ETHANOL TARGETING NELAVS: IMPLICATIONS FOR NEURAL STEM CELL MATURTION

A Dissertation

by

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ABSTRACT

Neurological deficits caused by fetal exposure to ethanol remains prevalent and are recognized as a serious public health issue. The effects of fetal alcohol exposure are multifaceted and is characterized by multiple structural malformations and cognitive deficiencies, however the basic molecular mechanisms central to these defects are not thoroughly understood. A central factor in embryonic development is post-transcriptional gene regulation. Post-transcriptional regulation governs all aspects of development and is an area of vulnerability that is targeted by ethanol. nELAVs RNA binding proteins are important post transcriptional regulators involved in RNA translocation and stability. Elucidating ethanol's effects as a teratogen on these regulators their target transcripts and binding partners will enable us to implement strategies to diminish several long-term effects of fetal ethanol exposure.

DEDICATION

This Dissertation is dedicated to my sons and husband who have ventured selflessly on this interesting and challenging journey with me.

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First and foremost thanks be to God for sustaining me on this academic journey. The experience gained while undertaking my studies, both in academia and personally has taught me to face the future with confidence and certainty. I would like to thank all my amazing family for their support, sacrifices and encouragements.

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CHAPTER I

INTRODUCTION

Neurological deficits caused by fetal exposure to ethanol remain prevalent and are recognized as a serious public health issue. Fetal alcohol exposure can result in a multifaceted disorder that is characterized by numerous structural malformations and cognitive deficiencies, the basic molecular mechanisms central to these defects are not thoroughly understood. Gene regulation occurs both transcriptionally and post transcriptionally. Post-transcriptional regulation is fundamental for embryonic development and regulates transcript-processing mechanisms such as translocation, stability and translation efficiency. Because of the regulatory processes involved in embryonic development, post-transcriptional regulation is an area of vulnerability that is targeted by teratogens such as ethanol. Two significant post-transcriptional regulators are the RNA binding proteins and the non-coding microRNAs. Elucidating ethanol's effects as a teratogen on post-transcriptional gene regulation will enable us to implement strategies to diminish several long-term effects of fetal alcohol exposure. This dissertation seeks to promote our understanding of the effects of ethanol exposure on post-transcriptional gene regulators at critical time points during embryonic development.

Fetal alcohol spectrum disorders

The maternal fetal environment is critical for the development of the embryo. In-utero exposure to varying levels of teratogens result in developmental malfunctions leading to neurological deficits and structural abnormalities (Fernandez et al., 2004, Vajda et al., 2013). Maternal alcohol consumption during pregnancy varies widely among individuals (May et al., 2013b) and as such the effects of alcohol exposure on the developing fetus can be very profound with varying degrees of severity. Fetal alcohol exposure results in a collection of mental and physical deficits, collectively termed Fetal Alcohol Spectrum Disorders (FASD) (Bertrand et al., 2005, Autti-Ramo et al., 2006). Prenatal alcohol exposure has long been implicated as being detrimental to the developing fetus (Sullivan, 2011), however, it was notably clinically characterized by Jones and Smith in 1973 (Jones and Smith, 1973). Specifically FASD includes neuropsychological deficits, craniofacial abnormalities, growth and bone deficiencies, cardiac anomalies and several other congenital defects (Jones and Smith, 1973, Paintner et al., 2012, Thanh and Jonsson, 2014). Because FASD is a complex disorder, characterization is complicated, however, a set of criteria for diagnosis was determined, and include Fetal Alcohol Syndrome (FAS), Partial Fetal Alcohol Syndrome (pFAS) (Moore et al., 2001, Bertrand et al., 2005), Alcohol Related Neurodevelopment Disorder (ARND) and Alcohol Related Birth Defects (ARBD) (Hoyme et al., 2005, May et al., 2013a). FAS is at the severe end of the spectrum with confirmed prenatal exposure, along with neurological damage and dysfunction, growth abnormalities, both prenatally and postnatally along with the characteristic craniofacial facial dysmorphologies first

described by Jones and Smith (Jones and Smith, 1973). pFAS is characterized by confirmed fetal exposure with some evidence of the characteristic facial dysmorphology seen in FAS, in addition to abnormal brain morphology, cognitive deficits and other neurological impairments (Hoyme et al., 2005). ARND is characterized by confirmed maternal alcohol use during pregnancy and presentation of minimal brain structure impairment and minimal characteristic dysmorphologies compared to what is seen in FAS and pFAS, but there are deficits in executive functioning, lack of acceptable social behavior and other cognitive deficits (Chudley et al., 2005, May et al., 2007). ARBD is characterized by subtler neurodevelopmental deficiencies and minor dysmorphologies resulting from prenatal alcohol exposure (May et al., 2007). ARBD characterization is more difficult as other congenital malformations can occur that are not attributed to prenatal alcohol exposure (O'Leary et al., 2010, Wilkins-Haug, 1997).

Impact of ethanol on fetal developmental mechanisms

Fetal alcohol exposure results in a range of deficits with the main area of vulnerability being neurological, specifically affecting the fetal brain. Because FASD is a spectrum disorder, the severity is variable and it is theorized that the time of exposure during development determines the variability in severity (Coulter et al., 1993). In fetal brain development, neural stem and progenitor cells undergo proliferation and maturation between the end period of the first trimester and start of the second trimester. Exposure to teratogens such as alcohol at this critical developmental period is known to cause neurological deficits and brain dysmorphologies. However, the molecular

mechanisms of ethanol exposure that renders the developing brain vulnerable remains unknown. In addition to gestational age of exposure, the quantity of maternal alcohol consumption is also of significance. When alcohol is consumed during pregnancy, there is evidence that the fetal amniotic fluid concentration equals that of the maternal blood within two hours post consumption (Nava-Ocampo et al., 2004), and also took a considerably longer time to be metabolized from the amniotic fluid (Nava-Ocampo et al., 2004). Interestingly, this pharmacokinetic model by Nava-Ocampo et al., gives insight into ethanol exposure at the crucial gestational age time point for neuronal development around the early second trimester period. In addition, cortical neuronal migration in humans also begins in the second trimester period (Bielas et al., 2004). This second trimester is very significant in embryogenesis and brain development, as two critical events, neurogenesis and migration takes place during this period. This second trimester period represents the focus of my research as the events occurring make the developing fetal brain particularly vulnerable to teratogens and may be the time point during development that the teratogenic effects of alcohol will be greatest. Even though we focus on the second trimester period it must be noted that the deleterious effects of alcohol exposure during pregnancy occur in all three trimesters therefore there is no safe period or lower limit for alcohol consumption during pregnancy. Fetal alcohol exposure very early in embryogenesis before maternal realization of pregnancy has been shown to be very detrimental to the developing fetus. Magnetic Resonance Microscopy of mouse models of ethanol exposure shows that in early first trimester equivalency periods, exposure at mouse gestational day seven and eight, the beginning of gastrulation and

neural plate formation, ethanol administration resulted in several characteristic FAS features such as facial dysmorphologies and cerebral cortical heterotopias (Parnell et al., 2009, Godin et al., 2010, Parnell et al., 2014). In the second trimester, exposure to alcohol affects the proliferation and maintenance of neuronal precursor cells causing aberrant migration of cortical neurons (Camarillo and Miranda, 2008). Synaptogenesis and establishment of neural circuitry occurs in the third trimester, during this period the brain may be more susceptible to apoptosis due to alcohol consumption (Ikonomidou et al., 2001). Ethanol has been shown to decrease mRNA levels of several antioxidant enzymes such as glutathione peroxidase and superoxide dismutase in the fetal brain but not the fetal liver (Drever et al., 2012), this may mean that the protection afforded by these antioxidants is diminished leading to oxidative damage of nucleotides or transcription and post transcription factors. Alcohol disrupts several genes in the developing brain (Drever et al., 2012, Khalid et al., 2014). Alcohol affects inhibitory pathways in the central nervous system (Toso et al., 2006). Alcohol affects blood flow to the fetal brain (Bake et al., 2012), alcohol exposure causes epigenetic reprogramming (Veazey et al., 2013) and alter vulnerable neural stem cell populations (Tingling et al., 2013). As the evidence indicates, fetal alcohol exposure disrupts developmental programs that regulate embryonic brain development. Because ethanol's effect on the developing fetal brain is deleterious, this presents a formidable challenge for us to understand the complexities underlying teratogenicity of ethanol on the developing brain. In the context of neurological disorders observed in FASD, alcohol can be

considered to be exerting its effects on core gene regulatory factors that govern neurogenesis, neural migration and maturation.

Neurogenesis, neuronal migration and differentiation

Neurons communicate by forming extensive and complex networks following differentiation. Neuron morphology is the basis of neurological function. Neural progenitor and stem cells ability to proliferate and differentiate into functional progeny in a spatiotemporal manner are tightly regulated by several transcriptional and post transcriptional mechanisms (Lee et al., 2000, Ohkubo et al., 2002, Cho et al., 2013, Girdler et al., 2013). In the developing cortex, progenitor cells in the ventricular zone exits the cell cycle and start to differentiate and migrate into the sub-ventricular zone, intermediate zone and cortical layer (Polleux et al., 2002, Ang et al., 2003, Hack et al., 2007, Hashimoto-Torii et al., 2008, Huang, 2009). During nervous system development, complex mechanisms determines the fate of the neuron, these involve interactions both transcriptionally and post-transcriptionally to determining final cell fate and functional refinement.

Post transcriptional gene regulation and RNA stability

Post-transcriptional mechanisms are responsible for regulation of growth and development. Post-transcriptional regulation incorporates all mechanisms involved in RNA processing from transcription to post transcriptional processing and translation. Post-transcriptional gene regulation has emerged as a key player in organizing the

complex relationship between genes, proteins and the cellular environment. One of the most critical post-transcriptional mechanisms controlling the expression of a large number of genes responsible for diverse processes is RNA stability. Two major regulators of RNA stability are the RNA binding proteins (RBP) and the non-coding RNAs, of which microRNAs (miRNAs) are the best characterized.

RNA binding proteins are involved in RNA stability, stem cell maintenance and differentiation and development. Neuronal RNA binding proteins such as HuB, HuC and HuD modulates the translation of developmental regulators involved in neural growth and development. Post-transcriptional regulation is dependent on the activity of RNA binding proteins acting as trans acting factors for mRNA transcripts (Pascale et al., 2008). miRNAs regulate translation by binding to sites in the 3' untranslated region (3'UTR) of their target genes and also act in conjunction with sequence specific RNA binding proteins. The interplay between non-coding RNAs and RNA binding proteins on target RNAs can modulate target expression depending on the requirement of the cell. In principle the stability, translation efficiency and final gene outcome following transcription is dependent on the interaction between the mRNA transcript and its regulatory binding partners.

