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Post-transcriptional regulation of BDNF and the Cdk5 pathway by the neuronal RNA binding protein HuD

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B.S., The University of Georgia, 2010

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Abstract

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By Megan Elizabeth Allen

Establishment of the neuronal network is essential for brain function. This multifaceted process requires intricate regulation of gene expression through both transcriptional and post-transcriptional mechanisms. In particular, post-transcriptional regulation of mRNAs offers the advantage of rapid and local control of the cellular proteome. Such sophisticated regulation depends on both cis-regulatory elements and trans-acting factors, such as RNA-binding proteins (RBPs) and miRNAs. One key factor for neuronal development and function is the RBP, HuD, which binds target mRNAs to regulate stability and translation. The list of HuD ligands is rapidly expanding. However, how HuD regulates mRNA targets *in vivo* to coordinate sophisticated biological paradigms is unknown.

In this dissertation we uncover HuD-dependent post-transcriptional regulation of two key pathways for neuronal development and function. We show that HuD selectively binds and stabilizes an mRNA isoform of the brain derived neurotrophic factor (BDNF) and therefore promotes expression of BDNF protein to be transported to hippocampal mossy fiber axonal boutons. We also discovered that post-transcriptional regulation by a HuD-miRNA molecular loop selectively up-regulates a Cyclin-dependent kinase 5 (Cdk5) activator called p39. This selective regulation occurs concurrently with histone-acetylation-dependent transcription of p39, which enhances Cdk5 activity in the postnatal brain. We reveal essential functions of the Cdk5 activator p39 in neuronal network formation. Moreover, HuD-regulated Cdk5 signaling governs the projection of hippocampal mossy fiber axons.

Given the functional interplay between BDNF and Cdk5 signaling, the cooperative regulation of both pathways by HuD revealed by our studies provides an intriguing example of higher-order coordination of gene networks. Thus, these studies advance our understanding of how post-transcriptional regulation is employed to govern multifaceted aspects of neuronal function in the brain.

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List of Abbreviations

AD: Alzheimer's disease

ALS: Amyotrophic Lateral Sclerosis

APA: Alternative Poly-Adenylation

APP: Amyloid Precursor Protein

ARE: Adenosine-uridine Rich Element

BACE1: Beta-secretase 1

BDNF: Brain-Derived Neurotrophic Factor

CamkIIα: Calcium/calmodulin-dependent protein kinase type II alpha chain

Cdc2: Cell division cycle protein 2 homolog

Cdk5: Cyclin-dependent kinase 5

chIP: chromatin Immunoprecipitation

DG: Dentate Gyrus

DGCs: Dentate Granular Cells

DISC1: Disrupted In Schizophrenia 1

DNA: Deoxyribonucleic Acid

eIF4F: eukaryotic translation Initiation Factor F

eIF5α: eukaryotic translation Initiation Factor 5α

ELAV: Embryonic Lethal Abnormal Vision

ELAVl: Embryonic Lethal Abnormal Vision-like protein

nELAV: neuronal Embryonic Lethal Abnormal Vision

FMRP: Fragile X Mental Retardation Protein

FTLD: Frontotemporal Lobar Degeneration

Fus: Fused in sarcoma

GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase

GAP-43: Growth Associated Protein 43

HDAC2: Histone Deacetylase 2

hnRNP-Q1: heterogeneous nuclear Ribonucleoprotein Q1

KSRP: KH-type Splicing Regulatory Protein

MAP1B: Microtubule Associated Protein 1B

meCP2: methyl CpG binding Protein 2

m7G cap: 5' 7-methylguanylate cap

mHTT: mutant Huntington's

miRNA: micro Ribonucleic Acid

mRNA: messenger Ribonucleic Acid

mTOR: mammalian Target Of Rapamycin

NLS: Nuclear Localization Signal

NMD: Nonsense Mediated Decay

NMDA: N-Methyl-D-Aspartate

NMDAR: N-Methyl-D-Aspartate receptor

NR2B: N-Methyl-D-Aspartate receptor subunit 2B

OL: Oligodendroglia

PABP: Poly-A Binding Protein

PKC: Protein Kinase C

pre-mRNA: pre-messenger Ribonucleic Acid

polyA tail: poly-Adenosine tail

qRT-PCR: quantitative Real Time Polymerase Chain Reaction

RBP: Ribonucleic acid Binding Protein

RISC: RNA-Induced Silencing Complex

RNA: Ribonucleic Acid

RNP: Ribonucleotide Particle

RT-PCR: Reverse Transcription coupled Polymerase Chain Reaction

RRM: RNA Recognition motif

siRNA: small-interfering Ribonucleic Acid

SMA: Spinal Muscular Atrophy

SMN: Survival of Motor Neuron

SNP: Single Nucleotide Polymorphism

TDP-43: TAR DNA-binding Protein 43

TrkB: Tropomyosin receptor kinase B

tRNA: transfer Ribonucleic Acid

UTR: Untranslated Region

UV-CLIP: Ultraviolet Cross-Linking Immunoprecipitation

WAVE1: Wiskott-Aldrich syndrome protein family verprolin homologous protein 1

ZBP: Zipcode Binding Protein

Chapter 1

General Introduction

1.1 Post-transcriptional regulation of cellular mRNAs is essential for the development and function of brain neurons

The transcription of protein-coding genes, by the enzyme RNA Polymerase II, into messenger ribonucleic acid (mRNA) and the subsequent translation of template mRNA into a polypeptide encompasses the central dogma of biology (1). The journey from transcription to translation, however, is complex and involves many layers of sophisticated post-transcriptional regulation to ultimately determine the abundance and asymmetric cellular distribution of many different mRNA species (2). Post-transcriptional regulation of mRNA (**Figure 1-1**) is a broad term which begins with nuclear mRNA processing events such as 5' end mRNA capping, splicing, alternative polyadenylation, and nuclear export (3-8). After these complex nuclear processing steps, cytoplasmic mRNAs are further regulated at the levels of mRNA stability, the localization of mRNAs to sub-cellular compartments, and the translation of mRNA into protein (9-12). Such sophisticated post-transcriptional regulation allows for the higher-order temporal and spatial control of the cellular proteome (3, 9).

1.1.1 Molecular mechanisms which govern cellular mRNA behavior in neurons

Molecular mechanisms which determine the temporal and spatial cellular distribution of mRNAs after nuclear export are particularly important in highly differentiated cells which require sophisticated, and often rapid, control of cellular protein abundance (13-15). Neurons are highly specialized cells of the central nervous system which develop polarized extensions, called axons and dendrites that meet and form synapses in order to transmit the chemical signals that underlie proper brain function (16). Thus the differential localization of mRNAs to sub-cellular compartments

is imperative in brain neurons (14). Moreover, neurons undergo extensive differentiation during development and must often remain highly plastic upon maturity due to a demanding and continually changing environment (16, 17). Therefore, post-transcriptional mechanisms offer neurons an additional layer to govern complex gene expression and the power to rapidly produce protein locally at functional sites, i.e. developing axons, dendrites, and neuronal terminals, without having to rely on *de novo* transcription and RNA processing (18, 19).

Each aspect of post-transcriptional regulation is governed by both cis-acting and trans-acting elements. Cis-acting regulatory elements refer to specific sequence or structural elements within an mRNA species that can serve as interaction domains for various trans-acting factors. The initial processing of pre-mRNAs, such as alternative splicing or alternative polyadenylation, can result in the inclusion or elimination of cis-regulatory sequences that influence the final fate or abundance of an mRNA (6, 7). Trans-acting factors refer to the specific subset of RNA binding proteins or non-coding RNAs that can interact with an mRNA. Two foundational tenants in the RNA regulation field are: 1) mRNAs are never alone within a cell, but rather are escorted at all times by associated trans-acting factors, and 2) mRNAs are almost never linear, but rather are assembled in-vivo into secondary and tertiary structures mediated by cis- and trans-acting factors (2).

The importance of regulating the decay of eukaryotic mRNAs to control eukaryotic mRNA cellular abundance is increasingly appreciated (12). Awareness that mRNA stability is a major contributor to levels of mRNA within the cell originated when large scale studies demonstrated that altered decay is responsible for almost half of the

alterations in mRNA expression in response to particular cellular signals (20). In neurons, many reasons exist for modulating mRNA stability. Most importantly, changes in RNA stability affect the steady state pool of mRNA available for translation in the cell (12, 21). Alternately, destabilizing elements within an mRNA or associated destabilizing factors allow mRNA turnover, which can limit translation of a factor that is not physiologically needed for an extended period (22).

Major mechanisms that trigger mRNA decay include: 5'Cap and 3' poly-A tail removal, surveillance by nonsense-mediated decay (NMD), or sequence specific elements which recruit degradation enzymes (23, 24). There are several roads to degradation of eukaryotic mRNA, as illustrated in **Figure 1-2** (23). mRNAs can be deadenylated by deadenylases allowing: 1) degradation in the 3'-5' direction by the exosome and associated proteins and/or 2) the removal of the 5' m7G cap by decappping enzymes and degradation in a 5'-3' manner by exonucleases. These processes are termed deadenylation-dependent degradation. Alternatively, mRNAs are subject to deadenylation-independent degradation when 1) de-capping triggers 5'-3' degradation or 2) sequence specific endonuclease cleavage leads to decay by 5'-3' or 3'-5' exonucleases (23). Thus, mechanisms which regulate mRNA decay can be broad or specific to an mRNA species. One of the best characterized cis-acting element that control mRNA stability, found in nearly 20% of neuronal mRNAs, is the A/U-rich element (ARE) (12, 18). Classically, AREs are located in the 3' untranslated region (UTR) of an mRNA and can recruit degradation factors to the mRNA in which they are present (25). A/U-rich elements also serve as potential binding sites to ARE-binding proteins which can protect the mRNA from destabilization, thus extending the half-life of the mRNA species (26,

27). Many families of RNA binding proteins (RBPs) can bind mRNA sequence elements to modulate target transcript stability (12).

Differential subcellular localization of mRNAs to distal regions of cells where they can be translated on site contributes to the asymmetric distribution of proteins within the cell (19). This phenomenon is particularly pronounced in neurons which go through extensive differentiation and develop lengthy polarized processes, called axons and dendrites. Neuronal axons protrude from the cell body and form synapses with dendritic spines of neighboring neurons to form a complex structural and functional network. To accommodate such specialized circuitry development, differential distribution of molecules within the soma, axons, dendrites, and at the synapse, requires regulated mRNA transport (10, 14, 16, 19).

Similar to other post-transcriptional mechanisms, mRNAs can contain cis-acting sequences that cooperate with trans-acting RNA binding proteins to signal the localization of an mRNA within the cell body and/or to different neuronal processes (10, 14, 19, 28). One example of a cis-acting localization sequence is found within the mRNA which encodes Brain Derived Neurotrophic Factor, BDNF, a factor that critical for development of neurons and synaptic plasticity (29). BDNF is subject to alternative polyadenylation (30). Use of the proximal polyadenylation site results in a BDNF mRNA species containing a short 3'UTR. Usage of the distal polyadenylation site results in a BDNF mRNA species with a long 3'UTR that contains the entire short 3'UTR plus additional sequence (30, 31). Sequence specific to the long BDNF 3'UTR results in de-stabilization of the transcript (32) and also directs BDNF mRNA to dendrites where BDNF mRNA can be translated in an activity-dependent manner (33, 34). In contrast,

BDNF mRNA possessing the short 3'UTR, and thus lacking the long 3'UTR specific sequence, are sequestered within the cell body (34). Importantly, the discrete cis-sequence located in the BDNF long 3'UTR likely provides a platform for unique trans-acting regulatory factors. Thus, in addition to complex regulation at the level of transcription, BDNF is subject to sophisticated post-transcriptional regulation which provides an additional layer of temporal and spatial control over BDNF distribution within neurons. In addition to the BDNF mRNA, many other examples exist of regulatory sequence within mRNAs required for the localization of transcripts (28).

The trafficking of mRNAs to distal processes requires complex coordination (14). Multiple-protein complexes are assembled with mRNA in the nucleus and these mRNPs are then transported to distal portions of axons and dendrites (35, 36). One of the most widely studied examples of this phenomenon is β-actin mRNA which contains a specific sequence, or "zipcode", in the 3'UTR which serves as a binding site for the zipcode binding protein (ZBP) and directs the mRNA to the axonal growth cone, where it can be locally translated (37-39). Directing mRNAs, rather than proteins, to distal portions of the neuron allows for a rapid increase, through local translation, of proteins to accommodate functional needs within the neuronal arbor and at neuronal terminals. In fact, there is growing appreciation for the physiological presence of translationally repressed mRNAs at the synapse which harbor the ability to be de-repressed upon stimulation to regulate synaptic plasticity (17, 40).

Each upstream post-transcriptional regulatory step can influence the availability of a particular mRNA for translation, the ultimate step of protein production. Eukaryotic translation can be divided into three stages: initiation, elongation, and termination (11,

41). Initiation begins when the pre-initiation complex (composed of the small ribosomal subunit, the initiator tRNA and initiation factors) is recruited to the 5'cap of the mRNA, through the eIF4F complex (42). The small ribosome subunit scans the mRNA for a start codon where elongation commences when the large ribosomal subunit joins the small subunits to begin the formation of the first peptide bond. Subsequently, translation elongation cycles by positioning of a charged tRNA to the A site of the ribosome, formation of the peptide bond with the peptidyl t-RNA that carries the nascent polypeptide chain, and the shifting of the mRNA to the next codon (11, 41). Translation termination occurs when a stop codon is encountered by the ribosome thereby catalyzing the release of the polypeptide chain and dissociation of the ribosomal subunits (11, 41). It is important to remember that actively translating mRNAs are often associated with multiple simultaneously translating ribosomes, termed polyribosomes (43). One example of miRNA and/or RBP translational inhibition is through interference of the 5' cap and eIF4E binding. RBPs can also bind the 3'UTR and/or 5'UTR of an mRNA to enhance translation initiation. Structural and sequence-specific components in the 5'UTR of an mRNA can also affect the efficiency of translation (44, 45).

In neurons, ribosomes and translation machinery are present outside of the cell body within distal regions of developing and mature axons and dendrites (46-48). Thus, protein synthesis can occur far from the soma in distal axonal and dendritic processes through local protein translation (19, 49, 50). Local translation at neuronal synapses is a crucial aspect of the development of the neuronal circuitry and the function of synapse (51, 52). For example, protein synthesis in axonal growth cones directs developing axonal extensions to their proper locations in the neuronal circuit *in vivo*. Moreover,

recent studies have shown transport of mRNAs which are locally translated within in mature axons *in vivo* (50, 53, 54). Local translation in the dendritic compartment is also important for the formation and maintenance of mature dendritic spines and at the post-synaptic sites of the functional synapse (51). Activity-dependent local translation, a crucial component governing synaptic plasticity, can occur following neuronal excitation or in response to synaptic stimuli (17, 51, 52). Plasticity refers to the ability of a synapse, or a collection of synapses within a circuit, to modulate synaptic strength over time in response to activity changes. Changes in synaptic plasticity are important for higher-order cognitive function in the brain, such as learning and memory, which requires activity dependent protein synthesis (17, 52).

1.1.2 Trans-acting factors governing post-transcriptional control of mRNA abundance

In addition to the cis-acting sequences within an mRNA, the varied protein "coat" associated with each mRNA species over the course of its lifespan is critical for determining cellular mRNA fate. These trans-acting factors, as described below, can regulate nuclear export, the stability and degradation, the assembly and transport, and the translational efficiency of an mRNA. RBPs can also coordinate with miRNAs to regulate mRNA stability and translation. Proper control by RNA binding proteins at each of these steps is crucial for the correct temporal and spatial distribution of mRNAs within the cell (21, 55, 56).

A variety of elements can serve as RBP recognition motifs. On one hand, RBPs can bind to either general or specific cis-acting regulatory sequences within the target mRNA (21). RBPs can also recognize secondary structures, which may or may not be

dependent on linear mRNA sequence. One example of this is the g-quadruplex RNA structure, which can serve as a binding site for RNA binding proteins. One recent example is the g-quadruplex located in the 5'UTR of the GAP-43 mRNA which interacts with hnRNP-Q1 to mediate GAP-43 translational repression (57). On the other hand, protein domains within RBPs can bind mRNA with stringent sequence selection or via broad recognition with little specificity (21). The most common RNA interacting domain located within RBPs is the RRM (RNA Recognition Motif), which can mediate RNA-protein interaction (58).

RBPs often play many different roles in directing mRNA regulation and resulting downstream neuronal functions. In fact, RBPs can govern multiple co- and post-transcriptional steps at different stages of the mRNA lifespan (21, 56, 59). Some RBPs, for example the ELAV family of proteins, can even regulate the abundance of the mRNAs encoding RBPs within their own protein family, which adds an additional layer of regulation to RBP function (56, 59). Adding further complexity to mRNA regulation, RBPs can also compete or cooperate with one another to bind an mRNA. Competition events often occur to regulate the mRNA in dynamically opposing ways (13). One example of this in neurons is HuD and KSRP, which compete for the same target sequence in the GAP-43 mRNA (60). HuD binding to GAP-43 stabilizes the transcript, whereas KSRP binding results in destabilization. Thus HuD and KSRP cooperate to determine the GAP-43 mRNA levels during neuronal development. (60).

The ability of RBPs to bind and regulate mRNAs at multiple points during mRNA metabolism allows for downstream functional control of overlapping signaling pathways which must be regulated simultaneously (21, 56, 59). The emergence of high-throughput

in vivo binding techniques such as crosslinking immunoprecipitation (CLIP) followed by high-throughput sequencing have been an instrumental step forward in advancing our understanding of the RBP-RNA interactome. However, identification of RBP ligands still leaves questions regarding the function of such interactions (61-64). Moreover, how RBPs regulate numerous RNA targets yet maintain the specificity required for the multifaceted processes that they govern in the cellular environment is an ongoing research challenge.

miRNAs can also serve as trans-acting regulatory elements governing mRNA post-transcriptional regulation. miRNAs are approximately 22nt long non-coding RNAs which bind to mRNA targets with incomplete sequence complementarity (55, 65, 66). miRNAs are transcribed as pri-miRNAs and processed by Drosha into the pre-mRNA stem loop inside of the nucleus. Pre-miRNAs are then exported into the cytoplasm where they are cleaved from a stem-loop pre-mRNA structure into a 22nt duplex by Dicer. The mature miRNA incorporated into the RISC complex is called the guide strand and the other strand, the passenger strand, is usually degraded. miRNAs can also be transcribed within introns and processed into pre-miRNAs after splicing occurs (67). The model of miRNA-dependent mRNA regulation is two-fold: a miRNA can mediate translational efficiency or mRNA degradation (65). However mechanisms which determine miRNA function, alone or in conjunction with other trans-acting factors, are still not fully understood.

Approximately 2000 miRNAs have been identified in the human genome and the brain has the highest numbers of miRNA species. In the nervous system, miRNAs are important for cell fate and for patterning during development (66, 68, 69). In neurons

specifically, miRNAs have been shown to play roles in many important aspects of neuronal function, including differentiation, development, and synaptic plasticity (70, 71). Emerging evidence suggests that miRNAs are also present in dendrites and many models postulate that they are heavily involved in synaptic activity-dependent translation (72, 73).

1.1.3 Coordination between mRNA binding proteins and miRNAs

Untangling the discrete effects of miRNAs and RBPs on post-transcriptional regulation of cellular mRNA abundance can be challenging as many mechanisms of regulation are inherently linked. Moreover, RBPs can regulate the abundance of miRNA species within the cell, through regulating splicing of sequences encoding miRNAs, the expression mRNAs encoding the miRNA processing proteins, or by regulating stability or processing of miRNA precursors (13, 55). Likewise, transcripts encoding RBPs can be post-transcriptionally regulated by miRNAs (13).

miRNAs and RBPs can also work cooperatively (74-76) or antagonistically (77-79) to regulate the same mRNA transcript (**Figure 1-3**) (55). When the RBP and the miRNA have non-overlapping sequence recognition motifs within the same target, the associated RBP can either positively or negatively regulate the miRNA-RISC complex binding. The miRNA and RBP can also compete for the same binding site, such that a bound RBP may cover a miRNA target site and thus stabilize or translationally de-repress the mRNA species. Furthermore, binding of an RBP can change the secondary structure of the mRNA molecule and thus hide or expose a potential miRNA binding site. Finally, RBPs can recruit other proteins to the mRNA through indirect association which can positively or negatively regulate miRNA-mRNA recognition. The list of miRNA-RBP

cooperation is growing while the functional implications of these interactions for neuronal pathways are being uncovered (55). However, our understanding of the molecular mechanisms which regulate these interactions *in vivo*, especially in neurons, is still incomplete.

One interesting functional example of miRNA-RBP interaction is the ubiquitous ARE binding protein, HuR. On one hand, HuR binding can expose the miRNA recognition element for let-7 on c-myc mRNA in HeLa cells (76). On the other hand, HuR can stabilize nucleolin mRNA by preventing mir-494 mediated repression (80). Thus, HuR mediates opposing mechanisms, by either promoting or inhibiting mRNA expression, depending on the mRNA target the coordinating miRNA species. Interestingly, many miRNAs have seed sequences that are A/U rich, and thus may be able to compete with ARE binding proteins to regulate stability of ARE containing mRNAs (81).

1.1.4 RBPs and miRNAs in neurological disease

Because of their essential roles in governing neuronal development and function, dysregulation of RBPs and miRNAs is prevalent in a growing list of neurological diseases (13, 72, 82-84). One of the most well-known examples of RBP deficiency in human disease is Fragile X Syndrome (FXS), the most frequent familial intellectual disability in the world, caused by the loss of Fragile X Mental Retardation Protein (FMRP) (85). The best characterized function of FMRP is repressing translation through inhibition of elongation at the resting state, thereby also offering a means of activity-dependent de-repression (86). The current understanding of FMRP function in controlling neuronal network development helps explain some of the cognitive impairments seen in

FXS patients. FMRP is associated with ribosomes in the cytoplasm and at dendritic spines (85, 87, 88). At the synapse, activity dependent translation of repressed FMRP mRNA targets allows for rapid bursts of protein synthesis through local translation in response to stimulation (89). Activity dependent de-repression of mRNA targets can be mediated by FMRP phosphorylation. Phosphorylation of FMRP on S499 in mice modulates translational repression by FMRP (90). This site can be de-phosphorylated by protein phosphatase-2A (PP2a) in an activity dependent manner to allow translation of FMRP-bound mRNAs (91). Degradation of FMRP caused by synaptic stimulation can also result in translational de-repression of mRNA targets (90). Interestingly, FMRP is associated with miRNA machinery and several miRNAs (85). One clear demonstration of cooperation between FMRP and miRNAs, controlled by phosphorylation, at the synapse is the reversible phosphorylation of FMRP which promotes miR-125a translational inhibition of PSD-95 (92). Future work will likely expand the list of miRNAs which cooperate with FMRP. The RNA binding protein HuD also regulates translation and cooperates with miRNAs to regulate mRNA targets, but such a mechanism is far from understood at the moment (93, 94).

The abundance of several miRNAs is altered in schizophrenia patients (83). Several chromosomal deletions and copy number variations associated with schizophrenia affect the miRNA biogenesis pathway, pointing to the potential of broad miRNA dysregulation (83, 95, 96). One specific example is the gene locus that encodes miR-137, which contains SNPs associated with an increase schizophrenia risk (97-99). Moreover, miR-137 was recently found to play a role in presynaptic plasticity and regulate target genes which have also been implicated in schizophrenia (97).

Dysregulation of RNA-binding proteins also occur in schizophrenia. Disrupted in schizophrenia 1, DISC1, is a strong susceptibility factor for psychiatric disorders, especially Schizophrenia (100). Recent reports demonstrate that one molecular function of DISC1 is to regulate the transport of mRNAs that control synaptic plasticity to dendrites (101, 102). How loss of DISC1 contributes to the multifaceted etiologies observed in schizophrenia, however, remains unknown. Taken together, although the symptoms of schizophrenia are quite complex, likely involving dysregulations of multiple sophisticated pathways, the potential for miRNA malfunction and RBPs to contribute to the schizophrenia pathology is supported by several compelling lines of evidence.

Neurodegenerative disorders comprise a group of diseases which share similar pathological characteristics including the progressive death and loss of neurons. These disorders are often linked to dysfunction of mRNA binding proteins and are increasingly associated with post-transcriptional regulation of mRNAs (103, 104). For instance, a common example is that of the RBPs TAR DNA-binding protein 43 kDa (TDP-43) and Fused in sarcoma (FUS), which have been genetically linked to the neurodegenerative diseases Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Lobar Degeneration (FTLD) and whose dysfunction has been hypothesized as a pathological driver for the progressive degeneration observed in ALS and FTLD (105). In these diseases, the predominantly nuclear TDP-43 and FUS are found in ubiquitin-positive inclusions in the cytoplasm (106, 107). Although dysregulated abundance and dysfunction of many other RNA-binding proteins is observed in models of degeneration, our understanding of the role that they play in such disorders remains incomplete (103).

Because miRNAs are also broad regulators of gene networks, it is not surprising that there is accumulating evidence for miRNA dysfunction in ageing related neurodegenerative disorders (108). For example, in a mouse model that displays conditional loss of the miRNA processing protein, Dicer, in forebrain neurons, neuronal loss was observed in the hippocampus and tau is hyper-phosphorylated (109). Additionally, miRNAs can regulate many of the proteins associated with neurodegenerative diseases, such as the regulation of amyloid precursor protein (APP) expression by miR-101 (110). Several ageing related miRNAs, such as miR101 and miR107 are also down-regulated in Alzheimer's disease (AD), perhaps leading to abnormally increased expression of protein from the mRNA targets of these miRNAs (111, 112). Although many links between miRNA regulation and neurodegenerative disorders have been examined, the potential roles of miRNA dysregulation in diseases such as AD remain to be untangled.

1.2 HuD is a specific RNA-binding protein that governs neuronal development and function

HuD is a neuron-specific member of the highly conserved Embryonic Lethal Abnormal Vision-like (ELAVl) family of RBPs that govern a multitude of processes essential for normal neuronal function (59). HuD binds a variety of mRNA ligands whose encoded proteins govern broad aspects of neuronal development and function (113). Regulation of mRNA targets by HuD occurs at nearly every post-transcriptional step from splicing to translation (59). However, our understanding of the *in vivo* functional consequences of HuD-mediated mRNA regulation is more limited. In particular, expanding our understanding of the temporal and spatial context of HuD-dependent post-

transcriptional regulation is an important prerequisite to establishing how HuD governs multi-faceted aspects of neuronal function during complex biological paradigms.

1.2.1 Evolutionary conservation and identification of the ELAV family of proteins

The embryonic lethal abnormal vision (ELAV) gene was originally discovered in an embryonic lethal mutant screen in Drosophila melanogaster (114). In flies, the ELAV protein is predominantly nuclear and specifically expressed in the nervous system, where it plays important roles in splicing and polyadenylation of mRNA (115). There are four human ELAV orthologue genes, named ELAV like (ELAVl) 1-4 (115). These genes encode the Hu- family of proteins, HuA/HuR (ELAVl1), HuB (ELAVl2), HuC (ELAVl3), and HuD (ELAVl4) (115). The Hu proteins were first identified as antigens in samples from human patients suffering from the rare neurological disorder, paraneoplastic neurological syndrome (Hu-syndrome), which arises as a comorbidity with cancer due to abnormal immune responses (116, 117). This ELAV genes are highly conserved during evolution in all metazoan species, suggesting an important cellular function (118).

All mammalian Hu proteins share protein domain homology with drosophila ELAV (dELAV) (118). Mouse Hu protein sequences are conserved with nearly 50% identity to dELAV (118). **Figure 1-4** shows the conserved protein domains of each Hu family member. Murine Hu proteins share three highly conserved RRM domains, which display over 80% sequence homology with one another (118). RRMs canonically function to bind RNA, underscoring their important roles in RNA regulation(58). RRMs 1 and 2 are thought to bind mRNA targets (119, 120), while RRM3 may serve to stabilize the protein-RNA complex through Poly-A tail binding (121-123). Notably, the Hu

members differ from one another most significantly in their hinge regions, which is the protein sequence thought to lend specificity to the mRNA target selection and thus downstream function of each Hu member (59, 118).

In vertebrates, two of the Hu- proteins, HuC and HuD, are expressed only in neurons, while HuB is expressed in neurons and gonads (124). The three neuronal Hu-proteins are predominantly cytoplasmic. In contrast, HuR is expressed ubiquitously, and is primarily detected in the nucleus at steady state, although cytoplasmic shuttling of HuR and cytoplasmic function of HuR has been reported (125-127). Although neuronal ELAV proteins (nELAV) are all expressed in brain neurons, they exhibit non-overlapping temporal and spatial patterns (59, 124, 128, 129). For example, expression of all nELAV members is induced during neuronal differentiation. However, in the developing mouse and rat nervous system, HuD and HuB gene expression is most abundant between E16 and P0 of development, although their specific patterns vary by brain sub-regions (118, 128), and persists in certain neuronal populations into adulthood (18). HuC is expressed abundantly and consistently throughout many brain regions between about embryonic day 10 through adulthood (118, 128). Additionally, in the developing neocortex, the neuronal Hu proteins are expressed to different extents in different cortical layers (118, 128). Expression of nELAV members is markedly reduced after the first two weeks of postnatal brain development in mice and is limited to specific neuronal populations in adulthood (59, 130). Thus, it is thought that, despite certain shared functions in directing neuronal development, nELAV members may pay non-overlapping roles to govern discrete aspects of neuronal function.

1.2.2 HuD function in neuronal development and in the adult brain

The expression of HuD in the developing and adult brain does not completely overlap with other Hu proteins. During mouse brain development, HuD is abundantly expressed, peaking at embryonic day 16, until about two weeks after birth (59, 113, 118, 128). During embryogenesis, HuD is expressed in neurons of the ventricular zone, the cortical plate, the developing hippocampus, and the sensory and motor neurons (113, 131). Upon brain maturity, HuD expression is limited to discrete neuronal populations. In the hippocampus, HuD is abundantly expressed throughout the hippocampus during development but becomes restricted to CA1-CA3 regions in the adult, except under stimulation (113, 130).

The temporal and spatial expression of HuD underscores the functional role for HuD in neuronal development (128). HuD has been noted as one of the earliest markers of the neuronal cell fate and several lines of evidence suggest HuD may be a key regulator in the transition from neuronal stem cell progenitors to differentiating neurons (113, 131). During neuronal differentiation HuD inhibits proliferation and promotes neuronal-identity factors. Thus, loss of HuD prevents morphological differentiation and neural-circuitry formation and overexpression of HuD can induce neuronal differentiation and drive axogenesis (132-136). Within the neuron, HuD is enriched at axonal growth cones during differentiation and is detectable in both axons and dendrites upon neuronal maturity (59). The mRNAs of several key transcription factors and cytoskeletal proteins crucial for neuronal development are regulated by HuD, including c-myc, c-fos, tau, and GAP-43 (113, 131). HuD also governs axonal outgrowth, dendritic arborization, and establishment of the neuronal circuitry through regulation of GAP-43 and other mRNA targets (135-137).

HuD is present at the pre- and post- synaptic terminals, and is upregulated in dendrites upon neuronal stimulation (59, 130, 131, 138). The conclusion that HuD directs synaptic plasticity arose from several lines of evidence. First, HuD is upregulated and localized to dendrites upon neuronal stimulation through an NMDAR-dependent mechanism, where it is associated with PABP and transcripts undergoing local translation, such as homer, GAP-43, and CAMKIIα (138, 139). HuD is also important for the activity dependent protein expression of synaptic BDNF (140). In fact, accumulating evidence suggests HuD regulates mRNA targets involved in learning and memory (141, 142). Supporting this idea, HuD protein, and its target GAP-43, are transiently upregulated in the CA1, CA2, CA3, and CA4 areas of the hippocampus 24 hours after spatial learning tasks (142). HuD knockout mice suffer from spatial learning impairment as assessed in the Morris water maze and transgenic mice where HuD expression is elevated have deficits in the acquisition and retention of memory during spatial learning (134). These phenotypes suggest an important but unclear role for HuD in learning and memory.

1.2.3 Molecular mechanisms of HuD regulation

Different molecular mechanisms are employed by HuD to govern target mRNA abundance in neurons (59). Within a neuron, HuD is predominantly cytoplasmic at the steady state (59). However, HuD does contain a putative NLS and can shuttle in and out of the nucleus. Often, RNA binding proteins shuttle between the nucleus and the cytoplasm in different biological scenarios to perform distinct co- and post-transcriptional functions (21). While HuD is most appreciated for the role that it plays in mRNA stabilization and mRNA localization, HuD has also been suggested to regulate alternative

splicing, alternative polyadenylation, and nuclear/cytoplasmic shuttling of mRNA (59). Additionally, recent studies report that HuD can oppose the active mTORC1-dependent miR-129-mediated translational repression of Kv1.1 mRNA by enhancing translation of Kv1.1 when mTORC1 is inactive (93). The proposed mechanism for this regulation is that inactive mTORC1 results in the degradation of high affinity HuD mRNA ligands and thus frees HuD to bind Kv1.1 mRNA (93). However, mechanisms by which HuD may regulate mRNA translation through miRNA cooperation are not completely understood. Taken together, the molecular mechanisms which mediate HuD functions in neurons are varied, suggesting important global roles for HuD in neuronal function.

The observation that HuD can bind to intronic and exonic regions of pre-mRNAs was the first indication of a nuclear role for HuD and implied that HuD could potentially regulate splicing (143). Subsequent work clearly demonstrated that HuD and other nELAV members can, in fact, regulate alternative splicing by enhancing either exon inclusion or exon exclusion (144). ELAVl family proteins are able to bind RNA Polymerase II and inhibit HDAC2 to regulate exon exclusion by altering the acetylation of certain genomic regions (145). Other roles for Hu-proteins in the nucleus include alternative polyadenylation (146). Although all Hu- members can regulate polyadenylation, this has been mainly a documented function for HuR. One example where all Hu- members, including HuR, can regulate alternate polyadenylation is for HuR mRNA (147). Hu- proteins bind to and block an upstream polyadenylation site and thus promote usage of a downstream site, resulting in a less stable transcript, and decreasing HuR protein levels (147). This example has been postulated to be one mechanism of reducing HuR expression in neurons, once the neuronal Hu proteins are

expressed (59). Finally, another function of HuD, which has a putative NLS, is the shuttling of mRNAs out of the nucleus, potentially through binding and cooperation with nuclear export complexes (61, 148). Thus, in addition to its well-known role of governing mRNA abundance in the cytoplasm, HuD can regulate the nuclear processing and export of mRNAs.

Although, HuD can regulate mRNAs within the nucleus, the most extensively studied functions of HuD are cytoplasmic, where HuD can control mRNA stability, localization, and translation (59). To regulate mRNA stability, HuD binds sequence elements in target mRNA transcripts (61, 120). A recent study reported that AREs comprise only about 45 percent of HuD target sequences (61). This study identified other RNA motifs for HuD binding (**Figure 1-5**), namely U-rich and G/U rich sequence (61). Identification of new HuD binding motifs may expand our understanding of the HuD-mRNA interactome and the signaling pathways that HuD regulates in neurons. Nonetheless, other factors must contribute to HuD-mRNA specificity besides sequence alone, as HuD does not bind all ARE element containing mRNAs or all U-rich elements.

Postulated molecular mechanisms for HuD-mediate stabilization are through either 1) competing with destabilizing factors or 2) masking destabilizing elements in the mRNA. In fact, HuD is one of the most well-studied stabilizing RBPs in neurons, binding and extending the half-life of a plethora of substrate mRNAs which govern neuronal development (12, 113). Multiple groups have performed high-throughput sequencing studies to identify HuD target mRNAs in neurons (61, 132, 149). These studies demonstrated that HuD can bind to mRNAs in multiple functional groups in neurons including: neuronal differentiation factors, other RNA-binding proteins, translation

factors, mRNP components, and cytoskeletal associated proteins (61). The most extensively investigated HuD target is the GAP-43 mRNA. HuD binds GAP-43 *in vitro* and *in vivo* through an A/U-rich element in the GAP-43 3'UTR and increases the half-life of the transcript, which promotes neurite extension during neuronal differentiation (135-137). While an increasing number of mRNAs are identified as HuD binding targets, our knowledge of how HuD regulates mRNA targets to orchestrate complex brain function *in vivo* is more limited.

