation system to ensure that the observed effects on BACE1 were directly related to CNBP and not caused by non-specific cellular events such as increased stress due to protein overexpression. The addition of spiked recombinant CNBP protein significantly increased ^{35}S-cysteine/methionine labeled BACE1 protein in a dose dependent manner in the cell-free system (**Fig 4.2C**). The increase in BACE1 occurred via a post-transcriptional mechanism as we did not detect an increase in BACE1 mRNA with increased CNBP added to the system. Taken together, these data clearly indicate that CNBP is a positive regulator of BACE1 expression and that this regulation is conserved across multiple cell types and species.

CNBP Knockdown Reduces BACE1 Protein Levels Variably

Since CNBP overexpression increased BACE1 protein levels, we next wanted to determine if the reverse was true. Does knocking down CNBP decrease BACE1 protein? To do this, we first transiently transfected H4 cells with CNBP shRNA or control shRNA (empty or scrambled) vectors for 72 hours, followed by Western blot for protein expression. We found that transient expression of CNBP shRNA significantly reduced CNBP protein levels and also reduced BACE1 protein levels by a similar amount when compared to the scrambled control shRNA (**Fig 4.3A**). Results were similar when comparing knockdown levels to an empty vector control.

Because we could not achieve any higher knockdown of CNBP with transient transfection (transfection efficiency was usually less than 50%), we created pooled stable H4 cell lines that expressed the shRNA constructs (empty vector, scrambled shRNA, or

CNBP shRNA). We then repeated our knockdown analysis using expression data grouped from the pooled stable cells and from transiently transfected cells. Surprisingly, even though CNBP levels were significantly reduced in the CNBP shRNA cells compared to the control shRNA cells, BACE1 levels were not significantly reduced in this larger analysis (**Fig 4.3B**). This may indicate that BACE1 reduction in response to sustained CNBP knockdown is less robust than the increases observed with overexpression. Alternatively, we may have simply selected for a population of cells that were more resistant to decreases in BACE1 expression in our pooled stable cells. Transient overexpression of CNBP in the CNBP shRNA pooled stable cell line resulted in a significant increase in BACE1 protein, indicating that this mechanism of regulation was still viable (**Fig 4.3B**). Altogether, these results imply that a reduction in CNBP can cause a variable decrease in BACE1 protein levels in cultured cells.

CNBP Expression Levels Differentially Affect BACE1 mRNA

To determine if overexpression or knockdown of CNBP had an effect on BACE1 mRNA levels, we transiently transfected HEK293T, H4, or SH-SY5Y (neuroblastoma) cells with CNBP (24 hr transfection) or CNBP shRNA (72 hr transfection) along with appropriate negative controls, followed by RNA extraction, purification, and analysis using real-time quantitative PCR. We were able to significantly overexpress CNBP in all three cell types as indicated by increased CNBP mRNA levels; however, CNBP overexpression did not significantly increase BACE1 mRNA levels in any of the three cell lines tested (**Fig. 4.4, *top***). This indicates that CNBP regulation of BACE1 likely occurs post-transcriptionally, as we suspected. Interestingly, knockdown of CNBP

decreased BACE1 mRNA levels slightly, although this reduction was only significant in SH-SY5Y cells (**Fig. 4.4*, bottom***).

To confirm that CNBP overexpression did not increase BACE1 protein or mRNA half-life, other members in our lab carried out steady-state metabolic labeling or pulse-chase experiments in H4 or HEK293T cells, followed by BACE1 protein or RNA analysis (MPM and RLW, unpublished data). The results indicated no obvious effect of CNBP overexpression on BACE1 protein or mRNA half-life. Taken together, our expression data indicate that an increase in CNBP likely regulates BACE1 through enhanced translation.

CNBP Associates with BACE1 mRNA

CNBP is an RNA binding protein and we suspected that it may interact with BACE1 mRNA to exert its translational regulation. In order to study this, we used RNA immunoprecipitation (RIP) to determine if CNBP associated with BACE1 mRNA at endogenous concentrations from cell lysates. Preliminary RIP experiments were done in whole cell lysates of H4 cells or whole tissue lysates from mouse pup brain. Poly-A binding protein (PABP) and β-Actin mRNA were used as a positive control for the RIP experiment to ensure the overall experimental conditions were sufficient. The PABP/β-Actin combination is used as a positive control in several commercially available RIP kits (Millipore, Sigma-Aldrich). We found that BACE1 mRNA was enriched in H4 or mouse brain lysates immunoprecipitated (IP) with our CNBP polysera compared to samples immunoprecipitated with negative control pre-immune serum (**Fig 4.5A**). Western blots

were run from the various IP fractions to confirm that only the CNBP polysera, and not the pre-immune serum, immunoprecipitated endogenous CNBP (**Fig 4.5B**).

RIP experiments carried out using whole cell lysates are routinely used for detecting interactions between protein and target mRNA but may result in non-physiological false positives due to compartment mixing once the cells are lysed [269]. In addition, weak or transient interactions between an RNA binding protein and its RNA targets may be missed. Thus, we wanted to determine if the CNBP/BACE1 mRNA interaction was confirmed when cells were crosslinked before lysing. Crosslinking of cells provides a "snapshot" of events that are taking place at any given time in the cell and can indicate interactions between two molecules that are in extremely close proximity [267]. We attempted two types of crosslinking: (1) crosslinking with formaldehyde that can be reversed by heating, and (2) irreversible crosslinking with UV irradiation that creates permanent covalent bonds between proteins and RNA molecules. We obtained similar results from both crosslinking experiments and thus pooled the data together for our crosslinked RIP analysis. Our results confirmed the association between CNBP and BACE1 mRNA and also identified some additional mRNAs that may be targets of CNBP as well (**Fig 4.5C**). Along with BACE1, we found that β-Actin, small ribosomal protein 17 (RPS17), bridging integrator (BIN1), and ODC1 mRNAs were all enriched in CNBP pull-down samples compared to negative control pull-down samples. Conversely, tau (MAPT), APP, GAPDH, and CNBP mRNAs were not immunoprecipitated by CNBP.

The BACE1 5' UTR Mediates CNBP Translational Regulation

Translational regulation of BACE1 is largely mediated by its 5' UTR which is long, highly structured, and contains several uORFs. Therefore, we hypothesized that CNBP mediates its interaction with BACE1 mRNA through the BACE1 5' UTR. The BACE1 3' UTR is also extremely long, but does not appear to affect expression [146]. To determine the effect of the BACE1 UTRs on CNBP-mediated protein translation, we expressed four different secreted alkaline phosphatase (seAP) reporter constructs in a cell-free, *in vitro* transcription / translation system (IVTT) with or without recombinant CNBP spiked in. The seAP vectors contained the seAP gene flanked by either the human BACE1 5' UTR, 3' UTR, both UTRs, or no UTRs driven by a T7 promoter.

Expression of these constructs in the IVTT system yielded several protein products (**Fig 4.6A**). Several of these bands may be truncated proteins but are produced from all four vectors and indicate *de novo* protein synthesis in this system. Comparison of band intensities by eye between constructs containing the BACE1 5' UTR with those lacking it illustrated the inhibitory nature of the BACE1 5' UTR as described previously [146] (**Fig 4.6A**). When recombinant GST-CNBP$_{WT}$ was added to the system, an increase in protein expression was observed and this effect was most prominent when the BACE1 5' UTR was present (**Fig 4.6A,B**). We chose to highlight one band as a representative of the others to illustrate that the BACE1 5' UTR appears critical for the CNBP-mediated increase in translated product (**Fig 4.6B,C**). These results imply that although the 3' UTR of BACE1 may be important for other regulatory mechanisms, CNBP likely exerts its translational regulation through the 5' UTR. As a result, we chose to focus the remainder of our studies on this region of the BACE1 mRNA.

CNBP Binds Directly to the BACE1 5' UTR

Our results from the RIP studies showed that CNBP is associated with BACE1 mRNA in cell lysates, but those experiments could not indicate whether the interaction is direct or indirect. Because CNBP is an RNA binding protein, we suspected that CNBP would bind directly to the BACE1 5' UTR. In order to test this, we used electrophoretic mobility shift assays (EMSA) using recombinant CNBP and various RNA probes. EMSA experiments are widely used to determine if a protein binds directly to a specific nucleic acid sequence (*eg.* an RNA probe), which is detected by running the complex on a polyacrylamide gel [270]. RNA that is bound to protein will migrate slower through the gel compared to free RNA, indicated by a shift in the bands.

Because the entire BACE1 5' UTR is long and complex, we first tested CNBP binding to a short, 31-nt region of the BACE1 5' UTR that was most similar in sequence and GC content to the 5' UTR of the *X. laevis* L4 ribosomal protein. The L4 probe was also 31-nt in length and contained the entire 5' UTR (plus start codon) of L4 minus the 5' TOP sequence; CNBP directly binds this segment [202]. We obtained consistent shifts with the L4 probe and CNBP under a variety of assay conditions (**Fig 4.7B,C**); however, we never observed binding of CNBP to the 31-nt BACE1 probe (**Fig 4.7D**). CNBP binds to regions of unpaired G residues constrained by a structured environment [215] and an analysis of the predicted secondary structures of the two 31-nt probes using the *mfold* web server ([271]; http://mfold.rna.albany.edu/?q=mfold) revealed that the L4 probe contained more unpaired Gs than the BACE1 probe, even though they are predicted to

have a similar hairpin-like structure (**Fig 4.7A**); this likely accounts for the differential binding observed.

Next, we created a longer, radiolabeled RNA probe containing the entire human BACE1 5' UTR as well as a portion of the N-terminal BACE1 ORF using *in vitro* transcription. Combining this probe with increasing amounts of recombinant CNBP resulted in direct binding of CNBP to the probe as indicated by EMSA (**Fig 4.8**). This was achieved with both GST-$CNBP_{WT}$ and with recombinant human CNBP containing a C-terminal DDK tag (Origene). Several previously published reports showed that GST alone did not bind various RNA probes in EMSA experiments [194, 215, 226]. Furthermore, a mutant CNBP protein (GST-$CNBP_{1-\Delta RGG}$), which lacks the RGG region important for RNA binding [194], failed to bind the probe. These results indicated that CNBP bound directly to an RNA probe that contained the BACE1 5' UTR *in vitro* and further suggested that this region is a likely site for CNBP-mediated translational regulation.

CNBP Increases in AD and Correlates with BACE1

We next sought to establish if CNBP was increased in the AD brain, which could account for the changes in BACE1. We examined a disease affected (the superior and middle temporal gyri; SMTG) and unaffected (the cerebellum; CB) region of the brain from a cohort of AD cases and age-matched controls for CNBP and BACE1 protein expression (**Fig 4.9A**). In both regions, the amount of CNBP and BACE1 protein were positively correlated (Spearman's ρ: SMTG = 0.423, $p<0.01$; CB = 0.316, $p<0.05$; **Fig 4.9C**). We next performed an analysis of CNBP and BACE1 protein levels using age,

post-mortem interval, and GAPDH or β-Actin levels (the loading controls) as covariates, and including gender as a variable. 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 (**Fig 4.9B**). 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 (**Fig 4.9B**). Neither CNBP nor BACE1 mRNA changed significantly in the disease, although CNBP mRNA trended towards the changes observed in protein levels (**Fig 4.10**).

We also examined a small number of MCI cases, looking for changes in CNBP in the earliest stages of disease. We detected a significant increase in CNBP in MCI cases ($F_{1,20}$ = 5.65, $p<0.03$) (**Fig 4.11, *left***). Although BACE1 was not increased in these cases, CNBP and BACE1 were correlated ($p<0.001$) (**Fig 4.11, *right***). Others in our lab found no relationship between CNBP and age, or CNBP and Aβ, in either the human or mouse brain, indicating that CNBP does not likely increase as a consequence of normal aging (MPM, unpublished data). Together, these data show that CNBP increases from an early stage of the disease process, and that this increase likely precedes significant increases in BACE1.

DISCUSSION

The BACE1 5' UTR appears to be the main *cis*-acting element regulating BACE1 translation, resulting in inefficient translation under normal, physiological conditions. It may be that certain *trans*-acting factors play a significant role in enhancing BACE1 translation, as seen during the progression towards AD. The experiments described in this

chapter implicate the RNA binding protein, CNBP, as an important and novel *trans*-acting factor in the regulation of BACE1 translation.

Our results indicate that CNBP protein is expressed at high levels in both primary cortical astrocytes and neurons from rat brain. The evidence that BACE1 translation is inhibited in primary rat astrocytes by an unknown mechanism [268] indicates that CNBP does not positively regulate BACE1 translation in this cell type, at least during early development. It also suggests that BACE1 translation is differentially regulated in various cell types and at different ages, possibly through other mechanisms such as noncoding RNAs. Because BACE1 in reactive astrocytes may play an important role in AD (reviewed in [116]), determining if there are significant differences in BACE1 translational regulation between neurons and astrocytes needs to be further studied.

Subcellular fractionation of primary cortical rat neurons indicated that CNBP exists in both the cytoplasm and nucleus, consistent with its role in transcriptional processes during development, and possibly suggests a shuttling mechanism for CNBP. Shuttling of CNBP between the nucleus and cytoplasm has been observed in the embryogenesis of the toad species *Bufo (Chaunus) arenarum* [220]. Alternatively, various pools of CNBP monomer and dimer may exist in different cellular locations; indicative of its binding activity [215]. The role of CNBP in translational regulation during development has not been investigated. Other members of our lab have looked at CNBP expression in young rodent brain using immunohistochemistry and found that CNBP was mainly localized to the cytoplasm; however, distinct staining could be detected in the nucleus as well (MPM, unpublished data). Interestingly, subcellular fractionation of aged whole rat brain tissue showed CNBP localization only in the

cytoplasmic fraction. This indicates a shift in CNBP to a mostly cytoplasmic function in the adult brain, consistent with a primary role in translation. With a molecular weight of around 20 kDa, CNBP is small enough to pass through the nuclear pore complex by simple diffusion [272]; however, our results suggest this may not happen readily in the aged brain. CNBP contains a conserved putative PKA phosphorylation site at its C-terminus and it is tempting to speculate that it could be involved in regulating shuttling between the nucleus and the cytoplasm. Additionally, specific cellular conditions in the aged brain may be conducive for CNBP to form large ribonucleoprotein complexes, keeping it largely localized to the cytoplasm.

CNBP is a translational regulator of several seemingly unrelated mRNA targets and we have shown in this chapter that CNBP is a *trans*-acting regulator of BACE1. Overexpression of CNBP in cultured cells or in primary neurons increased BACE1 protein production and knocking down CNBP had the opposite effect, indicating that CNBP is a positive regulator of BACE1 expression. Although overexpression of CNBP seemed to have no effect on BACE1 mRNA levels or stability, decreased CNBP expression below physiological normal levels reduced BACE1 mRNA expression. This pattern indicates that a threshold level of CNBP may be needed for the stability of its target transcripts and is characteristic of other RNA binding proteins, such as TDP-43, which sustains the normal levels of its target RNAs [273, 274]. Since under normal circumstances these proteins may be sufficiently bound to their cellular targets to affect stability, overexpression will not necessarily lead to further increases in RNA target abundance.

An observed increase in protein levels post-transcriptionally can only occur through enhanced mRNA or protein stability (*i.e.* increased half-life or decreased degradation) or through increased translation. Only one group has suggested changes in BACE1 degradation in AD via increased BACE1 half-life mediated through the lipid second messenger ceramide [155]. However, we detected no changes in BACE1 protein or mRNA half-life (MPM, RLW unpublished data) or in total BACE1 mRNA with CNBP overexpression, confirming that CNBP most likely enhances BACE1 translation. We have replicated our expression findings in various cell types (human embryonic kidney, neuroglioma, neuroblastoma, primary neurons) and from different species (hamster, rat, human), indicating that this method of regulation is highly conserved.

We found that CNBP associated with BACE1 mRNA at endogenous concentrations in cellular lysates through RNA immunoprecipitation studies. Besides BACE1, our RIP analysis identified several additional potential mRNA targets of CNBP, including β-Actin, BIN1, RPS17, and ODC1. Both RPS17 [184] and ODC1 [204] mRNAs have previously been shown to be direct targets of CNBP and served as positive controls to validate our results. Although we did not see any changes in β-Actin expression in cell culture experiments with CNBP modulation, we have noted changes in its expression in several *in vivo* experiments (MPM and RLW, unpublished data), indicating that it may be a *bona fide* target of CNBP regulation. BIN1 is involved in endocytosis [275] and membrane curvature [276] and has been implicated as the second highest genetic risk factor, behind ApoE4, for the development of sporadic AD [277, 278]. Currently, its role in AD pathogenesis is unknown. BIN1 has also been implicated in DM2 through alterations in splicing patterns [279]. Thus BIN1 is a particularly

interesting target that links CNBP to both DM2 and AD. Additional expression studies will be needed in order to validate β-Actin and BIN1 mRNAs as functional CNBP targets.

CNBP failed to bind mRNAs for GAPDH, APP, MAPT, BACE2, or CNBP itself. GAPDH is an abundant protein that exclusively uses cap-dependent translation and has been previously used as a negative control in CNBP RNA immunoprecipitation experiments [204]. APP and MAPT are critical AD-related proteins and interestingly both have been suggested to contain IRES elements in their 5' UTRs [280, 281]. Thus, it was anticipated that one or both would be enriched in our RIP experiments. The observation that neither was immunoprecipitated by CNBP may indicate that CNBP's interaction with IRES elements is selective and/or requires other regulatory proteins. We have shown in Chapter Three that BACE1 and BACE2 activities and proteins are highly correlated in the progression of AD, and this is suggestive of a shared mechanism of regulation. We speculated that CNBP might be this common regulator, but our RIP results indicated that CNBP does not bind BACE2 mRNA. Although this finding is interesting, it is not altogether surprising, given that the BACE1 and BACE2 transcript sequences are widely dissimilar except for their coding regions [282]. Finally, other RNA binding proteins, such as FMRP [283], TDP-43 [284], and PABP [285], have been shown to bind their own mRNAs, but according to our results this may not be the case with CNBP.

While these RIP experiments provided new and interesting information about possible CNBP targets, they should be evaluated with caution due to the inherent limitations of the experiment. RIP experiments, both native and crosslinked, may lead to

false-positives and/or false-negatives. The success of the RIP experiment is affected by the structural and sequence complexity of the binding sites and the accessibility of the protein to its target mRNAs. In addition, the choice of buffer may not accurately reflect the physiological conditions needed for binding, which may include formation of multi-protein complexes [286]. For example, in our studies we included EDTA, which is important for reducing background and gaining access to epitopes by disrupting ribosomes from mRNP complexes [266]. Since CNBP interacts with the translation machinery [204-207], we wanted to minimize this interaction. However, because the CNBP zinc fingers enhance interactions with RNA [194], chelators such as EDTA likely reduce CNBP binding activity, possibly causing us to miss some targets. The antibody binding site may also affect RIP efficiency; our polyclonal CNBP antibody recognizes the last 20 amino acids of the C-terminus which partially overlaps the last CCHC zinc finger and also the putative PKA phosphorylation site. Masking of these regions could severely hinder CNBPs ability to effectively bind target mRNAs, resulting in low immunoprecipitation efficiency. We did try a RIP experiment with a commercial N-terminal CNBP antibody (Abcam), but did not get pull-down of native CNBP from a cell extract. Thus, optimization of experimental conditions is required to obtain the most accurate results and any identified targets must be validated by secondary methods.