RNA binding proteins

RNA binding proteins post-transcriptionally controls the expression of a large number of genes responsible for diverse processes regulating neural organization. In the cell, RNAs do not exist as stand alone molecules, instead they are stably assembled as

part of ribonucleoprotein complexes (RNPs) (Kim et al., 2009). RNA binding proteins (RBPs) are proteins that bind RNA and regulate the outcome of all gene expression mechanisms (Sakakibara and Okano, 1997, Maris et al., 2005, Clery et al., 2008). RBPs are involved in RNA stability, in which assembly of RBPs on target RNAs results in either a repressive or enhancement effect. RNA binding activity can rapidly modulate gene expression in response to changing environmental conditions (Miller and Olivas, 2011). RNA binding proteins are also involved in RNA splicing, RNA decay, subcellular localization and translation (Kim et al., 2009). RNA binding proteins recognize and bind to target mRNAs in a sequence dependent manner to cis-elements usually confined to the 3'-UTR of mRNAs, but can also be in non-coding sequences and in the 5'UTR. (Pascale et al., 2008). These cis-elements associate with the RBPs in specific domains called RNA Recognition motifs.

RNA recognition motifs of the RNA binding protein

RNA binding proteins bind to regions of their target mRNAs at specific loci within the protein known as the RNA-recognition motif (RRM), the RNA Binding Domain (RBD) or Ribonucleoprotein domain (RNP domain). The RRM is found in all life forms (Maris et al., 2005). It is one of the most abundant RNA binding domains in higher vertebrates (Maris et al., 2005, Clery et al., 2008). An RRM consist of approximately 90 amino acids arranged in a $\beta_1\alpha_1\beta_2\beta_3\alpha_2\beta_4$ topology that forms four-stranded β-sheets packed against two α-helices (Maris et al., 2005). It is the involvement of the b-hairpin that has been strongly suggested to be heavily involved in RNA binding.

Structural studies show that amino acids of the b-hairpin are directly hydrogen-bonded to bases of nucleic acid targets (Clery et al., 2008). However, the loops connecting the b sheets and the a helices are also crucial for nucleic acid recognition. RRMs bind a variable number of nucleotides ranging from a minimum of two to a maximum of eight for some mRNAs. Typically most RRMs has three aromatic side chains located in the β_3 and the b_1-strand (Clery et al., 2008) that interacts with the bases such that the 5' and the 3' nucleotides stack on the aromatic ring located in b1 and in b3 positions (Clery et al., 2008). Note that the same RRMs will also interact with other proteins and not only RNAs. It is the conformation and hydrogen-bonding pattern for each RNA base that allows the RBPs to make specific interactions through a very small number of contact points (Morozova et al., 2006)

ELAVL/Hu proteins and their targets

ELAV/Hu proteins are homologs of Drosophila's Embryonic Lethal Abnormal Vision ELAV (ELAVs). They were first identified in drosophila as part of a gene mutation that gives rise to an embryonic lethal phenotype with numerous structural defects and hypotrophy of the CNS (Campos et al., 1987, Robinow et al., 1988, Yao and White, 1994). In humans ELAV proteins were initially identified as autoimmune antigens in paraneoplastic neurological disorders (Szabo et al., 1991, Ross et al., 1997, King et al., 1994), hence the later name Hu proteins. Hu proteins consist of four mammalian homologs, HuB, HuC, HuD and HuR. HuB, HuC and HuD are expressed in neurons and are also collectively termed neuronal ELAVs (nELAVs), whereas HuR is

ubiquitously expressed (Kasashima et al., 1999, Hinman and Lou, 2008). Hu proteins are necessary for neural development (Bolognani et al., 2010, Bolognani et al., 2006) and are characterized as mRNA stabilizing factors playing a significant role in promoting differentiation of neural progenitors (Mobarak et al., 2000, Anderson et al., 2001, Bolognani et al., 2010). Hu proteins have been identified as an early marker for commitment to the neuronal lineage (Kim et al., 1996, Wakamatsu and Weston, 1997) Hu proteins have three RNA recognition motifs RRMs that are highly conserved. Two RRMs are aligned near the N-terminus separated from the third at the C-terminus by a hinge region of 54-80 amino acids (Yannoni and White, 1999). In Hu proteins it has been shown that the N-terminus RRMs interact with the ARE whereas the C-terminus RRM interacts with the poly-A tail of the mature transcript (Ma et al., 1997).

The four vertebrate Hu proteins are characterized by a high degree of sequence homology (70-91%) even across species (Okano and Darnell, 1997, Yannoni and White, 1999, Samson, 2008). They are 40kDa in size and contain three approximately 90 amino acid-long RRM domains (Samson, 2008). The hinge regions contain the cis elements responsible for Hu nucleocytoplasmic shuttling. Hu proteins are distributed in both nuclear and cytoplasmic region of the cell (Pascale et al., 2008). Hu proteins function by binding to adenine uridine rich elements (AREs) in the 3'UTR of their target mRNA and transports them to the cytoplasm for translation (Mansfield and Keene, 2012).

Hu proteins targets and increase transcript stability of several mRNAs that are involved in development. Some mRNA targets of Hu proteins include Musashi (MSI), GAP-43 and Id mRNAs. Musashi is a well characterized, evolutionarily conserved RNA

binding protein (Pascale et al., 2008), that function as a regulator of mRNAs that play critical roles in stem cell maintenance and self-renewal (Nakamura et al., 1994, Kaneko et al., 2000, Okano et al., 2005). Msi is highly expressed in the developing nervous system precursor cells that generate both neurons and glia during embryonic and postnatal development (Ohyama et al., 2012). Gap-43 is neuron specific and growth cone localized, is essential for neuritogenesis and also functions in refining neural connections (Sakakibara et al., 1996, Sakakibara and Okano, 1997, Good et al., 1998, Sakakibara et al., 2001, Okano et al., 2005). GAP-43 is expressed very early in developing neurons and is a target of HuD (Sanna et al., 2014, Beckel-Mitchener et al., 2002). Inhibitor of DNA binding (ID) proteins are helix loop helix proteins that lack the basic amino acid domain (Perrone-Bizzozero et al., 2011), they interact with basic helix-loop-helix bHLH transcription factors forming a protein interaction that sequesters the bHLH proteins and ultimately inhibit transcription by preventing them from binding to DNA (Andres-Barquin et al., 2000).

MicroRNAs

MicroRNAs (miRNAs) are short non-protein coding RNAs that regulate gene expression through translation repression of their target mRNAs (Tzeng and de Vellis, 1998, Lyden et al., 1999). MicroRNAs are approximately 18-25 nucleotides in length, small non-protein coding RNAs involved in regulating diverse cellular mechanisms through sequence specific binding to target RNAs (Djuranovic et al., 2012, Sano et al., 2012, Bukhari et al., 2012). MicroRNAs are classified as either intergenic or intragenic

depending on the genomic location (Pillai et al., 2004, Yoo et al., 2011, Treiber et al., 2012). Intergenic miRNAs are synthesized from their own promoters, whereas intragenic miRNAs are synthesized along with the host's genes, using the hosts transcriptional start sites (Ballarino et al., 2009, Hinske et al., 2010). MiRNAs are generated from double stranded precursors and interacts with members of the Argonaute (AGO) protein family to form the miRNA-induced silencing complex (miRISC) also referred to as the micro-ribonucleoprotein complex (miRNP) (Gu et al., 2006, Xu et al., 2010, He et al., 2012). MiRNAs regulate gene expression by binding to sites in the 3' untranslated region (3' UTR) of their target genes and also act in conjunction with sequence specific RNA binding proteins. MiRNA translation repression occurs if the regulatory site on the target mRNA is partially complementary to the miRNA (Lee et al., 2004, Treiber et al., 2012). Nucleotides 2-8 of the miRNA is known as the seed region and is essential for the miRNA to interact with the target mRNA (Treiber et al., 2012). AGO is fundamental in the translation repression by miRNAs, and is the protein that is in closest association with the paired miRNA-mRNA interactions, other proteins are associated more peripherally. The AGO family of proteins can be therefore seen as specialized small RNA binding proteins (Lewis et al., 2005, Zorc et al., 2012). miRNAs regulate diverse cellular mechanisms during translation by being an adapter that guides the miRISC, through interaction with AGO to the sequence specific mRNA (Ender and Meister, 2010, Wei et al., 2012b). Some microRNAs target the actual translation initiation machinery by directly repressing some eukaryotic translation initiation factor (eIF) mRNAs, as an example Yoo et al., 2011 showed that hsa-miR-5787 targets and

suppresses eIF5 (Yoo et al., 2011). MiRNAs regulate neuron development, neural survival, maturation and plasticity. MiR-9 is highly expressed in the brain and is involved in neural stem cell differentiation (Coolen et al., 2012, Sun et al., 2013). In neural maturation, miR-124a is required for axonal development of hippocampal neurons (Zhao et al., 2009). Double mutant mouse for MiR-9-2 and miR-9-3 show reduced numbers of Cajal-Retzius and early born neurons plus aberrant misrouting of thalamocortical and corticofugal axons (Sanuki et al., 2011).

Long non-coding RNAs

Long non-coding RNAs (lncRNAs) consist of a minimum of 200 nucleotides (Wei et al., 2013, Garitano-Trojaola et al., 2013, Wang et al., 2014). A numerous amount of lncRNAs are identified in the vertebrate genome (Kapranov et al., 2007). Like miRNAs they are transcribed from intergenic and intragenic regions of the transcript (Li et al., 2012, Pauli et al., 2012). lncRNAs partially base pairs with their target mRNAs in a similar fashion as miRNAs. Long non-coding RNAs expression is developmentally regulated and associated with neural stem cell differentiation (Clark et al., 2012). Long non-coding RNAs are localized to specific subcellular compartments (Mercer et al., 2008) and affect a large amount of biological processes (Clemson et al., 2009). Long noncoding RNAs such as HOTAIR plays a role in cancer invasion and metastasis (Bian and Sun, 2011), XIST is expressed only from the inactive X-chromosome thus important in regulating development (Geng et al., 2011) and SOX2OT (sox2 overlapping transcript) SOX2OT is a stable transcript that is upregulated during mesoderm-lineage

differentiation (Brown et al., 1991). lncRNAs can act as a repository for generating endogenous siRNAs that trigger degradation of targets (Amaral et al., 2009). LncRNAs are emerging as having key roles in translation regulation of gene networks.

Eukaryotic translation

Translation is the process by which the sequence of nucleotides in an mRNA molecule directs the incorporation of amino acids into proteins within the ribosome (Kapranov et al., 2007). Although the final product of some genes is an RNA molecule itself most RNA molecules serve as intermediaries in the protein synthesis pathway (Alberts, 2002). Translation is a multistep process involving several molecular factors. Translation is usually thought of as occurring in a linear fashion, from activation-initiation-elongation- termination, however translation is in fact a cyclical process in that each round of translation is preceded by a previous termination event except in cases where there are newly assembled 40S subunits (Alberts, 2002). Translation initiation is the most complex component in this multi step process and as such most regulatory events takes place at the initiation step. Eukaryotic mRNAs has a 5'-cap and a 3' polyadenylate (PolyA) tail that are both required for efficient translation (Jackson et al., 2012).

Amino acid activation is the first step in the translation process (Imataka et al., 1998), chief factor in this step are the amino acyl tRNA synthetases (Novelli, 1967). Amino acyl tRNA synthetases, one for each tRNA, regulate activation by precisely pairing tRNAs with their cognate amino acids via a covalent bond (Cusack, 1997). The

bound tRNA is referred to as a charged tRNA (Cusack, 1997, Splan et al., 2008, Park et al., 2008). The aminoacylation of a tRNA with its associated amino acid by an aminoacyl-tRNA synthetase is crucial in determining the fidelity of protein biosynthesis (Splan et al., 2008). Aminoacyl-tRNA synthetases also have amino acid editing capabilities (Klipcan and Safro, 2004). The aminoacyl-tRNA synthetase is in fact one of the most important component of the translation machinery as it truly defines the final outcome of protein translation (Sankaranarayanan and Moras, 2001).