HuD also plays roles in localization and translation of mRNAs in both the soma and the neurites (59). HuD binds to a number of mRNP transport molecules (150-152). Perhaps the most well-studied mRNP interacting partner of HuD is the spinal motor neuron (SMN) complex (153). The colocalization of SMN and HuD in actively transported mRNA granules within motor neuron axons has been demonstrated, highlighting the putative role for HuD in mRNA trafficking (153). HuD abundance can also be concentrated to dendrites upon neuronal stimulation (138). An example of activity-dependent expression of BDNF at dendrites, controlled by HuD, has recently been revealed (140). Additionally, there are examples of both translational repression (94) and cap-dependent translational enhancement (121) for HuD mRNA targets. HuD can also indirectly regulate translation of the dendritic ion channel Kv1.1 by competing with miR-129 to regulate the abundance of Kv1.1 expression, demonstrating at least one example of HuD-miRNA coordination (93). Therefore, outside of the well-established role for HuD in modulating mRNA stability, emerging evidence suggests additional roles for HuD in mRNA localization and regulation of translation.

1.2.4 HuD and neurological disorders

Microarray studies found that HuD and a subset of its mRNA targets were elevated in Schizophrenic patients, suggesting that HuD-dependent post-transcriptional dysregulation may contribute to Schizophrenic etiology (154). Human single nucleotide polymorphisms in HuD have also been linked to Parkinson's disease (155). However, whether or how HuD plays a role in Parkinson's disease has not been further investigated. Because of the many identified targets of HuD important for neuronal function, one can imagine many scenarios for HuD-mediated abnormalities in neurological disorders like Schizophrenia and Parkinson's disease.

The role for HuD in RNP localization has been suggested to be a factor in spinal muscular atrophy (SMA) and amyotrophic lateral sclerosis (ALS) (151, 153), suggesting a common role in motor neuron disease. HuD interacts with the SMN (Spinal Motor Neuron) complex and the two translocate together down axons (153). Interestingly, exogenous expression of HuD together with the RBP IMP1 ameliorates some disease related defects in SMN deficiency models of SMA (151). Although many advances in this field have broadened our understanding of the molecular basis of motor neuron disease, molecular mechanisms underlying the specificity of motor neuron degeneration have not been completely elucidated, and more studies will be required to determine the full contribution of HuD and other RNA-binding proteins.

The dysregulation of both HuD and HuD-regulated mRNA targets have been reported in AD (156, 157). Two mRNAs, APP and BACE1 stabilized by HuD, have strong links to AD (156). Cleavage of APP, or amyloid precursor protein, yields Aβ. It is the aggregation of Aβ which forms the hallmark plaques characteristic of AD (158). Notably, HuD, APP, and BACE1 are elevated in AD patients (156). Although these

observations create intriguing links between AD and the HuD pathway, whether and how HuD directly contributes to neurodegeneration and AD pathogenesis remains to be answered.

1.3 Cdk5 signaling is critical for brain development and is dysregulated in neurological disease

Normal brain function relies heavily on the migration of neurons to their proper location, the integration of neurons into the neuronal circuitry, and on the establishment of functional synapses. Activity of the Cyclin-dependent kinase 5 (Cdk5), a non-canonical member of the Cyclin dependent kinase family, is critical for these post-mitotic neuronal processes to occur properly during embryogenesis and in the postnatal brain (159, 160). The dysregulation of Cdk5 activity, either loss or abnormal elevation, occurs in a variety of neurodegenerative diseases, thus underscoring the importance of finely tuned physiological regulation of Cdk5 activation (159, 161-163). Therefore, a major challenge in understanding the role for Cdk5 in normal neuronal function and neurological disease is delineating molecular mechanisms underlying the precise control of Cdk5 activity. To mediate such sophisticated control of Cdk5 activity in neurons, post-transcriptional mechanisms may provide a temporal advantage over transcription, perhaps when rapid upregulation of Cdk5 activity is required, and may also offer the ability to differentially modulate Cdk5 activity at the sub-cellular level.

1.3.1 The discovery of Cdk5, p35, and p39

Cdk5 is a non-canonical member of the Cdk family because it is most active in differentiated cells and does not play major roles in progressing the cell cycle (159). Although some of the first groups to report the discovery of Cdk5 identified the kinase

because of its high sequence homology to other Cyclin-dependent kinases, particularly Cdk1 (cdc2) (164, 165), discovery of Cdk5 was reported by multiple groups within the same year (164-166). A few years later Tsai et al. isolated a 35kDa protein and named it p35, which could bind and activate Cdk5 (167). Tang et al. reported a second, homologous 39kDa activator, p39, a year later, which could also activate the Cdk5 kinase (168). Notably, p35 and p39 are non-cyclin activators, and do not share primary sequence identity with other cyclins, although their tertiary structure when bound to Cdk5 is postulated to be similar to the canonical Cyclin-Cdk5 binding (169). The binding of either p35 or p39 to Cdk5 is sufficient for kinase activation (159). No additional phosphorylation of Cdk5 has been found to be necessary for activation of the complex (169), although this is a hotly debated topic. Cdk5 activation is, however, regulated by its ability to phosphorylate both p35 and p39, which leads to their degradation (170, 171).

It is important to note that both p35 and p39 can be cleaved through calcium-dependent calpain-mediated N-terminal cleavage to the lower molecular weight proteolytic fragments, p25 and p29, respectively (172-174). Such cleavage can be induced by neurotoxicity and neuronal activity and results in the mis-localization of Cdk5 activators away from the cell membrane due to the loss of the N-terminal myristoylation sites located in the full-length p35 and p39 proteins (171-173). Moreover, p25 and p29 have a longer protein half-life than their full-length counterparts (171, 172, 174). Thus, many studies have postulated the pathological effects of Cdk5 activator cleavage products in over-activation of Cdk5 (159). Notably, p39 is more resistant to this cleavage event than p35 (171). While N-terminal antibodies toward p35 exist, there are, to our knowledge, no specific commercially available N-terminal p39 antibodies. Thus,

pathological production and function of p25 has been the major focus of Cdk5 activator cleavage studies (159).

The question of why neurons express abundant amounts of two Cdk5 activators has persisted in the Cdk5 field. Initial studies suggested that p35 and p39 have unique temporal and spatial expressions, and perhaps unique functions. However, the severe cortical lamination defects of the p35-/- mouse and the lack of an overt phenotype in the p39-/- mouse led to the original hypothesis that p35 was the main functional activator of Cdk5, with p39 serving as a back-up copy (175, 176). However, it is only the double knockout mouse in which both p35 and p39 are lost that approaches the severe perinatal lethality of Cdk5-/- mice (176, 177). This observation hinted that p39 and p35 are both needed to govern normal brain development. Furthermore, as p35-/- mice do not fully recapitulate the loss of Cdk5 phenotype, this evidence was the first clue that p35 and p39 could have non-overlapping roles in directing Cdk5 function (175, 176).

Once activated, Cdk5 phosphorylates protein substrates to control neuronal development (159). Cdk5 is a proline-directed serine threonine kinase and early studies discovered that the Cdk5 consensus motif was X(S/T)PX(H/K/R), with X indicating any amino acid (178). One of the first identified phosphorylation targets of Cdk5 was the cytoskeletal protein, tau, which inspired an original name for the kinase as Tau Protein Kinase (179-181). Pioneer experiments investigating Cdk5 function observed the p35/Cdk5 complex at axonal growth cones (182, 183), leading to early studies which established a role for Cdk5 in governing neurite outgrowth (183). Following these initial functional studies, appreciation for the role of Cdk5 in the extension of axons and dendrites has grown rapidly (184-187).

1.3.2 Cdk5 activity regulates normal brain function

A wealth of literature following these foundational studies revealed that Cdk5 controls a multitude of brain functions through phosphorylation of a plethora of different protein substrates (**Figure 1-6**) (159). Besides neuronal migration and neurite outgrowth, which were the earliest indicated functions of Cdk5 (159), Cdk5 is a key player governing neurogenesis, neuronal survival, neuronal-network formation, neurotransmitter release, synaptic maturation, and synaptic plasticity (**Figure 1-6**) (160, 185, 187-192). Cdk5 phosphorylates a broad spectrum of protein targets in neurons which control the many signaling cascades important for such intricate neuronal function (159, 160, 191).

The role for Cdk5 in neuronal network establishment originated from the cortical layering defects present in the Cdk5-/- and p35-/- knockout mice (175, 177). When the cortex is formed during embryogenesis, neurons migrate through established layers to the top cortical layer, as they undergo differentiation (193). This process is abnormal in mice lacking p35 or Cdk5 (175, 177). Cdk5 phosphorylates several protein targets which govern this process and orchestrate the precisely ordered morphological changes required for cortical lamination (194-196). Notably, Cdk5 loss also disrupts the CA1 to CA3 layering in the hippocampus as well as layering in the cerebellum, pointing to an encompassing role for Cdk5 in neuronal migration and layering, although the mechanisms for neuronal migration and layering outside of the cortex remain to be elucidated (197).

Cdk5 also phosphorylates several substrates that control cytoskeletal dynamics, which is crucial for neuronal differentiation and neuro-circuitry formation (159). For example, inhibition of Cdk5 stabilizes microtubules and prevents the dynamic extension

and retraction required for morphology changes during axonal outgrowth during brain development (198). During neuronal differentiation, Cdk5 is essential for neurite outgrowth, as inhibition of Cdk5 activity or loss of Cdk5 impairs the differentiation of neurons in culture (183). Likewise, induction of Cdk5 activity leads to neurite extension. Recently it was also reported that the activity-dependent differentiation and growth of dendrites in culture is controlled by Cdk5 accumulation in the nucleus and Cdk5-mediated regulation of transcriptional cascades through MeCP2 phosphorylation (187). Thus, the role for Cdk5 in neurite outgrowth is well-established.

During neuronal development, small protrusions, eventually called dendritic spines, extend from dendrites that undergo maturation to form the post-synaptic portion of the synapse (199). Spines receive input from axons, transmit chemical signals, and are regulated to determine synaptic strength and plasticity. In particular, the number of dendritic spines per segment of dendrite and the distinctive specialized morphology of spines is critical for the propagation of neuronal signals within the network and is often dysregulated in disease (200-203). CDK5 plays sophisticated roles in controlling dendritic spine density (160, 204). In fact, in different biological paradigms, Cdk5 can either positively or negatively regulate spine formation (205-207). For example, CDK5 activation reduces dendritic spine density by modulating actin dynamics through phosphorylation of WAVE1 (207). Likewise, NMDA-receptor activation leads to degradation of p35 and de-phosphorylation of WAVE1, which enhances spine formation (208). Thus, Cdk5 can negatively regulate spine density. However, CDK5 activity is required for dendritic spine remodeling in response to stimulation by glutamate and the brain-derived neurotrophic factor (BDNF) through phosphorylation of the BDNF

receptor TrkB (205). Similarly, Cdk5 phosphorylation of S6K is also required for neuronal activity stimulated spine morphogenesis (209). Therefore, Cdk5 can also positively regulate spine density. How CDK5 activation leads to such diverse effects on dendritic spine morphology is not understood.

The role of Cdk5 in synaptic plasticity, a key aspect governing learning and memory, is also complicated. Conditional loss of CDK5 in neurons of the forebrain impairs fear conditioning and spatial learning (210). In contrast, the inducible loss of CDK5 in the whole brain enhances formation of memories through stabilization of the NMDA receptor NR2B (211, 212). The role for p25, which is the proteolytic cleavage fragment of p35 protein (174), in learning and memory is also quite complex. Learning and memory paradigms stimulate the production of p25 (213). However, the balance between physiological and pathological roles of p25 in cognitive function is intricate. Transient transgenic expression of p25 enhances learning and memory but prolonged p25 production results in reduced dendritic spine density and cognitive impairment (214). Together, these studies imply that the role for Cdk5 in neuronal development and higher order cognitive function is certainly multi-faceted. However, what regulates the discrete aspects of Cdk5 function in these paradigms is not understood. Cdk5 activity in learning and memory likely involves multiple layers of regulation and cooperation of many signaling pathways.

Although it is outside the scope of this work, it is important to note that recent studies investigating Cdk5 function point to roles for Cdk5 in non-neuronal cells (215, 216). While Cdk5 activity was highest in the brain (182), of the tissues originally examined, later studies have shown roles for Cdk5 function in other tissues, such as in

muscle and the pancreas. In these tissues, it seems that Cdk5 performs some similar functions as in brain neurons, contributing to cell death and migration but also regulating inflammation and cellular metabolism (215, 216). Although a few studies have claimed that Cyclin I can bind and activate Cdk5 in non-neuronal cells (217), if and what role p35 and p39 have in non-neuronal cell types to direct Cdk5 activity remains unknown.

1.3.3 Cdk5 activity is dysregulated in neurological disease

Dysregulation of CDK5 activity, either deficiency or exacerbation, is implicated in a growing list of human brain disorders (159, 161, 162). Reduced Cdk5 expression is reported in schizophrenic and Huntington's disease patients, where Cdk5-dependent phosphorylation is postulated to be neuro-protective by phosphorylating HTT (Huntington) and suppressing mHTT (mutant Huntington) toxicity (218-221). In contrast, Cdk5 activity is increased and regarded as pathological in neurodegenerative diseases, stroke, and epilepsy (163, 222, 223) . Moreover, mutations in the *CDK5* gene and the 3'UTR of p35 affecting p35 expression have been linked to intellectual disability (224). Unfortunately, the underlying molecular mechanisms of Cdk5 dysfunction in neurological disease remain understudied. However, these observations support the idea that tightly controlled Cdk5 activity in necessary to maintain a physiological range of Cdk5 function.

Cdk5 has been linked to neurodegeneration since its discovery. In fact, Cdk5 activity is a double-edged sword, where too much activity or two little activity can impair neuronal survival (222). Excitotoxicity, oxidative stress, and ischemia can all lead to production of p25, which has a longer half-life than p35 and can thus cause aberrantly prolonged activation of Cdk5 (173, 213, 223). Overexpression of p25 induces neuronal

death and long term transgenic expression of p25 leads to hallmark characteristics of neurodegeneration as seen in FTLD and AD (213, 214). Both excessive tau phosphorylation and p25-dependent Aβ production mediated by Cdk5 have been posited as Cdk5 contributing factors to pathology in AD (159, 213, 225). Moreover, with the increasing recognition of synaptic abnormalities in AD (226), whether and how Cdk5's multifaceted role in the morphogenesis of dendritic spines contributes to the cognitive abnormalities in AD remains to be answered.

Dysregulation of Cdk5 signaling also has numerous links to epilepsy. However, whether Cdk5 contributes to the induction of epilepsy or whether Cdk5 is protective against epileptogenesis remains unclear as different reports imply different roles for Cdk5 in seizure and epilepsy. In the brains of human epilepsy patients, for example, increased neuronal Cdk5 is detected (227). In addition, inhibition of Cdk5 activity counteracts the excitotoxic damages of kainic-acid induced seizure (163). However, mice lacking p35 or Cdk5 have increased susceptibility to seizure, suggesting p35-Cdk5 function may suppress seizure (175). One hypothesis is that p35-Cdk5 and p39-Cdk5 may have different function roles in seizure paradigms and thus mediate discrete aspects of Cdk5 function in epileptogenesis. However, the role for p39 in seizure has not been explored. Thus, whether and how p39 underlies the inducing role of Cdk5 function in epilepsy still remains unknown.

In summary, there are inconsistencies regarding whether Cdk5 activity is beneficial or pathological in different disease models. However, the molecular mechanism underlying Cdk5 dysregulation in such paradigms are often unclear. Therefore, understanding molecular mechanisms that control CDK5 activity and function

in normal brain function is a crucial prerequisite for developing novel strategies against brain disorders caused by deficiency or exacerbation of CDK5 activation.

1.3.4 The available amount of Cdk5 activators determines Cdk5 activity

Cdk5 is ubiquitously expressed. However, the highest Cdk5 activity is observed in the brain due to the abundance of p35 and p39 in neurons (176, 182). Thus, Cdk5 activity is limited by the available amount of Cdk5 activators. Evidence that the abundance of p35 and p39 is rate-limiting to Cdk5 activation comes from studies which observed changes in Cdk5 activity through genetically manipulating the dosage of Cdk5 activators, but not the kinase itself (176, 228). During development, Cdk5 activity is increased to accommodate neuronal circuitry formation and is elevated in adulthood in response to neuronal and synaptic activity changes (160, 167, 214). A prevailing challenge in the field is understanding how p35 and p39 expression in regulated to accommodate the sophisticated, and sometimes even conflicting, functions of Cdk5 in neurons. Importantly, p35 and p39 are extremely labile proteins in neurons, with a half-lives of around 1 hour for p35 and 5 hours for p39 (171). Thus, mechanisms which govern their replenishment are imperative for controlling Cdk5 activation and uncovering mechanisms which underlie expression and replenishment of Cdk5 activators within neurons is a vital prerequisite to understanding the intricate regulation of Cdk5 function.

p35 and p39 have distinct temporal expression patterns in the developing brain. p35 is abundantly expressed in embryonic brain neurons, where it directs Cdk5 function in governing neuronal migration (229, 230). In contrast, p39 expression is scarce during embryonic development, yet abundant in the adult brain (231, 232). These temporal expression pattern offer a potential explanation for why p39-/- mice do not suffer from

impaired cortical neuron layering during embryonic development as observed in p35-/- mice. A recent report demonstrated that p39 is the primary activator expressed in oligodendroglia (OL), the myelinating glia of the brain, and that p39 governs OL differentiation, whereas p35 is negligible in OLs (233). Fractionation studies demonstrate that the sub-cellular distribution of p35 and p39 protein do not overlap completely, but rather have distinct difference. However, a precise comparison of sub-cellular localization of Cdk5 activators at different points in development and under neuronal stimulation has not been elucidated (170, 171, 234). For example, both activators can translocate into the nucleus upon synaptic activation, yet p39 seems to be preferentially translocated into nuclei (170). p35 and p39 are also located at mature neuronal synapses, although it appears that p39 is enriched to a greater extent than p35 in synaptic fractions derived from adult brains (234).

Different temporal and spatial expression patterns of p35 and p39 support the notion that Cdk5 activators may direct Cdk5 to non-overlapping sets of phospho-targets, and thus may differently direct Cdk5 function. For instance, p39, but not p35, is necessary and sufficient for Cdk5-dependent phosphorylation of myosin regulatory light chain (235). In addition, p39 is preferentially localized to lamellipodia and neuronal growth cones where p39-dependent Cdk5 activity suppresses lamellipodia formation by reducing Rac1 activity (236).

p35 and p39 also exhibit differing biochemical properties. p39 is more resistant to proteosomal degradation than p35 and also harbors a significantly longer half-life as compared with p35 (171). In addition, both Cdk5 activators are myristoylated at the N-terminus, which enables plasma membrane association and triggers proteosomal

degradation and calpain-dependent cleavage (171). Calpain-dependent N-terminal cleavage of p35 and p39, which can be triggered by neurotoxicity or changes in cellular calcium, produces the C-terminal protein fragments, p25 and p29 respectively. These fragments harbor higher stability than their full-length protein derivatives and display mislocalization to the cytoplasm due to the loss of N-terminal myristoylation sites (172-174). Importantly, in addition to higher protein stability, p39 is also more resistant to calpain-dependent proteolytic cleavage than p35 (171, 172). Thus p35 is more likely to be cleaved to form p25 than p39 is to form p29. Very few studies have investigated the role of p29 in neurons, partly due to the lack of N-terminal antibodies which would detect p29. Rather, p25 has been extensively studied for its pathological role in neuronal death, neurodegeneration, AD, and stroke (173, 213, 214, 223). Thus, the pathological effects of p25 production are well-appreciated.

While little is known about the molecular mechanisms underlying the expression of p35 and p39, a few groups have uncovered transcription factors which affect the expression of p35 and p39. However, these studies primarily refer to general transcription factors, such as the ubiquitous sp1/sp3 and neuronal Brn-1/2, which can regulate the abundance of p35 and p39 (237, 238). Most reports do not directly test transcription, and the underlying mechanisms for differential expression of p35 and p39 therefore remains unsolved. Moreover, considering the polarity of neurons and the sophisticated temporal and spatial fine-tuning of Cdk5 activation, transcriptional regulation alone cannot accommodate the need for finely tuned or rapid regulation of Cdk5 activity.

1.3.5 Emerging evidence suggests post-transcriptional regulation of the Cdk5 activator, p35, by HuD.

Emerging evidence suggests that p35 can be regulated post-transcriptionally. The Riva group reported that the human p35 3'UTR contains elements which regulate the stability of p35 expression in model cell lines (239). In addition, RNA segments of p35 3'UTR bind to nELAV proteins *in vitro* and the nELAV protein member, HuD, can regulate expression of p35 protein in non-neuronal cells in culture (240). The potential functional link between the HuD and Cdk5 pathways is intriguing considering their overlapping functional spectrums in directing neuronal development and synaptic function. Additionally, these studies set the stage for important questions regarding the potential of post-transcriptional regulation of Cdk5 activators in neurons. However, whether p39 is also subject to such regulation and whether and how Cdk5 function is controlled by post-transcriptional means in neurons are questions to be addressed.

1.4 Summary and prevailing questions

In this dissertation, we explore how the post-transcriptional regulation of cellular mRNAs governs neuronal development and function. In particular, we investigate the hypothesis that: ***HuD governs complex brain function through post-transcriptional regulation of key pathways that control neuronal development and establishment of the neural-circuitry***. Our studies examine HuD-mediated post-transcriptional regulation of BDNF mRNA as well as Cdk5 activator mRNAs in hippocampal neurons (**Figure 1-7**).

The functions of BDNF in neuronal survival, neuronal circuitry development, and synaptic plasticity are well-recognized and dysregulation of BDNF is observed in neurological disease (29, 30, 241). Thus, regulation of BDNF expression is subject to many layers of control. The BDNF gene is subject to alternative polyadenylation resulting in BDNF mRNAs that harbor either a short or a long 3'UTR (31). The long

BDNF 3'UTR containing mRNA also has a shorter half-life than the short 3'UTR and long 3'UTR specific sequence localizes BDNF to dendrites and mediates activity-dependent translation of BDNF (32-34). However, trans-acting factors which differentially regulation BDNF long 3'UTR and BDNF short 3'UTR isoforms remain elusive. In this work, we investigate post-transcriptional mechanisms and trans-acting factors that preferentially regulate the long BDNF 3'UTR.

Likewise, the molecular signaling pathways governed by Cdk5 activity also control many crucial aspects of normal brain function and neurological disease (159). Despite intensive investigation, significant gaps remain in our understanding of how Cdk5 activity is regulated in sophisticated physiological paradigms. However, an important clue comes from the potential post-transcriptional regulation of the Cdk5 activator, p35, by the neuronal RBP HuD. The following unanswered question are prevailing gaps in knowledge regarding regulation of Cdk5 function: 1) how are Cdk5 activators regulated in neurons to mediate Cdk5 activity in multifaceted biological phenomena? 2) why do neurons express two distinct protein activators to control Cdk5 activity? 3) do CDK5 activators play synergistic or opposing roles in directing Cdk5 function in normal neuronal development and pathological conditions? We investigate these fundamental questions in this dissertation.

In **Chapter 2**, we introduce the RNA binding protein HuD and demonstrate how differential post-transcriptional regulation of BDNF isoforms by HuD influences abundance of BDNF in distinct neuronal populations. This molecular mechanism has important implications for regulation of BDNF production in neurons and for the shared function of HuD and BDNF in controlling plasticity of the neuronal circuitry. **Chapter 3**

explores post-transcriptional regulation of the Cdk5 pathway by HuD. Specifically, we uncover molecular mechanisms which underlie the differential regulation of p35 and p39 expression in neurons and discover that HuD drives p39-dependent Cdk5 activity and function in the hippocampus. In **Chapter 4**, we uncover epigenetic mechanism that specifically control abundance of p39 during neuronal differentiation and thus underlie Cdk5 activation during postnatal neuronal development. It is in this chapter that we reveal the essential and distinct functions of p39 in directing Cdk5 function during post-natal neuronal development and in the excitation of the hippocampal circuitry. Taken together, we unveil two key pathways for neuronal development and function under control of HuD-dependent post-transcriptional regulation. Thus, we demonstrate the important of post-transcriptional control of gene networks in coordinating the crucial processes required for normal brain function.

Figure 1-1: Post-transcriptional control of cellular mRNA abundance

Protein coding genes are transcribed in the nucleus by RNA Polymerase II. A 5' 7-methylguanylate (m7G) cap is added to the nascent pre-mRNA before transcription termination and the mRNA is then subjected to splicing by the spliceosome and associated proteins. As transcription terminates, the poly adenosine (poly A) tail is added to the pre-mRNA. Spliced mature mRNA is exported from the nucleus with associated RNA binding proteins. In the cytosol, mRNA can subjected to several additional regulatory processes. RNA binding proteins (RBPs) can stabilize mRNAs by competing with other RBPs or miRNA bindings sites. mRNAs and RBPs packaged into messenger ribonucleoprotein complexes (mRNPs) can be trafficked as granules on microtubules to distal processes. Finally, mRNAs are subjected to translational control in the soma and at the synapse where translation initiation is highly regulated by miRNAs and/or RBPs to control protein production (41). Figure adapted from *Molecular Cell Biology, Sixth Edition* (2008).

Figure 1-1

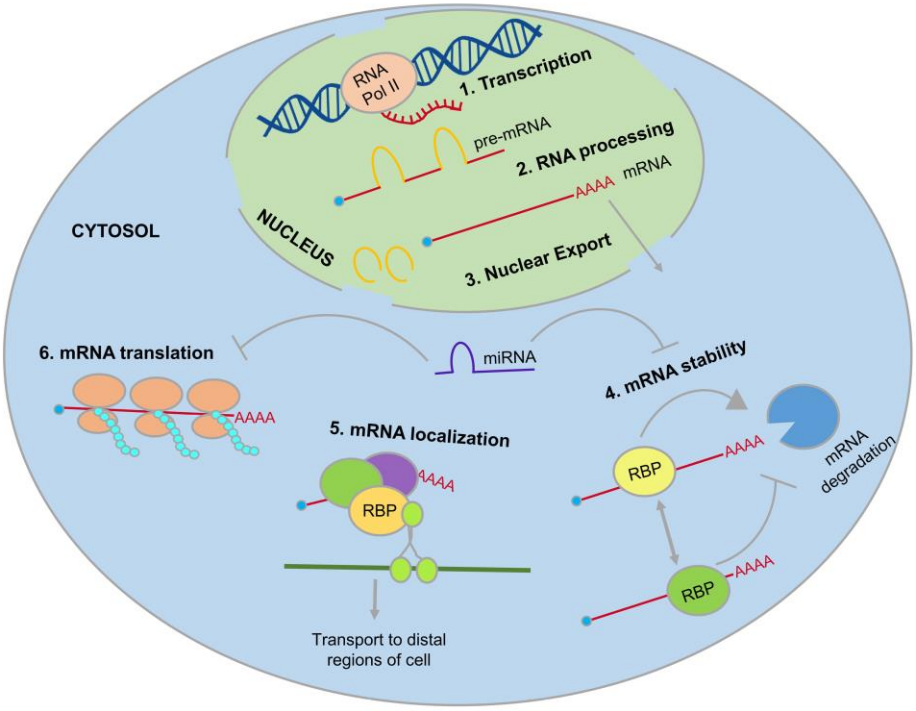

RNA Pol II
1. Transcription
pre-mRNA
2. RNA processing
AAAA
mRNA
CYTOSOL
NUCLEUS
3. Nuclear Export
6. mRNA translation
miRNA
4. mRNA stability
AAAA
5. mRNA localization
RBP
mRNA degradation
Transport to distal regions of cell

Figure 1-2: Eukaryotic mRNA degradation pathways

Multiple mechanisms exist for degradation of eukaryotic mRNA (22, 23). (A) Deadenylation-dependent mRNA occurs when the poly-A tail is removed by deadenylases which can be followed by 3'-5' decay by the exosome complex and/or the removal of the 5' m7G cap by decappping enzymes 5'-3' exonuclease decay. (B) Deadenylation-independent degradation occurs when either de-capping triggers 5'-3' degradation or sequence specific endonuclease cleave the mRNA followed by 5'-3' or 3'-5' exonuclease degradation. Elements of this figure adapted from Wu & Brewer (2012) and Beelman et al., (1995).

Figure 1-2

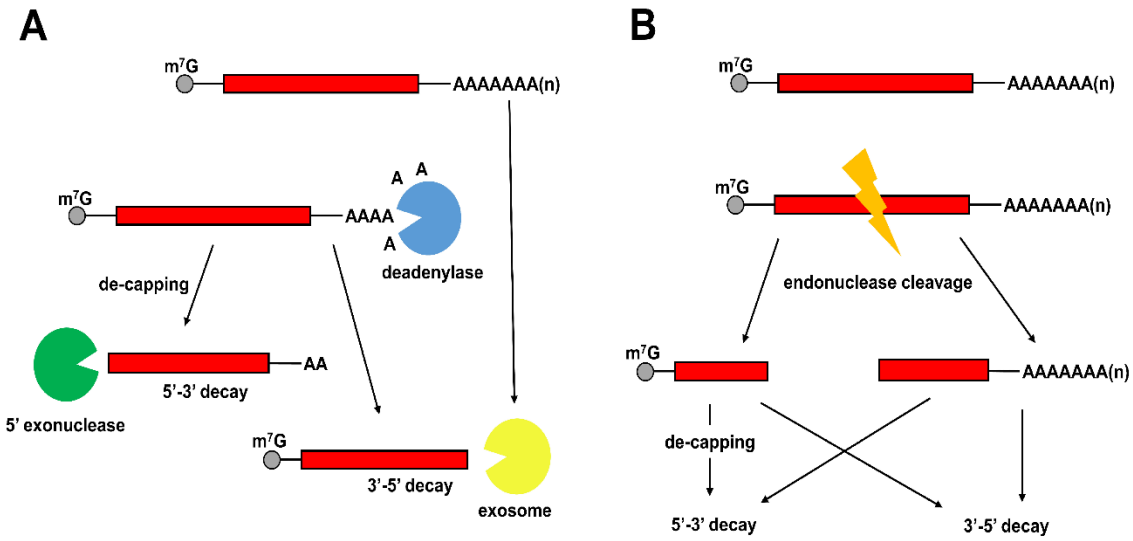

A
m7G
AAAAAAA(n)
m7G
AAAA
A
A
A
deadenylase
de-capping
AA
5'-3' decay
5' exonuclease
m7G
3'-5' decay
exosome
B
m7G
AAAAAAA(n)
m7G
AAAAAAA(n)
endonuclease cleavage
m7G
AAAAAAA(n)
de-capping
5'-3' decay
3'-5' decay

Figure 1-3: Coordination between miRNAs and RNA-binding proteins

Coordination between miRNAs and RNA-binding proteins (RBPs) occurs through either cooperation (A-B) or antagonism (C-D) (55). An mRNA may contain separate binding sites for the RBP and the miRNA, which may allow them to either cooperate (A) in destabilization or antagonize (C) one another in binding and regulating the mRNA. (B) Alternatively, the miRNA and RBP may have separate sequence binding regions, one of which is masked by secondary structure until the other trans-acting factor is bound. (D) The miRNA and RBP can also compete for the same binding site to mediate opposing functions. Figure adapted from Iadevaia & Gerber (2015).

Figure 1-3

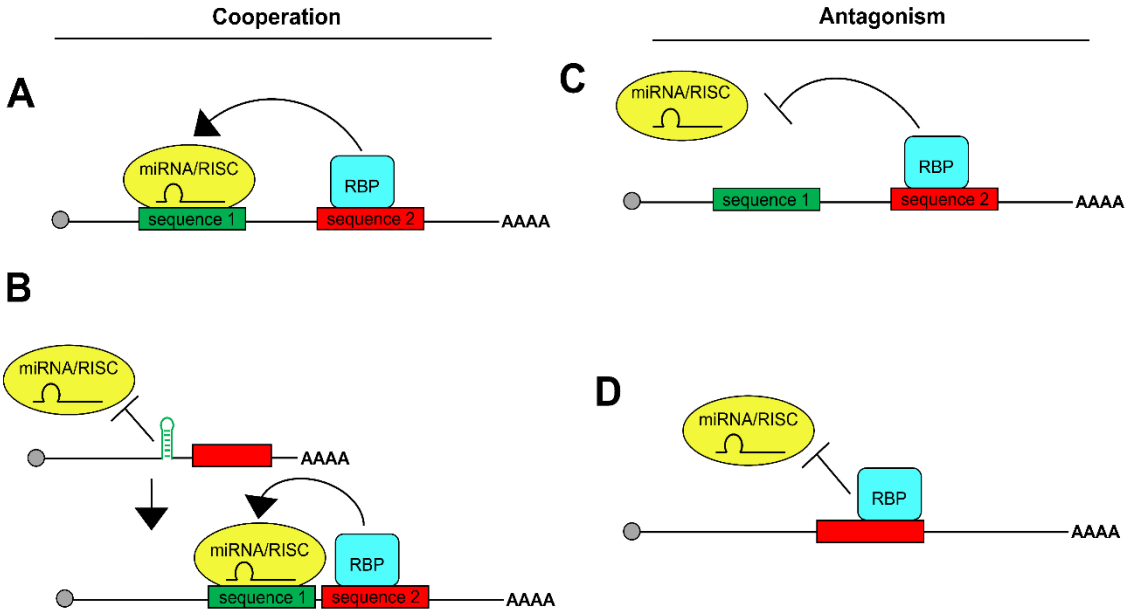

Cooperation
Antagonism
A
B
C
D
miRNA/RISC
RBP
sequence 1
sequence 2
AAAA

Figure 1-4: Protein domains of the conserved ELAV family of proteins

Protein domain structures of the drosophila Embryonic Lethal Abnormal Vision (ELAV) protein and the conserved mouse ELAVl family members. Conserved domains are colored in green (RNA recognition motif 1), blue (RNA recognition motif 2), and red (RNA recognition motif 3) (118). Protein amino acid (a.a.) number is displayed to the right of each member. Figure adapted from Okano & Darnell (1997).

Figure 1-4

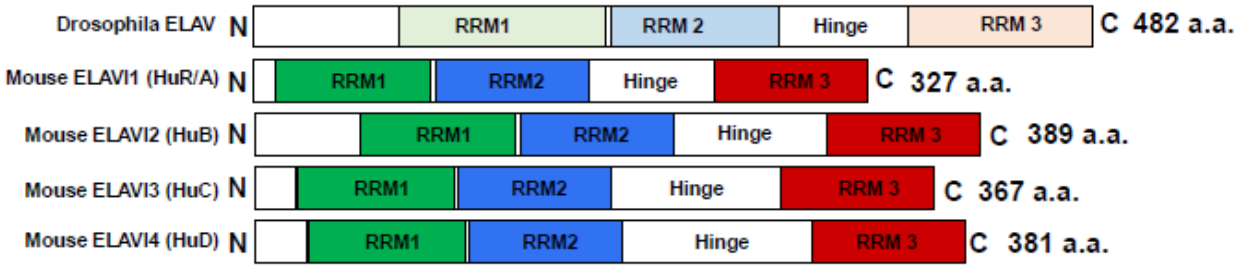
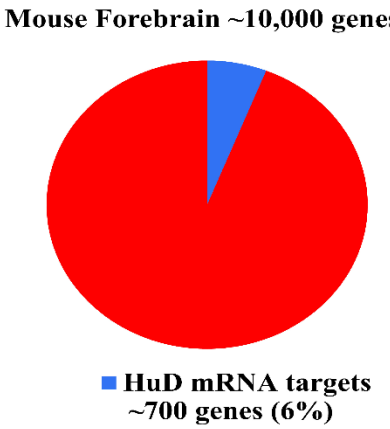

Drosophila ELAV
N
RRM1
RRM 2
Hinge
RRM 3
C 482 a.a.
Mouse ELAVl1 (HuR/A)
N
RRM1
RRM2
Hinge
RRM 3
C 327 a.a.
Mouse ELAVl2 (HuB)
N
RRM1
RRM2
Hinge
RRM 3
C 389 a.a.
Mouse ELAVl3 (HuC)
N
RRM1
RRM2
Hinge
RRM 3
C 367 a.a.
Mouse ELAVl4 (HuD)
N
RRM1
RRM2
Hinge
RRM 3
C 381 a.a.

Figure 1-5: HuD binds to different motifs to regulate mRNA targets

Mouse forebrain neurons encode about 10,000 genes, of these about 700 (or 6%) were identified as HuD targets (pie chart, right) (61). About 20% of genes encoded by murine forebrain neurons contain A/U-rich elements (AREs). However, less than half of all identified HuD binding targets contain A/U-rich elements, suggesting some selectivity in HuD recognition and the potential for alternate binding motifs (shown in table, right), uncovered by (61).