Our RIP experiments indicated that CNBP associated with BACE1 mRNA, and we showed that this interaction is direct through EMSA experiments. CNBP was able to bind the RNA probe containing the BACE1 5’ UTR, which is the most likely site for translational regulation. However, because the probe contained a portion of the BACE1 coding region, we cannot rule out that important interactions are limited to the 5’ UTR.

Similarly, although we showed that CNBP likely exerts its translational effects through the BACE1 5' UTR in our seAP IVTT assays, we cannot rule out that CNBP may functionally bind to portions of the BACE1 3' UTR as well. Further studies will be needed to address these questions.

The results from our human tissue analysis suggest that CNBP is increased in a disease-affected region of the brain (SMTG) and is highly correlated with BACE1 expression. Interestingly, both CNBP and BACE1 were decreased in the disease-unaffected cerebellum in this set of samples, which could underscore important pathogenic discrepancies in different brain regions. Neither BACE1 nor CNBP mRNA levels significantly changed in AD, although CNBP mRNA trended towards explaining protein levels. Finally, and most importantly, we found that CNBP was increased early in the disease process as we would expect if it plays a role in increasing BACE1 translation in AD.

Overall, we have shown in this chapter that CNBP positively regulates BACE1 translation, but determining how this regulation occurs and what causes CNBP to increase in AD are still unanswered questions. In the next chapter, we will begin to explore these questions in order to better understand the mechanism of CNBP translational regulation of BACE1 in the brain and its consequences for disease.

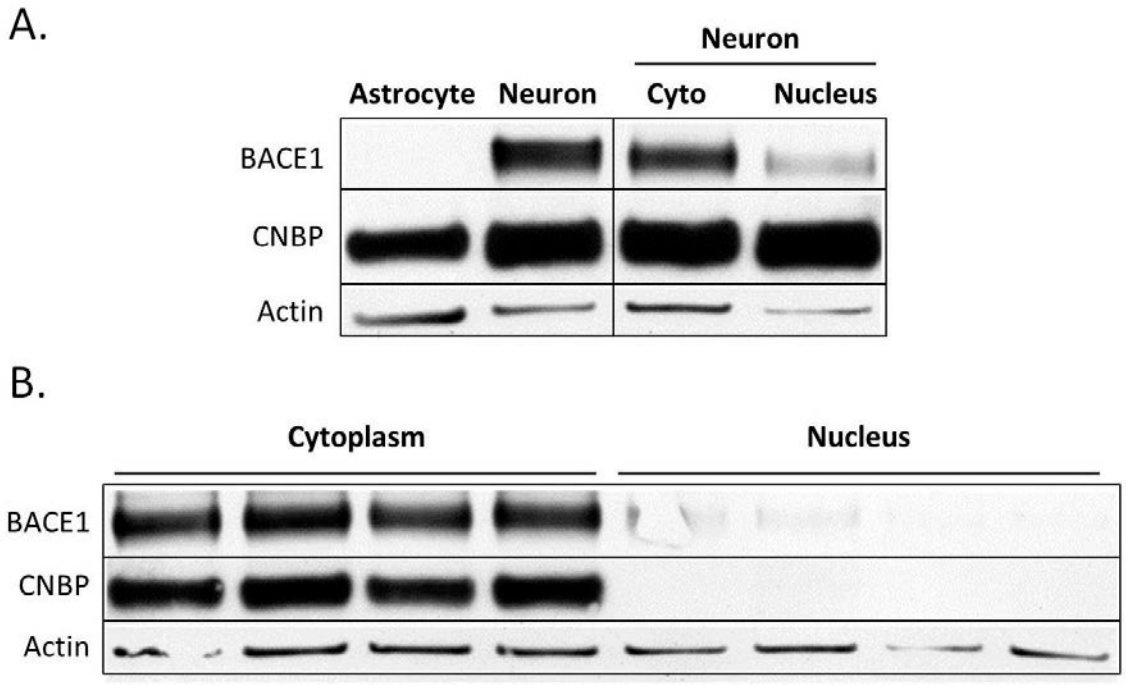


Figure 4.1 CNBP Expression and Localization in the Brain. (A) Approximately 8 µg of total protein was separated by SDS-PAGE followed by Western blotting. CNBP was present in high levels in whole cell lysates from both rat primary cortical astrocytes and neurons (*left*). A translational block of BACE1 occurs in primary rat astrocytes [268]. Subcellular fractionation of rat primary cortical neurons (*right*) revealed that CNBP was located in both the cytoplasm and nucleus in developing neurons. The presence of some BACE1 (D10E5) in the nucleus may represent partial carry over from the cytoplasmic fraction but did not account for the large portion of CNBP found in the nucleus. (B) Four 20-month old rat brains were fractionated into cytoplasmic and nuclear pools and evaluated for protein expression. Each lane was loaded with 8 µg of total protein. CNBP was found exclusively in the cytoplasm consistent with BACE1 localization. For these blots, β-Actin was used as a loading marker.

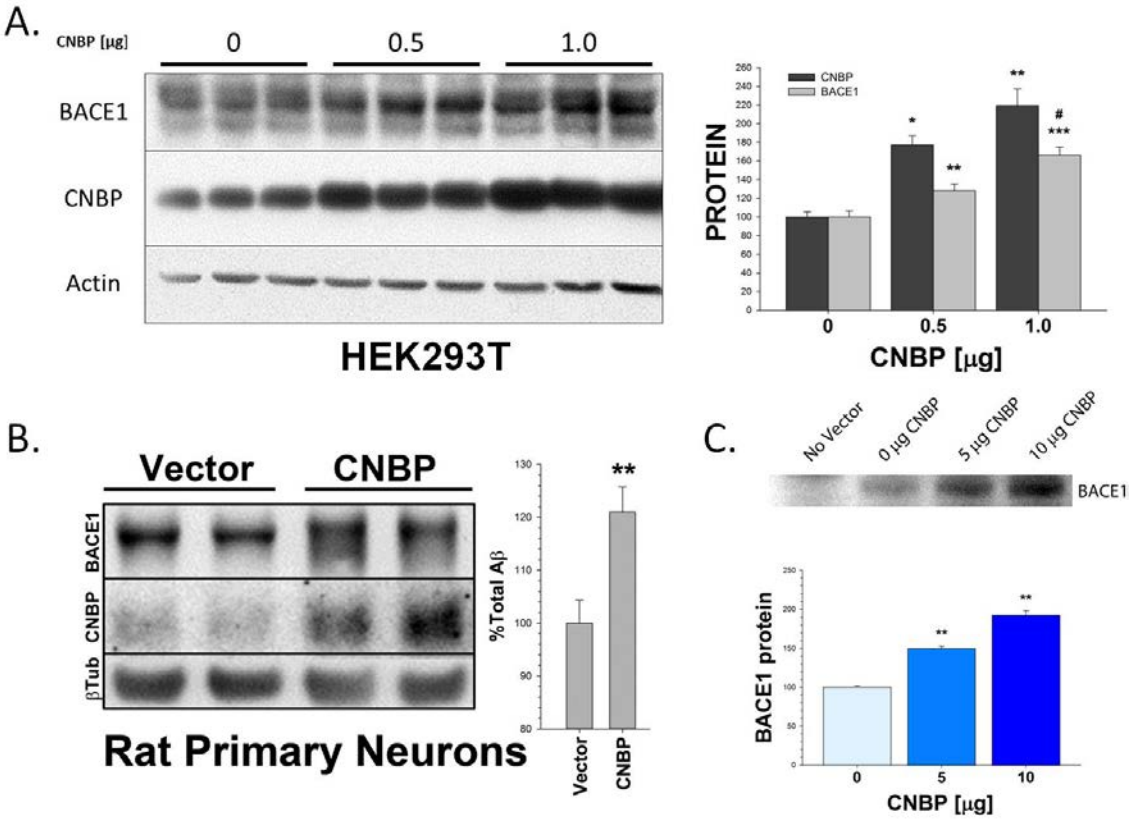

A.
CNBP [µg]
0
0.5
1.0
BACE1
CNBP
Actin
HEK293T
PROTEIN
CNBP
BACE1
CNBP [µg]
B.
Vector
CNBP
BACE1
CNBP
βTub
Rat Primary Neurons
%Total Aβ
Vector
CNBP
C.
No Vector
0 µg CNBP
5 µg CNBP
10 µg CNBP
BACE1
BACE1 protein
CNBP [µg]

Figure 4.2 CNBP Increases BACE1 Protein Expression. (A) Transient overexpression of CNBP with increasing amounts of CNBP cDNA lead to corresponding increases in BACE1 expression (EPR3956) in HEK293T cells. CNBP ($F_{2,27}$ = 6.46, $p<0.005$) and BACE1 ($F_{2,27}$ = 14.89, $p<0.001$) cDNA dose response (3 experiments, n = 8-11 replicates / dose); Dunnett's test vs. control, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$; vs. 0.5 µg CNBP, # = $p<0.05$. (B) Overexpression of CNBP in rat primary cortical neurons using AAV2 increased both BACE1 (D10E5) and total Aβ (*t*-test, ** = $p<0.01$). The sample size (n = 3) was too small to obtain significance in protein levels, although the virus had similar effects on human neuroblastoma cells (not shown). (C) CNBP significantly increased BACE1 protein production (*t-test*, ** = $p < 0.01$; n = 3 independent replicates) in a cell-free *in vitro* transcription / translation rabbit reticulocyte lysate system. The increase in BACE1 was dependent on the amount of CNBP added up to 10 µg; higher concentrations of CNBP (15 µg; n = 1, not shown) appeared to have no additional effect on BACE1 protein levels. BACE1 protein was normalized to BACE1 mRNA levels extracted from each sample, which showed a slight decrease in mRNA with increasing amounts of CNBP protein. Error bars represent SEM.

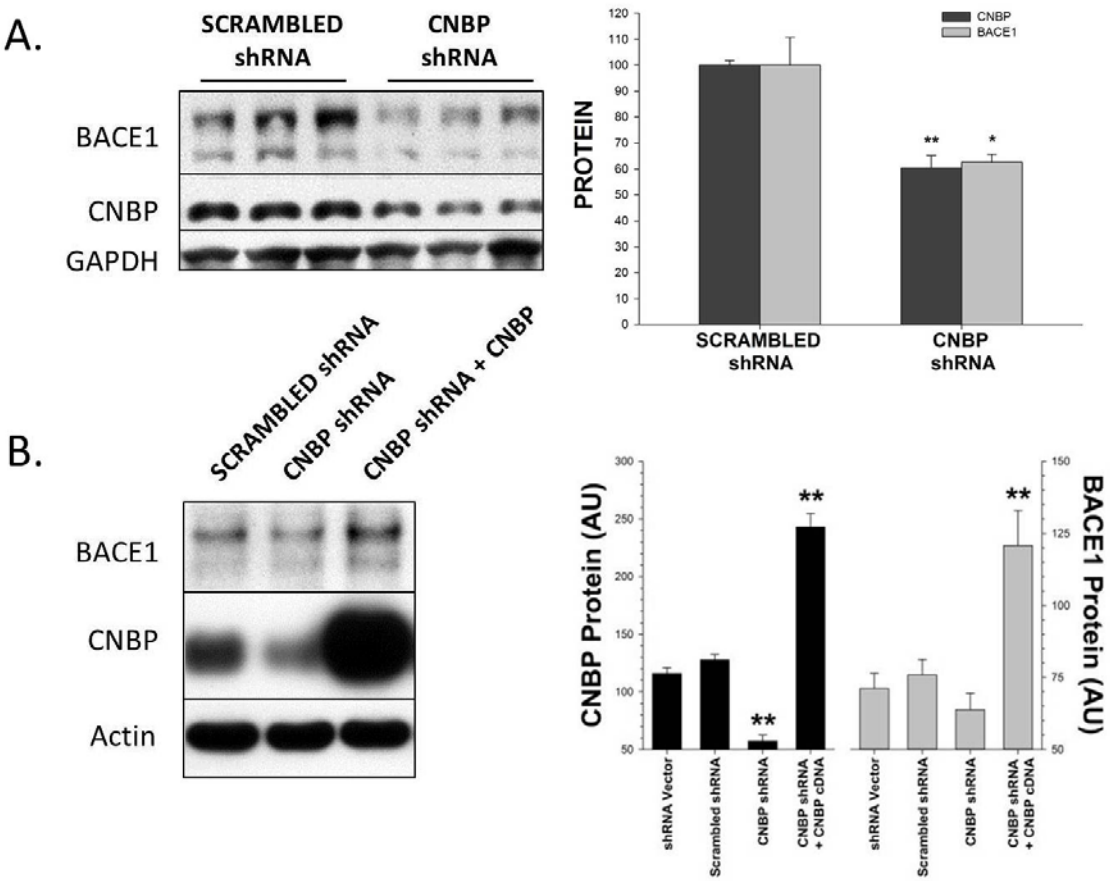

A.
SCRAMBLED shRNA
CNBP shRNA
BACE1
CNBP
GAPDH
CNBP
BACE1
PROTEIN
120
110
100
90
80
70
60
50
40
30
20
10
0
**
*
SCRAMBLED shRNA
CNBP shRNA
B.
SCRAMBLED shRNA
CNBP shRNA
CNBP shRNA + CNBP
BACE1
CNBP
Actin
CNBP Protein (AU)
BACE1 Protein (AU)
300
250
200
150
100
50
150
125
100
75
50
**
**
**
shRNA Vector
Scrambled shRNA
CNBP shRNA
CNBP shRNA + CNBP cDNA
shRNA Vector
Scrambled shRNA
CNBP shRNA
CNBP shRNA + CNBP cDNA

Figure 4.3 CNBP Knockdown Reduces BACE1 Protein Variably. (A) Transient shRNA knockdown of CNBP with an shRNA directed against its 3' UTR (Origene HuSH) in H4 neuroglioma cells lead to a reduction in BACE1 (EPR3956) (*t*-test, * = $p<0.05$; ** = $p<0.01$). (B) In pooled stable H4 cell lines, CNBP can be knocked down and then replaced by cDNA complementation (*left*). In the overall evaluation of knockdown experiments (*right*), the BACE1 reduction only trended lower ($p<0.13$) when CNBP was knocked down, even though the increase was substantial when CNBP was overexpressed (3 experiments, n = 9-12 replicates / construct; combined pooled stables and transient transfection; Scheffe test for multiple pairwise comparisons, ** = $p<0.01$, relative to scrambled shRNA). This could indicate that BACE1 reduction in response to CNBP knockdown may be less robust than the increase observed in response to overexpression. All knockdown results were the same when compared to an empty vector control. Error bars represent SEM. AU, arbitrary units.

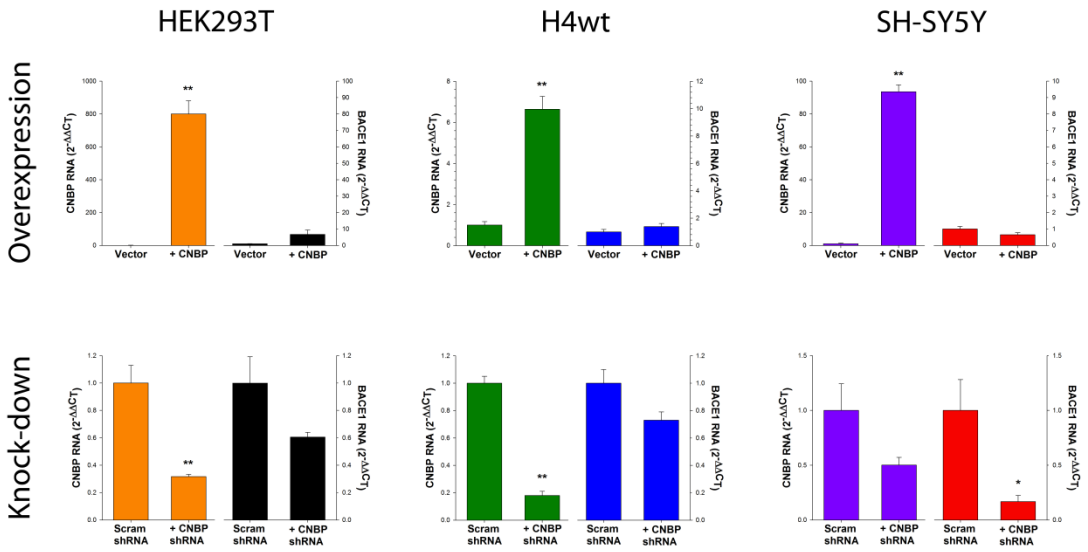


Figure 4.4 CNBP Overexpression or Knockdown Differentially Affects BACE1 mRNA Levels. CNBP overexpression (*top row*) in HEK293T, H4, or SH-SY5Y cells did not increase BACE1 mRNA expression. CNBP knockdown (*bottom row*) using shRNA trended towards a reduction in BACE1 mRNA levels overall and may indicate a threshold level of CNBP is needed for target mRNA stability. Geometric means were normalized to expression levels of β-Actin and GAPDH and compared to controls; *t*-test, * = $p<0.05$, ** = $p<0.01$. Results are the average of three individual experiments. Error bars represent SEM.

