THE ROLE OF HUD, A POST-TRANSCRIPTIONAL REGULATOR, IN THE DEVELOPMENT AND FUNCTION OF THE MURINE NEOCORTEX

By

ERIK MICHAEL DEBOER

A Dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

and

The Graduate School of Biomedical Sciences

University of Medicine and Dentistry of New Jersey

in partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Neuroscience

Written under the direction of

Mladen-Roko Rasin, M.D., PhD

and approved by

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New Brunswick, New Jersey

January 2014

ABSTRACT OF DISSERTATION

THE ROLE OF HUD, A POST-TRANSCRIPTIONAL REGULATOR, IN THE DEVELOPMENT AND FUNCTION OF THE MURINE NEOCORTEX

By ERIK MICHAEL DEBOER

Dissertation Director:
Mladen-Roko Rasin, M.D., PhD

The neocortex is a unique six-layered brain region composed of an array of morphologically and functionally distinct subpopulations of primary projection neurons forming complex circuits across the central nervous system. The developmentally progressive specification, differentiation, and signaling of these distinct subpopulations of neocortical projection neurons is critical to mammalian cognitive and sensorimotor abilities.

Recent research points to mRNA metabolism as a key regulator of this development and maturation process. Hu antigen D (HuD), an RNA binding protein has been implicated in the establishment of neuronal identity and neurite outgrowth *in vitro*. Therefore, we investigated the role of HuD loss of function on neuron specification and dendritogenesis *in vivo* using a mouse model. We found that loss of HuD early in development results in a defective early dendritic overgrowth phase as well as pervasive deficits in neuron specification in the lower neocortical layers, as well as defects in dendritogenesis in the CA3 region of the hippocampus. Subsequent behavioral analysis revealed a deficit in

performance of a hippocampal dependent task: the Morris water maze. Further, *HuD* knockout (KO) mice exhibited lower levels of anxiety than wild type counterparts, and were overall less active. Last, we found that *HuD* KO mice are more susceptible to auditory-induced seizures, often resulting in death.

I have also discovered that HuD itself is heavily regulated at the post-transcriptional level, and is expressed in four transcript variants which encode 4 functionally distinct protein isoforms. Specifically, my data indicate that HuD4 is translated during early neocortical neurogenesis when lower layers are formed, where HuD3 is specifically translated during late neocortical neurogenesis. Further, early HuD3 overexpression drives the production of upper layer neurons, where HuD4 overexpression drives the fate of lower layer neurons. Using a conditional transgenic line as well as in-vitro cell cycle analysis, I also determined that the translational regulation of HuD3 is dependent upon NT-3 arriving from the thalamic afferents to the neocortex. This trophic source appears to only affect those stem cells distal to the ventricle when they are in S-phase.

Acknowledgements

I am tremendously grateful to my thesis advisor, Dr. Mladen-Roko Rasin for his mentorship, hands-on training, guidance and direction throughout my doctoral research. My experience in Roko's lab has bolstered my career passion for neuroscience and allowed me to discover a deep interest in cortex and evolution. I also have great esteem and gratitude for my doctoral committee: Dr. John Pintar, Dr. Renping Zhou and Dr. Li Cai. Their guidance and our scientific discussions together with Roko have been instrumental in shaping my project and broadening my perspective on neuroscience. Additionally, our Neuroscience department is extraordinary, and replete with talented scientists and bright minds who I also must thank. Our department is also chaired by a progressive and visionary scientist, Dr. Cheryl Dreyfus, who I admire very much and who ultimately played a very important role in making my project possible.

I must also acknowledge my friends, lab mates and colleagues who provided scientific input and collaborations that have added value and depth to my experience and a memorable experience in graduate school. In particular, Dr. Althea Stillman for many hours of training, collaboration and scientific discussion. Mathew Kraushar, Aditi Dubey as scientific collaborators, friends and general support. I have also benefited from wonderful, motivated and talented undergraduate students. Notably, Ricardo Azevedo for his countless hours reconstructing Golgi stains in the HuD constitutive knockouts. Also Taylor Vega, Miguel Paredes for their work on HuD in specification and behavior. In addition,

the Rutgers joint program in neuroscience including Joan Mordes, Betty Wheeler and Perry Dominguez.

Last, and certainly not least I thank my family. My wife Maru, for her support and faith in my direction, even through the late nights and the trips to visit family without me. My son James and my daughter Clara who patiently waited for me to come home and always greeted me with a smile, a hug and a kiss. Also my brother Nathan, perhaps the finest academic mind I know for his inspiration to approach science in the view of epistemology. My father, Michael and my mother, Debe, who have encouraged me, backed my decisions and thought highly of me perhaps even when it was unwarranted! Thank you all for your support, this accomplishment is also yours.

I must also acknowledge that chapters 1-3 are published as my original work with coauthors (DeBoer et al., 2013). Chapter 4 is a manuscript under review in Journal of Neuroscience, post revision. Chapter 6 is, in part, composed of work I authored as part of a book chapter (DeBoer and Rasin, 2013).

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Chapter 1: General Introduction - Function and Development of the Neocortex

A. Introduction

The adult neocortex is the central circuit of consciousness, complex cognition, language and the coordination of voluntary motor activity in mammals (Weiler et al., 2008, Lui et al., 2011) . Throughout mammalian evolution, the neocortex is the brain region that has exhibited the greatest expansion in mass relative to body weight (i.e., encephalization) (Shultz and Dunbar, 2010). In this way, the neocortex can be thought of as the evolutionary foundation for cognitive advances, including the uniquely human "theory of mind" and language. However, with these advances, human-specific ailments such as schizophrenia, autism spectrum disorders, Parkinson's disease, Alzheimer's disease, and Amyotrophic Lateral Sclerosis have also developed (Garey, 2010, Wegiel et al., 2010, Morgen et al., 2011, Ozdinler et al., 2011, Rapoport and Nelson, 2011, Yang et al., 2011). Therefore, understanding the molecular and cellular mechanisms underlying neocortical formation, maintenance, and dysfunction is critical not only for furthering basic neuroscience knowledge of brain development and architecture but also for better understanding neuropsychiatric disorders. In addition, these efforts may improve current therapeutic approaches to these neocortical ailments.

Neocortical function relies on precise interactions among an array of cell types, which can be broadly divided into epithelial cells, glia and neurons. Neocortical neurons belong to two main classes: interneurons and primary

projection neurons. Interneurons are inhibitory GABAergic cells that have short processes forming local circuits. Interneurons migrate tangentially into the developing neocortex from the lateral, medial and caudal ganglionic eminences and can be delineated from projection neurons based on their morphology and expression of markers such as parvalbumin, somatostatin, vasoactive intestinal peptide, neuropeptide Y and cholecystokinin (Corbin et al., 2001, Tanaka and Nakajima, 2012, DeFelipe et al., 2013, van den Berghe et al., 2013).

By contrast, primary projection neurons are excitatory glutamatergic cells which carry out the mainstay of the signaling in the neocortex and extend processes over long distances. Importantly, of all of the neurons that populate the neocortex, 75-85% are excitatory projection neurons. The earliest systematic investigation of the neocortex by Santiago Ramón y Cajal revealed that these neurons have characteristic morphological features, including a pyramidal-shaped cell body, many basal dendritic processes, a single apical dendrite oriented toward the pial surface of the neocortex that gives rise to a variable number of oblique branches, and a single axon that usually stems from the base of the cell body or proximal parts of the basal dendrites (Ramón y Cajal S., 1988). Later seminal studies demonstrated histological differences in the density and size of neocortical cell bodies, which define what are now recognized as six distinct layers (Caviness, 1975, Ramón y Cajal S., 1988, Brodmann K., 2006, Hevner, 2006). The target of each projection neuron is related to its position within the six neocortical layers (I-VI) (Figure 1-1). Lower-layer (V-VI) neurons mainly project subcortically, with axons often terminating in the thalamus, brain

stem and spinal cord, although numerous collaterals for intermediate targets also exist (Floeter and Jones, 1985, Zhang and Deschenes, 1997). Upper-layer (II-IV) neurons exclusively project intracortically, either within the ipsilateral hemisphere or reaching the contralateral hemisphere via the corpus callosum.

The delineation of the neocortex into six layers arose from neuroanatomical and electrophysiological evidence. More recent work, initially in rodents, has defined subgroups of neurons based on of the expression of transcription factors (TFs) (Molyneaux et al., 2007, Kriegstein and Alvarez-Buylla, 2009). For example, subcortically projecting neurons selectively express Ctip2, Fezf2, and Tle4, whereas intracortically projecting neurons selectively express Cdp/Cux1 and Satb2 (Hevner et al., 2006, Molyneaux et al., 2007). These findings were recently extended to human and non-human primates, with gestational and postnatal investigations showing that the specificity of TF expression in neocortical projection neurons is at least partially conserved across species. As particular TFs correspond to differences in dendritic complexity and axonal projections and, hence, the function of distinct neuronal subpopulations (Kwan et al., 2012b), there are continuing efforts to identify additional markers of neuron subtypes. Moreover, ongoing studies continue to elucidate the molecular and cellular mechanisms underlying TF specification of neocortical neuron subpopulations.

The remaining text of this chapter will be dedicated to reviewing the current understanding of neocortical development with a focus on neural stem

cells, projection neurons, the use of state-of-the-art transcriptome analyses and the emerging field of the role of posttranscriptional processing steps.

B. Neural Stem Cells in the Developing Neocortex

All functionally-distinct subgroups of neocortical projection neurons are generated prenatally through a highly-orchestrated set of developmental processes. Projection neurons emerge from a pool of neural stem cell progenitors called radial glia (RG) that divide at the ventricular zone (VZ) surface (Figure 1-2). Lower-layer, subcortically projecting neurons are born first, followed by upper-layer, intracortically projecting neurons. The laminar organization of newborn cells results in the arrangement of distinct columns of functionally related neurons spanning different layers (Mountcastle et al., 1957, Hubel and Wiesel, 1962). According to the radial unit hypothesis (Rakic, 1988), the cytoarchitecture of these columns is the outcome of neuroblasts migrating along basal RG processes from the VZ of the prenatal neocortex. This hypothesis was later confirmed using retroviral green fluorescent protein (GFP) transfection, allowing the tracking of daughter cells from dividing RG (Kornack and Rakic, 1995). Thus, the organization of the mature neocortex arises prenatally, where prenatal neurogenesis is believed to produce all of the diverse subgroups of neocortical projection neurons (Casanova and Trippe, 2006, Kriegstein and Alvarez-Buylla, 2009, Kwan et al., 2012b). Therefore, the basis for advanced neocortical functions during adulthood is largely determined by the complex spatiotemporal control of changes in gene expression early in life, starting from neural stem cells (NSC).

As recent molecular and cellular work on NSCs has primarily used mice because of their amenability to genetic manipulation, the embryonic time points mentioned here are specific to mouse neocorticogenesis. NSCs share characteristics of other stem cells, such as self renewal and pluripotency, but have diverse progeny ranging from other types of progenitors to neurons, glia and ependymal cells. However, neocortical NSCs are highly polarized cells with basal and apical processes that span the neocortical wall and attach to tissue surfaces. During the prenatal period, neocortical NSC nuclei undergo a unique process called interkinetic nuclear migration (INM), during which nuclei move toward and away from the lateral ventricle along radial processes. During INM, NSC nuclei are exposed to extracellular cues that may be proliferative, neurogenic, or gliagenic (Taverna and Huttner, 2010, Kosodo, 2012). In this view, INM may influence NSC fate and lead to pseudostratification, a characteristic feature of proliferative region of the neocortex (Taverna and Huttner, 2010). Importantly, INM motions correspond with NSC cycle stages; M-phase and cytokinesis occur when the nucleus approaches the apical epithelial surface, and S-phase and DNA synthesis occur when the nucleus moves away from the ventricle.

The functional significance of INM is advancing rapidly. Recent studies show that disruption of this process is associated with aberrations in NSC cycling and that INM may influence the fate of dividing progenitors (Ueno et al., 2006, Taverna and Huttner, 2010, Yang et al., 2012). Furthermore, species-specific differences in NSC cycle length suggest that INM is involved in evolutionary

changes in neocortical neurogenesis (Kornack and Rakic, 1998, Breunig et al., 2011). Finally, abnormalities in INM are implicated in human developmental disorders like lissencephaly (Hatten, 2005). Therefore, regulation of RG cell cycle and INM during neocorticogenesis is important for maintaining the progenitor population and influencing the products of their divisions.

C. Neural Stem Cell Lineages in the Developing Neocortex

The earliest phase of neocorticogenesis begins with the proliferation of NSCs lining the lateral ventricle of the dorsal telencephalon (Figure 1-2). As the origin of these cells is epithelial, the earliest lineage of these progenitors is known as neuroepithelial cells (NECs), which are characterized by the expression of Nestin, Prominin-1 (CD133), and ZO-1 (Committee, 1970, Bystron et al., 2008). NECs maintain contact with both the pial (basal) surface of the developing neocortex and the apical epithelial lining of the lateral ventricle via radial processes that progressively elongate throughout neocorticogenesis. NECs of the dorsal telencephalon proliferate prior to embryonic day 10 (E10) and undergo multiple rounds of symmetric division, producing two daughter cells per cycle that expand the pool of NSCs for later neurogenesis (Kriegstein and Alvarez-Buylla, 2009). This phase is known as the “expansion phase” (Galli et al., 2002, Bishop et al., 2003, Noctor et al., 2004), which later transitions into the neurogenic phase of neocortical development. Importantly, only those NECs occupying the dorsal telencephalon region of the lateral ventricle cavity will give rise to later lineages of NSCs that ultimately produce distinct subpopulations of neocortical projection neurons (Sidman et al., 1959).

At approximately E10.5-12, while Nestin is still expressed but expression of CD133 is declining, neocortical NSCs begin to express markers similar to those of glial cells, such as the glutamate aspartate transporter (GLAST) and brain lipid binding protein (BLBP) (Rakic, 2003, Molyneaux et al., 2007, Kriegstein and Alvarez-Buylla, 2009). Although they maintain a polarized morphology, their nomenclature and the products of their division change; RG progenitors of NSC origin begin to undergo asymmetric divisions, producing one daughter self-renewing RG and one postmitotic neuron. In the earliest stages of neurogenesis, the postmitotic product of these divisions predominantly migrates directly into the cortical plate (CP) through an elegantly defined series of four phases (Noctor et al., 2004). Postmitotic neuroblasts derived from RG initially migrate rapidly along radial processes basally into the subventricular zone (SVZ) adjacent to the VZ, where they pause for approximately 24 hours. Progeny then undergo retrograde migration apically toward the VZ and finally turn back toward the CP. As these progeny remain in the CP without further division, this phase of neocorticogenesis is called "direct neurogenesis." The earliest born neurons project subcortically and occupy the deepest neocortical layer (VI), whereas subsequently born neurons migrate past the deepest layer and into the more superficial layer (Vb). Interestingly, postmigratory CP neurons are positioned in a way that they possibly split a pre-existing matrix structure called the preplate into the basal marginal zone, containing the most specialized layer of Cajal Retzius cells, and the subplate, a monolayer of cells below the CP (Meyer, 2010, Nichols

and Olson, 2010) for review, see (Kostovic I, 1990, Allendoerfer and Shatz, 1994).

After the formation of the deep neocortical layers predominantly through direct neurogenesis, a new phase of “indirect neurogenesis” begins to take place (Figure 1-2). This occurs in mice around E14.5, when asymmetric divisions of RG at the ventricular surface begin to predominantly produce a specialized cell subtype known as an intermediate progenitor cell (IPC) or basal progenitor cell (Noctor et al., 2004). Some controversy about the contribution of IPCs to neocorticogenesis exists, however, as there is evidence that IPC progeny may contribute to all neocortical layers (Pontious et al., 2008, Kowalczyk et al., 2009) and not just superficial layers. Nevertheless, IPCs migrate away from the proliferative VZ and populate the adjacent SVZ. There, they undergo symmetric divisions giving rise to at least two postmitotic neuroblasts that will become part of the superficial neocortical layers (II-IV) and project intracortically (Kriegstein and Alvarez-Buylla, 2009). In this manner, IPCs serve to amplify the output of a single RG.

IPCs can be distinguished from RG not only by their position and division type and final product but also by their morphology and molecular identity (Pontious et al., 2008). IPCs have a multipolar morphology and maintain no connection with either the pial or epithelial surface. Furthermore, transcriptional programming of the two stem cell populations is mutually exclusive; RG express Sox2 and Pax6, whereas IPCs express Tbr2, which is essential for IPC formation

and maintenance (Englund et al., 2005, Pontious et al., 2008, Sessa et al., 2008).

After the formation of the most superficial neocortical layers (II/III), neurogenesis ceases and the SVZ becomes less populated, reducing in size around E18 (Knoblich, 2008, Kriegstein and Alvarez-Buylla, 2009). Final terminal neurogenic divisions take place at the VZ surface, where RG divide symmetrically and produce postmitotic neurons, thereby reducing the available pool of progenitors.

In addition to neurons, other neocortical cell types include microglia, astrocytes, oligodendrocytes, and endothelial cells. Many neocortical astrocytes, oligodendrocytes and endothelial cells arise from the lineage of precursors aligning VZ as primary neurons, although at later stages of neocorticogenesis (Mission JP, 1991, Sun et al., 2005, Li et al., 2012). Thus, when neurogenesis ceases, the remaining RG give rise to astrocytes and oligodendrocyte precursors, and ependymal cells, which is beyond the scope of the current review.

D. Are RG in the Developing Neocortex Homogenous?

Historically, RG have been thought to be relatively homogenous in nature and to respond to temporal cues in the generation of distinct subpopulations of projection neurons (Molyneaux et al., 2007). However, recent evidence suggests that the situation is more complex. A morphologically similar class of NSCs in the VZ, short neural precursors (SNPs), has been found to contribute to

neocorticogenesis in a similar manner as RG (Gal et al., 2006). These cells have a short basal process of variable length that further shortens during mitotic division. Numbers of SNPs and RG are equivalent except at E14.5 during the "direct" to "indirect" neurogenesis transitioning time point, when SNPs outnumber RG. Although SNPs are morphologically distinct from RG, they appear to follow similar patterns of cell cycling and generation of postmitotic progeny.

More recent correlative evidence points toward a subpopulation of RG-like cells sporadically distributed across the VZ that express *Cux2* mRNA (Franco et al., 2012). It is possible that these cells are identical to SNPs; although, subsequent work is required to further unravel the morphological and molecular signatures of different NSC subpopulations. *Cux2*-expressing RG may predetermine their neuronal subtype cell-autonomously during asymmetric division at the ventricular surface as early as E10.5 (Franco et al., 2012). Importantly, *Cux2* is also expressed in the SVZ, where IPCs predominately give rise to upper-layer neurons (Zimmer et al., 2004). Using a transgenic reporter mouse with FLEx (Flip-Exclusion) technology, morphologically similar RG were distinguished by Cux2 expression though dTomato (Cux2-negative) or GFP (Cux2-positive) labeling (Franco et al., 2012). Cux2-positive cells generated upper-layer neurons in both *in vivo* and *in vitro* conditions and were more likely to re-enter the cell cycle, whereas Cux2-negative cells were more likely to terminally divide in symmetric fashion. These findings indicate that cell fate is programmed into the transcriptomes of the neocortical progenitor pool very early in neurogenesis. Future loss- and gain-of-function studies in which Cux2 is

directly manipulated may determine whether this molecule is necessary for narrowing RG fate and further identify possible overlaps and discrepancies between Cux2-positive RG and short NSCs. Thus in the RG populated VZ, there may be either a single type of progenitor that progressively differentiates or there is a co-existence of multiple progenitor subtypes (Franco and Müller, 2013). However, in either case, progenitors must respond promptly to spatiotemporally regulated extracellular cues, as summarized below.

E. Similarities and Differences between Human and Mouse Neocortical NSCs

Although the mouse neocortex does not fully reflect the remarkable complexity of the folded human neocortex (see (www.brainmuseum.org, 2012), the basic molecular mechanisms of neocorticogenesis in mice have been confirmed in humans (Bayatti et al., 2008), including the spatiotemporal specification of the six neocortical layers (Hevner, 2007, Fertuzinhos et al., 2009, Koopmans et al., 2010, Zhu et al., 2010, Saito et al., 2011, Huang et al., 2012, Kwan et al., 2012a). In humans, however, the molecular and cellular processes of brain development are more complex and the proliferative regions are proportionally larger than in mice (Figure 1-2). Generation of the human neocortex takes place over the entire course of gestation, with neurogenic divisions starting around gestational week 9-11 (Rakic P, 1968a, Sidman RL, 1973, Zecevic et al., 2005, Fish et al., 2008, Lui et al., 2011, Malik et al., 2013). During later stages of neurogenesis, the SVZ of humans and non-human

primates is significantly increased in thickness compared with other developing zones/layers and compared with mice (Cheung et al., 2010).

Another proliferative region outside the SVZ was recently discovered in humans (Hansen et al., 2010) and subsequently described in both carnivores and rodents (Fietz et al., 2010, Hansen et al., 2010, Wang et al., 2011a). This region, called the outer subventricular zone (oSVZ), is populated by RG-like neurogenic NSCs called outer radial glia (oRG) (Figure 1-2). oRG are found basally to Tbr2-positive cells in the SVZ, express Pax6 and Sox2, undergo several cell cycles, and maintain a basal process to the pial surface. TFs or other markers specific to oRGs and the specific postmitotic neural progeny that oRG contribute to the developing neocortex remain to be identified. Existing evidence, however, suggests that the oSVZ may be the primary region of proliferative expansion corresponding to the evolutionary advancement of neocortical size and function.

Sophisticated *in vitro* techniques now enable the modeling of remarkable steps of human cortical development in culture. This system uses human induced pluripotent stem cells (hiPSCs) and has been successful in mimicking the progression of neocorticogenesis — from NECs to RG and the subsequent generation of deep- and upper-layer neurons (Mariani et al., 2012, Espuny-Camacho et al., 2013). Initial studies using this technique demonstrate the ability of cultured hiPSCs exposed to distinct extracellular cues to aggregate in a sphere-like structure with a central cavity. RG-like stem cells line the inner opening, whereas postmitotic progeny expressing cortical TFs are found more

superficially. Importantly, the sequential birth of subpopulations of projection neurons is also preserved. Remarkably, these *in vitro* subpopulations of human projection neurons can be transplanted into a mouse neocortical slice culture, where they become electrophysiologically active and integrate into functional circuits. This new finding could begin to bridge a gap between *in vitro* and *in vivo* work and aid the translation of mouse-based research to humans.

F. Molecular and Cellular Mechanisms of Neocortical NSC Differentiation

During CNS development, the formation of the telencephalon, which contains the neocortex, is induced by a dynamic interplay of multiple intrinsic and extrinsic cues (Rallu et al., 2002). The progressive differentiation of neocortical NSCs and the transition from the expansion of a mostly homogenous population of NEC precursors to the specification of RG and ultimately specialized neuronal subpopulations occurs within a narrow time frame (Shen et al., 2006, Okano and Temple, 2009, Seuntjens et al., 2009, Siegenthaler et al., 2009). Therefore, neocortical NSCs appear to have intricate intrinsically programmed molecular systems that dictate differentiation while responding to extrinsic cues (Shen et al., 2006, Okano and Temple, 2009, Seuntjens et al., 2009, Siegenthaler et al., 2009).

G. Intrinsic Mechanisms Regulating Neocortical NSCs

Elegant *in vitro* analyses, first in mice and later in humans, indicated that timed developmental mechanisms are intrinsic to neocortical NSCs (Mariani et al., 2012, Shi et al., 2012, Espuny-Camacho et al., 2013). A seminal study using mouse neocortical NSC lineages showed that the sequential generation of

cultured neurons mimics the *in vivo* temporal order (Shen et al., 2006); as each cortical layer arises, cultured NSCs lose their potency and become restricted in their generation of different neurons. Similarly, cultured human NSCs first generate early neuron subtypes followed by later neuron subtypes (Mariani et al., 2012, Shi et al., 2012, Espuny-Camacho et al., 2013).

At perhaps the deepest intrinsic level, an open chromatin structure influences pluripotency and differentiation of ES cells (Hajkova et al., 2008, Gaspar-Maia et al., 2009). As neocortical neurogenesis progresses, the chromatin structure of DNA in RGs becomes more condensed, with an increase in the High Mobility Group A (HMGA) protein as neurogenic stages progress (Kishi et al., 2012). This protein group is associated with modulating chromatin structure and accessibility to transcription factors through DNA cross-linking (Vogel B, 2011). When MGA proteins were silenced in neural progenitor cells (i.e., NSCs) *in vitro* and *in vivo*, reduced levels of these proteins led to more differentiated states of transfected cells. This conclusion was supported by a greater proportion of cells expressing Beta III tubulin or exhibiting a loss of cell cycling. Over-expression of HMGA proteins produced the opposite outcome, with cells more likely to express proliferative markers or to incorporate the S-phase-labeling thymidine analog EdU. These findings indicate that the intrinsic mechanism of chromatin remodeling, which is clearly at work in other stem cell types, also influences neurogenic phases in NSCs.

Open chromatin states likely increase the accessibility of TFs to genomic regions, where they play a role in determining NSC fate. Further, there is also

evidence of direct TF influence over the chromatin state in development (Magklara et al., 2011, Guo et al., 2012). The neocortex occupies the dorsal part of the telencephalon and is characterized by specific expression of several TFs in NSCs, such as Empty spiracles homologue 2 (Emx2), Paired box 6 (Pax6), and Forkhead box G1 (Foxg1) (Muzio et al., 2002, Hanashima et al., 2004). These TFs prevent the expansion of ventral and medial neurogenic regions of the telencephalon, which correspond to future basal ganglia and hippocampi, respectively.

A subgroup of TFs, Forkhead box (Fox) TFs, which are mainly described as transcriptional repressors, have been studied in the context of neural stem cell maintenance (Rousso et al., 2012). Over-expression of Foxp2 or Foxp4 has redundant effects, with either protein sufficient to promote differentiation. Conversely, Foxp4 knockout (KO) mice show a lower number of differentiated neurons at early developmental stages and a greater proportion of cells positive for Ki67, an indicator of cell cycling. Mechanistically, this study implicates Foxp2/4 in the disruption of adherens junctions, which are critical for the maintenance of stem proliferative fates (Stepniak et al., 2009). Within these junctions, a host of proteins have been found to be responsible for junction maintenance, including N-Cadherins (Kadowaki et al., 2007, Rasin et al., 2007, Bultje et al., 2009, Stepniak et al., 2009). Foxp4 specifically down-regulates N-Cadherin mRNA without affecting other factors in the junctions. These data suggest an intricate interplay of intrinsic molecules in progressive NSC differentiation.

Finally, T-brain gene-2 (TBR2) TF constitutive depletion is lethal. However, conditional forebrain silencing of Tbr2 revealed its function in IPC formation and maintenance (Englund et al., 2005, Sessa et al., 2008). Intriguingly, early Tbr2 depletion resulted in reduced production of all neocortical layers, suggesting that indeed Tbr2 contributes to lower layer, subcortically projecting neurons as well as those of upper layers (Sessa et al., 2008). Strikingly, Tbr2 overexpression resulted in ectopic SVZ regions within the RG niche of the VZ. These findings determined Tbr2 is a key intrinsic molecule for identity and proliferation of IPCs.

Collectively, this brief overview on TF functions in NSCs clearly indicates the significance of complex intrinsic regulation of NSC proliferation and differentiation (for additional reviews see (Hevner, 2007, Molyneaux et al., 2007)). However, intrinsic pathways can be regulated by timed extrinsic cues, as follows.

H. Notch as an Extrinsic Regulator of Neocortical NSCs

Perhaps the best studied example of extrinsic influence over intrinsic gene expression is notch signaling. Notch signaling takes place through a receptor-ligand relationship involving the surface contact of two cells (Artavanis-Tsakonas et al., 1999, Yoon and Gaiano, 2005, Louvi and Artavanis-Tsakonas, 2006, Kopan and Ilagan, 2009, Ables et al., 2011). Binding of ligand components, such as Jagged or Delta proteins, to notch receptors (subtypes notch1-4) causes a γ-secretase cleavage of the intracellular notch receptor domain (ICN). ICN translocates to the nucleus, where it interacts with one of several TFs. The

activated protein displaces a repressor complex varying by cell type, thereby activating transcription (Cau and Blader, 2009, Latasa et al., 2009). The result of this process is, with few exceptions, to promote a stem cell fate and repress differentiation (Gaiano et al., 2000).

Although the main molecular players of the notch signaling system are found in the neocortex, evolution has conserved the pathway while modifying some aspects of its regulation. A recent neocortex-specific example concerns the Numb and NumbL proteins, which polarize RG through maintenance of Cadherin-based adherens junctions at the epithelial surface (Rasin et al., 2007). Through a putative association with Numb/NumbL proteins, mPar3, a conserved protein that is asymmetrically distributed during RG division, serves to enhance notch signaling (Bultje et al., 2009). Furthermore, notch signaling in the proliferative VZ/SVZ is distributed such that tight spatial regulation of RG is required for access to the signal (Del Bene et al., 2008, Sessa et al., 2008, Sessa et al., 2010). In particular, there is an apical enrichment of Notch signaling, which promotes NSC renewal (Buchman and Tsai, 2008, Del Bene et al., 2008). These examples strongly implicate INM in this signaling cascade, with RG progenitors contacting distinct members of notch pathway when they are closest to or either furthest away from the VZ surface.

Also, Tbr2-positive IPCs may express the notch ligand Jagged and thereby influence RG potency. Depletion of Tbr2 not only ablates IPC populations in the SVZ due to Notch interactions between IPCs and RG but also results in an early depletion of RG progenitors due to premature differentiation

(Mizutani et al., 2007). Further investigations of this phenomenon revealed that *mind bomb-1* is expressed in a subset of IPCs and newly born postmitotic neuroblasts, where it promotes endocytosis of ubiquitinated Notch ligands and mediates the fate choices of RG during both symmetric and asymmetric divisions (Koo et al., 2005, Yoon et al., 2008). Interestingly, findings in zebrafish indicate that Par3 selectively distributes mind bomb-1 to the self-renewing cell of an asymmetric pair (Dong et al., 2012). In this manner, neurogenesis is regulated in part by the selective spatial expression of a basally positioned, IPC-specific Notch signal modifier.

Of note, however, some degree of lack of conservation in notch signaling appears to exist among species. In zebrafish, Par3 in the neural tube is asymmetrically localized to the neural-fated progenitor, and loss of Par3 function results in a significant increase in symmetric divisions that generate two progenitors (Alexandre et al., 2010). In mouse neocortical neurogenesis, however, over-expression of mPar3 increases the number of progenitor-producing symmetrical, progenitor producing divisions, where RNA interference more often drove symmetrical neuron generating divisions (Bultje et al., 2009).

I. Other Extrinsic Cues Regulating Neocortical NSCs

Of the many extracellular influences on neocorticogenesis, perhaps one of the most striking recent examples is the role of the trophic factor Fgf10 in the NEC-to-RG transition (Sahara and O'Leary, 2009). In $Fgf10^{-/-}$ mice, a marker of RG (BLBP) was diminished at E11.5, 12.5, and 13.5, indicating a late shift in the transition from the expansion phase of symmetric NSC division to the

appearance of mature neurogenic RG. Subsequent experiments support this finding, showing an increased thickness of the rostral cortex during the postnatal period, whereas caudal regions were unaffected. Together, these results indicate that Fgf10 is a key mediator of regionally selective early NSC differentiation in the developing neocortex.

Extrinsic regulatory cues also can originate from outside the cortex and brain. In one example, loss of the meninges, sheets covering the developing neocortical wall, reduced the production of both neurons and IPCs, indicating less asymmetric divisions (Siegenthaler et al., 2009). Several subsequent elegant approaches revealed that retinoic acid is a powerful extrinsic cue derived from meninges driving proper NSC differentiation from symmetric to asymmetric divisions. For example, *in utero* retinoic acid treatment rescued the effect of depleting meninges on asymmetric divisions. Thus, these findings indicate that meninges can provide extracellular cues for the neocortical NEC to RG transition.

Interestingly, immature neurons may also send extrinsic cues that provide feedback and maintain differentiation of NSCs. For example, conditional deletion of a transcription factor Sip1 (also known as Zfhx1b) in young neurons regulates the production of subcortically projecting deep-layer neurons and intracortically projecting upper-layer neurons in a non-cell-autonomous manner (Seuntjens et al., 2009). Specifically, Sip1 deletion in early born neurons destined to reside in deep layers induced the premature production of upper-layer neurons and even glial precursors. In this way, Sip1 regulates the timing of cell fate switches during neurogenesis and the total number of neocortical projection neurons.

There is also evidence of the extrinsic influence of maternal endocrine signaling on neocorticogenesis. Maternal thyroid hormone (MTH) in pregnant dams induces profound changes in the maintenance and cycling of cortical progenitors and affects cortical thickness in developing rat embryos (Mohan et al., 2012). Pups from MTH-deficient dams had *Pax6*-deficient neocortices at E14, although this early deficit was corrected by E18. However, levels of Tbr2, which are indicative of upper-layer-generating IPCs, progressively declined in pups from hormone-deficient dams. This effect was only partially rescued by exogenous supplementation of MTH, indicating a coordination of other relevant factors. Nonetheless, this striking case confirms the importance of extrinsic factors to neocortical neurogenesis, even when they are generated outside the developing neocortex.

Collectively, these findings provide a platform of multiple converging extracellular factors on the intrinsic fate choices of NSCs in the developing neocortex. However, there is an additional intricate set of steps to ultimately define distinct subpopulations of neocortical projection neurons, as follows.

J. Postmitotic Differentiation and Specification of Subpopulations of Neocortical Projection Neurons

After NSC progeny commit to a postmitotic fate, nascent neuroblasts migrate along the basal radial processes of RG for review, see (Casanova and Trippe, 2006, Rakic P, 2007, Metin et al., 2008, Molnar Z, 2012). The diversity of neocortical projection neurons suggested the importance of accurately timed intrinsic programming in postmitotic differentiation. Indeed, precisely timed

changes in functional gene expression must occur for the progenies of RG division to produce the hundreds of distinct subtypes of neurons and glia that populate the mature neocortex and contribute to its proper function (Molyneaux et al., 2007). Generally, neocortical layer VI will predominantly project to thalamus via corticothalamic axons. Layer Vb will predominantly project to the brain stem and spinal cord via corticobulbar and corticospinal tracts, respectively. Superficial layers will project intracortically and to a smaller extent into superficial parts of the striatum. There is growing evidence of unique regulation at the DNA level by specific neuron subtypes.

A recent meta-analysis, together with a loss-of-function of coup-TF (chicken ovalbumin upstream promoter transcription factor)-interacting protein 2 (Ctip2) mutant, showed that corticospinal motor neurons (CSMNs) and callosal projection neurons (CPNs) express several similar but mutually exclusive factors (Arlotta et al., 2005). Expression patterns vary across neocorticogenesis, with TF expression becoming exclusive to each group of cells as they begin to differentiate. When Ctip2 is silenced, the subcortically projecting subset of layer Vb neurons fail to differentiate, indicating that this factor is necessary for their proper formation. When Ctip2 is repressed by the DNA-binding protein special AT-rich sequence-binding protein 2 (Satb2), which is required for the generation of CPNs, later-born upper-layer neurons differentiate into a separate subset of projection neurons (Alcamo et al., 2008, Britanova et al., 2008).

In addition, several groups simultaneously discovered the role of FEZ family zinc finger 2 (Fezf2) TF in CSMN axonal projections (Chen et al., 2005a,

Chen et al., 2005b, Molyneaux et al., 2005). Fezf2 is both necessary and sufficient for proper formation of subcortical projections. Developmental silencing of *Fezf2* prevented corticospinal tract axons reaching the spinal cord. In contrast, overexpression of *Fezf2* in upper layer neurons results in ectopic subcortical projections (Chen et al., 2005b, Chen et al., 2008). In addition, *Fezf2* can alter upper layer specification into early postnatal life (Rouaux and Arlotta, 2013), and is sufficient to alter the fate of progenitors from the basal telencephalon when overexpressed (Rouaux and Arlotta, 2010). In addition, Fezf2 expression in lower-layer neurons drives the expression of the lower-layer TF Tbr1. Collectively, these findings indicate Fezf2 as potent regulator of deep layer neuron projections and specification.

Just as the axonal projections of CPN and CSMNs are regulated by Satb2, Ctip2 and Fezf2 TFs, Cux1 (CDP) and Cux2 are important for the formation of dendritic trees in these upper-layer neurons (Cubelos et al., 2010). The lack of Cux1 and Cux2 results in fewer dendritic branches, smaller post-synaptic densities, and reduced excitatory post-synaptic currents, which are all indicators of differentiation failure. Similarly, Fezf2 expression in lower-layer neurons is necessary for normal dendritic architecture of layer 5 projection neurons (Chen et al., 2005b)

These findings were recently extended by an elegant study using double-mutant Fezf2, Ctip2, or Satb2 mice (Srinivasan et al., 2012). Using a beta galactosidase (LacZ) labeling system, the McConnell group discovered networking of TFs in mutual repression and derepression that ultimately

determine postmitotic fates of projection neurons in the developing neocortex. Briefly, upper layer neurons with a conditional EMX1 promoter-driven deletion of Satb2 projected ectopically to subcortical structures, but double knockout of Ctip2 and Satb2 lead only to a partial restoration of LacZ-positive intracortical axon projections. In mice with a EMX-Cre driven deletion of Fezf2, subcortical Fezf2-placental alkaline phosphatase (PLAP)-labeled axons were reduced, as expected. However, double knockout of Fezf2 and Satb2 does not restore this loss. Importantly, Ctip2 is downregulated in Fezf2 mutants, but restored in Fezf2/Satb2 double mutants, indicating that Fezf2 represses Satb2 expression, which in turn represses Ctip2 expression.

Corticothalamic projections are lost when Tbr1 expression is developmentally ablated. In Fezf2 mutants, the loss of corticospinal projections is paralleled by an increase in corticothalamic innervation and an expansion of Tbr1 expression (Hevner et al., 2002, McKenna et al., 2011). In addition, Tbr1 represses Fezf2 expression in layer 6 to restrict axons to corticothalamic tract (Han et al., 2011). Beside these roles in the formation of subcortical projections, Tbr1 overexpression was found to rescue intracortical projections in Satb2 mutants. In a conditional EMX1-Cre driven deletion of *Satb2*, Tbr1 overexpression at E15.5 was sufficient to rescue the intracortical callosal projections of transfected neurons. This, taken together with previous findings that $Tbr1^{-/-}$ mice show abnormalities in callosal connectivity, suggests that Tbr1 has an early role in specifying layer VI neurons, but also plays a role in establishing the connectivity of superficial layers (Hevner et al., 2001).