Following activation the next step is translation initiation. Translation initiation is the rate-limiting step of protein synthesis (Cusack, 1997, Splan et al., 2008) and as such is the step that is most regulated. The canonical pathway of eukaryotic translation initiation is divided into several stages regulated by essential proteins called eukaryotic initiation factors (eIFs) (Nukazuka et al., 2008). Translation initiation in eukaryotes begins with the identification and assembly of the 43S preinitiation complex (PIC) (Majumdar and Maitra, 2005, Majumdar et al., 2003, Lang et al., 2002, Sangthong et al., 2007). The 43S preinitiation complex is made up of the 40S subunit and an initiator methyionyl tRNA (met-$tRNA_i^{met}$) in association with GTP bound eukaryotic initiation factor 2 (eIF2) (Valasek et al., 2002, Pestova et al., 1998). Initiation factors eIF1, eIF1A, eIF4, eIF5 and the eIF3 complex all activates assembly of the 43S complex (Valasek et al., 2002). The attachment of the 43S preinitiation complex to the 5' m^7GTP cap of the mRNA is facilitated by the eIF4F complex which is made up of eIF4A, eIF4E, eIF4G, and the polyA binding protein (PABP) (Jivotovskaya et al., 2006, Majumdar and Maitra, 2005, Lomakin et al., 2003). Next, the assembled preinitiation complex scans the mRNA

for the initiation codon AUG, using complementarity base pairing with the anticodon of the initiator tRNA at the P site of the 40S subunit (Hellen, 2009, Ramirez et al., 2002). The molecular mechanism of scanning and start codon selection is critical to the formation of the preinitiation complex (Pestova and Kolupaeva, 2002, Singh et al., 2005, Battiste et al., 2000, He et al., 2003); this scanning mechanism has been validated by several studies and was first proposed by Kozak in 1978 (Kozak, 1978, Asano et al., 2000). Upon the first AUG recognition, the scanning is stopped and the GTPase activating protein eIF5 causes the irreversible hydrolysis of GTP bound eIF2 resulting in the formation of a stable 48S preinitiation complex (Kozak, 1978). The conventional rule of the scanning mechanism is that the 43S preinitiation complex (PIC) will stop scanning when it recognizes the initiation codon which is the first AUG triplet, located at a favorable site from the 5' end (Hinnebusch, 2011, Hellen, 2009, Pisarev et al., 2006), there are however exceptions to the first AUG rule (Hellen, 2009). The importance of the formation of this PIC is to assemble a stable closed loop structure that will enhance the fidelity of the translation machinery. DEAD-box helicases such as Ded1 assist in the scanning process by melting the mRNA structure so they can pass through the preinitiation complex (Kozak, 1989). The 48S preinitiation complex is then joined to the 60S subunit to form the active 80S initiation complex in a reaction catalyzed by eIF5B and eIF2 (Hellen, 2009).

Following translation initiation at the first codon by the 80S ribosome, the next step is elongation stage where the peptide chain is increased one amino acid at a time. In translation elongation the tRNA bound amino acids are incorporated into the growing

polypeptide chain according to the respective mRNA template (Dever et al., 2001, Lee et al., 2002, Hellen, 2009, Pestova et al., 2000). Translation elongation factors are responsible for the efficient polypeptide synthesis on the 80S ribosomes (Lang et al., 2001). Two distinct elongation factors required for translation elongation are elongation factor 1 (eEF-1) and elongation factor 2 (eEF-2) (Yaremchuk et al., 2012), along with corresponding isoforms (Proud, 1994, Nilsson and Nygard, 1986). Each amino acid is added to the C-terminal end of the growing polypeptide by means of a cycle of three sequential steps: aminoacyl-tRNA binding, followed by peptide bond formation, followed by ribosome translocation (Saha and Chakraburtty, 1986). The mRNA molecule progresses codon by codon through the ribosome in the 5′-to-3′ direction until one of three stop codons is reached.

The final step is termination where the completed protein is released from the ribosome (Greganova et al., 2011). Termination of translation occurs when a termination or stop codon (either UAA, UAG or UGA) enters the ribosome A-site following the last coding sequence (Lang et al., 2001, Stansfield et al., 1995). Termination requires two GTP bound Eukaryotic Releasing Factors (eRFs) eRF1 and eRF3, which are bound to the A-site; eRF1 is responsible for codon recognition (Lang et al., 2001, Inagaki and Ford Doolittle, 2000). Following entry of the stop codon GTP is hydrolyzed by eRF3, which results in the eRF1 triggered hydrolysis of the ester bond of the peptidyl-tRNA located in the P-site releasing the newly synthesized polypeptide chain (Frolova et al., 2000).

Translational regulation

Translational regulation represents an important level in gene regulatory networks and is important in development. Translational control is an extremely complex process requiring continuous integration of multiple molecular mechanisms. Most translational control is exerted at the level of initiation, regulated by eukaryotic initiation factors (eIFs). A key translation initiation factor responsible for cap-dependent initiation is the multiprotein eIF4F complex, which is made up of eIF4G and eIF4A. The eIF4F complex promotes the formation of the 48S preinitiation complex (Prevot et al., 2003). eIF4A is a DEAD-box protein that binds to the 5'UTR of mRNA, it is a RNA-dependent ATPase and RNA helicase (Gingras et al., 1999, Ozes et al., 2011, Rogers et al., 2002). eIF4G is a cap binding scaffolding protein with binding domains for both eIF4A, eIF4E, eIF3 and polyA binding protein (Gingras et al., 1999, Andreou and Klostermeier, 2014)

Regulatory sequences in the 3' UTR of the mRNA

Phylogenetic assessments among yeast and mammalian genomic sequences have revealed that untranslated regions (UTRs) of many mRNAs are where refinement of selective gene expression is carried out (Zhouravleva et al., 1995, Alkalaeva et al., 2006). The adenylate uridylate rich element (ARE) in the 3' UTR of mRNAs is one such regulatory sequence that mediates translational control (Hogan et al., 2008) by RNA binding proteins. How important are these sequences of mRNAs directly related to translational control? The hallmark study that first proposed AREs as regulators, was

carried out by Shaw and Kamen in 1986 (Shaw and Kamen, 1986). They showed that when nucleotide sequence from granulocyte-macrophage-colony stimulating factor (GM-CSF) was inserted into the 3' UTR of β-globulin mRNA it was destabilized. They proposed that the AU sequences were recognition signals for mRNA processing that degrades mRNAs for certain lymphokines, cytokines and proto-oncogenes (Shaw and Kamen, 1986). Subsequent studies (Prasad et al., 2008, Wang et al., 2008, Charlesworth et al., 2006) show that the 3' UTR is definitely a significant regulator of gene expression working in conjunction with trans acting factors such as RNA binding proteins and noncoding RNAs. AREs are composed of variable numbers of copies of the AUUAU pentamer or UUAUUUAUU nonamer (Lagnado et al., 1994, Jing et al., 2005, Franks and Lykke-Andersen, 2007, Deshpande et al., 2009, Mayr and Bartel, 2009, Hatipoglu et al., 2009, Cairrao et al., 2009). AREs are categorized into three classes. Class I is based on the presence of one to three pentamers located in the 3'UTR in close proximity to a uridine rich region, Class II AREs have at least two overlapping copies of the UA nonomer and Class III AREs do not contain any pentamers but have U-rich regions in close proximity (Zhang et al., 2002)

Regulatory roles of the 5'UTR

The 5'UTR contains regulatory elements that controls translation initiation (Zhang et al., 2002) and possesses several binding sites for trans acting elements that controls translation. However, unlike the 3'UTR region with specific cis-sequences that confer stability/instability, the 5'UTR region has no known specific cis sequences that

confer any type of translational control. Instead areas of regulation in the 5'UTR has to do with mechanisms of translation initiation factors, the 5' cap structure and steric hindrances caused by stem loop secondary structures located upstream of the start codon (Barrett et al., 2012). There has been one potential cis-sequence regulation consensus sequence -GCCACCAUGG- that flanks the start codon and appears to improve translation initiation, mutations in this sequence seems to result in the 40S subunit skipping the first AUG (Kozak, 1981, Curtis et al., 1995, Kozak, 2000, Barrett et al., 2012). During cellular stress and apoptotic conditions some mRNAs contain a cis-regulatory element in the 5" UTR called internal ribosome entry segments IRES that allow for cap independent translation initiation (Kozak, 1997). Translation regulation at the 5' UTR is most significantly determined by factors that influence translation initiation factors. The flow of information from the transcriptome to the proteome involves several regulatory factors such as RNA binding proteins and non-coding RNAs. Below is a representative schematic of interactions involved in post-transcriptional regulation involved in transcript stability and translation processing (Figure 1).

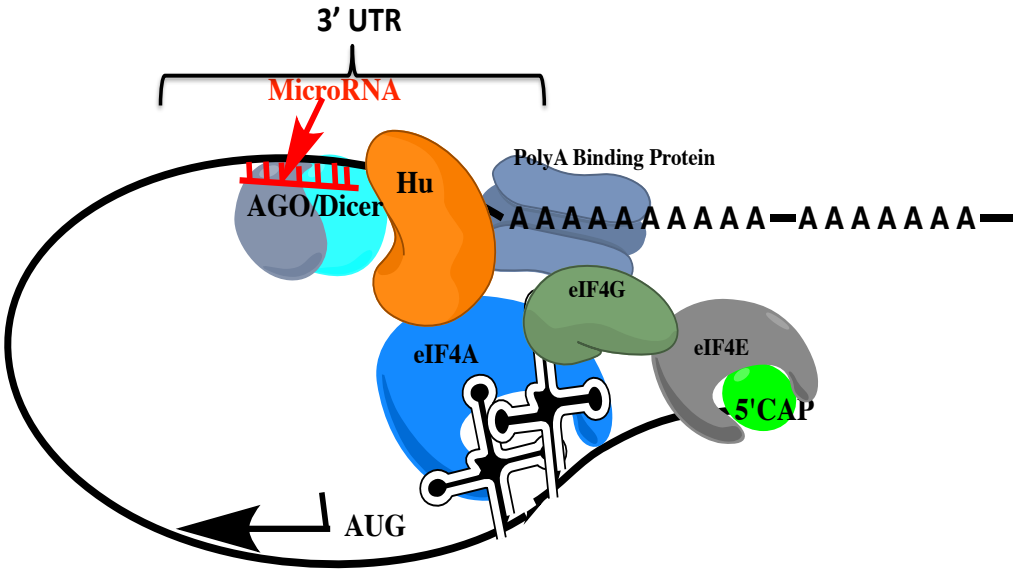


Figure 1. Model of transcript processing. RNA binding proteins and miRNAs interacting with the 3'UTR and translation initiation factors for post-transcriptional regulation.

Hypothesis

Neural stem cells exposed to alcohol prior to neuritogenesis results in morphological and neurocognitive deficits. Several studies have proposed mechanisms that may be targeted by alcohol during development, however the underlying cause of fetal alcohol spectrum disorders is not thoroughly understood. We hypothesize that alcohol exposure disrupts neuronal morphology by disrupting the functional dynamics of the Hu family of RNA binding proteins.