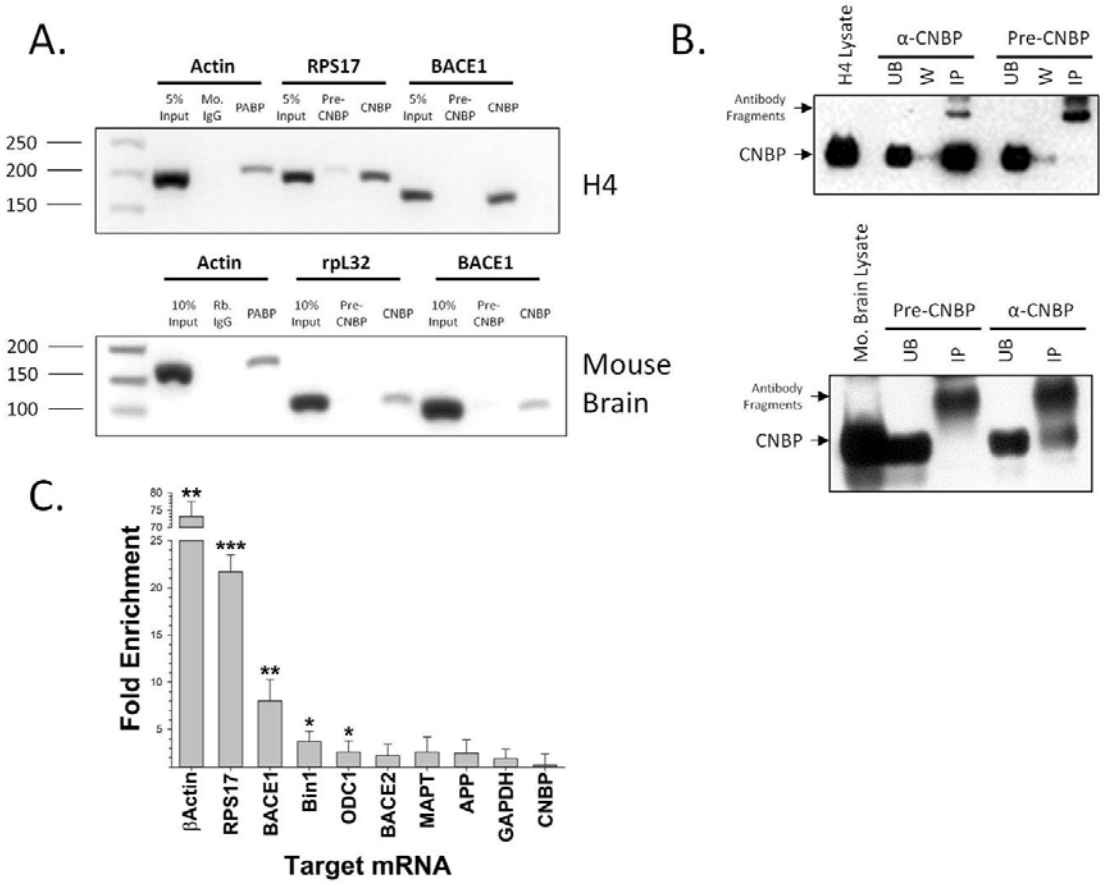
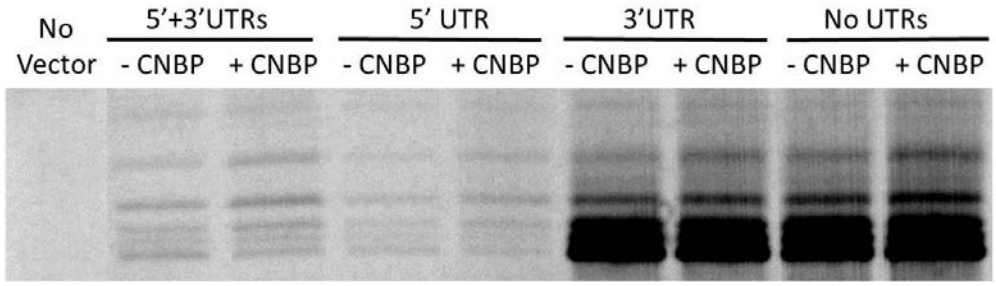
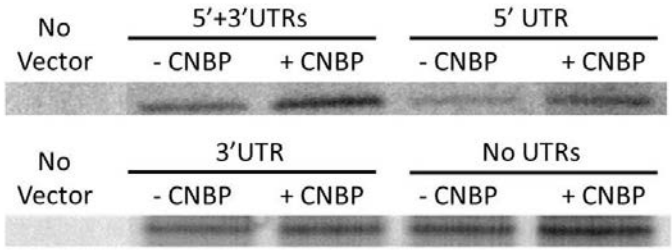

A.
Actin
RPS17
BACE1
5% Input
Mo. IgG
PABP
Pre-CNBP
CNBP
250
200
150
H4
rpL32
10% Input
Rb. IgG
100
Mouse Brain
B.
H4 Lysate
α-CNBP
Pre-CNBP
UB
W
IP
Antibody Fragments
CNBP
Mo. Brain Lysate
C.
Fold Enrichment
βActin
RPS17
BACE1
Bin1
ODC1
BACE2
MAPT
APP
GAPDH
CNBP
Target mRNA

Figure 4.5 CNBP Associates with BACE1 mRNA in Cell Lysates. (A) RNA immunoprecipitation (RIP) followed by RT-qPCR from H4 whole cell lysate or whole mouse brain lysate showed that CNBP associated with BACE1 mRNA at endogenous concentrations. PCR products were run on a 2% agarose gel to confirm correct product size and enrichment in the specific protein pull-down sample (PABP or CNBP) compared to the negative control pull-down sample (IgG or pre-CNBP). PABP/β-Actin was used as an overall positive control for the RIP experiment. RPS17 (human) or rpL32 (mouse), both identified mRNA targets of CNBP, were used as positive controls for CNBP pull-down. (B) IP-Western blots confirmed the immunoprecipitation of CNBP protein by CNBP polyserum (α-CNBP) and not by the pre-immune serum (Pre-CNBP). UB = unbound fraction, W = wash fraction, IP = immunoprecipitated fraction. (C) Examination of select mRNAs (of varying abundance) from RIP crosslinking experiments in H4 cells showed that in addition to BACE1 and RPS17, CNBP was able to pull down mRNAs for β-Actin, BIN1 and ODC1, but not BACE2, MAPT, APP, GAPDH or the mRNA for CNBP itself (Mann-Whitney Test, adjusted for multiple comparisons: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$; 3-4 independent experiments, 3 – 11 replicates / target mRNA). Fold enrichment represents the signal above background level and was calculated as 2^[Ct(mock) – Ct (IP)], where Ct represents the cycle threshold.

A.


No Vector
5'+3'UTRs
- CNBP
+ CNBP
5' UTR
- CNBP
+ CNBP
3'UTR
- CNBP
+ CNBP
No UTRs
- CNBP
+ CNBP

B.


No Vector
5'+3'UTRs
- CNBP
+ CNBP
5' UTR
- CNBP
+ CNBP
No Vector
3'UTR
- CNBP
+ CNBP
No UTRs
- CNBP
+ CNBP

C.

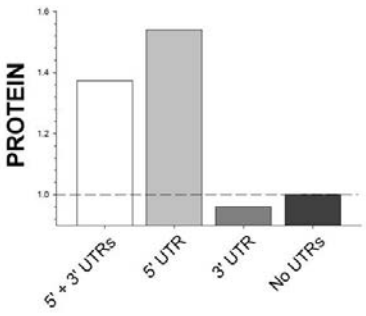
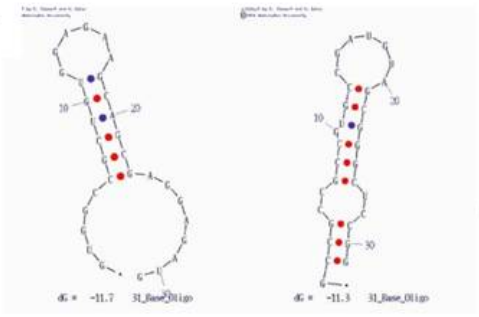

PROTEIN
1.6
1.4
1.2
1.0
5' + 3' UTRs
5' UTR
3' UTR
No UTRs

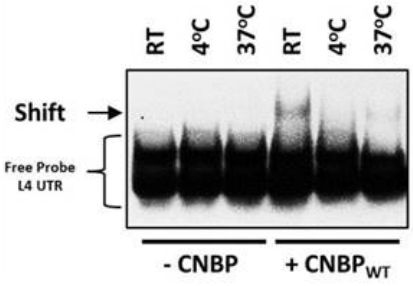
Figure 4.6 The BACE1 5' UTR Mediates CNBP Translational Regulation. (A) seAP vectors containing various combinations of BACE1 UTRs or no UTRs were translated in a cell-free lysate with or without recombinant GST-$CNBP_{WT}$ spiked in; protein products were radiolabeled with ^{35}S-cysteine/methionine and detected by SDS-PAGE and autoradiography. The same several protein products were produced from all four vectors used (compare to No Vector control, relocated from blot directly to the left of the protein lanes). The constructs containing the BACE1 5' UTR display a striking inhibition of overall protein production compared to the vectors lacking the BACE1 5' UTR, consistent with its described inhibitory nature. (B) A single protein band was further analyzed for expression levels with or without recombinant CNBP added to the system. (C) Densitometric quantification of this single band illustrated that CNBP increased protein expression only in vectors containing the BACE1 5' UTR when compared with the vector containing no UTRs. The BACE1 3' UTR had little effect on protein production in the presence of excess recombinant CNBP. This same general effect was observed for each individual band or when all bands were combined, implicating the BACE1 5' UTR as the most likely site of CNBP translational regulation.

A.

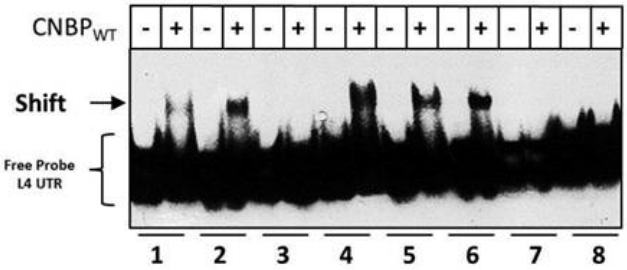


L4 BACE1

B.

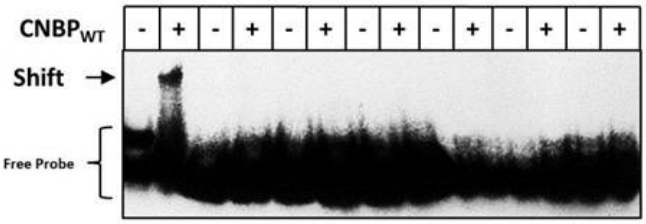


C.



List of buffer components:	Reference:
1.) 20mM HEPES-NaOH (pH 8.0), 100mM KCl, 0.5mM DTT, 20% glycerol	Pellizoni et al., 1997
2.) 10mM HEPES-NaOH (pH 8.0), 150mM KCl, 2mM $MgCl_2$, 1 mM DTT, 10% glycerol	Armas et al., 2004
3.) 20mM HEPES-NaOH (pH 8.0), 50mM KCl, 2mM $MgCl_2$, 10% glycerol	Gerbasi et al., 2007
4.) 20mM HEPES-NaOH (pH 8.0), 10mM $MgCl_2$, 1mM DTT, 10% glycerol	Lombardo et al., 2007
5.) 12mM HEPES-NaOH (pH 8.0), 4mM Tris-HCl (pH 8.0), 60mM KCl, 1mM DTT, 12% glycerol	Fink et al., 1992 and 1995
6.) 50mM Tris-HCl (pH 8.0), 50mM KCl, 1mM $MgCl_2$, 0.5mM DTT, 5% glycerol	McCarthy et al., 1999
7.) 10mM Tris (pH 7.4), 150mM KCl, 0.1mM DTT, 0.1mM EDTA, 10% glycerol	Invitrogen EMSA 5x binding buffer
8.) 10mM Tris (pH 7.5), 50mM KCl, 1mM DTT, 10% glycerol, 100µM $ZnCl_2$	Pierce 10x binding buffer + Zinc

D.



	L4-UTR		BACE1-5'UTR											
Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Probe (fmol)	50	50	50	50	50	100	100	100	50	50	50	100	100	100
rCNBP (ng)	0	50	0	50	100	0	50	100	0	50	100	0	50	100
Buffer #	6		4						6					

Figure 4.7 Identification of EMSA Binding Conditions for CNBP. (A) A known CNBP target (*X. laevis* L4 ribosomal protein RNA) was used to develop binding conditions, along with its closest *in silica* match (a 31-nt site with 58% identity, at position 210-240 in the BACE1 5' UTR). The predicted secondary structures (*mfold* program) for the two 31-nt oligos at their most favorable free energies (L4: ΔG^o = -11.7 kJ, BACE1: ΔG^o = -11.3 kJ) indicated similar hairpin structures. (B) CNBP bound the L4 probe most favorably at room temperature (RT) compared to 4°C or 37°C. Binding reactions were carried out in 1× binding buffer (10 mM Tris, pH 7.5, 50 mM KCl, 1 mM DTT, 10% glycerol, 10U RNase inhibitor) for 30 minutes with 50 ng (~100 nM) of recombinant human $CNBP_{WT}$-DDK and 0.5 ng (50 fmol; 2.5 nM) of L4 oligo. (C) Several buffer conditions (#1, 2, 4, 5, and 6) were reasonable for CNBP binding the L4 test probe; $CNBP_{WT}$-DDK: 50 ng (~100 nM); L4 oligo: 0.5 ng (50 fmol; 2.5 nM). (D) The most similar region of the BACE1 5' UTR compared to the L4 probe did not bind CNBP under the most favorable EMSA conditions. This indicated that the binding region for CNBP is not specific to this region, but likely binds a more generic motif such as structured G-rich regions with an adequate number of unpaired guanines [215, 231].

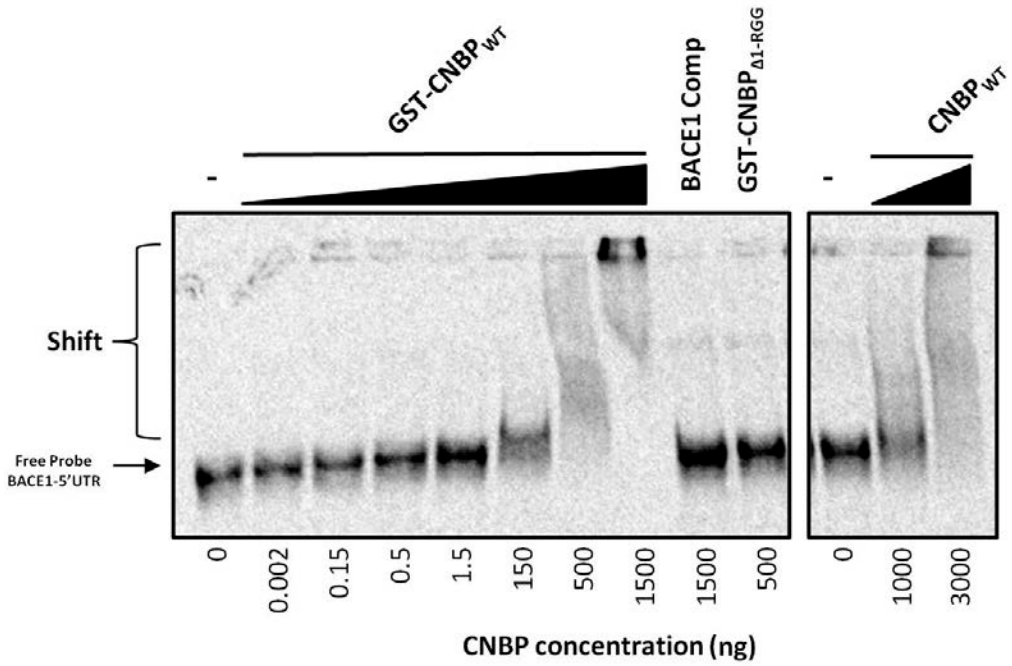
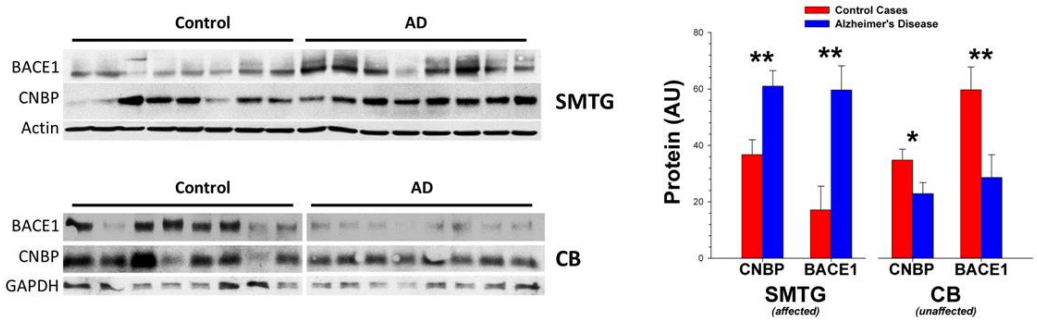


Figure 4.8 CNBP Binds Directly to the BACE1 5' UTR. Electrophoretic mobility shift assays showed that CNBP (both GST-CNBP$_{WT}$ and human CNBP$_{WT}$-DDK) bound directly to the labeled BACE1 5' UTR (6.14 ng; 1.08 nM per lane). Binding was blocked by an excess (300×) of unlabeled UTR competitor; a CNBP mutant (GST-CNBP$_{1\text{-}\Delta RGG}$) missing the RGG box (critical RNA recognition motif [194]) showed no binding and indicated the GST tag was not responsible for binding.

A.

	N (M/F)	Age	Brain Weight (g)	PMI (h)	Braak	MMSE
Control	19 (6/13)	86.0 ± 1.4	1200 ± 33.7	2.5 ± 0.15	1.0 ± 0.19	29 ± 0.36
AD	22 (6/16)	83.0 ± 1.3	1075 ± 20.6	3.0 ± 0.13	6.0 ± 0.00	8 ± 1.54

B.



C.

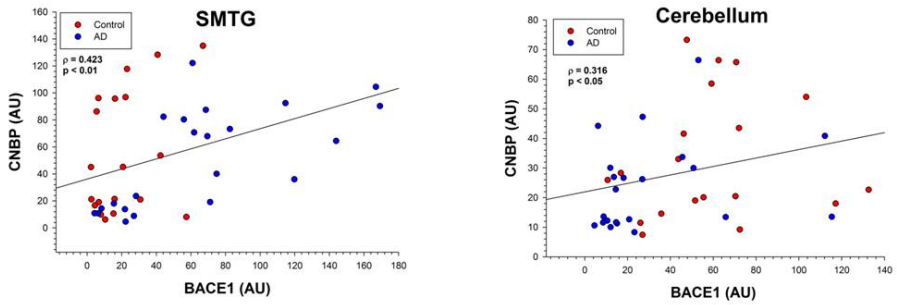


Figure 4.9 CNBP Increases With BACE1 in AD. (A) Human case statistics used for Western blot analysis. (B) *Left*, representative Western blots for BACE1 (Epitomics, EPR3956) and CNBP protein in AD-affected (the SMTG) and unaffected (the CB) regions. *Right*, in AD, CNBP and BACE1 were significantly higher in the SMTG, and significantly lower in the CB. (C) CNBP and BACE1 levels were significantly and positively correlated in both the SMTG ($p<0.01$) and the CB ($p<0.05$). * = $p<0.05$, ** = $p<0.01$. Error bars represent SEM. AU, arbitrary units.