Reports of nascent neurons with bifurcated axons—one putatively bound for an intracortical target and the other bound for a subcortical target (Garcez et al., 2007)—are further evidence of fate repression. These migrating neuroblasts are found in the intermediate zone (IZ) and become CPNs during early neocorticogenesis. A later study found that some neuroblasts migrating through the IZ co-express Ctip2 and Satb2, which is preserved in later postnatal stages (Lickiss et al., 2012). However, although a large population of bifurcated cells was identified through retrograde DiI labeling, these cells were never found to co-express Ctip2 and Satb2. Findings such as these convey a theme in neocortical development — that substantial decisions in postmitotic differentiation occur via both the promotion of specific cell fates and the active inhibition of alternative fates.

In humans, much less has been demonstrated about differentiation and specification of pyramidal neurons. However, subpopulation and layer specific markers identified in mouse neocortex are reproducible in human neocortices. Even though the number of molecular identity markers for human projection neurons is increasing, there is a need for significant work to fill gaps (Hevner, 2007). Substantially more is known on dendritic differentiation in some prefrontal neocortical regions. Initially it was found that during the perinatal period projection neurons of the human prefrontal cortex have a phase of rapid dendritic growth (Mrzljak L, 1992). This was recently extended by findings in newborn to 91 year old specimens. Here, layer 3 neurons showed a biphasic pattern of growth during early postnatal life, with about a year of stagnation in growth. Layer

5 neurons reached stable adult values sooner (Petanjek et al., 2008). These findings, along with the even more complex developmental pace of synaptogenesis in human neocortices (Petanjek et al., 2011), suggest differential molecular mechanisms behind dendritogenesis and perhaps human specific neurological and psychiatric diseases where neocortical circuits are disrupted.

Clearly, unique proteins mediate the differentiation of NSCs and the postmitotic specification of distinct subpopulations of neocortical projection neurons. Although these discoveries have begun to unravel the complexity of transcriptional control in the developing neocortex, the binary nature of this regulation still does not completely explain the subtle differences among neocortical neuronal subtypes. It is unequivocal that these TFs regulate numerous targets and work in concert with many other factors to hone and specify their functional genetic readout. As we will describe further, recent state-of-art global screens have begun to reveal the transcriptomic effects of these developmental regulators.

Figure 1 1

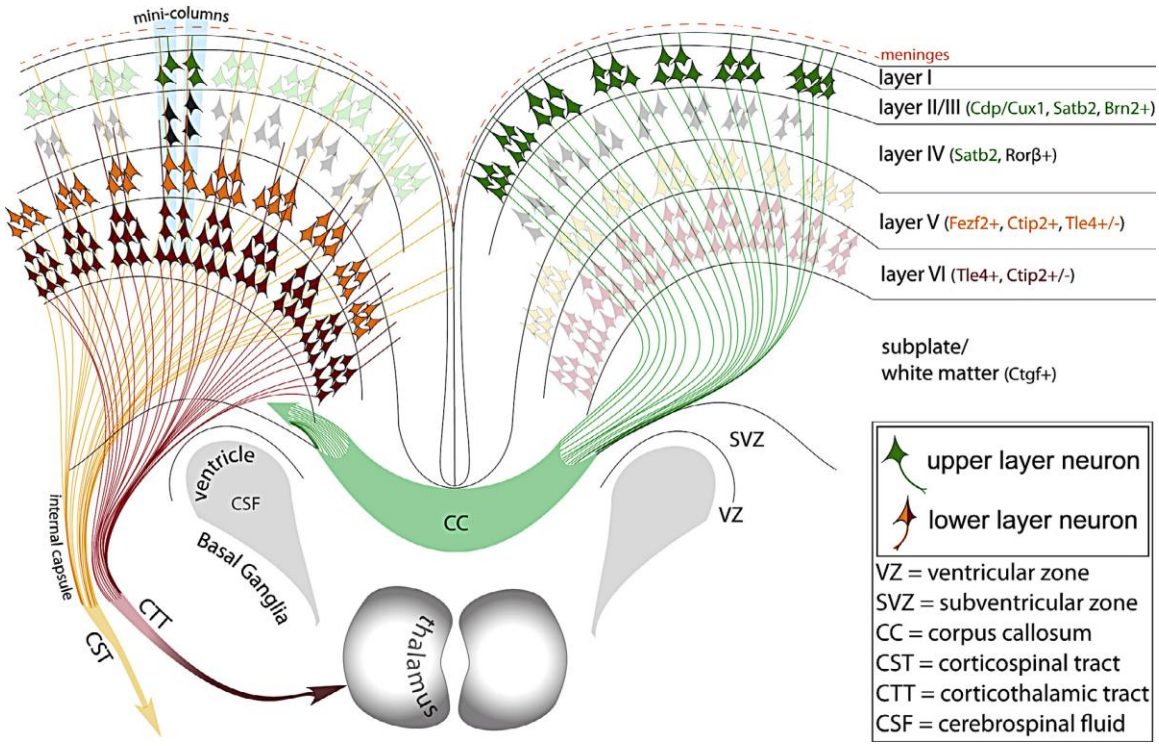

Figure 1-1.: A simplified schematic of the postnatal organization and projections of neocortical projection neurons. The neocortex is highly organized in both horizontal and vertical dimensions. Horizontally, six layers are defined by highly organized subpopulations of glutamatergic projection neurons, which represent approximately 85% of all neocortical neurons. These subpopulations of projection neurons are characterized by specific molecular identities, dendritic morphologies and terminal targets corresponding to each layer. Projection neurons that are born during later stages of prenatal neurogenesis will be predominantly placed in upper layers II–IV (green neurons). These neurons express specific transcription factors like CDP/ Cux1, and project solely intracortically forming the corpus callosum that connects the two hemispheres. However, there is also a smaller portion of intracortically projecting neurons placed in lower layers too (not shown). In contrast, earlier born projection neurons will be placed in lower layers V–VI (orange and red neurons). These subpopulations will express transcription factors like TLE4 and FEZF2, and will project subcortically to form long range tracts across the central nervous system like the corticothalamic tract (CTT) originating mainly from layer 6, and somewhat from layer 5, or corticospinal tract (CST) originating solely from layer 5. Within the subventricular zone (SVZ) of the corticostriatal junction, adult progenitors are found giving rise to olfactory cortex neurons.

Figure 1 2

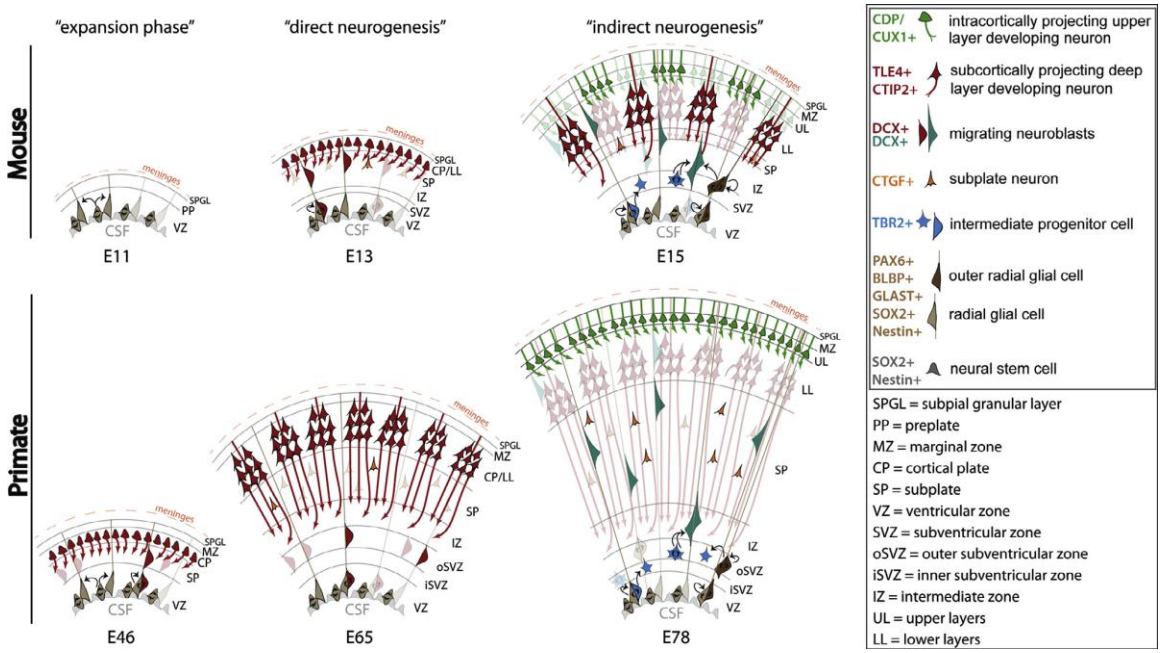

Figure 1-2. Schematic of distinct stages of neocortical neurogenesis in developing mouse and primate neocortices. The first ‘‘phase’’ of neocorticogenesis in mouse and primates is characterized by symmetric divisions of neural stem cells called neuroepithelium cells (NEC), which amplifies the number of neocortical progenitors at the ventricular zone (VZ) surface (left panels). This initial phase is accordingly called the ‘‘expansion phase’’. NECs will then transition into a different lineage of neocortical neural stem cells called radial glia (RG), which divide asymmetrically and first predominantly produce neuronal progeny. This phase was named ‘‘direct neurogenesis’’ (middle panels). As the neurogenic phase progresses, RG continue to undergo a series of asymmetric divisions, but they predominantly produce another progenitor subtype, intermediate progenitor cells (IPCs) and outer radial glia (oRG) (right panels). IPCs and oRG will divide in the subventricular zone (SVZ), which in primates is divided into inner (iSVZ) and outer (oSVZ) portions. Importantly, IPCs terminally divide symmetrically and produce at least two neural progenies. However, oRG will self renew and give rise to neural progenies and IPCs. In this way, both IPCs and oRGs amplify the output of RG, and thus, this later stage of neurogenesis was named ‘‘indirect neurogenesis’’. These progressive changes in differentiation of neocortical neural stem cells define the birth of distinct subpopulations of projection neurons. Deep-layer neurons that will project subcortically into thalamus, brain stem and spinal cord will be born before upper-layer intracortically projecting neurons. (Adapted from Angevine and Sidman, 1961; Rakic, 1974; Smart et al., 2002; Molna´ r et al., 2006).

Chapter 2: Post-Transcriptional Regulation in Neocorticogenesis

A. Neocortical Transcriptomics in Mice

From the scale of whole neocortices to single cells, transcriptome diversity is increasingly being investigated. Transcriptomics of the entire developing neocortex, however, are complicated by the discrete regions and subpopulations of cells contained therein. Therefore, transcriptomics are being used as a tool to investigate subcompartments and even single cell types in the developing and mature neocortex with great specificity, providing a transcriptional "signature" of regions and cellular subtypes. Greater precision of cellular subtype segregation, however, will allow even more specific conclusions to be drawn. For example, RG of the VZ, IPCs of the SVZ, and subpopulations of laminarly-organized neurons are distinct in morphology, TF identity and likely transcriptome, but have been challenging to separate for individual analysis. However, findings from transcriptome screens of the whole neocortex are typically confirmed with quantitative reverse transcription polymerase chain reaction (RT-PCR), *in situ* hybridization analyses and/or immunohistochemistry, which ultimately reveals the cells expressing the gene of interest.

To this end, a recent study sought to investigate laminar-specific transcriptomic signatures of the developing neocortex was performed using laser capture microdissection to isolate discrete neocortical regions from embryonic mice (Ayoub et al., 2011, Fietz et al., 2012). E18-P7 neocortices were microdissected into VZ, SVZ/IZ, and CP; RNA was harvested; and deep RNA sequencing (RNASeq) was performed. The findings confirmed expression of

subregion-specific transcripts and implicated a host of newly-identified and differentially expressed candidates. Interestingly, splicing was found to play a major role in the diversity of subregion transcriptomes, with splicing levels between regions often not agreeing with overall total transcript levels for a given gene. Of genes with two or more splice variants, 15.7% were differentially expressed in the CP, 11.8% in the VZ, and 12.8% in the SVZ. For example, Mfge8 has two variants expressed in the neocortex at E14.5 showing variant 1 selectively enriched in the VZ. These and other findings suggest that post-transcriptional trait-like splicing is a major contributor to neocortical complexity (Black, 2000, Grabowski and Black, 2001, von Holst et al., 2007).

Transcriptomic analysis of distinct subpopulations of projection neurons was recently achieved by elegant retrograde labeling of CSMNs, corticotectal neurons, and CPNs. Labeled neurons were isolated from the neocortex using fluorescent activated cell sorting (FACS) coupled to transcriptomic analysis (Molyneaux et al., 2009). This technique revealed numerous differences in the transcriptomes of distinct subpopulations of projection neurons that were subsequently confirmed by *in situ* hybridization and immunohistochemistry, including Ctip2. Comparisons of transcriptomes of upper versus lower neocortical layers also yielded discovery of differentially expressed genes, including *Fezf2* (Chen et al., 2005b). As neocortical development has been studied extensively in mice, there is already a large body of work in this species that characterizes the compartmentalization and enrichment of transcripts specific to different regions (Molyneaux et al., 2007, http://www.genepaint.org, 2012, Kwan et al., 2012b,

www.brain-map.org, 2012). In addition, large scale *in situ* profiling has been performed on developing mice, with results for many genes of interest publicly available at several websites, including GenePaint (http://www.genepaint.org/), Allen Brain Atlas (http://www.brain-map.org/), and Eurexpress (http://www.eurexpress.org/ee/), some of which were used in recent publications and as part of this review (Yi et al., 2010, Shim et al., 2012).

This type of investigation is beginning to delineate the transcriptional signatures of mouse neocortical subregions, layers, and proliferative versus non-proliferative compartments (Han et al., 2009, Ayoub et al., 2011, Belgard et al., 2011, Fietz et al., 2012). Analyses have also extended to pharmacological and fluorescent reporter transgenic animal models of disease states such as Attention deficit hyperactivity disorder, MDMA use, Alzheimer's disease, and maternal neglect (Bordner et al., 2011, Fernandez-Castillo et al., 2012, Kim et al., 2012, gensat.org, 2013, Lempp et al., 2013). These studies also demonstrate that the transcriptome is plastic (Peter et al., 2012). Importantly, efforts are also being made to synthesize neocortical transcriptome data from mice, non-human primates, and humans in both normal and adverse prenatal states (e.g., fetal alcohol exposure) (Wang et al., 2010b, Hashimoto-Torii et al., 2011, Kojima et al., 2013).

B. Neocortical Transcriptomics in Humans

Transcriptomes of brain subregions are also increasingly being used to describe a region's genetic signature in humans. The transcriptional load of one subregion or condition can be compared against another to determine the specificity or

enrichment of transcript complement. These techniques allow for the quantitative differentiation of regions in developing or evolutionarily disparate brains (Bernard et al., 2012).

Using exon-array screen technology on neocortical regions of the mid-gestational human brain, it was demonstrated that not only the expression of distinct transcripts varies between regions, but that they are differentially spliced or expressed asymmetrically between hemispheres (Johnson et al., 2009). Differentially spliced variants were in many cases selectively expressed in (e.g., LIMK2a, CPVex5-6, ROBO1b, and ANRKD32b) or absent from (e.g., NTRK2b, LIMK2b, CPVLex2, ROBO1a, and ANKRD32a) the neocortex. Many of the transcripts that are differentially expressed or spliced have known roles in neocortical development, specification of neuronal subtypes, and axonal outgrowth, and were associated with neurodevelopmental disorders such as autism.

In another study, the human brain transcriptome was investigated at 15 time points across the lifespan, from 5 weeks post-conception to 82 years of age (Kang et al., 2011). Brains from both males and females were subject to Affymetrix Human Exon array to examine genes that were differentially expressed and spliced between sexes and across development. These findings extended previous work and created a partial spatiotemporal map of the human transcriptome. Interestingly, many of the genes profiled showed differential exon inclusion in the neocortex either temporally (88.7%) or spatiotemporally (28.9%). Most differential splicing occurred during embryonic development, indicating that

much of this precise genetic control occurs at posttranscriptional level during the specification of primary neurons, RG cycling, and neuroblast migration.

In a study of 269 subjects ranging from before birth to over 70 years of age, next-generation sequencing technology revealed that rates of transcriptional change were high during the prenatal period and taper off in the first half-year of postnatal life, reaching a roughly steady state level by the second decade of life. This steady state persists for some time—until approximately the fifth decade of life—when transcriptional changes begin to progressively increase. Further investigation, coupled with several protein-level analyses, has recapitulated this pattern of splicing changes across age in two brain regions—the hippocampus and the neocortex (Mazin et al., 2013). Future analyses of this kind can aid in the creation of a transcriptomic “signature” of psychiatric disorders, which could facilitate the advancement of translational research.

Human prefrontal neocortex (PFC) complexity, generated at least in part through transcriptional diversity, is a hallmark of humans. Transcriptional signatures of the human prefrontal cortex (PFC) are reproducible across ages and ethnicities (Colantuoni et al., 2011). Specificities in PFC transcriptomes are of special interest given the role of this brain region in higher cognitive function and its involvement in human-specific disorders (Goldman-Rakic, 2002, Diamond, 2011, Janga and Mittal, 2011, Arnsten et al., 2012). Indeed, recent work shows differences in the PFC transcriptome of humans, chimpanzees, and macaques, whereas evolutionarily older brain structures (e.g., hippocampus and caudate) show conserved transcriptional profiles. Furthermore, several genes are

enriched only in humans, such as *CLOCK*, which is implicated in psychiatric disease and circadian rhythms, and *FOXP2*, which is implicated in language (Feuk et al., 2006). Interestingly, more than 200 genes are differentially expressed in the mouse and human prefrontal cortex (PFC), (Lai et al., 2001, Feuk et al., 2006, Vernes et al., 2008). Although differences in gene expression have clear consequences for functional gene output, these findings suggest that intricate differences in post-transcriptional splicing may be a key mechanism through which evolution has honed neocortical development and function.

The laminar specific transcriptome screens in mouse were recently extended to the human developing neocortical wall (Fietz et al., 2012). Interestingly, this screen did not find significantly different transcriptome in the oSVZ, which may be due to presence of both RG and IPCs in both regions. However, in mice the SVZ and cortical plate were more similar in transcriptome signature than to VZ. In contrast, human developing neocortices have more similar oSVZ and iSVZ than the developing cortical plate. This study also pinpointed significance of differentially expressed distinct mRNAs that encode the extracellular matrix proteins.

An additional broad set of screening work known as the ENCODE Project Consortium characterizes functional genetic elements in multiple cell types (Gerstein, 2012). These studies profile genomic regions of transcription factor association, chromatin states, and histone modifications combined with DNA and RNA sequencing to detail the properties of transcriptional activity. This study has generated data that may be instructive for further investigation of neuronal

circuits. In addition, genomic analysis at the single-cell level has been refined by isolating cells from the human PFC and caudate nucleus by FACS. Researchers used high-fidelity multiple displacement amplification methods and successfully generated material for whole genome sequencing from single cells sufficient (Evrony et al., 2012). This “single-cell fingerprinting” approach revealed that retrotransposon insertion rates were low in human neural cells of cortex and caudate nucleus and unlikely to account for cell heterogeneity in these regions. Retrotransposition is a remarkable process were transcribed pieces of the DNA are ultimately re-integrated into genome. These pieces of DNA that will be transcribed and then re-integrated are called retrotransposons, of which the best studied example is Long interspersed element-1 (LINE1 or L1) (Thomas et al., 2012) . This L1 retrotransposon was shown to be active specifically in neuronal progenitor cells (NPCs) *in vitro* and *in vivo* (Muotri et al., 2005) . In particular, Sox2 repressed L1 transcription. levels of Sox2 are decreased in postmitotic progenies, the L1 expression is increased in them and inserts preferentially into genes encoding neuronal mRNAs. These data suggested that retrotransposon elements contribute to the development of nervous system, which due to the dynamics of retrotransposition may or may not be masked in adults. Indeed, in the adult human cortex and caudate, "single-cell fingerprinting" did not support L1 as a major source of neuronal diversity in adult cortex and caudate ((Evrony et al., 2012). Nevertheless, the "single-cell fingerprinting" this approach was further used in a patient with hemimegalencephaly to map the mosaic *AKT* mutation in a lineage of cortically derived cells (Evrony et al., 2012). Results

showed that a subpopulation of both neuronal and non-neuronal cells carried this mutation, suggesting that its etiology is within an early multi-potent precursor. These analyses have changed the field and allowed for highly specific diagnostic tools with clinical utility.

Single-cell analysis can also be extended to the transcriptome level (Hashimshony et al., 2012). The highly scalable “CEL-seq” system assigns each isolated cell’s RNA a 5’ barcode and Illumina adaptor during reverse transcription, and then cDNAs from multiple cells are pooled for *in vitro* transcription and sequencing via a modified Illumina assay. This system was successfully applied to single cells isolated from *C. elegans* embryos as a proof of principle. The implications for using this technology to dissect the transcriptional character of neocortical circuits are profound. Although these are powerful tools to analyze the neocortex at the genomic and transcriptional levels, the field awaits a proteome-level analysis of the neocortex.

C. Post-Transcriptional Processing

As the transcriptome of NSCs and postmitotic neuronal subgroups comes into sharper focus, greater attention must be paid to the functional protein expression levels of these transcripts. Given that there are often disconnects reported between the transcriptome and proteome, the transcript complement of cell subtypes must be interpreted as a “first step” in the segregation of neocortical cell subtypes (Chang and Stanford, 2008, Taniguchi et al., 2010, Day et al., 2011). Indeed, the transcriptome level of resolution cannot directly be interpreted as functional genetic makeup. Therefore, correlating transcriptional data with

protein levels and the understanding of regulatory posttranscriptional steps will allow researchers to determine the cellular potential for rapidly translating and increasing protein content. Given the highly polarized, rapidly differentiating and functionally-specific cells of the developing neocortex, these regulatory processes may occur disproportionately to those of other brain regions.

Regulation at the post-transcriptional level may fill some of the gaps in the understanding of neocorticogenesis and rapid changes in functional gene expression. The traditional notion that DNA encodes RNA, which in turn encodes protein, assumes a passive role of mRNA in translation. It is now appreciated that mRNA itself is heavily regulated and can be targeted post-transcriptionally at many levels. After mRNA is transcribed, it can be subject to alternative splicing, sequestered or exported from the nucleus, transported throughout the cell, and/or selectively degraded or translated (Keene, 2007) (Figure 2-1).

The mechanism of canonical translation is well described. The pathway starts with initiation, when an mRNA is activated via binding to the 5' untranslated region (UTR) of the EIF4F eukaryotic initiation cap complex, composed of EIF4E, EIF4G, and EIF4A. The activated mRNA is then joined by the 43S pre-initiation complex, consisting of the small 40S ribosomal subunit and a ternary complex of eukaryotic initiation factor 2 (EIF2)-GTP-tRNAMet, which screens for the initiation AUG codon. In the late stages of the translation initiation/pre-elongation steps, the ternary complex is removed from the small 40S subunit, and the 60S ribosomal subunit is recruited to start formation of the actively translating 80S ribosomal polysomes. This is followed by eukaryotic elongation factor 2 (EEF2)-

dependent elongation, which is critical in the post-initiation phase for progression from A to P to E sites after the 40S and 60S subunits form the 80S ribosomal polysomes. Ultimately, termination and ribosomal recycling occurs (Figure 2-2) (Jackson et al., 2010, Kong and Lasko, 2012).

One well-studied example of post-transcriptional regulation is microRNA (miRNA) antisense silencing of mRNA translation. Broadly, miRNAs are a class of non-protein-coding RNA that function through translational repression of mRNA targets (Bartel, 2004). Typically, they are approximately 21 nucleotides in length in their mature form, and although they have similarities to other non-coding RNAs, they are distinct in target, synthesis, and their location in the genome (Bartel, 2004, He and Hannon, 2004, Bartel, 2009). The importance of post-transcriptional regulation in the neocortex was illustrated in a study in which gene encoding Dicer, the enzyme responsible for the maturation of both miRNAs and siRNAs, was floxed, and Cre was driven by the Emx promoter, which begins expression around E9.5 and is specific to the dorsal telencephalon (De Pietri Tonelli et al., 2008). Conditional Dicer KO embryos show a striking loss of almost all upper-layer neurons, which are preferentially born after E14.5. These neurons, however, are derived from a homogenous population of NSCs at the ventricular surface, indicating that intrinsic molecular programming achieved through the expression of miRNAs sharpens the fidelity of gene expression to allow for a properly formed and functional neocortex. As the upper layers of the neocortex are the newest in an evolutionarily sense (Cubelos et al., 2010), it is fascinating that Dicer as post-transcriptional regulator may play a central role in

the formation of layers associated with higher-level cognitive function. In this way, post-transcriptional regulation can be seen as directly necessary for the advancement of cortical function and the generation of upper layers.

Recently developed methods have aided in addressing the gap between transcriptome and proteome via ribosomal profiling on Bacterial Artificial Chromosomes (BAC) (Gong et al., 2002, Gong et al., 2003, Yang et al., 2006, Gong et al., 2010). Given that the genomic sequence is very long and contains regulatory sequences such as promoter and enhancer regions that are located many kilobases away from the poly(A) tail, traditional transfection techniques cannot always accurately address the amount of expression of individual transcripts. BACs offer the advantage of being able to drive cell subtype-specific expression of ribosomal proteins while containing a GFP tag. The GFP tag can then be purified to isolate the mid-translation profile of transcripts with cell types (TRAP) (Heiman et al., 2008, Dougherty et al., 2010, Gong et al., 2010, Dougherty et al., 2012). This technique has successfully revealed expression patterns of several genes (Head et al., 2007, Heiman et al., 2008). In combination with transcriptomic profiling of cell subtypes, this method can allow the functional genetic output of cell subtypes to be weighed against their transcriptional profile to assess their potential verses realized genetic complement. In developmental studies, this technique can be used to discriminate the TRAP of stem and newly postmitotic cells. This information can then be instructive for comparison against disease states or to extrapolate evolutionary characteristics or region-specific profiles.

D. Post-Transcriptional Regulatory Elements are Differentially Expressed in Space and Time Across Neocortical Neurogenesis

Post-transcriptional regulation is clearly one of the key players in generating neocortical cellular diversity and function. However, studies investigating the regulation of translation, the final step of post-transcriptional regulation, are only in their infancy. Nevertheless, as early as the 1960s, researchers already found clusters of ribosomes in neuronal soma, dendrites, and dendritic spines (Bodian, 1965, Giuditta A, 1977, Giuditta A, 1980, Steward O, 1982, Giuditta A, 1991, Koenig and Martin, 1996). Follow-up studies confirmed these active sites of translation (polysomes) in distant neuronal processes (Giuditta A, 1991, Koenig and Martin, 1996). These findings suggest the existence of localized protein synthesis and multiple post-transcriptional regulatory steps during neuronal development. miRNA's may be functional in this role as they are co-transported with target mRNA and compartmentalized in the cell, regulating translation at the synapse (Pichardo-Casas et al., 2012). However, there is no current screen focused on post-transcriptional processing elements and their significance may be overlooked.

Therefore, we proceeded to demonstrate changes in genes associated with the post-transcriptional regulation during murine neurogenesis. We performed a microarray analysis of RNA harvested from whole neocortices at 4 key developmental time points: E11 was chosen to outline expression during the onset of neurogenesis; E13 to investigate direct neurogenic processes giving rise to predominantly lower layer subcortically projecting neurons; E15 to outline the

predominant shift from birth of subcortically to intracortically projecting neurons; and E18 to investigate the final stages of neocortical neurogenesis (Figure 2-1). Analysis of transcriptomics was performed using GeneChip Mouse Exon 1.0 ST Array (Affymetrix; n=2 per developmental stage or experimental condition) coupled with bioinformatics in BioConductor/R using the oligo and limma packages (GK., 2005, Carvalho and Irizarry, 2010). In R, analysis was performed first with the oligo package to interpret exon data using the "core" transcript annotations with the highest confidence and then with the limma package to identify significant differences between groups using an F-test. Our initial analysis focused on transcripts associated with the post-transcriptional analysis steps of mRNA localization, degradation, stability and distinct steps of translation.

The colors in the heatmap (Figures 2-1 to 2-5) represent an RMA scaled gene summary data for the listed gene symbols. The patterns of colors depict changes in expression of the functional groups of genes shown. For example, genes were selected by their annotation with gene ontology biological function codes as follows: splicing (GO:0000398), mRNA localization (GO:0006406), mRNA decay (GO:0006402; also named mRNA breakdown), mRNA stability (GO:0048255), mRNA translation (GO:0006412), translation initiation (GO:0006413), translation elongation (GO:0006414), translation termination (GO:0006415), and RNA binding proteins (GO:0003723). For assignment of particular RBD and their number, such as 1 to 4 RRM and/or KH domains, each RBP was manually assessed for RBP domain using UNIPROT on-line (http://www.uniprot.org) and heatmap for each subset was produces as above.

Notably, for each step of the post-transcriptional processing and RBP subset we found at least one candidate regulatory member to be differentially expressed throughout neocortical neurogenesis. In addition, the greatest number of transcripts associated with post-transcriptional processing that were differentially expressed across neocorticogenesis are involved in translational control (Figure 2-2). This is not surprising, as mRNA translation is a complex regulatory point that is composed of several tightly controlled steps. Remarkably, when analyzed for different steps of translation (Figure 2-3), we again found differentially expressed mRNAs important for initiation, elongation and termination. These findings suggest rapid and precise spatiotemporal control of functional protein expression during progressive neocorticogenesis, particularly at the level of translation.

The array analysis was followed by qRT-PCR for one gene from each subgroup. Stable housekeeping reference genes are critical for credible qRT-PCR results. However, housekeeping genes for developmental neocortices have not been previously detailed in depth and therefore, we tested several previously used housekeeping genes (Gapdh, Pgk1, Rps18, and Rns18) and several new candidates that were unchanged in our across-development microarray analysis of neocortex (Rps13, Rps6kb1, Mrps6, and Pdcl2). The qRT-PCR results using these probes from E13, E15, and E18 neocortices were analyzed using Biogazelle qbasePLUS2 software to determine which housekeeping genes and how many of them should be optimally used, as described (Biogazelle; Zwijnaarde, Belgium) (Pinto F, 2012). Based on this approach, we found that

optimally at least four housekeeping genes must be used together to accurately determine the dynamics of mRNA expression levels across neocortical development: Mrps6, Rps13, Rps18, and Gapdh. Thus, the subsequent developmental qRT-PCR results were normalized to these four reference targets per stage and then to E13 to obtain relative mRNA levels across development. A change of $p < 0.05$ was considered significant using one-way analysis of variance (ANOVA). Finally, the expression sites of these mRNAs were assessed using on-line in situ hybridization databases Euroexpress and Genepaint (http://www.genepaint.org, 2012, www.euroexpress.org, 2012).

The follow up qRT-PCR of whole developing neocortices corroborated array data that post-transcriptional regulatory elements present dynamic changes in their expression levels across neocortical neurogenesis. For example, the mRNA decay regulator, the expression of *Zinc finger protein 36, C3H type-like 1* (*Zfp36l1*) decreased as neurogenesis progressed (Figure 2-1). Even more remarkable is its spatially restricted enriched expression in the VZ where RG cell bodies are residing, suggesting its role in neocortical progenitors (Figure 2-1). Interestingly, even mRNA translation regulators showed enriched expression in distinct compartments of developing neocortices. For example, mRNAs encoding initiation factor *Eif4E* and termination factor *Etf1* are enriched in VC and CP, suggesting dynamic control of distinct steps of mRNA translation during neocortical development in these two compartments.

Figure 2 1

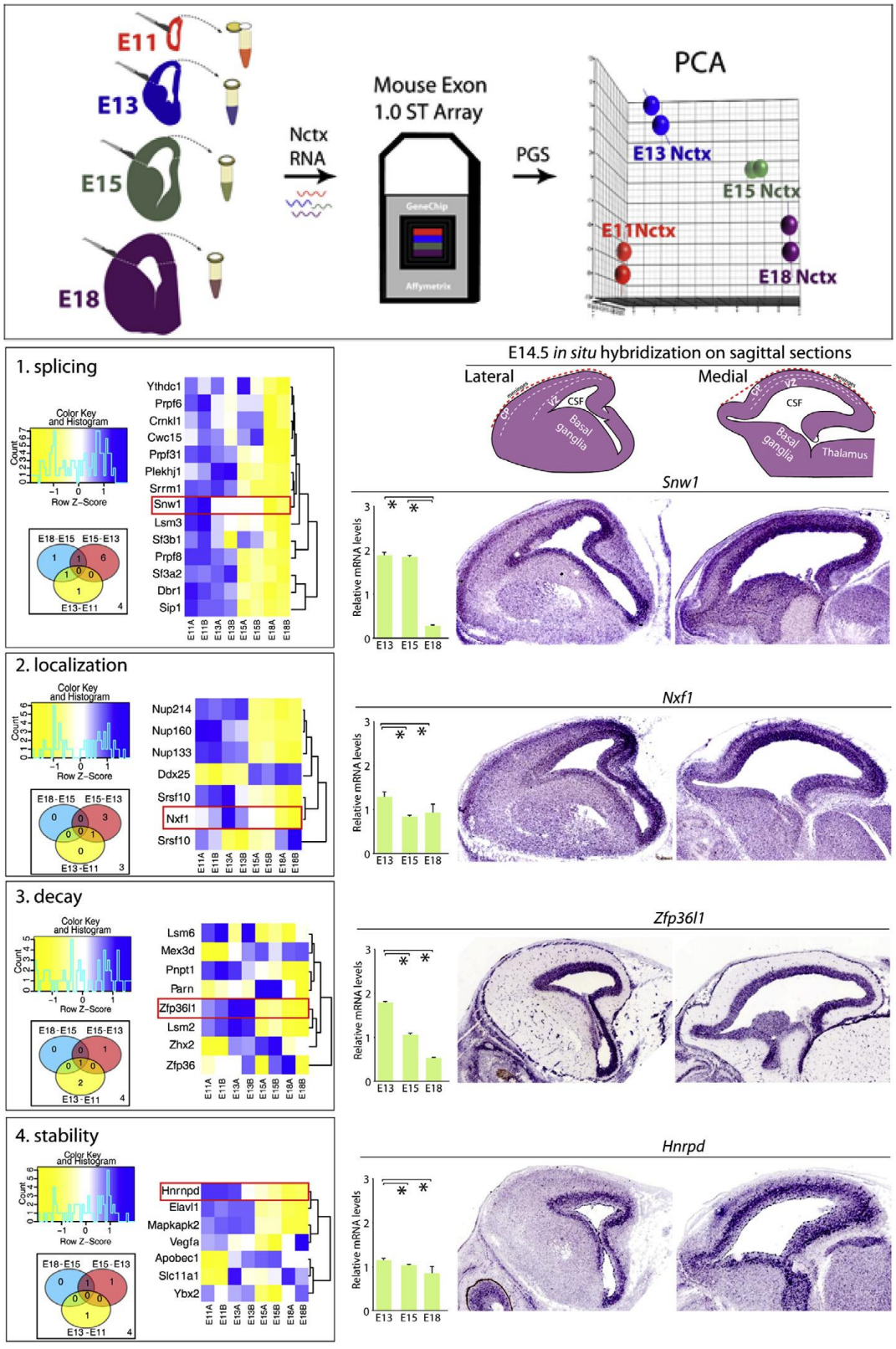
E11
E13
E15
E18
Nctx RNA
Mouse Exon 1.0 ST Array
GeneChip
Affymetrix
PGS
PCA
E13 Nctx
E15 Nctx
E11Nctx
E18 Nctx
1. splicing
2. localization
3. decay
4. stability
Color Key and Histogram
Row Z-Score
Count
E18-E15
E15-E13
E13-E11
Ythdc1
Prpf6
Crnkl1
Cwc15
Prpf31
Plekhj1
Srrm1
Snw1
Lsm3
Sf3b1
Prpf8
Sf3a2
Dbr1
Sip1
Nup214
Nup160
Nup133
Ddx25
Srsf10
Nxf1
Srsf10
Lsm6
Mex3d
Pnpt1
Parn
Zfp36l1
Lsm2
Zhx2
Zfp36
Hnrnpd
Elavl1
Mapkapk2
Vegfa
Apobec1
Slc11a1
Ybx2
E11A
E11B
E13A
E13B
E15A
E15B
E18A
E18B
E14.5 in situ hybridization on sagittal sections
Lateral
Medial
CP
CSF
Basal ganglia
Thalamus
Snw1
Nxf1
Zfp36l1
Hnrpd
Relative mRNA levels
E13
E15
E18

Figure 2-1. Transcriptome analysis of molecules involved in post-transcriptional mRNA processing steps within the developing mouse neocortex. (top) Neocortex (Nctx) from embryonic days 11 (E11), E13, E15, and E18 was dissected, and RNA was isolated then assayed using Mouse Exon 1.0 ST Arrays, and analyzed using Partek Genome Suite (PGS) and R/Bioconductor. Principal component analysis (PCA) revealed clustering among replicates and distinct differentiation among developmental stages. (Left) Gene ontology (GO) analysis of whole developing mouse neocortices during key steps of the neurogenesis for distinct steps of post-transcriptional mRNA processing: splicing (1), localization (2), decay (3), and stability (4). GO analysis is presented as heatmap (blue=higher expression; yellow=lower expression; normalized by gene) and corresponding Venn analyses. Heatmaps include all genes on the Affymetrix Mouse Exon array annotated with the listed GO term, and Venn diagrams depict numbers of genes having significant contrasts between adjacent time points. (Right) qRT-PCR of whole developing neocortices for sample genes annotated with red boxes on the heatmaps. Corresponding in situ hybridization of lateral (middle panels) and medial (right panels) sagittal neocortical sections of E14.5 neocortices were obtained from www.genepaint.com. Remarkably, besides temporally distinct expression levels, the post-transcriptional regulatory elements also show restricted enrichment in different compartments of developing neocortices. For example, expression of the decay regulator Zfp36l1 decreased during neurogenesis, while its expression is highly enriched in the VZ where RG reside. In contrast, expression of a splicing regulator, Snw1, dramatically decreased at E18 when neurogenesis ceases, but is enriched at E14.5 in both progenitor characterized compartment VZ and postmitotic compartment CP. All qRT-PCR values were normalized to four housekeeping genes Gapdh, Mrps6, Rps13, and Rps18, and then scaled to average. $p<0.05$.