CHAPTER II

ETHANOL EXPOSURE DISRUPTS STEM CELL MATURATION BY CHANGING THE DYNAMICS OF HU PROTEINS

Overview

Fetal alcohol exposure results in a cluster of birth defects termed Fetal Alcohol Spectrum Disorders (FASD). We have identified a family of RNA binding proteins, nELAVs/Hu proteins that regulate RNA stability and are important for neuronal stem cell maintenance and maturation. The mechanism by which alcohol affects neural development is unknown. We hypothesize that as a teratogen ethanol is disrupting the dynamic relationship between the Hu proteins and their targets, leading to altered progression of fetal stem cell maturation. Our data showed that ethanol affects neuron morphology and Hu proteins exhibits varying sensitivity to ethanol exposure. This data provides further information for elucidating the mechanisms by which ethanol exposure alter stem cell stage-specific neural RNA binding proteins and disrupt stem cell maturation.

Introduction

Fetal exposure to varying levels of teratogens has significant implications for the fidelity of fetal development programs. Fetal alcohol exposure results in an array of neurological and physical defects called **F**etal **A**lcohol **S**pectrum **D**isorders (**FASD**)

(Jones and Smith, 1973, Astley and Clarren, 2000). FASD encompasses craniofacial dysmorphologies such as smooth philtrum, neurological deficits, growth deficiencies, cardiac and other abnormalties (Bertrand et al., 2005, Riley and McGee, 2005). At the severe end of the FASD spectrum we have Fetal Alcohol Syndrome (**FAS**). FAS is defined by confirmed prenatal exposure, neurological and growth deficits and the characteristic craniofacial facial dysmorphologies as outlined by the Institute of Medicine (IOM) (Medicine, 1996, Hoyme et al., 2005). **P**artial **F**etal **A**lcohol **S**yndrome (**PFAS**) (Moore et al., 2001), **A**lcohol **R**elated **N**eurodevelopment **D**isorder (**ARND**) and **A**lcohol **R**elated **B**irth **D**efects (**ARBD**) (Hoyme et al., 2005, May et al., 2013a) all describes cases where not all characteristics of FAS are present but there is presence of neurological deficits and underlying structural abnormalities of brain and other organ systems.

Neuron positioning during embryonic brain development occurs in a spatio-temporal manner and is regulated by several genes (Ohkubo et al., 2002, Lee et al., 2000, Cho et al., 2013, Girdler et al., 2013). In the developing cortex, progenitor cells in the ventricular zone exits the cell cycle and differentiate and migrate into the sub-ventricular zone, intermediate zone and cortical layer (Polleux et al., 2002, Ang et al., 2003, Hack et al., 2007, Huang, 2009, Hashimoto-Torii et al., 2008). Developmental timing of exposure has been implicated as a determining factor in the severity of FAS (Coulter et al., 1993). It is believed that a higher incidence of fetal alcohol exposure tends to occur within the first trimester when it is believed that most pregnant women are unaware of their pregnancy status or they abstained from alcohol use later during the pregnancy

(O'Leary et al., 2010). Proliferation and maturation of neural stem cells occur between the end of first trimester and start of second trimester period. Fetal exposure to alcohol at this critical development period leads to brain dysmorphology and neurological deficits (Riley and McGee, 2005, Ervalahti et al., 2007, Roussotte et al., 2012). Specifically it has been shown that alcohol exposure leads to microcephaly and gray matter volume reductions (Riley et al., 2004, Nardelli et al., 2011). In addition to timing of exposure, concentration of alcohol per exposure is also important. Alcohol readily crosses the placenta and as such it has been shown that fetal amniotic fluid concentration equals that of the maternal blood within two hours post consumption (Nava-Ocampo et al., 2004) and is also slower to be metabolized from the amniotic fluid (Nava-Ocampo et al., 2004).

We know that ethanol does not kill neural stem cells but affects different subpopulations of stem cells (Santillano et al., 2005, Tingling et al., 2013) causing craniofacial dysmorphologies and neurological disorders. Because of this it is theorized that ethanol is exerting its effect by disrupting gene regulatory mechanisms. Development is controlled by differential gene regulation. Post-transcriptional gene regulation is critical for development as it is where refinement of final gene outcome occurs. RNA binding proteins are a major class of post- transcriptional regulators. RNA binding proteins bind to transcripts and regulate stability translocation, polyadenylation, splicing and translation. RNA binding proteins are involved in stem cell maintenance, differentiation and regulate all gene expression mechanisms (Sakakibara and Okano, 1997, Maris et al., 2005, Clery et al., 2008). RNA binding proteins are particularly

relevant in the nervous system as their activity can rapidly modulate gene expression in response to changing conditions (Miller and Olivas, 2011). The Hu family of RNA binding proteins, homologs of Drosophila's ELAVs, were identified as important for neuronal stem cell development (Wakamatsu and Weston, 1997, Kasashima et al., 1999, Mobarak et al., 2000, Anderson et al., 2001, Bolognani et al., 2006, Perrone-Bizzozero et al., 2011). Hu proteins consist of four mammalian homologs, HuB, HuC, HuD and HuR. HuB, HuC and HuD are restricted to neurons whereas HuR is ubiquitously expressed. Target mRNAs that Hu proteins bind include genes for neural stem cell maintenance and axon guidance. RNA binding proteins containing RRMs such as Hu proteins interact with their mRNA targets by binding to sequences in the 3'UTR.

The molecular mechanism of ethanol exposure that renders the developing brain vulnerable remains unknown. To understand ethanol's effect on neural stem cell developmental programs involving Hu proteins, we utilize an in vitro model of the early stages of neuronal maturation using mouse embryonic derived neural progenitor cells along with moderate to heavy ethanol exposure paradigms.

RNA binding proteins are pleiotropic factors that act as nodes modulating several genes specifically related to neuronal maturation. Ethanol's effect on Hu family of RNA binding proteins will have a cascading effect on multiple genes responsible for neuronal differentiation and organizational patterns. Defects in neuronal differentiation and organizational patterning leads to several craniofacial abnormalities and mental deficits comparable to those observed in FASD. Evidence has shown that ethanol exposure

promotes aberrant and premature maturation and affects differentiation in neural stem cell populations (Santillano et al., 2005, Prock and Miranda, 2007).

We found that ethanol influences the number of neural stem and maturing cells transcriptional activity and therefore hypothesized that as a teratogen, ethanol disrupts stem cell maturation by changing the functional dynamics of nELAV/Hu proteins.

Methods

Ethics statement

Timed-pregnant C57Bl/6 mice (Harlan, Texas) were housed in an AAALAC-approved facility at TAMHSC. All protocols were conducted with the approval of the Institutional Animal Care and Use Committee at TAMHSC (Approval number AUP2010-197).

Neural precursor cultures

Neural precursor cells were obtained from the dorsal telencephalic vesicles of gestational day 12.5 C57BL/6 fetal mouse based on methods by Santillano et al., 2005 (Santillano et al., 2005). Isolated precursor cells were maintained as neurospheres in serum free mitogenic media, containing Dulbecco's Minimal Essential Medium DMEM/F12 Lifetechnologies cat. no. 23017-015), 20 ng/ml Basic Fibroblast Growth Factor bFGF (BD Biosciences cat. no. 354060), 20ng/ml human Epidermal Growth factor hEGF (PreproTech cat. no. 100-15), 0.15 ng/ml leukemia inhibitory factor LIF

(Alomone labs cat. no. L-200), Insulin-transferrin selenium-X ITS-X (Lifetechnologies cat. no. 51500-056), 5ug/ml Heparin (Sigma-Aldrich cat. no. H4784), 50uM Progesterone (Sigma-Aldrich: cat. no. P7556) and Penicillin/Streptomycin (Life technologies cat no. 15140-122).

In-vitro differentiation

To study the effects of ethanol exposure on early stem cell maturation, we utilized an experimental model according to protocol by (Camarillo et al., 2007). Neurosphere cultures containing approximately 2 x 10^6 cells were assigned as either control or ethanol treated group with doses of 120mg/dl (26mM) ethanol or 320mg/dl (70mM) ethanol (Sigma Aldrich cat no., E7148) for a total of 5 days with refreshment of media on day 3. For stem cell maturation, cells were later transferred to either of two ethanol-free mitogen withdrawal differentiation media (Camarillo et al., 2007). For differentiation flasks were prepared by coating with 1mg/ml laminin (Life technologies cat no. 23017015) for two hours at room temperature prior to use. Immediately before use excess laminin was carefully removed, without disturbing the coating and the flask gently washed once with 1X PBS solution. Cells were prepared for differentiation by centrifuging at 1000 rpm for 5 minutes to remove ethanol containing mitogenic media. Cells were then resuspended in either a partial mitogenic media containing DMEM/F12, 5ug/ml Heparin, 1ml 100X ITS-x, 50uM progesterone, 1ml Penicillin/Streptomycin and 2mg/ml bFGF to model an early period of differentiation or to a mitogen free media containing DMEM/F12, 5mg/ml Heparin, 100X ITS-x, 50μM progesterone and

Penicillin/Streptomycin) modeling a later period of neural differentiation. The resuspended cells were then transferred to the freshly prepared laminin coated culture flasks and incubated for 72 hours in 5% CO_2 at 37°C. Cells plated in the early differentiation model are called Early Differentiated cells (EDCs) and cells plated in the later differentiation model are called (LDCs). Non-differentiated cells are called Neural Stem Cells (NSCs).

Flow Cytometry

To detect changes in RNA levels resulting from ethanol exposure, 5-Ethynyl Uridine (EU), incorporation was measured using CLICK-IT® RNA assay protocol (Life technologies cat no. C10329) and standard flow cytometric methods. Briefly, cells were incubated for two hours in media containing 5-Ethynyl Uridine on final day of culture, as outlined in manufacturers protocol. Non-adherent, cells were collected and centrifuged at 1500 rpm for 5 minutes at 4°C, washed once with ice cold PBS and immediately fixed using ice-cold methanol. For adherent cells, media was carefully removed and replaced with ice-cold methanol; a cell-scraping tool was used to gently remove the cells, which were then centrifuged at 1500 rpm for 5 minutes at 4°C. Following fixation, manufacturers protocol was employed followed by flow cytometric analysis. Population subtypes, debris and dead cells were gated using forward and side scatter profiles. Statistical analysis of flow cytometric data was done from triplicates of 10,000 events each.

Immunofluorescence analysis

Indirect immunofluorescence was carried out on cells grown on glass coverslips for 72 hours following ethanol treatment. Cells were incubated overnight with primary antibodies against a combined HuB/D, MAP-2, and Musashi-1. Following incubation, antigenic sites were localized with goat anti-rabbit or anti-mouse FITC or TRITC conjugated secondary antibodies. Nucleus was counterstained with DAPI. Images were captured on Nikon AR Confocal Microscope (Nikon Instruments Inc.)