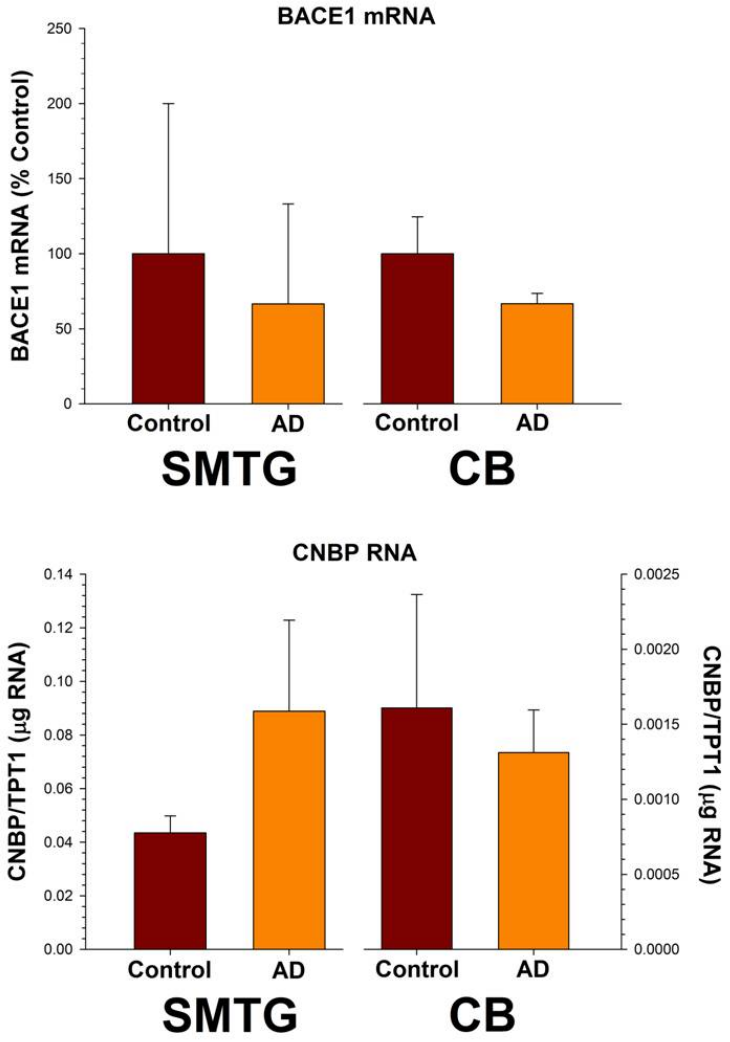


Figure 4.10 BACE1 and CNBP mRNA Levels are Unchanged in AD. BACE1 (*top*) and CNBP (*bottom*) total mRNA levels were not significantly different between Control and AD cases in either the SMTG or CB (BACE1: $p>0.2$; CNBP: $p>0.4$). The mRNA for CNBP trended towards an increase in the SMTG and a decrease in the CB, mirroring protein levels. mRNA data was only available for a subset of the cases, (10 AD, 9 control; BACE1 values were standardized to the geometric mean of GAPDH and TPT1. CNBP values were represented as a ratio of the CNBP/TPT1 standard curves; no effect was observed for alternative methods of standardization). Error bars represent SEM.

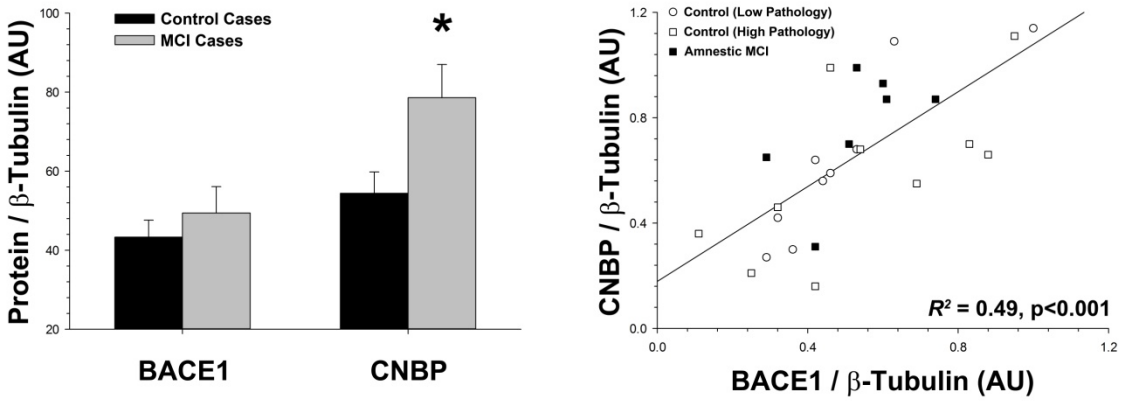


Figure 4.11 CNBP is Increased in Early-Stage AD. PBS extracts from a set of human tissue (SMTG region; Control, low pathology: n = 9; Control, high pathology (PCAD): N = 10, mild cognitive impairment (MCI): n = 7) were run on spot blots and probed for CNBP, BACE1 (Epitomics, EPR3956), or β3-Tubulin (Abcam). Three blots were run for each protein (9 total blots) for consistency and to avoid potential problems with incomplete stripping and re-probing of the same membrane. Protein spots were quantified by densitometry using Scion Image and the median value for each sample was used. β3-Tubulin values did not vary significantly across all samples or blots. Control cases (low pathology and high pathology) were grouped and compared to MCI cases. CNBP was increased in MCI (*left*), widely considered the earliest clinical stage of AD; BACE1 and CNBP were also significantly correlated (*right*) in this case series. * = $p<0.05$. Error bars represent SEM. AU, arbitrary units.

CHAPTER FIVE: CNBP is Induced by Cellular Stress and Increases Cap-Independent Translation of BACE1

INTRODUCTION

We have shown in Chapter Four that CNBP regulates translation of BACE1 and that this may play an important role in the etiology of sporadic Alzheimer's disease. CNBP is a positive regulator of BACE1 expression and increases early in the disease process. These results lead to two additional interesting questions: (1) how is CNBP expression regulated and (2) how might CNBP enhance BACE1 translation? This chapter will begin to address those two questions with the goal of discovering more about the functional role of CNBP in the brain and how it may regulate translation of its targets, specifically BACE1.

Impaired energy production in the brain has been suggested to play a major role in the development of sporadic AD. Positron emission tomography imaging studies revealed lower glucose utilization in AD brains compared to age-matched controls [131, 287-289]. Reduced brain glucose metabolism has also been reported in patients with MCI, indicating one of the earliest events that may occur in the brain leading to disease [290-292]. Importantly, inhibiting energy metabolism *in vitro* increases amyloidogenic APP processing [293-296]. Recently, it was shown that energy inhibition increases translation of BACE1 via the phosphorylation of the eukaryotic initiation factor eIF2α (eIF2α-P) [132, 170], although the mechanism of how this occurs is still unclear.

In addition to glucose deprivation, oxidative stress is another hallmark of the AD brain (reviewed in [297]). Oxidative stress occurs from an imbalance in the production and neutralization of reactive oxygen species (ROS), which are produced during energy

metabolism reactions. The accumulation of ROS in the form of free radicals and peroxides cause damage to DNA, RNA, protein, and lipid molecules in the cell and disrupt normal cellular signaling processes (reviewed in [298]). It is unclear what causes an increase in ROS in AD, but increased oxidative damage occurs in individuals with MCI [299, 300], and suggests a causal role in the disease. Oxidative stress may increase BACE1 expression transcriptionally ([136], reviewed by [301]) or through a post-transcriptional mechanism [171]. Furthermore, a feedback loop between Aβ production, oxidative stress, and secretase (α, β, γ) functions has been proposed [301] which may be evolutionarily conserved [302].

Most transcripts, including BACE1, are believed to be translated in the canonical 5' cap-dependent manner under normal cellular conditions. Cap-dependent translation is achieved through the recognition and binding of the 7-methylguanylate cap by a number of eukaryotic initiation factors and the small (40S) ribosomal subunit which combine to form a 43S pre-initiation complex. This complex then scans the transcript in a 5' to 3' direction in search of the first AUG start codon, where the large ribosomal subunit (60S) is recruited to form the 80S ribosomal complex and initiate translation. However, cap-dependent translation is impaired when cells undergo various forms of stress (reviewed in [303] and [304]). Cells often respond to physiological and pathophysiological stress (*eg.* nutrient deprivation, oxidative stress, viral infection, apoptosis, unfolded protein response, cell cycle transitions) by downregulating global protein synthesis to conserve energy and resources; this is achieved in part by the phosphorylation of eIF2α [304, 305].

Paradoxically, certain transcripts are preferentially translated during stress conditions and BACE1 appears to be one of these. These mRNAs encode proteins that

carry out crucial functions during times of cellular stress and many rely on alternative methods of translation initiation. The most commonly studied method is a cap-independent process that involves direct recruitment of the 40S ribosome close to the initiator start codon through an internal ribosome entry site (IRES) (Reviewed in [305]). IRESs are *cis*-acting structured elements that are usually located in the 5' UTR and were first discovered in picornaviruses, but have since been characterized in other viruses as well as a growing number of eukaryotic cellular mRNAs (reviewed in [306] and [307]). IRES sequences are highly variable and form complex structures, and their presence must be individually verified experimentally. It is unclear how IRES elements directly recruit ribosomes, but it has become apparent that a number of non-canonical initiation factors called IRES *trans*-acting factors (ITAFs) are involved in IRES-mediated translation (reviewed in [308] and [309]). Although there has been no evidence to suggest that the BACE1 5' UTR can support cap-independent translation through an IRES, it displays many features (extremely long, high GC content, multiple uORFs, activated by stress) common amongst other cellular transcripts that do contain proposed IRES elements [303]. Thus, further study is warranted to determine whether BACE1 can support cap-independent translation, especially during times of cellular stress.

To date, there have been no studies on how various cellular conditions affect CNBP expression, although several lines of evidence suggest that CNBP may be involved in stress-induced translational regulation. CNBP has been implicated in the translational regulation of two seemingly unrelated types of mRNAs: 5' TOP containing mRNAs and mRNAs harboring an IRES. However, both of these types of mRNAs are highly regulated by stress conditions which may be a common characteristic of CNBP

targets. In two separate studies, CNBP was described as an ITAF that promoted the IRES-mediated cap-independent translation of ODC1 [204] and also as a nucleic acid chaperone that could catalyze the rearrangement of nucleic acid secondary structures [215]. Interestingly, many other ITAFs have been shown to possess RNA chaperone activity ([310, 311] and reviewed in [312]). These clues indicate that CNBP could be broadly involved in stress-induced translational regulation.

In this chapter, we carried out a set of experiments to probe the possible mechanism for the observed enhanced translation of BACE1 by CNBP. We first used several different cell lines to show that various cellular stresses stimulate CNBP expression. We then used nuclease-EMSAs and circular dichroism (CD) to study properties of RNA structure upon CNBP binding and showed that it is unlikely CNBP enhances translation of BACE1 by decreasing global RNA secondary structure. Finally, we used various dicistronic reporter constructs in both cell culture and cell-free systems to show that increased levels of CNBP may mediate translation of BACE1 via a cap-independent mechanism.

METHODS

Nuclease EMSA

EMSAs were done essentially as described in Chapter Four, with some important additions. For nuclease treated samples, RNase T1 (1 U, Fermentas; for the L4 T1 experiment, the concentration was 20 U to ensure complete digestion), RNase A (1 U, Promega), or RNase V1 (0.1 U, Ambion) were added to the binding reactions for the last 5 minutes to allow nuclease digestion to occur. Samples were run on a non-reducing 5%

TBE polyacrylamide gel. Recombinant GST-$CNBP_{WT}$ was added at a 100:1 (CNBP:probe) molar ratio unless otherwise specified.

Circular Dichroism (CD) Spectroscopy

The entire unlabeled 5' UTR of BACE1 was generated by *in vitro* transcription as described in Chapter Four. Recombinant GST-$CNBP_{WT}$ (10 µM) was added to BACE1 5' UTR RNA probe (50 nM) in a total volume of 200 µL EMSA binding buffer and allowed to incubate for 15 minutes at room temperature prior to CD spectroscopy. CD spectra were recorded over a wavelength range of 250-350 nm with a Jasco-810 spectrapolarimeter using a quartz cell of 1-cm optical path length, and an instrument scanning speed of 50 nm/min with a response time of 8 s. The reported CD spectrum of each sample represented the average of four accumulations taken at room temperature. Blank cuvette and buffer background CD spectra were generated and determined to be free of noise. The spectral contribution of GST-$CNBP_{WT}$ (10 µM) alone was subtracted from the bound spectra.

Stress Experiments (Glucose Deprivation or H_2O_2 Treatment)

H4, HEK293T, or N2a cells were grown in 6-well or 12-well plates to 60-80% confluence as described in Chapter Two. For glucose deprivation experiments, media was removed from all wells and replaced with DMEM media containing 4500 mg/L D-glucose (referred to as high glucose in figures; Gibco) or no glucose (referred to as no glucose, low glucose, or glucose deprivation in figures; Gibco). The plates were incubated at 37ºC for the indicated amount of time, after which the media was removed,

cells were washed with 1× PBS, and lysed in 1× RIPA with PIC+EDTA, followed by brief sonication. Lysates were cleared by centrifugation and total protein was measured using BCA assay. For glucose deprivation experiments in primary cells, rat cortical neurons were harvested as described in Chapter Two and plated in 35 mm dishes at 1.35 million cells / dish. At 7 DIV, neurons were treated for 8 hr in 0.45-µM filter sterilized Locke's solution (5 mM HEPES, pH 7.4, 154 mM NaCl, 5.6 mM KCl, 1 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$) with or without 20 mM D-glucose (Sigma) [170, 313] and then harvested as described previously for biochemical analysis.

To induce oxidative stress, H4 cells were treated with 0, 50, 100, 200, 300 or 500 µM hydrogen peroxide (H_2O_2; Sigma, 30% stock) in fresh DMEM media (no sodium pyruvate, no FBS; Gibco) for 10 minutes at 37ºC. Afterwards, the media was removed and replaced with fresh DMEM media containing no H_2O_2 followed by an additional incubation for 1 hour at 37ºC. At the endpoint, cells were lysed and processed as described above for biochemical analysis.

Dual Luciferase Assay

H4 or HEK293T cells were grown in 6-well plates to 50-70% confluence and transfected with the human ODC1-IRES [205], RP-null, RPh-null, RP-BACE1, or RPh-BACE1 dual luciferase constructs [144] along with pCMV6-XL5 empty vector or pCMV6-XL5 CNBP using FuGene HD as described in Chapter Two. Cells were harvested after 24 hours and dual luciferase assays (DLR Reporter Assay System; Promega) were performed in white opaque 96-well microplates (Costar) according to the manufacturer's instructions using a BioTek Synergy HD plate reader set to monitor luminescence. *In vitro* transcription / translation assays were carried out as described in

Chapter Four (0.5 µg of plasmid and 10 µM GST-CNBP$_{WT}$ were used) and 5 µL of each cell lysate reaction was used for dual luciferase assays. Results were presented as a ratio of Firefly luciferase activity to Renilla luciferase activity compared to appropriate negative controls.

RESULTS

Cellular Stress Increases CNBP Expression

We first sought to answer the question of why CNBP expression may increase in the early AD brain. Since CNBP has been implicated in the translation of two different sets of stress-regulated mRNAs (5' TOP- and IRES-containing) and since BACE1 translation is regulated by various cellular stresses, we hypothesized that cellular stresses common to AD may increase CNBP expression as well. To test this, we performed studies in multiple cell lines and primary neurons grown under conditions of low glucose, mimicking the state of energy deprivation in the AD brain [170]. Glucose deprivation for 2-8 hours and in several cell types lead to a robust increase in both CNBP and BACE1 expression overall (**Fig. 5.1A-C**), and this effect was partially blocked by a shRNA directed against CNBP (**Fig. 5.2**). A breakdown of the results indicated that all cell lines showed increased CNBP and BACE1 expression (clearly visible on Western blots) with glucose deprivation, but only HEK293T (set 2) and primary neurons reached statistical significance (**Fig. 5.1D**). These results reflected inconsistencies in Western blot data, even within the same samples run multiple times, resulting in a large variance. A larger set of samples with more replications would likely result in significant increases for all cell types. It is important to note that BACE1 and CNBP protein levels remained highly

correlated in these experiments as well (**Fig. 5.1E**). Although brief H_2O_2 exposure was also able to induce CNBP expression by up to 2-fold at 500 µM (**Fig. 5.3**), we did not observe a corresponding increase in BACE1 under these conditions. Nevertheless, these results propose a mechanism for increased CNBP levels observed in AD and indicate that prolonged cellular stress, as seen in the progression of AD, may be linked to a pathogenic role of CNBP.

CNBP Does Not Alter BACE1 5' UTR Global Secondary Structure

The high degree of stable secondary structure in the BACE1 5' UTR has been proposed to inhibit BACE1 translation by impeding ribosome scanning [146]. Since CNBP can rearrange nucleic acid secondary structure through its nucleic acid chaperone activity, we first considered the possibility that CNBP may improve BACE1 translation by reducing the global secondary structure of the BACE1 5' UTR. This would likely require CNBP to "coat" the RNA molecule when present in excess quantities, a characteristic that has been attributed to other RNA chaperone proteins [314]. Coating of the RNA molecule by CNBP could theoretically allow for more efficient ribosomal scanning in order for the translational machinery to reach the authentic AUG codon and may also explain why CNBP increased BACE1 protein expression in a dose-dependent manner.

We first evaluated the ability of CNBP to bind the BACE1 5' UTR probe in the presence of various RNA nucleases using EMSA. RNase T1 cuts only after unpaired G nucleotides, RNase A cuts only after unpaired C or U nucleotides, and RNase V1 cuts only double stranded RNA (dsRNA). Since CNBP binds unpaired G nucleotides, we

hypothesized that CNBP should partially protect these sites of the from RNase T1 activity, preserving a shift using EMSA. On the other hand, RNase A was expected to freely digest the RNA regardless of CNBP binding. Finally, if CNBP plays a role in reducing RNA secondary structure by coating the mRNA (binding to most unpaired Gs), then CNBP should retain partial binding ability to the BACE1 5' UTR in the presence of RNase V1.

We first showed that CNBP offered some RNA protection in the presence of RNase T1 using the L4 31-nt probe (**Fig. 5.4A**). Next, we performed EMSAs using the radiolabeled BACE1 5' UTR probe along with recombinant CNBP and RNase T1, A, or V1. Just as with the L4 probe, CNBP was able to partially protect the BACE1 5' UTR from digestion by RNase T1, presumably through binding unpaired G residues (**Fig. 5.4B**). Alternatively, RNase A was able to fully digest the RNA probe, even with CNBP bound, as expected (*data not shown*). Finally, CNBP-bound BACE1 5' UTR RNA was not protected from RNase V1 digestion (**Fig. 5.4C**), indicating that CNBP does not likely unfold global secondary structure of the BACE1 5' UTR at high concentrations.

We attempted to confirm this result through CD spectroscopy measurements of the unlabeled BACE1 RNA probe alone or bound to CNBP, looking for drastic changes in the CD spectra between the two which might indicate a global change in secondary structure. Our results indicated that the CD spectras of free probe and CNBP-bound probe were largely identical (**Fig. 5.4D**), further suggesting that a global unfolding of the RNA molecule is unlikely upon the presence of excess quantities of CNBP.

CNBP Enhances Cap-Independent Translation of BACE1

Recent studies indicated that CNBP directly binds the ODC1 IRES to mediate cap-independent translation, possibly as part of a larger complex of ITAFs [204, 205]. We designed a set of experiments to test the possibility that CNBP regulates cap-independent translation of BACE1 using dicistronic luciferase reporter constructs (**Fig. 5.5**). Dicistronic reporter constructs contain two non-overlapping ORFs separated by a stop codon immediately after the 5' cistron and a linker region (the sequence to be tested) preceding the 3' cistron, and are considered good models to test for cap-independent translation [315]. A hairpin placed at the 5' end of a dicistronic construct inhibits cap-dependent translation of the upstream cistron and is often used to confirm that any activity of the downstream cistron results from cap-independent initiation. The rat BACE1 5' UTR sequence in these constructs is highly similar to the human BACE1 5' UTR [58, 144, 146] and has been confirmed to not contain a cryptic promoter, nor is it cleaved into two monocistronic transcripts [144, 147] which could result in false-positive readings of cap-independent luciferase activity.