Figure 2 2

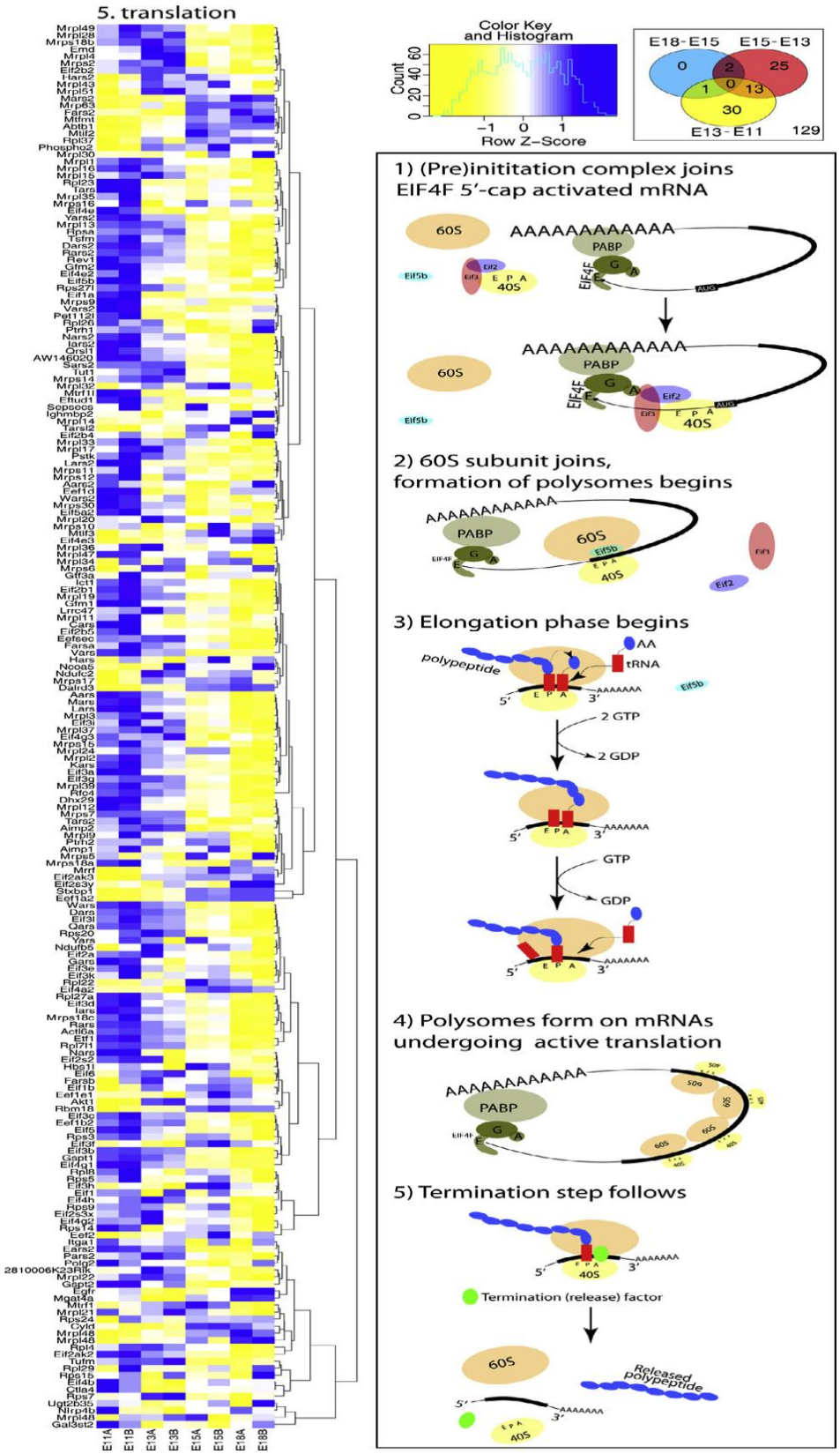
5. translation
Color Key and Histogram
Count
Row Z-Score
E18-E15
E15-E13
E13-E11
129
1) (Pre)initiation complex joins EIF4F 5′-cap activated mRNA
60S
AAAAAAAAAAAA
PABP
EIF4F
Eif5b
Eif2
Eif3
40S
AUG
2) 60S subunit joins, formation of polysomes begins
3) Elongation phase begins
polypeptide
tRNA
AA
2 GTP
2 GDP
GTP
GDP
4) Polysomes form on mRNAs undergoing active translation
5) Termination step follows
Termination (release) factor
Released polypeptide
E11A
E11B
E13A
E13B
E15A
E15B
E18A
E18B

Figure 2-2. Transcriptome analysis of developing neocortices during prenatal neurogenesis for mRNA translation reveals numerous mRNA clusters showing dynamicexpression patterns. (Right) Canonical process of translation and points of regulation. 50-cap activated mRNA carries EIF4F complex (EIF4A+EIF4G+EIF4E)boundtothe50 untranslated region(UTR)(step 1, top right animation).EIF4Gfrom the complex is associating with PABP bound to the 3' UTR making the active mRNA into a loop (step 2). On these mRNAs the pre-initation complex (eIF2–40S ribosome-Eif3) joins the 50UTR and screens for the start codon. Then the 60S ribosomal subunit joins the 40S to form the 80S monosome. This step is partially regulated by Eif5b, while Eif2 and EIf3 are removed from the 40S(step3).This initiation
phase then transitions into the elongation phase when active translation is being governed by polysome assembly and polypeptide elongation (step 4). Once the polypeptide is finalized by reaching the stop codon, the termination step dissociates the80Sribosome back into40S and60S subunits (step 5, bottom right animation). Each step of translation is regulated by distinct molecules, as shown in the next figure.

Figure 2 3

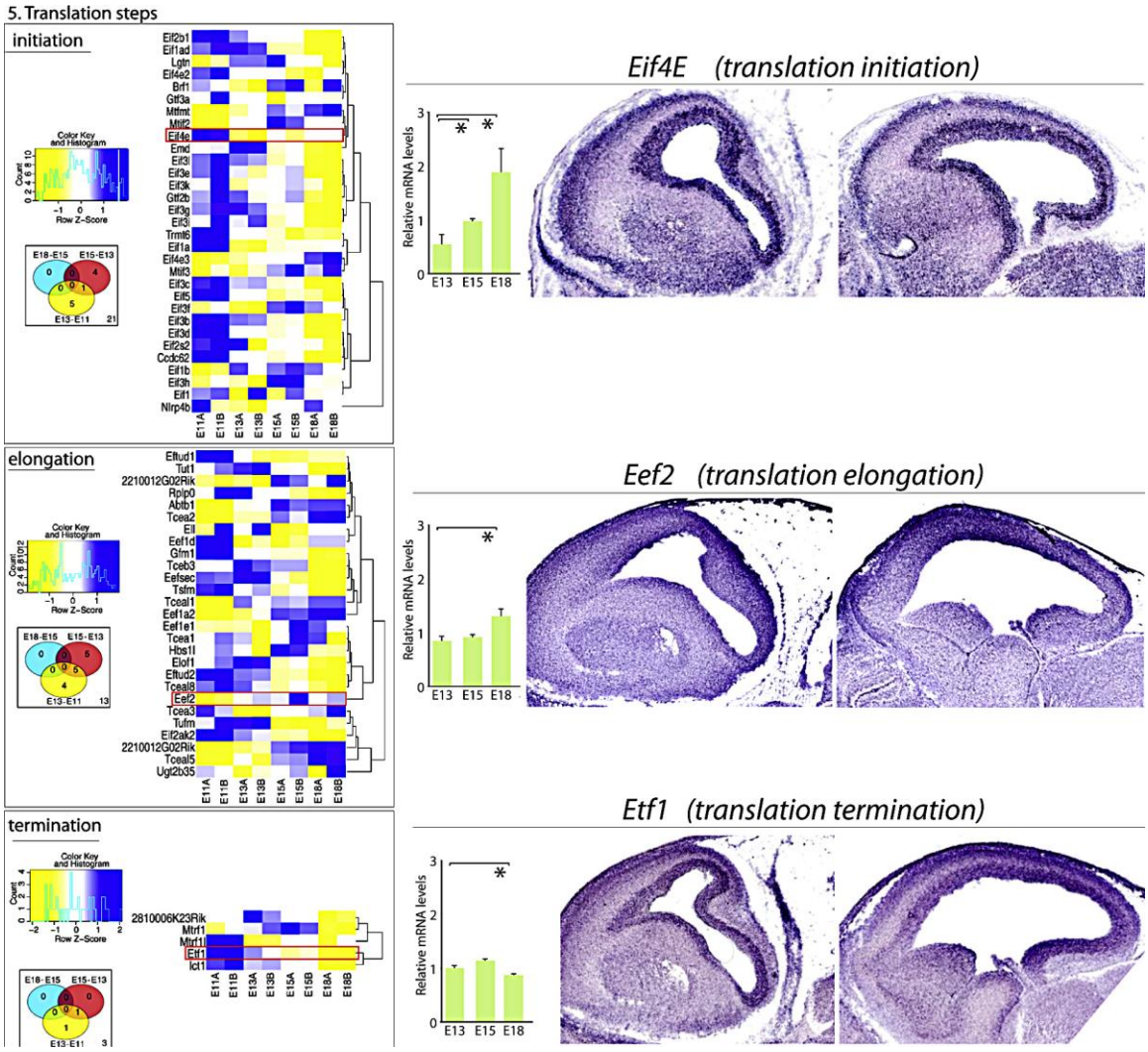
5. Translation steps
initiation
elongation
termination
Eif4E (translation initiation)
Eef2 (translation elongation)
Etf1 (translation termination)
Relative mRNA levels
E13 E15 E18

Fig. 2-3. Transcriptome analysis of the developing mouse neocortex for mRNAs encoding regulators of distinct steps of mRNA translation. (Left) Remarkably, even mRNAs encoding regulators of mRNA translation show dynamic changes in their expression during neurogenesis. GO analysis is again presented as heatmap (blue=higher expression; yellow=lower expression, normalized by gene) together with corresponding Venn analyses. (Right) qRT-PCR of whole developing neocortices for genes corresponds to relative gene expression in the heatmaps (red boxes). Corresponding in situ hybridization of lateral and medial sagittal neocortical sections of E14.5 neocortices were obtained from www.genepaint.com. Interestingly, initiation factor EIf4E and termination factor, Etf1, are both enriched in the VZ and CP, suggesting highly dynamic regulation of these two regulatory steps in RG progenitors and postmitotic differentiating neurons. All qRT-PCR values were normalized to four housekeeping genes (Gapdh, Mrps6, Rps13, and Rps18), and then scaled to average. $p<0.05$.

Chapter 3. RNA Binding Proteins in Neocorticogenesis

The study of perhaps the most dynamic and ubiquitous of all post-transcriptional regulators, RNA binding proteins (RBPs), has only recently commenced in the context of neocorticogenesis. These proteins are prime candidates for post-transcriptional regulation given that they rapidly influence all steps: the stabilization, degradation, transport, splicing, and translation of mRNA cues (Keene, 2007). Thus, they represent a unique regulatory interface between transcriptional programming and functional protein expression and affect virtually every level of RNA processing. Therefore, the dynamic activity of RBPs may be of great importance for the rapid and specific gene expression events that are disproportionately required by the developing neocortex.

Binding of target RNA occurs at an RNA-binding (RBD) domain of which there are almost 40 subtypes known to date (Lunde et al., 2007). RBPs have one or several RBDs, with greater numbers associated with increased specificity of RNA binding. RNA-recognition motifs (RRMs) are the most common example of RBDs in eukaryotes. RRMs provide both RNA and protein binding capacity. RRM can be situated that this single type of RBD is responsible for the specific binding of many RNA subtypes (Lunde et al., 2007, Clery et al., 2008). For example, the third RRM of the drosophila RBP, ELAV, is multifunctional and aids in both protein-protein and RNA-protein interactions, thereby influencing splicing events (Toba and White, 2008). In addition to RRMs, there are many other types of RBDs, such as RNP K homology (KH) and piwi domains. The unique quaternary structure of RBPs may allow for the specialized presentation of distinct RBDs. In

this manner, RBP-RNA binding can be modified by many of the factors known to modify proteins post-translationally. The molecular mechanisms of these interactions, however, are not well elucidated.

In many cases, RBPs bind co-transcriptionally in the nucleus and begin forming ribonucleoprotein (RNP) complexes varying in structure and composition that ultimately mediate RNA fate (Kishore et al., 2010). RBP-RNA binding occurs through a specific or semi-conserved sequence, by secondary structure, or both. An early study showed that a sequence of 3' UTR was necessary for localization of *Bicoid* RNA in Drosophila oocytes (Macdonald and Struhl, 1988). Further studies showed that within this region there are several stem loop structures required for sequential and increasingly specific localization of the transcript (Ferrandon et al., 1997). Sequence mutations of the secondary structures maintaining the stem loop were sufficient to preserve mRNA localization. Also, a cis-acting element within the 3' UTR of *Bicoid* was required for RNA dimerization of the transcript itself, and this too was necessary for transcript transport. Therefore, even within a single transcript, there are multiple variations of RBP binding activities that may influence grouping in disparate RNPs.

A. RBP Roles in Neocorticogenesis

RBP activity is critical to the developing and mature brain. Some of the earliest studies of mRNA localization show that *β-actin* contains a "zip-code" cis-element located in the 3' UTR that is responsible for the dendritic localization of the message (Kislauskis et al., 1994). Later research showed that this element preferentially exists in the *β-actin* 3' UTR, whereas the *γ-actin* 3' region lacks the

essential zip code (Willis et al., 2011). This and subsequent work solidified the view that an RBP Zipcode Binding Protein 1 (ZBP1) acts to bind this cis element and that of *GAP-43*, transporting these messages to the outgrowing neurites of developing neurons in the central and peripheral nervous system (Donnelly et al., 2011). Interestingly, a phosphorylation-deficient ZBP1 mutant failed to release mRNA cargo in the dendrites and initiate translation of *β-actin*. Therefore, RBPs are involved in mRNA transport and are affected in a spatially-specific manner by post-translational modifications that ultimately influence translation of mRNA cargo.

During neocortical neurogenesis, mRNA distribution in progenitors is dependent upon Staufen2, an RBP. Staufen2 localizes asymmetrically within dividing RG (Kusek et al., 2012). Loss of Staufen2 function via shRNA both in vitro and in vivo demonstrated role in Pax6+ RG maintenance and Tbr2 suppression. Further in vivo results demonstrated that Staufen2 serves to regulate cell cycle re-entry. In particular, silencing of Staufen2 promoted cell cycle exit and differentiation. Early overexpression of Staufen2 in vivo resulted in aberrant pockets of differentiated heterotopias of variable size and cell type, further suggesting its role in stem-maintenance. Interestingly, Staufen2 binds a subset of mRNAs whose role was associated with asymmetric divisions. These findings indicate that the machinery for segregating mRNA between dividing cells is critical for the maintenance of progenitors and the spatiotemporally appropriate generation of neocortical layers.

Alternatively, bound mRNA can be regulated during and after splicing events by RBPs as a regulatory step between transcription and translation. One example in the neocortex is *Magoh*, which binds at the exon junction complex of target mRNAs (Silver et al., 2010). In this study, the *Magoh*^{mos2} allele was found to cause a putative frame shift in the Magoh protein, resulting in a truncated protein. This factor is homologous between mice and humans at 100% of its amino acids. Importantly, haploinsufficiency in Magoh$^{Mos2/+}$ neocortices lead to a significant decrease in cortical mass, mimicking microcephalic neuropathology. Further investigations found proper numbers of Pax6-positive RG progenitors and a significant reduction in numbers of IPCs. As a result, the neocortex was disorganized and lacking many lower- and upper-layer markers, with lower numbers of Tbr2-positive IPC progenitors. Also, Tuj1- and DCX-positive postmitotic neurons are generated at an increased rate early in neurogenesis, indicating premature cell cycle exit and differentiation. This microcephalic phenotype demonstrates the role of an RBP in neocortex formation, and future studies will parse out the downstream transcripts involved.

Although the Magoh^{mos2} allele mediates the proliferation and generation of neurons from IPCs in the cortex, another RBP, Nova2, modulates an alternatively-spliced isoform of Dab1—an adapter of the Reelin pathway associated with proper placement of neocortical projection neurons (Sheldon et al., 1997, Yano et al., 2010). Nova2 binds Dab1.7bc in the cortex and cerebellum and selectively controls splicing of the Dab1.7bc isoform. Loss of Nova2 function prevents the selective exclusion of this exon, and the anatomical consequences

are similar to the Reeler phenotype, with disorganized and ectopic cortical layers, especially those formed by later-born cohorts of neurons. *In utero* electroporation of wild-type Dab1 rescues the effects of Dab1.7bc expression in Nova2 KO mice. Furthermore, acute over-expression of Dab1.7bc via *in utero* electroporation also causes cortical disorganization when assessed at E14.5. Interestingly Nova, another member of this protein family, has a function apart from its splicing role in the nucleus. That is, it appears to be involved in translocating *GIRK2* mRNA to the dendrites of spinal cord neurons and inhibitor synapses, thereby putatively regulating differentiation or plasticity (Racca et al., 2010). In this manner, the post-transcriptional event of alternative splicing, which is modulated by an RBP, is sufficient to regulate neuronal migration and differentiation in the neocortex.

In addition, splicing has been directly implicated in the self-renewal versus differentiation choice of RG in a polypyrimidine-tract-binding (PTB) protein-2 (Ptbp2) loss-of-function study (Licatalosi et al., 2012). PTBs are RBPs that play an important role in alternative splicing functions (Sawicka K, 2008). Ptbp2 is heavily expressed during the embryonic phases of neurogenesis and greatly reduced after P7. Using the recently generated HITS-CLIP technique (discussed in detail later), researchers found that targets of Ptbp2, including Depdc5, Sphkap, Erbb4, Dzip1, Ank3, Braf, Ppp3cb, and the basal progenitor maintenance factor, *Numb*, are more likely to be alternatively spliced in $Ptbp^{-/-}$ mice. Functionally, this failure to inhibit splicing results in the premature and aberrant differentiation of RG into DCX-positive neuroblasts. Failure to exclude the alternative third exon of Numb is coincident with this developmental

abnormality. Furthermore, INM is perturbed, with BrdU-positive S-phase cells found ectopically at the epithelial surface of the VZ, whereas phospho-histone 3-positive M-phase RG divide aberrantly, away from the LV.

B. RBPs characterized by KH, piwi and RRM are Differentially expressed during Neocortical Neurogenesis.

Our microarray analysis coupled to bioinformatics of developing neocortices revealed a large number of RBPs that have dynamic expression changes across neocortical neurogenesis (Figure 3-1). Interestingly, several clusters of RBPs are highly expressed during "direct neurogenesis", while others are highly expressed during "indirect neurogenesis." These data suggest the influence of RBPs across neocorticogenesis and a high level of complexity in their combinatorial roles. Dividing the RBPs based on their domain type, we found that that the most numerous subgroups of RBPs in developing neocortices are characterized by RRM domain, followed by KH (Figure 3-2). Interestingly, within the RRM subgroup the number of RRMs per molecule range from 1 to 4 for all but *RNA binding motif protein 19* (*Rbm19*) which has 6 RRMs. Each of the subgroups contain members which either decrease or increase in their expression across neocortical neurogenesis suggesting their temporally distinct roles. Furthermore, assessment of available on-line *in situ* hybridizations of developing neocortices at E14.5 from www. genepaint.com and/or www.euroexpress.org revealed also spatial specificity in their expression (Figure 3-2). These findings suggest the dynamic spatiotemporal regulation of RBP's as well as their function. Further, these data also point to their selective

spatiotemporal control of neocortical post-transcriptional processing steps. Taken together previous findings, our screen suggests a significant role of posttranscriptional regulatory elements in neocortical development, and possibly evolution.

C. Fragile X Mental Retardation Protein

Perhaps the best described example of the functional involvement of an RBP characterized by KH domain in brain development is Fragile X mental retardation protein (FMRP). In the clinic, FMRP mutations result in Fragile X mental retardation, an autism spectrum disorder, and are the most common monogenic cause of autism. The gene *Fragile X Mental retardation 1 (FMR1)* was identified by positional cloning (Verkerk et al., 1991), whereas encoded FMRP was recognized as an RBP somewhat later (Ashley CT Jr, 1993). Indeed, many of the genes disrupted in autism are associated with FMRP (Iossifov et al., 2012). The FMR1 RBP mutation involves increasing numbers of hereditary CGG repeats in the 5' UTR of the *FMR1* transcript encoding FMRP (Verheij et al., 1993). This may ultimately occur because the promoter region of *FMR1* lies within this locus (Khalil et al., 2008). Functionally, FMRP is responsible for the mass exodus of nascent mRNA transcripts from the nucleus and preferential translocation to the dendrites, involving as much as 4% of total mRNA (Santoro et al., 2012). These recent studies show that the lack of FMRP results in underdeveloped synapses and dendritic spines. New research demonstrates that FMRP may be a downstream effector of mTORC1 and may mediate synaptogenesis and dendritic spine maturation through control of *GluR* and *PSD-95* mRNA translation (Liu-

Yesucevitz et al., 2011). Remarkably, wild-type offspring nurtured by *Fmr1* mutant dams develop hyperactivity, a common trait of autism mouse models, implicating FMRP as a maternal environmental factor that disrupts neurological development (Zupan and Toth, 2012).

At the cellular level, compared with wild-type mouse cortical neurospheres, Fmr1-deficient E13 and P6 neurospheres generate three-fold more neurons and 15% fewer glia cells (Caldwell et al., 2001, Castrén et al., 2005). Neurospheres from humans with Fragile X syndrome (FXS) human embryos showed higher five-fold increase in neurons and a 70% decrease in glial cells. This shift is also found *in vivo,* where knockdown of Fmr1 by *in utero* electroporation of shRNA increases IPC production at the expense of RG (Saffary and Xie, 2011).

In the neocortex specifically, FMRP has been implicated in evolutionary differences between mice and humans in the expression of NOS1, a gene associated with synaptogenesis and schizophrenia (Kwan et al., 2012a). FMRP selectively binds *NOS1* mRNA in the mid-gestational human neocortex, whereas the homologous mouse protein does not effectively bind *Nos1*. Furthermore, in Fragile X mental retardation, there is a significant decrease in human NOS1 protein levels, whereas protein levels are preserved in FMRP KO mice. These findings indicate that there is a species-specific role of FMRP in neocortical development. In addition to offering direct evidence linking FMRP and NOS1 expression to FXS, these findings demonstrate a difference in post-transcriptional regulation of NOS1 and suggest that FMRP may have a critical

species- and region-specific role in the translation and function of this molecule. A recent study extends these findings by showing that FMRP is necessary for RG self-renewal by suppressing differentiation into TBR2-expressing IPCs (Saffary and Xie, 2011).

Recent examination of the FMRP mechanism has elucidated its role in the translation of bound targets through regulation at the elongation step (Darnell et al., 2011). In particular, the direct targets of FMRP have been identified through *in vivo* crosslinking-IP (CLIP). CLIP crosslinks protein and RNA molecules that are in direct contact. The subsequent IP can be subject to sequencing analysis (HITS-CLIP) to determine broad RBP-mRNA interactions (Darnell, 2010). Using this methodology, FMRP was found to bind a subset of mRNA targets, most often within their coding regions and frequently to those that are loaded with ribosomes and stalled in translation. Deletion of FMRP using multiple techniques, however, does not restore bound targets to active translation as assessed by sucrose gradient fractionation. Although the specific action of FMRP remains elusive, these findings suggest a dynamic role for this protein, allowing target mRNAs to be loaded with ribosomes and staged for translation while perhaps protecting them from degradation. Many FMRP mRNA targets are also involved in synaptic plasticity and are candidate autism genes, such as mGluR subunits that are altered in FXS (Cruz-Martin et al., 2012). In summary, FMRP is a mass regulator of functional gene expression that appears to be tightly regulated and highly specific. Studies on FMRP shed light on the ability of a single protein to cause multiple types of mental retardation. However, furthering our knowledge of

the distinct steps and developmental time windows of FMRP activity are essential for better understanding FMRP-associated disorders and possible pharmacotherapeutics.

D. Elav RBPs

The Embryonic Lethal Abnormal Vision-Like (ElavL) proteins characterized by 3 RRM domains were identified as autoantibodies of patients with paraneoplastic neurological encephalomyelitis and paraneoplastic sensory neuropathy (Szabo A, 1991, Lövblad KO, 1993, Deschênes-Furry et al., 2006). Mammalian ElavL is homologous to Drosophila Elav, an RBP that is neuron-specific and required for nervous system development (Campos et al., 1987, Robinow and White, 1988, Robinow S, 1988). In mammals, ElavLs are also known as Human Antigen (Hu) family proteins and consist of four members: HuR (Elavl1), HuB (Elavl2), HuC (Elavl3), and HuD (Elavl4). These Hu proteins are mostly neuron-specific with the exception of HuR. They promote the stability and translation of its mRNA targets (Szabo A, 1991, Park et al., 2000).

Perhaps the best studied Hu proteins, HuD, is well conserved across species. HuD and other Elav family members have three RRMs: two at the N-terminus, followed by an intervening “linker” or “hinge” region, and then a C-terminus RRM (Fukao et al., 2009). In general, Elav proteins are known to stabilize transcripts, and HuD specifically does so through binding to AU-rich instability elements at the 3’ UTR, which are the targets of RBPs and exonucleases that preferentially degrade mRNA. HuD stabilization occurs through the competitive inhibition of these factors through binding at the same

sequences (Park-Lee et al., 2003). A recent study shows that the first two RRMs have the most efficient RNA binding capacity but that the third RRM is essential for binding the long poly A tail of some neuron-specific transcripts (Bolognani et al., 2010), which preferentially promotes stability and translation of HuD targets. Furthermore, this study identifies members of an RNP that aid the involvement of HuD in translation, including eiF4G, eif4A, eiF4E, and PAPB. Subsequent analysis using RNAase show that only the eiF4A interaction is protein-dependent and that other members bind to an RNA bridge. This study also demonstrates that the linker region and third RRM of HuD are required for protein-protein interaction and the presence of HuD in translating polysome fractions of a sucrose gradient. These results suggest that HuD functions in neocorticogenesis by selectively binding to translational protein complexes and influencing the translation of target transcripts.

Through transcript stabilization and promoting translation, HuD influences the differentiation of neurons and the proliferation of NSCs . For instance, an assessment of proliferation through *in vitro* analysis of a constitutive HuD KO mouse revealed a reduction in number of neurospheres, indicating a deficit in proliferation or differentiation of NSCs (Akamatsu et al., 2005). In the behaving mouse, these effects read out to a lack of motor control, evidenced by poor rotorod performance and abnormal paw clasping. In agreement with these results, *in vitro* studies demonstrate that HuD binds the 3' UTR of *GAP-43,* and its over-expression is sufficient to drive the increased stability and expression of *GAP-43* and the formation of neurites (Chung et al., 1997a, Anderson et al.,

2001). Other *in vitro* studies examining HuD expression in PC12 cells describe an increased expression of HuD in cells that are actively extending neurites. Inhibition of HuD via RNA interference completely stunts neurite outgrowth without affecting existing processes (Dobashi et al., 1998, Aranda-Abreu et al., 1999). As such, HuD is implicated in the initial stages of neurite outgrowth but not the stability of already-formed dendrites. The mechanism of this action appears to be an association of GAP-43 with HuD granules at growth cones that is dependent on translation, although this evidence come from co-localization rather than direct polysome analysis (Smith et al., 2004). Related behavioral studies show that learning tasks, such as the Morris water maze, up-regulate HuD levels and HuD co-localization with Gap-43 in rat hippocampi (Quattrone et al., 2001, Pascale et al., 2004). Thus, HuD is also active in the adult animal, seemingly to modulate plasticity through Gap-43 regulation similar to the mechanism at work in neurite outgrowth. Interestingly, gene ontology studies of HuD binding reveal that approximately 7% of bound targets are mRNAs of other RBPs such as Musashi 2 (Bolognani et al., 2010).

Misregulation of HuD, furthermore, is implicated in a host of diseases. For example, at least two single nucleotide polymorphisms in the ELAVL4 gene locus are associated with the age of onset of Parkinson's disease (PD), a motor and cognitive disorder, in a clinical study of 1,223 members of 643 families (Noureddine et al., 2005). This study was replicated several years later by genotyping the two previously reported risk alleles for PD and discovering a third minor risk allele, confirming and extending ELAVL4's link to PD (DeStefano et al.,

2008). An interceding study of PD and control patients from the United States, Norway, and Ireland narrowed a possible genetic founder to an Irish population while confirming the locus and identity of the two previously reported risk alleles (rs9675852 and rs3902720) (Haugarvoll et al., 2007). Further investigations implicate HuD, through its role in alternative splicing, to a well-studied lymphoblastic leukemia known as the Philadelphia Syndrome, which is caused by a chromosomal translocation resulting in the BCR-ABL fusion protein (Bellavia et al., 2007, Mullighan et al., 2008). In this disorder, the TF Ikaros is deleted in the vast majority of patients with chronic myelogenous leukemia. HuD functions as an intermediary between notch3 signaling and Ikaros. Notch3 signaling up-regulates HuD and promotes alternative splicing of a non-transcriptionally active form of Ikaros, which disallows its contribution to lymphoid leukemias. HuD is implicated in human malignant neuroblasts due to its abnormal stabilization of the transcript of *N-myc* pre-RNA during nuclear processing (Cho and Noguchi, 1997, Darina L Lazarovab, 1999). Curiously, HuD mRNA is also increased in the blood of patients with small cell lung cancer (D'Alessandro et al., 2008), and several studies have implicated HuD as a marker for small cell lung cancer at the protein level (King, 1997). More recent *in vitro* studies using SH-SY5Y cells transfected with either HuD over-expression constructs or antisense RNAi vectors show correlated expression of HuD and N-myc. Furthermore, there is evidence for the loss of an HuD allele that is located on chromosome 1p in patients with neuroblastoma, particularly those with the worst prognoses (Grandinetti et al., 2005).

Clearly, the regulation of HuD expression is critical to the development and steady-state function of biological systems. Furthermore, several studies demonstrate that *HuD* itself is under heavy translational and post-transcriptional control. For example, HuD is edited by the adenosine deaminase that acts on RNA1 and 2 enzymes (Enstero et al., 2010). RNA editing is a process through which enzymatic activity acts on double-stranded RNA to edit adenosines to inosines, which are interpreted as guanosines by the ribosome during translation. Therefore, the peptide product can be changed. In the previously mentioned study, *HuD* was found to have five editing sites within its coding region, indicating its variable post-transcriptional regulation. It should also be noted that HuD is a target of alternative splicing, and there is evidence that this is an auto-regulatory event governed by other Hu family proteins. A recent study demonstrated that up-regulation of Hu proteins in HeLa cells promotes the inclusion of HuD exon 6 (Wang et al., 2010a). RNAi against Hu expression, however, decreases the rate of exon6 inclusion. So far, HuD is known to be expressed as four distinct protein isoforms in the mouse (NCBI), each which may have a distinct role in neocorticogenesis.

Other mouse studies implicate HuC/D in steady-state levels of transcripts as well as splicing (Ince-Dunn et al., 2012). Using HITS-CLIP technology to assess splicing and steady-state levels in Elavl3$^{-/-}$ and Elavl4$^{-/-}$ mice, consensus binding was predominately found in the 3' UTR of mRNA targets. In pre-mRNA targets, however, Elavl3 was often bound and regulating splicing of bound cargo. Furthermore, Elavl3 targets are often involved in synaptic plasticity and signaling.

Elavl3/4$^{-/-}$ mice exhibit a 50% loss of cortical glutamate. In the Elavl3$^{-/-}$ single KO, an alternative splicing site is occupied by Elavl3 on the pre-mRNA of the glutaminase enzyme. This enzyme is largely responsible for generating cortical glutamate, and Elav3 controls the generation of two isoforms of this protein. A significant decrease in one isoform (Gls-I) but not the other (Gls-s) may be responsible for this change in excitation. Finally, investigators electrophysiologically detected active seizure patterns in Elavl3 mutant mice. Collectively, members of the Hu family appear to be involved in many complex disorders. Although these disorders are most likely not the outcome of a single causative gene, studies of Hu family proteins may help provide insights in disease mechanisms and potential pharmacotherapeutics.

E. RBP Musashi and Regulation by HuD

The RBP Musashi is highly conserved across species. Initially discovered in Drosophila, it was found to be required for asymmetric divisions of sensory organ progenitors (Nakamura et al., 1994, Kaneko et al., 2000). In vertebrates, Musashi isoforms Musashi1 and Musashi2 are expressed in both adult and embryonic NSCs. Musashi regulates Numb and p21, a cyclin-dependent kinase (CDK) inhibitor (Imai et al., 2001, Battelli et al., 2006, Nishimoto and Okano, 2010), both which are implicated in neocortical NSC differentiation and cell cycle progression. Musashi appears to maintain stem cell self-renewal and undifferentiated state by disrupting recruitment of the large ribosomal subunit and other factors for polysome assembly. These mechanisms are still not well understood, and more focused research is needed to identify Musashi's

biochemical pathway. Musashi expression levels decrease as differentiation progresses, making it undetectable in postmitotic neurons (Kaneko et al., 2000).

Interestingly, HuD stabilizes *Musashi* mRNA, and this effect may be critical in the transition from stem cell proliferation to neural differentiation (Ratti et al., 2006). Recent studies show Musashi-Notch signaling to be a key regulator in chronic myeloid leukemia (Griner and Reuther, 2010, Ito et al., 2010). In relation to the CNS, Musashi-1 is ectopically expressed in a large majority of neurons exhibiting neurofibrillary tangles or Pick bodies (Lovell and Markesbery, 2005). Finally, *Musashi* KOs show phenotypes similar to FMRP-deficient animals (Sakakibara and Okano, 1997, Sakakibara et al., 2002). Collectively, these findings suggest an important role of Musashi in normal development and the pathogenesis of many neurological and psychiatric disorders.

F. Modulation of HuD activity by Neurotrophic factors

HuD expression has been described as being downstream of well-described signaling pathway molecules as well. For example, in SH-SY5Y cells, for example, neuron specific Elav proteins (HuB, C and D) were found to be positively regulated and recruited by Protein Kinase C (PKC) upon pharmacological stimulation of this protein using Daceylglycorol analogs (Pascale et al., 2005). Other studies have shown that PKC-induced neurite outgrowth is dependent upon HuD, particularly because of its stabilization of the *GAP-43* transcript (Mobarak et al., 2000). In this study, NGF was used to stimulate PKC activation in PC12 cells, which normally results in neurite outgrowth. IN the absence of HuD, however, GAP-43 levels were reduced and

NGF did not induce neurite outgrowth, illustrating HuD's position in a well-described neurotrophin signaling pathway, discussed in detail below. Another well-described signaling protein, Akt, has also been implicated in modulating HuD expression. Akt signaling is functionally associated with HuD's linker region and is required for HuD-induced translation and reads out functionally as neurite outgrowth in PC12 cells (Fujiwara et al., 2011). Furthermore, in the presence of a dominant negative form of Akt which fails to bind to HuD, PC12 cells do not exhibit outgrowth. Given that PKC, NGF and Akt, all molecules known to reside within the Neurotrophin signaling pathway, it is unsurprising that HuD has also been linked to BDNF-induced neurite outgrowth in cultured hippocampal neurons (Abdelmohsen et al., 2010). These findings show that HuD is again a post-transcriptional target, this time of miR-375, which targets the 3'UTR of HuD and degrades the transcript. When miR-375 was overexpressed in primary hippocampal neurons, HuD levels were diminished and BDNF failed to induce neurite outgrowth. Therefore, HuD and Neurotrophin function are mechanistically linked.

An introduction to the biological significance and function of neurotrophin signaling-

The class of proteins known as Neurotrophins is a highly homologous family of secreted molecules around 13 kDa in size which have been implicated in a host of processes. Their discovery began as a series of experiments in which excised tumors were found to excrete a factor into medium which influenced the positive outgrowth of spiral ganglion explants (Stanley Cohen, 1954, Cohen, 1956, Levi-

Montalcini, 1956, Cohen and Levi-Montalcini, 1957). These seminal experiments identified the first of the known proteins in this family, Nerve Growth Factor (NGF). These early investigations were the subject of the 1986 Nobel prize in medicine and began a cascade of knowledge which has added seminally to the concepts of trophic support, cell survival, cell outgrowth and stem cell differentiation (Levi-Montalcini, 1987, Moses V, 1992, Hory-Lee et al., 1993, Cohen A, 1994, Segal et al., 1995). The remaining members of this family have since been discovered and classified (in order): Brain Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3) and Neurotrophin-4 (NT-4). The knowledge of these proteins' activity has grown vastly in the interceding years, and it is now know that neurotrophins are the most upstream members of an intricate and diverse array of downstream signaling effectors.

An essential mediator of all biological activity of the neurotrophin family is binding to a surface receptor. The primary class of these surface receptors are known as the "tropomysin-receptor-kinase" family and are named for its binding to the tropomysin molecule in human colon carcinoma cells (Martin-Zanca et al., 1986). This family of receptor tyrosine kinases was subsequently characterized in 1991, again in the context of cancer (Barbacid et al., 1991). There are three members of this well characterized family that have preferential binding for each of the neurotrophins. TrkA, the first discovered receptor, preferentially binds NGF, whereas TrkB has a higher affinity for BDNF and NT-4, and TrkC for NT-3 (Kaplan DR, 1991, Bibel and Barde, 2000, Huang and Reichardt, 2003). While this is certainly not an exhaustive list of the binding receptors for these widely-

expressed trophic factors, they are representative of the most widely-characterized. Trk receptors are highly homologous and have several key biochemical features. The most characteristic feature of this family of receptor is the intracellular kinase domain. This is followed by a transmembrane domain and an extracellular domain with characteristic leucine motives and two immunoglobulin-like domains (Schneider R, 1991). Ligand binding to the extracellular domains influences activation of the intracellular kinase domains through receptor dimerization which enables the receptor to recruit adapter proteins for downstream signaling (Stephens et al., 1994, Huang and Reichardt, 2003). Additionally, the $P75^{NTR}$ receptor binds to each of the neurotrophins and serves to modulate the signaling sensitivity of the Trk receptors, affects axonal outgrowth and has cell survival consequences, both pro and anti apoptotic (Clary and Reichardt, 1994, Mukai et al., 2000, Roux et al., 2001).