Immunofluorescence analysis was carried out on both neural stem cells and differentiated neurons to determine subcellular localization of the HuB/D, Musashi and MAP2. Differentiated neurons were grown on glass coverslips (VWR cat. No. 72196-25) in 6-well plates (Fisher Scientific cat no. 353047) for 72hrs following treatment. Immunofluorescence analysis using antibodies against MAP2 (EMD Millipore cat no. AB5622) Elavl2 (HuB)+Elavl4 (HuD) (Abcam cat no. ab72603) and Musashi-1 (EMD Millipore cat no. 04-1041)). Non-differentiated cells were subjected to the same staining protocol as differentiated cells.

Western blot analysis and cell fractionation: whole cell and nuclear and cytoplasmic components

Total proteins were extracted from neural stem cells and differentiated cells using cell extraction buffer containing 50mM Tris pH 7.4 (Life Technologies cat. no.15504-020), 150mM NaCl (Sigma-Aldrich cat. no. S-3014), 10% Glycerol (Fisher Scientific cat. no. BP229-1), 1mM EGTA (Fisher Scientific cat. No. 02783-100), 1mM Na-

Orthovanadate, pH 10 (Sigma-Aldrich cat. no. S-6508), 5μM ZnCl2 (Sigma-Aldrich cat. No. Z-48750), 100mM NaF (Sigma-Aldrich cat. No. S-7920), 10ug/ml Aprotonin (Sigma-Aldrich cat. No. A-1153) 1ug/ml Leupeptin (Sigma-Aldrich cat. No. L-9783), 1%Triton X-100 (Fisher scientific cat.no. BP151-100) and distilled water. On day of use of extraction buffer 1mM Phenylmethylsulfonylfluoride was added to buffer. Samples were centrifuged at 15000-18000 rpm for 10 minutes; the soluble protein in the extract the supernatant was collected and quantified using the Pierce® BCA protein Assay Kit; cat no. 232225 followed by spectrophotometric measurements on TECAN® plate reader or NanoDrop® 2000 spectrophotometer.

To obtain the subcellular distribution of Hu and Musashi, proteins were extracted from separate nuclear and cytoplasmic fractions according to manufactures protocol using the NE-PER Nuclear and Cytoplasmic extraction kit (Thermo Scientific cat. No 78833). Samples containing 15ug of protein was mixed with a 5X loading dye,(6.25 ml TRIS-HCl pH 6.8, Glycerol, β-mercaptoethanol, SDS, Bromophenol blue) and heated at 95 °C for 7-8 minutes and then resolved using the NuPage Novex 4-12% Bis Tris Minigels (Life Technologies cat. no NP0336BOX) in a 1X NuPAGE® MOPS running buffer. Proteins were transferred to polyvinylidene (PVDF) membranes using the Life technologies iBlot® system. The membranes were washed 3 times in 1X TTBS (TBS-Tween) and blocked for 1 Hr. with 5% non-fat dry milk in 1X TTBS at room temperature. Membranes were each incubated with primary antibodies against HuB (dilution 1:200) (Sigma-Aldrich cat.no. H1538), HuC (dilution 1:300) (Abcam cat. no. Ab78027), HuD (dilution 1:500) (Abcam cat no. ab72603), Elavl2 (HuB)+Elavl4 (HuD)

(Abcam cat no. ab72603) and Musashi (1:200) (EMD Millipore cat. no. 04-1041), overnight at 4°C. Following incubation membranes were washed 3 times in 1X TTBS and incubated with horseradish peroxidase conjugated with (appropriate secondary antibodies) with goat anti-mouse IgG (BD Pharmingen 554002) or goat anti-rabbit IgG (Santa Cruz Sc-2004) dilution 1:2000 at room temperature for 1Hr. Bands were detected with ECL Western Lightening Western Blot Detection System (Pierce cat. no. 32109). Blots were imaged on the FluorChemQ® imaging equipment and quantified using the alphaView® analysis software. The optical density of each band was corrected by α-tubulin or Anti-Histone core antibody (Abcam cat. no ab7832).

Real time qPCR

For mRNA expression analysis, total mRNA was isolated using mirVana RNA isolation kit (Life technologies cat. no. AM1561) total RNA extraction protocol, mRNA was quantified on the NanoDrop 2000 spectrophotometer (Thermo Scientific) and then reverse transcribed into cDNA using qScript^{TM} cDNA Supermix (Quanta Biosciences cat. no. 95048). Primers to HuB, HuC, HUD, Musashi and GAP43 were designed using NCBI PrimerBlast and USCSC Genome BLAT browsers. (List of forward and reverse primers: Table 1). Real Time qPCR was carried out using the Applied Biosystems 7900HT Fast Real-Time PCR System and Quanta Biosciences Perfecta SYBR® Green Supermix (Quanta Biosciences cat. no. 95073-012).

Table 1. List of mRNA primer sequences.

Gene	Forward primer	Reverse primer
HuB	5'-ACACAGCCAATGGTCCAACC -3'	5'- GGTGAGGAGCAGTTGTTGTTTA-3'
HuC	5'- AATCCTGCAAGTTGGTTCGGG-3'	5'-GAGTAGTTCACAAACCCATAGCC-3'
HuD	5'-GCCTCAGGTGTCAAATGGACC-3'	5'-CCATACCCTAAACTCTGTCCTGT -3'
Musashi-1	5'- GCTACTGCCTGTCCCTCAAC-3'	5'- GGGTAGGGCAACTGGCTAAT-3'
Gap-43	5'-CATCAGCCCCGCCTTAGAG-3'	5'-CCACCAGTTCAGGGACTTCTT -3'
18S	5'-ATGGCCGTTCTTAGTTGGTG -3'	5'-CGCTGAGCCAGTCAGTGTAG -3'

Cell culture and transfection

A short sequence of 19 nucleotides targeting HuB location 306 and a short sequence of 19 nucleotides targeting HuD location 890 was constructed into OmicsLink short hairpin RNA (shRNA) expression clones, cat no. MSH043651-1-CH1 (Elavl2), and cat no. MSH029419-3-CH1 (Elavl4), purchased from Genecopoeia, Rockville, MD, USA) to knockdown the expression of HuB and HuD respectively. Neural stem cells were cultured according to our standard protocol outlined above. 24h before transfection cells from either ethanol treated and control group were transferred to antibiotic free medium. For transfection, glass coverslips in 6-well plates were coated with 1mg/ml laminin for 1hr. Excess laminin was removed, wells washed with 1X PBS and warm antibiotic free medium added to each well. Cells were trypsinized with 0.5% Trypsin-EDTA at 37°C for 3 min. Fresh warm medium was used to stop the enzyme reaction and cells were pelleted by centrifugation at 1000 RPM for 5 min. Cells were then

resuspended in 1ml fresh medium and 10 ml was removed for counting using Countess® automated cell counter (Life technologies cat. no. C10310). Remaining cells were pelleted and resuspended in transfection buffer of volume to make a final cell concentration of 1.5-2.5 million cells /10 ml per well of 6-well plate. 2.5 mg of plasmid per 1 million cells was added to the cells in the transfection buffer. Cells were transfected using the NEON® transfection system (Life technologies cat. no MPK5000) according to manufacturers protocol with settings at 1200V for 20 milliseconds and 2 pulses. Following electroporation cells were then added to the prepared 6-well plates and incubated for 72 hrs at 37°C and 5% CO_2 incubation. All vectors contained eGFP or mCherry fluorescent proteins to enable visualization and analysis of transfection efficiency. Transfection efficiency was analyzed by flow cytometric analysis.

Statistical analyses

Standard statistical analysis (ANOVA) was applied to each experimental data set. $p < 0.05$, is considered statistically significant.

Results

Neural stem cell development model

We modeled the fetal neural stem cell niche using Neurosphere cultures obtained from the early second trimester-equivalent mouse. When cultured in defined culture medium containing Epidermal (EGF) and Fibroblast (FGF) growth factors as well as

leukemia inhibitory factor (LIF) neural cells displaying a spherical morphology after 2-3 days called neurospheres. Cells within these cultures express a number of stem cell markers such as Sox 9, Sox3 and c-Kit (Tingling et al., 2013). For maintenance and renewal of neural stem cells. Neurosphere cultures do not express neuronal Neurofilament and NeuN (Camarillo et al., 2007). Cultures were then driven towards neuronal differentiation by withdrawal of EGF, FGF and LIF following the provision of extracellular matrix in the form of laminin. Two distinct phenotypes are obtained; namely, Early and Late differentiated cells respectively (Camarillo et al., 2007). Early differentiated cells (EDCs) displayed a flattened morphology with mainly bipolar neurons or less than 5 neuritic processes. This model (Figure 2) establishes the in-vitro equivalent of early maturing neurons migrating to the sub-ventricular zone during development. Late differentiation cells (LDCs) exhibit a flattened morphology with star-like neuritic processes. LDCs express NeuN and Neurofilament but not Nestin signifying a later maturation state (Camarillo et al., 2007). This model allows us to measure the immediate and persistent effects of ethanol as a teratogen affecting neuronal development. Neural stem cells are exposed to ethanol and the immediate effect is evaluated. Following ethanol withdrawal cells were allowed to differentiate to later maturation states and the persistent effect is evaluated.

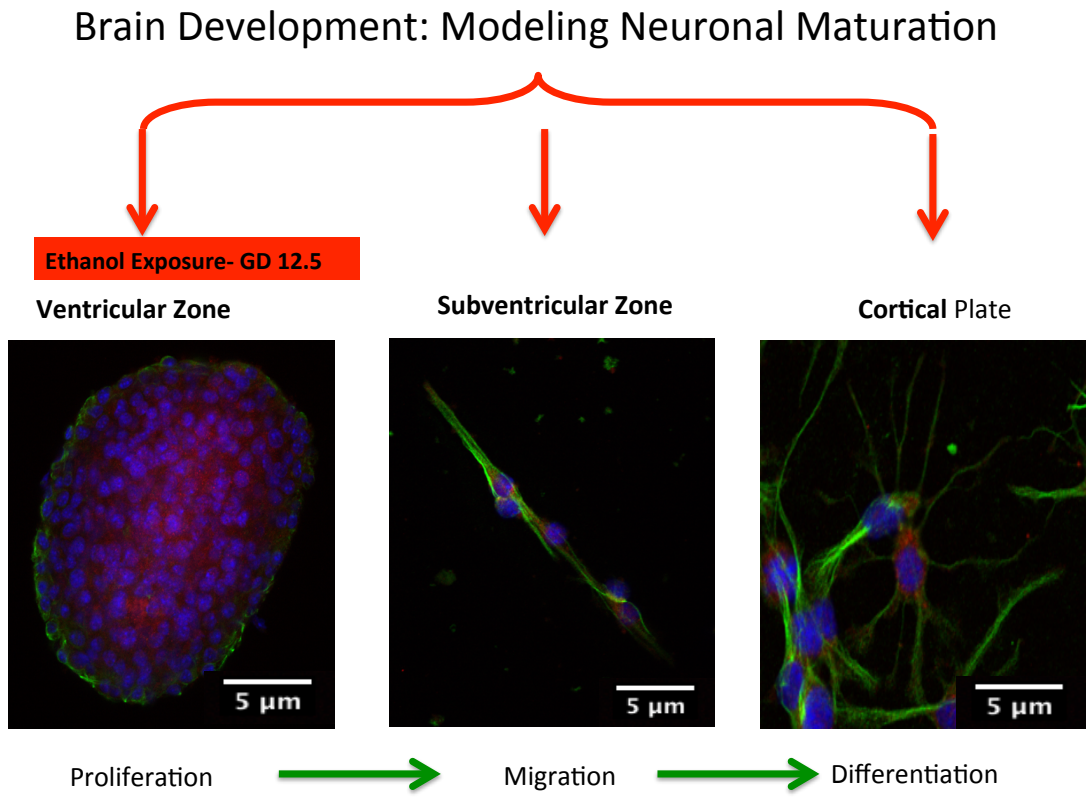


Figure 2. Schematic and representative images of neural stem cell developmental model.