Figure 1-5



HuD target sequences:

Target Sequence	Example
ARE, Class I	AUUUA
ARE, Class II	overlapping $(AUUUA)_n$
ARE, Class III	(U-rich)
Consensus Motif I	CCCUCCCUCUCUC
Consensus Motif II	UUUUGUUUUGUUU
Consensus Motif III	UUUUUUUUUUAAA

Bolognani et al. (2010)

Figure 1-6: Cdk5 signaling plays important roles in neuronal function

Cdk5 phosphorylates many protein substrates to govern neuronal function. Here, major functional pathways controlled by Cdk5 are depicted (text), with important phosphorylation targets surrounding (green circles) (159). This figure was adapted from Su & Tsai (2010).

Figure 1-6

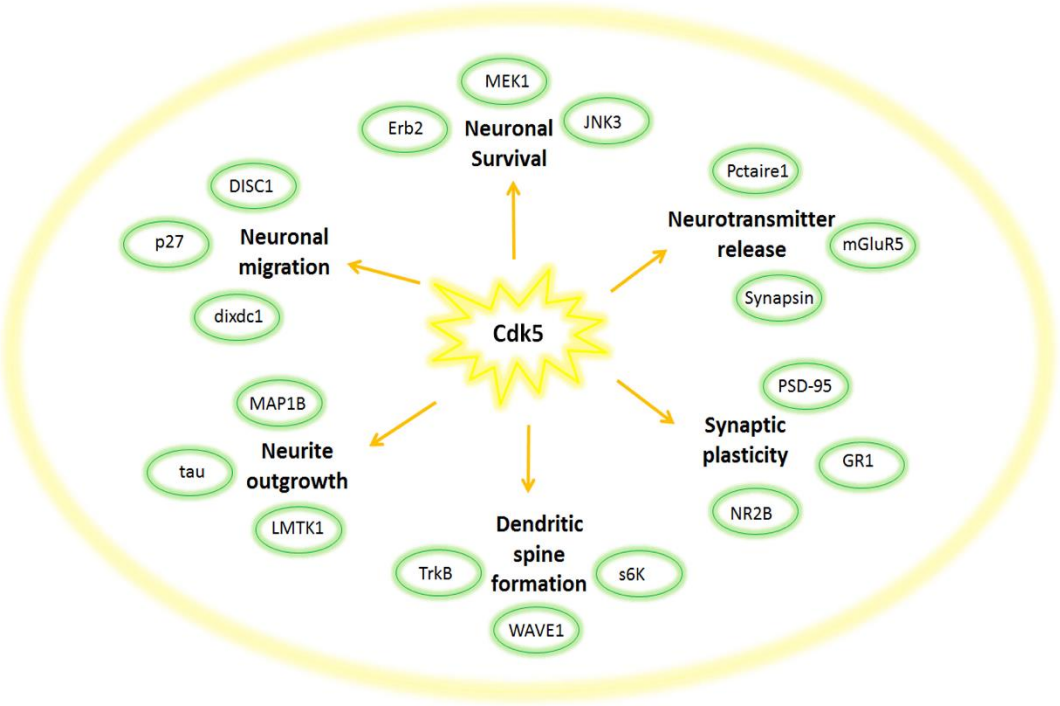
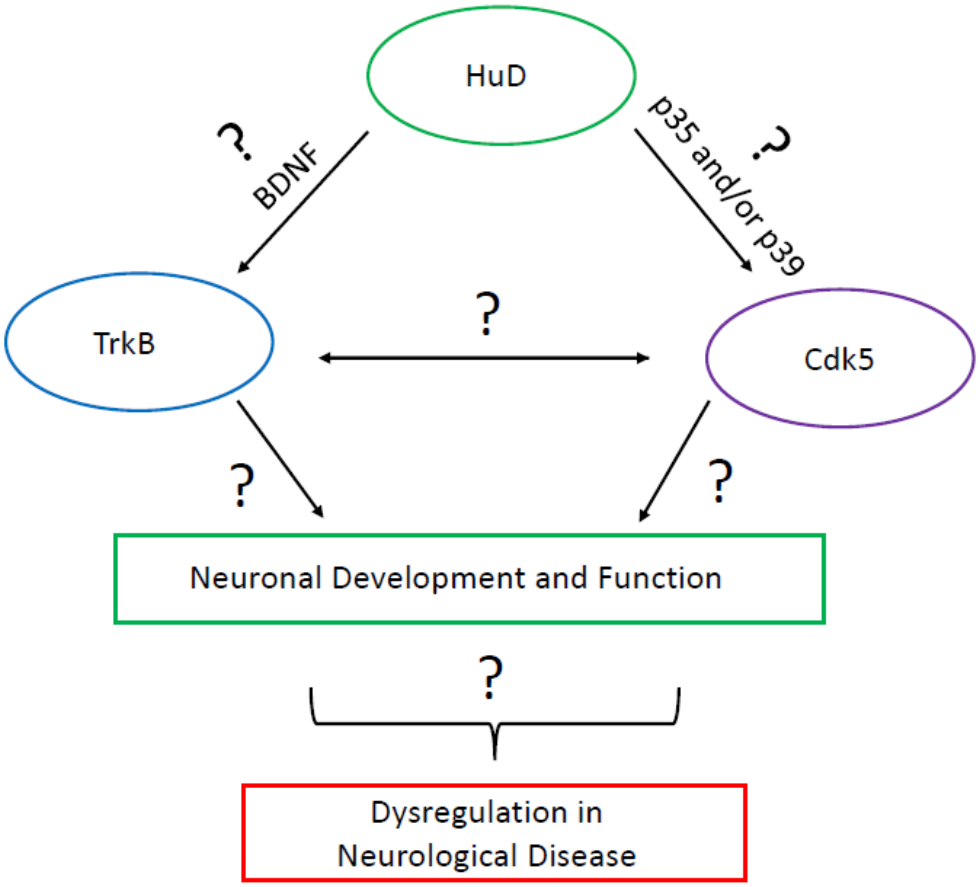

MEK1
Erb2
Neuronal Survival
JNK3
DISC1
p27
Neuronal migration
dixdc1
Pctaire1
Neurotransmitter release
mGluR5
Synapsin
Cdk5
MAP1B
tau
Neurite outgrowth
LMTK1
PSD-95
Synaptic plasticity
GR1
NR2B
Dendritic spine formation
TrkB
s6K
WAVE1

Figure 1-7: Questions to be investigated in this dissertation

Here we show several key players in neuronal development and function, HuD, Cdk5, and BDNF. The goal of this dissertation is to better understand the potential links and undiscovered regulatory steps between each player, indicated by question marks. We hypothesize that: ***HuD governs complex brain function through post-transcriptional control of key pathways that control neuronal development and establishment of the neural-circuitry.***

Figure 1-7


HuD
?
BDNF
?
p35 and/or p39
TrkB
?
Cdk5
?
?
Neuronal Development and Function
?
Dysregulation in
Neurological Disease

Chapter 2

HuD Promotes BDNF Expression in Brain Neurons via Selective Stabilization of the BDNF Long 3′UTR mRNA

This chapter is published as:

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*denotes equal contribution to this work

2.1 Introduction

Brain-derived neurotrophic factor (BDNF) plays pivotal roles in governing a broad spectrum of brain functions including neuronal survival, neural network development, and synaptic plasticity. To accommodate such intricate functions, BDNF expression is under precise regulation. Furthermore, dysregulation of BDNF is indicated in the pathogenesis of various neurological and psychiatric diseases (241, 242). Transcription of BDNF can be initiated from at least eight different promoters in mammals (31, 243), which underlies distinct responses to various stimulation cues (31, 244, 245). Besides the sophisticated transcriptional regulation, alternative polyadenylation of the BDNF transcripts results in two pools of BDNF mRNAs that carry either a short or a long 3'untranslated region (UTR), regardless of which promoter drives BDNF transcription (31, 246). While the entire sequence of the short 3'UTR is contained within the long 3'UTR, it is the unique sequence in the BDNF long 3' UTR that mediates differential regulation of BDNF production through multiple post-transcriptional mechanisms. For instance, the long 3'UTR, but not the short 3'UTR, suppresses BDNF translation in the resting brain and mediates neuronal activity-dependent translation of BDNF (33). The long 3'UTR can also target BDNF mRNA into dendrites thereby governing dendritic BDNF protein abundance (33, 34). Furthermore, the short and long 3'UTR differentially affect BDNF mRNA stability, with the long transcript having a much shorter half-life than its short counterpart (32). Mechanistically, the unique sequence in the BDNF long 3'UTR provides binding sites for trans-acting RNA-binding proteins (RBPs) and/or microRNAs to achieve differential posttranscriptional regulation

of BDNF mRNA isoforms (247). However, no previous studies have identified any RBPs that specifically bind and regulate the BDNF long 3'UTR mRNA in brain neurons.

We report here that HuD, a neuron-specific RBP that plays critical roles in governing neuronal circuitry development and plasticity via controlling mRNA stability and/or translation (131), selectively binds the BDNF long 3'UTR but not the short 3'UTR. A highly conserved A/U-rich element (ARE), located at the distal end of the BDNF long 3'UTR, mediates direct interaction with HuD in vitro and in transfected cells, which is necessary for HuD to stabilize reporter RNAs that harbor the BDNF long 3'UTR. Moreover, shRNA-mediated HuD knockdown results in overall reduction of the BDNF long 3'UTR mRNA in the soma and processes of hippocampal neurons whereas over-expression of HuD leads to selectively enhanced expression of the BDNF long 3'UTR mRNA. Finally, we show that over-expression of HuD from a transgene increases BDNF long 3'UTR mRNA levels primarily in the hippocampal dentate granule cells (DGCs) and BDNF protein accumulation in the hippocampal mossy fiber (MFs) axon terminals projected from DGCs. Together, our findings identify a novel mechanism for posttranscriptional regulation of distinct BDNF mRNA isoforms, which reveals a functional link between the pathways under HuD and BDNF, both playing key roles in controlling plasticity of neuronal circuitry.

2.2 Results

2.2.1 HuD selectively enhances expression of the BDNF long 3'UTR reporter through a highly conserved ARE

AREs are prominent motifs found in mRNA 3'UTRs that recruit various ARE-binding proteins (ARE-BPs) to stabilize or destabilize target mRNAs (27). AREs can be

divided into three different classes: Class I AREs contain a core penta-nucleotide AUUUA flanked by A/U, Class II AREs contain overlapping AUUUA motifs, and Class III AREs do not contain typical AUUUA motifs but long stretches of U-rich sequences. Using the ARED-Organisms database (http://brp.kfshrc.edu.sa/ARED/), we identified a highly conserved Class I ARE specifically located in the BDNF long 3'UTR immediately up-stream of the distal polyadenylation site (**Figure 2-1A**), suggesting that ARE-BPs may preferentially regulate stability of the BDNF long 3'UTR mRNA.

HuD is a neuronal specific ARE-BP (248) that displays a functional spectrum substantially overlapping with that of BDNF (18). To explore whether HuD interacts with the BDNF long 3'UTR via the ARE which in turn stabilizes the mRNA, we generated luciferase reporter constructs that carry the short BDNF 3'UTR (S-3'UTR), the full-length BDNF long 3'UTR (L-3'UTR) or the BDNF long 3'UTR lacking the ARE (dARE). The parental luciferase construct that does not harbor predictable ARE (PCLuc) and the aforementioned BDNF 3'UTR reporters were transfected individually into the immortalized brain neuronal cell line, CAD. Three sets of specific primers were employed in RT-PCR reactions to detect expression of the aforementioned reporter mRNAs, with primer A in the luciferase coding sequence (CDS), primer B located in the proximal portion of the long 3'UTR, and primer C located immediately up-stream of the ARE. As shown in **Figure 2-1B**, primer A detects comparable levels of all luciferase reporter mRNAs, whereas Primer B and C only detect the full-length BDNF long 3'UTR and the Δ-ARE reporter mRNA, confirming the expected expression of reporter mRNAs. Next, BDNF reporter constructs carrying L-3'UTR, ΔARE L-3'UTR and S-3'UTR were co-transfected with a c-myc-tagged HuD construct (133), followed by UV-cross linking

immunoprecipitation (CLIP) using an anti-myc antibody and qRT-PCR. Successful immunoprecipitation of myc-HuD is shown in **Figure 2-1C**. Due to the presence of endogenous BDNF transcripts in CAD cells, primer A was used in this experiment to specifically address association of myc-HuD with the reporter mRNAs that carry various sequence segments in the BDNF 3'UTRs. As shown in **Figure 2-1D**, the long 3'UTR reporter mRNA is preferentially enriched in immunoprecipitated HuD complex compared to the short 3'UTR reporter mRNA. Furthermore, removing the ARE in the long 3'UTR significantly attenuated the association of the reporter mRNA with HuD.

We next tested whether HuD can regulate reporter mRNA expression selectively via the BDNF long 3'UTR in an ARE-dependent manner. As shown in **Figure 2-2A**, myc-HuD significantly increased luciferase reporter expression from the full length BDNF long 3'UTR construct, but not the BDNF short 3'UTR construct. In addition, myc-HuD leads to a moderately reduced decay of the BDNFL-3'UTR reporter mRNA in the presence of the transcription inhibitor actinomycin D (**Figure 2-2B**). Furthermore, removing the ARE in BDNF long 3'UTR abolished the effect of HuD on luciferase reporter activity (**Figure 2-2C**). Importantly, the increase of BDNF long 3'UTR reporter mRNA by myc-HuD was also abolished when the ARE was removed (**Figure 2-2D**), recapitulating the HuD response measured by luciferase activity (**Figure 2-2C**). Thus, although HuD can also promote translation initiation (121), its regulation mediated by the BDNF long 3'UTR ARE most likely occurs at the level of mRNA stability.

2.2.2 HuD directly binds and stabilizes RNA that carries the ARE segment in the BDNF long 3'UTR

To test whether HuD can indeed directly bind the ARE segment in the BDNF long 3'UTR, we performed RNA-mobility shift assays using recombinant GST-HuD or GST, which is a commonly used approach for demonstrating direct interaction between an RNA-binding protein and the sequence element within its target mRNA. As shown in Figure 3A, GST-HuD bound the BDNF-ARE segment with high affinity. A mobility shift of the RNA can be clearly visualized with only 25 ng of GST-HuD in the presence of high concentrations of non-specific RNA competitor (tRNA, 0.25 mg/ml) and BSA (0.25mg/ml). In contrast, GST alone did not bind the RNA at any concentrations examined. The specificity of HuD-ARE interaction was further demonstrated by the displacement of the radiolabeled RNA with a cold ARE competitor (**Figure 2-3A)**, right panel).

Although myc-HuD increased the BDNF L-3'UTR mRNA in an ARE-dependent manner (**Figure 2-2D**), this experiment could not exclude the possibility that HuD may also regulate other trans-acting factors which in turn stabilize BDNF L-3'UTR mRNA. To further examine whether interactions between HuD and the BDNF-ARE alone could result in RNA stabilization, we used *in vitro* transcribed capped and polyadenylated BDNF-long 3'UTR ARE RNA and GST-HuD for *in vitro* decay assays (**Figure 2-3B**). Addition of GST-HuD to brain extracts resulted in a significant stabilization of the RNA (Two way ANOVA, $F = 4.68206$. $DFn=1$ $DFd=30$, $p=0.03857$), with an almost 2-fold reduction in the initial rate of decay. These data clearly indicate that HuD is capable of direct binding to the ARE in the BDNF long 3'UTR, which in turn leads to stabilization of the bound RNA.

2.2.3 HuD expression levels selectively regulate the abundance of the endogenous BDNF long 3'UTR mRNA

We next examined whether HuD abundance can govern the expression levels of endogenous long-BDNF mRNA. HuD was successfully knocked down in CAD cells using a previously published siRNA (**Figure 2-4A**), which leads to a significant reduction of the endogenous BDNF long 3'UTR mRNA (**Figure 2-4B**). We then performed shRNA-mediated HuD knockdown in primary cultured hippocampal neurons that express high levels of HuD and BDNF (136, 249). Considering the dendritic localization of the BDNF long 3'UTR mRNA (34), we performed fluorescent in situ hybridization (FISH) using a BDNF long 3'UTR-specific probe and quantitatively measured the IF signals in the soma and dendrites of transfected neurons marked by co-expression of GFP. As shown in Figure 4C and D, shRNA-mediated HuD knockdown leads to significant reduction of long 3'UTR BDNF mRNA in both soma and dendrites, suggesting that HuD governs the level of the long 3'UTR BDNF mRNA in the entire neuron.

Reciprocally, we treated cortical neurons with HSV vectors to achieve forced expression of exogenous HuD (136). The control virus that expresses the LacZ gene was used to treat parallel cultures. As shown in **Figure 2-5A**, over-expression of HuD significantly increased the levels of the BDNF long 3' UTR mRNA, whereas the pan BDNF mRNA levels were unaltered (**Figure 2-5B**), in which the short BDNF mRNA is the major isoform (33). Together, these results suggest that HuD abundance indeed governs neuronal BDNF long mRNA expression.

2.2.4 A HuD transgene selectively up-regulates BDNF long 3'UTR mRNA and BDNF protein in the hippocampal MF pathway

Finally, we tested whether elevated HuD expression can regulate neuronal BDNF production *in vivo* through the long 3'UTR. Quantitative FISH was performed using brain slices of HuD transgenic mice (HuD-Tg) that express myc-tagged HuD under the CamKIIα promoter (137). Non-transgenic WT littermates were processed in parallel as baseline controls. Consistent with the preferential increase of HuD from the transgene in HuD-Tg hippocampal DGCs (137), BDNF long 3'UTR mRNA is significantly up-regulated in the DGCs of HuD-Tg (**Figure 2-6A** and **2-6B**). In contrast, BDNF long 3' UTR mRNA was not increased in CA3 or CA1 neurons (**Figure 2-6**), conceivably explained by the fact that the HuD transgene is not significantly over-expressed in CA3 and CA1 pyramidal neurons (137) .

BDNF protein synthesized in DGCs is primarily transported to and stored in hippocampal MF axons that project through the hilus to form large synapses with CA3 dendrites (250). Consistent with the enhanced expression of BDNF long 3'UTR mRNA in DGCs (**Figure 2-6**), significantly increased BDNF protein immunofluorescence was detected in the hilus and the CA3 strata lucidum and radiatum of HuD-Tg as compared to the WT control (**Figure 2-7A** and **2-7B**). Transgenic HuD expression also increased BDNF protein in cells of the DG subgranular zone (SGZ) that is enriched of adult neural stem cells (251), particularly in the characteristic long processes extended into the DGC layer (arrows in **Figure 2-7A**). In contrast, BDNF protein levels in HuD-Tg CA1 neurons are not significantly altered (**Figure 2-7A** and **2-7B**). Given the vigorous regulation of endogenous HuD in DGCs (130), HuD-dependent stabilization of BDNF

long 3'UTR mRNA is a novel mechanism that controls BDNF production in the hippocampal MF pathway, a critical circuitry that governs hippocampal excitatory activities in physiological and pathological plasticity during epileptogenesis (250, 252).

2.3 Discussion

Our studies identify HuD as the first RBP that selectively binds to and stabilizes the BDNF long 3'UTR mRNA but not the short BDNF mRNA. The interaction and function of HuD is mediated by an ARE in the distal end of BDNF long 3'UTR, which is highly conserved in mammalian species. Furthermore, we provide evidence that the cellular abundance of HuD in neurons preferentially regulates the expression levels of the BDNF long 3'UTR mRNA isoform in culture and *in vivo*. Considering the vigorous regulation of HuD and BDNF during neuronal differentiation and neuronal activation (29, 139, 242), the functional connection between HuD and BDNF uncovered by our studies provides a novel mechanism that may synergistically govern normal brain development and accommodate functional changes in the brain.

In a recent report, HuD was shown to associate with several neurotrophic factor mRNAs, including BDNF (249). In addition, manipulating HuD expression correlated with altered expression of these neurotrophic factor mRNAs. However, whether the long or the short BDNF 3'UTR interacts with HuD was undetermined. Whether HuD controls stability of BDNF mRNA and whether a specific ARE in BDNF 3'UTR is required for HuD to regulate BDNF is unknown. Moreover, whether HuD abundance can regulate BDNF mRNA *in vivo* remains elusive. These are important questions given the two distinct BDNF 3'UTRs differentially regulate BDNF translation and dendritic localization in response to neuronal activation (33, 34) In this study, we demonstrated

that a highly conserved ARE in the distal end of the BDNF long 3'UTR primarily mediates the association of the reporter mRNA with HuD in transfected cells and directly interacts with recombinant HuD *in vitro* with high specificity and affinity. We also show that recombinant HuD can stabilize an mRNA that contains the ARE in the BDNF long 3'UTR in brain lysates. Furthermore, HuD preferentially regulates the endogenous BDNF long 3'UTR mRNA in both the somatic and dendritic compartments of brain neurons. In contrast, the short BDNF3'UTR reporter mRNA does not appear to be regulated by HuD. Noticeably, mRNAs carrying AREs in the 3'UTR often display short half-lives (27, 253). Consistent with his view, the BDNF long 3'UTR mRNA is less stable than the BDNF short 3'UTR mRNA (32), likely due to recruitment of undefined destabilizing RBPs and/or miRNAs by the unique sequence in the long 3'UTR. Such instability provides a practical opportunity for stabilizing RBPs, represented by HuD, to selectively increase the abundance of BDNF long 3'UTR mRNA. This is of particular interest, considering the fact that the long 3'UTR, but not the short 3'UTR, mediates activity-dependent translation of BDNF in brain neurons (33).

Despite its primary localization in the neuronal soma, the BDNF long 3'UTR mRNA is also delivered to dendrites, which is critical for controlling dendritic BDNF protein levels and synaptic maturation (34). Thus, molecular mechanisms governing dendritic BDNF mRNA abundance have attracted enormous attention. Interestingly, HuD is also detected in dendrites, although the majority of HuD is present in the neuronal soma (139, 249, 254). We show that shRNA-mediated HuD knockdown reduces the long BDNF mRNA isoform in both the somatic and dendritic compartments of hippocampal

neurons in culture, suggesting that HuD plays essential roles to govern the stability of the BDNF long 3'UTR mRNA in both compartments.

Importantly, HuD expression is vigorously regulated during neuronal development, nerve regeneration and functional changes of the brain (113). HuD protein is highly expressed in the embryonic and neonatal brain, but reduced to low levels in the adult brain where it is subjected to marked up-regulation upon neuronal activation in multiple plasticity paradigms (18, 130, 139, 141, 142, 254). We found that over-expression of HuD in primary cultured cortical neurons selectively increases the BDNF long 3'UTR mRNA, but not the pan BDNF mRNA. Thus, the previously reported function of HuD in up-regulating BDNF must be specifically mediated by the BDNF long 3'UTR. It is important to point out that HuD is differentially regulated in distinct neuronal subpopulations in the brain in various functional paradigms (18, 118). Along with the distinct expression patterns of the short and long BDNF mRNA isoforms in various brain regions (34), HuD-dependent selective stabilization of the BDNF long 3'UTR provides a novel posttranscriptional mechanism that increases the complexity of temporal and spatial regulation of BDNF to accommodate brain development and function.

A particularly interesting case is the regulation of HuD in hippocampus. HuD protein is not normally detected in adult hippocampal DGCs (130). However, upon seizure induction, HuD is drastically up-regulated in DGCs. In contrast, although HuD is present in CA1 and CA3 neurons at rest, it is only moderately increased after seizures. Recapitulating the robust increase of HuD in DGCs in response to seizure, resting HuD-Tg mice display the highest fold increase of HuD protein in DGCs (132, 137), although HuD transgene expression can be detected in all forebrain neurons. The relatively lower

level of over-expression of the HuD transgene in CA1 explains the lack of significant increase of BDNF protein in these neurons. In contrast, BDNF protein expression is preferentially increased in DGCs, which is transported and stored in the axonal terminals of the MFs. Considering the well-known effects of BDNF in promoting the growth of neuronal processes (252), increased BDNF expression in hippocampal MFs is likely a contributing factor for the MF over-projection in HuD-Tg (132). Conceivably, the seizure-induced HuD up-regulation in DGCs could contribute to the well-characterized preferential increase of BDNF in the MF terminals and MF sprouting during epileptogenesis (250, 255).

Taken together, our study reveals a novel mechanism that controls BDNF expression through HuD-dependent stabilization of the BDNF long 3'UTR mRNA, which would impact various neuronal populations that harbor differential regulation of HuD in response to neuronal activity changes. However, because conventional knockout of HuD results in abnormal neuronal development (256), definitive demonstration of the biological consequence of HuD-dependent BDNF mRNA stabilization through the long 3'UTR is a challenging question that may only be addressed once inducible knockout of HuD can be achieved. Besides controlling mRNA stability, HuD was recently reported to enhance translation initiation through unwinding structural 5'UTRs (121). Noticeably, the long 3'UTR BDNF mRNA is repressed for translation initiation in resting neurons but drastically activated upon neuronal and synaptic stimulation (33). Thus, although the ARE in BDNF long 3'UTR appears to mediate HuD effect primarily through mRNA stability in resting neurons, whether HuD can also enhance BDNF translation upon neuronal activation, perhaps involving interaction between the long 3'UTR and distinct

5’UTRs in BDNF mRNA isoforms, is an intriguing question to be answered by future studies.

Figure 2-1: HuD enhances luciferase reporter expression through an ARE in the BDNF long 3'UTR

(A) A highly conserved cluster of Class I ARE in the BDNF long 3'UTR, with a consensus core sequence of ***AUUUA*** flanked by symmetric A or U (underlined). Triangles indicate alternative polyadenylation sites in the BDNF primary transcript. Primers used for RT-PCR to detect reporter mRNAs are illustrated underneath. (B) RT-PCR using multiple primers illustrated in (A) confirms expression of the expected RNA sequence in each reporter. (C) Immunoblot (IB) showing expression of myc-HuD in the input (Inp) of transfected CAD cells and successful immunoprecipitation (IP) of myc-HuD. Two different anti-myc antibodies were used for IP and IB. (D) CLIP-qRT-PCR quantification of reporter mRNAs co-immunoprecipitated with HuD relative to the corresponding mRNA levels in the input. * indicates $P<0.05$ by Student's t-test (n=3). Due to the presence of endogenous BDNF mRNA, primers specific for the luciferase coding region were used to detect reporter mRNAs. BDNF long 3'UTR reporter mRNA is preferentially enriched over short 3'UTR mRNA in immunoprecipitated HuD complex. Deletion of the ARE in the long 3'UTR significantly reduced the association or reporter mRNA with HuD.

Figure 2-1

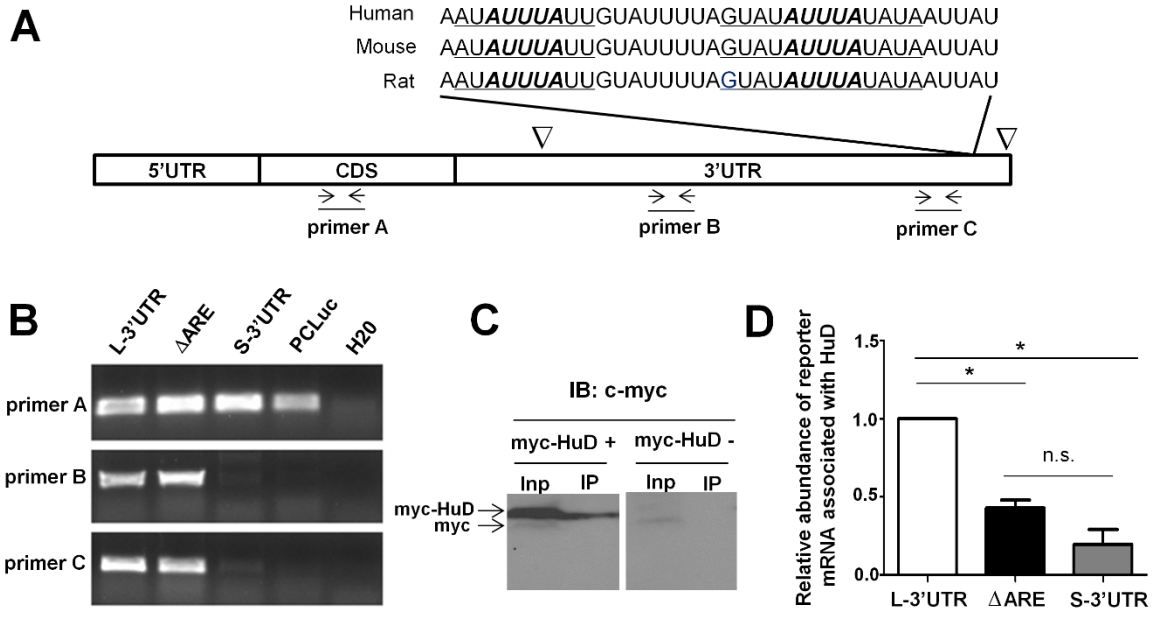

A
Human AAUAUUUAUUGUAUUUUAGUAUAUUUAUAUAAUUAU
Mouse AAUAUUUAUUGUAUUUUAGUAUAUUUAUAUAAUUAU
Rat AAUAUUUAUUGUAUUUUAGUAUAUUUAUAUAAUUAU
5'UTR
CDS
3'UTR
primer A
primer B
primer C
B
L-3'UTR
ΔARE
S-3'UTR
PCLuc
H20
primer A
primer B
primer C
C
IB: c-myc
myc-HuD +
myc-HuD -
Inp
IP
Inp
IP
myc-HuD
myc
D
Relative abundance of reporter mRNA associated with HuD
1.5
1.0
0.5
0.0
*
*
n.s.
L-3'UTR
ΔARE
S-3'UTR

Figure 2-2: HuD selectively enhances expression of the luciferase reporter that harbors the BDNF long 3'UTR in an ARE-dependent manner

CAD cells were co-transfected by 0.1 μg of reporter construct together with either pcDNA-HuD (HuD) or the pcDNA parental vector (PC). 20 ng of pRL-CMV Renilla luciferase construct was included in each transfection. Twenty-four hours after transfection, cells were harvested and subjected to dual luciferase reporter assay or mRNA extraction followed by DNAase-treatment and RT-qPCR. (A) myc-HuD enhances expression of the luciferase reporter that carries the BDNF long 3'UTR but not the short 3'UTR. (B) myc-HuD reduces decay of the BDNF long 3'UTR reporter mRNA in co-transfected CAD cells in which transcription is inhibited by actinomycin D. (C) Loss of the ARE in the BDNF long 3'UTR abolished the repose to HuD-dependent enhancement of reporter expression. (D) HuD regulates reporter mRNA expression mediated by the ARE in the BDNF long 3'UTR. * indicates $P<0.05$ by Student's t-test (n=3).

Figure 2-2

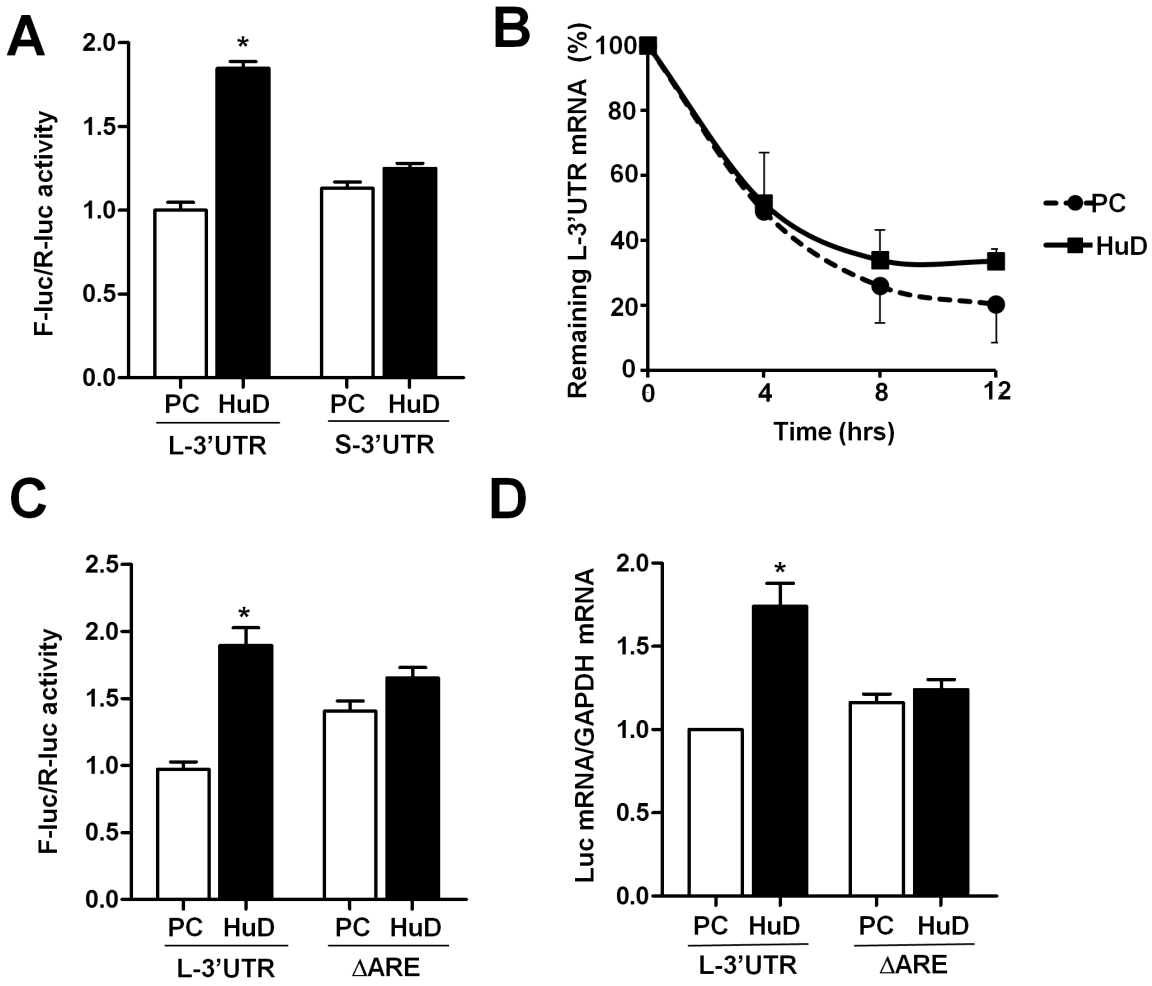

A
F-luc/R-luc activity
2.0
1.5
1.0
0.5
0.0
*
PC HuD
L-3'UTR
PC HuD
S-3'UTR
B
Remaining L-3'UTR mRNA (%)
100
80
60
40
20
0
0
4
8
12
Time (hrs)
PC
HuD
C
F-luc/R-luc activity
2.5
2.0
1.5
1.0
0.5
0.0
*
PC HuD
L-3'UTR
PC HuD
ΔARE
D
Luc mRNA/GAPDH mRNA
2.0
1.5
1.0
0.5
0.0
*
PC HuD
L-3'UTR
PC HuD
ΔARE

Figure 2-3: Binding of HuD to the ARE in the BDNF-long 3'UTR increases the stability of the mRNA

(A) Specific binding of HuD to the ARE in BDNF-long 3'UTR was demonstrated by REMSA using recombinant proteins and radiolabeled RNA along with a 10-fold molar excess of cold ARE competitor. (B) *In vitro* decay assay of capped and polyadenylated RNA containing the BDNF-ARE with left lane showing RNA molecular weight markers. Analysis of the rate of decay in three independent experiment revealed that the mRNA is stabilized in the presence of HuD. Half-life for GST-treated mRNA is 7.0 ± 0.90 min, and for GST-HuD-treated mRNA is 12.0±1.0 min. ** indicates $p<0.05$ by Two way ANOVA with a quantitative factor (n=3 separate experiments).