The cascade of signaling consequent to Trk receptor activation is not fully understood. Broadly, Trk receptors become dimerized once a ligand binds to the extracellular domain of the protein. Then, the kinase domains become activated and recruit docking proteins, adaptor proteins and scaffolding proteins in order to activate various arms of a diverse downstream series of pathways modulating a wide array of cellular function. One such pathway is the well-described Phosholipase C stream, which functions to convert IP2 to IP3 which is released from the plasma membrane and becomes a discrete signaling molecule (Hisatsune et al., 2005). IP3 activates DAG which is implicated in adjusting intracellular calcium stores and activation of protein kinase C, one member

upstream of the well described MEK pathway resulting in multiple phosphorylation targets and gene transcription through phosphorylation of CREB (Stephens et al., 1994, Van Der Hoeven et al., 2000, Carles GIL1, 2003). NGF signaling involving PLC is known to induce neuronal differentiation as well as to maintain long-term potentiation in hippocampal neurons (Obermeier A, 1994, Minichiello et al., 2002). This is particularly interesting because it is an example of Trk signaling in an adult process, while most data for the Trk family is developmental in nature. The TrkB receptor also signals to PLC through a phosphorylated docking site and adapter protein to influence the proper migration and lamination of neocortical neurons and oligodendrocites (Medina et al., 2004).

Other arms of signaling downstream of Trk are PI3 kinase, which signals to other proteins including the well described Akt, which itself activates mTOR through inhibition of the TSC1/2 complex and facilitates cell growth (Obermeier A, 1994, Inoki et al., 2002, Zhang et al., 2003). Another consequence of this signaling pathway is the activation of 14-3-3 which serves to phosphorylate and inactivate Bad, an apoptotic protein, promoting cell survival (Zhang et al., 1999). It is interesting to note that 14-3-3 also signals to PLC, demonstrating the complexity of assessing discrete portions of Trk signaling (Van Der Hoeven et al., 2000). Furthermore, the aforementioned $P75^{NTR}$ receptor also activates Akt, promoting its activation and reducing apoptosis (Roux et al., 2001). There are some data to include this pathway in the guidance of growth cones of Xenopus axons responding to gradients of BDNF (Markus et al., 2002, Ming et al., 2002) . Another clever study dissected the contribution of PI3 to axonal outgrowth by

transfecting dominant negative forms of signaling proteins downstream of PI3 kinase into spinal cord neurons lacking the pro apoptotic factor Bax (Markus et al., 2002). This study cleverly demonstrated that NGF signaling required Akt and downstream members of this signaling cascade in order to produce 4 fold increases in axonal outgrowth.

Even as there is a diversity of downstream signaling venues in the Trk pathway, virtually any discussion of neurotrophin signaling to the Trk receptors will involve their best characterized role as trophic factors. Trk signaling has been implicated in neuronal proliferation as well as neurite outgrowth, differentiation as well providing trophic cues to prune neuronal redundancies after initial bursts of developmental neuronal expansion (Moses V, 1992, Hory-Lee et al., 1993, Cohen A, 1994, Obermeier A, 1994, Segal et al., 1995, Heinrich et al., 1999, Zhang et al., 1999, Bibel and Barde, 2000, Zhang et al., 2000, Markus et al., 2002, Medina et al., 2004). This work also implicates Trk signaling in multiple tissue types, in both the peripheral and central nervous systems mediating diverse processes even within a tissue subtype (such as proliferation and differentiation in the neocortex). As noted above, however, there are roles for Trk signaling in the developed brain including signaling and signal potentiating (Clary and Reichardt, 1994, Minichiello et al., 2002). Given the diversity of function of Trk receptors, clearly there is need for better clarity the fidelity of this signaling at the receptor level.

Figure 3 1

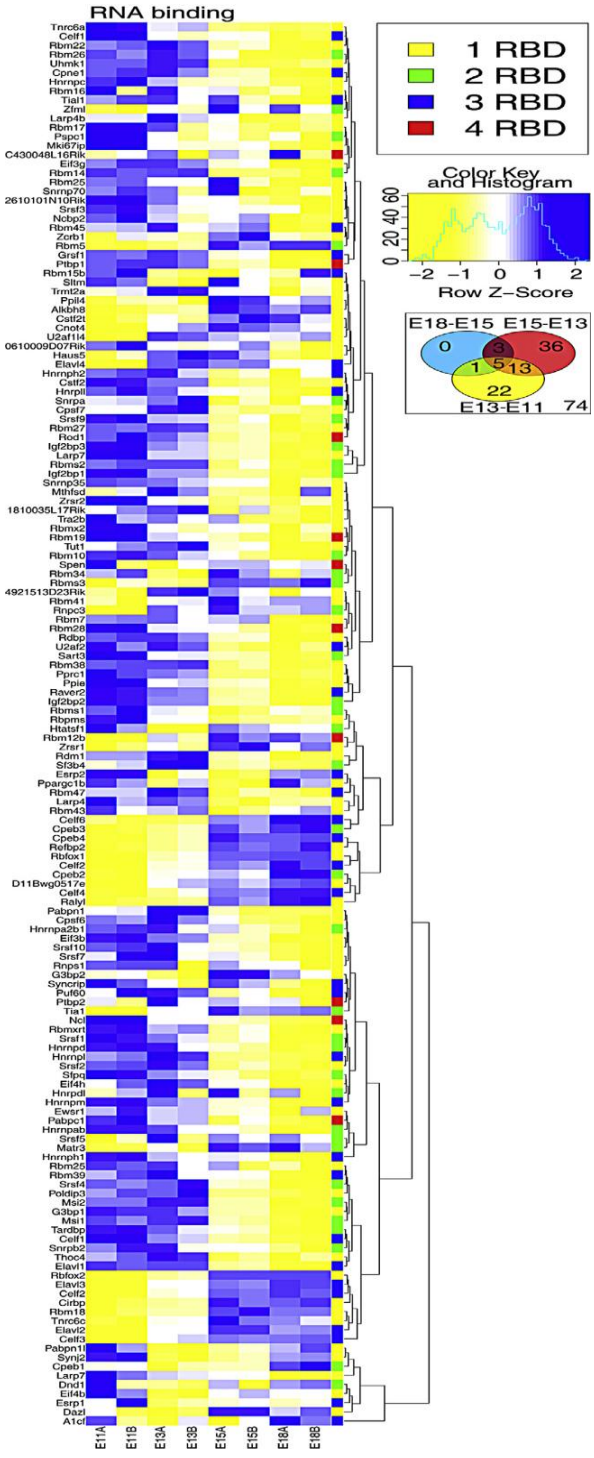
RNA binding
1 RBD
2 RBD
3 RBD
4 RBD
Color Key and Histogram
0 20 40 60
−2 −1 0 1 2
Row Z−Score
E18-E15 E15-E13
0
3
36
1
5
13
22
E13-E11
74
E11A
E11B
E13A
E13B
E15A
E15B
E18A
E18B

Fig. 3-1. Transcriptome analysis of the developing mouse neocortex for RNA binding proteins (RBPs). (left) GO analysis for RBPs revealed their substantial enrichment in developing neocortices, again with a predominant switch in expression levels occurring around E15. Each RBP has one or more RNA binding domains (RBDs) like KH, piwi or anRNA recognition motif (RRM). Total number of RBDs per RBP are presented with the color key (upper right corner).

Figure 3 2

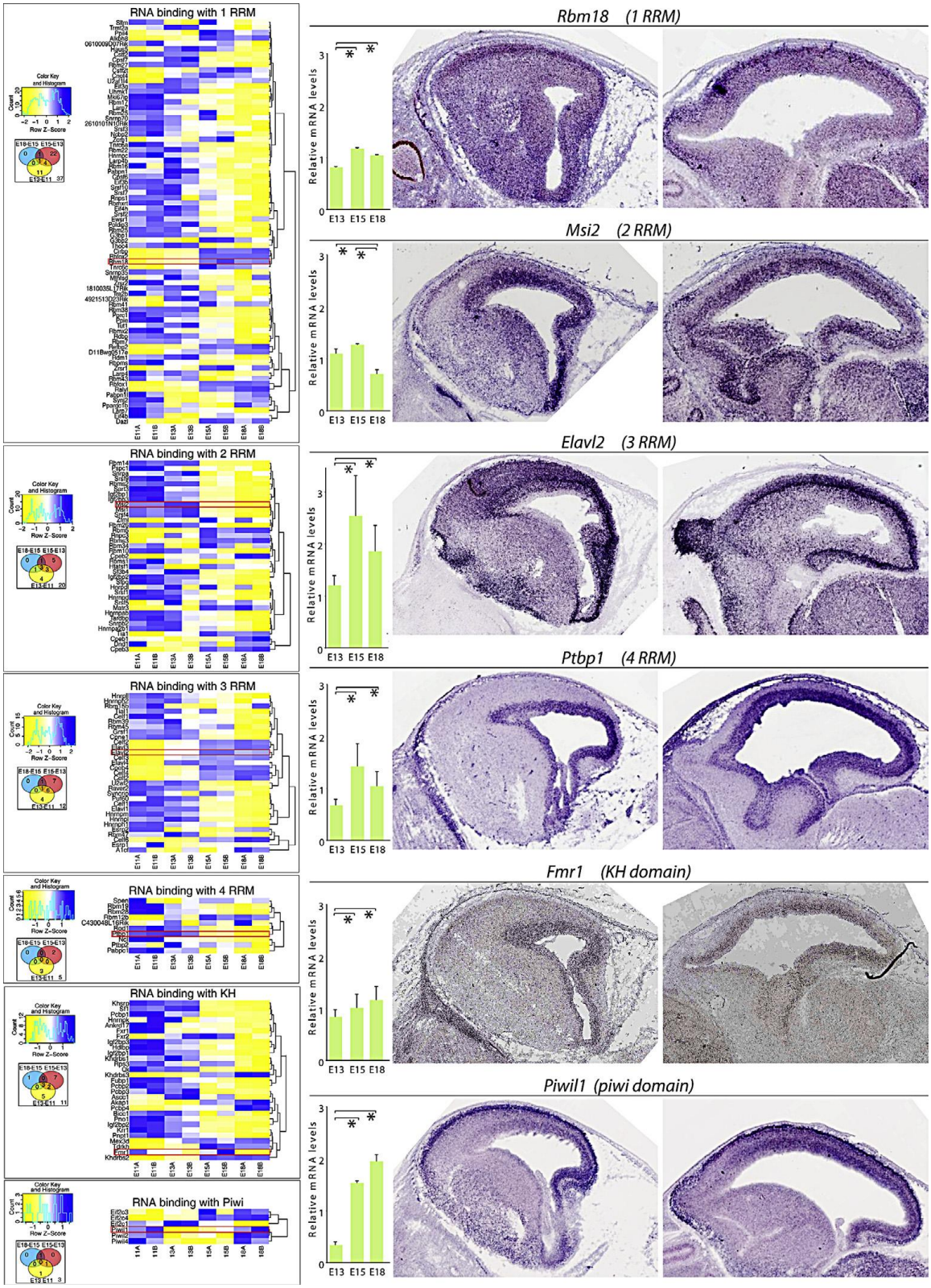
RNA binding with 1 RRM
RNA binding with 2 RRM
RNA binding with 3 RRM
RNA binding with 4 RRM
RNA binding with KH
RNA binding with Piwi
Rbm18 (1 RRM)
Msi2 (2 RRM)
Elavl2 (3 RRM)
Ptbp1 (4 RRM)
Fmr1 (KH domain)
Piwil1 (piwi domain)
Relative mRNA levels
E13 E15 E18
Color Key and Histogram
Row Z-Score
E11A E11B E13A E13B E15A E15B E18A E18B

Fig. 3-2. Transcriptome analysis of the developing mouse neocortex for RBPs with RRM, KH or piwi domains. (Left) RBPs with distinct RBD signatures show differences in their number and temporal expression. For example, RBPs with one RRMs are the most numerous group. Corresponding Venn diagrams are provided below the heatmap key. (Right) qRT-PCR and in situ hybridization from www.genepaint.com revealed dynamic spatiotemporal expression of distinct RBPs. One example for each RBP group from the heatmap on the left is presented. Ptbp1, which is characterized by four RRM domains is highly enriched in the VZ, while Rbm18 characterized by one RRM seems to be in postmitotic neurons of CP, and some signal is detected in the migratory zone between VZ and CP. Piwil1 shows high signal in the VZ and CP, but is somewhat decreased in the anterolateral VZ. All qRT-PCR values were again normalized to four housekeeping genes Gapdh, Mrps6, Rps13, and Rps18, then scaled to average. $p<0.05$.

Chapter 4: Prenatal depletion of HuD Disrupts Cortical Circuit Formation and Behavior

A. Introduction:

Proper functions of the neocortex and hippocampus are required to carry out spatial learning, memory and complex motor activities (Bystron et al., 2008, Lui et al., 2011, Arnsten, 2013). This functionality is established during an intricate developmental process when excitatory glutamatergic neurons within these regions are born, specified into functionally-distinct subpopulations, and organize themselves spatially (Bystron et al., 2008, Kriegstein and Alvarez-Buylla, 2009, DeBoer et al., 2013). Once positioned, neurons create axonal connections with targets either proximal or very distal, while also developing complex arbors of dendrites to receive signals from other neuronal afferents. Glutamatergic projection neurons typically exhibit a pyramidal cell body shape, a single apical dendrite which may branch several times before its terminal tuft, as well as an array of basal dendrites, all of which extend spines to receive input. Signaling from axonal afferents and neocortical circuit functions are, therefore, greatly dependent upon the ability of appropriately positioned neurons to receive input through properly-developed dendrites and spines. Disruptions in this process create aberrations in final circuitry, and ultimately undermines the function of these regions resulting in cognitive and motor deficits as well as seizure (Melzer et al., 2012). Similarly, perturbations in dendrite and spine morphology are hallmarks of many human disorders such as epilepsy and autism spectrum

disorders including fragile X (Kitaura et al., 2011, Anderson et al., 2012, Clement et al., 2012).

In order for the largely asymmetrical and polar neurons of the neocortex and hippocampus to develop properly, mRNA important for dendritogenesis must be transported and locally translated (Zivraj et al., 2010, Donnelly et al., 2013). As such, the maturation of dendrites may be mediated by RNA binding proteins (RBP) which bind RNA and mediate transcript metabolism (Akamatsu et al., 2005, Keene, 2007, Ince-Dunn et al., 2012, DeBoer et al., 2013). A large body of work has implicated Hu antigen D (HuD), a uniquely brain-expressed RBP, in neurite outgrowth *in vitro* (Dobashi et al., 1998, Aranda-Abreu et al., 1999, Anderson et al., 2000, Mobarak et al., 2000, Anderson et al., 2001, Abdelmohsen et al., 2010). For instance, in cultured PC-12 cells and hippocampal neurons, HuD silencing resulted in decreased growth of dendrites; the main recipients of axonal afferents (Aranda-Abreu et al., 1999, Akamatsu et al., 2005, Abdelmohsen et al., 2010). Further, genetic mutations in HuD were associated with movement disorders in human (Noureddine et al., 2005, Haugarvoll et al., 2007, DeStefano et al., 2008), and *HuD* depletion in a rodent model resulted in deficiencies in rotorod tested motor performance (Akamatsu et al., 2005). The role of HuD in the establishment and maturation of dendritic arbors in neocortices *in vivo* and the impact this has on cortical circuitry, however, has not been investigated.

Therefore, using a mouse loss-of-function model, we evaluated the impact of early HuD depletion on the specification, arborization and function of neurons

in the adult neocortex and hippocampus. Our findings below demonstrate HuD's specific role in the identity and differentiation of a subpopulation of cortical glutamatergic neurons which underlie cognition, spatial memory and appropriate circuit function.

B. Experimental Methods

Subjects:

HuD WT and KO mice were bred as littermates from heterozygous parents described previously (Akamatsu et al., 2005). HuD-GFP reporter mice were purchased from genesat.org. We analyzed mice at P28 and P90 using a Golgi method for dendrites; all other analyses were performed at P60-P90. All studies were run blind with respect to subject genotype. Genotyping was performed as described (Akamatsu et al., 2005). All procedures were in compliance with Rutgers University Robert Wood Johnson Medical School IACUC protocols. 72 mice of either sex were used for analysis.

Immunohistochemistry:

Experimental sections were collected and perfused with 150 ml 4% PFA (pH = 7.4) and post-fixed up to 24 hours at +4^{o}C. Fixed brains were sectioned coronally using a Leica vibrotome at 70 µm. Immunohistochemical experiments were performed as described . Primary antibodies used in dilution: 1:100 Rabbit anti Cdp (SCBT M22 Cat# SC-13024), 1:250 Mouse anti-Tle4 (SCBT E10 Cat# SC-365406), 1:1000 Chicken anti-GFP (Aves Cat# GFP-1020), 1:1000 rabbit anti GFAP (Abcam Cat# ab7260), 1:100 Mouse anti CC1 (Calbiochem Cat# OP80),

1:100 Mouse anti Parvalbumin (Swant Cat# 235), 1:250 mouse anti NeuN (Millipore Cat# Mab377) and 1:250 mouse anti Gad67 (Millipore Cat# MAB5406). Secondary antibodies, Cy2, Cy3 and Cy5 used 1:250 (Jackson Immunoresearch). Confocal imaging was performed using FV1000MPE Olympus using 10x and 60x Olympus objectives.

Cell Specification Analysis:

Confocal images of immunostained cortical plate of WT and *HuD* KO 70 µm coronal sections were taken. The cortical plate was then equally subdivided into columns of 10 virtual bins from Layer II (bin 1) to subplate (bin 10). Total DAPI+ nuclei were counted, as well as Cdp+ or Tle4+ neurons and NeuN+ neurons. Analysis was performed by counting total number of DAPI+ cells in each column of 10 bins. The proportion of each column's DAPI+ cells that were either Tle4+, Cdp+ or NeuN+ was noted for each bin. Three columns were counted per brain, and means were compared for each bin between genotypes using a student's t-test. Equal rostral caudal levels were chosen for analysis using the Allen Brain Atlas. N=4 per genotype. $P<0.05$ was considered significant.

Golgi Staining and Analysis:

HuD WT and KO brains were taken from P28 and P90 mice and processed as per the manufacturer's protocol (FD Neurotechnologies rapid Golgi kit). Fully processed brains were sectioned at 270 µm and coverslipped as per manufacturer's protocol. Z-stack images (2 µm step) were taken of 5 upper and 5 lower layer neocortical projection neurons from the middle of the slice as well as 5 CA3 pyramidal neurons per brain N=3 brains per genotype per age. All

images were taken using a Leica DMRE brightfield microscope with openlab software. Multi-tiff Z-stack images of neurons were reconstructed using off-site Neurolucida software and analyzed as described (Rasin et al., 2011).

qRT-PCR:

Cortices were manually dissected from E15, P7 and P90 mice, and hippocampi were manually dissected from P7 and P90. Whole RNA was extracted using the RNeasy kit from Qiagen. Resulting RNA was DNAsed using Ambion Turbo DNAse kit. qRT-PCR was performed using the Invitrogen onestep qRT-PCR thermocycler. HuD mRNA quantified using custom TaqMan® PN 4331348 probe order AIRR9ZJ and normalized to Gapdh Mm99999915_g1 TaqMan® probe using TaqMan® RNA-to-CT™ 1-Step Kit. N=2 brains per age.

Electroporation and Cell Culture:

In-utero electroporations were performed using control shRNA and HuD shRNA plasmids (origene sku TG501025) co-electroporated with CAG-RFP or CAG-GFP respectively as described at E13.5 (Rasin et al., 2007). Electroporated pups were allowed to gestate for 4 hours before they were removed from the dam and the transfected neocortices were manually dissected (Figure 4-6). Dissected cortices were then dissociated and cultured for either 1 or 3 days in-vitro (DIV) on laminin and poly-L-ornithine plates using Neurobasal media supplemented with B27, glutamax, sodium pyruvate and Penicillin-Streptomycin as described (Sestan et al., 1999). After 1 or 3 DIV, primary cultures were fixed in 4% paraformaldehyde, imaged with confocal microscope and reconstructed

using Neurolucida software as above. 1 DIV Ctrl N=5, 1 DIV *HuD* shRNA N=8, 3 DIV Ctrl N=5 3 DIV *HuD* shRNA N=4.

Motor Activity:

Mice were placed into one of two Behavioral Spectrometer chambers (Behavioral Instruments, Hillsborough NJ). Each chamber consisted of a 40 cm by 40 cm arena equipped with floor and wall sensors. All vibration, locomotor and rearing movements were captured by acceleration, weight and photo-beam sensors (respectively). Sensor readings were recorded, analyzed and compared to a master list of 23 built-in behaviors. Categorization of behavior was achieved using a best-fit procedure such that every second of the session was scored (http://www.behavioralinstruments.com/). Mice were placed in the center of the arena and data was collected for 30 minutes. Between sessions the chambers were wiped with 70% ethanol. Behavioral data collected from the Behavioral Spectrometers was subjected to a two-way ANOVA (behavioral category repeated) with genotype and behavioral category as the two factors. *Post-hoc* comparisons between genotypes on each behavior were made using a Student-Newman-Keuls procedure; $P<0.05$ was considered significant.

Water Maze:

The water maze consisted of a circular tub 71 cm in diameter and 29 cm in height, painted white on the interior and filled ¾ full with water maintained at 23-26°C and made opaque with white non-toxic latex paint. A starting point was determined randomly from one of four equally-spaced quadrants. For visible

platform testing, an 8.0 cm diameter black platform was placed in one quadrant of the maze with the platform 1.5 cm above the surface. This procedure was repeated 5 times, and a mean of each trial was generated for each genotype (N=5 WT and 8 KO). In the hidden platform, an identical platform painted white sat 2 cm below the surface of the water. Animals received five trials each day and each animal was allowed a maximum of 60 seconds to reach the escape platform. A total of four trial days were performed. The position of the hidden platform remained constant throughout the experiment and the room was illuminated and extramaze cues were present. If the animal did not reach the platform in 60 seconds, a score of 60 was recorded and the animal was gently guided to and placed on the platform. During the intertrial interval of 30 sec, all animals rested atop the platform until the next trial began. Mean latency times were collected for each genotype and compared using a one way ANOVA and $P<0.05$ was considered significant. N=5 WT and 8 KO.

Elevated Plus Maze:

The elevated plus maze consisted of two long closed arms (65 cm long and 8 cm wide), two short open arms (30 cm long and 9 cm wide), and a central square of 5 cm by 5 cm; the maze was 60 cm above the floor. Each mouse was placed in the center square and observed for 10 minutes. The number of times the animal crossed into a closed arm or an open arm was recorded as was the total time in each arm. The maze was washed with 70 % alcohol between trials. Mean proportion number of seconds and total proportion of time spent in each arm was collected for each genotype and compared using a student's t-test.

Seizure:

Seizure susceptibility was assayed in mice individually housed in their home cage using a 30 second metallic, auditory stimulus of 60 decibels was generated 6 inches above the top of the cage. Convulsion was noted if tonic-clonic seizure was provoked. Proportion of animals experiencing seizure was compared using a standard Z-test.

C. Results:

HuD is expressed in mature hippocampal and cortical neurons

Largely *in vitro* work has demonstrated HuD as a marker of differentiated neurons (Szabo A, 1991, Lee et al., 2003, Darsalia et al., 2007). To address whether HuD is involved in neuronal subtype specification and circuit functions in adults *in vivo*, we first assessed specificities in HuD's expression in two cortical regions rich in diverse neuronal subtypes which are implicated in learning, memory and higher cognitive functions; the hippocampus and neocortex. To do this, we obtained an HuD-GFP mouse; a transgenic line which expresses Green fluorescent protein (GFP) under the control of the HuD promoter. Through immunohistochemical analysis at postnatal day 28 (P28), we found that GFP is not expressed in the Cdp+ upper-layer intracortically projecting neocortical neurons (layers II-IV), whereas expression is robust in Tle4+ subcortically projecting lower layer neurons (Figure 4-1A-E). This cortical expression pattern was confirmed through DAB staining for GFP in the HuD-GFP transgenic (Figure 4-1F, F' and F'') (Gong et al., 2003). Upon investigation at 60x magnification, we found colocalization at 60±10.5% of HuD+ for Tle4+ (layers V and VI), and

46.6±6.7% of Tle4+ neurons were HuD+ in subcortically projecting lower layer neurons (Figure 4-1G). These findings suggest that HuD may be a novel molecular marker for a subpopulation of lower layer neurons in adult neocortices.

Next, we assessed HuD-GFP expression in mature hippocampal regions CA1-3 as well as the dentate gyrus (Figure 4-2A-D). We found robust expression in all of these hippocampal regions' cell bodies consistent with the location of glutamatergic neurons. Given that we found HuD expression in Cdp+ and Tle4+ neocortical primary neurons, we next assessed if HuD is expressed in glutamatergic, excitatory neurons and not GABAergic interneurons (DeFelipe et al., 2013). To this end, we performed immunohistochemistry with antibodies detecting glutamate decarboxylase (Gad67), as well as the calcium binding protein parvalbumin. These proteins identify all GABAergic interneurons and subset of interneurons, respectively. We found no colocalizations of either marker of interneurons with HuD-GFP in the neocortex or hippocampus (Figure 4-2E-L). Further analysis of HuD expression with CC1, a marker of mature oligodendrocytes, and glial fibrilary acidic protein (GFAP), a mature astrocyte marker also yielded no colocalizations (data not shown). Therefore, these data indicate that HuD is expressed robustly in a subpopulation of glutamatergic excitatory neurons of lower neocortical layers and the hippocampus.

HuD is critical for the balanced expression of Tle4 in lower layer neocortical projection neurons

As the neocortex contains a multitude of neuronal subtypes, the specification and maintenance of neuronal identity is critical to the laminar structure of the

neocortex as well as its function (Arlotta et al., 2005, Guillemot, 2005, Molyneaux et al., 2007, Bithell et al., 2008, Shoemaker and Arlotta, 2010, Kwan et al., 2012b, DeBoer et al., 2013). Previous findings indicate that HuD deletion induces cell death in NSC's as well as cell identity changes *in vitro* (Akamatsu et al., 2005). *HuD* KO mice, however, survive to adulthood and are viable. Therefore, we aimed to investigate the role of *HuD* depletion from the earliest developmental period on the identity of adult neocortical projection neurons. To do this, we bred *HuD* KO mice and performed immunohistochemical analysis of neuronal subtypes identified by Cdp and Tle4, (Figure 4-3A-B) and quantified their distribution in 10 equal bins from layer II (bin 1) to the subplate (bin 10). Upon analysis of the proportion of cells (DAPI+) in each bin that were either Tle4+ or Cdp+, we found that there was a significantly lower proportion of DAPI+ cells which were Tle4+ in all but 1 of the bins from bin 6-10, which correspond to lower neocortical layers (mean proportions 0.006, 0.016, 0.041, 0.043, 0.045 for WT respectively vs. 0.004, 0.008, 0.03, 0.037, 0.031. P=0.043, 0.005, 0.03, 0.223 and 0.01) (Figure 4-3C). Conversely, we found a modest upregulation of Cdp+/DAPI+ cells in the *HuD* KO brains only in bin 5, which is a transition between upper and lower neocortical layers generally without robust expression of either marker (mean proportion= 0.009 for WT and 0.003 for KO, P=0.006) (Figure 4-3C). No differences in total DAPI+ number were observed. When the total proportion of Cdp and Tle4+ cells in each column were considered, we found a slight but insignificant increase in proportion of Cdp+ cells in the KO cortex, and a significant downregulation of Tle4+ cells (mean proportion= 0.456

0.325 for WT and KO respectively, P=0.0004) (Figure 4-3D-E). Investigation using a pan-neuronal marker, NeuN, revealed no changes in NeuN+/DAPI+ neurons per neocortical bin or total neocortical NeuN+/DAPI+ neurons (Figure 4-3 F and G and data not shown). These results suggest that HuD is involved in the specification and/or maintenance of a subpopulation lower layer neocortical neurons that predominantly project subcortically, which may impact the function of this portion of the neocortical circuit.

Dendritogenesis at P28 is impacted in the hippocampus and lower, but not upper neocortical layers of the HuD KO

Previous studies have implicated HuD in neurite outgrowth *in vitro* (Chung et al., 1997b, Dobashi et al., 1998, Aranda-Abreu et al., 1999, Anderson et al., 2000, Mobarak et al., 2000, Anderson et al., 2001, Pascale et al., 2004, Smith et al., 2004, Fukao et al., 2009, Abdelmohsen et al., 2010). To assess if HuD deletion disrupts cortical dendritogenesis *in vivo*, we performed a quantitative Golgi analysis on P28 WT and *HuD* KO neurons of lower and upper neocortical layers as well as the CA3 region of the hippocampus. Previous investigation has demonstrated that HuD is involved in circuit formation and function in CA3 (Figure 4-4A and D) (Tanner et al., 2008, Perrone-Bizzozero et al., 2011). At P28, dendritic arbors are nearly fully formed but the young animals have had very little exposure to confounding variables which modify dendritic arbors, such as handling, social activity and sexual maturity (Gibb and Kolb, 2005, Pitchers et al., 2010). Using 3D reconstruction with Neurolucida software in double blind fashion, we found decreased dendritic complexity in the lower cortical layers

(layers V and VI) in *HuD* WT and KOs (Figure 4-4B,C,E and F). Within these data, we found that a proportion of neurons show similar branching between WT and KO, while a subset of neurons showed significant differences. When we analyzed the entire quantified population as a group we found that lower layer neurons had fewer basal branch points (mean WT=9.067 branches, SE=2.4 vs. KO=6 branches, SE=1.6; p=0.03), had fewer branch endings (mean WT=16 vs. KO=12.7 P=0.05), and basal dendrites were shorter in total length (mean WT=4193 µm, SE=948 vs. KO=247 8µm, SE=211; p=0.029). Apical dendrites of lower layer neurons were also affected and had fewer branch points (mean WT=3.3 branches, SE=0.506 vs. KO=1.8, SE=0.381; p=0.01), had fewer dendritic endings (mean WT=11.7 vs. KO=9.07 P=0.04), and were shorter in total length (mean WT=3566 µm SE=403 vs. KO=2031µm, SE=279; p<0.01) (Figure 4-4A and C). The CA3 region of the hippocampus showed decreased differentiation similar to the lower neocortical layers where CA3 neurons had fewer basal branch points (mean WT=9.73 branches vs. KO=4.6 P=0.007), had fewer dendritic endings (mean WT=13.5 vs KO=7.8 P=0.01), and were shorter in total length (mean WT=4127 µm vs. KO=1694 P=0.0005) (Figure 4-4D, E and F). Apical dendrites of CA3 neurons were also affected, and exhibited fewer branch points (mean WT=9.8 vs. KO=4.6 P=0.0007), had fewer dendritic endings (mean WT=10.8 vs. KO=5.67 P=0.001), and were shorter in total length (mean WT=4436 µm vs. KO=2048 P=0.0002). Interestingly, upper neocortical layers as a group were comparatively unaffected and had similar numbers of basal branch points (mean WT= 7.3, SE=1.16 vs. KO mean=8.4, SE=1.14; p=0.22), basal

branch length (mean WT= 3765 µm, SE=376 vs. KO mean=3720 µm SE=518; p=0.047), apical branch points (mean WT= 3.133, SE=0.68 mean vs. KO= 4.5, SE=0.85; p=0.082), and apical branch length (mean WT= 3145 µm, SE=403 vs. KO mean= 3500 µm, SE=451; p=0.26) (data not shown). These data indicate that constitutive loss of *HuD* early in development has a pervasive effect on the establishment of cortical circuits, particularly on dendritic arborization in a subpopulation of neurons in the adult neocortex and hippocampus.

Dendritic morphology deficits in the hippocampus persist in the adult *HuD* KO

We followed our P28 dendritic morphology analysis with an assessment of the P90 adult neocortex and CA3 hippocampus. At this age in we sought to determine if the deficits we found in developing dendritic arbors persisted in the mature, behaving animal. To this end we again performed quantitative Golgi on P90 animals and reconstructed neurons in the CA3 hippocampus and lower neocortical layers (Figure 4-4A and D). Our findings in the neocortex indicate that overall basal and apical length decreased from P28 to P90 in WT animals (to 69 and 72% of P28 length, P=0.06 and 0.002 respectively) which is consistent with previous findings of a lifespan of dynamic changes in dendrite length (Metzger, 2010). KO lengths however, did not change discernibly (P90 length= 92 and 90% of P28 length of basal and apical dendrites, respectively). Neocortical arborization in lower layers at P90 did not reach significance for the metrics of apical dendritic length (mean WT=11.6 SE=0.76 vs. KO=11.3 SE=0.89 and P=0.4), dendritic branches (mean WT=2.8 SE 0.4 vs KO=2.4 SE=0.4 and

p=0.29), or dendritic endings (mean WT=11.6 SE=0.76 vs. KO=11.3 SE=0.89 and p=0.4) (Figure 4-4B). Basal branching was persistently deficient in neocortical lower layer neurons (mean WT=7.7 SE=0.97 vs. KO=5.4 SE=0.59 and P=0.03), and while other indices showed some reduction, significance was not reached in basal length (mean WT=2906 µm, SE=300 vs. KO=2696 µm, SE=228; p=0.28), or basal branch endings (mean WT=13.9 SE 1.23 and KO=12.2 SE=0.67 and P=0.1) (Figure 4-4C). These data suggest that HuD is required for the expansion/overgrowth of dendrites during early postnatal development in mice.

Analysis of the CA3 hippocampus at P90 showed no significant change in the apical region for branch length (mean WT=1923 SE=261 vs KO=2138 SE 343 and p=0.31), branches (mean WT=7.3 SE=1.2 vs. KO=7.8 SE=1.8 p=0.29) or branch endings (mean WT=10 SE=1.18 and KO=10.6 SE 1.7 and p=0.36) (Figure 4-4E). However, persistent defects in basal branch length (mean WT=5890 µm SE=716 vs. KO=3431 SE=457 p=0.004), basal branching (mean WT=19.1 SE=2.1 vs. KO=13.2 SE 1.8 p=0.02), and basal branch endings (mean WT=26 SE=2.2 vs. KO=18 SE=2 p=0.004) were noted (Figure 4-4F). In sµm, these findings demonstrate that HuD is involved in the development of apical and basal dendrites in lower neocortical layers and the hippocampal CA3 region, and that many of these deficits persist in the mature animal suggesting circuit deficits.

HuD **levels decrease from E15 to adult in the neocortex and hippocampus**

In order to ascertain the relationship between HuD expression and the selective arborization defects we discovered at P28 and P90, we next investigated the

developmental expression of HuD across the timeframe of our studies. Therefore, we accessed the gensat database for developmental confirmatory images of the HuD-GFP reporter mouse we characterized here (Figure 4-5A-C) (Gong et al., 2003). These images show HuD-GFP DAB signal decreases from E15 to P7 and becomes more regionally restricted to lower layer neurons (Figure 4-5A and B). HuD signal decreases again in the adult, and is restricted only to lower layers (Figure 4-5C). To confirm this expression, we accessed the St. Jude research hospital *in-situ* database and searched for *HuD in-situ* images complementary to the HuD-GFP DAB staining (Magdaleno et al., 2006). *HuD* mRNA expression closely resembled the HuD-GFP DAB pattern, particularly at P7 and adult (Figures A'-C'). It should be noted that the untagged GFP molecule is free to diffuse into any compartment of the cell, which may explain DAB signal in regions where mRNA is not detected. Subsequently, we quantitatively confirmed *HuD* mRNA expression decrease from E15 to adult by performing qRT-PCR for HuD at E15, P7 and adult in hippocampal and neocortical tissue (Figure 4-5D and E). Our results demonstrate a significant decrease in *HuD* mRNA from E15 to P7 in both hippocampus and neocortex (normalized to E15 neocortex SE=0.08; mean fold change to P7 neocortex=0.054 SE=0.001; mean fold change to P7 hippocampus= 0.034 SE=0.003 and $p<0.01$ for all measures). Closer examination of the relationship between P7 *HuD* expression and adult reveals a subsequent decrease in *HuD* expression from P7 to adult in either cortical region (normalized to P7 neocortex *HuD* expression (SE=0.03 fold), P7 hippocampus mean fold change=0.64

SE=0.05; mean fold change to adult neocortex=0.62 fold SE=0.05; and mean fold change to adult hippocampus=0.34 SE=0.004 and P<0.01 for all measures) (Figure 4-5E).

HuD depletion disrupts dendritic outgrowth in cultured neocortical neurons

We next asked how early HuD is required in dendritic arborization. In Figure 4-5A we found that HuD is highly expressed in the developing neocortical plate during neocorticogenesis in agreement with previous studies (Okano and Darnell, 1997, Gong et al., 2003, Magdaleno et al., 2006). Therefore, we performed in-utero electroporation of Ctrl shRNA/RFP or an efficient HuD shRNA/GFP plasmid at E13, when lower layer neocortical neurons are born (Figure 4-6A-B´) (Rasin et al., 2007, DeBoer and Rasin, 2013). We then dissociated the transfected neocortices and cultured them for 1 and 3 days before fixing and reconstructing the resulting, transfected neurons (Figure 4-6C-D). We found no significant difference in the number of dendrites per cell, or the length of dendrites at 1 DIV (Mean dendrites Ctrl = 3.4 ± 0.81 vs *HuD* shRNA = 2 ± 0.65, mean dendritic length Ctrl = 11.1 ± 2.31 vs *HuD* shRNA = 9.11 ± 2.13µm, Figure 4-6 E, G, I and K). While there was no significant change in dendrite number at 3 DIV, we noted a significant decrease in dendritic length when compared to control (mean dendrites Ctrl = 4 ± 1 vs HuD shRNA = 4 ± 1, mean dendritic length Ctrl = 88.92 ± 19.26 vs HuD shRNA = 28.25 ± 8.17µm P=0.016, Figure 4-6F, H, J and L). Therefore, silencing of HuD at E13.5 reduced neurite outgrowth significantly 3 days after transfection. These findings support that HuD is critically involved in the earliest stages of dendrite outgrowth in the neocortex.

HuD KO mice are less active than WT

To determine the effect of possible specification and circuitry deficits on the behavior of *HuD* KO mice, we employed a novel device which generates a broad, spectral analysis of HuD WT and KO littermates. The Behavioral Spectrometer reads photobeam breaks as well as vibrations of mice and extrapolates a multitude of behaviors. Figure 4-7 shows the effect of the KO manipulation on unconditioned behavior emitted by the mice in an open field. In general, the KO mice significantly apportioned more of their time in low energy expending activities (stationary) and less in the high energy activity of locomotion ($P=0.012$). Additionally, within the four categories of behavior (stationary, orienting, rearing and moving) the KO mice engaged significantly more ($p<0.05$) in relatively less energetic actions like “still”, “sniff”, “clean limb” and “shuffle” and significantly less ($p<0.05$) time in energetic actions like walking, running and trying to climb the walls (i.e. “rear_climb”). Typically, still behavior such as remaining prone or by freezing indicate an anxiety response, suggesting that *HuD* KO mice may have a greater propensity to anxiety-induced behaviors (Crawley, 1999, Lau et al., 2008).