Cell morphology and differentiation

Because nELAVs are important regulatory RNA binding proteins for neuronal maturation and differentiation and because neuronal morphology is very specific we wanted to examine if ethanol exposure affects differentiation and morphology. Cell size is functionally significant in neurons and is very important for synaptic function and neurotransmitter release. In addition dendritic morphology is also very important for neural communication especially for polarized cells that transmit information along long

distances. Morphological features such as nuclear area and perimeter and cytoplasmic area and perimeter were assessed using confocal microscopy imaging for multidimensional measurements (Figure 3**)**. We observed a significant interaction effect between ethanol exposure and differentiation state for cytoplasmic area $F(2,94) = 6.26$ $p<0.002$, for nuclear area $F(2,94) = 44.36$ $p< 2.61\ E^{-14}$, for cytoplasmic perimeter $F(2,94) = 4.62$, $P<0.01$ and nuclear perimeter $F(2,94) = 153$, $P<2.39E^{-30}$. Cytoplasmic area was significantly less in ethanol exposed cells at 320mg/dl in EDCs compared to controls $p=1.44E^{-5}$. In LDCs cytoplasmic area was significantly reduced in both 120mg/dl and 320mg/dl exposed cells $p<0.002$ and $p< 1.05E^{-5}$ respectively.

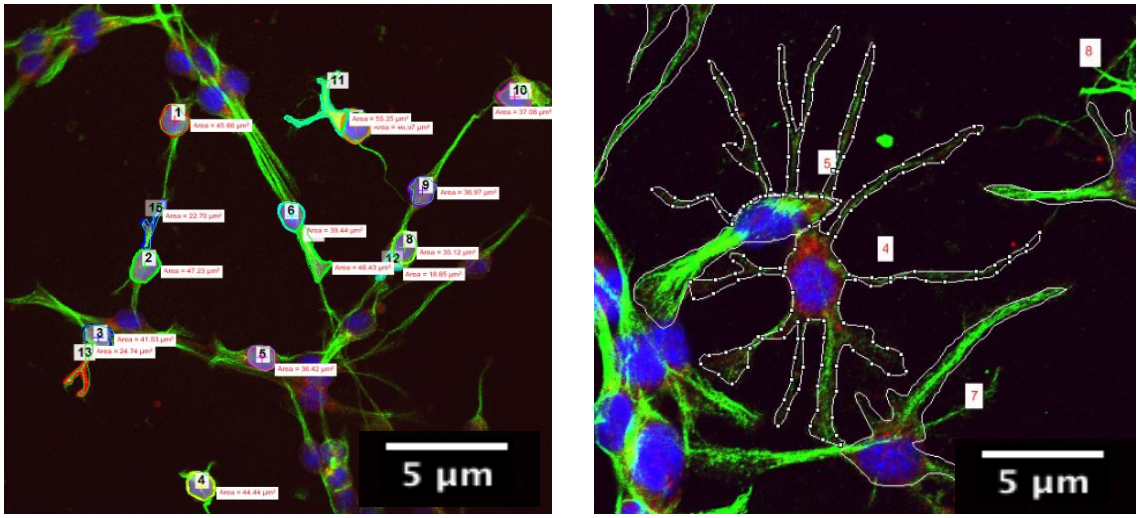


Figure 3. Representative image showing nuclear and cytoplasmic sampling. Immunolabelled images depict localization of Microtubule associated protein-2, MAP2 (green) and HuB/D (red), Nuclear area represented by DAPI (blue)

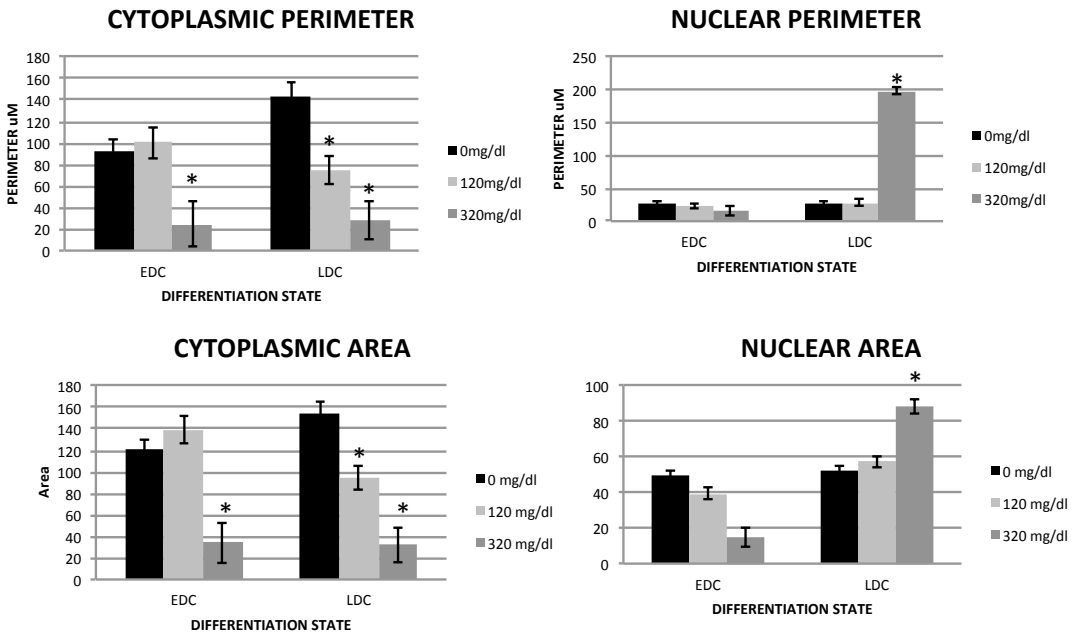


Figure 4. Morphological analysis of differentiating neurons following ethanol exposure.

In cytoplasmic perimeter we observed an increase in cytoplasmic perimeter following differentiation. In EDC Ethanol exposure at 320mg/dl exposure levels caused a significant reduction in cytoplasmic perimeter compared to control $P<3.5E^{-5}$. There was also a significant decrease in 320mg/dl compared to 120mg/dl $P<0.001$, suggesting a dose dependent interaction. This decrease persisted as the cells mature to LDCs where ethanol exposure at both 120mg/dl ($p<0.01$) and 320mg/dl ($p<0.001$) reduced cytoplasmic perimeter. In nuclear perimeter there was no observed difference in nuclear

perimeter in EDCs or following exposure. However in LDCs ethanol exposure at 320mg/dl significantly increased nuclear perimeter $P<2.9E^{-13}$ compared to control and also compared to exposure at 120mg/dl, (Figure 4**).**

Ethanol exposure regulates global transcriptional activity in developing neurons

Gene expression can be tightly controlled at the transcription level, which represents the first step in the flow of information from genome to the proteome (Nagore et al., 2013). First we investigated ethanol's effect on transcriptional activity and mRNA synthesis. Global mRNA synthesis was measured by incorporation of 5-ethynyl uridine (EU), an alkyne modified nucleoside analog into nascent mRNA. 5-EU is actively incorporated into nascent RNA but not DNA. As a true teratogen the consequences of ethanol exposure is persistent even after it is removed from the environment of the developing neural stem cells. To evaluate immediate and any persistent changes that would affect transcriptional activity and RNA synthesis following ethanol exposure, cells were cultured under ethanol-exposed conditions for 5 days followed by complete removal of ethanol. Cells were then maintained as neural stem cells or allowed to differentiate according to our differentiation protocol. Each culture was maintained under ethanol free conditions for 72h. EU incorporation was measured by flow cytometric analysis. Transcript levels were assessed following biosynthetic EU incorporation into newly synthesized RNA using flow cytometric analysis.

Our data indicates that there is significant heterogeneity in both neural stem cells and in differentiated cells. Flow cytometric analysis allowed us to define 3 subpopulations of

cells in each maturation state, based on forward and side scatter analysis. These three subpopulations were designated as population 1, population 2 and population 3, (Figure 5**)**. We observed in population 1 that transcriptional activity significantly increases as neural stem cells mature from stem cells to EDCs and LDCs $F_{(2,27)} = 80.53$, $p<4.00E^{-12}$. NSCs had a higher transcriptional activity than EDCs $p< 0.015$ and LDCs $p<0.013$. Ethanol exposure did not alter transcriptional activity in population 1. In population 2 there was a significant interaction between ethanol exposure and differentiation state $F_{(4,27)} = 3.80$, $p<0.014$. There was increased transcriptional activity as the cells mature from NSCs to LDCs $F_{(2,27)} = 73.6$, $p<1.20E^{-11}$. Transcriptional activity in EDCs and LDCs were significantly less than in NSCs, $p<0.002$ and $p<0.013$ respectively. Ethanol exposure did not affect transcriptional activity of NSCs in population 2, however there was a significant increase in transcriptional activity in EDCs that were previously exposed to ethanol at a concentration of 120mg/dl, $p<0.04$. Transcriptional activity in EDCs exposed to 320mg/dl ethanol was not significantly altered. In LDC there was a similar trend as in EDC towards higher transcriptional activity following exposure at 120mg/dl and no effect at exposure of 320mg/dl.

In population 3 we observed a statistically significant decrease of transcriptional activity as cells mature form NSC to EDCs and LDCs, $F_{(2,27)} = 16.02$, $p<2.58E^{-05}$. As previously observed in subpopulations 1 and 2, there was a significant increase in transcriptional activity in population 3 as the cells mature. Ethanol exposure did not have any significant effect on transcriptional activity in the NSC or LDCs. However ethanol exposure at 320mg/dl reduced transcriptional activity in EDCs $P<0.03$.