Insertion of the BACE1 5' UTR between the two luciferase genes enabled cap-independent translation of firefly luciferase when expressed in HEK293T cells (**Fig. 5.6A**). Importantly, BACE1 5' UTR mediated firefly luciferase expression increased under conditions of glucose deprivation (**Fig. 5.6B**), and when CNBP was overexpressed (**Fig. 5.6C**). We were able to replicate the effect of CNBP on the dual luciferase reporters in an *in vitro* transcription / translation assay (**Fig. 5.6E**). As a positive control for these experiments, we included a dual luciferase construct containing a portion of the ODC1 5' UTR that contains an IRES [205] in place of the BACE1 5' UTR and showed that CNBP

overexpression enhanced cap-independent translation comparable to that observed with the BACE1 constructs. CNBP was overexpressed to similar levels in these experiments, as confirmed by Western blot (**Fig. 5.6D**). Finally, CNBP overexpression did not affect cap-dependent translation of *Renilla* luciferase (**Fig. 5.7**). Taken together, these results suggest that CNBP enhances cap-independent translation of BACE1 possibly through a previously undiscovered IRES element, and that this regulation may be dependent on CNBP protein levels, which are increased by cellular stress.

DISCUSSION

In this chapter, we showed that CNBP can be induced by cellular stressors designed to mimic some aspects of the environment in the AD brain, such as glucose deprivation and oxidative stress. We observed similar results in several cell lines and in primary neurons, indicating a conserved mechanism of regulation. In the glucose deprivation experiments, we did not detect a significant increase in CNBP prior to BACE1 over several time points; pulse-chase experiments over shorter time frames may be useful in determining a more detailed expression profile. Also, even though we showed that H_2O_2 exposure could increase CNBP expression, we did not detect a corresponding increase in BACE1 expression as had been reported previously [171]. The discrepancies between results may be explained by differences in experimental methods (cell lines, treatment conditions, antibodies used) and requires further study.

It is not surprising that CNBP may be involved in stress-induced translational regulation. Numerous RNA binding proteins are involved in important cellular activities under stressed conditions. These include the previously identified ITAFs heterogeneous

ribonucleoprotein (hnRNP) I/PTB (polypyrimidine tract binding protein; [316]), hnRNP E2/PCBP2 (poly r(C) binding protein 2; [317]), La autoantigen [318, 319], and unr (upstream of N-ras; [320]) as well as proteins involved in neurodegenerative diseases such as FMRP [321] and TDP-43 (reviewed in [322]) among others. The roles of these proteins vary from transcriptional and translational regulation to mRNA localization and splicing. CNBP has already been linked to La [202, 230] and PCBP2 [204] in translational regulation and may function as part of a complex of proteins needed for coordinated response to cellular stress. In addition, an analysis of the mouse CNBP promoter [190] indicated several putative binding sites for transcription factors that have been implicated in neuronal stress response such as Sp1 [323], AP-1 (reviewed in [324]), and CHOP-C/EBP [325], which indicate that CNBP transcription may be upregulated by stress. Although we only evaluated protein expression in these stress experiments, RNA analysis from our human tissue experiments in Chapter Four at least hinted that CNBP transcription may change in the AD brain.

CNBP has recently been characterized as a nucleic acid chaperone protein [215]. Nucleic acid chaperones bind single-stranded DNA or RNA and catalyze the rearrangement of secondary structures in an ATP-independent manner, influencing cellular processes such as transcription, translation, splicing, and stability (reviewed in [314]). A role for CNBP chaperone activity in c-Myc transcription has recently been described, in which CNBP was shown to catalyze the formation of G-quadruplex (G4) structures through rearrangement of ssDNA bases [231]. This process used the DNA melting and annealing activities of CNBP; it may be that CNBP could also rearrange the secondary structure of RNA molecules to influence translational regulation.

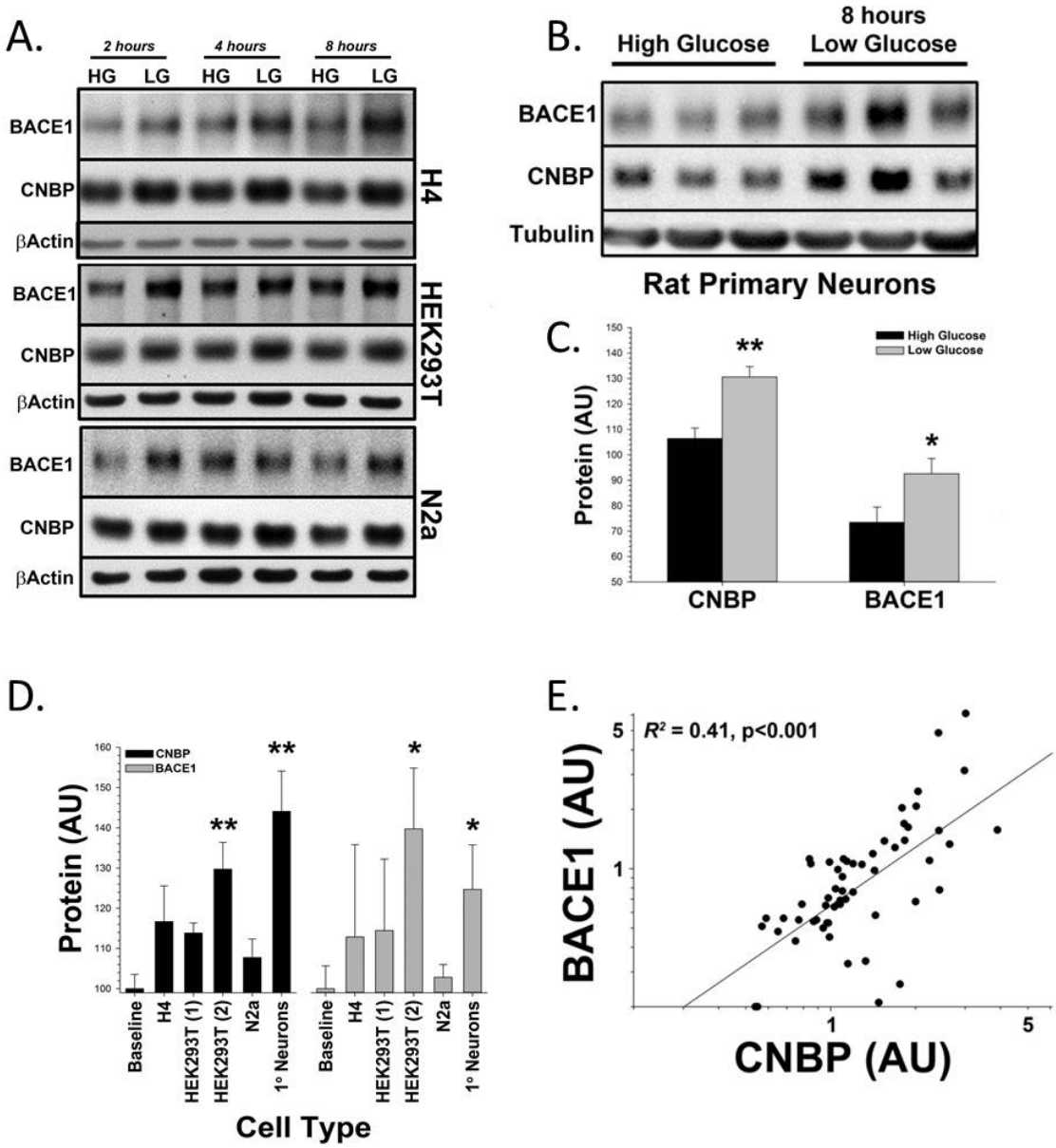
It has been suggested that RNA chaperone proteins, in excess quantities, may "coat" a nucleic acid target, thereby destabilizing global secondary structure (reviewed in [314]). Helix destabilizing assays utilizing various RNA nucleases are typically used to test for this activity. Our nuclease-EMSA and CD results indicated that it is unlikely CNBP reduces global secondary structure of BACE1 mRNA, although it may be that numerous CNBP proteins may associate with the mRNA, depending on the amount of unpaired G's available and the surrounding structured environment. These results do not rule out the possibility that certain cellular conditions or additional cellular factors may be needed to see a global rearrangement of secondary structure, or that CNBP may be involved in rearrangement of local secondary structures to influence translational regulation.

Our results indicated that the BACE1 5' UTR facilitates cap-independent translation and that this activity is enhanced when CNBP levels are increased either directly (by CNBP overexpression) or indirectly (by glucose deprivation). We observed an increase in cap-independent translation by CNBP both in cell culture and in a cell-free system. The ODC1 IRES was used as a positive control for CNBP-mediated cap-independent translation and we saw comparable results with previously published data [204, 205] as well as with our own results for CNBP-mediated cap-independent translation of BACE1. It is interesting to note that ODC1, which provides the rate-limiting activity in polyamine synthesis, is increased after CNS injury [326] and is also increased in AD brain tissue [327, 328] where it shifts to a more cytoplasmic localization [329]. Thus in AD, increased ODC1 may be mediated by CNBP as well.

The results from our dual luciferase experiments makes it tempting to speculate that the BACE1 5' UTR contains a true IRES element. There has been no evidence to support the idea that the BACE1 5' UTR harbors an IRES to support cap-independent initiation of BACE1 translation, although only two groups have looked. Rogers *et al*. reported no evidence of cap-independent translation from a dicistronic luciferase construct containing the rat BACE1 5' UTR expressed in cultured rat cells; however, they did not show any data to support their claim [144]. De Pietri Tonelli *et al*. expressed BACE1 discistronic vectors in SK-N-BE (human neuroblastoma) or HeLa cells and found no evidence of cap-independent translation either, but once again no data was shown [148]. This group also used an MVA-T7pol vaccinia virus system to express their reporter constructs which allows for transcription to occur solely in the cytoplasm; however, it has recently been suggested that a "nuclear experience" may play a role in BACE1 translation, suggesting that cytoplasmic expression systems may not be physiologically relevant [147]. Importantly, both of these studies only looked for cap-independent translation under normal cellular conditions in which cap-dependent translation predominates. Although we did observe cap-independent expression of our BACE1 constructs in unstressed cells, this may be attributed to cell type specific factors or standardization methods. The most important result is that we observed an increase in cap-independent expression above control levels under stressed conditions and when CNBP was overexpressed. It is interesting to note that some limited evidence indicates that the 5' UTRs of both APP [280] and MAPT [281] may contain IRES elements, but CNBP did not bind either of these mRNAs in our RIP experiments (Chapter Four). Although this does not prove the absence of a BACE1 IRES, it does suggest that CNBP

probably does not bind indiscriminately to all IRES elements. Propensity to bind and regulate certain IRES elements but not others is a common trait amongst ITAFs and is most likely dictated by the formation of specific RNP complexes to allow for greater combinatorial control (reviewed in [307]). It is important to note that cap-independent translation and IRES-directed translation initiation are not synonymous. It has recently been proposed that some cellular mRNAs may employ cap-independent translation that is 5' end- and scanning-dependent in the absence of a 5'cap or an IRES, or when eIF4E, which binds the 5' cap and is required for cap-dependent initiation, is inhibited (reviewed in [330]). Therefore, it is plausible that CNBP could direct cap-independent translation of BACE1 in an IRES-independent fashion.

Although the data presented in this chapter begins to describe how CNBP might regulate the translation of BACE1 during the progression of AD, many details are still left unanswered. The next, and final chapter, will serve to provide an overall conclusion to this body of work as well as to discuss some of the interesting questions generated by this study, including specific recommendations for future experiments.


A.
2 hours
4 hours
8 hours
HG
LG
HG
LG
HG
LG
BACE1
CNBP
βActin
H4
BACE1
CNBP
βActin
HEK293T
BACE1
CNBP
βActin
N2a
B.
High Glucose
8 hours
Low Glucose
BACE1
CNBP
Tubulin
Rat Primary Neurons
C.
High Glucose
Low Glucose
Protein (AU)
**
*
CNBP
BACE1
D.
CNBP
BACE1
Protein (AU)
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*
Baseline
H4
HEK293T (1)
HEK293T (2)
N2a
1° Neurons
Baseline
H4
HEK293T (1)
HEK293T (2)
N2a
1° Neurons
Cell Type
E.
R² = 0.41, p<0.001
BACE1 (AU)
CNBP (AU)

Figure 5.1 Glucose Deprivation Increases Both CNBP and BACE1 Expression. Representative Western blots of CNBP and BACE1 (D10E5) expression in (A) cultured cells (H4, HEK293T, N2a) and (B) primary cortical rat neurons over various time points under conditions of glucose deprivation (low glucose) (HG = high glucose, LG = low glucose). (C) Overall, there was a significant increase in both CNBP ($F_{1,64} = 17.0$, $p<0.1$) and BACE1 ($F_{1,64} = 6.4$, $p<0.02$) expression in cultured cells subject to glucose deprivation up to 8 hours. (D) Breakdown of effects of glucose deprivation in multiple cell types (5 independent experiments; replicates in brackets, ½ grown in high glucose conditions): H4 (n = 8), HEK293T (experiment 1, n = 8), HEK293T (experiment 2, n = 36), N2a (n = 8), rat primary neurons (n = 6); Baseline = high glucose measurements. Dunnett's test, * = $p<0.05$, ** = $p<0.01$. (E) CNBP and BACE1 were also strongly correlated in these experiments. Results were unchanged when standardized to loading control (β-Actin, GAPDH, β3-Tubulin) levels. Error bars represent SEM. AU, arbitrary units.

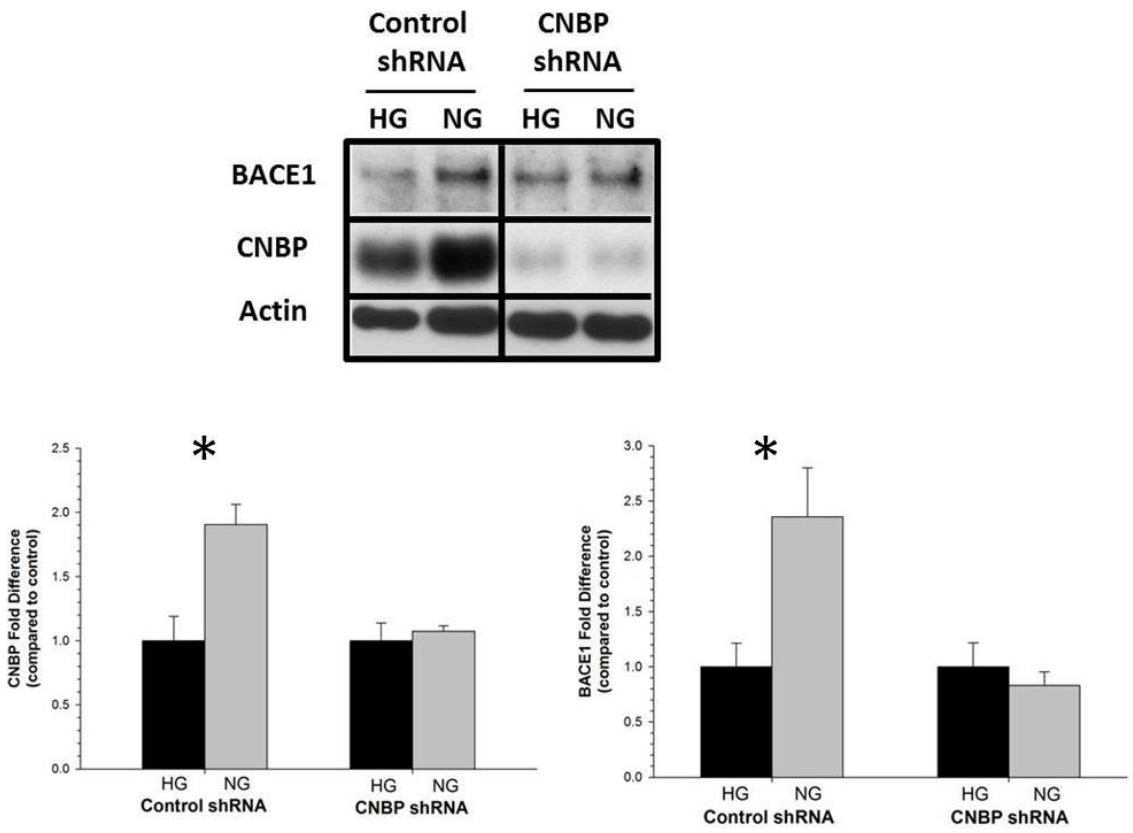

Figure 5.2 CNBP May Mediate Stress-Induced BACE1 Protein Expression. Stable expression of a shRNA directed against CNBP in H4 cells at least partially blocked the effects of glucose deprivation (2 hour glucose deprivation) on BACE1 expression relative to the control shRNA vector (n = 3); *t*-test, * = $p<0.05$. HG = high glucose, NG = no glucose (glucose deprived). Error bars represent SEM.

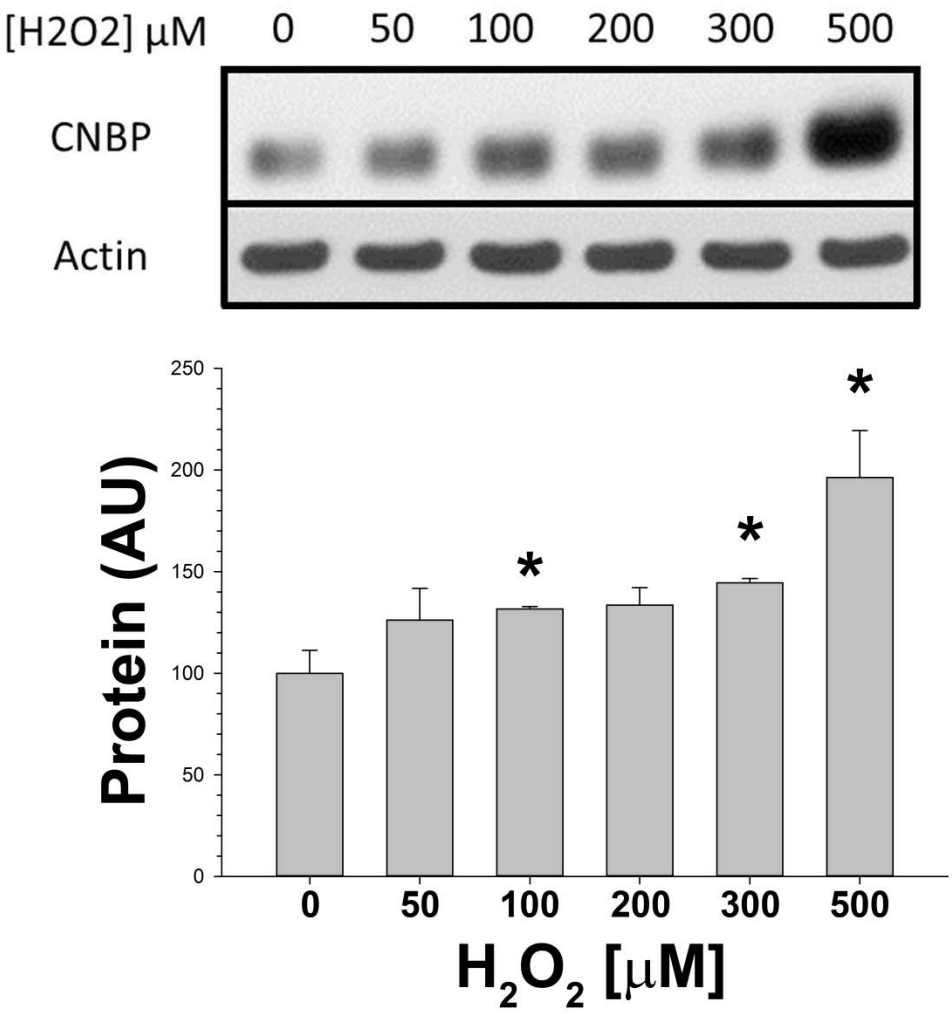

Figure 5.3 H_2O_2 Increases CNBP Protein Expression. Brief (10 minute) exposure to H_2O_2 followed by a 1 hour incubation lead to increased CNBP expression ($F_{5,12}$ = 4.3, $p<0.02$) in H4 cells (n = 2-5 replicates / concentration) in a dose-dependent manner, although we did not detect a change in BACE1 in this cell type under these conditions; Dunnet's test, * = $p<0.05$. Error bars represent SEM. AU, arbitrary units.