HuD KO mice display abnormalities water maze and elevated plus maze

Previous research has demonstrated motor deficits in HuD KO mice, however cognitive deficits have not been assessed (Akamatsu et al., 2005). To determine if the deficiencies in hippocampal differentiation in Figure 4-4 affected the function of this circuit, we performed a behavioral analysis associated with this circuit, the Morris water maze. Previous work has demonstrated that HuD is

upregulated in the hippocampus after the Morris water maze challenge (Pascale et al., 2004). In this task, mice swim toward a platform in an opaque bath and use visual cues surrounding a circular tub in order to orient themselves within the opaque bath to find a platform hidden below the water's surface. First, we tested the ability of WT and KO mice to find and swim toward a visible platform (Figure 4-8A). By the 3rd of 5 trials, HuD KO mice swam to and mounted the platform as quickly as WT, and there was no significant difference between the genotypes. Subsequently, mice were challenged to find the platform when it was submerged (hidden trial). Here, we found that HuD KO mice took significantly longer to locate and mount the platform, especially in days 3 and 4 of this trail (WT means=13.6, 10.8, 6.8 and 5 seconds for trial days 1-4 respectively vs KO means= 17.1, 12.8, 13.2 and 9.4 seconds, P=0.02, 0.42, 0.02 0.03 for each trial respectively). (Figure 4-8B). These data indicate that HuD KO mice have difficulty learning how to orient themselves in the water maze environment, particularly as they performed more poorly in the later trials.

Hippocampal and neocortical circuits are also part of the limbic system and involved in anxiety (Packard, 2009). Therefore, we assessed the levels of anxiety in the *HuD* KO mice by employing the elevated plus maze. This maze contains an open arm (no walls) which mice avoid (Holt et al., 1988). Therefore, WT mice often spend most of the time in the closed portion of the maze. When compared to WT littermates, however, *HuD* KO mice spent a greater amount of the trial time in the open arm (mean WT=59 vs KO=118.5 seconds P=0.011 Figure 4-8C), as well as a greater proportion of the trial in the open arm(mean

WT=11.7% vs KO=27.3% P=0.041 Figure 4-8D). These data indicate an aberrant response to anxiety-producing environments after HuD depletion, or an inability to perceive the open arm as threatening.

HuD loss of function predisposes mice to auditory-induced seizure

Hu proteins have been previously implicated in governing total cortical glutamate levels and neuronal excitability by mediating the expression of glutamine synthase in the cortex (Ince-Dunn et al., 2012). Further, our previous findings demonstrate that HuD controls aspects of circuit formation in the neocortex and hippocampus, two areas heavily implicated in the generation of seizures . Therefore, we assessed if *HuD* KO mice were more susceptible to auditory-induced seizures than WT littermates. To this end we presented a metallic, auditory stimulus to KO animals and their WT littermates for 30 seconds (Halladay et al., 2006). Remarkably, 62.5% of KO animals responded with full-body convulsion, and 37.5% of those tested died subsequently (Fig. 4-9). In contrast, no WT animals experienced convulsion during this stimulus. These findings indicate that loss of HuD disrupts neuronal excitability *in vivo*.

D. Discussion

Upon initiating our study, we surmised that constitutive loss of HuD function would affect cortical circuit form and function in adults *in vivo*, and that this would read out as cognitive and behavioral deficits. Our findings support this theory, and demonstrate that HuD determines the identity of a subpopulation of deep layer projection neurons of the adult neocortex. Further, we have demonstrated that HuD is involved in the dendritic arborization of a subset of lower layer neocortical projection neurons, as well as those in the CA3 region of the hippocampus. These findings are in agreement with studies performed *in vitro* strongly implicating HuD in dendritogenesis processes (Dobashi et al., 1998, Mobarak et al., 2000, Smith et al., 2004, Bolognani et al., 2007, Tanner et al., 2008, Perrone-Bizzozero et al., 2011). However we also found in-vivo HuD is required for the appropriate dendrite overgrowth expansion phase in neocortical layers V and VI at P28, since by P90WT length decreased to KO levels. We found that these molecular and neuroanatomical changes are accompanied by learning deficits in Morris Water Maze, a hippocampal dependent task. We also noted that HuD KO mice are less anxious through our findings in the behavioral spectrometer and elevated plus maze experiments. These results demonstrate a possible global role for HuD in the specification of neuron identity as well as circuit formation of the central nervous system, starting from stem cells (Akamatsu et al., 2005).

The study of glutamatergic neuronal subtypes in the neocortex is a field of intense scientific interest given that they are functionally distinct and underlie the

multitude of complex neocortical functions (Molyneaux et al., 2007, Leone et al., 2008, Kwan et al., 2012b, DeBoer et al., 2013). As these studies progress, new molecular markers with increasing and overlapping specificity are desired to delineate the multitude of neocortical projection neuron subtypes. Our findings indicate that HuD is expressed in a subset of Tle4+ lower layer neocortical projection neurons. However, we found that HuD expression is more restricted to deeper layers than Tle4, and that colocalization between these two markers is only 60±10.5% (Figure 4-4-1). In this way, HuD-GFP could be particularly useful as a new molecular marker of a subpopulation deep layer cortical neurons in the adult neocortex. This pattern of expression is consistent with previous reports, where *HuD* mRNA is expressed more deeply in the cortex than *HuC* (Okano and Darnell, 1997, Magdaleno et al., 2006). It is possible that there are differences between our HuD reporter expression and HuD protein expression. However, this is unlikely given that previous findings at the mRNA level show similar expression. HuD is more ubiquitously expressed throughout the cortex in development and expression becomes more limited and regionally-specific in the adult. Our findings as well as previous data demonstrate a decrease in HuD expression from P7-Adult (Bolognani et al., 2007). Therefore, HuD may have active roles in the establishment and maturation of cortical circuits.

Interestingly, our analysis showed specificity in HuD effects on cortical neurons. We found that only the dendritic arborization of a subset of lower neocortical layers were affected by HuD loss of function. Newly formed dendrites will expand and overgrow due to growth factors, which will later be fine tuned by

activity to decrease the length (Metzger, 2010). Our data demonstrate that lower neocortical dendrites fail to efficiently extend dendrites at P28 in the KO when compared to similar aged WT littermates. When analyzed again at P90, *HuD* KO dendrites are of similar length to P28 KO, whereas WT dendrites of P90 animals have retracted significantly. Early overgrowth followed by a subsequent retraction period is a known phenomenon in neocortex, and may be a critical phenomenon for the formation of balanced, mature cortical circuitry (Judaš et al., 2003, Metzger, 2010, Petanjek et al., 2011), which may be evolutionary also adapted (Petanjek et al., 2008). Our data also demonstrate pervasive deficits in neuronal complexity in both lower neocortical layers and the hippocampal CA3 subregion of *HuD* KOs. In this way, HuD may have a dual effect on normal process of circuitry establishment as well as circuit maintenance in the neocortex and hippocampus. To our knowledge, HuD is the first RBP to be described as having a specific role in initial dendritic overgrowth in the neocortex. Given the extensive literature on reciprocal maturation of brain circuits, these defects may also extend to circuit development aberrations and functional deficiencies in regions afferent to the neocortex or hippocampus.

Previous work has demonstrated that the molecular players in the differentiation of these two neocortical subregions are different (Chen et al., 2005b, Chen et al., 2008, Gyorgy et al., 2008, Cubelos et al., 2010, Srinivasan et al., 2012). Although the mechanism of HuD and many of its targets have been elucidated, HuD binds promiscuously and likely mediates the metabolism of a multitude of transcripts which coordinately carry out neuronal maturation in the

cortex (Ince-Dunn et al., 2012). HuD is also known to be involved in several stages of mRNA life, from nuclear transport, subcellular transport, stabilization and translation (Kasashima et al., 1999, Bolognani et al., 2007, Fukao et al., 2009, Fallini et al., 2011). Further, given our data that HuD is involved in the process of neuronal specification as well as arborization, it is likely that the targets of HuD may be differential throughout cortical neurogenesis, postmitotic specification, and dendritogenesis.

The lower layer neocortical neurons in which we noted HuD expression project subcortically, primarily to the thalamus, brain stem and spinal cord (Chen et al., 2005b, Kriegstein and Alvarez-Buylla, 2009, McKenna et al., 2011, DeBoer et al., 2013). The corticospinal and corticothalamic neurons are found in this region, and control complex sensorimotor behavior. Our finding that HuD loss of function inhibits the specification and differentiation of these lower layers, suggesting disruption in motor circuits guiding fine motor movements in rodents. These findings are consistent with previous reports that HuD KO mice are less able to perform well on the rotorod challenge (Akamatsu et al., 2005).

One of our central findings is that HuD KO mice are more susceptible to seizure induced by an auditory stimulus than WT littermates. This novel finding also follows previous research that Hu proteins govern neuronal excitability given that hyperexcitibility of cortical circuitry is a hallmark of epilepsy (Ince-Dunn et al., 2012). These findings implicated HuC/D in the translation of glutamine synthase which ultimately controls cortical glutamate levels. Further, these studies demonstrated that the HuC knockout mouse has baseline abnormalities in EEG

with the appearance of seizure without convulsion. Our findings suggest that the loss of HuD function is predisposes mice to behavioral convulsion in the presence of an auditory stimulus and that a subset of glutamatergic neurons is disrupted. In concert, these findings suggest that RNA metabolism through Hu family proteins may be critical for appropriate cortical circuit function, and warrant subsequent investigation as an epileptic risk factors in the clinic. At the preclinical level, the mechanism of Hu proteins' involvement in convulsion must be further investigated. For example, subdissections of the HuD-GFP mouse in the WT and *HuD* KO background coupled to flowcytometric sorting and ribosomal footprinting or HITS-CLIP may elucidate the metabolism of HuD-regulated transcripts in a cell-specific fashion. These data may help investigators identify messages implicated in seizure which are governed by HuD in a cell subtype specific fashion. Further, the focal region of Hu loss of function seizures has not been investigated, and field potential recordings from the available knockouts may be instructive in this regard. In addition, we analyzed KOs that have HuD depleted very early in development, suggesting that early events may underlie convulsions in adults in agreement with previous findings (Wang et al., 2011b).

Clinical studies have associated single nucleotide polymorphisms in HuD with Parkinson's disease age of onset, however there is no established link with the epileptic movement disorders (Noureddine et al., 2005, Haugarvoll et al., 2007, DeStefano et al., 2008). Perhaps subsequent work will scrutinize data from these studies for the presence of epilepsy in populations with HuD mutations. Furthermore, cognitive deficits are a common comorbidity of epilepsy

(Perrine and Kiolbasa, 1999) which is in agreement with our Morris Water Maze findings. Taken together, this study implicates HuD in the generation and differentiation of cortical brain regions and their lifelong function. In concert with previous work, these findings implicate HuD as a uniquely brain expressed post-transcriptional regulator of mRNA metabolism involved in many key steps of cortical generation- from governance of early stem cell cycles to the excitability and function of cortical circuits.

Figure 4 1

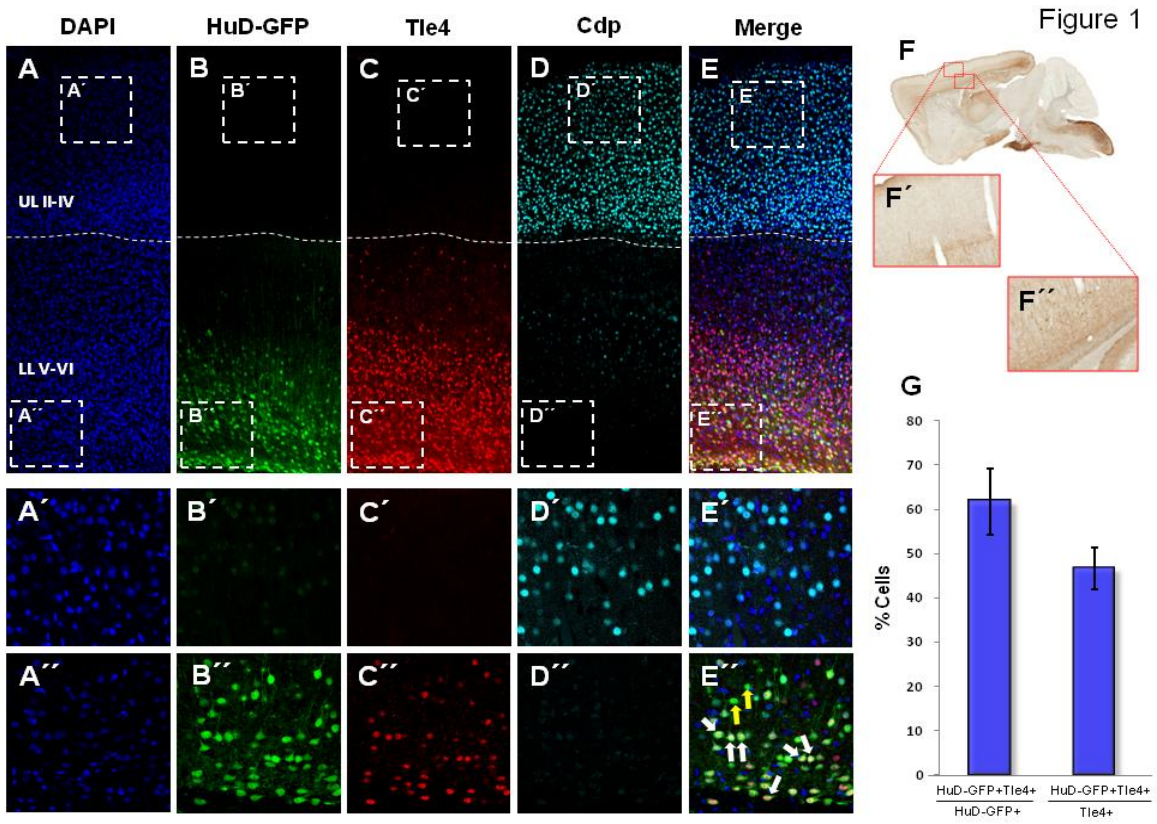

Figure 4-1. HuD-GFP is expressed in the lower, but not upper-layer primary neurons of the mature neocortex.

A-E: Representative 10x confocal images of the neocortical wall for DAPI (blue), HuD-GFP (green), Tle4 (red), Cdp (light blue) and merged channels, respectively. Dashed line demarcates upper vs lower neocortical layers. UL: Upper layers, LL: Lower layers.

A´ -E´: Insets in A-E, representative 60x confocal images of upper neocortical layers.

A´´- E´´: Insets in A-E, representative 60x confocal images of lower neocortical layers. White arrows indicate HuD-GFP/Tle4+ neurons. Yellow arrows indicate HuD-GFP+/Tle4- neurons.

F and F´: Representative light microscopy image of HuD-GFP mouse sagittal brain section using anti GFP-DAB staining (Gong et al., 2003).

G: Quantification of the proportion of HuD-GFP+ neurons colocalized with Tle4, (left) and Tle4+ neurons colocalized with HuD-GFP (right).

Figure 4 2

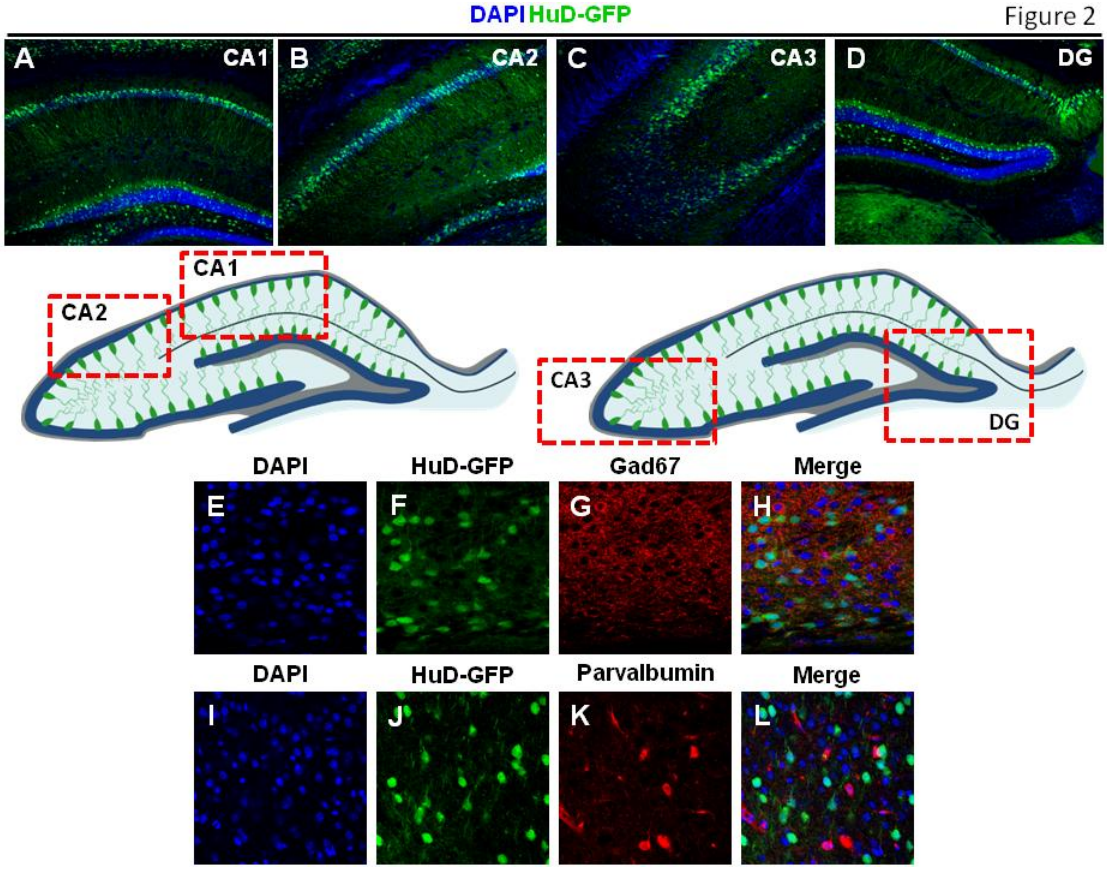

Figure 4-2. HuD is not expressed in Gad67 or Parvalbumin+ interneurons and is expressed in the CA1-3 and dentate gyrus of the hippocampus.

A-D: Representative 10x confocal images of hippocampal subregions CA1, CA2, CA3 and dentate gyrus, respectively.

Below: Schematic of HuD-GFP expression in the hippocampus. Red boxes denote regions where representative confocal images were captured.

E-H: Representative 60x confocal images of cortical of DAPI (blue), HuD-GFP (green), Gad67 (red) and merged channels, respectively.

I-L: Representative 60x confocal images of cortical of DAPI (blue), HuD-GFP (green), Parvalbumin (red) and merged channels, respectively.

Figure 4 3

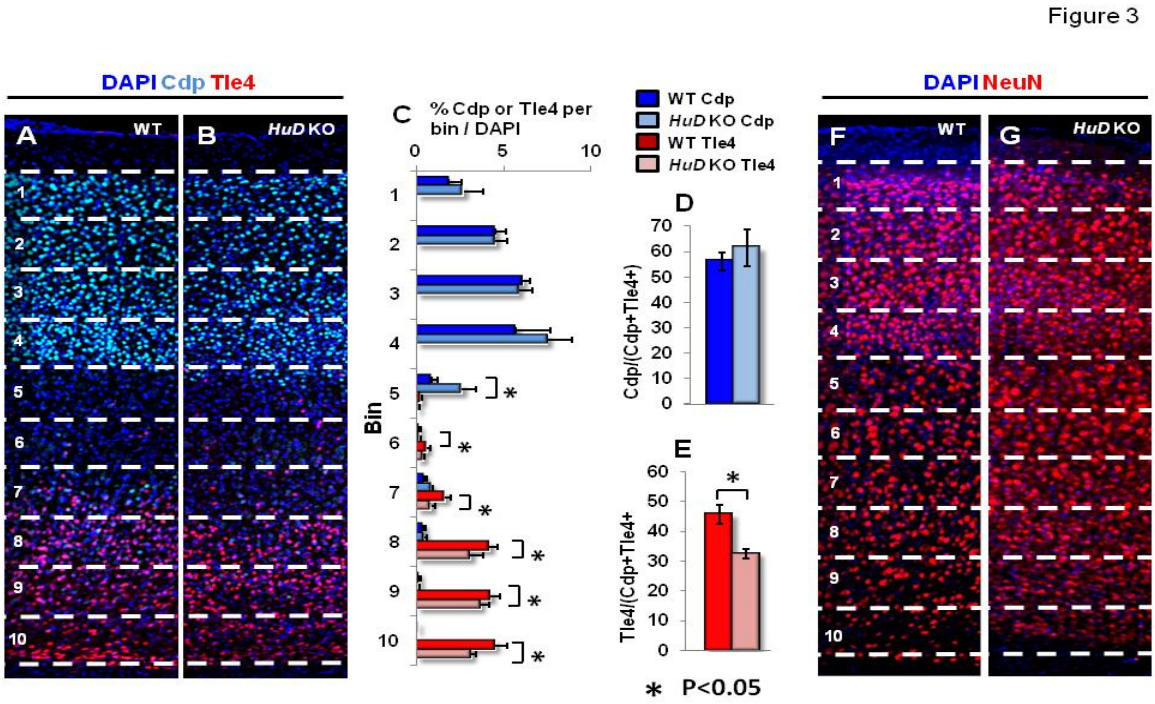

Figure 4-3. Early loss of HuD function disrupts the specification of lower layer neocortical primary neurons.

A-B: Representative confocal images of the neocortical wall of adult WT and *HuD* KO at P90. Numbers and dashed lines denote 10 equal bins for analysis, from Layer II (bin 1) to the subplate (bin 10). DAPI in dark blue, Tle4 in red and Cdp in light blue.

C: Quantification of the number of Cdp+ or Tle4+ cells in each bin/the number of DAPI+ cells in the column. Numbers reported as proportion DAPI+ cells which are Cdp+ or Tle4+. Mean bin proportion compared between WT and KO for each bin.

D: Quantification of the proportion of Cdp+ neurons from total labeled neurons (Cdp+Tle4).

E. Quantification of the proportion of Tle4+ neurons from total labeled neurons.

F and G: Representative confocal images of the cortical plate of adult WT and *HuD* KO as in A-B. DAPI in dark blue and NeuN in red.

Figure 4 4

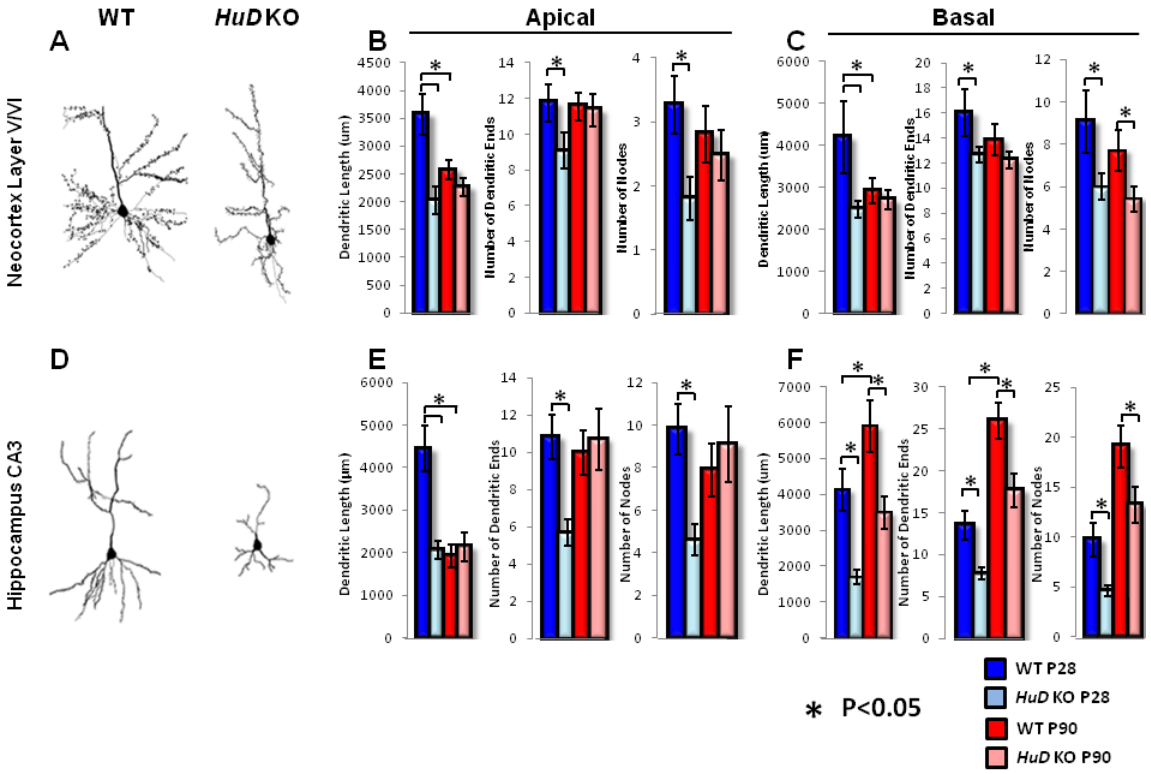

Figure 4-4. *HuD* loss of function disrupts dendritogenesis in deep neocortical layers and the CA3 region of the hippocampus.

A,D: Representative tracings of lower layer neocortical primary neurons (A) and hippocampal CA3 pyramidal neurons (D).

B. Quantification of apical dendrites' length, dendritic ends and nodes in lower layer neocortical neurons.

C. Quantification of basal dendrites' length, dendritic ends and nodes in lower layer neocortical neurons.

E. Quantification of apical dendrites' length, dendritic ends and nodes in CA3 pyramidal neurons.

F. Quantification of basal dendrites' length, dendritic ends and nodes in CA3 pyramidal neurons.

Figure 4 5

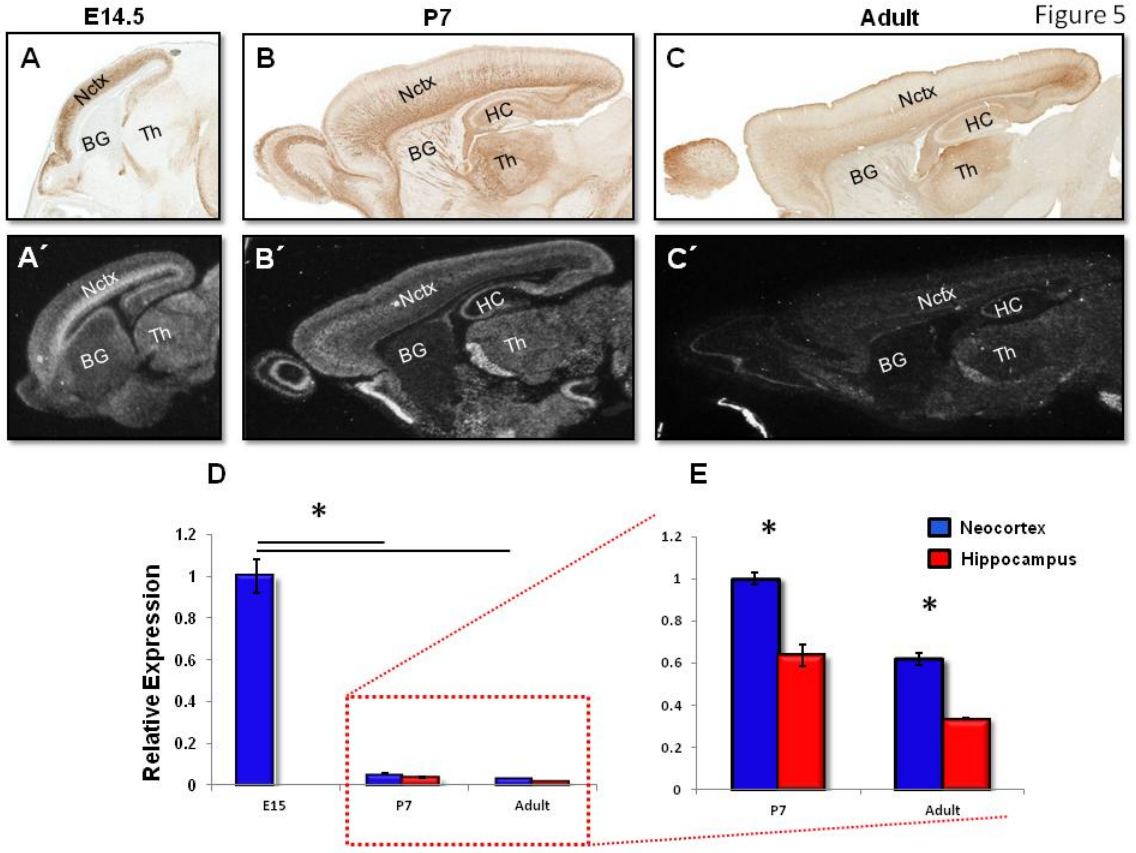

Figure 4-5. HuD expression decreases from E14.5 until adult, and becomes more regionally specific.

A-C. Representative images of HuD-GFP mouse sagittal brain sections stained with anti GFP DAB. E14.5, P7 and Adult, respectively (Gong et al., 2003).

A´-C´ Representative images of *HuD* ISH mouse sagittal brain sections. E14.5, P7 and Adult, respectively (Magdaleno et al., 2006).

D and E. qRT-PCR of developing neocortex at E15, P7 and adult. Hippocampus analysis at P7 and adult. Gapdh was used for normalization. Values were normalized to E15 neocortex in (D). Values were normalized to P7 neocortex in (E)

Figure 4 6

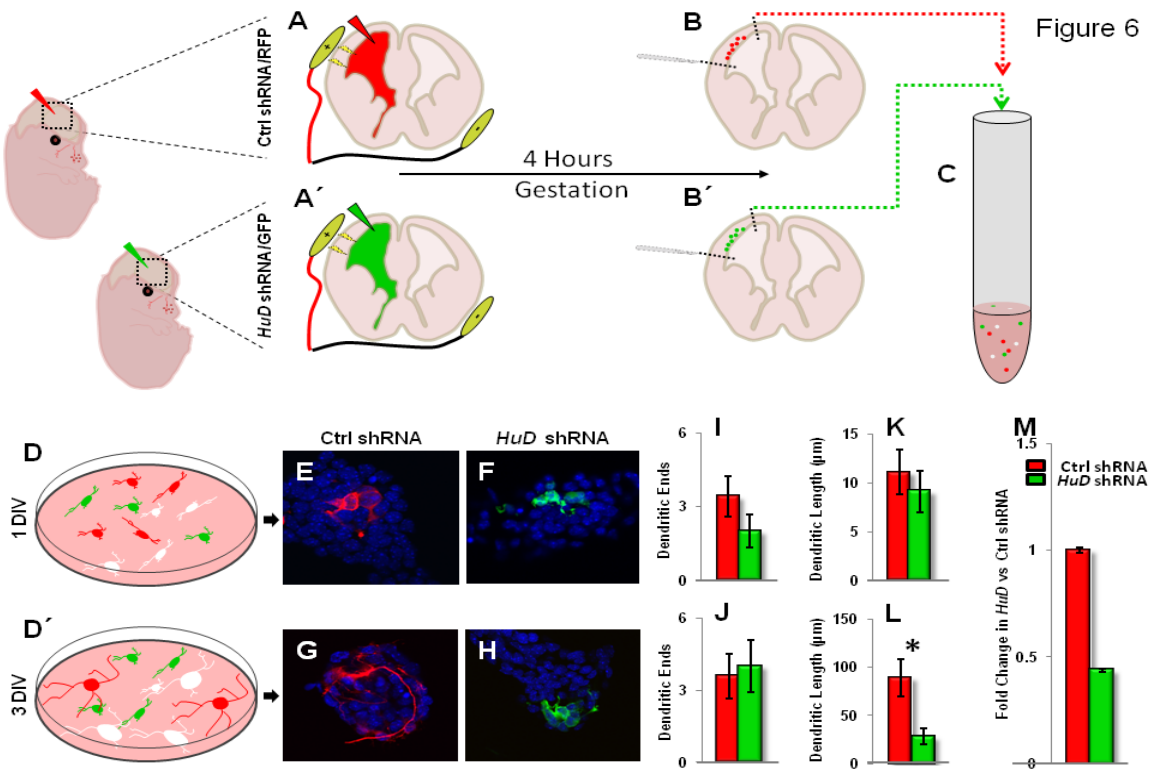

Figure 4-6. HuD controls the earliest stages of dendrite outgrowth.

A-B´. Schematic of In-utero electroporation and dissociation of Ctrl shRNA/RFP (top panel) and HuD shRNA/GFP in E13.5 developing neocortex. Developing neocortices were electroporated at E13.5 with either Ctrl shRNA (RFP) or HuD shRNA (GFP). After 4 hours neocortices were dissociated and cultured.

C. Schematic of dissociation of electroporated neocortices for primary cell culture.

D and D´. Schematic of cell cultures taken at 1 and 3 DIV for analysis.

E and F. Representative 60x confocal images of Ctrl and *HuD* shRNA transfected neurons at 1 DIV, respectively.

G and H. Representative 60x confocal images of Ctrl and *HuD* shRNA transfected neurons at 3 DIV, respectively.

I-L. Quantification of neurite endings in 1 and 3 DIV cell cultures.

M. qRT-PCR analysis of *HuD* shRNA efficiency in vitro. Gapdh was used as a normalization control.

Figure 4 7

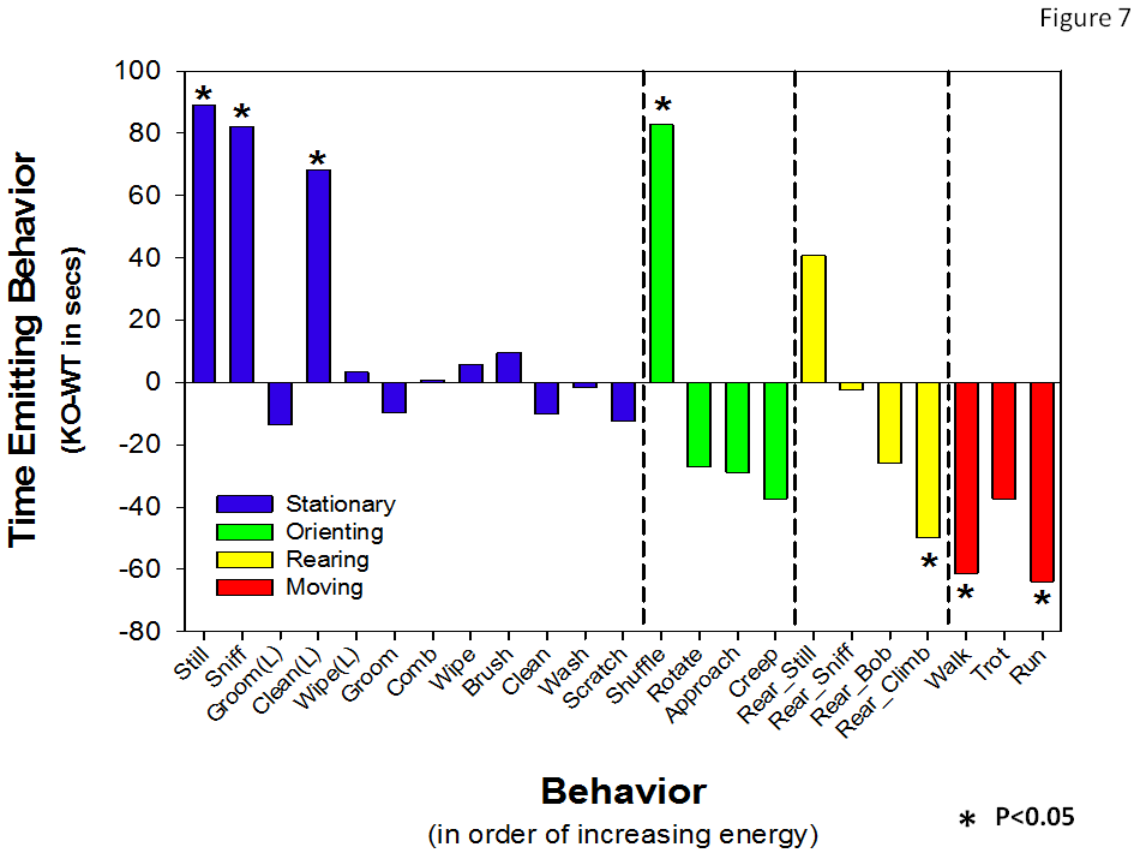

Figure 4-7. Spectral analysis of HuD KO shows reduced overall activity and increased stereotypic behaviors.

Spectral analysis of total time spent performing each behavior where *HuD* KO mean-WT mean for each. Analysis subdivided into 4 main categories; stationary, orienting, rearing and moving.

Figure 4 8

Figure 8

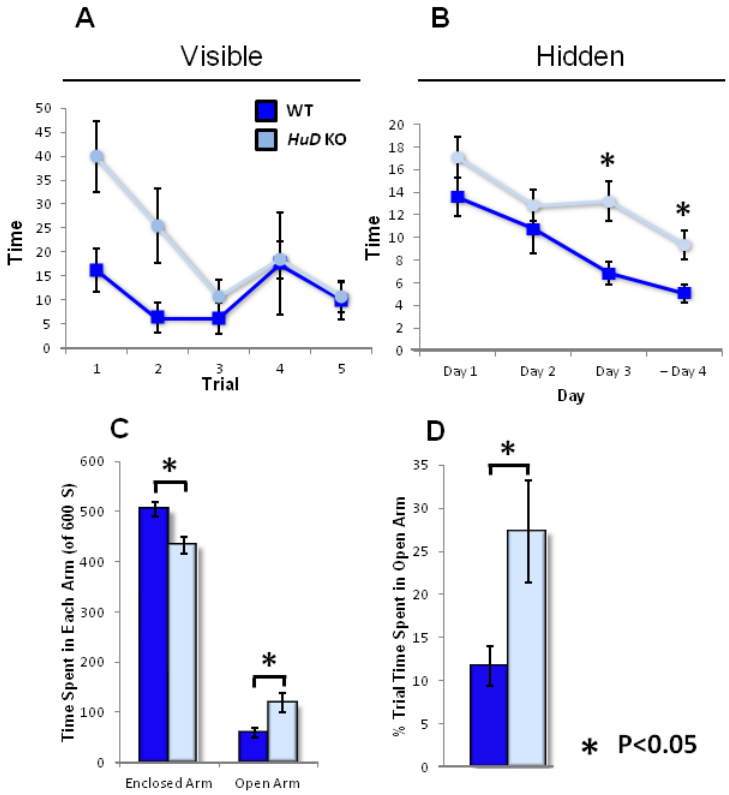

Figure 4-8. HuD KO mice perform poorly in Morris water maze and spend more time in the open arms of the elevated plus maze.