Figure 2-3

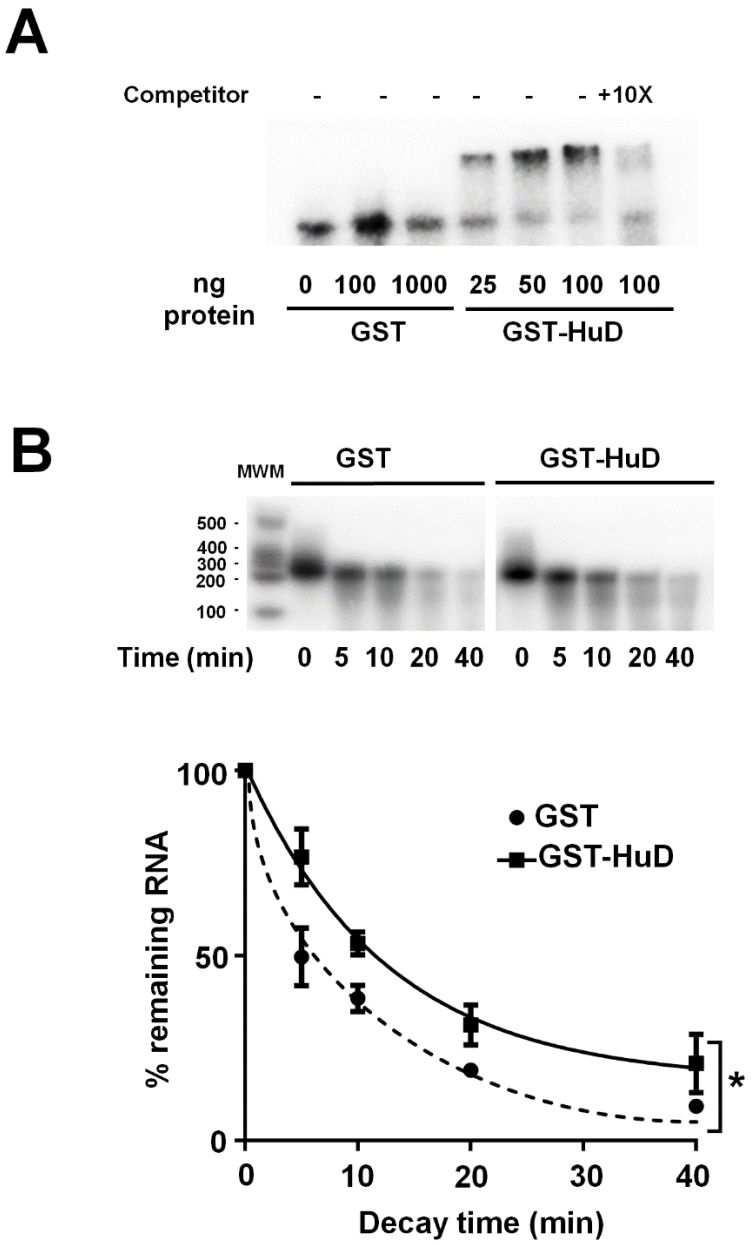

A
Competitor - - - - - - +10X
ng protein 0 100 1000 25 50 100 100
GST
GST-HuD
B
MWM
GST
GST-HuD
500
400
300
200
100
Time (min) 0 5 10 20 40 0 5 10 20 40
100
50
0
% remaining RNA
GST
GST-HuD
*
0 10 20 30 40
Decay time (min)

Figure 2-4: HuD knockdown reduced the levels of endogenous BDNF long 3'UTR mRNA

(A) siRNA knockdown of HuD in transfected CAD cells measured by qRT-PCR using a HuD-specific primer flanking the target site by the siRNA. (B) Reduction of endogenous BDNF long 3'UTR mRNA in CAD cells measured by qRT-PCR as a result of HuD knockdown. For (A) and (B), results were derived from 3 independent experiments (n=3), * indicates $P<0.05$. (C) Representative confocal images of L-BDNF FISH (red) in E17 hippocampal neurons transfected with either pEGFP control vector or pEGFP-shHuD plasmid. The transfected cells was marked by the green fluorescence and the number of FISH grains were counted in the cell bodies and processes (n=6 for each condition) and graphically displayed in (D). Arrows mark the FISH grains in the processes. Note that the number of ISH grains in the shHuD- treated cells decreased throughout the soma and neurite relative to control GFP-vector treated cells.

Figure 2-4

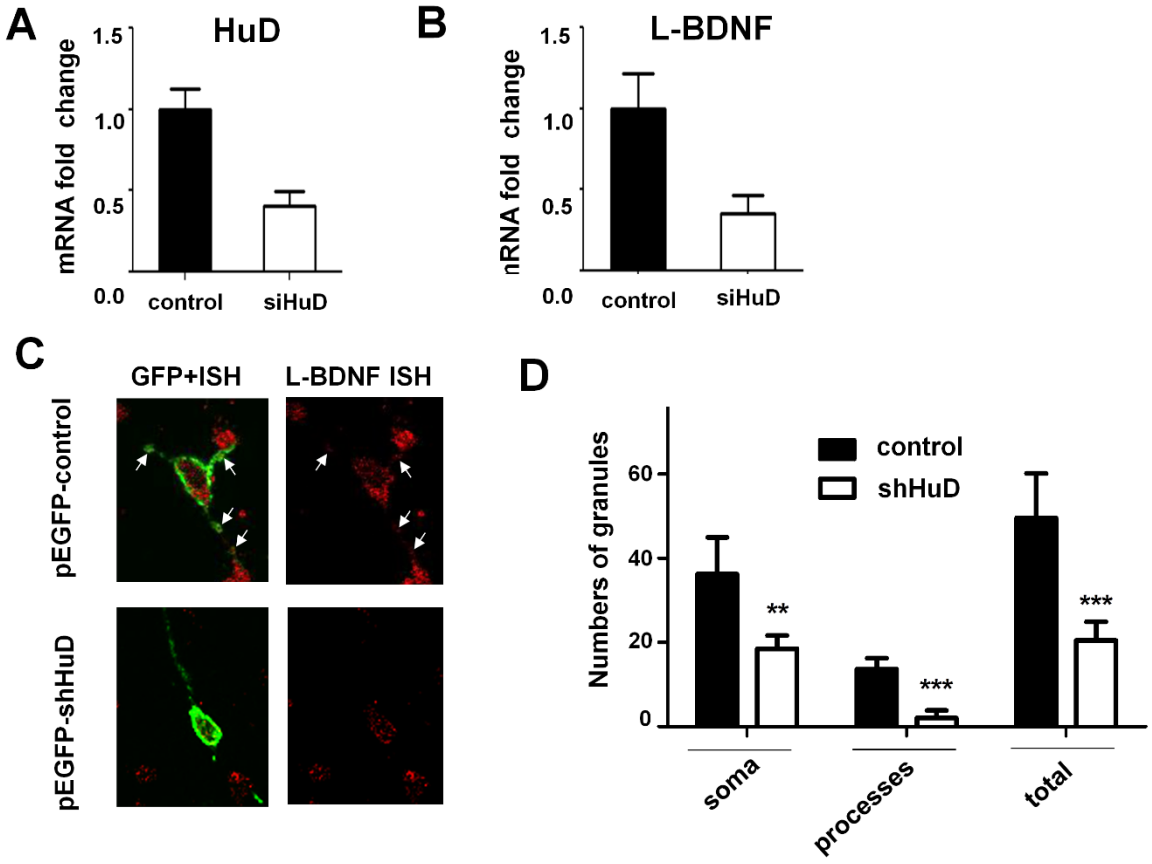

A
HuD
mRNA fold change
1.5
1.0
0.5
0.0
control
siHuD
B
L-BDNF
mRNA fold change
1.5
1.0
0.5
0.0
control
siHuD
C
GFP+ISH
L-BDNF ISH
pEGFP-control
pEGFP-shHuD
D
Numbers of granules
control
shHuD
60
40
20
0
**
***
***
soma
processes
total

Figure 2-5: Forced expression of exogenous HuD selectively enhanced expression of BDNF long 3'UTR mRNA in primary cultured neurons

E17 cortical neurons were cultured for 2 days and then infected with HSV-HuD or HSV-lacZ virus. Following 3 days in culture, the levels of long 3'UTR BDNF mRNA and pan BDNF mRNA were determined by qRT-PCR using primers specific in the long 3'UTR (A) and primers in the coding region that detects the pan BDNF mRNA (B). *$p<0.05$ (Student's t-test, n=4).

Figure 2-5

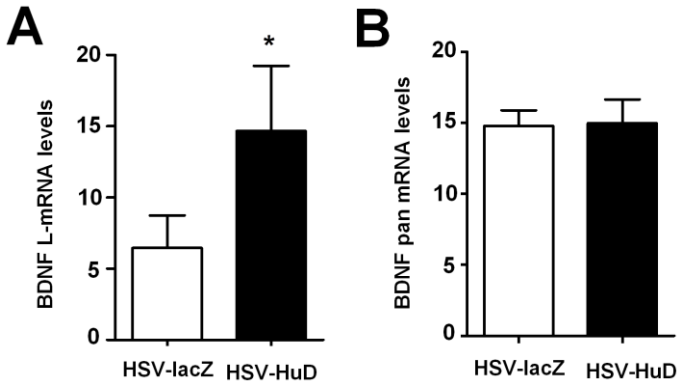

A
BDNF L-mRNA levels
20
15
10
5
0
*
HSV-lacZ
HSV-HuD
B
BDNF pan mRNA levels
20
15
10
5
0
HSV-lacZ
HSV-HuD

Figure 2-6: **A HuD transgene enhances expression of the BDNF long 3'UTR mRNA in hippocampal DGCs**

(A) Representative FISH images of fluorescent in situ hybridization of BDNF long 3'UTR mRNA in the neuronal soma layers of hippocampal DG, CA3 and CA1 of HuD-Tg and WT littermates (scale bar = 75 μm). (B) Quantification of FISH intensity in four pairs of HuD-Tg and WT controls. ** $p<0.01$ (Student's t-test, n=4).

Figure 2-6

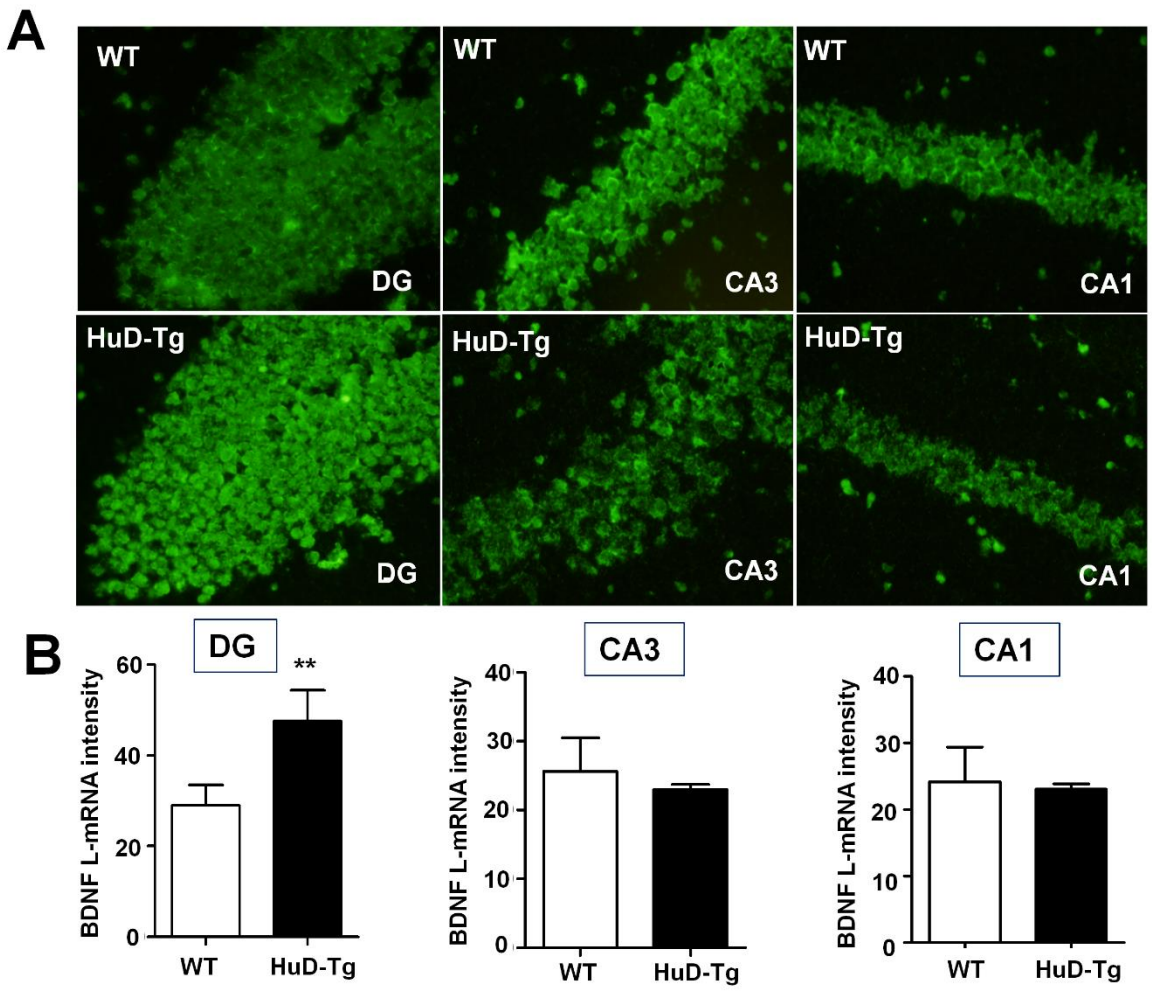
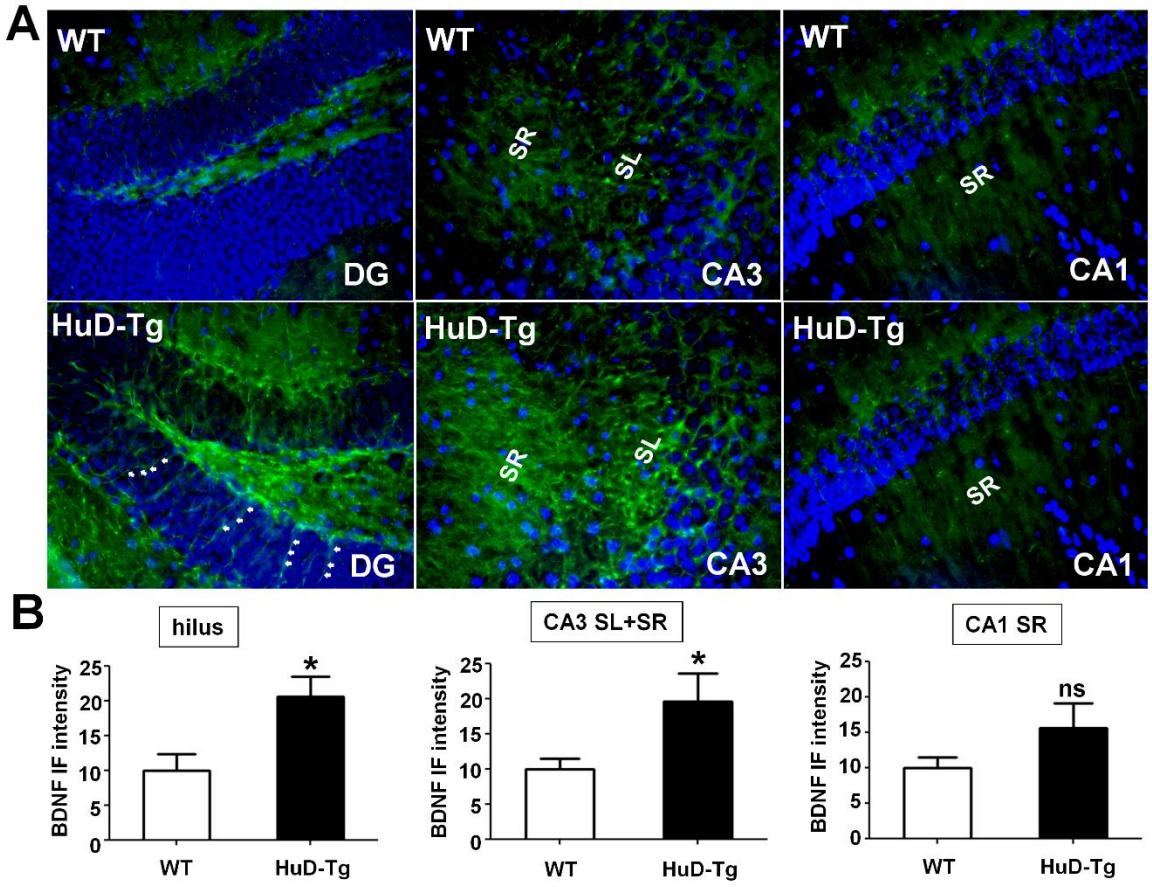

A
WT
DG
WT
CA3
WT
CA1
HuD-Tg
DG
HuD-Tg
CA3
HuD-Tg
CA1
B
DG
**
BDNF L-mRNA intensity
60
40
20
0
WT
HuD-Tg
CA3
BDNF L-mRNA intensity
40
30
20
10
0
WT
HuD-Tg
CA1
BDNF L-mRNA intensity
40
30
20
10
0
WT
HuD-Tg

Figure 2-7: The HuD transgene enhances BDNF protein levels in the hippocampal MF pathway

(A) Representative images of BDNF immunofluorescence (green) in the hippocampal hilus, CA3 and CA1 regions in HuD-Tg and WT littermates. DAPI stained nuclei (blue) mark the aforementioned principle neuron layers. Asterisks indicate enhanced BDNF signals in the long processes of adult neural stem cells in the SGZ. (B) Quantification of BDNF immunofluorescence in the hilus, CA3 strata lucidum (SL) and strata radiatum (SR) and CA1 stratum radiatum (SR) are graphically displayed. *$p<0.05$ (Student's t-test, n=3).

Figure 2-7


A
WT
DG
WT
SR
SL
CA3
WT
SR
CA1
HuD-Tg
DG
HuD-Tg
SR
SL
CA3
HuD-Tg
SR
CA1
B
hilus
BDNF IF intensity
25
20
15
10
5
0
*
WT
HuD-Tg
CA3 SL+SR
BDNF IF intensity
25
20
15
10
5
0
*
WT
HuD-Tg
CA1 SR
BDNF IF intensity
25
20
15
10
5
0
ns
WT
HuD-Tg

Chapter 3

HuD differentially regulates Cdk5 activators and neuronal Cdk5 function through post-transcriptional mechanisms

This chapter to be submitted for review as:

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*denotes equal contribution to this work

3.1 Introduction

Sophisticated post-transcriptional regulation by RNA-binding proteins controls the expression of an expanding list of genes that play key roles in neuronal development and function (21). For instance, the neuronal ELAV (nELAV) family of RNA-binding proteins governs multiple aspects of brain function by regulating the stability and translation of numerous mRNA targets (56, 59). HuD is a well-recognized nELAV family member, which is primarily localized to the cytoplasm and plays crucial roles in controlling neuronal network development and function (59, 113). As an early marker of neuronal differentiation, HuD is abundantly expressed in the embryonic and neonatal brain where it induces factors that govern neuronal lineage establishment and morphological differentiation (131, 256). In the adult brain, HuD expression is diminished in certain brain regions, represented by the dentate gyrus (DG) in the hippocampus (132), but is markedly upregulated upon neuronal stimulation to modulate synaptic plasticity (130). The loss of HuD prevents neuronal differentiation and results in spatial learning defects (134, 135). Moreover, human patients suffering from Schizophrenia and Alzheimer's disease display altered HuD expression (59, 156). The sophisticated roles of HuD in controlling mRNA stability and translation are mediated by the binding of HuD to A/U-rich elements (AREs) often present in the 3' untranslated regions (3'UTRs) of targets mRNAs (59, 113, 257). Recently, HuD function has also been linked with microRNAs (miRNAs) (13, 93). Although over 700 mRNAs are identified as HuD ligands (61), the list of functional HuD targets *in vivo* is far from complete.

One example is the human p35 mRNA, which encodes a protein activator for Cyclin-dependent kinase 5 (Cdk5), a serine/threonine kinase that shares substantially overlapping functions with HuD in normal and diseased brains (59, 159). In fact, Cdk5 activity is tightly regulated by two distinct labile protein co-factors, p35 and p39, which display distinct spatial-temporal expression profiles and play non-overlapping roles in directing Cdk5 function to control neuronal circuitry establishment and synaptic function (171, 176, 232, 233). Conceivably, post-transcriptional regulation of Cdk5 activators could offer a practical mechanism that governs the intricate functions of Cdk5. Indeed, the lengthy 3'UTR of human p35 contains destabilizing cis-acting sequence elements (239), miRNA target sites (258), and mutations that have been linked to non-syndromic intellectual disability (224). In addition, p35 mRNA was reported to associate with nELAVs *in vitro* and p35 protein levels can be regulated by manipulating HuD abundance in culture (240). These emerging lines of evidence strongly suggest post-transcriptional regulation of p35. Nonetheless, studies investigating the regulation of p35 by HuD are limited to human cell lines in culture (240). Thus, whether and how HuD indeed regulates Cdk5 activity and function in brain neurons still remains elusive.

We identified highly conserved AREs in the 3'UTRs of p35 and p39 mRNAs and found that HuD binds to and stabilizes not only p35 mRNA, but also p39 mRNA, in rodent brain neurons through AREs. However, to our surprise, elevated HuD expression in the mouse hippocampus preferentially increases the levels of p39 protein whereas p35 protein is unaltered. We found that HuD forms a molecular loop with miRNA-101a to maintain normal abundance of p35 protein production. Finally, we show that the selective up-regulation of p39 by HuD enhances phosphorylation of Cdk5 targets and promote

mossy fiber axon projection in the hippocampus. These studies provide the first *in vivo* evidence demonstrating post-transcriptional regulation of Cdk5 pathway function in brain neurons through HuD-dependent mechanisms.

3.2 Results

3.2.1 HuD stabilizes Cdk5 activator mRNAs in brain neurons through conserved 3'UTR AREs

Using the computer algorithm AREsite2.0, we identified a strong class II ARE (defined as overlapping AUUUA pentamers in the terminal portion of the p35 3'UTR and the p39 UTR, respectively (**Figure 3-1A, Figure 3-2**). The putative Class II AREs in both mRNAs are conserved in mouse, rat, and human. In addition, the rodent p35 3'UTR terminal region also harbors a U-rich region with a strong consensus sequence for a Class III ARE (61). Interestingly, despite the high levels of homology between rodent and human p35 3'UTR sequences, the strong Class III ARE is only present in the terminal region of the rodent p35 3'UTR. Considering the well-documented role of AREs in controlling mRNA stability (12), we examined the decay of the endogenous p35 and p39 transcripts during differentiation of primary cultured rat cortical neurons. Decay curves of p35 (**Figure 3-1B**) and p39 (**Figure 3-1C**) were generated based on reverse-transcription quantitative PCR (RT-qPCR) readings at various time points after blocking transcription by Actinomycin D. We found that the calculated half-lives of p35 and p39 mRNAs are increased approximately 2 fold in neurons which have undergone six days of differentiation *in vitro* (DIV) than those at DIV2. Notably, this is the developmental window when HuD is markedly upregulated in brain neurons (132).

To directly test whether HuD controls the abundance of p35 and p39 mRNAs in differentiating neurons, we knocked down HuD in primary cortical neurons using a previously validated siRNA (259). HuD mRNA levels were reduced by approximately 50%, which led to a ~50% reduction of p35 mRNA (**Figure 3-1D**) and p39 mRNA (**Figure 3-1E**), respectively. Thus, HuD is necessary for maintaining cellular abundance of p35 and p39 mRNA in brain neurons. In contrast, Cdk5 mRNA levels remained unaffected by the HuD-specific siRNA (**Figure 3-1F**).

To identify cis-acting elements in Cdk5 activator mRNAs that mediate the effect of HuD, we generated reporter constructs in which the ARE-containing segment of the p35 3'UTR or the full length p39 3'UTR were fused downstream of the firefly luciferase coding region. Myc-tagged human HuD (259) was co-transfected with each luciferase reporter construct (**Figure 3-2A**). The renilla luciferase construct was also co-transfected as an efficiency control. Expression of myc-HuD elicited increased luciferase activity from either the p35 or the p39 3'UTR reporter (**Figure 3-2B and 3-2C**) but not from the parental luciferase vector (**Figure 3-4**). We further tested whether the predicted AREs are necessary for HuD to stabilize reporter mRNAs. Deletion of the conserved Class II ARE in the p39 3'UTR completely abolished the effect of HuD (**Figure 3-2D**). In addition, although deletion of the Class II ARE alone in the p35 3'UTR reporter did not eliminate response to HuD (**Figure 3-2E**), deletion of both the Class II and Class III AREs ameliorated the reporter response to HuD (**Figure 3-2F**). These data suggest that the predicted AREs located in p35 and p39 3'UTRs mediate the effect of HuD-dependent mRNA stabilization.

3.2.2 HuD binds and increases the abundance of both p35 and p39 mRNAs but selectively up-regulates p39 protein in the hippocampus

HuD abundance declines and is differentially expressed in neuronal subpopulations in the adult hippocampus (130). To overcome the limitation of poor HuD antibodies and to test whether HuD interacts with p35 and p39 mRNAs *in vivo*, we performed UV-crosslinking immunoprecipitation (CLIP) using a transgenic mouse model (HuDtg+) where myc-tagged human HuD is expressed in all forebrain neurons (137). The abundance of myc-HuD is approximately two fold of the endogenous HuD (137), with the highest expression detected in the hippocampal dentate gyrus (DG) that harbors abundant p35 and p39 mRNAs (137). Successful immunoprecipitation (IP) of myc-HuD from HuDtg+ hippocampal lysates was confirmed by immunoblot with non-transgenic littermates (HuDtg-) serving as a negative control (**Figure 3-5A**). The mRNAs directly bound and cross-linked with myc-HuD were isolated, followed by qRT-PCR analysis to assess enrichment of HuD-bound transcripts normalized to that in the input. GAP43, a well-known HuD mRNA target (137) was used as an enrichment standard. As shown in **Figure 3-5B**, the enrichment of p35 mRNA in isolated HuD complex was comparable to GAP43 mRNA whereas p39 mRNA enrichment significantly exceeded GAP43 mRNA. In contrast, the GAPDH mRNA was not enriched. These results demonstrate that HuD directly binds both of the endogenous Cdk5 activator mRNAs in hippocampal neurons.

We next questioned whether ectopic expression of the myc-HuD transgene in the hippocampus increases expression of p35 and p39. qRT-PCR analysis revealed that both p35 (**Figure 3-5C**) and p39 (**Figure 3-5D**) mRNA levels are increased in the HuDtg+ hippocampus compared to HuDtg- littermate controls. In contrast, Cdk5 mRNA levels

were unaltered in the HuDtg+ hippocampus (**Figure 3-5E**), indicating that HuD only regulates expression of Cdk5 activator mRNAs but not the kinase itself. As expected, p39 protein was elevated 2-fold in the HuDtg+ hippocampus (**Figure 3-6A**). Surprisingly, despite elevated steady state levels of the p35 mRNA, p35 protein levels were unaltered by the myc-HuD transgene (**Figure 3-6B**). Cdk5 protein levels also remained unchanged (**Figure 3-6C**). Therefore, although HuD binds and stabilizes both p35 and p39 mRNAs, only p39 protein expression is enhanced by HuD, suggesting the existence of additional regulatory mechanisms by HuD that maintain steady p35 protein levels *in vivo*.

3.2.3 miR-101a is up-regulated by HuD and selectively targets the p35 3'UTR to inhibit p35 protein expression

Because human p35 mRNA is regulated by the miRNA 103/107 family (258) and the target sites are conserved in rodent p35 3'UTR (**Figure 3-7A**), we hypothesized that HuD may up-regulate miRNA(s) that target p35 3'UTR to suppress p35 translation. Through such a mechanism, HuD may increase p35 mRNA stability yet still maintain normal levels of p35 protein production. Because miR-103/107 are expressed in glia in addition to neurons, we dissected the DG from HuDtg+ hippocampi, which is enriched with neurons that express the highest levels of the transgenic myc-HuD (132). However, qRT-PCR did not detect changes in miR-103 or miR-107 in the DG of HuDtg+ mice (**Figure 3-7B, 3-7C**). We next searched for miRNAs predicted to target p35 but not p39 mRNAs using several computerized algorithms, including miRanda, PicTar, and Targetscan. Predicted binding energies were calculated using RNA hybrid. The miR-101a, which is predicted to target both the human and the mouse p35 3'UTR with optimal binding energy (**Figure 3-8A**), stood out as a top hit.

To test whether endogenous miRNA 101a suppresses p35 expression, we transfected anti-miR-101a into primary cultured cortical neurons and quantified endogenous p35 mRNA and protein. Antagonizing miR-101a increased the p35 proteins by immunoblot (**Figure 3-8B**) and the p35 protein to mRNA ratio (**Figure 3-8C**), suggesting that miR-101a is a natural suppressor for p35 translation. To test whether miR-101a directly targets the p35 3'UTR, we utilized the p35 3'UTR reporter which contains the putative miR-101a site. Co-transfection of miR-101a with this reporter construct resulted in decreased luciferase activity (**Figure 3-8D**), whereas a p35 3'UTR reporter lacking the putative miR-101a site failed to respond to miR-101a (**Figure 3-8E**). In addition, we showed that miR-101a does not suppress the reporter that carries p39 3'UTR (**Figure 3-8F**).

We next tested whether and how HuD may regulate miR-101a expression in the hippocampus. As shown in Fig. 5G, qRT-PCR revealed that the mature form of miR-101a was significantly upregulated in the DG of HuDtg+ mice compared to the HuD-tg- controls. In contrast, the levels of pri- and pre-miRNA101a in the HuDtg+ DG were unaltered (**Figure 3-8H** and **3-8I**). Thus, HuD-mediated up-regulation of mature miR-101a provides a molecular feedback loop that counteracts the increase of p35 mRNA by HuD, thereby maintaining steady p35 protein production while preferentially up-regulating p39.

3.2.4 HuD-dependent upregulation of p39 enhances phosphorylation of Cdk5 targets and mossy fiber axonal projection in the hippocampus

To test whether HuD-mediated upregulation of p39 protein expression ultimately leads to enhanced Cdk5 function, we performed immunoblot using phospho-specific

antibodies to quantify phosphorylation of Cdk5 target proteins. The microtubule associated protein 1B (MAP1B) is a well-documented Cdk5 target that promotes axonal extension during neonatal development (260, 261). We found that Cdk5-dependent mode-I phosphorylation of MAP1B (PI-MAP1B), detected by the SMI-31 antibody(262), was significantly increased in the neonatal HuDtg+ hippocampus as compared to that in HuDtg- littermates (**Figure 3-9A**). In addition, Cdk5-dependnent phosphorylation of the glucocorticoid receptor 1 (GR1) at S211 (263) was also elevated in Hudtg+ hippocampus at the age of one month (**Figure 3-9B**). Furthermore, the aberrantly increased phosphorylation of Cdk5 targets due to transgenic expression of HuD was rescued by genetic removal of the p39 gene in HuDtg+, p39-/- mice (**Figure 3-9C**). Notably, p35 is not reduced in the HuDtg+, p39-/- hippocampus. These results indicate that p39 is responsible for HuD-enhanced Cdk5 activity in the hippocampus.

PI-Map1B plays critical roles in controlling microtubule dynamics, which in turn advances axonal extension and pathfinding (260). In addition, HuDtg+ mice display overgrowth of hippocampal mossy fibers (MFs), which are axons originated from DG granule cells to form synapses with CA3 neurons (264). Thus, we questioned whether the enhanced p39-Cdk5 signaling *in vivo* contributes to the MF over-projection phenotype in HuDtg+ mice. The infrapyrimadal bundle (IPB) and the suprapyramidal bundle (SPB) of MF tracts are clearly labeled by immunofluorescence of Calbindin in the hippocampus of HuDtg-, p39+/+; HuDtg+, p39+/+; and HuDtg+, p39-/- mice (**Figure 3-10A**). The relative length of IPB to SPB is a well-established hall mark for accessing MF projection (ref). Consistent with a previous report, a significant IPB over-projection was observed in the HuDtg+ mice in the presence of Wt p39 alleles (HuDtg+, p39+/+) as compared to the

HuDtg- littermate controls (**Figure 3-10A and 3-10B**). Importantly, the MF over-projection was completely reversed by genetic removal of the p39 gene (**Figure 3-10A and 3-10B**). Together, these results indicate that p39-directed Cdk5 signaling plays essential roles in meditating the function of HuD in controlling MF projection and hippocampal micro-circuitry development.

3.3 Discussion

In this study, we provide the first evidence that post-transcriptional regulation governs Cdk5 activity and function in brain neurons via HuD-mediated stabilization of Cdk5 activator mRNAs. Furthermore, our studies reveal a novel mechanism where a HuD-miR molecular loop differentially regulates Cdk5 activators *in vivo*, which leads to preferential enhancement of p39 protein production, increased phosphorylation of Cdk5 targets, and hippocampal MF over-projection. These findings provide the first functional link between the HuD and Cdk5 pathways in establishing neuronal circuits. A working model that illustrates HuD-mediated post-transcriptional control of Cdk5 pathway is presented in Figure 8.

3.3.1 Highly conserved AREs in the 3'UTRs of Cdk5 activator mRNAs suggest that post-transcriptional regulation is a common mechanism underlying intricate Cdk5 function

The rodent and human p35 and p39 3'UTRs harbor 70% and 72% sequence identity, respectively, suggesting that post-transcriptional regulation of Cdk5 activators, and hence Cdk5 activity and function, is evolutionarily conserved. We show that the Class II ARE in the p39 3'UTR, which is 100% identical in mouse, rat and human, is responsible for HuD-dependent regulation of p39. Such conserved regulation by HuD is

further supported by its activity in stabilizing rodent p35 mRNA in our studies as well as human p35 mRNA in a previous report (240). The lengthy p35 3'UTR contains complex cis-acting RNA sequence motifs. The predicted Class II ARE and target sites for miR-103/107 and miR-101a in the p35 3'UTR are all conserved between rodents and human. Interestingly, removal of only the Class II ARE from the murine p35 3'UTR did not affect reporter response to HuD. However, when the long U-stretch within the mouse p35 3'UTR that contains a canonical Class III ARE was also removed, the effect of HuD was abolished, suggesting that HuD may interact with multiple RNA segments in the p35 3'UTR. In fact, the human p35 3'UTR also contains a U-rich element, although it is distantly separated from the conserved Class II ARE, which is reported to cooperate with hnRNPA2/B1 to repress reporter mRNA expression (240). Precise dissection of the cis-acting RNA motifs and the functional interplay between distinct RNA-binding proteins that regulate p35 is the next challenge in uncovering post-transcriptional mechanisms governing Cdk5 activators.

The ultimate question is the functional significance of post-transcriptional regulation of the Cdk5 pathway. The literature has demonstrated that Cdk5 activity is under tight control by the cellular abundance of p35 and p39, both of which are labile and degraded upon Cdk5 activation (171, 228, 265) . Thus, stabilization of Cdk5 activator mRNAs offers an effective mechanism to replenish Cdk5 activators independent of transcription. In addition, Cdk5 plays intricate roles in brain development and function, which governs neuronal migration, neuronal network assembly and activation, neurotransmitter release, and memory consolidation (159). In addition, both p35 and p39 proteins are detected at developing growth cones and neuronal synapses (234, 266). Thus,

post-transcriptional regulation could offer rapid and/or sustained regulation of Cdk5 activators, perhaps even in subcellular compartments of neurons, which is certainly a promising mechanism to support diverse Cdk5 functions.

3.3.2 A novel HuD-miRNA "checkpoint" allows preferential induction of p39-directed Cdk5 function in the brain

Although HuD can stabilize both Cdk5 activator mRNAs, transgenic expression of HuD preferentially increase p39, not p35, in mouse hippocampus. To this end, we discovered that HuD also increases the expression of the mature form of miR-101a, which targets the p35 3'UTR to suppress p35 translation. This HuD-miR regulatory loop acts as a "checkpoint" that maintains steady production of p35 protein while p39 protein expression is selectively increased. The functional interplay between HuD and miR-101a reveals a novel mechanism for HuD to achieve sophisticated post-transcriptional regulation of its functional targets beyond direct interactions with its mRNA ligands.

It is important to note that the two Cdk5 activators display distinct spatial-temporal expression profiles during brain development and differential subcellular distribution within neurons (179, 230, 234). In addition, emerging evidence suggests that Cdk5 activators may play non-overlapping roles in normal and diseased brains. In particular, p35 protein has a much shorter half-life than p39 and is more prone to calpain-dependent cleavage (171). Unlike the full length Cdk5 activators that associate with the plasma membrane through N-terminal myristolation, the 25 kD proteolytic cleavage product of p35, called p25, is mislocalized and thought to cause Cdk5 malfunction and neuronal damage under neurotoxic insult, such as in stroke and Alzheimer's disease (AD)

(159, 173, 213). In this regard, the HuD-miR-101a "checkpoint" may function to prevent the over-production of p35 and to limit the consequential damage to neurons upon insult.