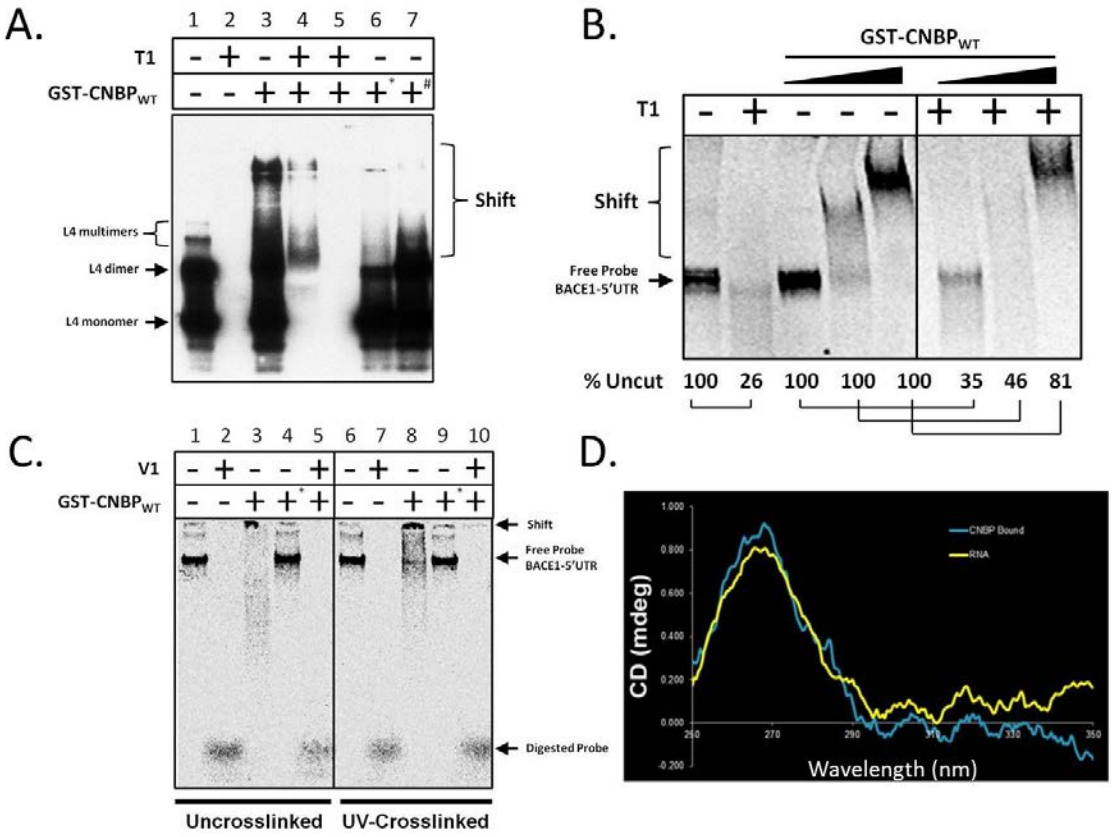
A.
1 2 3 4 5 6 7
T1
GST-CNBP$_{WT}$
Shift
L4 multimers
L4 dimer
L4 monomer
B.
GST-CNBP$_{WT}$
T1
Shift
Free Probe
BACE1-5'UTR
% Uncut 100 26 100 100 100 35 46 81
C.
1 2 3 4 5 6 7 8 9 10
V1
GST-CNBP$_{WT}$
Shift
Free Probe
BACE1-5'UTR
Digested Probe
Uncrosslinked
UV-Crosslinked
D.
CNBP Bound
RNA
CD (mdeg)
Wavelength (nm)

Figure 5.4 CNBP Binding has Minimal Effects on Global BACE1 RNA Secondary Structure. (A) Digestion of bound and unbound L4 probe (the CNBP positive binding control) with RNase T1 indicates partial protection of the probe (Lane 1, L4 probe; Lane 2, T1 digested L4 probe; Lane 3, L4 bound to CNBP; Lane 4, partially protected L4 probe; Lane 5, CNBP added after digestion does not bind the L4 probe; Lane 6, CNBP treated with excess EDTA (*) reduces binding to the L4 probe; Lane 7, heat denatured CNBP ($^{\#}$) reduces binding to the L4 probe). Molar ratio of CNBP:probe was 100:1. (B) BACE1 5' UTR similarly shows partial protection from RNase T1 digestion (note increase in % uncut with increasing CNBP added); this is consistent with CNBP binding regions of unpaired G's [331]. Molar ratios of CNBP:probe were 20:1, 150:1, and 750:1. CNBP binding did not offer any protection against RNase A digestion (RNase A cuts after C/U; *not shown*). (C) CNBP binding does not protect against RNase V1, which digests dsRNA (* = EDTA and heat treated CNBP); this suggests that CNBP binding does not significantly reduce the amount of dsRNA within the BACE1 5' UTR. Molar ratio of CNBP:probe was 100:1. (D) CNBP binding did not have a striking effect on the CD spectra of the BACE1 RNA.

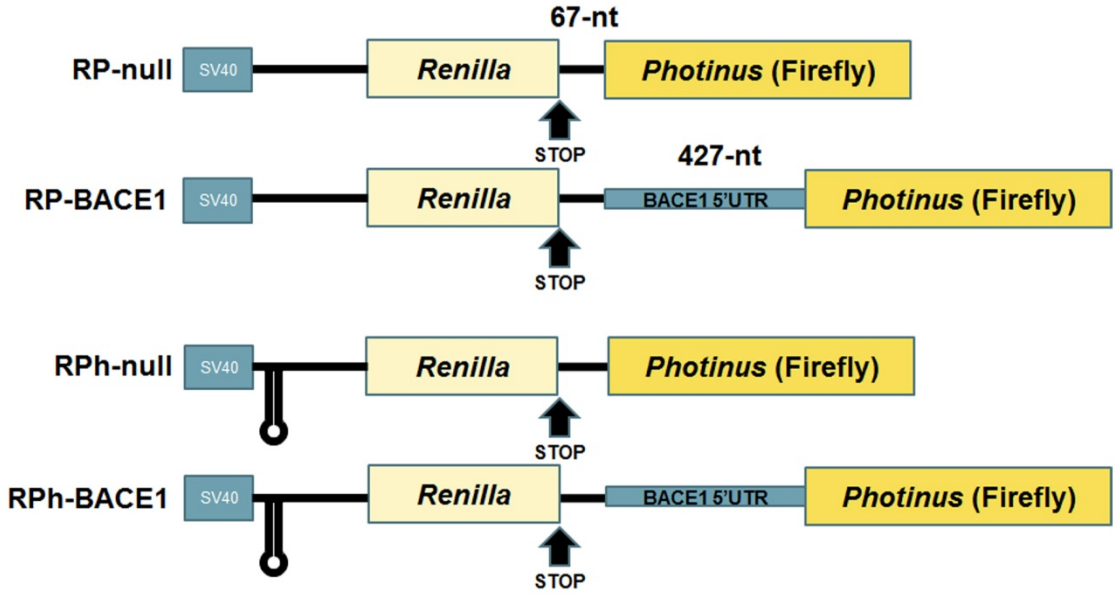

Figure 5.5 Schematic of Dual Luciferase Constructs. Dual luciferase constructs [144] contained an SV40 promoter with (RPh) or without (RP) a 5' hairpin to block cap-dependent translation, and with or without the BACE1 5' UTR inserted between the two luciferase genes. Since a STOP codon follows the *Renilla* luciferase gene, and since the BACE1 5' UTR remains intact and does not contain a cryptic promoter [144, 147], the *Photinus* (Firefly) luciferase can only be translated into active enzyme via a cap-independent mechanism. In these constructs, the rat BACE1 5' UTR is used, which is 427-nt long with four uAUGs (three uORFs) and is highly conserved with the human BACE1 5' UTR [58, 144, 146], indicating a conserved regulatory role.

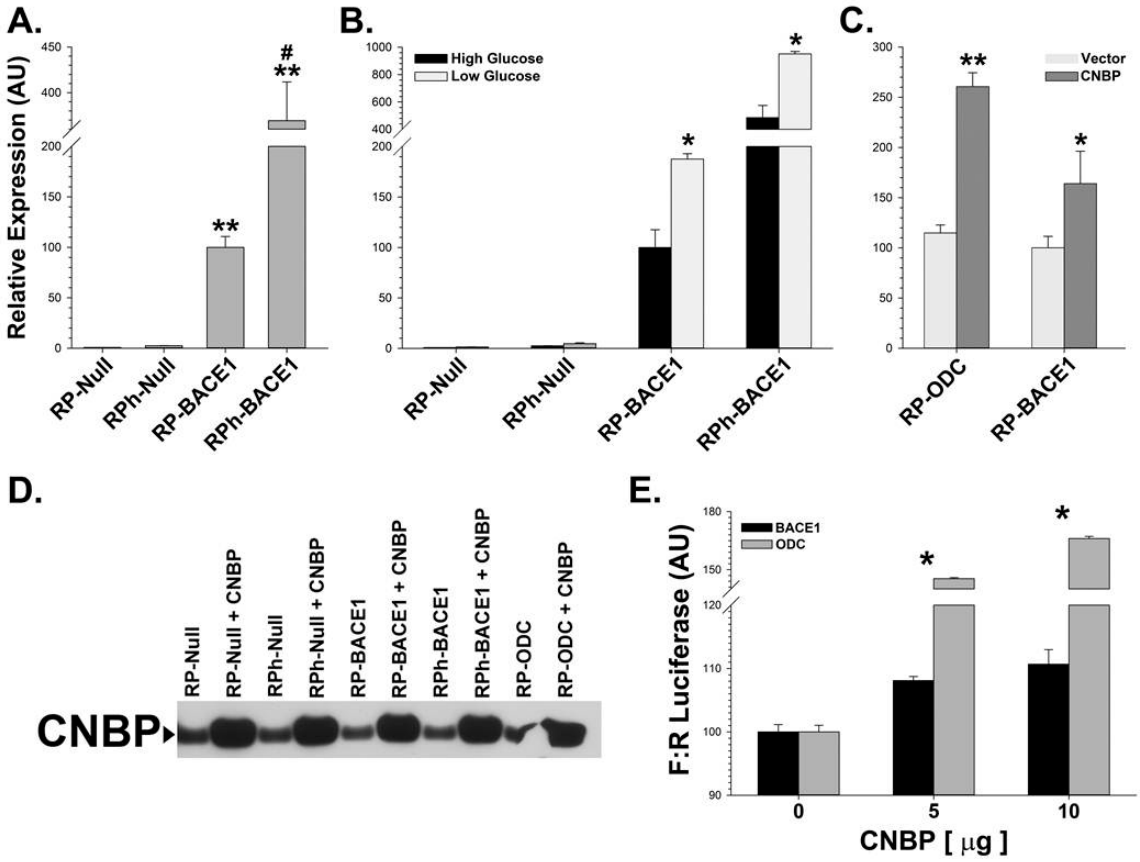
A.
Relative Expression (AU)
RP-Null
RPh-Null
RP-BACE1
RPh-BACE1
B.
High Glucose
Low Glucose
C.
Vector
CNBP
RP-ODC
RP-BACE1
D.
CNBP
RP-Null
RP-Null + CNBP
RPh-Null
RPh-Null + CNBP
RP-BACE1
RP-BACE1 + CNBP
RPh-BACE1
RPh-BACE1 + CNBP
RP-ODC
RP-ODC + CNBP
E.
BACE1
ODC
F:R Luciferase (AU)
CNBP [μg]

Figure 5.6 CNBP Drives Cap-Independent Translation of BACE1. (A) The BACE1 5' UTR showed cap-independent translation compared to the null constructs (since cap-dependent translation is partially blocked in the RPh-BACE1 construct, the relative increase in expression is larger). (B) Glucose deprivation further increased the relative magnitude of cap-independent expression. (C) CNBP overexpression caused a similar increase in RP-BACE1 cap-independent translation as compared to a known positive control, the ODC1 5' UTR. (D) CNBP overexpression in panel C was comparable for all constructs by Western blot. (E) CNBP increased relative cap-independent translation (F:*R* = Firefly:*Renilla* expression) of the BACE1 RPh vector and the hODC1-IRES vector in an *in vitro* transcription / translation system in a concentration dependent manner. *t*-test, * = $p<0.05$, ** = $p<0.01$; # = $p<0.01$, relative to RP-BACE1. In panels A, B, and C, the F:*R* expression ratios are shown for each construct standardized to RP-BACE1 which was set to 100. In panel E, F:*R* ratios are shown standardized to the 0 µg CNBP samples. Error bars represent SEM. AU, arbitrary units.

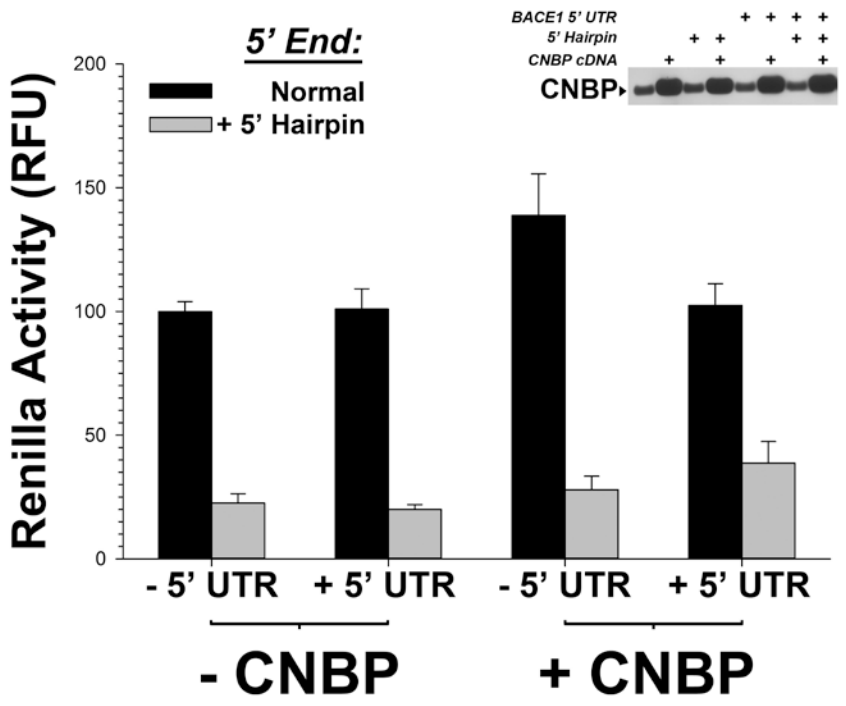

Figure 5.7 *Renilla* Activity is Not Affected by the BACE1 5' UTR or CNBP. As expected, the 5' hairpin was an exceptionally strong suppressor of cap-dependent *Renilla* expression (t_{46} = 19.1, p<0.0001). Insertion of the BACE1 5' UTR in between the *Renilla* and firefly luciferase genes had no impact on overall expression (p<0.7). CNBP overexpression (*insert:* blot indicating matched levels of expression) also had no effect on the *Renilla* expression from any of these constructs (p<0.3), indicating that CNBP did not have a generic effect on cap-dependent translation (2 independent experiments were performed in HEK293T cells, n = 6 replicates / construct, ½ with CNBP overexpression). Firefly luciferase expression (see **Fig 5.6**) was determined from the same experiments. Data are shown standardized to RP-BACE1, set to 100. Error bars represent SEM. RFU, relative fluorescent units.

CHAPTER SIX: Summary and Future Directions

SUMMARY

In Chapter Three, we evaluated the expression of BACE1 and the related enzyme BACE2 in the brain tissue of a cohort of human cases ranging from early AD (PCAD, MCI), to late-stage AD, as well as several FTD cases. We showed that BACE1 protein and activity are increased in the earliest stages of AD, indicating a possible causal role in the development of disease. These changes occurred in a disease affected region (the SMTG) of the brain, but not in a disease unaffected region (CB). An increase in BACE1 preceded significant accumulation of Aβ in the progression of AD, and we observed increased BACE1 in FTD cases as well which do not accumulate Aβ; this initial rise in BACE1 may be a response to enhanced cellular stress. We found similar results with BACE2 and showed that BACE1 and BACE2 expression are highly correlated in disease, suggesting a possible shared mechanism of regulation. A role for BACE2 in AD is currently unknown. In this study, BACE1 was weakly correlated with soluble Aβ measures and may not be a good predictor for amyloid load. Importantly, we verified that BACE1 protein is increased in the absence of changes to mRNA levels, suggesting that a post-transcriptional method of regulation is altered in disease.

Previously, our lab identified the RNA binding protein, CNBP, as a potential regulator of BACE1 expression (MPM, unpublished data). In Chapter Four, we presented evidence that confirmed CNBP is a positive regulator of BACE1. Overexpression of CNBP in cell culture models and in primary neurons increased BACE1 protein; knockdown of CNBP decreased BACE1 protein levels variably, possibly through decreased transcript stability. We were also able to stimulate BACE1 translation in a cell-

free system using coupled *in vitro* transcription / translation by spiking in recombinant CNBP; these results indicated direct regulation by CNBP. Interestingly, in both cell and cell-free systems, CNBP increased BACE1 protein levels in a concentration-dependent manner. We showed that CNBP overexpression did not increase BACE1 transcription, further suggesting a primary role in translation. This was verified by checking for alterations in BACE1 mRNA or protein half-life after CNBP overexpression; in these experiments no changes were observed (MPM, RLW; unpublished data).