A: Average latency to find a visible platform by genotype in 5 trials of the Morris water maze.

B: Average latency to find a hidden platform by genotype in 4 consecutive days of testing, 5 trials per day.

C: Average total time spent in the enclosed and open arms of the elevated plus maze, by genotype.

D: Proportion of total time spent in the open arms of the elevated plus maze, by genotype.

Figure 4 9

Figure 9

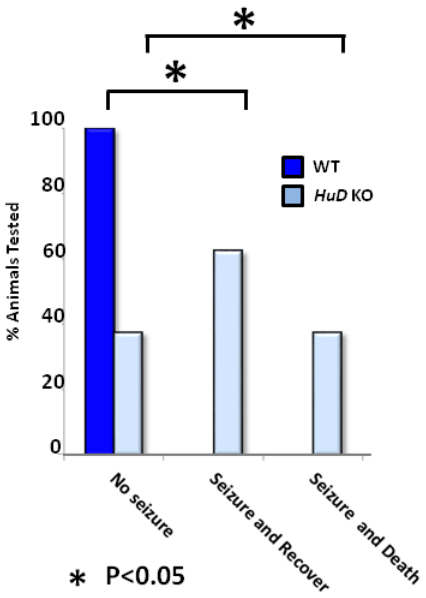

Figure 4-9. HuD KO mice are more susceptible to auditory induced seizure than WT.

Quantification of the proportion mice which did not experience seizure, experienced a seizure and subsequently recovered, and experienced seizure and immediately died.

Chapter 5. NT-3 Mediates the Isoform-Specific Role of HuD in Neocorticogenesis only in S-phase

A. Introduction

The mature neocortex is the seat of high-level cognition, learning, memory, and reasoning and is central in coordinating voluntary motor activities (Weiler et al., 2008, Pichardo-Casas et al., 2012). The mature neocortex composed of glutamatergic, excitatory projection neurons which are precisely organized into six discrete layers, as well as an array of inhibitory interneurons and glia. Each layer of projection neurons is generated sequentially from a pool of neural stem cells (NSCs) lining the lateral ventricle called radial glia progenitors (RGs) (Zhang et al.). In mouse, the genesis of projection neurons from the RG progenitors takes place over a very short time frame from embryonic day 11 (E11) to E18. The time point during the neurogenic phase when a projection neuron is born determines its location and transcriptional programming within the neocortical plate as well as its axonal connectivity and function.

Early neurogenic phases (E11-E14) produce predominantly lower layer projection neurons expressing the transcription factor marker Tle4 (Transducin-like enhancer of split 4, homolog of Drosophila E) and project axons through the basal ganglia to terminal contacts in the thalamus, brainstem, and spinal cord. Later phases (E14-E18) produce primarily Cut-like homeobox 1 (CDP)-expressing upper layer neurons and send axons to targets within the same hemisphere or to the opposite hemisphere through the corpus callosum. RG throughout development execute intrinsic programs of gene expression which

result in the appropriate production of lower and upper-layer neurons. However, these programs work in concert with those initiated by extrinsic factors such as those initiated by cell-cell contact or secreted molecules . The exposure of RG to extrinsic signals occurs in part through interkinetic nuclear migration, (INM) a process by which the nucleus of the RG is positioned away from the ventricle during S-phase, and is rests at the apical, epithelial surface of the ventricle during M-phase (Ueno et al., 2006, Pontious et al., 2008, Sessa et al., 2008, Taverna and Huttner, 2010, Mazin et al., 2013). These studies have demonstrated that INM is a critical mechanistic process of neocorticogenesis.

During development, the neocortex is dynamically influenced by subcortical structures developing in concert. At mid-neurogenesis, the cortex is innervated by afferent thalamic axons which establish the thalamocortical tract. Reciprocally, efferent fibers from deep neocortical layers (V and VI) "handshake" with ascending thalamic axons and innervate the thalamus creating the corticothalamic tract. These innervation events are a possible source of extrinsic factors to the developing neocortex. Previous findings indicate the development of ascending thalamic fibers is dependent upon NT-3, a secreted trophic factor (Hanamura et al., 2004, Yamamoto and Hanamura, 2005). Further evidence indicates that NT-3 is a critical trophic cue within the developing RG, influencing fate decisions in neocorticogenesis (Park et al., 2006, Bartkowska et al., 2007). Recent evidence indicates that post-transcriptional gene regulation may be particularly critical for the development and function of the neocortex (Black, 2000, Ayoub et al., 2011). In keeping with this idea, our preliminary analysis of

RNA metabolism events in the neocortex changes vastly at mid-neurogenesis when corticothalamic and thalamocortical tracts are first established (DeBoer et al., 2013).

Given that RNA Binding Proteins (RBPs) can participate at nearly every post-transcriptional stage, we scrutinized our screen for candidate RBP regulators of neocortical neurogenesis. To this end, we identified HuD (Elavl4) as a candidate RBP regulator of neocortical neurogenesis (DeBoer et al., 2013). Previous studies demonstrate that members of the ELAV protein family can also be influenced by extrinsic factors, such as neurotrophins (Akten et al., 2011, Fallini et al., 2011). In SH-SY5Y cells, neuron-specific ELAV proteins (HuB, C, and D) were found to be positively regulated and recruited by protein kinase C (PKC), a kinase effector downstream of neurotrophin signaling (Pascale et al., 2005). Other studies have shown that PKC-induced neurite outgrowth is dependent upon HuD, particularly because it stabilizes the *GAP-43* transcript (Mobarak et al., 2000). In addition, protein kinase B (also known as Akt) is required for HuD-induced translation and mediates functional neurite outgrowth in PC12 cells (Fujiwara et al., 2011). PKC and Akt are members of the neurotrophin signaling pathway. Indeed, HuD has also been linked to BDNF-induced neurite outgrowth in cultured hippocampal neurons (Abdelmohsen et al., 2010). These findings demonstrate that HuD may be a neurotrophin-dependent post-transcriptional target, where miR-375 targets the 3’ UTR of HuD to silence its expression. Therefore, HuD and the well-characterized neurotrophin signaling

pathway are functionally linked; however, it is not yet known how timed developmental neurotrophin signals regulate HuD function in neocorticogenesis.

Using the mouse neocortex as a model system, we identified the role of the source of developmental NT-3 on the function of HuD in the development of the neocortex. Our findings demonstrate that thalamic but not cortical NT-3 influences the isoform-specific translation of HuD. HuD isoforms are distinctly functional; HuD4 drives the production of lower layer neocortical projection neurons whereas HuD3 preferentially generates upper layers. Our data, both in-vitro and in-vivo, confirm these results and suggest HuD is a multi-functional post-transcriptional regulator of neocorticogenesis which is preferentially translated in an isoform-specific factor in the presence of a thalamic-produced trophic factor, NT-3.

B. Materials and Methods

This methods section is part of a manuscript in preparation for submission and equal credit for its authorship is due to Dr. Althea Stillman.

Animals

Experiments involving animals were carried out in accordance with UMDNJ Institutional Animal Care and Use Committee protocols. For timed pregnancies, adult female CD1 mice were purchased from Charles River Laboratories (Wilmington, MA). For all timed pregnancies, the day of vaginal plug discovery was considered embryonic (E) day 0.5. The following transgenic lines were purchased from Jackson Laboratories: for TrkC, 129S2-*Ntrk3*^{tm1Bbd}/J (stock #

002481); for *Crh-Cre* B6 (Cg- *Crh*$^{tm1(cre)Zjh}$/J (stock #012704); for *Rfp* reporter B6. Cg-*Gt(ROSA)26Sor*$^{tm9(CAG-tdTomato)Hze}$/J (stock #007909). The *Ntf3*flox line was a kind gift.

Fixation

Embryonic brains and postnatal day 0 (P0) were dissected and fixed by immersion for several hours to overnight in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), pH 7.4.

Antibodies

The following primary antibodies and dilutions were used for immunohistochemistry: mouse anti-HuD (SCBT, 1:20,000, sc-28299), rabbit anti-Pax6 (Covance, 1:250, PRB-278P), rabbit anti-Tbr2 (Abcam, 1:250, ab23345), rabbit anti-CDP (SCBT, 1:100, sc-13024), rat anti-Bcl11b (SCBT, 1:100, sc-98514), mouse anti-Tle4 (SCBT, 1:250, sc-365406), chicken anti-GFP (Aves, 1:1000, GFP-1020), rabbit anti-Tuj1 (Cell Signaling, 1:250, 2146S), rabbit anti-Dcx (Cell Signaling, 1:250, 4604S). The secondary antibodies used at a 1:250 dilution and were obtained from Jackson ImmunoResearch.

The following primary antibodies and dilutions were used for immunoblotting:

Mouse anti-Gapdh (Millipore, 1:5000, MAB374), Mouse anti-HuD (SCBT, 1:1,000). The secondary antibodies used at a 1:5000 dilution and were obtained from Jackson ImmunoResearch.

Immunohistochemistry

Standard methods were used for immunostaining as described previously (Rasin et al 2007). Briefly, fixed brains were blocked in 3% agarose/PBS and sectioned at 70µm using a vibratome. Subsequent sections were processed free-floating. First, sections were pre-incubated in blocking solution (BS) containing 5% normal donkey serum, 1% bovine serum albumin, 0.1% glycine, 0.1% L-lysine, and 0.04% Triton-X-100. Primary antibodies were added and incubated for 36 hours. Washes were performed with 1xPBS and secondary antibodies were incubated in BS without Triton for 2 hours at RT. After washes, DAPI was applied and sections were mounted with Vectashield (Vector Laboratories).

Laser Capture Microscopy

Whole brains were manually dissected from developing embryos at E13 and E16 in an RNAse-free environment and flash frozen. Brains were then sectioned to 60 uM in a cryostat at -20°C onto Molecular Machines & Industries (MMI) Membrane Slides. Sectioned tissue was allowed to dry thoroughly and stained with 0.1% Cresyl Violet Acetate for 45 seconds, rinsed with Nuclease-Free water and immersed for 1 minute in 95% ETOH. Subsequently dried slides were subject to microdissection using an MMI SmartCut Plus microscope. Neocortical compartments were determined by morphology of cresyl violet-stained cells. E13 brains were sectioned into two equal compartments. E16. RNA was harvested from developmental compartmentalized tissue using the RNAqueous-Micro kit LCM protocol (Ambion). At least 3 brains were analyzed per condition. Significant changes between conditions were analyzed using the ΔΔCT method.

A student's t-test was performed between conditions where $P<0.05$ was considered significant.

Western Blot Analysis

Protein samples were lysed in Tissue Protein Extraction Reagent (T-PER, Pierce) and concentration was determined using Pierce 660 nm Reagent in conjunction with a Nanodrop spectrophotomer. Protein samples were analyzed using the NuPAGE system (Life Technologies) per manufacturer's instructions and with 4-12% Bis-Tris gels and transferred to nitrocellulose membranes. The resulting membranes were blocked with 5% non-fat dried milk, 10% FBS in PBS with 0.3% Triton (PBST). Primary and secondary antibodies were incubated in 10% FBS in PBST and washes were done in PBST. Development of blots was performed with ChemiGlow West Chemiluminescence Substrate Kit (Proteinsimple) and visualized with Syngene Gbox and the software GeneSnap from Syngene. Quantification of band intensity was done with GeneTools from Syngene. Experimental protein was normalized to Gapdh levels. At least 3 biological replicates were performed for all analyses and a student's t-test was used to compare averages. $P<0.05$ was considered significant.

Quantitative Real-time PCR (qRT-PCR) and RT-PCR

RNA sample concentrations were determined using a Nanodrop 1000 spectrophotometer. For LCM samples 10-25 ng of total RNA was used per reaction. For all other reactions 50 ng per reaction was used. Applied Biosystems StepOne Real-Time System and reagents were used. Results were analyzed

using the ΔΔCt method with *Gapdh* as a normalization control, unless otherwise specified.

For RT-PCR of *Elavl4* variants, the following primer sets were used:

F1, GGCAGAAGAAGCCATCAAAG

R1, CGTAGTTGGTCATGGTGACG

F2, GGGATTCATCCGCTTTGATA

R2, AGGATTTGTGGGCTTTGTTG

Taqman probes used:

Probe name	**Description**	**NCBI Accession**	**Taqman Assay**
HuD1&4	mouse *Elavl4* variant 1 and 4	NM_010488, NM_001163399	Mm01263578_m1
HuD2	mouse *Elavl4* variant 2	NM_001038698	AIQJGG3 (custom)
HuD3	mouse *Elavl4* variant 3	NM_001163397	Mm00516018_m1
HuD4	mouse *Elavl4* variant 4	NM_001163399	Mm01263581_m1
pan-HuD	mouse Elavl4 variants 1,2,4	NM_010488, NM_001038698,	Mm01263580_mH

		NM_001163399	
pan-HuD ex4to5	all mouse *Elavl4* variants, exon 4 to 5	NM_010488, NM_001038698, NM_001163397, NM_001163399	AIRR9ZJ
Gapdh	mouse *Gapdh*	NM_008084.2	Mm03302249_g1

Constructs

The following constructs were used for this study.

Name	**NCBI accession number**	**Company**	**Catalog #**
HuD1 OE	NM_010488	GeneCopoeia	EX-Mm30154-M46
HuD2 OE	NM_001038698	GeneCopoeia	EX-Mm26736-M46
HuD3	NM_001163397	GeneCopoeia	EX-

OE			Mm29523-M46
HuD4 OE	NM_001163399	GeneCopoeia	EX-Mm29524-M46
HUD3 OE	NM_001144775.1	GeneCopoeia	EX-Mm6858-M46
HuD3 shRNA	NM_001144775.1	Origene	custom
HLUC		GeneCopoeia	EX-hLUC-M46
Control (*Ctrl*) shRNA		Origene	TR30012
Control (*Ctrl*) shRNA		Origene	TR30015

In Utero Electroporation

In utero electroporations were performed at E13.5 and analyzed at E16.5 or P0 as described (Rasin et al., 2007). Co-transfections of approximately 2 µL plasmid DNA mix containing 0.5 µg/µL of CAG-*Gfp* and 4 µg/µL of plasmid of interest. For rescue experiments, concentrated amounts (8 µg/uL) of shRNA and OE were combined and injected. Harvested brains were immunostained and imaged as above. For quantification, 10 equal bins within the sensorimotor neocortices were drawn spanning CP from the pial surface (bin 1) to the Subplate (bin 10). Three equal bins were drawn between the subplate and the VZ. Bin totals of GFP+ cells were divided by the total GFP+ cells in all 10 bins or 3 bins to create positional distributions of transfected cells. Also see chapter 4 and 6 of this document. N=3 for Ctrl, HuD 3 OE. N=2 for HuD 1, 2 and 4 OE. N=2 for HuD3 shRNA, Ctrl shRNA and HuD3shRNA+Human HuD3 rescue. The proportion of total GFP+ cells in each bin as a proportion of the total in all bins averaged between replicates and compared by a student's t-test for each bin. $P<0.05$ was considered significant. For transcription factor analysis, GFP+ also positive for the marker of interest (i.e. CDP or TLE4) were analyzed as a proportion of total GFP+ cells (i.e. (CDPandGFP+/total GFP+)). Proportions were averaged between biological replicates and compared between conditions using a student's t-test.

Confocal Microscopy and Quantification

Images were taken with an Olympus BX61WI confocal microscope and processed using Fluoview FV-1000 software. Quantification of immunostaining and co-localization was done with Neurolucida and Image J software.

Cell Culture/Transfections/FACS

N2a cells were grown in DMEM media containing 10%FBS, 1% Glutamax, 1% Sodium Pyruvate (Invitrogen). Cells were allowed to reach approximately 70-80% confluency before transfection with Lipofectamine per manufacturer's protocol (Invitrogen). Overexpression constructs were harvested 72 hours after transfection and shRNA constructs were taken 5 days after.

Cells were pelleted and resuspended in 1 mL of DMEM media containing 0.5% FBS and 1mM Hepes for Fluorescent Activated Cell Sorting (FACS). Cells were sorted with the BD FACSArray Bioanalyzer by the Analytical Cytometry/ Image Analysis Core Facility at Rutgers University.

Primary Cultures

Neocortices were manually dissected from CD1 mice at E13.5, dissociated and plated at 75,000/cM^2 as described (Sestan N, 1999). Primary neurons were cultured in Neurobasal media with B27 supplement (Gibco). At the time of plating, either recombinant human NT-3 (100 or 500ng/mL, R&D systems) or PBS vehicle control were added. For Trk inhibition analysis, K252a 2uM was used, or DMSO vehicle control were added. Media and treatments were replaced 24 hours after plating, and cells were harvested 2 DIV at 48 hours post-

plating. Harvested cells were divided for RNA and protein analysis. Protein was harvested with T-PER reagent and analyzed with western blotting as described above. RNA was harvested using a Qiagen RNeasy kit in conjunction with Ambion Turbo DNase as described previously.

Cell Stage Synchronization/Stalling

N2a cells were synchronized into specific phases of the cell cycle using 50 ng/mL Nocodazole for 18 hours as described (Zhang et al., 2012). Nocodazole arrests the cell cycle in M-phase as it disallows the breakdown of the mitotic spindle. Upon release from Nocodazole, previous findings have demonstrated that S-phase occurs 16 hours from release, where G1 can be assessed 8 hours post release. When NT-3 was used, stalling was performed without NT-3, which was added upon release from M-phase.

Polysome Isolation

The following protocol was developed by Kevin Thompson and the following three paragraphs describing sucrose fractionation are adapted from a manuscript written by the following scientists: **Matthew L. Kraushar, Kevin Thompson, H.R.Sagara Wijeratne, Barbara Viljetic, Ronald P. Hart, Mladen-Roko Rasin**

One or two days prior to fractionation, 10-50% sucrose gradients were made using 2 ml ultracentrifuge polyallomer tubes (#347357). Sucrose gradients were formed by creating 10, 20, 30, 40 and 50% densities of sucrose and injecting them underneith each other with a sterile syringe such that the 10% fraction is highest in the column (20 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl2)

supplemented with EDTA-free protease inhibitor (Santa Cruz Biotechnology #sc-29131), RNAase inhibitor (Invitrogen #100000840), 20 mM DTT (Invitrogen #NP0009), and 0.1 mg/ml cyclohexamide (Santa Cruz Biotechnology #sc-3508A)). Gradients were stored at 4°C.

Isolated neocortices or plated N2a cells were lysed in polysome extraction buffer (PEB; 20 mM Tris-HCl, 100 mM KCl, 10 mM MgCl2, 0.3% Igepal; Sigma-Aldrich #CA-630) supplemented with EDTA-free protease inhibitor (Santa Cruz Biotechnology #sc-29131), RNAase inhibitor (Invitrogen #100000840), 20 mM DTT (Invitrogen #NP0009), and 0.1 mg/ml cyclohexamide (Santa Cruz Biotechnology #sc-3508A) for 10 minutes on ice with movement to sufficiently lyse tissue. Insoluble fractions were removed from the PEB-lysed product by centrifugation (Sorvall Biofuge Fresco) for 10 minutes 4°C at ~13,000g. Cleared product was subject to 260/280 nm spectrophotometry to determine RNA concentration (NanoDrop ND-1000). 50 µg total RNA was loaded onto a 2 mL column.

Ultracentrifugation was performed on loaded columns at 39,000 rpm for 39,000 rpm for 50 minutes (Sorvall Discovery M120SE with S-55-S rotor and buckets). Samples were then immediately placed into a tube piercer (Brandel #621140007) connected to a syringe pump (Brandel syringe pump) and fractionated into 14 equal volume fractions while recording absorbance at 267 nm which measures protein and nucleic acid concentrations (Brandel UA-6 absorbance detector). RNA was extracted using Trizol-LS reagent according to

the manufacturer's protocol and stored at -80°C before qRT-PCR analysis as previously described.

qRT-PCR was performed on RNA isolated from fractionations. Plates were loaded in 2-3 technical replicates. Biological replicates were loaded on separate plates. CT values from each probe were converted to copy number in each fraction using this equation: CN=2^(40-CT). Total copy number of each analyzed probe was summed and the percentage of mRNA for analyzed transcripts per fraction was determined. Percentages were averaged between biological replicates. Translating fractions were then summed (9-13) vs non-translating (2-7). A t-test was performed on non-translating fractions between conditions (i.e. fractions 2-7 in E13 vs E16 for average percentage of copy number in those fractions for HuD3) and again for translating fractions. $P<0.05$ was considered significant.

C. Results

HuD is highly expressed in postmitotic projection neurons in the neocortex, and in a subset of RG at E15.

We previously identified HuD as a candidate post-transcriptional regulator of neocortical neurogenesis through an array of developing stages of neocortex (DeBoer et al., 2013). To assess the contribution of HuD to the process of RG differentiation and neurogenesis, we first asked where HuD is expressed at key developmental time points; E13, E15 and E18. To do this, we performed immunohistochemistry against HuD as well as Nestin, a marker of stem cells,

DCX, to stain nascent neuroblasts, and Tbr2, a marker of intermediate progenitors. We found that HuD is robustly expressed in the cortical plate (CP) where specified, postmitotic neurons are organized (Figure 5-1A-A´´). Interestingly, we also found HuD expression at the epithelial ventricular surface at mid-neurogenesis (E15) (Figure 5-1B). Apical HuD staining also colocalized with nestin, indicating that HuD is present at the apical feet of RG at this key developmental timepoint (Figure 5-1B inset and 5-1C-F .

HuD transcript variants are not differentially expressed in the VZ throughout neocortical development

HuD is expressed as 4 unique transcript variants, each encoding a unique protein isoform (Figure 5-2, http://genome.ucsc.edu/). Previous work has demonstrated that the ability of HuD to participate in translation of its bound mRNA targets is dependent upon a sequence of amino acids in the "linker" region of the protein, which is variable between the 4 isoforms (Figure 5-2, Red box) (Fukao et al., 2009). Given that we found HuD has a duel expression pattern in both RG and postmitotic neurons (Figure 5-1A), we reasoned that HuD may be carrying out two functions through expression of distinct transcript variants. Therefore, we performed laser capture microdissection (LCM) on E13 and E16 neocortices and isolated the prolific VZ (Figure 5-3A). We extracted whole RNA from the tissue and performed HuD variant-specific qRT-PCR (Figure 5-3B). Our findings indicate that there is no significant difference in expression of *HuD* transcripts between E13 and E16 VZ, and that differences in HuD protein

expression in the VZ cannot be explained by increased expression of any of the *HuD* variants.

HuD3 is translated during mid, but not early neurogenesis

As HuD protein is expressed only at E15 in the RG in the VZ and transcript levels are unchanged between E13 and E16 in the VZ, we reasoned that HuD may be differentially translated between early and mid-neurogenesis. In keeping, current studies are beginning to demonstrate that translational regulation may be a key regulator in generating neocortex-specific proteome complexity(Johnson et al., 2003, Birzele et al., 2008, Kwan et al., 2012a). Therefore, we evaluated the translation of each *HuD* transcript variant in E13 and E16 neocortices. To do this, we isolated polysome-stabilized whole RNA from E13 and E16 neocortices. Next, we subject the lysate to ultracentrifugation in a linear 5-50% sucrose gradient to separate low-mass, non-translating mRNA transcripts from heavy, polysome associated messages. qRT-PCR analysis of transcript levels in each of 14 fractions shows similar amounts of translating, polysome associated mRNA for *HuD* transcripts 1,2 and 4 (Figure 5-4). *HuD* transcript variant 3 (*HuD3*) however, is found in much greater quantity in translating fractions at E16 than E13. Non-translating and translating fractions were significantly different between E13 and E16 for *HuD3* analysis (P=0.04 for each comparison). These data indicate that *HuD3* translation is specifically increased at E16 vs. E13, and may be responsible for the increased HuD protein expression seen in RG at mid-neurogenesis.

HuD3 promotes the production of upper neocortical layers, where HuD4 promotes lower layer production

At mid-neurogenesis, a shift occurs in the output of RG, where divisions predominantly produce upper-layer neurons through the process of indirect neurogenesis (Noctor et al., 2004, Pontious et al., 2008, Sessa et al., 2008, Kowalczyk et al., 2009). Therefore, we obtained HuD isoform-specific overexpression vectors and confirmed their efficacy in-vitro (Figure 5-6A). Next, we co-electroporated either control or HuD isoforms individually along with a GFP overexpression vector into the developing neocortex of E13 pups. Brains were taken at P0 for analysis and subject to immunohistochemistry against Tle4, CDP and GFP. First, we analyzed the position of GFP+ cells in the CP by superimposing 10 equal bins from the subplate (bin 10) to the pia (bin 1) (Figure 5-6B). Remarkably, overexpression of HuD3 resulted in the majority of GFP+ cells being localized to upper bins (Figure 5-5A and B). Reciprocally, overexpression of HuD4 resulted in the majority of transfected cells remaining in bins associated with lower neocortical layers (bins 4-7, Figure 5-6A and B). Overexpression of HuD1 and 2 resulted in cell distribution similar to control (Figure 5-5A and B).

Analysis of layer-specific transcription factor markers demonstrated that GFP+ HuD3-transfected cells were indeed of upper layer identity; significantly more of these cells were found to be CDP+ than control, and many fewer of them were Tle4+. When analyzing HuD4-transfected cells, however, we found the opposite trend. Significantly more HuD4-transfected cell colocalized with Tle4,

where fewer colocalized with CDP. No significant changes were found in HuD1 and 2 overexpression analysis. In keeping with our layer marker findings, HuD3-transfected neurons exhibited very few subcortical axonal connections descending through the striatum, a hallmark of upper-layer neurons (Figure 5-7) (Molyneaux et al., 2007, Cubelos et al., 2010). Further, HuD4 overexpression produced a robust population of subcortically projecting cells (Figure 5-8) (Molyneaux et al., 2007, Kriegstein and Alvarez-Buylla, 2009, DeBoer et al., 2013). Taken together, these results suggest HuD isoforms promote distinct postmitotic specification fates in differentiating RG.

Silencing of *HuD3* stifles the production of upper layer neurons

The most surprising finding from our HuD isoform overexpression battery (Figure 5-6) was that early RG at E13, which typically produce lower layers, could be converted to upper-layer producing cells with HuD3 was overexpressed (Figure 5-6 and 5-7) (Kriegstein and Alvarez-Buylla, 2009, DeBoer et al., 2013). Therefore, we asked a reciprocal question; if HuD3 is silenced, will affected cells fail to become upper layer neurons when upper-layer neurons are typically born (E15-P0). Therefore, we obtained an HuD3-specific shRNA construct and determined its efficacy in vitro (Figure 5-9). Next, we co-electroporated the HuD3-shRNA or control vector along with a GFP construct into the developing neocortices of E13 pups. As above, we performed immunohistochemistry for layer-specific markers and bin analysis of resulting cells (Figure 5-10). Early loss of HuD3 reduced the number of GFP+ transfected cells in bins associated with upper layers (1-5) when compared to control. Further, these cells were more

often Tle4+ than control. To assess if the effect of HuD3 shRNA could be mitigated by rescue with this isoform, we next performed a rescue experiment where HuD3 shRNA was co-electroporated with GFP and an HuD3 overexpression construct which could not be targeted by HuD3shRNA (Figure 5-10). Resulting transfected neurons were found preferentially in upper neocortical layers through bin analysis. HuD3 rescue transfected cells colocalized less often with Tle4 and more often with CDP. These findings indicate HuD3 is an essential regulator of upper layer formation in neocorticogenesis.

HuD3 and 4 promote differentiation of RG

We next asked whether HuD isoforms 3 and 4 promote specification of upper and lower layer neurons through direct or indirect neurogenesis. We surmised that if HuD overexpression promoted neuronal specification through direct neurogenesis, transfected cells should less often colocalize with the RG marker Pax6. Similarly, if these neurons are specified through indirect neurogenesis, then we would expect to see a reduction in Tbr2 and a preservation of Pax6 levels in transfected stem cells. Therefore, we performed in utero electroporation of HuD3 and 4 at E13 and assayed the resulting neocortices at E16 (Figure 5-11). Through immunohistochemistry, we found that Pax6 levels were reduced in both HuD3 and 4 overexpression, while Tbr2 levels were unchanged (5-12 and data not shown). Therefore, we concluded that HuD3 and 4 promote the specification of upper and lower layers, respectively, through direct neurogenesis where HuD variants do not promote RG differentiation into IPCs, but rather into postmitotic neurons.

NT-3 increases protein levels of HuD in vitro

Our data demonstrate that translation of HuD3 during mid-neurogenesis promotes the production of upper layer neurons. Therefore, we next asked if an extrinsic cue was promoting the translation of HuD3 during this critical timeframe. Previous evidence has demonstrated that HuD is a downstream effector of Trk signaling in a variety of contexts. Further, overexpression of NT-3 was recently found to coincide of upper-layer production (Seuntjens et al., 2009). Therefore, we dissected neocortices from developing embryos at E13, when lower neocortical layers are being formed. We dissociated the tissue, generated primary cultures and exposed them to mock conditions, 100ng/ml NT-3 or 500ng/ml NT-3 (Figure 5-13). After 48 hours, cultures were harvested for HuD variant-specific qRT-PCR analysis as well as western blot (data not shown and Figure 5-13). We found a dose-dependent increase in HuD protein levels without significant change in abundance of any HuD transcript. Therefore, we concluded that NT-3 signals a translational increase in HuD, which may promote the production of upper layer neurons.

Thalamic ablation of NT-3 reduces HuD in RG during mid-neurogenesis

The development of the neocortex occurs in the greater context of the developing brain, and is likely influenced by developing subcortical structures. Previous findings have indicated that the thalamus influences areal formation within the neocortex, possibly through signals contributed through axonal afferents (Vue et al., 2013). NT-3 is known to be involved in the outgrowth of thalamic axons, which innervate the developing neocortex during mid-neurogenesis. Our

previous findings indicate that HuD-protein levels are increased in the presence of NT-3, and therefore, we generated a conditional thalamic deletion of NT-3 to assess the contribution of thalamic NT-3 on protein levels of HuD in the developing neocortex. To do this we examined conditional NT-3 KO (cKO) mice generated from Crh-Cre mice crossed with Nt-3-flox mice. The Crh promoter is found in the medial nuclei of the thalamus and is active by E11.5 in the thalamus (Figure 5-14) (www.brain-map.org, 2012). *Nt-3/Crh-Cre* cKOs were examined for HuD protein in mid-neurogenic RG through immunohistochemistry, and exhibited less HuD than WT while transcript levels of all HuD variants remained stable (Figure 5-15). These findings suggest that NT-3 contributed to the neocortex during mid-neurogenesis regulates the translation of HuD in RG, promoting the production of upper layers.

NT-3 may promote translation of *HuD3* in S-phase

Our in utero electroporation studies indicated that HuD3 is critical for upper layer formation, and our developmental translational analysis of neocortex demonstrated that this isoform is translated during mid neurogenesis, when upper layers begin to be formed. We also found that NT-3 contributed to the neocortex from the thalamus during mid-neurogenesis is critical for increasing HuD protein levels in RG. Thalamic afferents innervate the neocortex between the developing SVZ and the subplate, in the intermediate zone which later becomes the axonal tract composing the corpus callosum (DeBoer et al., 2013). As RG progress through INM, they are closest to this possible source of thalamic secretion during S-phase. Therefore we assessed if NT-3 promotes the

translation of HuD transcript variants during S phase as well as G1 phase as control. To this end, we obtained N2a cells which we synchronized in M-phase. Next, the cells were allowed to progress into G1 or S phase in the presence of 500ng/ml NT-3, or mock conditions. Next, cells were harvested for translational analysis as above. Next, fractionated RNA was subject to qRT-PCR using HuD variant-specific probes. Our findings demonstrate that *HuD1,2* and *4* are equally translated in the presence or absence of NT-3 in G1 and S phase (Figure 5-8). When HuD3 was equally translated in the presence or absence of NT-3 in G1 phase, but translation as significantly increased in the presence of NT-3 in S-phase. These findings suggest that *HuD3* is translationally regulated in S-phase by NT-3. These findings represent the final portion of my orignal data and have become part of a collaboration with other members of the Rasin lab. Given that this finding is part of ongoing study, a statement about significance cannot yet be made about this piece of data. However, taken together with our previous results, thalamic NT-3 may increase the translation of *HuD3* in the neocortex and promote the production of upper layers.

D. **Discussion**

Neocortical circuitry is the foundation of cognition, complex motor control and sensation, while malformations have been associated with a host of neuropsychiatric diseases. The excitatory neurons which form these complex and intricately-connected circuits are generated prenatally from a dynamic pool of stem cell progenitors. While recent descriptive studies have detailed that the neocortical transcriptome exhibits stage, species and region-specific expression

patterns, little work has investigated the mechanism of mRNA regulation governing NSC differentiation in neocorticogenesis. Here, we provide a first evidence of a network of posttranscriptional regulatory molecules influencing NSC fate and neocorticogenesis.

Neocortical neurogenesis produces the entirety of neocortical projection neurons in a very short time course. For this to occur, proliferative RG must rapidly shift the type of neuron being produced at progressive neurogenic stages. Our data demonstrate that HuD mediates the shift between the production of lower layer neurons and those of the upper neocortical layers in an isoform-specific fashion. Our LCM analysis provided evidence that HuD transcript variants are not differentially expressed between early and late neurogenesis in the RG population. However, subsequent investigation at the translation level indicates that HuD3 was specifically translated at E16 during late neurogenesis, but not at E13 when early neurogenesis occurs. Further, our electroporation studies show that early overexpression of HuD3 is sufficient to generate upper-layer neurons from stem cells which normally produce lower-layer neurons. Conversely, HuD4 expression prevented the production of upper layer neurons, where even late RG produced neurons of lower layers. These findings suggest a model in which HuD is regulated in an isoform-specific manner at the translation stage in RG.

Although there is strong evidence that post-transcriptional regulation underlies the staggering region-specific and functional complexity of the neocortex, the literature addressing the translation step of mRNA metabolism is

very limited (Johnson et al., 2009, Kang et al., 2011). HuD itself is a post-transcriptional mediator which is known to stabilize and influence the translation of its targets. Our studies suggest that HuD's four known isoforms exert differential influence over the fate decisions of RG in a temporally-specific manner. Evidence from bioinformatic platforms such as NCBI and the UCSC genome browser show that HuD3 and huD4 exhibit important differences in the "linker" region, between RRM 2 and 3 (Kent et al., 2002, McGinnis and Madden, 2004). Scientific precedent suggests that a single amino acid change in this region can ablate HuD's protein-protein binding to EIF4A, its association with polysomes and ultimately disruption of the translation of its targets (Fukao et al., 2009). Therefore, it is logical that HuD3 and HuD4 could promote such different fate outcomes in dividing RG. Future studies may evaluate the differences in translational machinery that these two isoforms are associated with, as well as possible differences in target binding given that the RRM's may be differently positioned in these two isoforms.

The temporally-distinct translation of HuD3 and HuD4 prompted us to assess a possible signal governing this transition. As neocorticogenesis transitions from early, lower-layer producing direct neurogenesis to late, upper-layer producing indirect neurogenesis, the developing cortical plate is innervated by ascending thalamic afferents (DeBoer et al., 2013). The development and pathfinding of these fibers is dependent upon NT-3 (Hanamura et al., 2004, Yamamoto and Hanamura, 2005). HuD has been well-described as downstream of this canonical trophic signaling pathway which can be initiated at the TRK

receptors (Abdelmohsen et al., 2010, Lim and Alkon, 2012, Allen et al., 2013). Our novel finding that HuD exerts differential control of RG fate through isoform-specific expression led us to question whether the translation of these variants is under the control of this pathway. However, even as a multitude of downstream effects of the Neurotrophin/TRK pathway have been described, there has been little investigation on the effects of trophic signaling on translation. Ablation of NT-3 from the thalamus decreased HuD levels at mid-neurogenesis in RG and stifled the progression of the cell cycle. Previous evidence had demonstrated that HuD governs the cell cycle through binding of P21 and P27 (Joseph et al., 1998, Kullmann et al., 2002). Further, these results marry logically with additional findings that RG cell cycle dynamics are critical to the formation of the neocortex. In this way, HuD may exert isoform and temporally-specific control of the RG cell cycle resulting in the production of the appropriate neuronal subtype.

Thalamic afferents arrive to the neocortex in a region of the developing CP between the SVZ and IZ. Therefore, as RG progress through the INM, they are closest to the source of thalamic-fiber generated NT-3 during S-phase (Kosodo, 2012). Our finding that HuD3 translation was induced by NT-3 only in S-phase confirmed our hypothesis that HuD's isoform-specific expression is critical not only to cell cycle progression but the fate decision of the RG receiving NT-3 input. Although several studies have addressed the effect of extrinsic factors on cell fate decisions, few have investigated the phase at which fate specification occurs (Yoon et al., 2008, Rash et al., 2011). To our knowledge, this study represents the first evaluation of the impact of trophic factor-induced isoform-

specific translation of a post-transcriptional mediator has on cell cycle progression and fate specification in the neocortex.

Our findings suggest extra-cortical developmental events heavily influence the intricate dynamics of INM and fate specification in the neocortex. Future studies of this kind will continue to elucidate the network of events and molecules which hone the evolutionarily-increasing complexity of the neocortex and its function. Our findings extend the implications of previous work indicating that the neocortex exhibits regional and stage-specific, transcription and splicing (Ayoub et al., 2011, Dillman et al., 2013, Mazin et al., 2013). Although one previous study have demonstrated species and region-specific translation in human, there remains a large body of work to determine the impact of translational regulation in neocorticogenesis (Kwan et al., 2012a).