In contrast, the higher stability of p39 protein and its resistance to proteolytic cleavage appears to be advantageous over p35 for enhancing Cdk5 activity at the physiological locations where Cdk5 functions within a neuron. Consistent with this idea, p39 is preferentially localized to axonal growth cones (266) and the selective up-regulation of p39 by HuD leads to increased PI-MAP1B, a functional substrate of Cdk5 which is also enriched in growth cones to control microtubule and actin dynamics and govern axonal extension and pathfinding (260). Thus, PI-MAP1B is a representative target for HuD-dependent regulation of p39-Cdk5 in neuronal network development; although the full spectrum of HuD regulated Cdk5 function still remains to be uncovered. Despite the fact that HuD-mediated over-projection of hippocampal MFs may involve multiple factors, the selective up-regulation of p39 by HuD is certainly a contributing mechanism, as genetic removal of p39 rescues the MF over-projection in HuDtg+ hippocampus.

3.3.3 Potential roles of HuD-mediated post-transcriptional regulation of the Cdk5 pathway in brain diseases

The functional spectrum of HuD and the Cdk5 pathway display substantial overlap not only during normal neuronal development but also in diseased brains. For instance, both Cdk5 and HuD are implicated in epileptogenesis (134, 139, 163, 175). In pharmacologically induced epileptic animal models, HuD is markedly up-regulated in the hippocampus, especially in the DG (132, 139). Hence, ectopic expression of myc-HuD in the HuDtg+ mice recapitulates seizure activity-induced HuD expression. In addition, the

aberrantly enhanced hippocampal MF projections by HuD-p39-Cdk5 is consistent with the well-documented hippocampal MF sprouting, a hallmark for pathological structural plasticity in epileptogenesis (267-269). Moreover, HuD stabilizes the mRNA that encodes brain derived neurotrophic factor (BDNF) and enhances BDNF expression (259), which activates TrkB receptor tyrosine kinase, a mechanism known to advance epileptogenesis (255, 270). Notably, Cdk5-dependent phosphorylation of TrkB also enhances BDNF-TrkB signaling (205). Thus, HuD-dependent enhancement of Cdk5 function may couple both pathways in epileptogenesis.

Emerging evidence also suggests a potential link between HuD and Cdk5 in Alzheimer's disease (AD). Cdk5 over-activation and Cdk5-dependent hyper-phosphorylation of tau were observed in the AD brain. Over-production of the p35 cleavage product, called p25, is a proposed mechanism for over-active Cdk5 (159, 173, 213). More recently, several studies also reported increased HuD expression in the AD cortex (156, 157). Presumably, aberrantly elevated HuD expression can stabilize p35 mRNA. In addition, miR101a, which suppresses p35 protein production as shown in our current study, is progressively increased in the aging brain of healthy individuals but is downregulated in the AD brain through undefined mechanisms (111, 271). This uncouples the "checkpoint" miR101a from HuD, which may increase p35 protein production and hence lead to hyperactivity of Cdk5 in AD neurons. It is worth mentioning that the checkpoint HuD-miR101a loop may also regulate amyloid precursor protein (APP), a critical factor in AD pathogenesis. HuD was recently discovered to bind the 3'UTR of and stabilize APP mRNA (156). In addition, miR101a also targets APP 3'UTR (110). Thus, the increase of HuD and reduction of miR101a in AD brain leads to

over-production of APP, which not only underlies amyloid plaque formation but also facilitates cleavage of p35 resulting in an additional contributor to hyper-function of Cdk5 (173). In this regard, defects in the miR101a "checkpoint" for HuD-mediated mRNA stabilization may affect multiple AD-risk factors.

In conclusion, our studies uncover novel post-transcriptional mechanisms in which coordination by HuD and miR101a differentially regulates Cdk5 activators and thus enhances Cdk5 activation and function to promote hippocampal MF projection *in vivo*. Such sophisticated regulation by HuD during neuronal development and activation may offer mechanisms to control the intricate roles of Cdk5, and thus accommodate the complex functional needs of brain neurons. Because the cis- and trans-acting factors underlying HuD-mediated control of Cdk5 are highly conserved in human, the post-transcriptional mechanisms revealed by our studies are likely employed by human neurons to govern normal brain function and may be dysregulated in the pathogenesis of various brain disorders.

Figure 3-1: HuD regulates the abundance of Cdk5 activator mRNAs in differentiating neurons

(A) Using ARESite2.0, we identified conservation of putative Class II and Class III A/U-rich elements (AREs) in the 3'UTR of p35 mRNA (top) and a conserved class II ARE in the 3'UTR of p39 p39 mRNA (bottom). (B-C) At DIV 2 and DIV 6 mRNA was analyzed from primary cortical neurons after treatment with Actinomycin D. Results are displayed as the percentage remaining of p35 mRNA (B) and p39 mRNA (C) at the indicated time-points post-treatment. Values were fit to a decay curve and are graphically displayed. $N \geq 3$ for all time-points. (D-F) siRNA knockdown of HuD was performed on DIV 3 and 4 and total mRNA was collected at DIV 5. The amount of p35 (D), p39 (E), and Cdk5 (F) mRNA was measured by qRT-PCR and normalized to the control GAPDH. $N \geq 3$ and panels D-F were subject to statistical analysis by the Student's t-test.

Figure 3-1:

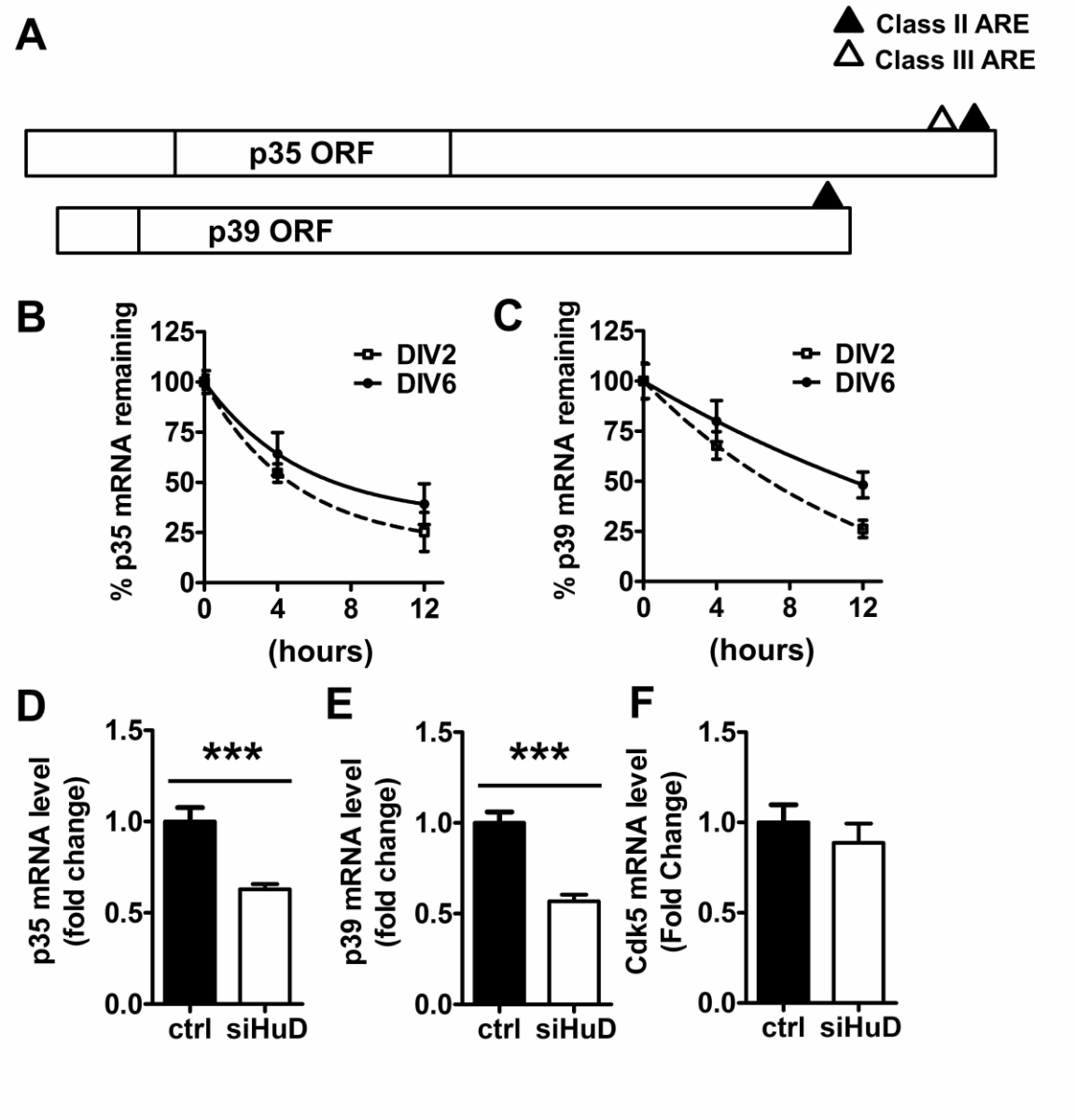

A
Class II ARE
Class III ARE
p35 ORF
p39 ORF
B
% p35 mRNA remaining
125
100
75
50
25
0
0
4
8
12
(hours)
DIV2
DIV6
C
% p39 mRNA remaining
125
100
75
50
25
0
0
4
8
12
(hours)
DIV2
DIV6
D
p35 mRNA level (fold change)
1.5
1.0
0.5
0.0
***
ctrl
siHuD
E
p39 mRNA level (fold change)
1.5
1.0
0.5
0.0
***
ctrl
siHuD
F
Cdk5 mRNA level (Fold Change)
1.5
1.0
0.5
0.0
ctrl
siHuD

Figure 3-2: Conservation of A/U-rich elements in the 3'UTR of p35 and p39 mRNA

The computer algorithm ARE site2.0 was utilized to identify putative Class II A/U rich elements (AREs) in the mouse p35 (A) and p39 (B) 3'UTRs. Additionally, a U-rich sequence corresponding to a Class III ARE was identified in the p35 3'UTR (A). Conservation of putative elements between rodents and humans is shown.

Figure 3-2:

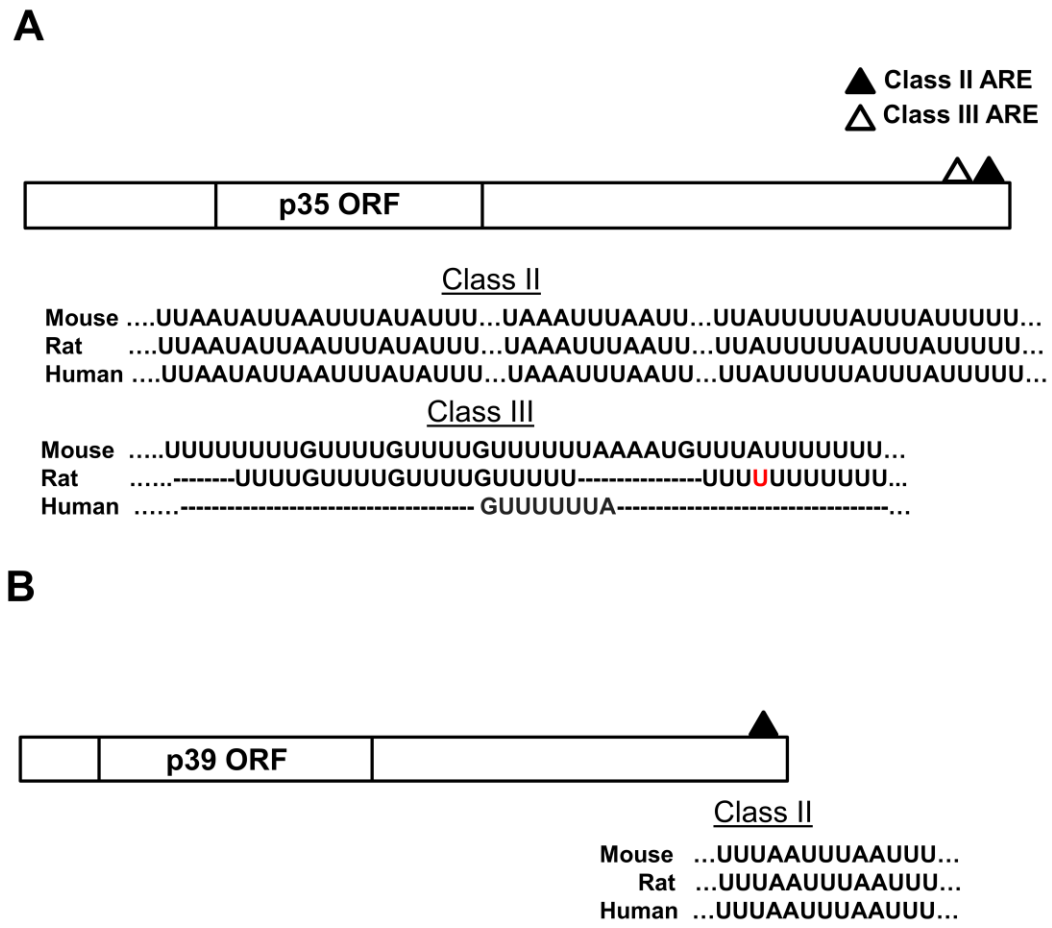

A
Class II ARE
Class III ARE
p35 ORF
Class II
Mouse ….UUAAUAUUAAUUUAUAUUU…UAAAUUUAAUU…UUAUUUUUAUUUAUUUUU…
Rat ….UUAAUAUUAAUUUAUAUUU…UAAAUUUAAUU…UUAUUUUUAUUUAUUUUU…
Human ….UUAAUAUUAAUUUAUAUUU…UAAAUUUAAUU…UUAUUUUUAUUUAUUUUU…
Class III
Mouse …..UUUUUUUUGUUUUGUUUUGUUUUUUAAAAUGUUUAUUUUUUU…
Rat ……--------UUUUGUUUUGUUUUGUUUUU----------UUUUUUUUUU...
Human ……---------- GUUUUUUA----------…
B
p39 ORF
Class II
Mouse …UUUAAUUUAAUUU…
Rat …UUUAAUUUAAUUU…
Human …UUUAAUUUAAUUU…

Figure 3-3: HuD stabilizes p35 and p39 mRNA in a 3'UTR dependent manner

(A) CAD cells were transfected with either myc-tagged HuD or the parental control vector pcDNA3.1. (B-C) Co-transfection with a Firefly Luciferase reporter that contains either the p35 (B) or the p39 (C) 3'UTR resulted in elevated Firefly Luciferase activity, which was normalized to the transfection control Renilla Luciferase. (D) HuD effect on luciferase activity is ameliorated by deletion of the Class II ARE from the p39 3'UTR reporter. (E) However, a reporter which lacks the Class II ARE sequence of the p35 3'UTR was unaffected. (F) Class II and Class II ARE deletion from the p35 3'UTR reporter abolishes HuD effect on luciferase activity. $N \geq 3$ for all panels and results were analyzed by the Student's t-test.

Figure 3-3

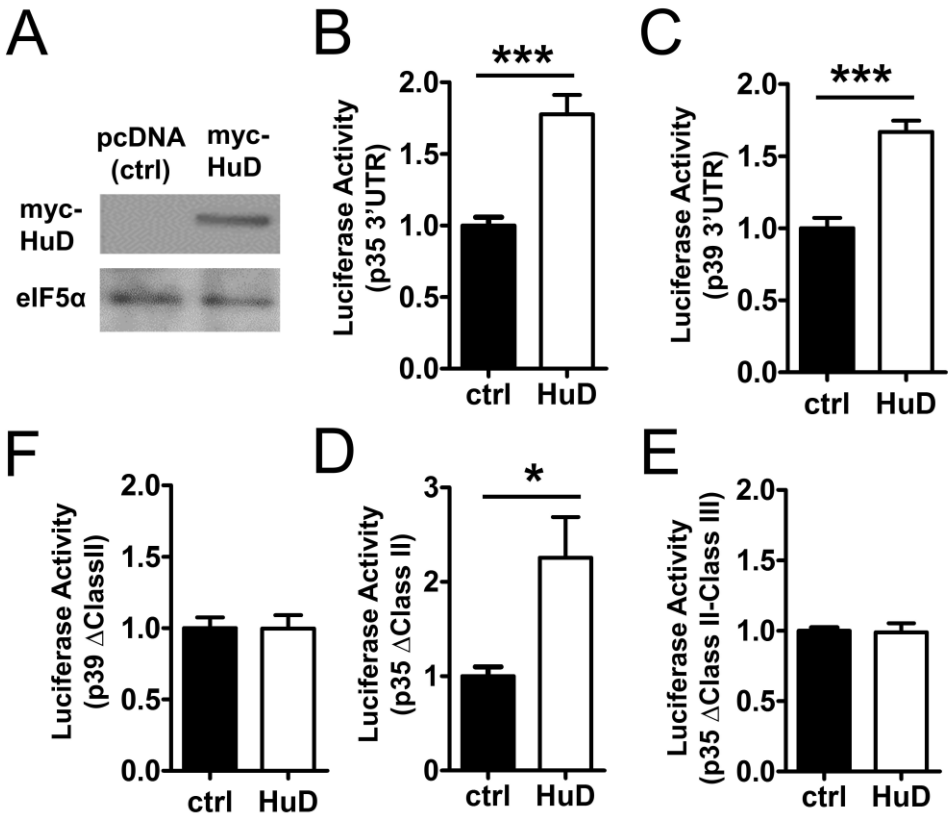

A
pcDNA (ctrl)
myc-HuD
myc-HuD
eIF5α
B
***
Luciferase Activity (p35 3'UTR)
2.0
1.5
1.0
0.5
0.0
ctrl
HuD
C
***
Luciferase Activity (p39 3'UTR)
2.0
1.5
1.0
0.5
0.0
ctrl
HuD
F
Luciferase Activity (p39 ΔClassII)
2.0
1.5
1.0
0.5
0.0
ctrl
HuD
D
*
Luciferase Activity (p35 ΔClass II)
3
2
1
0
ctrl
HuD
E
Luciferase Activity (p35 ΔClass II-Class III)
2.0
1.5
1.0
0.5
0.0
ctrl
HuD

Figure 3-4: HuD does not alter expression of the parental luciferase vector

Luciferase activity is unchanged when the luciferase parental vector is co-transfected with either myc-HuD or pcDNA3.1 vector control. N ≥3 and results were analyzed by the Student's t-test.

Figure 3-4

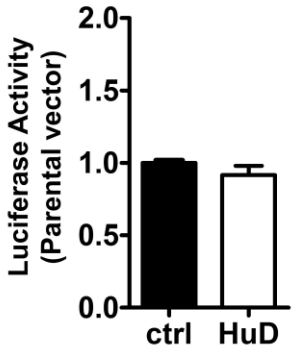


Figure 3-5: HuD binds to and increases levels of Cdk5 activator mRNAs in the HuD tg+ hippocampus

(A) UV-Crosslinking IP (UV-CLIP) was performed on hippocampal tissue from HuDtg+ mice using a c-myc antibody to immunoprecipitate transgenic myc-HuD. Successful IP is shown by immunoblot with a myc-antibody. (B) Co-immunoprecipitated mRNAs were analyzed by qRT-PCR with gene specific primers. mRNA abundance for each gene was normalized to input levels and expressed as a fold change to the positive control, GAP-43. Enrichment of GAPDH mRNA, p35 mRNA and p39 mRNA is displayed graphically. One-way ANOVA was used for panel B and N=5. (C-E) qRT-PCR measurement of p35 (C), p39 (D), and Cdk5 (E) mRNA in HuDtg+ hippocampi compared to littermate controls (HuDtg-). For panels C-D, N=7 for HuDtg-, N=13 for HuDtg+. N=3 for panel E. Results from panels C-E were subject to the Student's t-test.

Figure 3-5

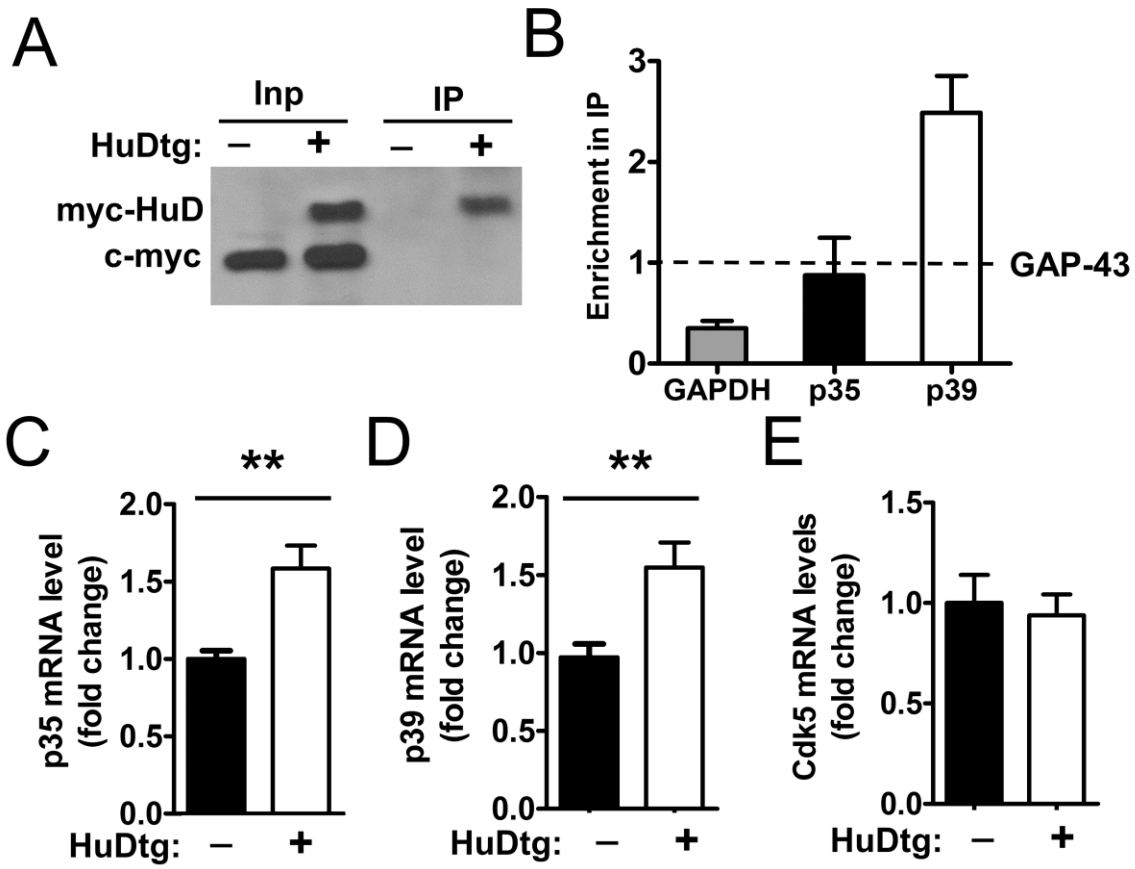

A
Inp
IP
HuDtg: – + – +
myc-HuD
c-myc
B
Enrichment in IP
3
2
1
0
GAP-43
GAPDH
p35
p39
C
**
p35 mRNA level (fold change)
2.0
1.5
1.0
0.5
0.0
HuDtg: – +
D
**
p39 mRNA level (fold change)
2.0
1.5
1.0
0.5
0.0
HuDtg: – +
E
Cdk5 mRNA levels (fold change)
1.5
1.0
0.5
0.0
HuDtg: – +

Figure 3-6: Elevation of HuD in the hippocampus selectively up-regulates p39 protein expression

Immunoblot shows levels of p35 (A), p39 (B), and Cdk5 (C) protein in the HuDtg+ and HuDtg- hippocampi. Protein was normalized to the loading control, eIF5α for each panel. Graphic quantification is shown below each immunoblot. N=11 for HuDtg- and N=15 for HuDtg+ for panel A. N=7 for HuDtg- and N=13 for HuDtg+ for panels B and C. All panels were subject to the Student's t-test.

Figure 3-6

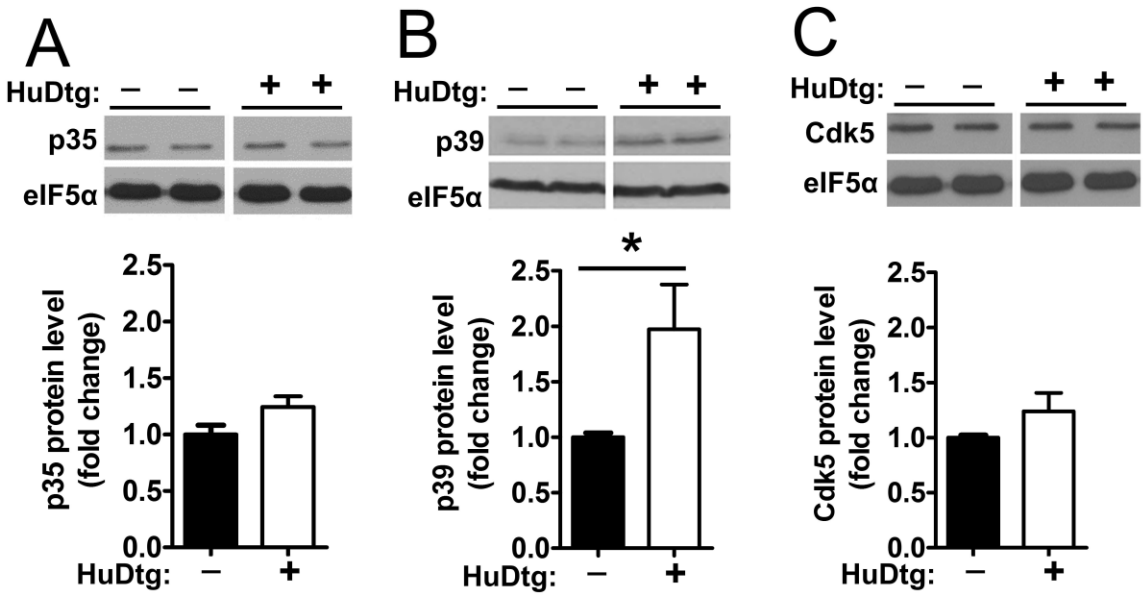

A
HuDtg: – – + +
p35
eIF5α
p35 protein level (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
HuDtg: – +
B
HuDtg: – – + +
p39
eIF5α
*
p39 protein level (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
HuDtg: – +
C
HuDtg: – – + +
Cdk5
eIF5α
Cdk5 protein level (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
HuDtg: – +

Figure 3-7: Conservation of miR-103 and miR-107 sites in the rodent and human p35 3'UTR and analysis of miR-103/107 levels in the HuDtg+ hippocampus

(A) RNAhybrid was used to calculate the predicted binding energy of miRNA-RNA duplexes of the putative miR-103 and miR-107 sites in the p35 3'UTR. Levels of miR-103 (B) and miR-107 (C) analyzed by miRNA specific qRT-PCR detection are shown. N=3 for miR-103 and N=6 for miR-107. Results were analyzed by the student's t-test.

Figure 3-7

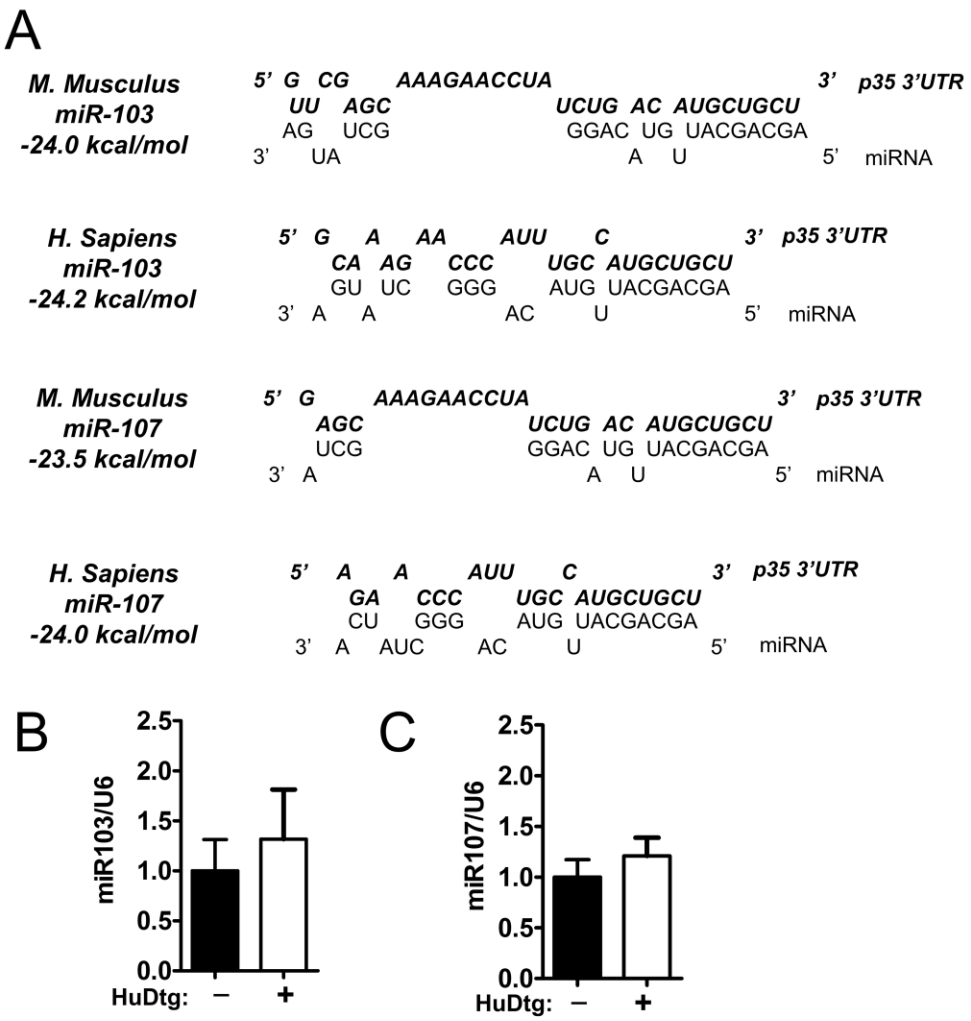

A
M. Musculus
miR-103
-24.0 kcal/mol
5' G CG AAAGAACCUA 3' p35 3'UTR
UU AGC UCUG AC AUGCUGCU
AG UCG GGAC UG UACGACGA
3' UA A U 5' miRNA
H. Sapiens
miR-103
-24.2 kcal/mol
5' G A AA AUU C 3' p35 3'UTR
CA AG CCC UGC AUGCUGCU
GU UC GGG AUG UACGACGA
3' A A AC U 5' miRNA
M. Musculus
miR-107
-23.5 kcal/mol
5' G AAAGAACCUA 3' p35 3'UTR
AGC UCUG AC AUGCUGCU
UCG GGAC UG UACGACGA
3' A A U 5' miRNA
H. Sapiens
miR-107
-24.0 kcal/mol
5' A A AUU C 3' p35 3'UTR
GA CCC UGC AUGCUGCU
CU GGG AUG UACGACGA
3' A AUC AC U 5' miRNA
B
miR103/U6
2.5
2.0
1.5
1.0
0.5
0.0
HuDtg: – +
C
miR107/U6
2.5
2.0
1.5
1.0
0.5
0.0
HuDtg: – +

Figure 3-8: Upregulation of miRNA 101a in the HuDtg+ hippocampus suppresses p35 protein expression

(A) Bioinformatics analysis revealed a conserved putative miR-101a-3p site in the p35 3'UTR. Predicted RNA pairing and association energy derived from RNAhybrid is display for mouse (top) rat (middle) and human (bottom). (B) The anti-miR of miR-101 or a control ant-miR (ctrl) were transfected into primary cultured neurons. The protein/mRNA ratio for each dish is graphically displayed to the right of the representative immunoblot. (C) miRNA-specific RT-qPCR detection was used to quantify the amount of miR-101a in the neuron rich dentate gyrus of the HuDtg+ hippocampus. Results for HuDtg+ and HuDtg- are displayed graphically. (D-E) qRT-PCR amplification using primers specific to the pri- (D) and pre- (E) precursors of miR-101a was performed. (F-G) Firefly Luciferase reporters harboring either the conserved terminal portion of the p35 3'UTR (F) or a p35 3'UTR reporter lacking the putative miR-101a site (G) were transfected into CAD cells in the presence of miR-101a or a control miR (ctrl). (H) The p39 3'UTR containing Luciferase reporter did not respond to miR-101a. All dishes were transfected with Renilla Luciferase as a transfection control. Firefly Luciferase activity normalized to Renilla Luciferase is displayed graphically. Student's t-test was performed for all panels. $N \geq 3$ for all panels.

Figure 3-8

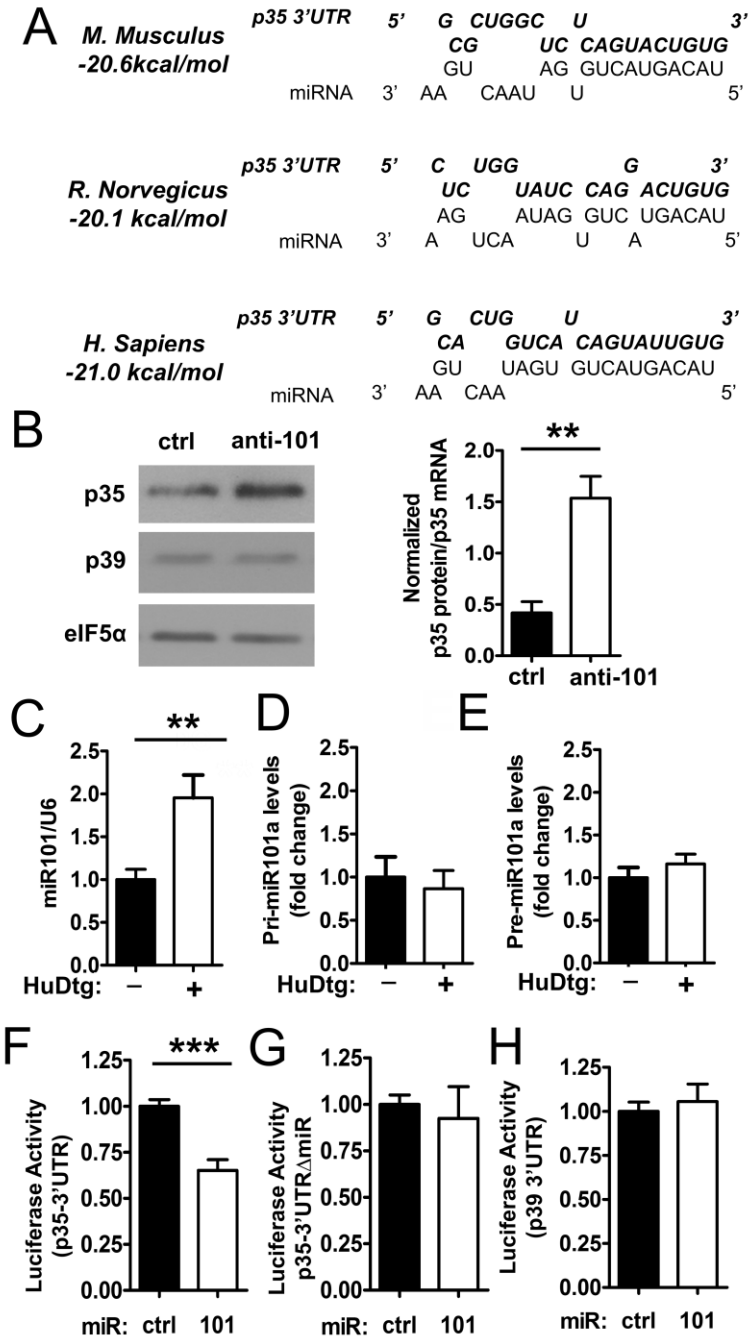

A
M. Musculus
-20.6kcal/mol
p35 3'UTR 5' G CUGGC U 3'
CG UC CAGUACUGUG
GU AG GUCAUGACAU
miRNA 3' AA CAAU U 5'
R. Norvegicus
-20.1 kcal/mol
p35 3'UTR 5' C UGG G 3'
UC UAUC CAG ACUGUG
AG AUAG GUC UGACAU
miRNA 3' A UCA U A 5'
H. Sapiens
-21.0 kcal/mol
p35 3'UTR 5' G CUG U 3'
CA GUCA CAGUAUUGUG
GU UAGU GUCAUGACAU
miRNA 3' AA CAA 5'
B
ctrl
anti-101
p35
p39
eIF5α
Normalized p35 protein/p35 mRNA
**
ctrl
anti-101
C
miR101/U6
**
HuDtg: – +
D
Pri-miR101a levels (fold change)
HuDtg: – +
E
Pre-miR101a levels (fold change)
HuDtg: – +
F
Luciferase Activity (p35-3'UTR)
***
miR: ctrl 101
G
Luciferase Activity p35-3'UTRΔmiR
miR: ctrl 101
H
Luciferase Activity (p39 3'UTR)
miR: ctrl 101

Figure 3-9: Phosphorylation of Cdk5 targets is elevated in the HuDtg+ hippocampus

(A) Immunoblot shows mode-I phosphorylation of MAP1B in neonatal HuDtg+ and HuDtg- hippocampi. eIF5α serves as a loading control. (B) Immunoblot of Glucocorticoid receptor 1 (GR1,S211) phosphorylation and total GR1 in the 1 month old HuDtg+ hippocampus, compared to HuDtg- control. The ratio of phospho/total protein is displayed graphically below A and B. $N \geq 3$ for A-B and results were analyzed by Student's t-test. (C) p39 was genetically deleted from HuDtg+ mice and phosphorylation of GR1 and MAP1B is compared between HuDtg-/p39+/+, HuDtg+/p39+/+, and HuDtg+/p39-/- littermates. Immunoblot of p39 and myc-HuD demonstrates correct genotypes.