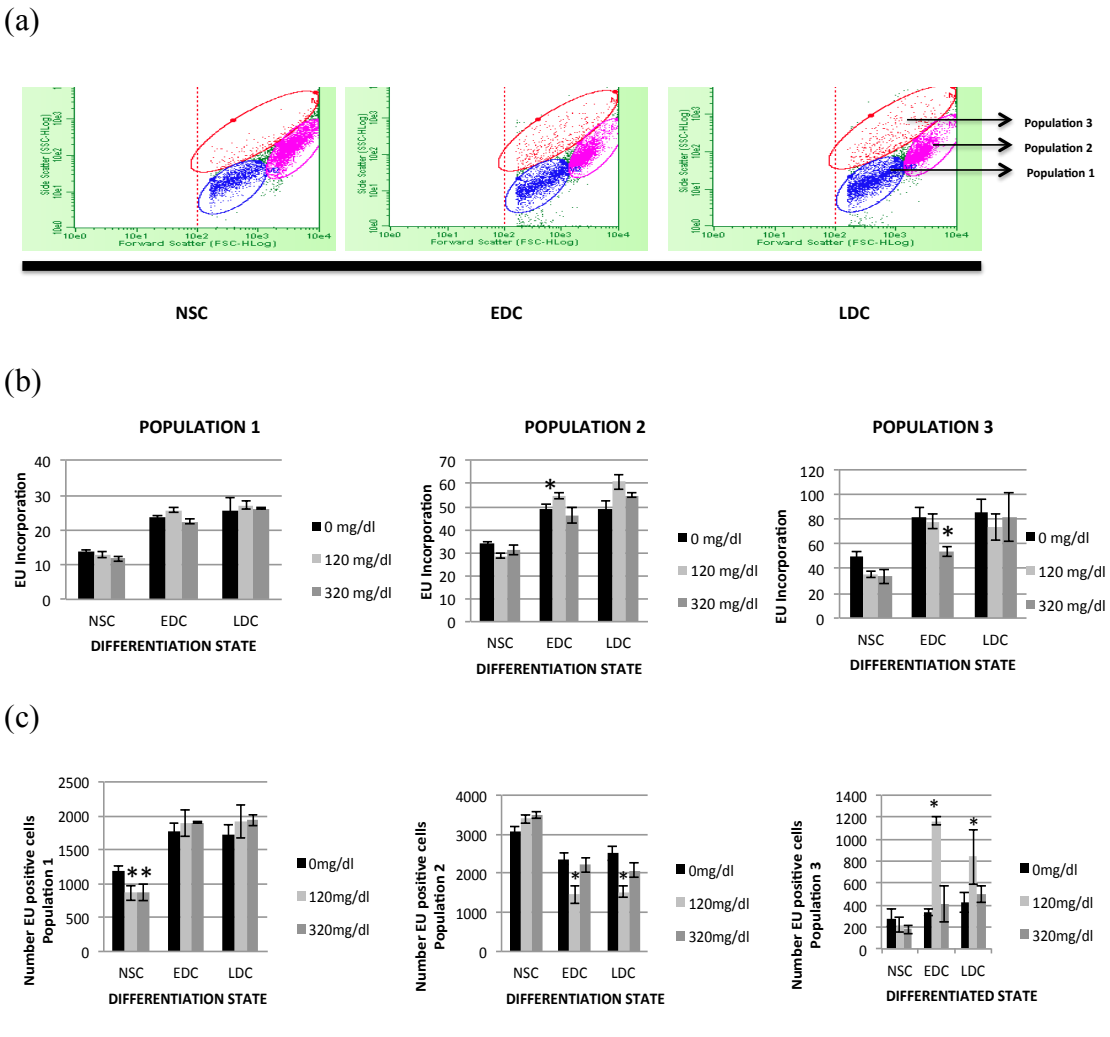


Figure 5. Flow cytometric analysis of following biosynthetic EU incorporation into nascent RNA. (a) Representative dot plots of three distict subpopulations of cells identified by flow cytometric analysis. (b) Histograms of mean fluorescent intensity for each population. (c) Histograms of proportion transcriptionally active cells

Here we see that transcriptional activity increases as neurons differentiate. This is important as molecular mechanisms that govern morphological characteristics and migration is switched on to allow for neurite formation. Transcription in EDCs appear to be more susceptible to ethanol exposure compared to NSCs and LDCs. EDCs represents

the transition from stemness to the early neuritogenesis and are undergoing significant programing that will determine later migration and functionality. Because active transcription levels were different for each population we next determined the proportion of cells that were transcriptionally active in each group and treatment conditions. Ten thousand cells were assessed in triplicate.

In population 1 the proportion of transcriptionally active cells were significantly different between differentiation states $F_{(2,27)} = 41.98$, $p<5.18E^{-09}$. Number of cells increased following differentiation from NSCs to EDCs ($p<0.004$) and LDCs ($p<0.009$). In population 1 NSCs ethanol exposure at 120mg/dl ($p<0.03$) and 320mg/dl ($p<0.04$) decreased the number of transcriptionally active cells. Ethanol exposure did not alter the proportion of transcriptionally active cells in EDC and LDCs of population 1.

Interestingly we observed that in population 2 in the EDC and LDCs at exposure of 120 mg/dl the proportion of cells that were transcriptionally active were significantly less than controls ($p<0.01$), even though the same group (EDCs previously exposed to ethanol at 120mg/dl) exhibited a higher level of EU incorporation (transcriptional activity) (Figure 5**).** This shows that exposure at 120mg/dl reduced the number of transcriptionally active cells compared to controls while simultaneously increasing transcriptional activity. That is, even though active cells were less, the level of activity was more. In population 3 in the EDC at ethanol exposure 120mg/dl we observed that the number of transcriptionally active cells were significantly more than control ($p<3.98E^{-06}$) and 320mg/dl ($p<0.004$) cells. In population 3 NSCs and LDCs ethanol exposure had no observed significant effect on proportion of EU positive cells.

Ethanol exposure counters the developmental decrease of Hu transcripts

Following our observation that transcriptional activity is affected by ethanol exposure, we wanted to determine specific effects on mRNA transcripts for each nELAV, HuB, HuC and HuD.

HuB

We observed a statistically significant interaction effect of both ethanol exposure and differentiation state on HuB mRNA expression $F_{(4,24)} = 6.87$, $P<0.002$. In controls HuB mRNA expression is highest in NSCs with a reduction following differentiation. Specifically HuB transcript levels in NSCs were significantly greater that in EDMs $p<0.03$ and in LDMs $p<0.02$ Figure 6. Ethanol exposure immediate effect was a premature transcript reduction in the NSCs at 320mg/dl exposure ($p<0.03$). Following ethanol exposure and differentiation we observe a significant increase in HuB transcripts in LDCs at both 120mg/dl ($p<0.045$) and 320mg/dl ($p<0.026$). This shows that ethanol is having an opposing effect on HuB expression levels by increasing its expression in more mature neurons.

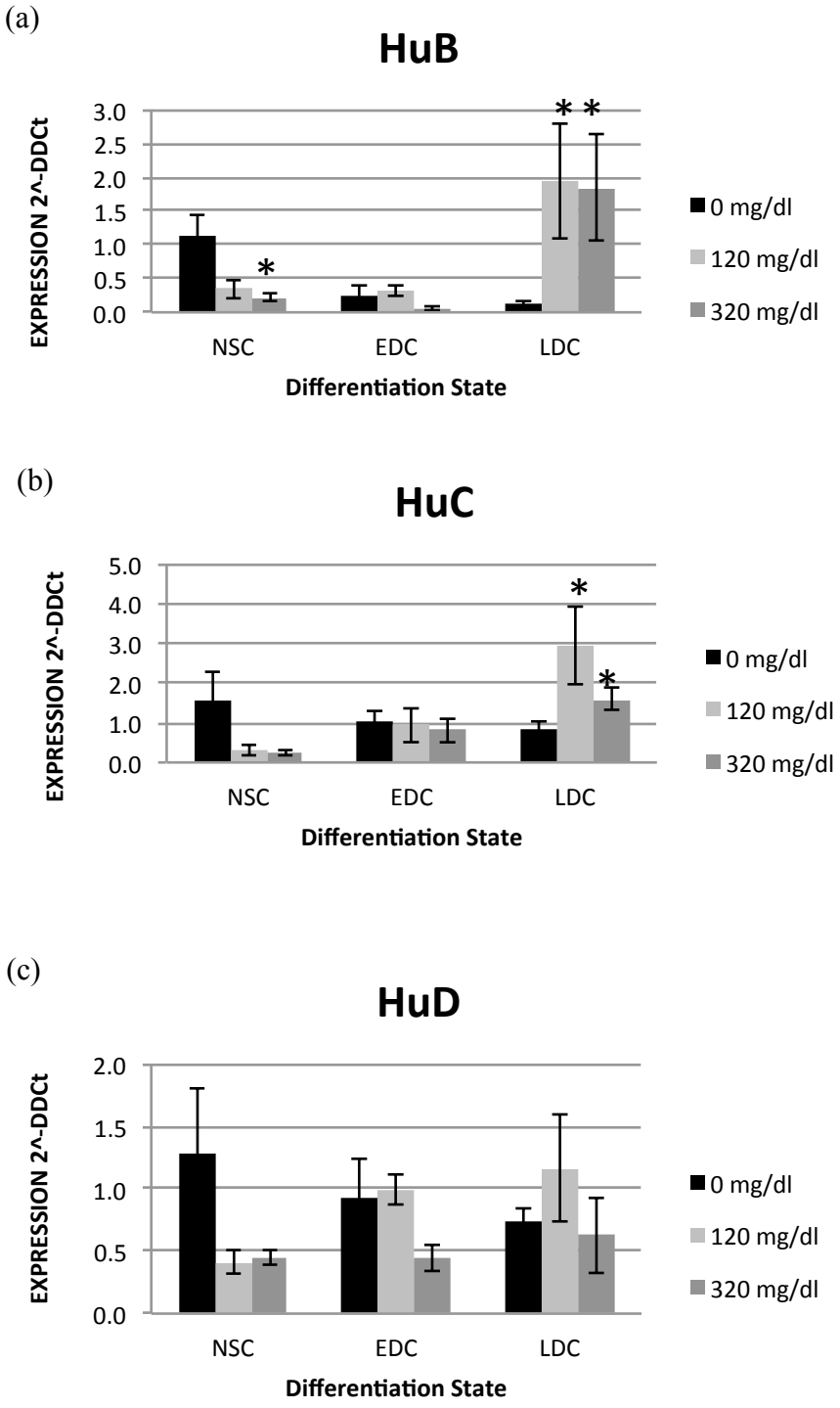


Figure 6. MRNA expression of nELAVs members in NSCs and differentiating neurons. (a) HuB. (b) HuC (c) HuD

HuC

We observed an interaction effect between ethanol exposure and maturation state on HuC mRNA expression $F_{(2,24)} = 4.62$, $p<0.02$. Under control conditions HuC expression remained relatively constant throughout the development period. In NSCs ethanol exposure at both 120mg/dl and 320mg/dl showed a trend towards decreasing HuC expression, this however was not significant (Figure 6**).** Again in EDCs, similar to HuB, we observed no significant effect of ethanol on HuC. Following ethanol exposure at 120mg/dl in LDCs there was a significant increase in HuC mRNA expression $p< 0.05$ and also an observed significant increase at 320mg/dl exposure ($p < 0.04$).

HuD

What we observed in HuD is there was no significant effect or change in HuD expression based on differentiation state or ethanol exposure. Ethanol exposure at 120mg/dl showed a trend towards an increase in HuD levels in LDCs compared to control but the increase was not significant (Figure 6**)**. Normally, transcript levels of Hu are reduced upon differentiation, Hu family members have been shown to have highest expression earlier in neuron maturation because it is required for differentiation, however following cell fate determination Hu expression is usually down regulated.

Expression of Hu protein

Since we observed an increase in Hu transcript mRNA expression following ethanol exposure, the next step was to determine if ethanol exposure caused any change

in protein expression. First we isolated protein from whole cell lysates from each differentiation state and ethanol exposure conditions. We then used an antibody that recognizes both HuB and HuD to assess protein levels. In addition later differentiated cells were not used for comparison in our protein analyses because expression was repeatedly below detectable levels by western blot. This problem was more pronounced following nuclear fractionation.