Figure 5 1

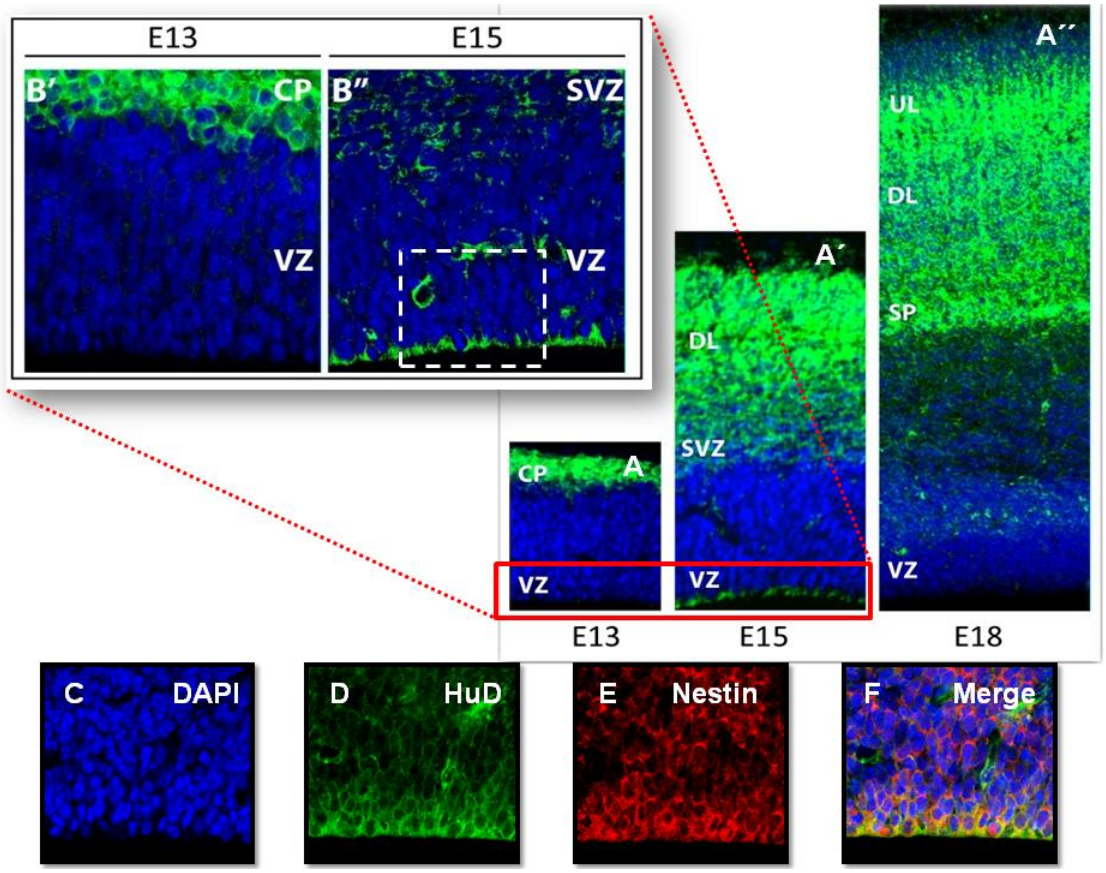

Figure 5-1: Representative confocal images of HuD in the developing mouse cortex (HuD in green, Dapi in blue). A-A´´: HuD is expressed in the postmitotic neurons of the cortical plate at all ages (right panels). B: HuD expression is found in the RG stem cells selectively at E15 (Left panels, E15). B, inset and C-F: HuD at E15 in the apical surface colocalizes with nestin, a stem cell marker.

Figure 5 2

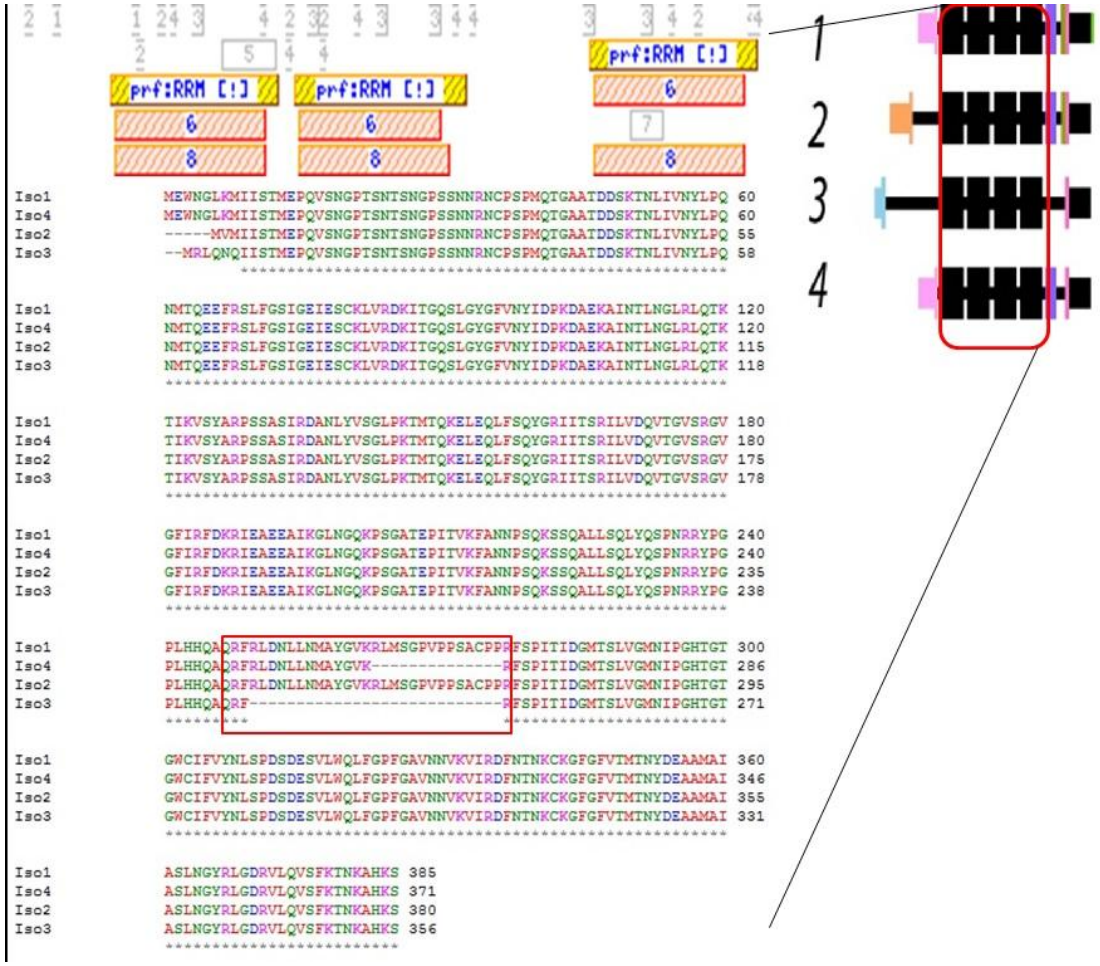

Figure 5-2. HuD is expressed in four known transcript variants (http://www.ucsc.com, top right schematic). Transcript variants encode four distinct protein isoforms, which exhibit significant changes in the linker domain (red box).

Figure 5 3

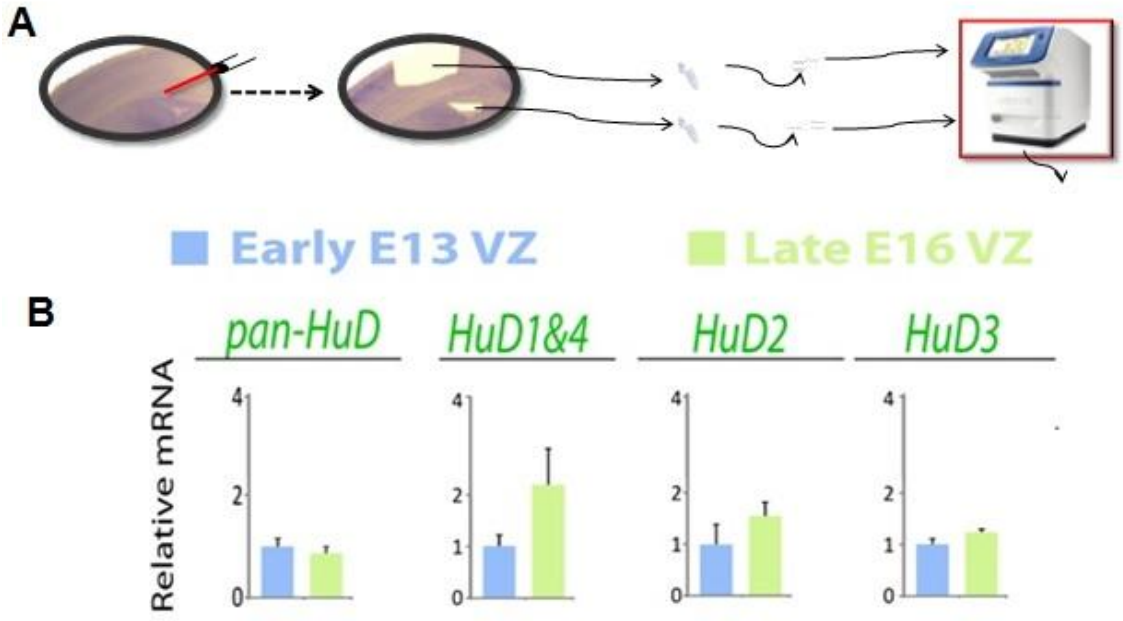

Figure 5-3. A. Cartoon of LCM tissue dissection and qRT-PCR analysis. **B.** qRT-PCR results indicate that HuD transcript variants are not significantly different in expression between early (E13) and late (E16) neurogenesis in the RG stem cell population.

Figure 5 4

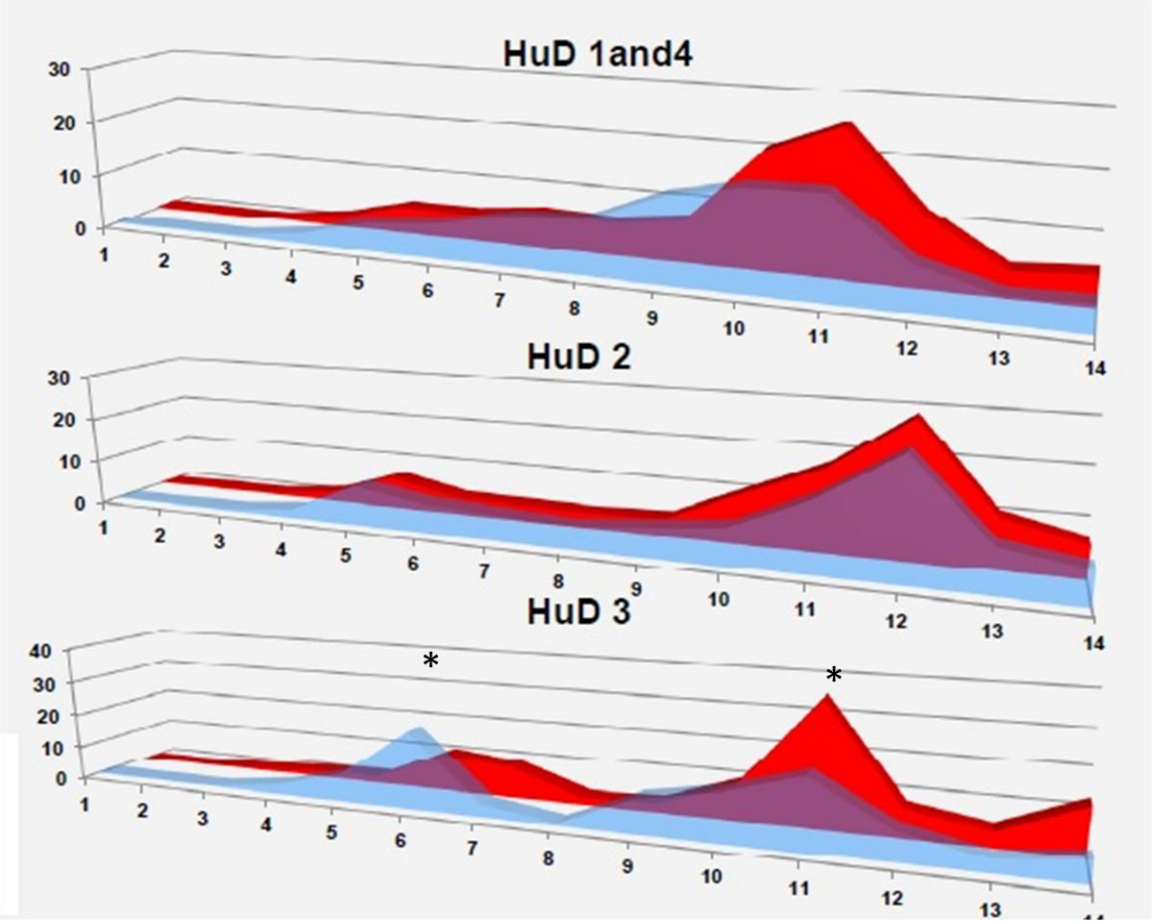

Figure 5-4. qRT-PCR analysis of E13 vs E16 neocortex mRNA separated by sucrose fractionation (E13 in Blue, E16 in Red). Light fractions (1-7) represent non-translating mRNA which is not bound to a high-mass ribosome. Heavy fractions (8-14) represent mRNA messages associated with 1 or several ribosomes and are undergoing active translation. Note HuD3 translation specially peaks at E16.

Figure 5 5

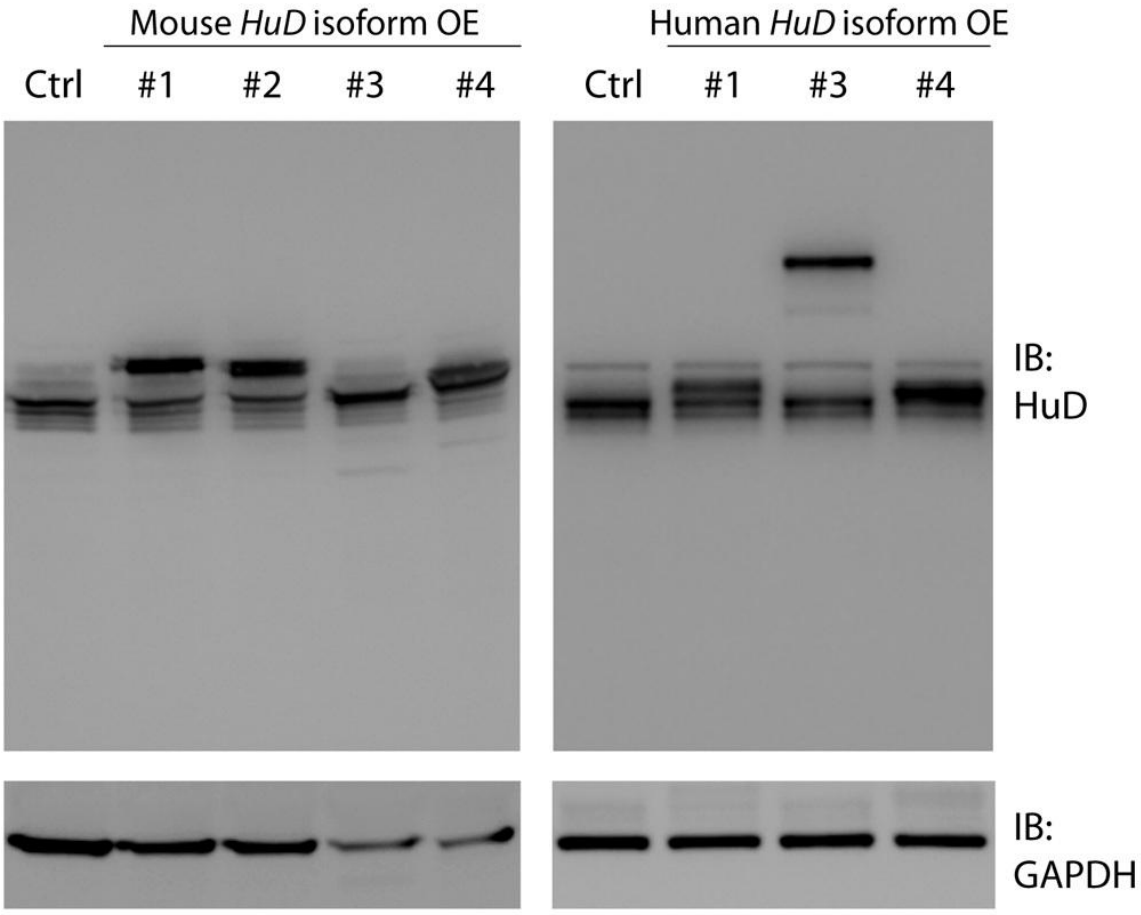

Figure 5-5. IB of HuD variant-specific overexpression vectors were obtained and lipofected into N2a cells. Note the variant-specific expression of mouse (left) and Human (right) HuD isoforms indicated by a pan-HuD antibody.

Figure 5 6

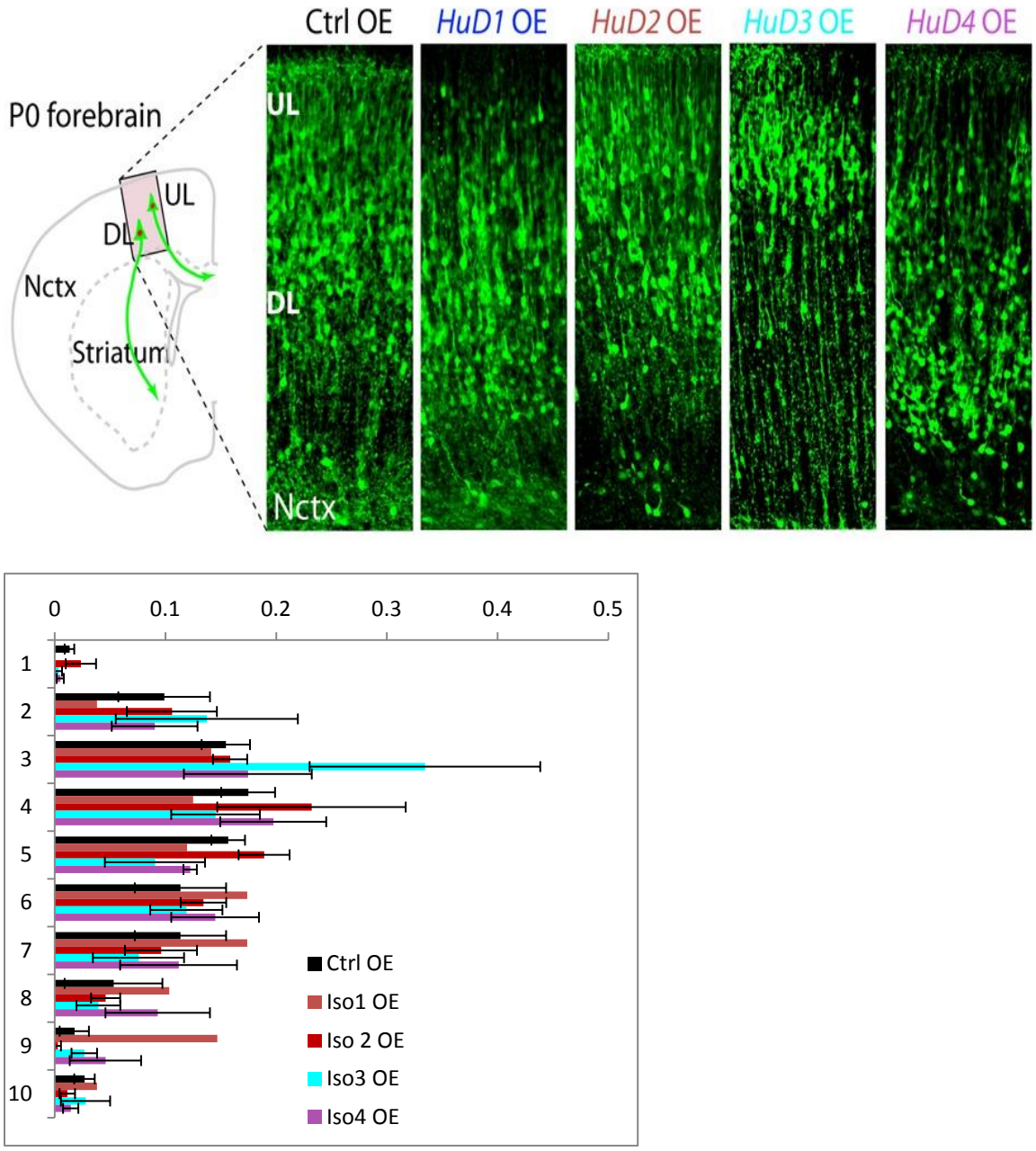

Figure 5-6. Representative confocal images of HuD-transfected GFP+ neurons in the neocortex (top). Note the cluster of cells found in upper layers in HuD3 overexpression, and the cluster of lower-layer cells found in HuD4 overexpression. Bottom: quantification of GFP per bin/total GFP in column. Again, note the large proportion of HuD3-transfected cells in upper bins, where HuD4 cells are predominately found in lower bins.

Figure 5 7

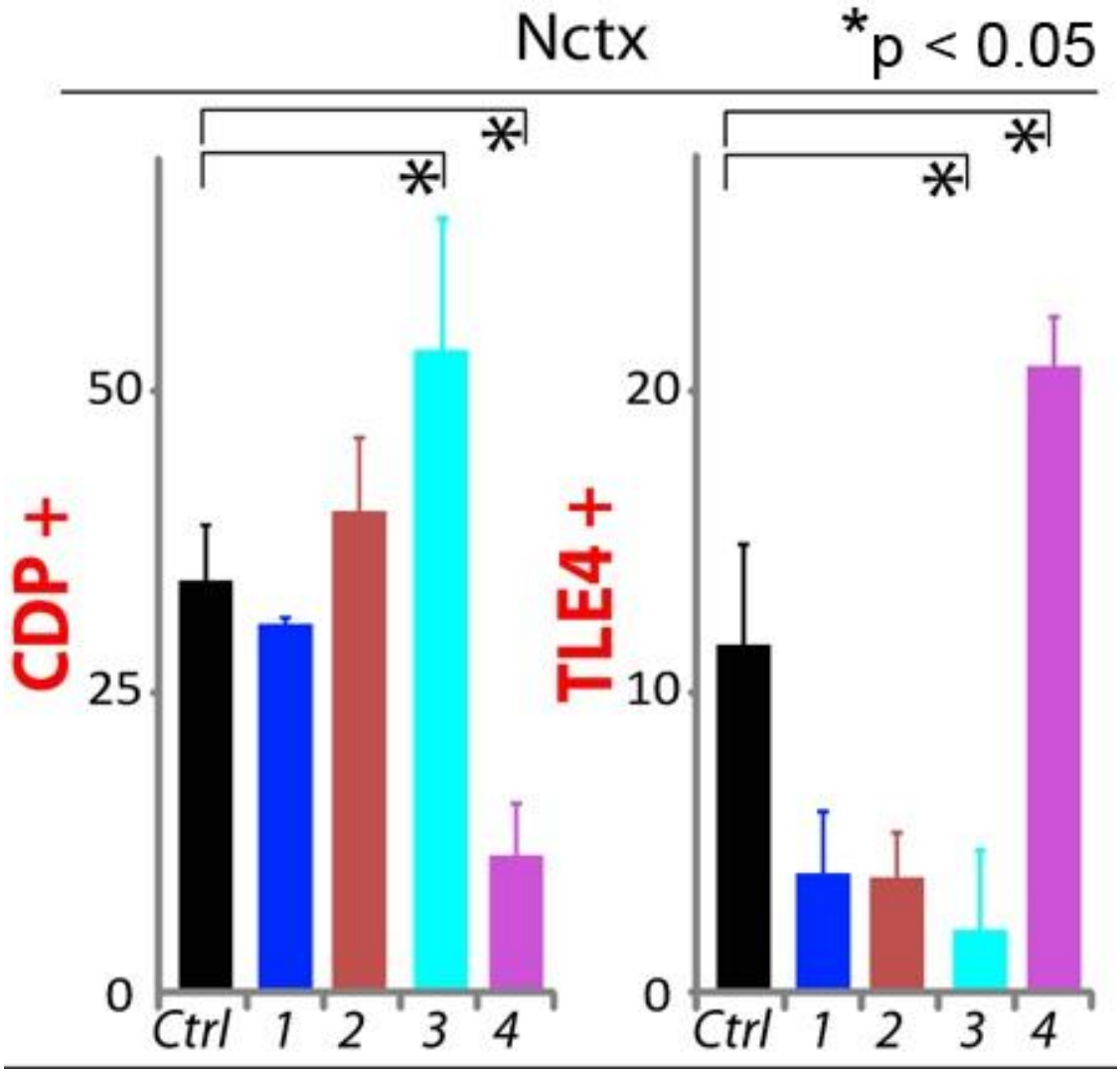

Figure 5-7. Transcription factor analysis of HuD-variant transfected neurons at P0. HuD3 transfected neurons demonstrated a significantly higher proportion of cells positive for CDP, an upper-layer marker when compared with control. (left panel). Conversely, HuD4 overexpression resulted in an increased proportion of cells positive for Tle4, a lower-layer marker (Right panel).

Figure 5 8

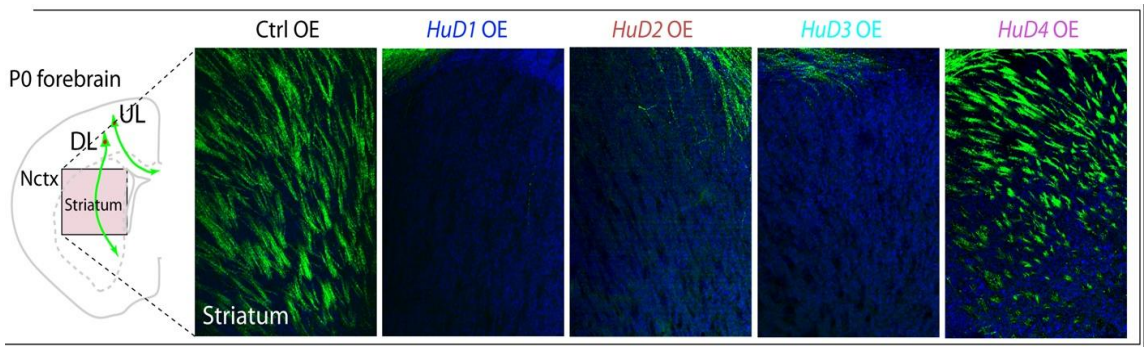

Figure 5-8. Representative confocal images of the basal ganglia of HuD-transfected brains at P0. Note the large number of HuD4+axons projecting to deep layers, while HuD3+ axons fail to project to lower layers; indicative of lower and upper layer fates, respectively.

Figure 5 9

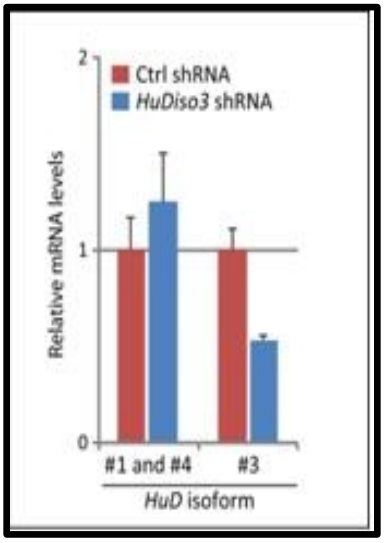

Figure 5-9. N2a cells were lipofected with shRNA vectors which were scrambled (Ctrl) targeted HuD variant 1 and 4 or HuD3. Variant-specific qRT-PCR analysis was conducted. Note the reduction of HuD3 specifically in the HuD3 shRNA condition while HuD variants 1 and 4 are preserved.

Figure 5 10

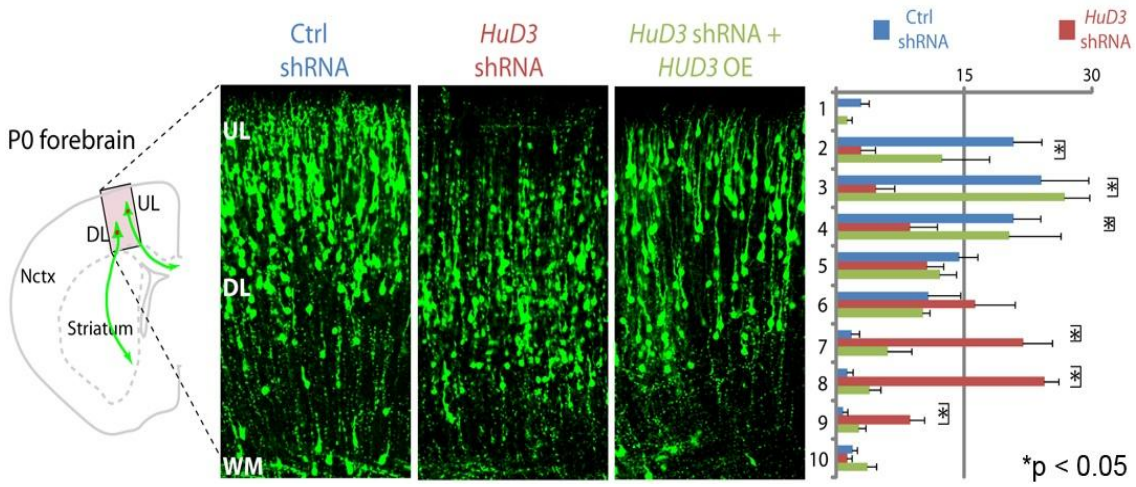

Figure 5-10. Representative confocal images of Ctrl shRNA, HuD3 shRNA and HuD 3 shRNA+ Human HuD3 OE rescue (left, middle and right panels, respectively). Note the high proportion of cells found in lower layers in the HuD3 shRNA condition, whereas HuD3 shRNA+ HuD3 HumanOE rescues this phenotype. Right panel: quantification of GFP+ cell proportions per bin/total GFP+ cells in each column.

Figure 5 11

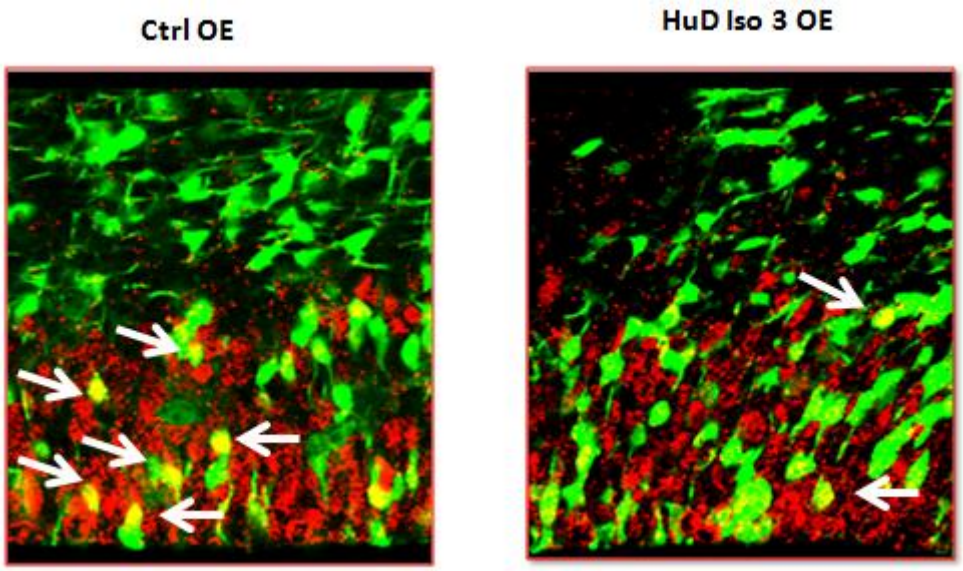

Figure 5-11. Representative confocal images of HuD variant-specific electroporation from E13 to E16 of Ctrl (left) and HuD3 (right). Many fewer colocalizations of GFP-transfected cells (Green) with Pax6 (red) were noted in the HuD3 OE condition than Ctrl.

Figure 5 12

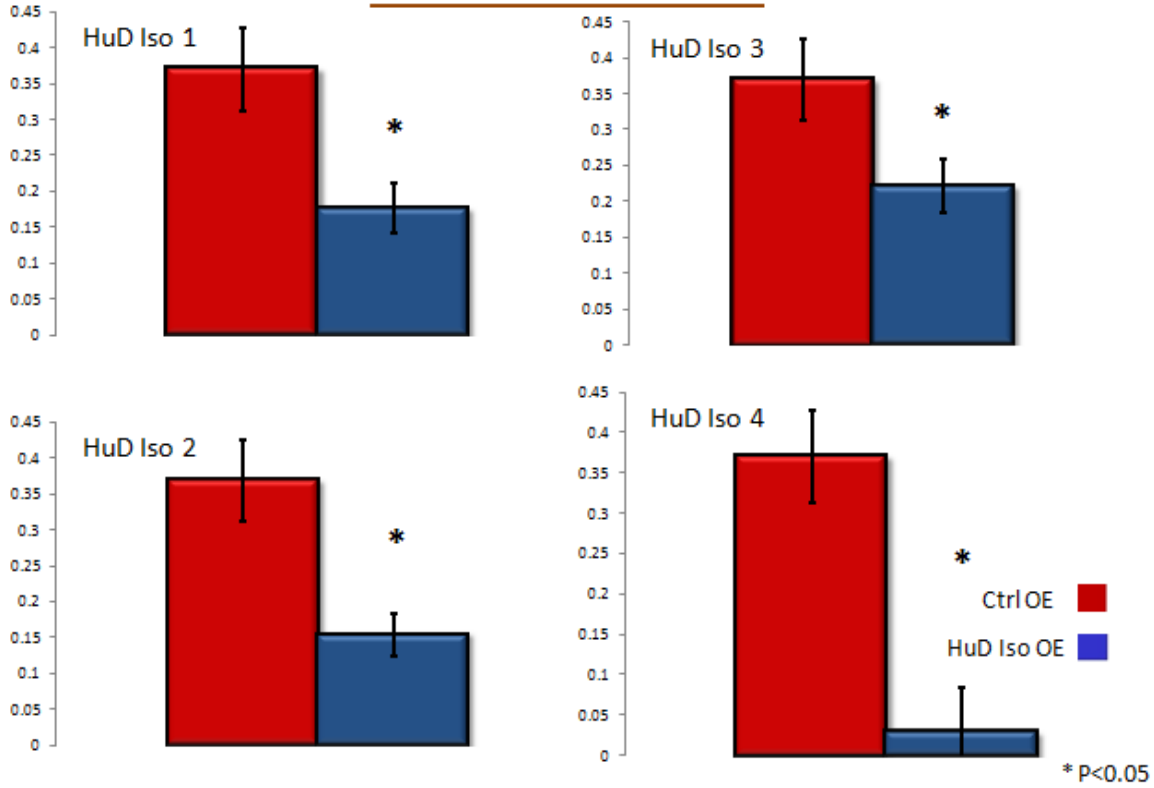

Figure 5-12. Quantification of HuD variant-transfected GFP+ cells colocalized with Pax6 in the VZ at E16.

Figure 5 13

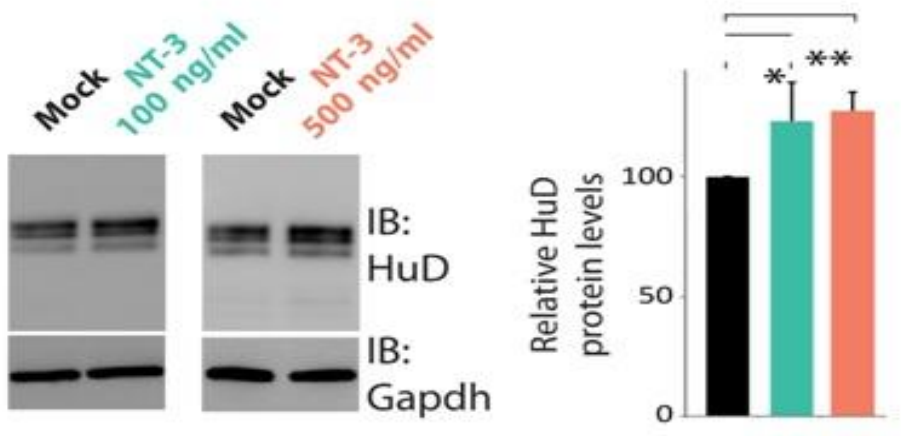

Figure 5-13. Western blot analysis of E13 primary cultures exposed to mock (normal media, black writing) 100 (green) and 500 (red) ng/ml NT-3 for 48 hours. Left, IB of HuD levels. Right, quantification of western blots, left. *P<0.05 **P<0.01.

Figure 5 14

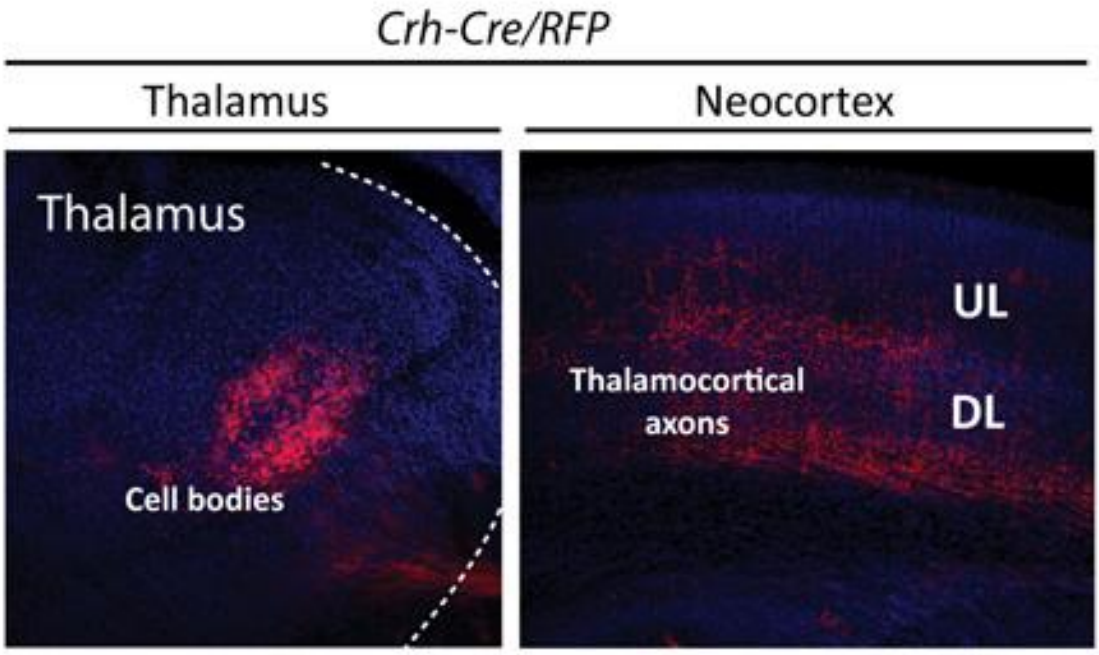

Figure 5-14. Representative confocal images of Crh expression at P0 in the thalamus (left) and Neocortex (right). Crh-Cre/RFP (red) Dapi (Blue). Mouse line developed and images taken by Althea Stillman.