Figure 3-9

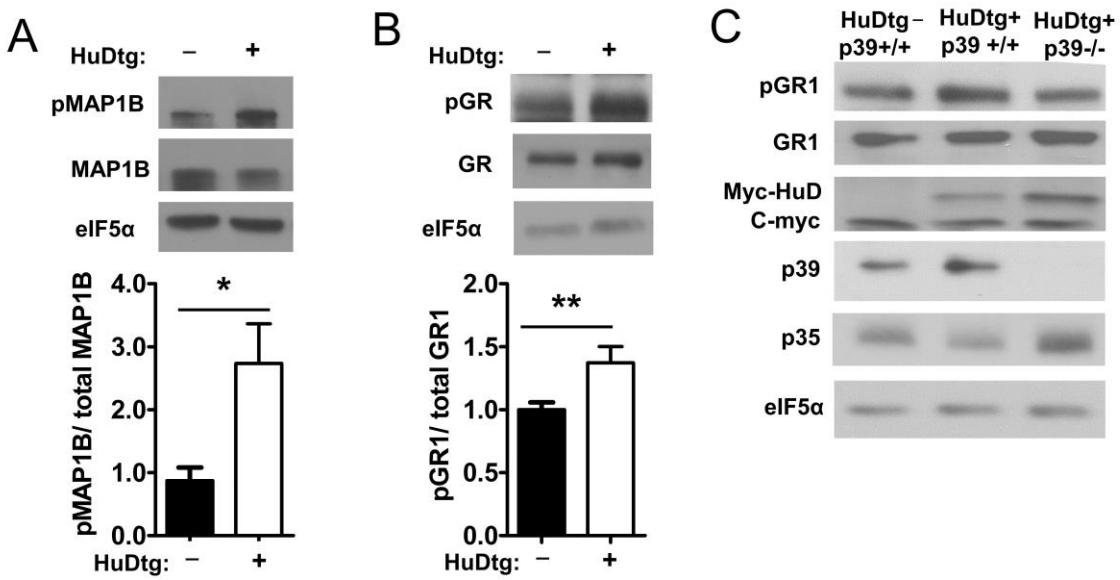

A
HuDtg: – +
pMAP1B
MAP1B
eIF5α
pMAP1B/ total MAP1B
4.0
3.0
2.0
1.0
0.0
*
HuDtg: – +
B
HuDtg: – +
pGR
GR
eIF5α
pGR1/ total GR1
2.0
1.5
1.0
0.5
0.0
**
HuDtg: – +
C
HuDtg – p39+/+
HuDtg+ p39 +/+
HuDtg+ p39-/-
pGR1
GR1
Myc-HuD
C-myc
p39
p35
eIF5α

Figure 3-10: Elimination of p39 ameliorates hippocampal circuitry abnormalities in the HuDtg+ mouse

(A) Representative IF of calbindin labeled hippocampal mossy fiber pathway at P30 for HuDtg- (top), HuDtg+ (middle), and HuDtg+/p39-/- (bottom) mice. (B) The length of the infrapyrimadal bundle (IPB) was normalized to that of the suprapyramidal bundle (SPB) and results graphically displayed below. N=4 for HuDtg- and HuDtg+/p39-/-; N=5 for HuDtg+. Results were analyzed by One-way ANOVA followed by Bonferroni's post-hoc test. Images in this figure obtained in collaboration with Guanglu Liu.

Figure 3-10

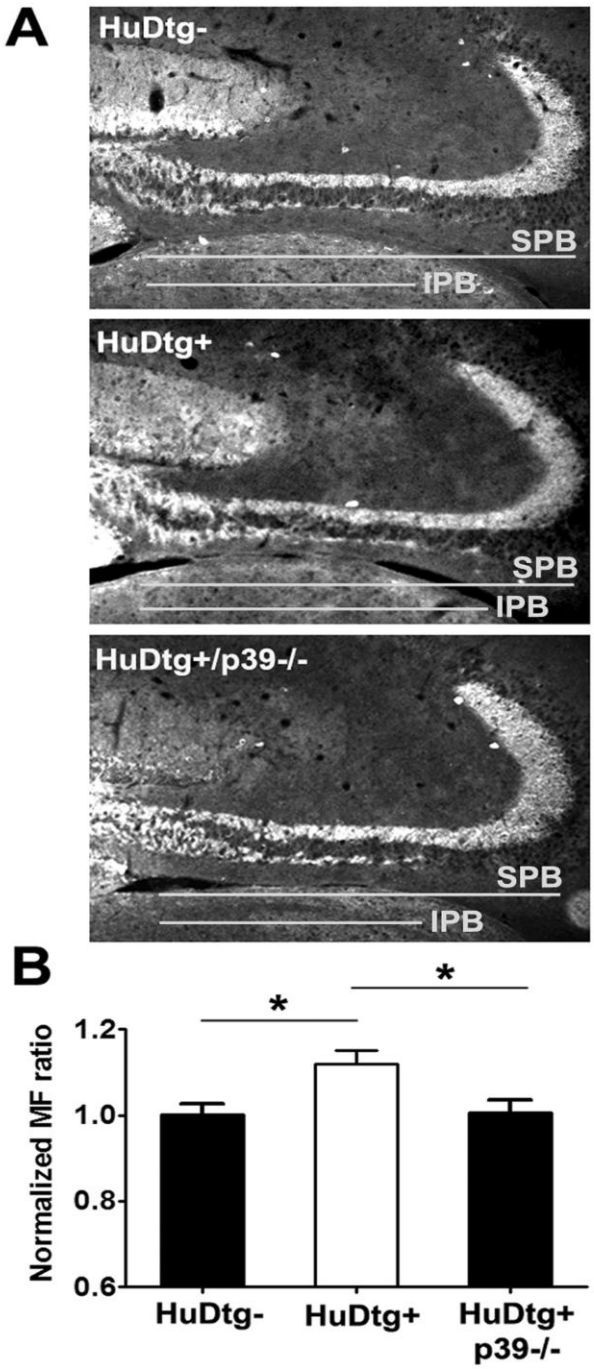
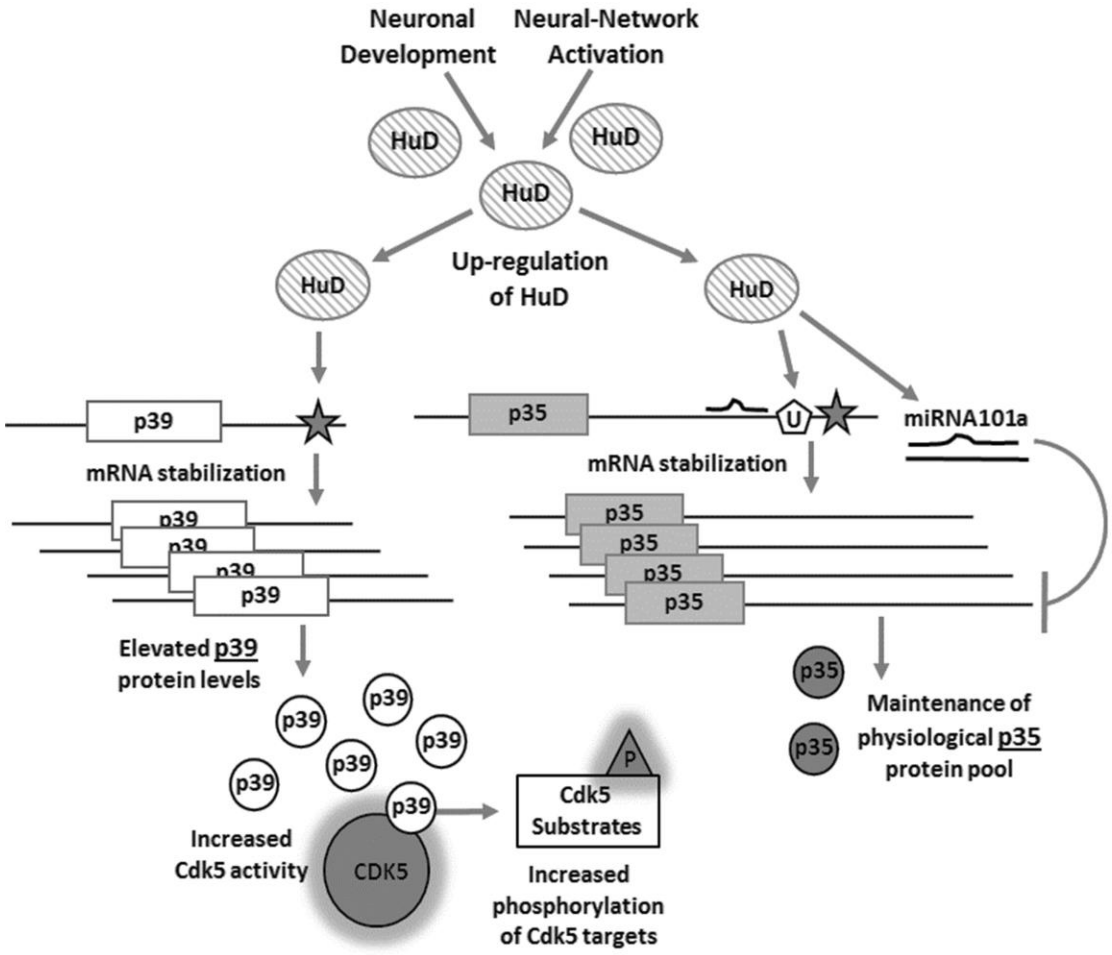

A
HuDtg-
SPB
IPB
HuDtg+
SPB
IPB
HuDtg+/p39-/-
SPB
IPB
B
*
*
Normalized MF ratio
1.2
1.0
0.8
0.6
HuDtg-
HuDtg+
HuDtg+
p39-/-

Figure 3-11: Model of HuD-dependent regulation of Cdk5 signaling

During neuronal development and at times of neuronal activation, HuD is upregulated to accommodate its role in regulating neuronal function. Our model suggests that HuD directly binds and stabilizes both p35 and p39 mRNAs via ARE element in the 3'UTRs of their transcripts. Stabilization of p39 mRNA results in increased steady state p39 protein availability. However, the elevation of miR-101a, which targets the p35 3'UTR and suppresses p35 protein production in response to HuD upregulation, leads unchanged steady state p35 protein levels. Therefore, elevated HuD-dependent elevation of p39 leads to increased Cdk5 activity and phosphorylation of Cdk5 targets, which mediates Cdk5 function in neuronal development and neuronal circuitry formation.

Figure 3-11


Neuronal Development
Neural-Network Activation
HuD
HuD
HuD
HuD
Up-regulation of HuD
HuD
p39
mRNA stabilization
p39
p39
p39
p39
Elevated p39 protein levels
p39
p39
p39
p39
p39
Increased Cdk5 activity
CDK5
P
Cdk5 Substrates
Increased phosphorylation of Cdk5 targets
p35
U
miRNA101a
mRNA stabilization
p35
p35
p35
p35
p35
p35
Maintenance of physiological p35 protein pool

Chapter 4

p39 drives Cdk5 activity increase in postnatal neurons and governs neuronal network formation and epileptic response

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*denotes equal contribution to this work

4.1 Introduction

Molecular pathways that play intricate roles in the brain are often regulated by orthologous factors that harbor similar biochemical properties yet serve differential biological functions. One such example is Cyclin-dependent kinase 5 (Cdk5), a proline-directed serine/threonine kinase that controls neuronal migration in the embryonic brain, neuronal circuitry formation in the neonate, and neurotransmitter release in the adult (159). Two distinct protein co-factors, called p35 and p39, display comparable efficiency for activating Cdk5 *in vitro* and can independently activate Cdk5 *in vivo* (176, 265). Although Cdk5 is ubiquitously expressed, its activity is limited by the available amount of p35 and p39 within a cell, both of which are rapidly degraded upon Cdk5 activation (171). Exacerbation and deficiency of Cdk5 activity have been found in a rapidly growing list of neurological diseases (159), indicating that precise control of Cdk5 activity is crucial for normal brain function. Nonetheless, how the expression of Cdk5 activators is regulated to govern Cdk5 function remains poorly understood.

During embryonic brain development, the essential function of Cdk5 in cortical layering is primarily mediated by p35, whereas p39 is scarcely expressed until after birth (167, 175, 176, 234). In the postnatal brain, both p35 and p39 are abundantly expressed in neurons and can be detected within neuronal arbors and synaptic terminals (183, 234). However, despite the intensive investigation of p35-dependent Cdk5 activity, p39 function is understudied and therefore poorly understood, partly due to the lack of an overt phenotype in p39-/- mice (176). Although p39 was found to act as the primary Cdk5 activator in oligodendrocytes (OLs) that mediates Cdk5 function to advance myelin development and repair (233), why neurons express abundant p39 in addition to p35 in the postnatal brain still remains a mystery. The complexity of Cdk5 function in

controlling dendritic spine density and synaptic plasticity (205, 206, 212) raises the intriguing question of whether p35 and p39 may differentially direct the diverse roles of Cdk5 in neurons. Importantly, during neonatal neuron development, Cdk5 activity is markedly increased (167) to advance axonal and dendritic arbor formation (272, 273). However, which Cdk5 activator is responsible for increasing Cdk5 activity in postnatal neurons is not understood.

We found that selective up-regulation of p39 is the underlying mechanism for the increased Cdk5 activity during neuronal differentiation. Loss of p39 in neurons not only attenuates overall Cdk5 activity but also differentially affects Cdk5 targets critical for neuronal network assembly, which leads to abnormalities in axonal growth and dendritic spine formation. Moreover, $p39^{-/-}$ mice display dysregulation of Cdk5 targets at synapses and ameliorated behavioral and molecular responses, which is opposite of the pro-epileptic phenotype of the p35-/- mice. These studies demonstrate that p39 plays essential roles in directing Cdk5 signaling and suggest that a functional interplay between Cdk5 activators is crucial for postnatal neuron development and brain function.

4.2 Results

4.2.1 Histone acetylation-mediated transcription selectively up-regulates p39 during neuronal differentiation

To determine which Cdk5 activator mediates increased Cdk5 activity in differentiating neurons, we examined p35 and p39 protein expression by immunoblot. A nearly ten-fold increase in p39 protein levels was detected in rat cortical neurons during the first week of differentiation *in vitro* (DIV) **(Figure 4-1A** and **4-1B**). In contrast, p35 protein levels were only slightly increased without statistical significance. Selective up-

regulation of p39 protein was also observed in the hippocampus during neonatal development (**Figure 2**). A similar fold-increase of p39 mRNA was observed, whereas p35 mRNA expression was unaltered (**Figure 1C**). Continued p39 up-regulation resulted in comparable numbers of p39 and p35 mRNA molecules in each neuron by DIV15, estimated by qRT-PCR based on known amount of cDNA (p39, 2.018 +/- $0.3534x10^{-24}$ moles/cell; p35, 2.766 +/- $0.4678x10^{-24}$ moles/cell). No apparent changes in protein stability of p39 were observed during neuronal differentiation (**Figure 3**). Thus, the increase of p39 mRNA underlies the selective up-regulation of p39 protein in neonatal neurons.

To investigate whether transcription of p39 is enhanced during neuronal differentiation, we performed a chromatin immunoprecpitation (ChIP) assay to assess recruitment of RNA polymerase II (Pol II). Pol II binding to the p39 promoter was significantly increased at DIV6 as compared to DIV1 (**Figure 4-1D**). In addition, treatment with the histone deacetylase (HDAC) inhibitor Trichostatin A (TSA) significantly increased p39 mRNA (**Figure 4-1E**), but not p35 mRNA (**Figure 4-1F**). TSA-induced up-regulation of p39 was accompanied by increased acetylation on lysine 9 of histone 3 (H3K9), a surrogate marker for histone-acetylation mediated transcriptional up-regulation (274), throughout the p39 gene (**Figure 4-1G**). In contrast, TSA did not induce H3K9 acetylation at the p35 promoter (**Figure 4-1H**). These data indicate that histone acetylation differentially regulates transcription of p35 and p39 in developing neurons.

4.2.2 Loss of p39 attenuates overall Cdk5 activity but differentially affects phosphorylation of Cdk5 targets in brain neurons

We performed acute knockdown of p39 by siRNA in cultured cortical neurons without affecting p35 or Cdk5 (**Figure 4-4**), which leads to significantly reduced activity of immunoprecipitated Cdk5 (**Figure 4-5A**). Furthermore, immunoblot analysis using two independent phospho-specific antibodies detected reduction of Cdk5-dependent mode I-phosphorylation of microtubule associated protein 1B (PI-MAP1B) (**Figure 4-5B**), a key player that controls microtubule dynamics, axonal extension and pathfinding, and dendritic spine formation (260). Interestingly, phosphorylation of WAVE1 at S397 (pWAVE1-S397), a well-recognized functional target of Cdk5 that also plays key roles in synaptic development (207), was not significantly affected by p39 knockdown (**Figure 4-5C**). To further test whether the loss of p39 affects phosphorylation of Cdk5 targets *in vivo*, we examined PI-MAP1B in the neonatal hippocampus of p39-/- mice and wild type (Wt) controls. Similar to cultured cortical neurons, PI-MAP1B was significantly reduced in the p39-/- hippocampus at the age of P5 and P10 (**Figure 4-5D**), the time window for hippocampal microcircuit formation (275) In contrast, pWAVE1-S397 was unaffected in the p39-/- hippocampus (**Figure 4-5E**). Moreover, Cdk5-dependent phosphorylation of the glucocorticoid receptor at S211 (pGR1-S211) (263), which is critical for adult brain function (276), was significantly reduced in the p39-/- hippocampus at the age of P48 (**Figure 4-5F**). Notably, expression of p35 was not significantly altered in the p39-/- hippocampus of neonates and young adults (**Figure 4-6**). These results indicate the functional importance of p39-dependent Cdk5 activity in the developing and the adult brain.

4.2.3 Essential function of p39 in advancing axonal development, dendritic spine formation, and synaptogenesis

We next questioned whether p39 is essential for axon development in hippocampal neurons that display a well-defined temporal profile of axonal growth and branching in culture (277). At the peak of rapid axon extension (DIV4), the maximum axon length of p39-/- hippocampal neurons was significantly shorter than Wt controls (**Figure 4-7A** and **B**). Meanwhile, the number of branches from the primary axon was increased in p39-/- neurons (**Figure 4-7C**). Consequently, the total length of axon was not altered by the loss of p39 (**Figure 4-7D**). To test whether the loss of p39 affects axonal development *in vivo*, we examined hippocampal mossy fibers (MFs), which are well-characterized axons of dentate granular cells that form synapses with CA3 pyramidal neurons, in p39-/- and Wt mice. Immunostaining of calbindin labels the supra-pyramidal bundle (SPB) and infra-pyramidal bundle (IPB) of MFs, respectively (**Figure 4-7E**). The relative length of IPB to SPB is a commonly used marker for assessing MF projection during hippocampal microcircuit formation (275). Consistent with the aberrant axonal growth in culture, the IPB was significantly shortened in p39-/- neonates (**Figure 4-7F**). Together, these data demonstrate that p39-dependent Cdk5 activity is required for proper axonal development of hippocampal neurons in culture and *in vivo*.

The role of Cdk5 in controlling dendritic spine density is complex in various biological paradigms (159, 205, 206). To address the function of p39 in dendritic spine development and to minimize compensatory adaptations caused by long-term p39 deficiency, we performed acute siRNA knockdown of p39 in DIV13 hippocampal neurons that undergo robust dendritic spine and synapse formation (278). The co-expressed eGFP identified transfected neurons (~5%) and provided a volume marker for imaging dendritic spines. p39 knockdown was achieved in distantly separated neurons

without affecting nearby presynaptic neurons. As a result, a significant reduction of cell-autonomous spine formation was observed (**Figure 4-8A**). Expression of Flag-tagged human p39 significantly increased spine density in p39-siRNA-treated neurons (**Figure 4-8B**). Furthermore, the density of mature spines labeled by PSD-95 (**Figure 4-8C**) and functional spines that formed synapses labeled by the presynaptic marker SV2 (**Figure 4-8D**) were also reduced by p39 knockdown. These data demonstrate that p39-mediated Cdk5 activity is essential for dendritic spine and synapse formation during neuronal network assembly.

4.2.4 Loss of p39 results in dysregulation of Cdk5 targets at synapses and ameliorated response to pharmacologically induced seizure

Because Cdk5, p35 and p39 are present at synapses (234), we next questioned how the loss of p39 may affect Cdk5 targets in synaptoneurosomes (SNS) isolated from the hippocampus. Surprisingly, Cdk5-dependent phosphorylation of synapsin I at site 7 [pSynI-S553 in mouse, (279)], a major player that regulates synaptic vesicle trafficking and neurotransmitter release (280), was significantly increased in p39 -/- SNS (**Figure 4-9A**). In contrast, P1-MAP1B in p39-/- SNS was unaffected (**Figure 4-9B**). To explore whether the loss of p39 causes dysregulation of p35, which may underlie the altered phosphorylation of SynI, we examined the full length p35 and its cleavage product p25 in SNS (**Figure 4-9C**). We found that the sum of p35 and p25 was increased ~30% in p39-/- SNS (**Figure 4-9D**). Moreover, p35 was increased ~two-fold (**Figure 4-9E**) concomitant with reduced p25, resulting in a four-fold increase of the p35 to p25 ratio (**Figure 4-9F**). Thus, the loss of p39 not only increased synaptic abundance of p35 but also reduced p35 cleavage. Given the fact that p35 is associated with membranes whereas p25 is released

from membranes, the dysregulation of synaptic p35 caused by the loss of p39 is predicted to preferentially enhance phosphorylation of membrane-associated Cdk5 targets, represented by pSynI-S553.

Considering the involvement of Cdk5 and p35 in epilepsy (175, 281), we explored whether and how the loss of p39 may affect epileptic responses upon kainic-acid (KA)-induced seizure (**Table 4-10**). Interestingly, unlike the pro-epileptic phenotype in p35-/- mice (175), p39-/- mice failed to maintain the duration of status epilepticus (SE) (**Figure 4-11A and 4-11B)**. Furthermore, the loss of p39 differentially affected expression of activity-induced immediate early genes. As shown in **Figure 4-11C**, KA-induction of c-fos mRNA, a commonly used molecular marker for neuronal activation (282), was enhanced in the p39-/- hippocampus in the early phase of SE and maintained at normal levels through the end. In contrast, induction of Arc and BDNF, both of which are known to control hippocampal excitatory responses and epileptogenesis (270, 283), was significantly ameliorated in the p39-/- hippocampus (**Figure 4-11D** and **4-11E**). These data indicate that p39 is essential for maintaining behavioral and molecular epileptic responses and further suggest that the functional interplay between p39 and p35 is critical for balancing the pro- and anti-epileptic roles of Cdk5.

4.3 Discussion

We discovered that selective up-regulation of p39 is responsible for the increase of Cdk5 activity in postnatal neurons, which accommodates the functional needs of Cdk5 in neuronal arbor formation and synaptogenesis. Furthermore, the complex dysregulation of Cdk5 targets, the aberrant axonal and dendritic spine development, and the failure in maintaining epileptic responses caused by the loss of p39 demonstrate essential roles of

p39 in neuronal development and brain function. Hence, our studies have ended the long debate regarding whether p39 is a backup of p35 and suggest that p39 and p35 play non-overlapping roles to achieve balanced Cdk5 function in a normal brain.

4.3.1 Transcriptional up-regulation of p39 is the driving force for enhancing Cdk5 activity in the postnatal brain

The markedly increased mRNA and protein of p39, not p35, clearly indicates that p39 is responsible for increasing Cdk5 activity during postnatal neuron development. Indeed, acute knockdown of p39 reduced approximately 50% of Cdk5 activity in cortical neurons. As p39 is also up-regulated to advance OL differentiation and myelin lesion repair (233), selective production of p39 is the commonly employed mechanism for postnatal enhancement of Cdk5 activation by both neurons and OLs. Given the fact that p39 has a longer half-life and is more resistant to proteolytic cleavage than p35 (171), selective up-regulation of p39 during postnatal brain development provides an advantage over p35 for sustained increase of Cdk5 activity. Mechanistically, histone acetylation of the p39 promoter can be regulated in differentiating neurons, which is an underlying mechanism for selective recruitment of RNA-Pol II to enhance p39 transcription. In contrast, histone acetylation at the p35 promoter appears to be maximized. This raises an intriguing possibility for selective modulation of p39 expression and therefore p39-dependent Cdk5 function in brain neurons.

4.3.2 The loss of p39 causes complex functional consequence on Cdk5 targets

Reduced Cdk5 activity and phosphorylation of Cdk5 targets upon acute knockdown of p39 in cultured neurons and in p39-/- hippocampus demonstrate the intrinsic function of p39 in activating Cdk5 in postnatal neurons. In fact, p39-dependent

Cdk5 activation is not limited to differentiating neurons, but is also important in the adult brain. Notably, not all Cdk5 targets are affected by the loss of p39. For example, p39 deficiency does not affect pWAVE1-S397 *in vitro* or *in vivo*, a well-documented target of p35-Cdk5 in modulating dendritic spine maturation (207). Considering the emerging evidence of differential distribution of p39 and p35 in nuclear-cytoplasmic compartments, neuronal growth cones, and cell membrane (170, 171), whether p35 and p39 direct Cdk5 to phosphorylate distinct targets in neurons is a challenging question to be addressed by future studies.

Besides deficiency of p39-dependent Cdk5 phosphorylation, the loss of p39 also results in dysregulation of p35 at synapses in the adult brain. The increased p35 abundance and reduced p35 cleavage at the p39-/- synapse lead to aberrantly elevated phosphorylation preferentially on membrane Cdk5 targets, represented by pSynI-S553 that controls the recruitment of resting synaptic vesicles into the recycling pool (280). Together, the complex phosphorylation changes of Cdk5 targets caused by p39 deficiency argue that p35- and p39-dependent Cdk5 activation achieve a functional balance for Cdk5 signaling, which governs normal brain development and function.

4.3.3 p39 is essential for promoting neuronal network development

Cdk5 phosphorylates numerous proteins crucial for neuronal morphogenesis (159) and pharmacological inhibition of Cdk5 or elimination of p35 abrogates axon formation (26, 35). We show here that p39 also plays essential roles in governing axonal extension and branching of hippocampal neurons. Thus, p39 and p35 likely cooperate to promote axonal growth. Interestingly, p39 is preferentially localized to neuronal growth cones (32). Moreover, knockdown of p39, but not p35, abrogates Cdk5-dependent regulation of

lamellipodia formation. Notably, the reduced maximal axon length and increased axon branching in p39-/- neurons recapitulates axonal abnormalities in the MAP1B hypomorph mutant (284). The abnormal MF projection in p39-/- neonatal hippocampus are also consistent with the roles of PI-MAP1B in axonal extension and pathfinding (260). Thus, PI-MAP1B is a conceivable functional target of p39-Cdk5 in driving axonal growth and pathfinding.

The function of Cdk5 in dendritic spine density is complex. Cdk5-dependent phosphorylation of Ephexin1 causes dendritic spine retraction (206, 207). Cdk5-p35 dependent phosphorylation of WAVE-1 also negatively regulates dendritic spines in striatal neurons (206, 207, 285). Conversely, Cdk5 phosphorylates TrkB, which underlies the activity-dependent increase in spine density and synaptic enlargement in hippocampal neurons (205). In addition, inhibition of Cdk5 activity suppresses dendritic spine outgrowth in the nucleus accumbens (286). We found that acute knockdown of p39 in postsynaptic neurons significantly reduces spine density and synaptogenesis, indicating the intrinsic function of p39 in advancing dendritic spine formation in hippocampal neurons. The fact that knockdown of p39 and p35 differentially affects Cdk5 targets (287) raises the intriguing question of whether p39 and p35 may direct Cdk5 to distinct phosphorylation substrates and thus differentially regulating spine density in various biological paradigms.

4.3.4 Cdk5 activators may play opposing roles to govern neuronal excitability and epileptic responses

Cdk5 expression is increased in postmortem brains of epilepsy patients (227). Additionally, the loss of either Cdk5 or p35 leads to increased seizure susceptibility (175,

288), suggesting that p35 mediates the anti-epileptic function of Cdk5. On the other hand, inhibition of Cdk5 activity counteracts KA induced excitotoxicity damages (163). We found that p39-/- mice fail to maintain SE upon KA-induced seizure, suggesting that p39 may mediate the pro-epileptic function of Cdk5. The opposing epileptic responses caused by the loss of p35 and p39 suggest a functional balance between Cdk5 activators in maintaining normal excitability of the neuronal circuitry.

At this point, how the loss of p39 causes failures in maintaining SE is undetermined, likely involving multiple layers of mechanisms. First, dysregulation of p35 at the p39-/- synapses leads to increased pSynI-S553, which is a main effector for Cdk5-mediated sequestration of synaptic vesicles in the resting pool and depletion of the recycling pool during long-lasting stimulations (280). In addition, because NMDA receptor activation triggers calpain-dependent cleavage of p35, the reduced p35 cleavage in p39-/- synapses may suggest deficiency of NMDA receptors which play key roles for the maintenance of epilepsy (289). Moreover, the loss of p39 selectively ameliorates KA-induced expression of BDNF and ARC, both of which are known to govern hippocampal neuron excitability (270, 283). The reduced BDNF induction in p39-/- brain is consistent with the recent discovery that transcription of BDNF is a target for activity-dependent nuclear function of Cdk5 (187) and the observation that p39 is more prone to nuclear translocation than p35 (170). Moreover, given the critical function of BDNF-TrkB signaling in epileptogenesis (270, 290), the ameliorated induction of BDNF in p39-/- contributes to the shortened duration of SE.

Taken together, our studies have uncovered novel means employed by neurons to control Cdk5 function through p39 in the postnatal brain. The selective regulation of p39

during postnatal neuron development and the dysregulation of a subclass of Cdk5 targets due to the loss of p39 suggest non-overlapping roles of Cdk5 activators, which offer possible explanations for the diverse and sometimes conflicting roles of Cdk5 in modulating brain function. Moreover, our studies provide evidence that argues for the functional requirement and interplay between p39 and p35, which are crucial for achieving balanced Cdk5 function in normal postnatal neurons.

Figure 4-1: Transcription and histone acetylation of p39 and p35 during neuronal differentiation

(A) Immunoblot of p39 and p35 protein in primary rat cortical neurons. (B) Densitometry quantification of p35 and p39 protein normalized to β-actin. (C) Quantification of p35 and p39 mRNA in rat primary cortical neurons by qRT-PCR. (D) Pol II ChIP-qPCR of primary cortical neurons using primers specific for the p39 promoter. (E and F) Quantification of p39 (E) and p35 (F) mRNAs in primary cortical neurons exposed to Trichostatin A (TSA) or DMSO by qRT-PCR. (G and H) Acetyl-H3K9 ChIP-qPCR on DIV 5 primary cortical neurons treated by TSA using primers for the promoter, coding region (ORF) and the 3'end of p39 (G) and the promoter of p35 (H). Two-way ANOVA followed by Bonferroni's post-hoc test was performed for all panels except D and H, where the Student's t-test was performed. Data in this figure obtained in collaboration with Wenqi Li and Li Ku.

Figure 4-1

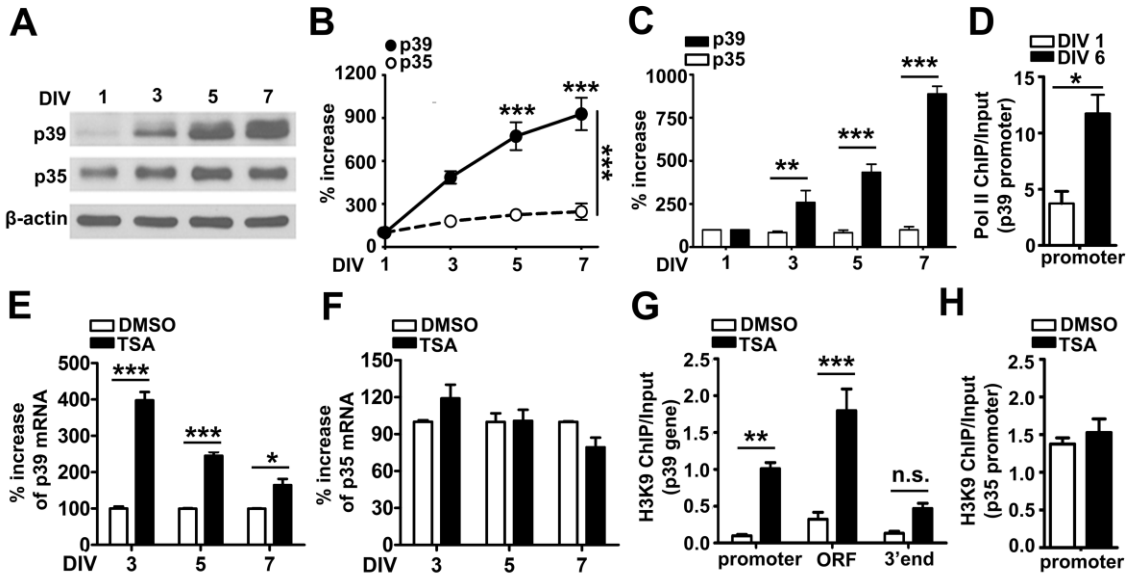

A
DIV 1 3 5 7
p39
p35
β-actin
B
p39
p35
% increase
DIV 1 3 5 7
C
p39
p35
% increase
DIV 1 3 5 7
D
DIV 1
DIV 6
Pol II ChIP/Input (p39 promoter)
promoter
E
DMSO
TSA
% increase of p39 mRNA
DIV 3 5 7
F
DMSO
TSA
% increase of p35 mRNA
DIV 3 5 7
G
DMSO
TSA
H3K9 ChIP/Input (p39 gene)
promoter ORF 3'end
n.s.
H
DMSO
TSA
H3K9 ChIP/Input (p35 promoter)
promoter

Figure 4-2: p39 protein is selectively upregulated in the mouse hippocampus during neonatal development

Immunoblot of p39 and p35 protein in mouse hippocampal lysates prepared at postnatal days (PD) 1, 3, 5, and 7 (N=5 for DIV 1,3,7, DIV 5, N=4). The percentage of increase at each time point is graphically displayed below. Two-way ANOVA followed by Bonferroni's post-hoc test was performed. Data in this figure obtained in collaboration with Wenqi Li.

Figure 4-2

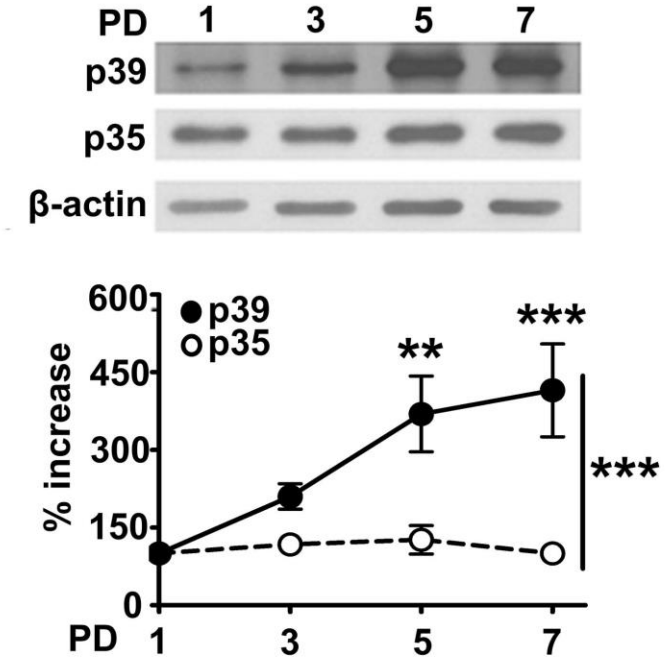

PD 1 3 5 7
p39
p35
β-actin
600
450
300
150
0
% increase
p39
p35
**
***
***
PD 1 3 5 7

Figure 4-3: p39 protein stability is not altered during neuronal differentiation

Immunoblot analysis of p35 and p39 protein in DIV3 and DIV 7 primary rat cortical neurons exposed to the translation inhibitor cyclohexamide (CHX, 100ug/mL) for 0 hours and 2 hours. β-actin serves as a loading control. Data in this figure obtained in collaboration with Wenqi Li.