The results from our RNA immunoprecipitation experiments showed that CNBP associated with BACE1 mRNA at endogenous concentrations and that CNBP bound directly to the BACE1 5' UTR *in vitro* using EMSA. Furthermore, the BACE1 5' UTR seemed to be necessary for an increase in CNBP-mediated protein production in an *in vitro* transcription / translation system. These studies further indicated that direct binding of CNBP to the BACE1 5' UTR may be needed to carry out its regulation. Finally, we looked at CNBP and BACE1 expression in the brain in a cohort of AD and age-matched control cases. Both proteins were increased in AD in the SMTG, but decreased in the CB. No significant changes in BACE1 or CNBP mRNA levels were observed between control and AD cases; however, CNBP mRNA levels trended towards predicting protein levels. We extended our studies to several early-stage AD cases and found that CNBP protein levels were significantly increased in MCI cases; significant BACE1 elevation was not observed in these cases, indicating that increased CNBP levels in the brain likely precede increased BACE1 levels, as we would predict. Importantly, in all of our human cases, CNBP and BACE1 proteins were significantly positively correlated.

In Chapter Five, we asked the question of why CNBP expression might increase in the progression of AD. Since BACE1 translation is increased by cellular stress and since known CNBP mRNA targets also tend to be regulated in this fashion, we speculated that cellular stresses common to AD might increase CNBP as well. Indeed, we found that both glucose deprivation and H_2O_2 exposure (to induce oxidative stress) increased CNBP expression in cell cultures and in primary neurons. This indicated that CNBP might be a critical regulator of BACE1 protein translation during times of enhanced cellular duress.

Finally, we wondered how CNBP might enhance BACE1 translation. Since little was known about how CNBP regulates translation of any of its targets, this was a daunting task. However, it was recently discovered that CNBP binds to an internal ribosome entry site in the ODC1 5' UTR to activate cap-independent translation [204]. Cap-independent translation is often employed by stress response proteins during times of heightened cellular strain when traditional cap-dependent translation is inhibited. Although cap-independent translation had never been observed for BACE1 before, its 5' UTR is typical of other cellular transcripts that do support this alternative method of translation initiation, usually through an IRES element. We tested the ability of the BACE1 5' UTR to support cap-independent translation in HEK293T cells using dual luciferase reporter constructs. Our results indicated that the BACE1 5' UTR could support cap-independent translation and most importantly that this activity was increased by CNBP either directly (through CNBP overexpression) or indirectly (through glucose deprivation). The BACE1 5' UTR also mediated cap-independent translation in a CNBP concentration dependent manner in a cell-free *in vitro* transcription / translation assay.

Taken together, these results indicate that cellular stress may increase CNBP protein levels which in turn enhance cap-independent translation of BACE1.

The goal of this project was to determine if the RNA binding protein, CNBP, is a regulator of the Alzheimer's disease β-secretase protein, BACE1, and if so, how this regulation might occur. The results presented in this thesis, as a whole, begin to shape a compelling story for the involvement of CNBP as a novel translational regulator of BACE1 with important implications for the development of sporadic AD (**Fig 6.1**). The data presented in this thesis complement previously published literature in the fields of BACE1 expression and CNBP molecular function, yet also propose interesting and novel insights that advance both fields of research. Although much has been learned from these studies, many questions remain to be answered.

CONCLUSIONS and FUTURE DIRECTIONS

CNBP Interactions with the BACE1 5' UTR

In Chapter Four, we showed that CNBP associated with BACE1 mRNA at endogenous concentrations in cell lysates through RNA immunoprecipitation experiments and that CNBP bound directly to the BACE1 5' UTR using EMSA and *in vitro* transcription / translation experiments. These studies indicated that direct binding of CNBP to the BACE1 5' UTR may be needed to carry out its regulation. Future experiments will be conducted to try and discern the exact location or locations where CNBP *functionally* binds to the BACE1 5' UTR. Since CNBP may bind to numerous unpaired Gs along the 5' UTR, truncated 5' leader sequences linked to a reporter gene could be used to determine which regions are necessary for CNBP-mediated translational

regulation. Alternatively, constructs in which the various uAUGs have been mutated could be used in the same manner. Many of these constructs have already been made by other labs and may be available for future studies [143-146].

CNBP is the first known *trans*-acting protein implicated in regulating BACE1 translation through the BACE1 5' UTR. Given the length and structural complexity of the BACE1 5' UTR, it is surprising that no one has reported other proteins that interact with this region. In the future, it will be important to determine if CNBP acts as part of a complex of proteins, as expected, to regulate BACE1 translation. Tandem affinity chromatography followed by mass-spectrometry analysis may be used to determine other RNA binding proteins that interact with the BACE1 5' UTR. In this manner, the BACE1 5' UTR could be used to "fish" out interacting proteins from a tissue or cellular extract, which could then be identified using mass spectrometry methods. A similar method was used to identify CNBP and PCBP2 bound to the ODC1 IRES element [204]. It would be particularly helpful if a narrow functional CNBP binding region of the BACE1 5' UTR was identified first.

CNBP and the Phosphorylation of eIF2α

It has been reported that phosphorylation of eIF2α (eIF2α-P) increases BACE1 translation after glucose deprivation [170] or oxidative stress [171]. We have shown that CNBP is involved in the stress-induced translational regulation of BACE1 and this suggests a link between CNBP and eIF2α-P (**Fig. 6.2**). Our results indicate that cellular stress increases CNBP protein levels and enhances cap-independent translation of BACE1, possibly through an IRES. IRES-mediated translation of some transcripts

requires phosphorylation of eIF2α [332], but can be active independent of eIF2α-P as well [333]. Therefore, it is tempting to speculate that upon phosphorylation of eIF2α during stress conditions, increased levels of CNBP enhance cap-independent translation of BACE1. CNBP could accomplish this by direct recruitment of ribosomes to the mRNA as part of an ITAF complex or through rearrangement of local secondary structures using its RNA chaperone activity. We did notice a potential block in the stress-induced increase of BACE1 in our CNBP shRNA pooled stable cells, indicating that CNBP may be critical for this process. However, in order to definitively determine if CNBP is a required *trans* factor responsible for increasing BACE1 translation during cellular stress, we would need to inhibit or completely knock out CNBP expression *in vivo*. Under these conditions, induction of cellular stress and eIF2α-P should not cause an increase in BACE1 protein. Unfortunately, there are no known inhibitors of CNBP and CNBP knockout cells do not exist, making this hypothesis difficult to test. It has been proposed that CNBP mutants lacking the RGG region act as dominant negatives *in vivo* [194]; these mutants could be useful for studying specific CNBP molecular functions in the future.

The phosphorylation of eIF2α, and subsequent reduction in levels of the eIF2-GTP/Met_i-tRNA pre-initiation complex, has been proposed to enhance ribosomal bypass of BACE1 uORFs through decreased reinitiation events [170]. Thus, a higher proportion of small ribosomal subunits may reach the authentic BACE1 start codon to initiate translation. It may be that CNBP plays a role in this hypothesis by enhancing ribosomal read-through of uORFs upon eIF2α-P (**Fig 6.2**). This could occur after cap-dependent or cap-independent initiation. In this model, CNBP may interact directly with the small

ribosomal subunit to enhance scan-through of uORFs; this may be mediated by CNBP's RNA chaperone activity which could remove impeding secondary structures. Alternatively, CNBP may simply act as a tether, keeping the 40s subunit associated with the mRNA longer. The evidence that CNBP associates with both RNA and proteins of the ribosomal subunits strengthens this proposed model. Finally, increased CNBP levels may mask sites near the uAUGs, allowing for more efficient ribosomal scan-through. In these hypothetical models, CNBP may be critically important for scanning and reinitiation events of BACE1 and other targets with uORFs. Much more work will be needed to clarify the link between CNBP and eIF2α-P during cellular stress and whether they are involved in the same mechanism of translational regulation or separate mechanisms altogether.

Additional CNBP Molecular Targets – RNA and Protein

A secondary aim of this project was to discover other mRNAs that may be functional targets of CNBP, either in muscle or in brain, with implications for involvement in myotonic dystrophy type 2 (DM2) or neurodegenerative disease, respectively. RNA purification followed by high-throughput sequencing analysis (RNA-Seq) is the most current and advanced method for analyzing a specific pool of RNAs (reviewed in [334]). RNA-Seq has been used to identify targets of other RNA binding proteins including TDP-43 [284, 335] and polycomb proteins [336]. RNA-Seq allows for the identification of individual RNA (mRNAs and non-coding RNAs) targets as well as providing specific sequence information which may shed light on consensus binding regions [337]. Our results indicate that RNA immunoprecipitation of CNBP may be

feasible for carrying out a RNA-Seq analysis if the experimental conditions can be further optimized. Ultimately, we anticipate that CNBP should pull down a large pool of mRNA targets given its broad binding site requirements; any interesting targets would need to be validated by expression studies.

There has been one published report of identified targets and a consensus binding site for the yeast protein Gis2p, an ortholog of human CNBP, using tandem affinity purification followed by DNA microarray analysis (RIP-Chip) [206]. This study identified hundreds of mRNAs with stretches of G(A/U)(A/U) trinucleotide repeats associated with Gis2p that included ribosomal RNA processing factors and muscle-related genes. We looked for this consensus sequence in the BACE1 5' UTR but were unable to find a match. It is important to note that Gis2p lacks the RGG domain between the first and second zinc fingers, which plays a major role in RNA binding [194]. Therefore, CNBP likely recognizes an overlapping set of targets with Gis2p, but possibly many more.

We selectively evaluated several transcripts as potential binding targets of CNBP using crosslinked RIP followed by real-time quantitative PCR. Along with several positive controls (RPS17, ODC1) and BACE1, we identified β-Actin and BIN1 mRNAs as potential targets of CNBP. Like BACE1, neither β-Actin nor BIN1 are known to harbor an IRES or 5' TOP structure in their 5' UTRs, and indicate that CNBP's recognition of mRNA targets may be much broader than initially thought. BIN1 is an especially interesting target given that it has been linked to both AD [277] and DM2 [279]. Preliminary experiments in our lab have indicated that BIN1 expression and/or splicing may be altered in AD (CJH, unpublished data). Future experiments should be

carried out to determine if CNBP affects BIN1 expression. In addition to RNA immunoprecipitation strategies, our lab is currently trying to identify CNBP targets through targeted overexpression or knockdown of CNBP *in vivo* in mouse brain and/or muscle using proteomics and RNA-Seq strategies.

Along with identifying mRNA targets of CNBP, it will be critical to learn more about proteins that interact with CNBP in order to determine whether it primarily functions as part of a larger RNP complex, as suspected. Thus far, there have been few studies that have identified possible CNBP-interacting proteins. A large-scale study of protein-protein interactions in human cells using a mass spectrometry-based approach identified some 40 proteins that interact with CNBP [209]. Among them were numerous ribosomal proteins (both small and large subunit), heterogeneous ribonucleoproteins (hnRNP C, A3, R, UL1), other RNA binding proteins (PURA, SYNCRIP), and various factors involved in RNA processing. The Saccharomyces Genome Database (SGD), which compiles data from large-scale yeast interactome analyses, suggests that Gis2p interacts with at least 28 other RNA binding proteins including translation initiation factors (Ygr054p; ortholog of eIF2A), cytoplasmic ATP-dependent RNA helicases (Dbp1, Dbp2, and Dhh1), and Lsm1 and Lsm6, which associate with the Pat1 decapping enzyme and the Xrn1 exoribonuclease involved in mRNA degradation [206]. A more focused study using tandem affinity purification identified CNBP and the previously characterized ITAF, PCBP2, as direct, functional binding partners of the ODC1 IRES sequence [204]. This study also identified many other ITAFs and RNA binding proteins as possible CNBP-interacting proteins, but none have been validated. Taken together, these studies suggest that CNBP is likely involved in various layers of post-

transcriptional regulation and may function in a complex combinatorial fashion with numerous other proteins.

Dual Roles of CNBP Translational Regulation

CNBP appears to control translation of a diverse set of mRNAs. These targets are involved in global protein translation (5' TOP-containing mRNAs) as well as specialized translation (IRES-containing mRNAs). In our studies, we did not observe a consistent striking alteration in total protein levels when CNBP was overexpressed or knocked down (CJH, unpublished data). In general, overexpression slightly reduced total protein levels and knockdown slightly increased total protein levels. A common theme amongst CNBP targets may be stress-induced regulation. Interestingly, amino acid starvation causes sequestration of 5' TOP mRNAs to stress granules [338], where translation is arrested at the initiation step, keeping global protein synthesis low [339]. Conversely, many IRES-containing mRNAs are preferentially translated during times of cellular stress (reviewed in [340]). Based on these data, it is tempting to speculate that during normal cellular conditions, CNBP mediates cap-dependent translation of one set of RNAs (e.g. 5' TOP mRNAs), but when cells become stressed, CNBP shifts to a primary role in cap-independent translation of a separate set of mRNAs (IRES-containing). Future studies focused on these two sets of mRNAs may confirm this hypothesis.

CNBP Structure and Function

There is still much to be learned about the CNBP protein itself. For example, what post-translational modifications does it undergo? Is it phosphorylated, and if so, what are the functional consequences? What is the significance of CNBP binding to RNA as a

dimer, as has been suggested [215], and do preformed pools of CNBP monomer and dimer exist? What are the important domains that mediate protein-protein interactions? Also, does CNBP get proteolytically cleaved at the PEST site in human tissues as described for other species, and how would this affect CNBP's cellular functions? Answering these questions will help shape our understanding of how CNBP functions in the cell and how it may contribute to human diseases such as myotonic dystrophy type 2 or Alzheimer's disease.

Altered CNBP Expression Levels as a Contributing Factor to Disease

CNBP has already been implicated in DM2 [178], and the results from our studies now suggest that CNBP may be involved in the development of sporadic AD as well. Interestingly, these two diseases may be linked to the expression level of CNBP, albeit in opposite directions. In DM2, haploinsufficiency of CNBP protein may play a role in the phenotype of the disease [182]. Our CNBP knockdown studies indicate that CNBP may be required at a threshold concentration for target transcript stability, as was indicated with BACE1. Further experiments are needed to see if CNBP knockdown decreases BACE1 mRNA half-life, which would help confirm a role in target transcript stability. Currently, our lab is working on expressing CNBP shRNA constructs in mouse quadriceps and gastrocnemius muscles using a lentiviral delivery system with the goal of discovering potential CNBP targets.

The results from this study indicate that an overexpression of CNBP may have pathogenic consequences as well. CNBP likely regulates the expression of many proteins and has been implicated in the control of cell proliferation and cell death (reviewed in

[224]). Thus, sustained elevated levels of CNBP, as we predict occurs in the progression of AD, could have dire consequences on global protein expression. Determining what proteins are altered upon CNBP overexpression will be critical in determining the extent to which CNBP may play a role in disease processes. These results, as a whole, imply that drastic perturbations in CNBP protein from normal levels may have pathogenic consequences for both DM2 (decreased levels) and AD (increased levels).

CNBP Mouse Model

We have clearly showed that CNBP affects BACE1 translation and this may have implications for sporadic AD. However, a more definitive way to link CNBP to a causal role in the development of AD would be to carry out experiments using a transgenic mouse model. For example, crossing a mouse line that overexpresses CNBP specifically in neurons with a well characterized mouse model of AD (for instance, the Tg2576 mouse model, which expresses the APPΔNL mutation [341]) would allow us to determine if CNBP overproduction increases Aβ production and deposition *in vivo,* presumably through increased BACE1 expression, but possibly through other unknown mechanisms as well. There are no mouse models that overexpress CNBP at this time; however, our lab has tried to overexpress CNBP in the brain of newborn mice using various viral delivery systems. Thus far, we have been unsuccessful in achieving CNBP overexpression *in vivo* (RLW, unpublished data); it may be that CNBP expression is tightly regulated at this stage since it has such critical functions in development. Thus, an inducible expression model may be necessary to overexpress CNBP at later ages.

Therapeutic Implications

We observed a moderate increase in CNBP protein levels in the progression of AD which may be linked to pathogenic consequences. Our results suggest that targeted knockdown of CNBP would be needed as a therapy for AD; however, reduction of CNBP by as little as 50% results in numerous problems associated with a DM2 phenotype [182]. Therefore the best strategy would be to employ a partial knockdown of CNBP, with an aim in returning protein concentrations to near basal levels. We have observed 20-30% knockdown of CNBP levels in cell culture using a lentiviral shRNA construct against CNBP (MPM, unpublished data); this may be sufficient to test as a therapy *in vivo*. We still need to learn more about CNBP's targets and its molecular functions in the cell in order to determine if it could be a suitable therapeutic target.

Another possible therapeutic intervention would be to block the interaction between CNBP and BACE1 mRNA. Of course, this would require the identification of a specific region or sequence that CNBP binds to, such as an IRES element, if one exists. Specifically, this strategy could prevent cap-independent translation of BACE1 (but possibly many other stress response proteins as well). Translational regulation is already a major therapeutic target in cancer [342, 343] and inhibition of cap-independent translation may be a novel avenue for the treatment or prevention of AD. In support of this, there is evidence that the predominant Alzheimer's disease drug, memantine, may act as an inhibitor of IRES-mediated translation initiation [344] which could help block the expression of APP, tau, and potentially BACE1. At this time, we are still a ways off from determining if CNBP and/or its molecular pathways could be suitable therapeutic targets for treatment of AD, but our results indicate that it is a an intriguing possibility.