Figure 5 15

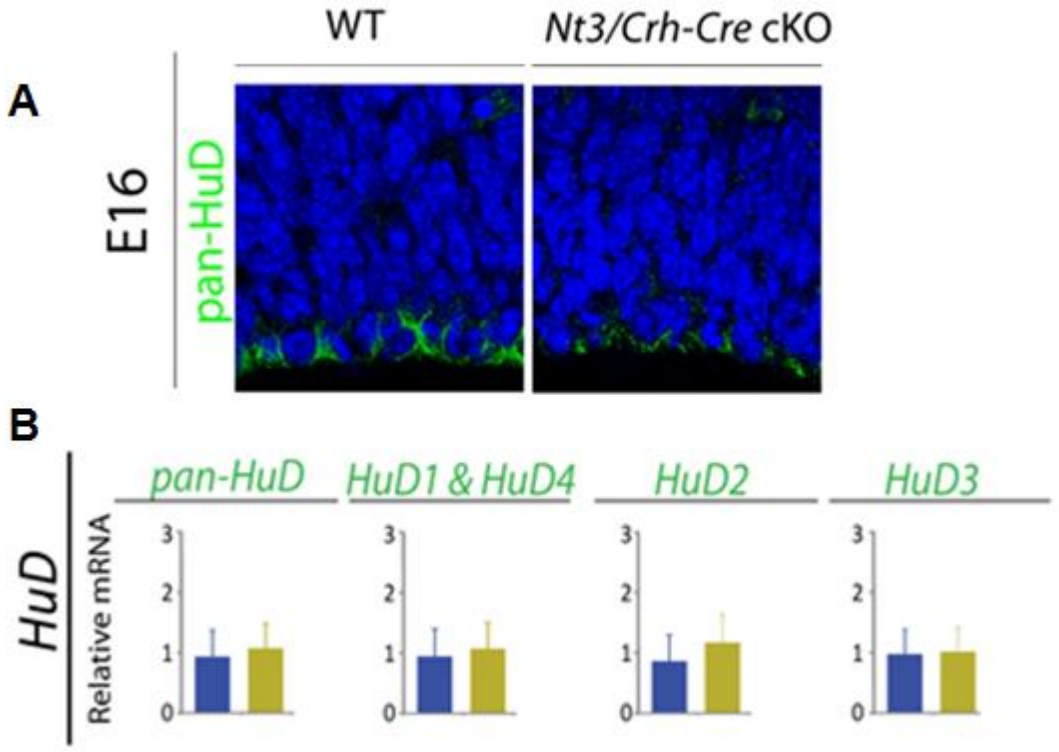

Figure 5-15. A. Representative confocal images of HuD immunostaining in WT and Nt3/Crh-Cre cKO VZ at E16. HuD in green, Dapi in blue. B. LCM-qRT-PCR of WT vs *Nt3/Crh-Cre cKO* VZ at E16 for all HuD variants. *Nt3/Crh-Cre cKO* mouse line developed and immunostaining/confocal analysis performed by Althea Stillman.

Figure 5 16

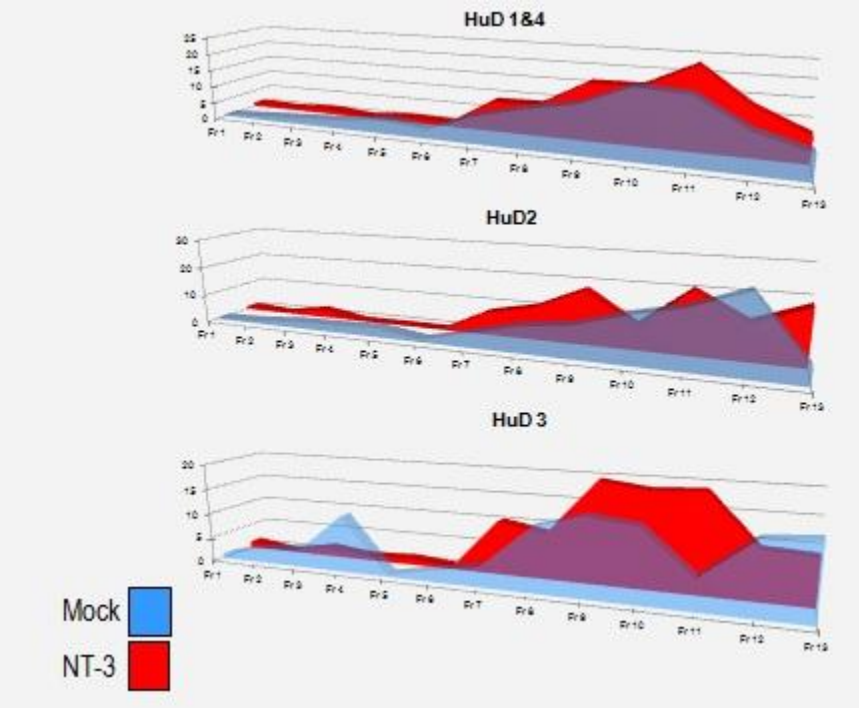

Figure 5-16. Translational analysis of HuD isoforms by qRT-PCR in N2a cells at S-phase in mock (Blue) or NT3+ (red) conditions. Note HuD3 expression increases in polysome fractions in the presence of NT-3 at S-phase.

Chapter 6: Special Methodologies and Techniques

1. Introduction to cortical nucleoside labeling

Some 35 years ago, the introduction of radioactive triturated thymidine (^{3}H-T) as a thymidine nucleoside analog allowed researchers to determine the birthdate of cells (Rakic P, 1968b). ^{3}H-T can be pulsed into a developing system, and will be incorporated into cells which are synthesizing a new copy of DNA during the S-phase of the cell cycle at that time. Importantly, the progeny of the ^{3}H-T labeled cells also carry the isotope and thus, can later be examined histologically through radiographic imaging of the resulting tissue.

Later, this technique was widely used in seminal studies investigating the developing central nervous system (CNS), including those which demonstrated the sequential spatio-temporal generation of distinct subpopulations of neocortical projection neurons. The neuronal progeny which take place in deeper neocortical layers (V-VI) are born from earlier divisions of neural stem cells (NSCs), whereas neurons that reside in the superficial layers (II-IV) are later-born neurons (Rakic, 1974, Nowakowski et al., 1975, Brand and Rakic, 1979, Crandall and Herrup, 1990, Polleux et al., 1997). These and others' data demonstrated a fundamental mechanism in the developing central nervous system; the timing of stem cell divisions dictates the fate and final placement of the postmitotic progeny (Dehay and Kennedy, 2007).

More recently, non-radioactive thymidine analogs were introduced, such as the halogenated deoxyuridine nucleoside analogs; bromodeoxyuridine (BrdU),

iododeoxyuridine (IdU) and chlorodeoxyuridine (CldU) (Miller and Nowakowski, 1988, Breunig et al., 2007, Rash et al., 2011). In addition, and most recently, 5-ethynyl-2'-deoxyuridine (EdU) (also referred to as "Click-it") technology has been developed, using an alkyne group to foster incorporation into DNA (Salic and Mitchison, 2008). Similar to ^{3}H-T, these small molecules are incorporated into the DNA of dividing cells during the S-phase of the cell cycle. BrdU, IdU, CldU or EdU can be pulsed into developing central nervous system in a similar manner to ^{3}H-T, but can also be applied through drinking water for continuous labeling of cell divisions (Zhao et al., 2003). Importantly, early validation studies demonstrated that incorporation of halogenated deoxyuridines can be used to distinguish proliferating cell populations in a similar manner to the earlier ^{3}H-T studies (Miller and Nowakowski, 1988, Crandall and Herrup, 1990). Given the unique chemical structure of the halogenated analogs, some can be subsequently labeled using distinct antibodies. Thus, an advantage of halogenated deoxyuridine labeling is that two analogs can be used to track progeny generated at different time points within the same developing tissue/region (Figure 6-1). A recent example of colabeling using CldU in conjunction with IdU has allowed researchers to track sequential birth and final placement of different subpopulations of neocortical neurons generated at discrete time points within normal and mutated developing neocortices (Rash et al., 2011). Of note, care should be taken when combining halogenated analog studies with EdU, as the chemistry involved in each and the resulting sensitivity of labeling is somewhat different (Salic and Mitchison, 2008).

In addition to their use in determining the birthdate and final position of postmitotic neuronal subpopulations, deoxyuridine nucleoside analogs can be used to evaluate cell cycle dynamics, exit, or re-entry of proliferating stem cell populations *in vivo* (Hansen et al., 2010, Rash et al., 2011). This is performed by pulsing two thymidine analogs closer together in time, such that stem cell populations can incorporate both analogs as they undergo subsequent S-phases. Even as they have some notable limitations such as dilution with each cycle (Breunig et al., 2007), halogenated deoxyuridines can be employed in this method to uncover the proportion of cells re-entering or exiting the cell cycle during distinct neurogenic phases of normal and mutated or lesioned central nervous system (Figure 6-2).

Using Nucleoside Analogs to Track the Differentiation of Cells that are Modulated Autonomously via *In Utero* Electroporation

The recently developed technique, *in-utero* electroporation (IUE) allows an investigator to modulate the gene expression of NSCs autonomously in the developing CNS *in vivo*, and subsequently to track the changes in the progeny of these cells throughout development (Bai et al., 2003, Saito, 2006, Rasin et al., 2007). For example, previous birthdating studies of developing neocortices using nucleoside analogs have elucidated the time points at which distinct subpopulations of projection neurons are born (Caviness VS Jr, 1973, Rakic, 1974, Nowakowski et al., 1975, Brand and Rakic, 1979, Miller and Nowakowski, 1988, Crandall and Herrup, 1990, Polleux et al., 1997, 1998). Therefore, the functional gene expression levels of each subpopulation of sequentially-

generated neocortical projection neurons can be manipulated by performing IUE at the time of their birth.

IUE involves first injecting a purified plasmid into the lateral ventricle(s) of a developing mouse pup, then transfecting the stem cells lining the ventricles with an mild electrical pulse. Usually, these plasmids encode an overexpression vector or a short hairpin RNA (shRNA) specific to a gene of interest, and separately a control overexpression or a control, non-specific scrambled or point mutation shRNA as described (Saito, 2006, Rasin et al., 2007). Usually a plasmid encoding a reporter fluorescent protein, such as eGFP is co-transfected, to allow for later visualization of transfected cells upon tissue processing (Bai et al., 2003, Saito, 2006, Rasin et al., 2007); for more details see section 2 of this chapter. After the IUE is performed, all progeny of the transfected NSCs will express the gene of interest/targeting shRNA and reporter fluorescent protein. The transfected progeny can then be pulse labeled using halogenated deoxyuridine nucleoside analogs, such as CldU and IdU, at separate time points (Figure 6-1). Using this approach, an investigator can evaluate the cell autonomous effect of altered gene expression on the resulting birthdates, differentiation, or cycling rates of cells in the neocortex (Kosodo et al., 2011, Rash et al., 2011).

Nucleoside labeling is often used in conjunction with other labeling techniques to examine a birthdate and lineage of a cell. In the developing CNS, several markers have been identified to detect delineate stem cells such as RG and IPCs, from subtypes of postmitotic neurons and glia (Table 6-1). In the

neocortex, molecules have also been described which can separate other cell cycle phases (PH-3, Cyclin D and E, Table 6-1).

2. In-Utero Electroporation

Transfection offers a means of changing the gene expression of a target cell typically through the use of cDNA plasmid expression vectors. These vectors most often contain a heavily-targeted promoter upstream of a coding sequence for a transcript of interest. Eukaryotic cells have a bilipid membrane which is negatively charged. given that DNA also carries a negative charge, eukaryotic cells will not normally take up DNA. Transfection is the process by which the cDNA can be driven into a cell with some expectation of efficiency and without destroying the target cell. Popular techniques include lipofection, where cDNA is coated with a cationic lipid creating small liposomes which can pass the negatively-charged lipid barrier (Strain, 2006, Koynova and Tenchov, 2011). This technique is especially suited to plated cells which are robust and resilient. Other techniques include plated electroporation, during which cDNA is driven toward a transient positive pole. The current of this circuit also porates, or creates small openings in the target cells. cDNA is then driven, physically, into the cells. The efficiency of this technique is lower than lipofection, but is suited to primary culture cells where lipofection media conditions are incompatible with the cells' requirements and the efficiency is low.

While electroporation has been employed for some time, recent progress has been made particularly by those studying neocortex by utilizing some of the aspects of plated cell electroporation in vivo. This is accomplished by typical

amplification of plasmid cDNA. cDNA is then super-concentrated to 4000ng/uL. In many cases, cDNA of interest is mixed with a fluorescent reporter cDNA such that the transfected cells can be visualized (where co-electroporation is assumed to be 90-95%) . These plasmids, along with a dye, are then loaded into a small glass pipette. Surgically, the pups of an anesthetized pregnant mouse dam are exposed. Plasmid cDNA is pippetted through the glass pipette into the lateral ventricle of the developing mouse embryo by puncturing the CP through the uterine wall. Once the ventricle has been injected with 1-2 uL of cDNA, a square electric pulse of 50mV is sent through the brain of the pup by external copper paddles. Typically the paddles are arranged such that the positive paddle is positioned over the dorsal surface of the pup's head, such that the cDNA will be driven from the ventricle into the cortical stem cells or neurons, depending on the age of the pup. When performed on pups in the anesthetized dam, this process is termed "In-utero electroporation (Figure 6-3)."

In-utero electroporation is an advantageous technique for those studying cortical development for several reasons. First, the direction of current and the injection point can be manipulated, such that the cells targeted for transfection can be somewhat isolated. Typically the cells targeted are those of the dorsal telencephalon between the ages of E11 and E16. As described in Chapter 1, these are the neurogenic progenitor RG. Second, this process allows the user to influence acute gene expression changes in the stem cell population which could be compensated for at the genetic level. Also, as each cell is transfected individually, the small percentage of the total stem cells that do become

transfected carry out their gene expression programs in a cell-autonomous manner. That is, each cell is individually manipulated, where neighboring, non-transfected cells are considered normal. This is important if the user is determining the effect of a transcript of interest in the context of an otherwise normally-developing cortex.

3. Laser Capture Microdissection

As the neocortex develops, several subregions become apparent. As discussed in Chapter 1 and Chapter 2, the proliferative regions of the neocortex in the dorsal telencephalon include the VZ, the SVZ and the oSVZ, which each contain different types of stem cells which are also differentially potent. Further, throughout development and during adulthood, the CP contains multitudes of different subtypes of excitatory projection neurons. In many cases there are also regional specializations within the neocortex, particularly in human. Therefore, because tissue subtypes are compartmentalized throughout development, subdissections of tissue have become necessary to discriminate one cell population from another for transcript and protein-level analysis. To do this, several groups have employed laser capture microdissection (LCM).

LCM is a technique by which unfixed cortical or other tissues are sectioned in one plane (horizontally, coronally or sagittal) typically onto a membrane slide. The membrane slide can then be stained with histological stains if desired (treatments are available for RNAase-free tissue treatments of this type). Next, the slide is placed on a microscope dissection machine, where a laser will cut sections of the tissue and either drop or stick-cap them into an

RNAase free collection tube (Figure 5-3 and Figure 6-4). Subsequent RNA analysis can then reveal regionally-specific expression patterns which are particularly of consequence in the neocortex. Further, some level of protein analysis is possible, but one must be mindful of the temperature conditions of the sample. Previous studies mention discontinuing sectioning after 30 minutes for RNA analysis.

Figure 6 1

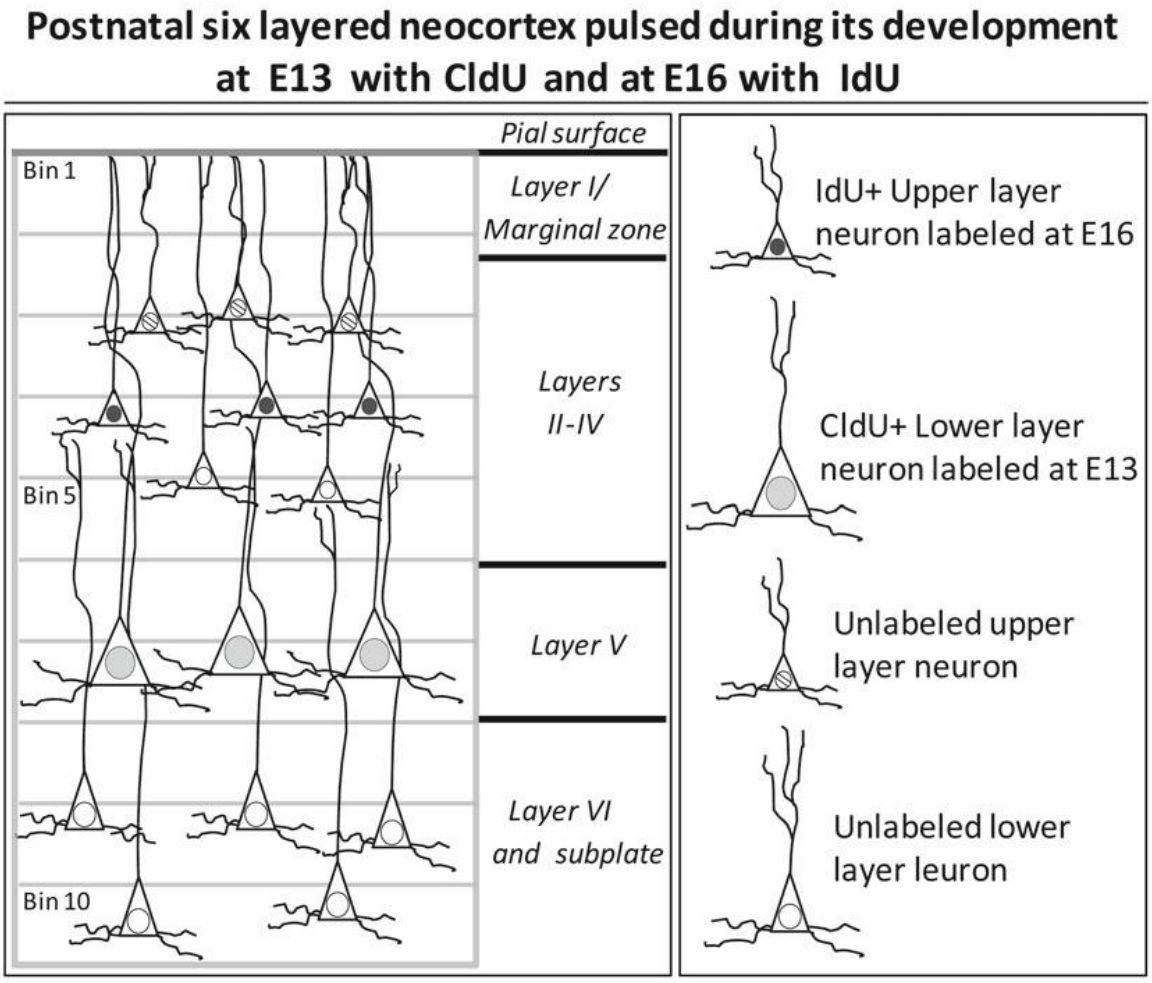

Figure 6-1. A schematic example of embryonic birthdating analysis in a postnatal neocortex. Note the presence of postmitotic neuronal progeny in neocortical layers II-VI. CldU pulsed at E13.5 preferentially labels Layer V neurons (light gray nucleus). IdU pulsed at E16.5, however, preferentially labels neurons in layers II-IV (dark gray nucleus). Note that cells which were not born at E13.5 or E16.5 are unlabeled. A grid of 10 bins is overplayed on the cortical plate for distribution analysis as described

Figure 6 2

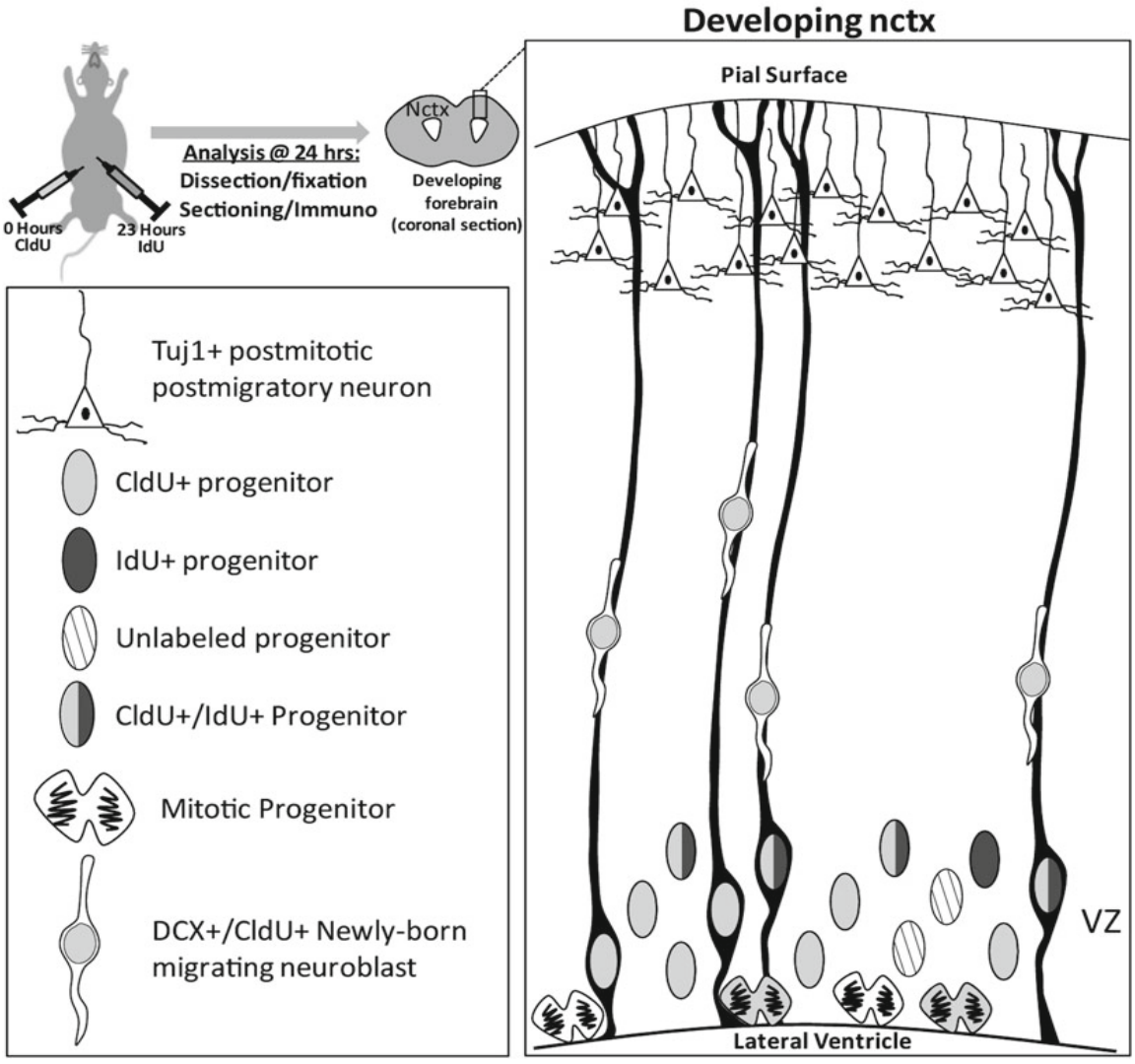

Figure 6-2. A schematic for analog pulse injections. A pregnant dam injected with CldU at starting time point 0 (*light gray*), 24 hours before embryo collection. Subsequently the dam is injected with IdU 23 hours after the CldU injection and 1 hour before collection. Processed tissue immunostained for CldU and IdU is imaged with a confocal microscope. This example depicts imaging the neocortex (*box*) for cell cycle analysis. CldU+/IdU negative (*light gray nucleus*) cells have exited the cell cycle, and are beginning to migrate and differentiate. CldU-negative/IdU+ (*dark gray nucleus*) are cycling at 24 hours but were not in S-phase at start time 0, and are progenitor cells. CldU+/IdU+ (*half light/dark gray nucleus*) cells have re-entered the cell cycle and are progenitor stem cells. Note the presence of some CldU+/DCX+ migrating neuroblasts with leading (thicker) and trailing (thinner) processes. These neuroblasts migrate along the representative black radial glia processes attached at pial surface. Finally, note the presence of mitotic progenitors at the ventricular surface and postmitotic, postmigratory Tuj1+ pyramidal neurons in the neocortex (Nctx). VZ = ventricular zone

Figure 6 3

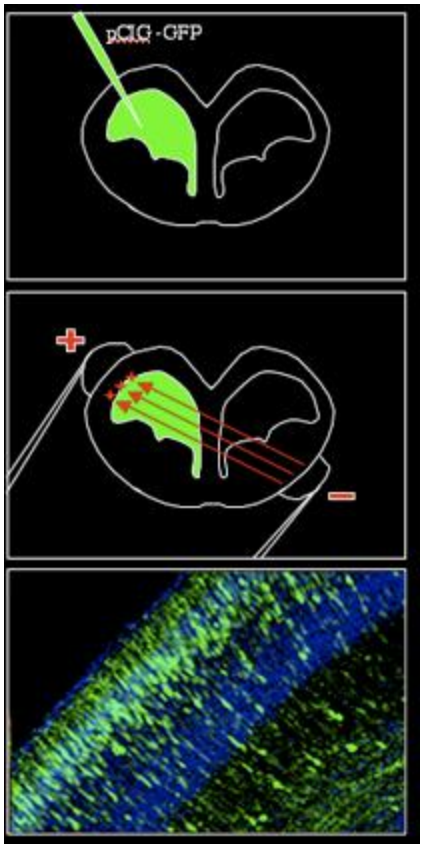

Figure 6-3. Schematic of In-Utero electroporation and resulting transfected tissue. Top panel: plasmid cDNA is injected into the lateral ventricle (in this case GFP plasmid is used). Middle panel: Current is applied through square pulses to influence the direction of transfection. In this case, the dorsal telencephalon VZ is being transfected. Bottom panel: Representative confocal image of an electroporated brain where GFP+ cells report the cDNA transfection. Picture obtained from http://www.pvdhlab.site.ulb.ac.be/?page_id=22.

Figure 6 4

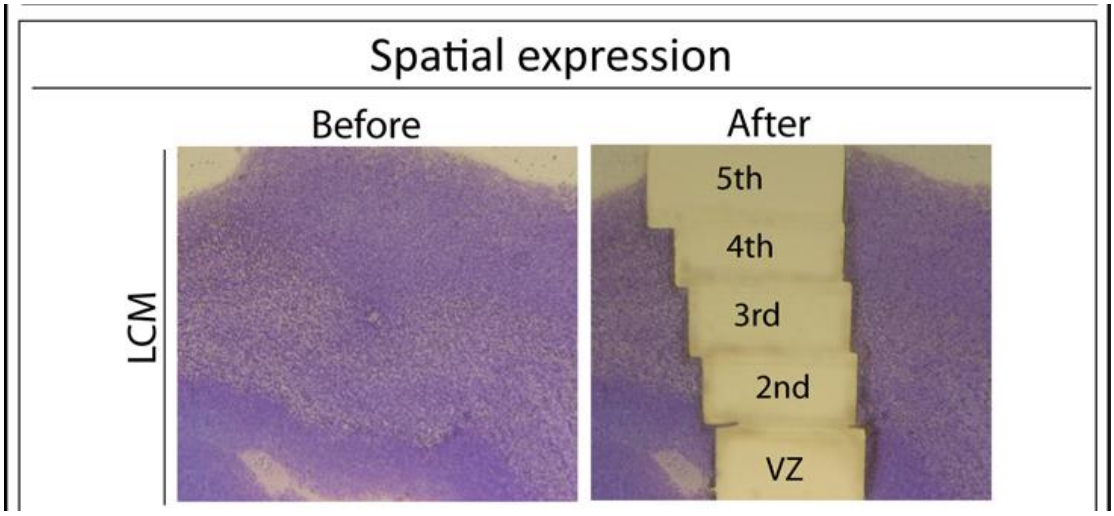

Figure 6-4. Representative image of crsyl-violet-stained coronally sectioned neocortex subdissectied through LCM into 5 regions from the VZ to the pia (5th section).

Table 6 1

Cortical Cell Subtype Markers		
Antigen	**Age/Stage Expressed**	**Population Labeled**
Pax6	E10.5-P0	RG and NSC
Sox2	E10.5-P0	RG and NSC
BLBP	E11.5-P0	RG
Glast	E11.5-P0	RG and Astrocytes
Tbr2	E14.5-P0	IPCs of Neocortex
Tbr1, Tle4	E12.5-	SP and Layer VI of Neocortex
Fezf2, Ctip2	E13.5-	Layers V (high) and VI (low) of Neocortex
Er81	E13.5-	Layer Va/b of Neocortex
Svet1, Cux1, Cux2	E14.5-	Layers II-IV of Neocortex
DCX	E12.5-P0	Migrating Neuroblasts
βIII tubulin	E12.5-	Mature, Postmigratory Neurons

Table 6 2

Cell Cycle Phase Markers	
Marker	**Phase labeled**
Ki67	Expressed in all actively cycling cells, and specifically localized to chromatin in M-phase
PCNA	Expressed increasingly in G1 and peaks in S-phase
BrdU, CldU, IdU, EdU	Incorporated during S-phase
PH3	Expressed in M-phase
Phospho-Vimentin	Expressed in cycling radial glia
Cyclins	Cyclin D (G1, S, G2 and M) Cyclin E (G1-S) Cyclin A (S-G2) Cyclin B (Late G2-M)
Tis21	Expressed during a neurogenic division (Attardo et al., 2010)

Chapter 7: Implications and Future Studies

A discussion of post-transcriptional regulation in future studies

Human evolutionary advantages of language, complex motor behavior, and advanced cognition can be traced to the morphological and functional expansion of the neocortex. Increased proliferative regions such as the SVZ and oSVZ can explain the dramatic increase in neuronal number, but this alone does not explain the vast functional differences among mammalian neocortices. Other neocortical regions also show evolutionary expansion, one of most important being the subplate, which is vastly expanded in human and non-human primates (Kostovic et al., 1989, Kostovic and Judas, 1998, 2010) (for review and psychiatric implications of this region, see (Kostovic et al., 2011)). Modern techniques, which permit a closer look at the transcriptomic architecture of neocortical compartments across development and species, reveal that the specificity of neocortical regions are dictated at least in part by vastly different transcript complements as well as uniquely expressed splice variants.

Given the relative homology of the mouse and human genomes and their similar number of protein-coding genes, it stands to reason that regulation at the post-transcriptional level may explain the disparity in complexity among mammalian neocortices. Of these post-transcriptional mechanisms, splicing is of particular interest, as it allows an expansion of proteome functionality and a narrowed fidelity without broad DNA-level changes. Investigations of human pre-mRNA demonstrate that 74% of these molecules are subject to alternative splicing (Johnson et al., 2003). A later study showed that these splicing events

produce functional isoforms, suggesting that proteins are robust molecules that tolerate excisions and insertions, perhaps allowing the evolution of the proteome (Birzele et al., 2008). It may not be unfounded to extrapolate that evolutionary changes in the neocortex may correlate with greater specificity in the proteome of neocortical compartments.

For the splicing of a target transcript to impact the activity of a cell, it must be translated. At this heavily regulated post-transcriptional step, there is already one species-specific example of transcript regulation by an RBP (Kwan et al., 2012a). However, profiling of the proteome to investigate compartmentalized and species-specific evolutionary changes in the neocortex is not yet complete. This is likely due to the technical difficulties involved in such analyses. The spatiotemporal genetic specificity necessary to generate defined subpopulations of neocortical projection neurons likely involves an interaction of transcript regulation events, such that uniquely spliced variants are transported and rapidly translated based on cellular demand. Subtle alterations in spatiotemporal expression at the post-transcriptional level result in a wide spectrum of neocortex-associated disorders. Clinical applications of post-transcriptional studies are vast, and promising findings already exist. Indeed, future studies could combine the increasingly precise definitions of psychiatric disorders in the Diagnostic and Statistical Manual of Mental Disorders with analyses of transcriptional profiles. Studies employing this approach are already beginning to characterize the transcriptional profiles of schizophrenia, autism, and suicide (Roussos et al., 2012, Sequeira A, 2012, Ziats and Rennert, 2012). Recent work

in rats also identifies changes in functional classes of transcripts in neurons that either do or do not “sprout” and regrow connections after a modeled stroke infarct (Li et al., 2010). By comparing animals of different ages, this study also shows that the vast majority of changed transcripts in successfully outgrowing neurons differed depending on age, suggesting treatments that may be tailored for particular age groups. A similar screening method in a study of the effect of maternal exposure to alcohol on mice and humans (Hashimoto-Torii et al., 2011) demonstrates a consistent down-regulation of the proliferative TBR2 transcript and protein as well as postmitotic specification markers, particularly those of upper-layer neurons. A screen of a mouse model of anxiety also confirms transcript changes in the hippocampus and cortex, pointing toward new targets for study (Virok et al., 2011). Furthermore, layer-specific profiling using BAC-TRAP shows translational changes in layer V projection neurons after antidepressant treatment (Schmidt et al., 2012). Finally, acute traumas can alter functions of distinct regulatory members of mRNA translation. For example spinal cord injury alters mRNA binding signature of EIF4E in neocortical neurons contralateral to the hemisection model (Thompson K, 2010), an initiation factor that in our screen came as part of GO:RNA binding. These data suggest that post-spinal cord injury mRNAs for possible regenerative efforts are acutely present in central neurons, but their translation is disrupted. Together, these types of studies demonstrate an increasing trend toward defining brain disorders based on transcript profiles and their posttranscriptional processing.

These types of studies could enhance our understanding of disease mechanisms within varying disease subtypes, leading to more specific and effective treatments. The understanding of the rich post-transcriptional contribution to disease states, however, must proceed to the final functional output of gene expression. The mere presence of a transcript cannot be equated to its proteomic function, and there is new evidence that the initiation and elongation steps of translation are heavily regulated, and perhaps aberrantly in disease (Darnell et al., 2011). The understanding of the metabolism of mRNA may be well served by greater study of RBPs in parallel with their mRNA targets of regulation. Therefore, future studies on the role of RBPs in neocorticogenesis and profiles of the developmental proteome are paramount to understanding the functional genetic complement of a tissue or cell.

Discrimination of cell subtypes in the developing and mature cortex

As previously discussed, the developing dorsal telencephalon is populated by several different subtypes of stem cells, such as RG, SNP's, IPC's and oRG (DeBoer et al., 2013). Throughout several cell cycles, these stem cell subtypes eventually give rise to all of the glutamtergic neurons of the neocortex. In RG and IPC specifically, the dynamics of the cell cycle have been worked out extensively . Although notch signaling and other cell-cell contact and extrinsic factors have been assessed as regulators of the cell cycle and resulting postmitotic specification and fate, post-transcriptioanal regulation during this process has been virtually unaddressed by modern neuroscientists (Gaiano et al., 2000, Mizutani et al., 2007, Bultje et al., 2009, Ables et al., 2011).

Future studies should make efforts to investigate the post-transcriptional changes occurring in the phases of the cell cycle, particularly in neocortex across stages of development. These types of studies will elucidate what families of transcripts are being metabolized, and at which stage of metabolism, throughout the cell cycle and across development. As described in chapter 5 of this dissertation, we have already obtained results that HuD3, for example, is translated only in S-phase, and only during late stages of neurogenesis. The next stage of this research will dissociate cycling stem cells in-vivo, and perhaps perform FACS to separate each stage. IN this way, cycling cells in G1, S and G2/M phases can be separated by DNA content in their nucleolus. Each separated cell phase can then be subject to microarray, sucrose fractionation or ribosomal footprinting to assess expression and translation of new targets for study. For increased resolution, FACS may include a transgene reporter, such as Pax6-RFP, where only Pax6 positive cells can be assessed. This process would separate RG, SNP and oRG from IPCs, especially in late stages of neurogenesis. Indeed, the post-transcriptional characteristics across a developmental time course in a stage-specific fashion between divergent stem cell classes would provide a wealth of data for future study. Further, because markers of cell subtypes are often assessed at the protein level especially in neocortex, a study such as this may provide new markers for analysis of stem-cell subtypes in the developing telencephalon (Molyneaux et al., 2009).

A variation of the study above could also be performed on postmitotic neurons in the cortex. Regions of interest can be manually dissected, and with

the use of one or more fluorescent reporters, the aspects of the development of these cells can be assessed. For example, early stages and dendritogenesis of upper vs lower layers of the neocortex from early postnatal stages until mature adult. Once these baselines have been established, similar studies can assess the impact of these processes when RBPs' expression are modulated. This may provide an accurate assessment of the contribution of each RBP to mRNA metabolism throughout the maturation of neuron classes in the neocortex.

Once the contribution of RBPs have been investigated, the mechanism they exert control over the development or function of the neocortex can also be interrogated by not only performing developmental, regional and stage-specific RIP-Chip, but also assessment through mass-spec of the protein-binding partners of a target RBP throughout the same developmental or regional investigation. Although RBP's act to metabolize RNA, they do so in concert with several other actors, which include partner RBP's eukaryotic initiation factors and translational machinery. This conglomerate is called a ribonucleoprotein cluster. It is therefore conceivable that a functionally-specific RBP's role may be developmentally modulated by the protein partners which aid in carrying out its function.

In sum, this document outlines some of the modern philosophy and techniques used to study neocorticogenesis from the stance of post-transcriptional regulation. As our understanding and the technology available to us has advanced, new, broad studies must be undertaken to elucidate the general and specific contributions of post-transcriptional regulation from the

earliest stem cell divisions to the mature function of the neocortex. Historically, and less so in recent times, mRNA has been viewed as a passive stage between protein-coding DNA and the proteome. However, an increasing body of evidence implicates the metabolic process of mRNA as mechanistic and contributory to the complexity of the proteome in the neocortex. Further, it is now understood that the non-coding RNA such as piRNA feed back onto the genome through modulation of retrotransposable elements. Through methylation and histone modification, the protein level also modifies DNA and ultimately mediates its accessibility to transcription factors. In this view, perhaps the next generation of understanding will cease to assess each level of regulation, and entertain a model in which regulatory elements work in both feed-forward and feed-back loops. These regulatory patterns perhaps govern networks of transcripts both through direct regulation of their metabolism and indirectly through regulatory means at other levels of expression.

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