Figure 4-3

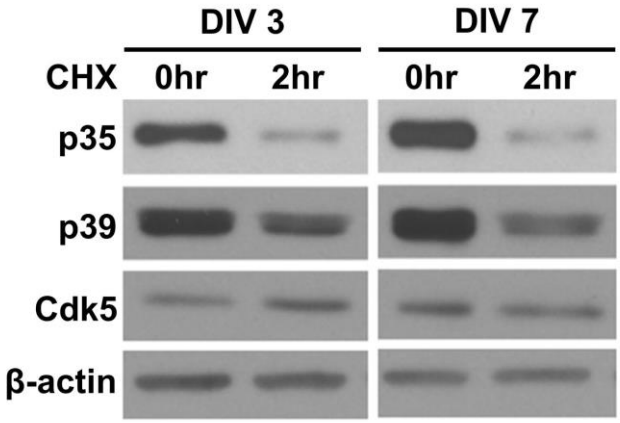


Figure 4-4: Specific knockdown of p39 without affecting p35 in primary rat cortical neurons

(A) Immunoblot of p35 and p39 protein 48 hours after the last transfection using p39-specific siRNA (sip39) or negative control siRNA (ctrl). Densitometry reading is normalized to the β-actin signal and the percentage of knockdown is graphically displayed on the right for p39 (B) and p35 (C). N=4 for each treatment and the student's t-test was performed. Data in this figure obtained in collaboration with Wenqi Li.

Figure 4-4

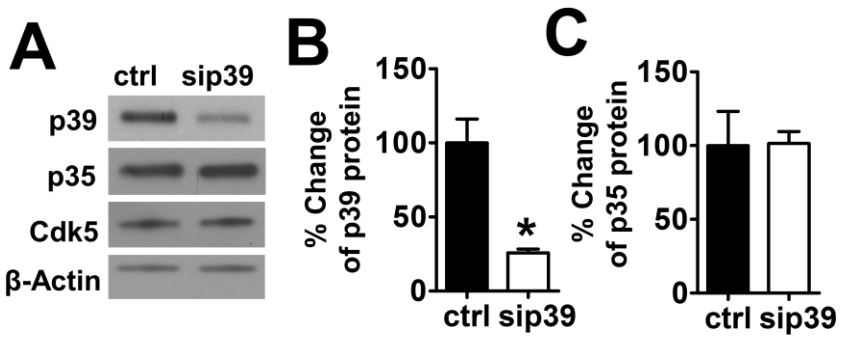
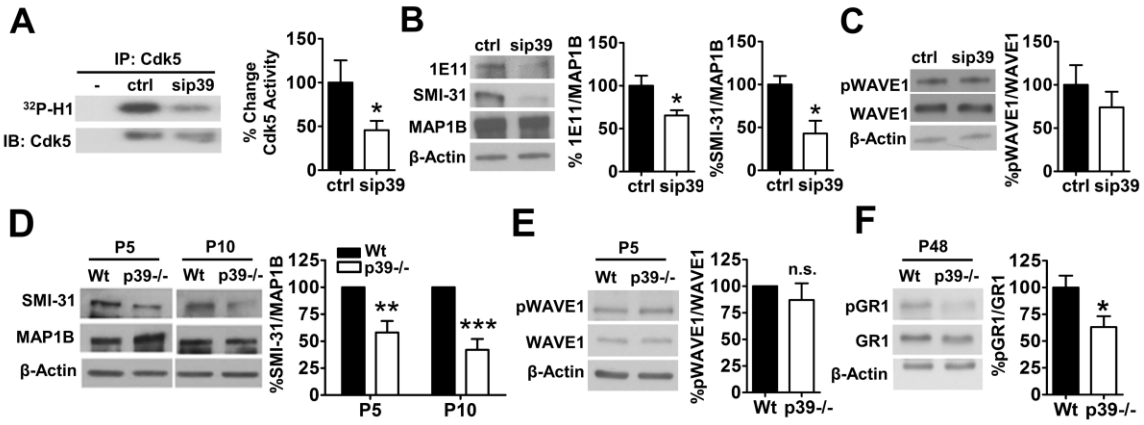

A
ctrl
sip39
p39
p35
Cdk5
β-Actin
B
% Change of p39 protein
150
100
50
0
*
ctrl sip39
C
% Change of p35 protein
150
100
50
0
ctrl sip39

Figure 4-5: Altered phosphorylation of Cdk5 targets in p39 siRNA treated rat cortical neurons and p39-/- mouse hippocampus

(A) *In vitro* kinase assay using Cdk5 complexes immunoprecipitated from DIV5 cortical neurons treated with sip39- or ctrl-siRNA and Histone H1 as the substrate in the presence of γ-^{32}P-ATP. Cdk5 activity is calculated by normalizing ^{32}P-H1 signal to Cdk5 protein level, detected by immunoblot (left), and is graphically displayed on the right (N=6). (B and C) Immunoblot analysis (IB) of Cdk5 targets upon siRNA-knockdown of p39 in cortical neurons. (B) Mode I phosphorylation of MAP1B (PI-MAP1B) using two different phospho-specific antibodies (1E11 and SMI-31) and total MAP1B by IB. (C),WAVE1-pSer397 and total WAVE1 by IB. (D-F) IB of Cdk5 targets in hippocampi of p39-/- mice and Wt controls. (D) PI-MAP1B and total MAP1B; (E) WAVE1-pSer397 and total WAVE1; and (F) GR1-pSer211 and total GR1. For B-F, densitometry reading of each phospho-protein was normalized to the corresponding total protein and graphically displayed on the right. Two-way ANOVA and Tukey's post-hoc test was performed for (D). All other panels were analyzed by the Student's t-test. Data from panels B-F obtained in collaboration with Wenqi Li.

Figure 4-5


A
IP: Cdk5
- ctrl sip39
32P-H1
IB: Cdk5
% Change Cdk5 Activity
150
100
50
0
*
ctrl sip39
B
ctrl sip39
1E11
SMI-31
MAP1B
β-Actin
% 1E11/MAP1B
*
ctrl sip39
%SMI-31/MAP1B
*
ctrl sip39
C
ctrl sip39
pWAVE1
WAVE1
β-Actin
%pWAVE1/WAVE1
ctrl sip39
D
P5
P10
Wt p39-/- Wt p39-/-
SMI-31
MAP1B
β-Actin
%SMI-31/MAP1B
Wt
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P5 P10
E
P5
Wt p39-/-
pWAVE1
WAVE1
β-Actin
%pWAVE1/WAVE1
n.s.
Wt p39-/-
F
P48
Wt p39-/-
pGR1
GR1
β-Actin
%pGR1/GR1
*
Wt p39-/-

Figure 4-6: p35 protein levels at P5 and P48 in the p39-/- hippocampus

Immunoblot shows hippocampal p35 proteins levels from p39-/- and Wt mice at postnatal days 5 (A) and 10 (B). p35 protein levels were normalized to β-actin protein and quantification is graphically displaced below the corresponding representative images. Student's t-test was performed for all panels. Seven pairs of mice were analyzed for P5 (N=7), and N=4 for P48. Data in this figure obtained in collaboration with Wenqi Li.

Figure 4-6

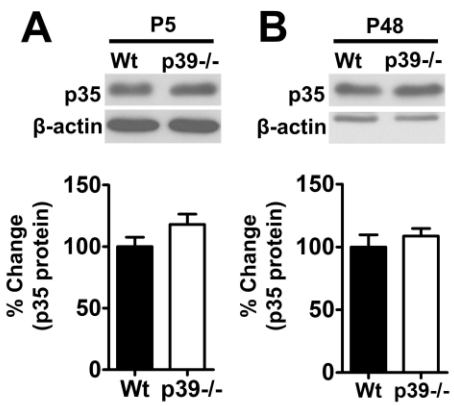

A
P5
Wt
p39-/-
p35
β-actin
B
P48
Wt
p39-/-
p35
β-actin
% Change (p35 protein)
150
100
50
0
Wt
p39-/-
% Change (p35 protein)
150
100
50
0
Wt
p39-/-

Figure 4-7: Aberrant axonal development of p39-/- hippocampal neurons in culture and mossy fiber projection *in vivo*

(A) Immunofluorescent staining (IF) of tau (green) and MAP2 (red) label axons and dendrites, respectively, of DIV4 hippocampal neurons derived from Wt or p39-/- mice. (B-D) Quantification of maximal axonal length (B), branch number (C), and total axonal length (D) (N=19 for WT, N=24 for p39-/-). (E) Representative IF of calbindin labeled hippocampal mossy fiber pathway at the age of P10. (F) The length of the infrapyrimadal bundle (IPB) was normalized to that of the suprapyramidal bundle (SPB) and graphically displayed (N=4). Student's t-test was performed for all panels. Images in this figure obtained in collaboration with Wenqi Li and Guanglu Liu.

Figure 4-7

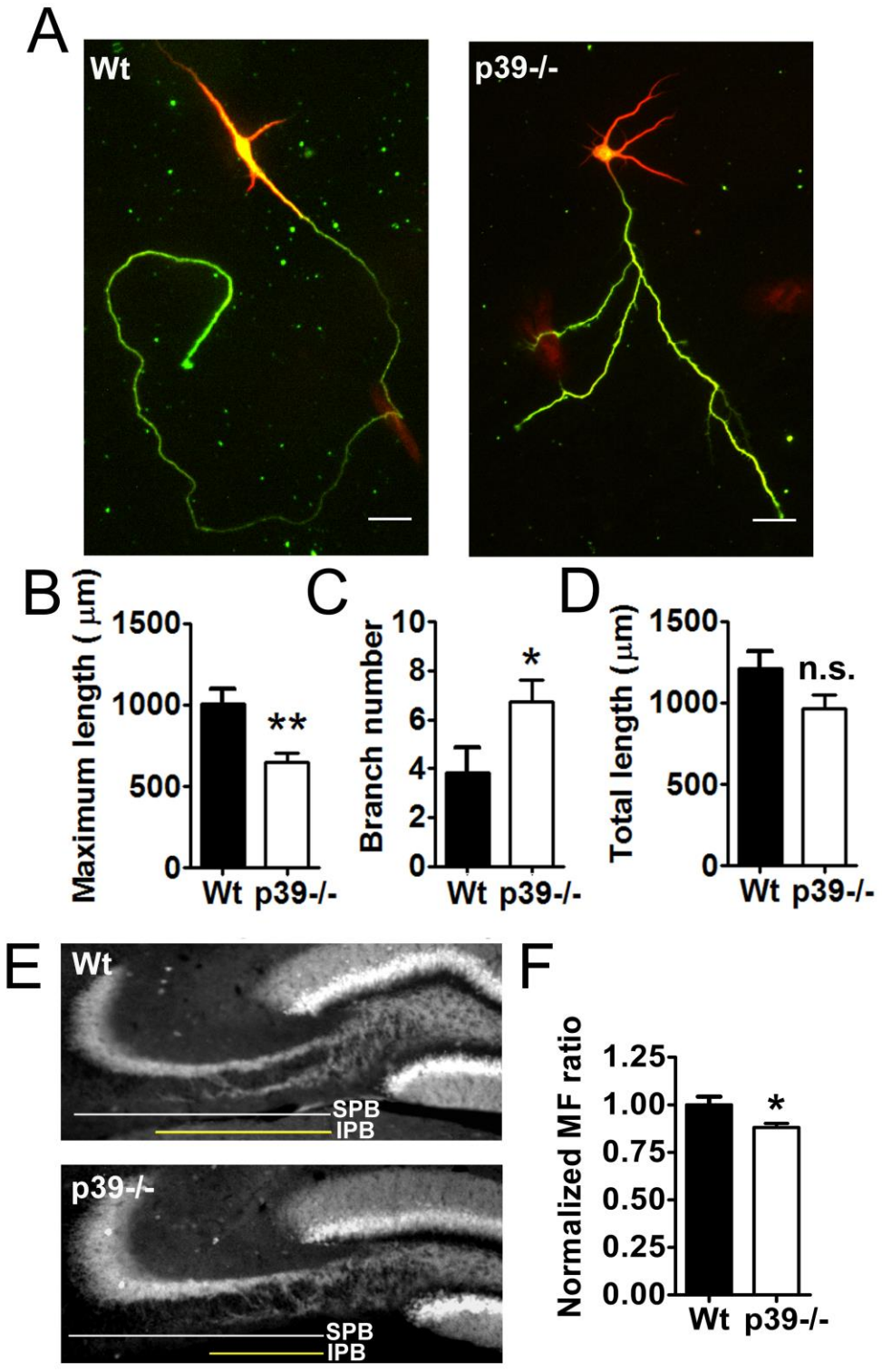

A
Wt
p39-/-
B
Maximum length (μm)
1500
1000
500
0
**
Wt p39-/-
C
Branch number
10
8
6
4
2
0
*
Wt p39-/-
D
Total length (μm)
1500
1000
500
0
n.s.
Wt p39-/-
E
Wt
SPB
IPB
p39-/-
SPB
IPB
F
Normalized MF ratio
1.25
1.00
0.75
0.50
0.25
0.00
*
Wt p39-/-

Figure 4-8: Impairment of dendritic spine and synapse formation upon acute knockdown of p39 in hippocampal neurons

Primary hippocampal neurons were co-transfected with eGFP (green) and control siRNA (ctrl) or p39-siRNA (sip39) (A, C, and D) or with Flag-p39 (B) at DIV13. At DIV 16, confocal imaging of dendritic spine was performed. Representative images of dendritic spines labeled by eGFP are displayed on the left with quantification of spine density on the right. The numbers of dendritic segments analyzed are indicated on the bar graphs. (A and B) p39 siRNA reduced dendritic spine formation (A), which was rescued by Flag-p39 (B). (C) p39 knockdown reduced mature dendritic spines that contain PSD95 (red). (D) p39 knockdown reduced dendritic spines that form synapses, marked by co-localization with the pre-synaptic terminal marker SV2 (red). The Student's t-test was performed for all panels. For (A) and (C), Welch's correction for statistically significant differences in variance was applied to the t-test. Data in this figure obtained in collaboration with Wenqi Li.

Figure 4-8

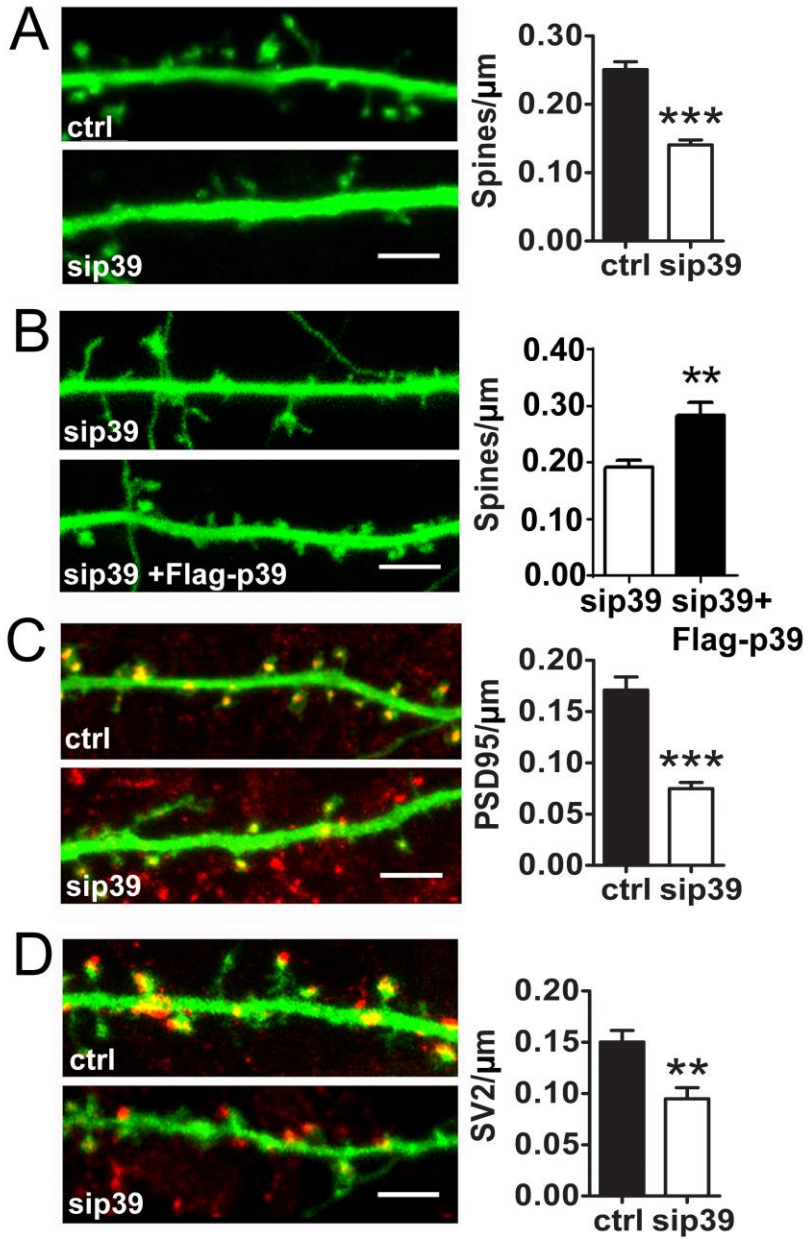

A
ctrl
sip39
Spines/µm
0.30
0.20
0.10
0.00
***
ctrl sip39
B
sip39
sip39 +Flag-p39
Spines/µm
0.40
0.30
0.20
0.10
0.00
**
sip39 sip39+ Flag-p39
C
ctrl
sip39
PSD95/µm
0.20
0.15
0.10
0.05
0.00
***
ctrl sip39
D
ctrl
sip39
SV2/µm
0.20
0.15
0.10
0.05
0.00
**
ctrl sip39

Figure 4-9: Dysregulation of synaptic Cdk5 targets and p35 in p39-/- hippocampal synaptoneurosomes

(A and B) IB of Cdk5-dependent phosphorylation of synapsin I at S533 (pSyn) (A) and PI-MAP1B (B) in SNS isolated from Wt and p39-/- hippocampi. Densitometry reading of each phospho-protein was normalized to the corresponding total protein and graphically displayed on the right. (C) IB of p35 and p25 in Wt and p39-/- SNS. (D-F) Densitometry analysis of the sum p35+p25 (D), full length p35 (E), and the ratio of p35/p25 (F) in Wt and p39-/- SNS (N=4). β-actin serves as a loading control for all experiments.

Figure 4-9

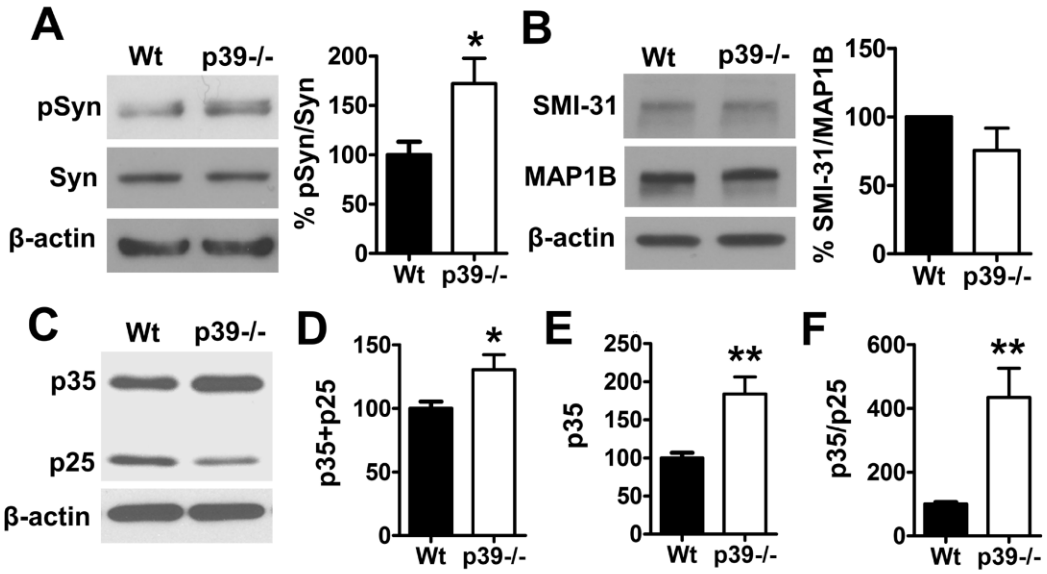

A
Wt
p39-/-
pSyn
Syn
β-actin
% pSyn/Syn
200
150
100
50
0
*
Wt
p39-/-
B
Wt
p39-/-
SMI-31
MAP1B
β-actin
% SMI-31/MAP1B
150
100
50
0
Wt
p39-/-
C
Wt
p39-/-
p35
p25
β-actin
D
p35+p25
150
100
50
0
*
Wt
p39-/-
E
p35
250
200
150
100
50
0
**
Wt
p39-/-
F
p35/p25
600
400
200
0
**
Wt
p39-/-

Figure 4-10: Behavioral and molecular response to kainic acid (KA)-induced seizure in p39-/- and Wt mice

(A) Seizure scores in ten minute intervals for Wt and p39-/- mice after KA injection. (B) SE duration, defined as the average amount of time spent in SE, in Wt and p39-/- mice. (C-E) qRT-PCR quantification of c-fos mRNA (C), ARC mRNA (D) and BDNF mRNA (E). Numbers of mice analyzed in each genotype are indicated in a table in the Materials and Methods. Two-way ANOVA and Bonferroni's Post-hoc test were applied for all panels except (B), where the Student's t-test was used. Data in panel A was obtained in collaboration with Wenqi Li.

Figure 4-10

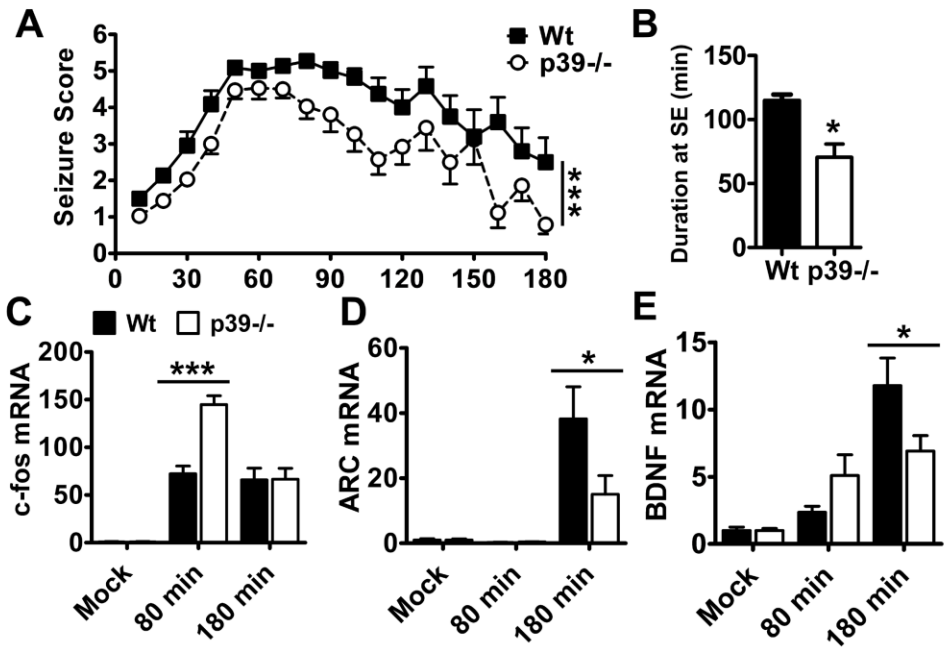

A
Seizure Score
Wt
p39-/-
***
B
Duration at SE (min)
*
Wt p39-/-
C
Wt
p39-/-
c-fos mRNA
***
Mock
80 min
180 min
D
ARC mRNA
*
Mock
80 min
180 min
E
BDNF mRNA
*
Mock
80 min
180 min

Table 4-11: Seizure behavior upon KA injection

Latency, defined as the amount of time from injection until onset of grade 5 seizure (SE, status epilepticus), was quantified for all Wt and p39-/- mice that entered S.E. Duration, defined as the length of time spent at grade 5 seizure (SE) was quantified for all p39-/- and Wt mice surviving from injection to 180 minutes. The total number of mice injected for each genotype (Total) are indicated at the bottom. The number and the percentage of mice that displayed grade 3 or higher seizure and those that died within 180 minutes of injection are also included. Student's t-test was performed to analyze duration and latency. *denotes $p<0.05$ by the student's t-test.

Table 4-11

	Wt	p39-/-
Latency to SE (min)	30.00 +/- 6.45	35.42 +/- 8.38
Duration at SE (min)	122.86 +/- 27.21*	72.50 +/- 34.28*
Animals Reaching SE	18 (81.8%)	24 (70.6%)
Animals Died	8 (36.4%)	7 (20.4%)
Total	22	34

Chapter 5

Discussion:

Concluding remarks, unresolved questions, and future directions

Advancements in our understanding of the many layers of post-transcriptional regulation underlying the intricacies of neuronal development and function promote a deeper understanding of neurobiology. In this dissertation, we uncovered two pathways, controlled by the neuronal RNA-binding protein HuD, which direct neuronal differentiation, the development of the hippocampal neural-circuitry, and neuronal activation (**Figure 5-1**). We demonstrate that through selective post-transcriptional regulation of BDNF and Cdk5 activator expression, HuD coordinates multiple pathways governing neuronal development and function. Thus, HuD is a critical factor which may synergistically control BDNF/TrkB signaling and/or Cdk5 signaling to regulate multifaceted biological paradigms important for normal brain function.

Our data demonstrate that HuD specifically binds to and stabilizes the BDNF long 3'UTR mRNA, thereby controlling the abundance of BDNF protein and BDNF accumulation in mossy fiber axon terminals within the hippocampus. HuD can also bind and stabilize Cdk5 activator mRNAs in neurons. Interestingly, HuD-mediated regulation of BDNF, p35, and p39 occurs through conserved Class II and Class III A/U-rich elements. We found that although the mRNA expression of both Cdk5 activators was elevated in response to HuD, p39 protein was selectively upregulated. We discovered a HuD-miRNA loop which selectively promotes p39 protein expression in hippocampal neurons through upregulation of miR-101a, a novel translational inhibitor of p35 expression. As a result, HuD governs p39-dependent Cdk5 target phosphorylation and promotes mossy fiber extension in the hippocampus. Thus, we disovered that p39 expression, and thus Cdk5 activity, in neurons is governed by both transcriptional and post-transcriptional regulatory mechanisms. Moreover, in contrast to the model where

p39 serves as back-up for p35, we show that p39 is an essential Cdk5 activator which underlies Cdk5 activity increase during neuronal development and that p39 promotes Cdk5 function during neuronal circuitry development and epileptogenesis. Our data support a model where p39 and p35 direct Cdk5 to a non-overlapping subset of phosphorylation targets and mediate distinct aspects of Cdk5 signaling to coordinate and balance Cdk5 activity during normal brain function. Thus, we found that HuD-mediated regulation of the BDNF and Cdk5 pathways accomplishes coordinated control of neuronal network assembly and function. However, the list of potential HuD ligands is rapidly expanding and thus future studies will be required to piece together the converging networks important for neuronal function under control of HuD-mediated regulation.

5.1 <u>What are the up-stream factors governing p35 and p39 expression in neurons?</u>

Soon after the discovery of Cdk5, the protein co-factors p35 and p39 were identified and it has been known for decades that their abundance determines Cdk5 activation (176, 228). However molecular mechanisms governing Cdk5 activator expression, and thus controlling Cdk5 function, have remained elusive. This gap in knowledge is responsible for the incomplete understanding of how Cdk5 activity is regulated to accommodate neuronal function in different biological paradigms and why dysregulation of Cdk5 activation is often observed in neurological disease (159, 220, 221, 223, 225). Through this work, we have uncovered factors that differentially govern transcriptional, post-transcriptional, and translational regulation of the two distinct Cdk5 activators, p35 and p39. In both the developing and mature brain neurons, our evidence points to preferential transcriptional and post-transcriptional regulation of p39 to mediate

or fine-tune Cdk5 activation, respectively. Moreover, in the postnatal brain, whether in differentiating neurons or oligodendrocytes, upregulation of p39 underlies elevated Cdk5 activation (233). Therefore, it seems brain cells employ modulation of p39 to underlie the alterations in Cdk5 activity required for physiological Cdk5 function.

The next step in understanding the regulation of Cdk5 activity in neurons will be the identification of the remaining regulatory factors governing p39 expression. For instance, epigenetic factors controlling acetylation of the p39 promoter, transcription factors which specifically govern p39 expression during development, and de-stabilizing factors that may compete or coordinate with HuD to maintain the steady state p39 mRNA pool still remain unidentified. Previous work from our lab showed that p39 is also up-regulated to mediate Cdk5 activation during oligodendroglia (OL) differentiation (233). Thus, whether p39 expression in developing OLs is controlled by acetylation-dependent transcription and whether similar or cell-specific factors govern these processes in OLs and neurons remains to be answered. Moreover, p39 is translationally active in OLs while p35 is translationally suppressed (233). Notably, miRNA101a, which suppresses p35 protein production in neurons, is also expressed in brain glia (291). Whether miRNA 101a or other miRNAs contribute to the suppression of p35 in OLs is an intriguing possibility for future studies. Moreover, future studies uncovering whether and how p35 and p39 are subjected to translational regulation in neurons and whether such regulation may be cell-type specific will aid in our understanding of how Cdk5 activator expression is governed, potentially during activity-dependent stimulation or by local translation.

Our data suggest that, in conjunction with each molecular mechanism of selective p39 upregulation in neurons, a “check-point” to prevent elevation of p35 expression

exists. It appears that saturation of histone acetylation at the p35 promoter prevents further increase of p35 transcription during neuronal development and translational inhibition by miR-101a occurs as a feedback mechanism to upregulation of p35 mRNA by HuD. Thus, it appears that p39 is the preferential Cdk5 activator to be modulated in postnatal neurons when changes to Cdk5 activation or Cdk5 function are biologically required. Conceivably, there are inherent advantages to upregulation of Cdk5 activity through p39 versus p35. For example, both p39 mRNA (**Figure 3-1**) and p39 protein have a longer half-life than p35 in neurons (171). Moreover, p39 protein is more resistant to calpain-dependent proteolytic cleavage (171). The benefits of this stability are multifaceted: a longer mRNA half-life allows for the increased potential of sub-cellular neuronal localization, transport, and greater temporal availability of p39 mRNA for translation. Localized p35 and/or p39 mRNA at the neuronal synapse has not been reported, yet seems inevitable considering the complex role of Cdk5 in governing synaptic plasticity and function (160). A longer p39 protein half-life allows for sustained Cdk5 activation, such as during neuronal development (167). The higher resistance to calpain-mediate cleavage of p39 may decrease the chances of producing the cleavage fragment, p29. Both p25 and p29 are mis-localized in the cell and display increased half-lives leading to aberrant Cdk5 activation (172, 174). However, it is p25 which is well-documented to mediate pathological effects on neurons (173, 213, 214, 223).

5.2 <u>What are the discrete aspects of p35-Cdk5 and p39-Cdk5 function in neurons?</u>

In addition to uncovering mechanisms which preferentially regulate p39 expression, our studies have ended the long debate of whether p39 is merely a back-up copy of p35. Our data demonstrate that p39 is essential for driving Cdk5 activity and

function during neuronal development. In addition to being essential for axon extension and spine development, it seems that, in mature neurons at the synapse, p39 may balance or coordinate with p35 to direct synaptic Cdk5 function. Moreover, p39 expression seems to be critical for the proper expression and cleavage of p35 at the synapse. Future studies which uncover roles of p39-Cdk5 at the synapse and delineate what underlies the connection between p39 expression and synaptic p35 dysregulation will likely aid our understanding of how Cdk5 function is regulated to control neurotransmitter release, synaptic vesicle recycling, neuronal activation, and learning and memory (160, 192, 204). As p35 is localized to the membrane and p25 is cytoplasmic (171, 174), the regulation of p35 expression and cleavage through p39 also has important implications for understanding what controls phosphorylation of Cdk5 targets at different parts of the synapse; i.e. the synaptic membrane versus the pre-synaptic or post-synaptic terminal. Understanding what directs target phosphorylation at the synapse is a critical prerequisite to understanding how Cdk5 governs synaptic function in different scenarios.

A historical limitation to studying p39 function was the lack of specific reagents to detect p39. This limitation has since been over-come with increasing interest in p39 function with antibodies that target p39's N-terminal region. Yet, a specific C-terminal antibody will be required to dissect the endogenous physiological and/or pathological function of p29, as has been elucidated for p25. Additionally, a remaining limitation to understanding the role that p39 plays in directing Cdk5 function in the adult brain, perhaps at the synapse under stimulation, is the lack of an inducible p39 knockout mouse model. Although the p39-/- mouse model has been a useful first approach to understanding p39 function, dissecting whether loss of p39 in neurons is cell autonomous

or how acute loss of p39 affects regulation of Cdk5 activation in neurons and glia will require a cell-type specific inducible knockout model. Likewise, no p39 transgenic model exists to study the reciprocal neuronal response to increased p39 *in vivo*. Likely these tools will come with the growing appreciation for the essential function of p39. In particular, comparing function effects of inducible neuronal loss of p39 versus inducible neuronal loss of p35 may untangle Cdk5 activator roles in complex paradigms such as upon neuronal stimulation or during ageing. Considering the pathological effect, a comparison of p25 and p29 cleavage in such scenarios would be particularly intriguing.

Consistent with the model of non-overlapping function for Cdk5 activators, we demonstrate that loss of p39 differentially affects Cdk5 target phosphorylation in developing neurons. Why loss of p39 leads to decreased mode I phosphorylation of MAP1B and decreased phosphorylation of S211 on GR1, yet does not affect phosphorylation of the known Cdk5 substrate, WAVE1, is a mystery, especially considering detection of normal p35 protein levels in the p39-/- brain at P5. One potential mechanism is different localization of p35 and p39 within neurons. Such non-overlapping sub-cellular distribution could occur through mRNA transport or through association of p35-Cdk5 or p39-Cdk5 with different cytoskeletal elements. For example, perhaps p39-Cdk5 preferentially associates with regulators of microtubule dynamics, while p35-Cdk5 may associate with protein regulators of the actin cytoskeleton, thus controlling different aspects of neuronal function. Different groups have attempted to address this question yet direct comparison of p35 and p39 association with different cytoskeletal elements is difficult to assess due to the differential sensitivity of p35-Cdk5 and p39-Cdk5 to buffer salt concentration and detergent (120, 265). Moreover, considering the distinct spatial and

temporal expression of p35 and p39, deciding which time and location to compare cytoskeletal association would be difficult.

Nonetheless, it appears that p39 is required for proper phosphorylation of Cdk5 substrates. To definitively answer the question of whether p35 and p39 direct Cdk5 to discrete subsets of *in vivo* phosphorylation substrates, future studies will likely employ cell-type specific phospho-proteomics. Such data has the potential to fill critical knowledge gaps in our understanding of the multifaceted signaling capabilities of Cdk5 kinase activity. Moreover, a greater understanding of which pathways may be governed by p35-Cdk5 and/or p39-Cdk5 could lend important functional insights into the seemingly enigmatic activity of Cdk5 in different biological paradigms.