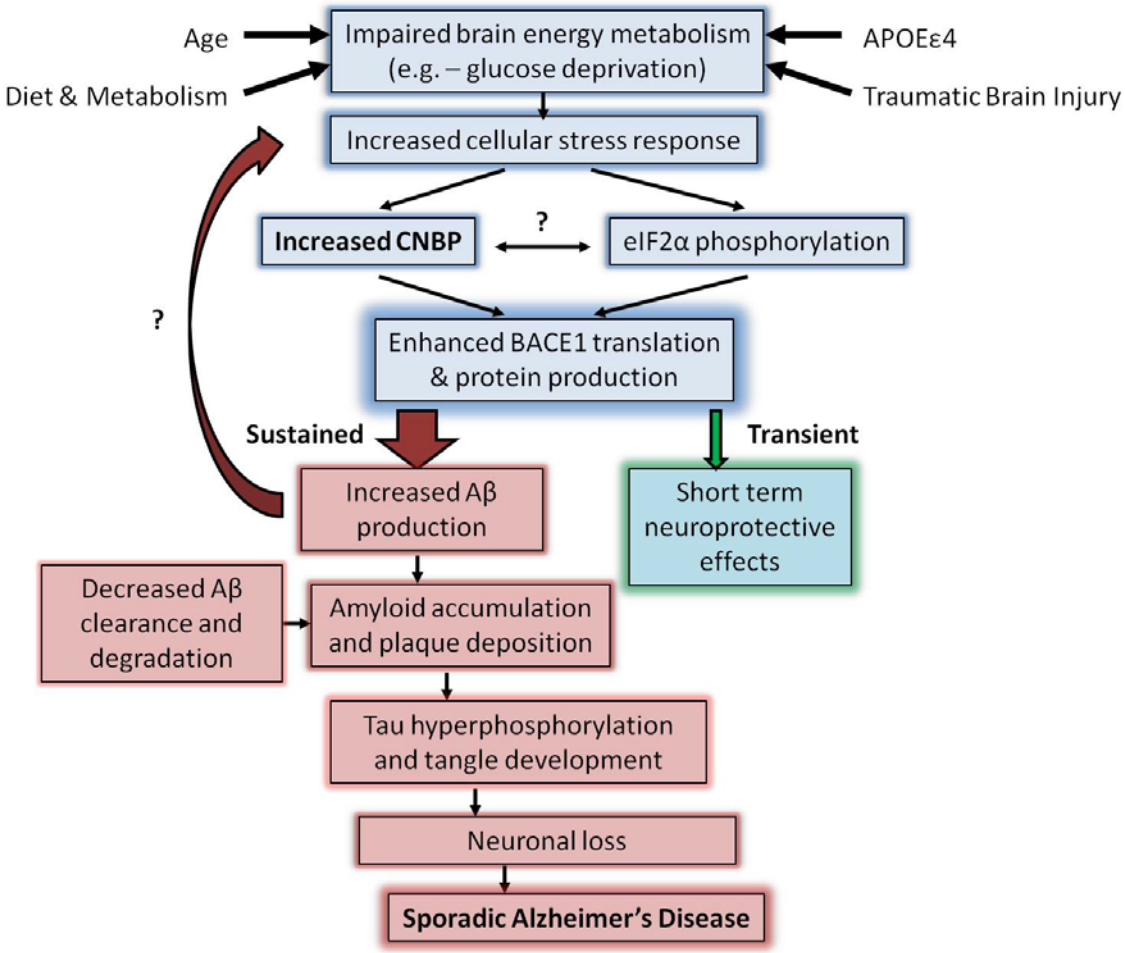
Age
Diet & Metabolism
Impaired brain energy metabolism
(e.g. – glucose deprivation)
APOEε4
Traumatic Brain Injury
Increased cellular stress response
Increased CNBP
?
eIF2α phosphorylation
?
Enhanced BACE1 translation
& protein production
Sustained
Transient
Increased Aβ
production
Short term
neuroprotective
effects
Decreased Aβ
clearance and
degradation
Amyloid accumulation
and plaque deposition
Tau hyperphosphorylation
and tangle development
Neuronal loss
Sporadic Alzheimer's Disease

Figure 6.1 Model of CNBP's Proposed Role in AD. In this generalized model, various AD risk factors such as age, diet and metabolism, ApoE4 genotype, or traumatic brain injury lead to gradual impairment in brain energy utilization beginning years or decades before onset of disease. Over time, reduced energy availability in the brain activates the stress response pathway in cells which upregulates CNBP expression and also causes the phosphorylation of eIF2α. This combination of events might work in tandem to enhance BACE1 translation though an unresolved mechanism. A transient increase in stress response proteins, such as BACE1, likely has neuroprotective effects. However, if stressed conditions persist, BACE1 levels may become chronically elevated. Over many years during the progression to AD, even a small increase in BACE1 could have significant impact on amyloid production. Increased Aβ levels may contribute to a feedback mechanism in which cells are further stressed and more BACE1 is generated. These events, coupled with impairments in amyloid clearance and degradation, lead to enhanced Aβ deposition and plaque formation in the brain. At some point, a threshold level of Aβ accumulation is reached that initiates downstream pathology including tau hyperphosphorylation, tangle formation, and neuronal loss, eventually leading to sporadic Alzheimer's disease.

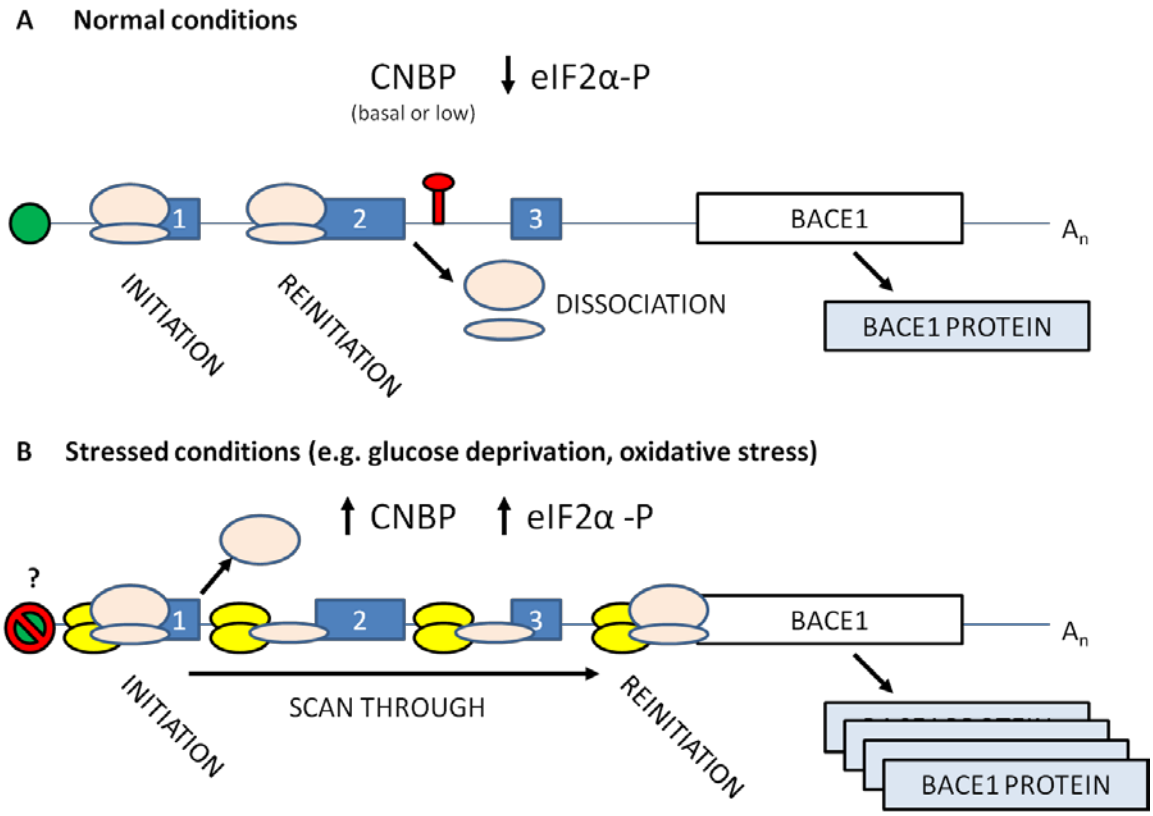
A Normal conditions
CNBP
(basal or low)
eIF2α-P
1
2
3
BACE1
An
INITIATION
REINITIATION
DISSOCIATION
BACE1 PROTEIN
B Stressed conditions (e.g. glucose deprivation, oxidative stress)
CNBP
eIF2α -P
?
1
2
3
BACE1
An
INITIATION
SCAN THROUGH
REINITIATION
BACE1 PROTEIN

Figure 6.2 Possible mechanisms of how CNBP regulates BACE1 translation. (A) Under normal cellular conditions, BACE1 translation is very inefficient due to a high degree of secondary structure (red loop; for simplicity) and three uORFs (blue numbered boxes) in the BACE1 5' UTR which impede the ribosome (pink ovals) from reaching the authentic BACE1 start codon. Since eIF2α is largely unphosphorylated, multiple rounds of translation reinitiation and dissociation may occur at uORFs. Low or basal levels of CNBP may transiently or weakly interact with the BACE1 5' UTR (for simplicity, no CNBP is shown bound) to influence transcript stability, but BACE1 translation is unaffected. (B) During cellular stress, CNBP levels (yellow ovals; shown bound as a dimer) are elevated and BACE1 translation is enhanced through several possible mechanisms. CNBP may recruit ribosomes directly near a start codon (uAUG or authentic), possibly through an undiscovered IRES element, to activate cap-independent translation. Since eIF2α-P levels are high, it is believed that enhanced ribosomal scan-through of the 5' UTR occurs due to a decrease in reinitiation events at uAUGs. CNBP may play a role in this by tethering the small ribosomal subunit (single pink oval) to the mRNA after termination of a uORF, keeping it associated with the transcript longer as it scans downstream. Additionally, CNBP may play a role in masking upstream uAUGs. In all scenarios considered, CNBP might use its RNA chaperone activity to alter local secondary structures which may be needed for direct recruitment of the ribosome or more efficient ribosomal scanning. The green circle indicates the 5' cap structure.

APPENDIX ONE: List of Abbreviations

5’ TOP	5’ terminal oligopyrimidine
AAV	Adeno-associated virus
Aβ	Amyloid-beta
AD	Alzheimer’s disease
ADAM	A disintegrin and metalloprotease
APLP	APP-like proteins
APP	Amyloid precursor protein
APPswe	APP Swedish mutation (ΔNL)
AICD	APP intracellular domain
APOE	Apolipoprotein E
ATP	Adenosine triphosphate
BACE(1/2)	β-site APP cleaving enzyme (1/2)
BCA	Bicinchoninic acid
BIN1	Bridging integrator 1
BME	β-mercaptoethanol
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CB	Cerebellum
CD	Circular dichroism
cDNA	Complementary DNA
CNBP	Cellular nucleic acid binding protein
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTF(α/β)	C-terminal fragment
DIV	Days *in vitro*
DM2	Myotonic dystrophy type 2
DMEM	Dubelcco’s modified eagle medium
DNA	Deoxyribonucleic acid
DR6	Death receptor 6
DTT	Dithiolthreitol
eEF(1A/2)	Eukaryotic elongation factor (1A/2)
eIF2α	Eukaryotic initiation factor 2 alpha
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
ER	Endoplasmic reticulum
FAD	Familial Alzheimer’s disease
FBS	Fetal bovine serum
FMRP	Fragile-X mental retardation protein
FTD	Frontotemporal dementia
G4	G-quadruplex
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gis2p	GIg suppressor 2 protein

GFP	Green fluorescent protein
GGA	Golgi-localized γ-ear containing ARF
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
HIV	Human immunodeficiency virus
hnRNP	Heterogeneous nuclear ribonucleoprotein
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IP	Immunoprecipitation
IPTG	Isopropylthio-β-galactoside
IRES	Internal ribosome entry site
ITAF	IRES trans-acting factor
IVTT	In vitro transcription / translation
LRP	Low density lipoprotein receptor-related protein
MAPT	Microtubule associate protein tau
MEM	Minimum essential medium
MCI	Mild cognitive impairment
MES	2-(N-morpholino)ethanesulfonic acid
MMSE	Mini-mental state examination
MOI	Multiplicity of infection
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
Na_v-β_2	β-subunits of voltage-gated sodium channels
NB	Neurobasal
NC	Nucleocapsid
NRG	Neuregulin
NSAID	Non-steroidal anti-inflammatory
ODC1	Ornithine decarboxylase
ORF	Open reading frame
PABP	Poly-A binding protein
PAGE	Polyacrylamide gel electrophoresis
PBS(T)	Phosphate buffered saline (+ Tween)
PCAD	Preclinical AD
PCR	Polymerase chain reaction
PEI	Polyethyleneimine
PEST	Pro, Glu, Ser or Thr-enriched
PKA	Protein kinase A
PLB	Polysome lysis buffer
PMI	Postmortem interval
PS(1/2)	Presenilin (1/2)
PSGL-I	P-selectin glycoprotein ligand-I
PVDF	Polyvinylidene fluoride
qPCR	Quantitative real-time PCR
RGG box	Glycine/arginine rich region
RIP	RNA immunoprecipitation

RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNA-Seq	RNA-high throughput sequencing
RNP	Ribonucleoprotein
ROS	Reactive oxygen species
RPS17	Human small ribosomal subunit 17
Rpl32	Rodent large ribosomal subunit 32
RTN	Reticulon
RT-PCR	Reverse transcription PCR
sAPP(α/β)	Soluble APP (α/β)
SDS	Sodium dodecyl sulfate
seAP	Secreted alkaline phosphatase
SEM	Standard error on the mean
SMTG	Superior middle temporal gyri
ssDNA	Single-stranded DNA
ST6Gal I	Alpha-2,6-sialyltransferase
TACE	TNF-alpha converting enzyme
TBI	Traumatic brain injury
TBS(T)	Tris-buffered saline (+Tween)
TDP-43	TAR-DNA binding protein 43
TGN	Trans-Golgi network
TPT1	Tumor protein, translationally controlled 1
uAUG	Upstream AUG
uORF	Upstream open reading frame
UTP	Uridine triphosphate
UTR	Untranslated region
UV	Ultraviolet
VHS domain	Vps-27, Hrs and STAM domain
WT	Wild-type
ZNF9	Zinc-finger protein 9

Symbols

Alpha, α
Beta, β
Gamma, γ
Kappa, κ
Mu, μ

APPENDIX TWO: PCR Primers

Primers Used for General qPCR Experiments

Gene	Symbol	Primer Identifier	Sequence (5' to 3')
β-site APP Cleaving Enzyme 1	**BACE1 (Human)**	BACE1 Forward	TATCATGGAGGGCTTCTACGTTG
		BACE1 Reverse	GTCCTGAACTCATCGTGCACAT
β-site APP Cleaving Enzyme 2	**BACE2 (Human)**	BACE2 Forward (3' UTR)	GCAACCATGAACTCAGCTATTAAGAA
		BACE2 Reverse (3' UTR)	AGAAAGCGCCACCATCGA
Cellular Nucleic Acid Binding Protein	**CNBP (Human)**	CNBP Forward	TCCTTCATGCAGGGTTCTGTCAGT
		CNBP Reverse	GACAAGGTTGGAATGTGCACAGCA
β-Actin	**β-ACTIN (Human)**	ACTIN Forward	ACCAACTGGGACGACATGGAGAAA
		ACTIN Reverse	TAGCACAGCCTGGATAGCAACGTA
Glyceraldehyde-3-Phosphate Dehydrogenase	**GAPDH (Human)**	GAPDH Forward	ACCACAGTCCATGCCATCAC
		GAPDH Reverse	TCCACCACCCTGTTGCTGTA
Tumor Protein Translationally Controlled 1	**TPT1 (Human)**	TPT1 Forward	GATCGCGGAACGGGTTGT
		TPT1 Reverse	TTCAGCGGAGGCATTTCC

Primers Used for RIP qPCR Experiments

Gene	Symbol	Primer Identifier	Sequence (5' to 3')
β-Actin	**β-ACTIN (Human)**	ACTIN Forward	ACCAACTGGGACGACATGGAGAAA
		ACTIN Reverse	TAGCACAGCCTGGATAGCAACGTA
Glyceraldehyde-3-Phosphate Dehydrogenase	**GAPDH (Human)**	GAPDH Forward	ACCACAGTCCATGCCATCAC
		GAPDH Reverse	TCCACCACCCTGTTGCTGTA

APPENDIX TWO continued

Ribosomal Protein S17	**RPS17 (Human)**	RPS17 Forward	ATGAAGCGAATTCAGAGAGGCCCA
		RPS17 Reverse	AGTGACCTGAAGGTTGGACAGACT
Amyloid β-Precursor Protein	**APP (Human)**	APP Forward (Exon 3)	AACCAGTGACCATCCAGAAC
		APP Reverse (Exon 4)	ACTTGTCAGGAACGAGAAGG
Microtubule-Associated Protein Tau	**MAPT (Human)**	MAPT Forward (Isoform 6)	ATTACTGCCAACAGTTTCGGCTGC
		MAPT Reverse (Isoform 6)	TAAGAAGGCCCATGGTGCTGAAGA
Ornithine Decarboxylase 1	**ODC1 (Human)**	ODC1 Forward	CTGCTTGATATTGGCGGTGGCTTT
		ODC1 Reverse	ATGCAACATAGTATCTGCCGGGCT
β-site APP Cleaving Enzyme 1	**BACE1 (Human)**	BACE1 Forward	TGCAGTCAAATCCATCAAGGCAGC
		BACE1 Reverse	TGATGCGGAAGGACTGGTTGGTAA
β-site APP Cleaving Enzyme 2	**BACE2 (Human)**	BACE2 Forward	GCAACCATGAACTCAGCTATTAAGAA
		BACE2 Reverse	AGAAAGCGCCACCATCGA
Cellular Nucleic Acid Binding Protein	**CNBP (Human)**	CNBP Forward	TCCTTCATGCAGGGTTCTGTCAGT
		CNBP Reverse	GACAAGGTTGGAATGTGCACAGCA
Bridging Integrator 1	**BIN1 (Human)**	BIN1 Forward (Isoform 1)	AGAGAACAACGACCTGCTGTGGAT
		BIN1 Reverse (Isoform 1)	TTGGCAATGCGTGACTTGATGTCG
β-Actin	**β-Actin (mouse)**	Mouse β-actin Forward	TTGCTGACAGGATGCAGAAGGAGA
		Mouse β-Actin Reverse	ACTCCTGCTTGCTGATCCACATCT

APPENDIX TWO continued

Ribosomal Protein L32	**rpl32 (mouse)**	Mouse rpl32 Forward	TCTGGTGAAGCCCAAGATCGTCAA
		Mouse rpl32 Reverse	GGGTTTCCGCCAGTTTCGCTTAAT
β-site APP Cleaving Enzyme 1	**Bace1 (mouse)**	Mouse Bace1 Forward	ATGTTGCCACTCTGCCTCATGGTA
		Mouse Bace1 Reverse	TGAGCAGGGAGATGTCATCAGCAA

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Vita

Christopher John Holler

Born: April 30th, 1983

Birthplace: Roanoke, VA

EDUCATION

2007 – Present	University of Kentucky Doctoral Candidate in Cellular and Molecular Biochemistry Lexington, KY
2005 – 2007	Virginia Polytechnic Institute and State University Master's of Science in Biological Systems Engineering Blacksburg, VA
2001 – 2005	Virginia Polytechnic Institute and State University Bachelor's of Science in Biological Systems Engineering Blacksburg, VA

ACADEMIC HONORS

University of Kentucky Presidential Fellowship, 2011-2012
University of Kentucky Predoctoral International Travel Award, Spring 2011
University of Kentucky Predoctoral Domestic Travel Award, Fall 2011
University of Kentucky Predoctoral International Travel Award, Spring 2009
American Society for Molecular Biology and Biochemistry (ASBMB) Predoctoral Travel Award, Spring 2009
University of Kentucky Graduate School Academic Fellowship, 2007-2008
Paul E. Torgersen Graduate Research Excellence Award (Most Outstanding Master's Project for College of Engineering), Virginia Tech, 2007
Graduated with High Distinction (Magna Cum Laude), Virginia Tech, 2005
W. Thomas and Barbara Robertson Scholarship, 2004-2005
NSF-REU Undergraduate Fellow, Summer 2004
Peter H. Fitzgerald Scholarship, Spring 2004
Roanoke County Coca-Cola Scholarship, 2001
T. Marshall Hahn Scholarship, 2001

PUBLICATIONS

Murphy, MP*, Webb RL*, **Holler CJ***, Rochette MJ, Smith TE, Beckett TL, Li F, Dowling ALS, Laux AL, Spinelli A, Baum K, Jansen KR, Niedowicz DM, Das P, Golde

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