One example may be the untangling the role of Cdk5 function in dendritic spine formation. pS397-WAVE1, which seems to be under control of p35-, but not p39-, dependent Cdk5 activity, provides one mechanistic explanation for the seemingly opposing roles that Cdk5 plays in spineogenesis, as the phosphorylation of WAVE1 and ephrin-A1 by p35-Cdk5 negatively regulates spine formation and spine density, respectively (206-208). Perhaps, p39-dependent Cdk5 phosphorylation targets mediate the enhancement of phospho-TrkB dependent dendritic spine growth by Cdk5 as seen in neurons under stimulation of BDNF (184, 205). Indeed, we observed that the loss of p39 hampers the induction of BDNF in response to neuronal stimulation by kainic acid, suggesting a potential link between p39-Cdk5 activity and BDNF mRNA production thus BDNF-TrkB signaling. A recent seminal study linking Cdk5 to TrkB function demonstrated a role for p35 in BDNF-induced LTP, but did not show a correlation between p35 and the phosphorylation of the S478 site on TrkB (205). In fact, in their

paradigm, p35 levels do not correlate with S478 phosphorylation levels. Therefore, although p35 plays an essential role in directing Cdk5 for synaptic plasticity under stimulation, whether p39-dependent Cdk5 activation underlies p-S478 TrkB, which was the Cdk5 site demonstrated to modulate activity-dependent dendritic spine remodeling, is an intriguing question to be answered by future phospho-proteomics.

5.3 How does HuD coordinate pathways that govern neuronal development and function?

Understanding how RNA-binding proteins facilitate intricate neuronal function through selective binding to multiple mRNA targets, often through seemingly degenerate sequence, is a prevailing challenge. Our studies demonstrate HuD-mediated 1) stabilization of Cdk5 activators yet selective production of p39 protein; 2) the specific binding and stabilization of the long 3'UTR isoform of BDNF mRNA, which contains unique sequence that targets BDNF to dendrites. Mechanistically, HuD preferentially binds to unique ARE sequence contained within the long BDNF 3'UTR, but not the short 3'UTR, in the BDNF mRNA to promote selective stabilization of BDNF mRNA that contains the long 3'UTR. Additionally, while HuD binds both p35 and p39 mRNA via AREs, HuD preferentially drives p39 protein production through control of a miRNA feedback loop. Thus, we found two examples of HuD-mRNA target functional specificity. However, further mechanisms governing HuD-target specificity remain to be uncovered. For example, CLIP studies show that HuD does not bind all ARE elements, as the percentage of neuronal mRNAs containing AREs is nearly 20% and identified HuD ligands make up only around 6% of murine neuronal mRNAs (61). Thus, more *in vivo* investigations into HuD-mRNA target regulation and the functional consequences of such

regulation will be necessary to identify the mechanisms underlying HuD-directed neuronal development and function. Additionally, whether HuD-miRNA feedback loops are a common mechanism employed for governing selective expression of neuronal protein among related functional players in a pathways and/or whether HuD coordinates with other miRNA species to regulate mRNA targets will be an interesting direction for future HuD studies.

Wiring the brain depends on the cooperation between signaling pathways during neuronal development. Our work links TrkB and Cdk5 signaling, which are both governed by HuD-mediated post-transcriptional mechanisms. Whether and how HuD may play a role to functionally coordinate the prominent roles of Cdk5 and BDNF/TrkB signaling during neuronal development remains to be investigated. Furthermore, HuD-mediated coordination between Cdk5 and TrkB signaling in mature neurons, during synaptic stimulation when HuD is dramatically up-regulated (138), is an interesting possibility to be examined by future work. In particular, although we examined the molecular mechanisms governing Cdk5 activator expression and BDNF long mRNA stabilization in resting neurons, whether and how regulation of HuD affects BDNF and/or Cdk5 activator expression under synaptic stimulation remains unknown. Moreover, whether HuD-mediated mRNA stabilization occurs in conjunction with dendritic trafficking of the long BDNF 3'UTR isoform and/or whether HuD-mediated regulation of Cdk5 activator mRNAs involves transport to distal processes is an unexplored possibility. In fact, although p35 and p39 proteins are detected at mature neuronal synapses (234), whether Cdk5 activator mRNAs are transported and locally translated is not known. Discovering whether Cdk5 activators are subject to local activity-dependent

translation, potentially through HuD-dependent regulation, would be an important question to address in order to understand synaptic Cdk5 signaling and how the HuD and Cdk5 pathways may converge to control plasticity, learning and memory, and neuronal excitation.

Recent studies have shown that HuD protein can be both phosphorylated and methylated and that such post-translational modifications can affect HuD-mRNA target interactions during neuronal differentiation (292). In fact, phosphorylation of HuD by Protein Kinase C (PKC) is required for local dendritic synthesis of reporters containing the BDNF long 3'UTR specific sequence (292). Future studies will be necessary to determine whether post-translational regulation of HuD affects regulation of p35, p39, or BDNF *in vivo* and what the functional consequences of such regulation might be.

5.4 What are the implications of neuronal HuD function for human health and disease?

Dysregulation of HuD, Cdk5, and BDNF/TrkB have all been linked to neurological diseases including Parkinson's disease, stroke, neurodegeneration, schizophrenia, and epilepsy (59, 159, 163, 241, 293, 294). However, whether there is coordination between these pathways to progress the etiology of human brain disorders is not known. A better understanding of whether and how HuD-mediated regulation of Cdk5 and BDNF are coupled may provide unique insights into the treatment of neuronal dysfunction that involves either factor. We provide the basis here by uncovering molecular mechanisms for HuD regulation of Cdk5 activator and BDNF expression in the hippocampus. However future work will be required to understand the molecular

intricacies of HuD-mediated regulation of neuronal function through BDNF and Cdk5, which may ultimately help to develop therapeutic strategies against neurological disease.

HuD mechanisms which differentially regulate Cdk5 activators might provide the means for therapeutic intervention in the future. Cdk5 activity is dysregulated in several neurological disorders (159, 220, 223). However this can occur in the form of either abnormally elevated or decreased Cdk5 activity. Thus, different disorders may involve discrete aspects of Cdk5 signaling, perhaps specifically mediated by either p35 or p39. In conjunction with our work describing differential mechanisms regulating Cdk5 activator expression, if distinct roles of p35 and p39 are uncovered to direct neuronal function and Cdk5 pathology in brain disease, the development of compounds targeting selective abundance of Cdk5 activators may be ideal therapeutic candidates for distinct cases of hyper- and hypo- Cdk5 function.

Alzheimer's disease (AD) was one of the first neurological disorders thought to involve Cdk5 dysfunction, as Cdk5 phosphorylates tau and hyper-phosphorylation of tau protein is observed in AD patients (222, 295). Several molecular mechanisms may contribute to the abnormal elevation of Cdk5 activity observed in models of AD. Both HuD and miR-101a expression decrease with age in normal neurons (271). However, while HuD levels are elevated in the cortex of patients with AD (156, 157), miR-101a is downregulated (111). Because HuD stabilizes p35 mRNA and miR-101a suppresses p35 protein production, the abnormal levels of these two trans-acting factors uncouples the miR-HuD checkpoint controlling p35 expression and is potentially a "double hit" contributing to over-production of p35. In cortical neurons, the presence of amyloid beta induces elevated calpain-mediated cleavage of p35 (173, 213) into the more stable

fragment, p25, which mediates aberrant Cdk5 activation. Coupled to increased p35 cleavage, dysregulation of trans-acting factors governing p35 expression is a conceivable contributor to pathological Cdk5 activation. In conjunction, miR-101a and HuD are both regulators of amyloid precursor protein (APP), the precursor to the cleavage fragment which accumulates in insoluble Aβ plaques (110, 156). Similar to p35, miRNA 101a suppresses APP production and HuD has been shown to bind and stabilize APP mRNA. Thus, dysregulation of HuD-mediated regulation of different mRNAs may converge as a contributor to neurodegeneration.

Epilepsy is another prevalent human disorder that involves dysregulated Cdk5 and BDNF (227, 294). Developing strategies to treat the underlying causes of epilepsy has proved challenging due to the multifaceted underlying etiologies. The mossy fiber pathway in the hippocampus forms a critical circuitry that governs excitability and activates physiological and pathological plasticity during neuronal excitation and the development of epilepsy (252, 264, 268, 269, 296). In this dissertation, we show that p39-dependent Cdk5 activation, mediated by HuD, contributes to the axonal extension of hippocampal mossy fiber neurons from DGCs. Moreover, HuD is responsible for the elevated levels of BDNF in mossy fiber terminals. Considering the well-documented role of BDNF in neurite outgrowth, HuD likely governs mossy fiber extension through coordination of both BDNF and Cdk5. Mossy fiber sprouting, or the abnormal over-extension of mossy fibers, is a hallmark characteristic observed in human and animal models of epilepsy (264, 268, 269, 297). HuD is dramatically upregulated in response to seizure in hippocampal DGCs and the inhibition of CDK5 is reported to counteract excitotoxic damages induced by kainic-acid stimulated seizure (163). Thus, excitation

stimulated expression of HuD and HuD-mediated regulation of Cdk5 and/or BDNF may govern mossy fiber sprouting and hippocampal neural-circuitry changes that underlie pathological effects of epilepsy. A mouse model in which inducible loss of HuD in hippocampal neurons can be achieved would be an ideal tool for understanding how stimulated expression of HuD may govern circuitry changes in seizure and neuronal activation paradigms. Which activator mediates the pathological effect of Cdk5 during seizure is unknown. During physiological stimulation it is likely that Cdk5 activators balance one another. However, because p35 knockout mice suffer from spontaneous seizures (175) and p39 knockout mice display an ameliorated seizure response, it is conceivable that p39 mediates the pathologic effect of CDK5 in epilepsy. Whether and how the balance of Cdk5 activators is disrupted to contribute to pathological synaptic and circuitry defects during seizure or in the development of epilepsy is an interesting question for future studies.

5.5 Concluding Remarks

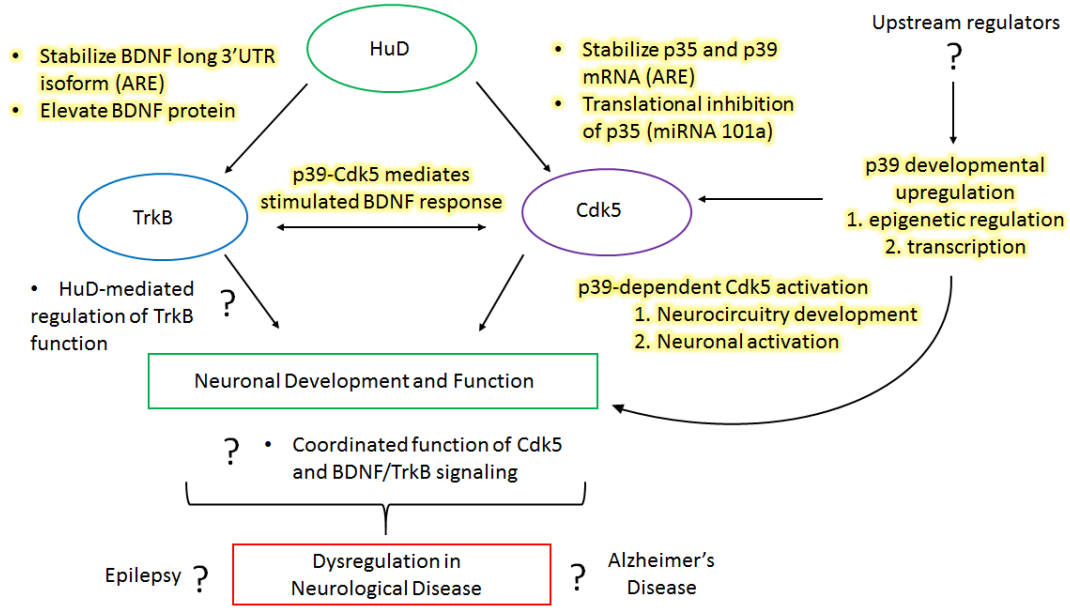
In neurons, post-transcriptional mechanisms offer the advantage of an additional regulatory layer governing the complex temporal and spatial distribution of the cellular proteome, without relying on transcriptional regulons governed by chromatin organization and pre-mRNA processing. The findings in this dissertation provide a basis for a better understanding of how factors governing post-transcriptional regulation, represented by HuD, can regulate normal neuronal neural-circuitry establishment through synergistic and coordinated regulation of multiple functional pathways. Here, we demonstrate HuD-mediated control of both the Cdk5 and the BDNF/TrkB pathways. Future studies building on this foundation will be necessary to determine how HuD

coordinates simultaneous Cdk5 and BDNF function in complex neuronal paradigms such as during learning and memory, aging, pathological stimulation, or neurodegeneration. Such future work may provide key insight for strategies to target dysfunction in the neurological disorders which involve HuD, Cdk5, and/or BDNF.

Figure 5-1: Current model and unanswered questions regarding HuD-dependent regulation of BDNF and Cdk5.

Here we show our current model of BDNF and Cdk5 activator regulation under control of HuD. Findings uncovered in this dissertation are shaded in yellow, with yet to be uncovered links and unanswered questions indicated by a question mark.

Figure 5-1


HuD
• Stabilize BDNF long 3'UTR isoform (ARE)
• Elevate BDNF protein
• Stabilize p35 and p39 mRNA (ARE)
• Translational inhibition of p35 (miRNA 101a)
Upstream regulators
?
TrkB
p39-Cdk5 mediates stimulated BDNF response
Cdk5
p39 developmental upregulation
1. epigenetic regulation
2. transcription
• HuD-mediated regulation of TrkB function
?
p39-dependent Cdk5 activation
1. Neurocircuitry development
2. Neuronal activation
Neuronal Development and Function
? • Coordinated function of Cdk5 and BDNF/TrkB signaling
Epilepsy ?
Dysregulation in Neurological Disease
? Alzheimer's Disease

Chapter 6

Materials and Methods

6.1 Materials and Methods: Chapter 2

Cell culture, plasmids and transfection:

The ARE at the distal end of BDNF long 3'UTR was removed by PCR amplification of the pcLuc-BDNFlongUTR plasmid (33) with primers of ccgctcgagTGGATTTATGTTGTATAGATTAT (forward) and gctctagaACATGGTGAATAATATCTTTACC (reverse), and subcloned between the XhoI/XabI sites to replace the full length BDNF 3'UTR in pcLuc-BDNF longUTR (pcLuc-BDNF longUTRΔARE). Luciferase reporter constructs were co-transfected with myc-HuD (298) or pcDNA into the immortalized mouse brain neuron cell line CAD using Lipofectamine 2000 (Invitrogen). The pRL-TK renilla luciferase construct was also co-transfected. Reporter expression was quantified using the Dual-Luciferase assay (Promega).

UV-crosslinking-immunoprecipitation (CLIP) and RT-PCR:

UV-crosslinking was performed as previously described (299) with modifications. Briefly, cells were exposed to UV light in a Stratalinker (Stratagene, 400 mJ). Cells were then lysed in ice-cold buffer containing 25 mM Tris, 150 mM NaCl, 0.5% Triton X-100, and protease inhibitors. The postnuclear supernatant was precleared with IgG-conjugated protein A-Sepharose beads in the presence of 0.001% SDS followed by immunoprecipitation using anti-c-myc antibody (1:500, SantaCruz) conjugated to protein A beads as described in our previous study (300). The immunoprecipitated complexes were proteinase K-treated before RNA extraction (301). RT-PCR was performed with Primer A: ATCAGGCAAGGATATGGGCTCACT (forward) and TCCAGATCCACAACCTTCGCTTCA (reverse); Primer B:

TGGCCTAACAGTGTTTGCAG (forward) and GGATTTGAGTGTGGTTCTCC (reverse); and Primer C: CAGTGGCTGGCTCTCTTACC (forward) and GGCCACAGACATTTACTTACAGTTT (reverse).

Actinomycin D treatment and reporter mRNA decay in transfectected cells:

The BDNF-L-3'UTR plasmid was transfected into CAD cells with either the myc-HuD construct or the parental vector control. 24 hours post-transfection, cells were treated with actinomycin D at the concentration of 8μg/mL and harvested at 4, 8, and 12 hours. qRT-PCR was performed using DNase-treated total RNA isolated from each time-point using luciferase primers. Percentage of remaining reporter mRNA at each time point was calculated by normalizing the qRT-PCR reading to that of time zero and plotted against time.

In vitro mRNA binding and decay:

A 164 bp fragment containing the BDNF ARE in the long 3'UTR (nt 2640-2746) was PCR-amplified and cloned into the XbaI/XhoI sites of pBSKII (Invitrogen). Sequencing results confirmed 100% identity as compared to published sequence. Radiolabeled BDNF-ARE RNA was prepared by *in vitro* transcription using ^{32}P-UTP as described (122). RNA electrophoretic mobility shift assay [REMSA, (302)] used 100,000 cpm of ^{32}P-UTP labeled BDNF-ARE RNA, increasing amounts of purified GST or GST-HuD (303), and 0.25 mg/ml yeast tRNA and 0.25 mg/ml of BSA to minimize non-specific interactions. Specific competition was carried out with a 10-fold molar excess of cold BDNF-ARE RNA. *In vitro* mRNA decay reactions were performed using ~200 fmol (100,000 cpm) of capped and polyadenylated radiolabeled BDNF-ARE RNA and 20

µg S100 protein from HuD-KO mice. Reactions were supplemented with either 50 ng of GST-HuD or GST and the half-life of the mRNA was calculated as described (130).

Treatment of Primary cultures of embryonic cortical and hippocampal neurons:

Animal treatment was in compliance to NIH regulations under the approval of IACUC by the Emory University and University of New Mexico. Neuronal cell cultures were prepared from E17 C57BL/6 mice (304), and were grown for 24 hours before infection with HSV-HuD or control HSV-lacZ as described (136). After 72 h, total RNA was isolated and BDNF long 3'UTR mRNA and pan BDNF mRNA were quantified by RT-qPCR using GAPDH mRNA as an internal reference.

For shRNA-mediated HuD knockdown, 100,000 E17 hippocampal neurons were grown for 4 days on poly-L lysine coated coverslips and then transfected with either pRetro-shHuD plasmid (305) and pEGFP (Clontech) or control pRetro vector and pEGFP using Lipofectamine 2000 (Life technologies). Following 48 h, cells were fixed in 4% PFA and prepared for FISH as described below. To knockdown HuD in CAD cells, 200 npmol of siHuD that harbors identical sequence to the targeting sequence in shHuD, or a negative control siRNA (Invitrogen), was transfected into CAD cells. Total RNA was collected 48 hrs after transfection, followed by qRT-PCR to quantify HuD and BDNF long mRNA respectively. GAPDH mRNA was used as an internal reference for normalization.

In situ hybridization and immunofluorescence:

Fluorescent in situ hybridization (FISH) was performed using a digoxigenin-labeled antisense oligonucleotide complementary to nucleotides 2508-2556 in the BDNF long 3' UTR (5' GGGTGTATACAATAACTTTTATCTGCAAACACGTTAGG-

CCATATTAC) as described (132). For FISH of brain slices, duplicate adjacent cryostat brain sections from HuD-Tg mice and non-transgenic wild type littermates (WT) were analyzed in parallel and images acquired using the same exposure (typically 50 ms with a 40X objective)using an Olympus BX-60 microscope with a DP71 CCD-digital camera (Olympus America). A similar protocol was used for FISH of hippocampal neurons in culture with the following modifications to detect both the signal from transfected GFP and L-BDNF mRNAs. After the hybridization with dig-labeled probes, cells were incubated with sheep anti-dig antibodies (Roche) and mouse anti-GFP antibodies (Millipore) followed by Alexa 488-conjugated anti-mouse secondary antibodies (Life Technologies) and Cy3 TSA reagent (Perkin-Elmer). Images were acquired in a Zeiss LSM 510 confocal microscope using a 63x objective and 0.8 μm optical slices.

To assess BDNF protein levels in the hippocampus, brain slices derived from HuD-Tg mice and WT littermates were subjected to immunofluorescent staining followed by image acquisition as described previously (33). Fluorescent signals within the mossy fiber tracts were quantified using ImageJ (NIH) and the density of IF was calculated by normalization of the IF signal to the area of measurement.

6.2 Materials and Methods: Chapter 3

Animal use and dissection of the hippocampus:

All animals used were treated in accordance with the regulations of the National Institute of Health and under approval of the Emory University Institutional Animal Care and Use Committee (IACUC). Mice were anaesthetized with isoflurane, subjected to cervical dislocation and hippocampi was dissected from mice at the indicated ages. Dissection of the dentate gyrus (DG) was performed as described in (57).

UV-crosslinking-immunoprecipitation (CLIP) and RT-PCR:

UV-crosslinking was performed as previously described (299) with modifications. Briefly, tissue was minced in 1XPBS and exposed to UV light in a Stratalinker (Stratagene, 400 mJ). Tissue was then homogenized in ice-cold lysis buffer containing 5mM Tris, .32M Sucrose, 1% Triton X-100, and protease inhibitors. The postnuclear supernatant was precleared with IgG-conjugated protein A-Sepharose beads in the presence of 0.002% SDS followed by immunoprecipitation using anti-c-myc antibody (1:500, SantaCruz, SC-789) conjugated to protein A beads as described in our previous study (300). Immunoprecipitated complexes were washed in lysis buffer, spun down, and treated with 4mg/mL proteinase K-treated before RNA extraction (301).

RNA isolation and quantitative RT-PCR (qRT-PCR):

Total RNA was extracted with Trizol (Invitrogen) and reverse transcribed using random primers (Promega) and the Quantitect Reverse Transcription Kit with DNAse treatment (Qiagen). qPCR was performed using Quanta SYBR Green FastMix for iQ kit (20117, Quanta) in a iQ5 Multicolor Real-time PCR detection System (Biorad). Relative quantification of each mRNA in neurons was determined based on the standard curve generated using corresponding primers and all relative concentrations were normalized to

the expression of GAPDH as an internal control. The primers used for amplification are as follows:

Name	**Forward 5'-3'**	**Reverse 5'-3'**
p39	AACCTGGTGTTCGTGTACCTGCT	AGATCTCGTTGCCCATGTAGGAGT
p35	AACAGCAAGAACGCCAAGGACAAG	ATGTTGCTCTGGTAGCTGCTGTTG
Cdk5	AACAGCAAGAACGCCAAGGACAAG	ATGTTGCTCTGGTAGCTGCTGTTG
HuD	GCAGAGAAAGCCATCAACACTTTA	GCTTCTTCTGCCTCAATCCTCT

Immunoblot analysis and antibodies used:

Whole cell lysates from cultured neurons, cultured cells, or hippocampal tissue were prepared by sonication as previously described (233). Proteins were separated by SDS-PAGE, and transferred to a PVDF membrane (GE Healthcare). All membranes probed with phospho-specific antibody detection were blocked for 1 hour in TBST with 5% BSA and 5mM sodium orthovanadate. All other membranes were blocked in PBST with 10% milk for 1 hour. Membranes were incubated with primary antibodies diluted to the concentration indicated below. For phospho-antibody detection, antibodies were diluted in TBST with 3% BSA and 5mM sodium orthovanadate. For total protein, primary antibodies were incubated in PBST with 2% milk. After incubation with HRP-conjugated secondary antibodies, membranes were washed and subjected to chemiluminescence detection.

Antibodies used are indicated below:

Name	**Company**	**Cat**	**Dilution**	**Method**
p35	Cell Signaling Tech	2680	1:1,000	IB
P39	Santa Cruz	sc-365781	1:1,000	IB
CDK5	Cell Signaling Tech	2506	1:1,000	IB

SMI-31 (p-MAP1B)	Calbiochem	NE1022	1:1,000	IB
Total MAP1b	Provided by Dr. I Fischer		1:10,000	IB
pS211-GR	Cell Signaling Tech	4161	1:1,000	IB
GR	Cell Signaling Tech	3660	1:1,000	IB
c-myc	Santa Cruz	Sc-40	1:500	IB

Primary Cell culture and transfection:

Primary rat cortical and hippocampal neurons were prepared from embryonic day 18 (E18), or from postnatal day 0 (P0) wild-type (Wt) or p39-/- mouse pups using well-established protocols (306). Cells were treated with sequential transfection using 200 nmol siHuD and Lipofectamine 2000 on DIV 3 and DIV4 and cell lysate was collected 24 hours later, at DIV 5.

Luciferase Assays and reporter constructs:

Various segments of the p35 or p39 3'UTR were inserted into the xho1 and xba1 cloning sites downstream of the luciferase coding region of the pcLuci vector (259). For the p39 ΔARE luciferase construct, deletion of the Class II ARE core "AUUUA" pentamer was performed using the Quickchange Lightening Site-directed Mutagensis Kit (Agilent) and confirmed through sequencing.

Primers used for cloning are as follows:

Name	**Forward 5'-3'**	**Reverse 5'-3'**	**Fragment size (bp)**
p39 3'UTR	CTCGAGagcactggactatgaacttgga	TCTAGAgcgcttccagcgctttatagtt	1248
p35 terminal 3'UTR	CTCGAGggcaactgaggaaagagc	TCTAGAgtgacggatggtcagtgc	832
p35 Δ Class II ARE	CTCGAGggcaactgaggaaagagc	TCTAGAcagtatccacagtttgg	649
p35 Δ Class II Class III ARE	CTCGAGggcaactgaggaaagagc	TCTAGAgcagtatcggatgtacagc	520

Luciferase reporter constructs were co-transfected with either myc-HuD (259) or the pcDNA vector control into the immortalized mouse brain neuron cell line CAD using Lipofectamine 2000 (Invitrogen) as indicated in the corresponding figure legends. The pRL-TK renilla luciferase construct was co-transfected for assessing transfection efficiency in luciferase assay. Reporter expression was quantified using the Dual-Luciferase assay (Promega).

Actinomycin D treatment:

DIV 2 or DIV 6 primary cortical neurons were treated with Actinomycin D at the concentration of 8 µg/mL and harvested at 0, 4, or 12 hours post-treatment. qRT-PCR was performed using DNase-treated total RNA isolated from each time-point using luciferase primers. Percentage of remaining mRNA at each time point was calculated by normalizing the qRT-PCR reading to that of 18s rRNA and plotted as % remaining from time (259).

Hippocampal slice preparation and immunohistochemistry:

Adjacent cryostat brain sections from p39-/- and Wt mice at 1 month of age were subjected to immunofluorescent staining using a calbindin antibody (Millipore, 1:50) and a FITC-conjugated secondary antibody. Parallel images were acquired for p39-/- and Wt control slides under the same exposure conditions using an Olympus IX-51 microscope equipped with a Retica digital camera. Quantitative analysis of the highlighted mossy fiber tracts was performed as previously described (275).

Statistical Analysis:

All error bars indicate standard error of the mean. In instances of two-sample comparison, the Student's t-test was performed. For multiple comparisons, one-way

ANOVA was performed followed by Tukey's post-hoc test or Two-way ANOVA was performed followed by Bonferrroni's post-hoc test, as indicated in each figure legend. For all experiments that are quantitatively represented, at least two multiple independent experiments were performed and the sample size for quantitation is noted. In each panel: * denotes a p-value of <0.05, ** denotes a p-value of <0.01, and *** denotes a p-value of <0.001.

6.3 Materials and Methods: Chapter 4

Animal treatment and behavior analysis:

The p39-/- mouse colony was previously described (176). Animal treatment was performed in accordance with NIH regulations under approval of the Emory University IACUC.

Pharmacological seizure induction and behavioral analysis:

6-8 week old male Wt (C57Bl/6) and p39-/- (Courtesy of Dr. James Bibb) mice were subjected to intraperitoneal injection of kainic acid (25mg/kg) or 1XPBS (mock). Seizure activity was monitored for up to 180 minutes. A modified Racine scale was used as described in (307). Duration of SE (time spent at or above seizure grade 5 between injection and 180 min) and latency to S.E. (time elapsed from injection until grade 5) were measured. The number of mice used for qPCR analysis of each immediate early gene in **Figure 6** is indicated below:

Primer	Mock, Wt	Mock, p39 -/-	80 min, Wt	80 min, p39-/-	180 min, Wt	180 min, p39-/-
c-fos	7	5	7	4	9	8
ARC	7	4	3	4	9	9
BDNF	7	5	3	4	8	10

Primary neuron culture and transfection:

Primary rat cortical and hippocampal neurons were prepared from E18 brains whereas Wt and p39-/- mouse hippocampal neurons were raised using P0 brains (306). Sequential transfections of p39 siRNA (233) or control siRNA were performed on DIV 3 and DIV4 using lipofectamine (Invitrogen), and neurons are harvested at DIV5 for Cdk5 kinase assay as previously described (233). For dendritic spine analysis, rat hippocampal

neurons were transfected with eGFP and p39 siRNA on DIV13 as described (308) and processed at DIV16 for immunostaining and confocal imaging.

Cdk5 Activity Assay:

The Cdk5 kinase assay was performed as previously described (233). Briefly, primary cultured rat cortical neurons transfected with sip39 or sictrl (Invitrogen) were lysed in a buffer containing 0.1% NP-40, 50 mM Tris-HCl (pH 7.5), 25mM NaCl, 1 mM EDTA, 5 mM sodium orthovanadate, 5 mM sodium fluoride, and protease inhibitors. Lysates containing equal amounts of total protein in each sample were incubated with 2.5μg of anti-Cdk5 antibody (Millipore) for immunoprecipitation of Cdk5 complexes. The complexes were washed and suspended in a kinase buffer [20 mM MOPS (pH 7.0), 5 mM $MgCl_2$, 100 μM EDTA, 100 μM EGTA, and protease inhibitors], and incubated with 100 ng/μl histone H1 in the presence of 25 μM cold ATP and 10 μCi γ-^{32}P-ATP at 30°C for 30 minutes. Proteins from each reaction were resolved by SDS-PAGE and transferred to PVDF membranes. Phosphorylation of Histone H1 was detected by autoradiography and immunoprecipitated Cdk5 was detected by immunoblot on the same membrane for quantitative analysis.

Dendritic spine analysis:

Confocal imaging of dendritic spines from eGFP-transfected neurons were performed as previously described (308). Mature and synapse-forming spines were marked by PSD95 and SV2 immunofluorescence, respectively. Spines were counted and the density was manually calculated. Images were captured using a Nikon C1 system and analyzed using ImageJ (NIH). Dendritic spines were defined as described in (309), and secondary or tertiary dendritic segments were chosen to ensure a uniform population.

Spine numbers were counted on the entire dendritic segment and the density per unit of dendritic segment length were manually calculated. For identification of mature and functional spines, neurons were subjected to immunostaining of SV2 and PSD95 as pre-and-post synaptic markers, respectively (310) , followed by staining with a Texas Red-conjugated goat antibody. Experimenters were blind to treatment during acquisition and analysis. All experiments were from a minimum of three independent cultures and at least six transfected neurons per dish.

Axon length and branch measurement in culture:

At DIV4, cultured mouse hippocampal neurons were subjected to dual immunofluorescent staining of tau-1 and MAP2. Confocal imaging was performed and single-axon neurons were selected for quantification. Axons were traced with the "Simple Neurite Tracer" plugin (ImageJ, NIH). Length and branch number of every axon was quantified using the ImageJ software (311).

Analysis of mossy fiber (MF) tracts:

Cryostat brain sections from p39-/- and Wt mice were subjected to parallel immunofluorescent staining for calbindin. Fluorescent signals on the MF tract were captured and quantified as previously described (275).

Quantitative analysis of RNA:

Total RNA was extracted with Trizol (Invitrogen) and reverse transcribed using random primers (Promega) and the Quantitect Reverse Transcription Kit with DNAse treatment (Qiagen). qPCR was performed using Quanta SYBR Green FastMix for iQ kit (20117, Quanta) in a iQ5 Multicolor Real-time PCR detection System (Biorad). Relative quantification of each mRNA was determined based on the standard curve generated

using corresponding primers and all relative concentrations were normalized to the expression of β-actin as an internal control.

Primers used for RT-qPCR analysis

Name	**Forward 5'-3'**	**Reverse 5'-3'**
p39	AACCTGGTGTTCGTGTACCTGCT	AGATCTCGTTGCCCATGTAGGAGT
p35	AACAGCAAGAACGCCAAGGACAAG	ATGTTGCTCTGGTAGCTGCTGTTG
c-fos	AGAAGGGGCAAAGTAGAGCAG	CGCAGACTTCTCATCTTCAAGT
ARC	CCTACAGAGCCAGGAGAATGA	CTTCAGGAGAAGAGAGGATGGT
BDNF	GCCGCAAACATGTCTATGAGGGTT	TTGGCCTTTGGATACCGGGACTTT

Chromatin immunoprecipitation (ChIP):

For ChIP, hippocampi were dissected from Wt and p39-/- mice at the indicated ages, minced and fixed in 3.4% formaldehyde 1XPBS buffer in the presence of protease inhibitors for 10 minutes. For cultured neurons, formaldehyde was added to the media to a concentration of 3.4% and incubated for 10 minutes at 37°C. Each sample was washed with 0.125M Glycine for 5 minutes followed by two 1XPBS washes before being sonicated 4 times in 10 seconds intervals. The genomic DNA fragment size in each sample was examined by ethidium bromide stained agarose gel and total DNA concentration was determined using a spectrometer. For each sample, lysate containing 50□g of DNA was subjected to IP using ChIP Assay Kit (Millipore). 4□g of antibody to H3K9 (Rabbit, Millipore, 07-352) or RNA Polymerase II (Rabbit, Santa Cruz Biotechnology, sc-899) or IgG control were used in the corresponding ChIP.

Primers used for Chromatin IP-qPCR analysis

Name	**Forward 5'-3'**	**Reverse 5'-3'**
p39 promoter	TGCCAAGGAGAAAGGGTGCATTTG	TCCAAGCCGCAGTATAGAAGCCAT
p39 ORF	AACCTGGTGTTCGTGTACCTGCT	AGATCTCGTTGCCCATGTAGGAGT
p39 3' end	ACAGGACTTGGCTTTCAC	GTTGACTGCGCCAAATTC
p35 promoter	GGGCTGAGAACCATCTTGTTT	TGTGGCCTTTCGATAGCTGGG

Protein extracts, immunoblot analysis and antibodies used:

Whole cell lysates from cultured neurons or brain tissues were prepared by sonication as previously described (233). Proteins were separated by SDS-PAGE, and transferred to a PVDF membrane (GE Healthcare). All membranes for phospho-specific antibody detection were blocked for 1 hour in TBST with 5% BSA and 5mM sodium orthovanadate. All other membranes were blocked in PBST with 10% milk for 1 hour. Membranes were incubated with primary antibodies diluted to the concentration indicated below. For phospho-antibody detection, antibodies were diluted in TBST with 3% BSA and 5mM sodium orthovanadate. For total protein, primary antibodies were incubated in PBST with 2% milk. After incubation with HRP-conjugated secondary antibodies, membranes were washed and subjected to chemiluminescence detection.

Synaptoneurosome preparation:

Synaptoneurosome (SNS) preparation was performed essentially as previously described (312)with brief modifications. Hippocampi from 6 week old mice were used. Hippocampi from each mouse were homogenized in HEPEs buffer without sucrose and EGTA. Samples were passed first through two 100 µm nylon mesh filters, followed by one 5 µm SLSV025LS Millipore filter and centrifuged for 15 minutes at 3,500g, 4°C. The pellets containing SNSs were re-suspended in the same buffer described above, lysed and subjected to immunoblot analysis.

Antibodies used for immunoblot and immunofluorescence:

Name	**Company**	**Cat**	**Dilution**	**Method**
p35	Cell Signaling Tech	2680	1:1,000	IB
P39	Santa Cruz	sc-365781	1:1,000	IB
CDK5	Cell Signaling Tech	2506	1:1,000	IB
b-actin	Sigma-Aldrich	A5441	1:5000	IB
WAVE1	Millipore	MABN503	1:1,000	IB
SMI-31 (p-MAP1B)	Calbiochem	NE1022	1:1,000	IB
1E11 (p-MAP1B)	ENZO	ALX-804-168-c100	1:1,000	IB
Total MAP1B	Provided by Dr. I Fischer		1:10,000	IB
pS211-GR	Cell Signaling Tech	4161	1:1,000	IB
GR	Cell Signaling Tech	3660	1:1,000	IB
Synapsin Ia/b	Santa Cruz	sc-8295	1:1,000	IB
p-Synapsin Ia/b	Santa Cruz	sc-12913r	1:1,000	IB
SV2	DSHB	AB_2315387	1:2,000	IF
Tau1	Millipore	MAB3420	1:200	IF
MAP2	Millipore	AB5622	1:2,000	IF
Calbindin	Millipore	AB1778	1:50	IF
PSD95	Thermo	MA1-045	1:200	IF

Statistical Analysis:

Error bars indicate S.E.M. Student's t-test was used for two-sample comparisons. For multiple sample comparisons, one-way ANOVA and Tukey post-hoc test or two-way ANOVA analysis and Bonferroni's post-test were used. In each panel: * denotes a p-value of <0.05, ** denotes a p-value of <0.01, and *** denotes a p-value of <0.001.

Chapter 7

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