In whole lysate NSCs, we observed a significant interaction effect between differentiation state and ethanol exposure $F_{(2,12)} = 7.33$, $p < 0.008$, (Figure 7). There was an increase in HuB/D protein levels following ethanol exposure at 320mg/dl $p<0.05$ in the NSC. Even though there were overall effects of ethanol in whole lysates we wanted to examine more specific details in different cellular components. This is important because interactions and stability in the nuclear compartment may be different than in the cytoplasmic compartment. In the nuclear fraction ethanol of NSCs exposure at 120mg/dl caused a significant increase in protein levels compared to control, $p<0.02$. However in the EDC protein levels were significantly decreased compared to control in both 120mg/dl ($p<0.004$) and 320mg/dl ($p< 0.003$). In the cytoplasmic fraction, there was a trend towards decreased protein levels following maturation from NSCs to EDCs, $p<0.02$. There was a significant decrease in protein expression following ethanol exposure in the NSCs at 320mg/dl ($p<0.03$) compared to control, in EDCs there was an increase in protein levels following ethanol exposure at 120mg/dl ($p< 0.007$) compared to control (Figure 7).

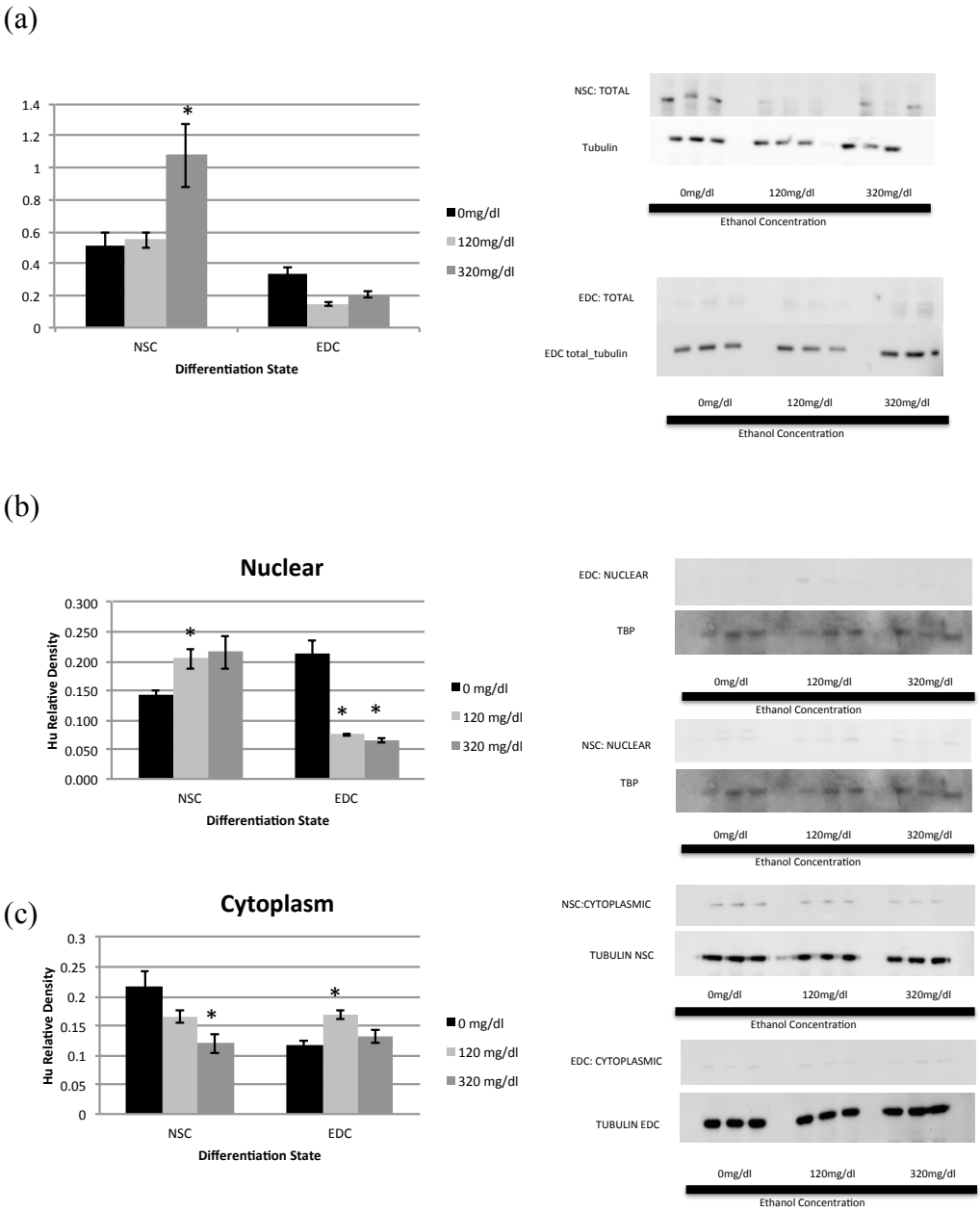


Figure 7. Ethanols effect on protein expression. (a) Protein quantification of HuB/D in (a)Whole Lysates, (b) Nuclear fraction (c) Cytoplasmic fraction by western blotting. Densitometry values normalized for loading with β–tubulin for cytoplasmic fractions and TATA binding protein TTBP for nuclear fractions.

Transfections

Following an observed general decrease in levels of Hu levels following ethanol exposure using different measurement parameters, and specifically seeing that ethanol exposure at 320mg/dl resulted in decreases in both nuclear and cytoplasmic areas and perimeter. Taken together our data suggests that ethanol is generally causing a reduction in Hu expression levels and that reduction may account for morphological changes, have implications for neurogenesis, migration and cell communication. To evaluate this idea we transfected by electroporation a validated shRNA against HuB and HuD and carried out a Sholl analysis for morphological assessments (Figure 8).

HuB knockdown

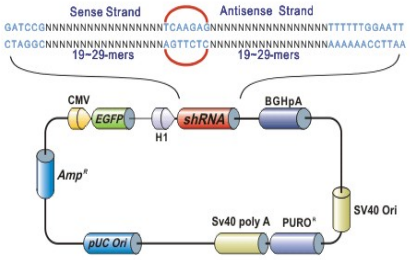
In HuB knockdown there was no significant difference in overall dendritic length. There was however a statistically significant decrease in dendritic complexity in HuB knockdown compared to control $F_{(1, 38)} = 41.77$, $p<1.33E^{-07}$. In the control group ethanol exposure at 320mg/dl increased dendritic complexity ($p<3.53E^{-05}$), however there was no significant effect of ethanol exposure on dendritic complexity in the HuB knockdown group (Figure 9**).**

HuD knockdown

We observed a statistical significant decrease in dendritic length in HuD knockdown cells compared to controls $F_{(1, 38)} = 28$, $P<5.31E^{-06}$. Ethanol exposure had no effect on dendritc length in the control group, however in the HuD knockdown cells

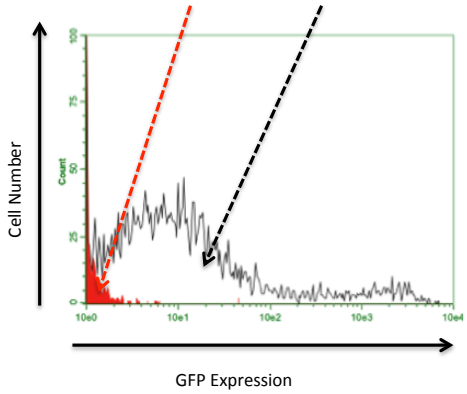
ethanol exposure at 120mg/dl significantly increased dendritic length ($p<0.04$) (Figure 9**).**

(a)



Gene	Target Sequence
Elavl2_HuB	ggccaacttgcaataacac
Elavl4_HuD	ccgctttgataagaggatt

(b)



(c)

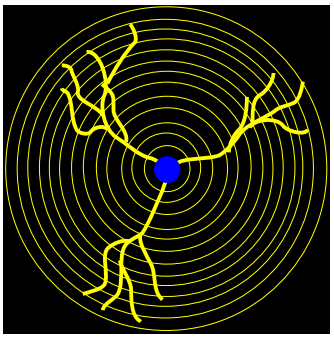


Figure 8. Hu gene knockdown. (a) Vector information for HuB and HuD (b) Flow cytometric assessment of transfection efficiency (c) Schematic of Sholl Analysis

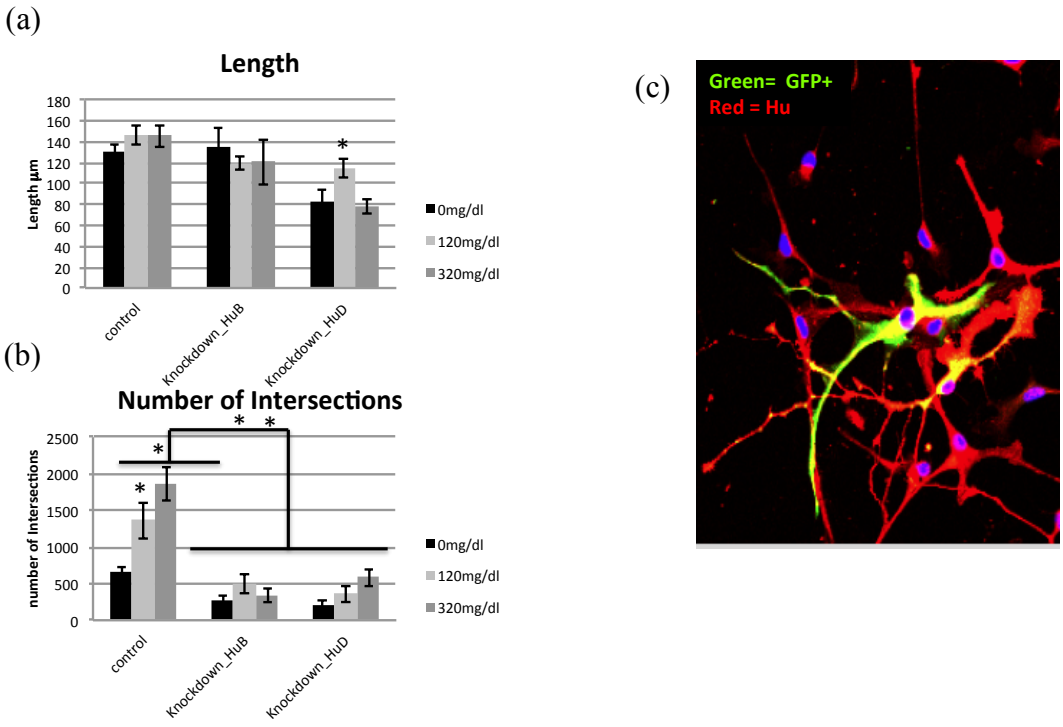


Figure 9. Sholl analysis. (a) Quantitative analysis of dendritic length and (b) number of intersections (c) Representative image of fluorescence microscopy of transfected cells. Immunofluorescence stain shows transfected cells expressing eGFP (green) with GAP-43 (red) counterstain

Dendritic complexity was significantly decreased in HuD knockdown compared to control $F_{(1, 38)} = 22.7$, $P < 2.68\ E^{-05}$. In addition in the control group ethanol exposure at both 120mg/dl ($p < 0.03$) and 320mg/dl ($p < 0.01$) resulted in significant increase in neuron complexity. In the knockdown group ethanol exposure resulted in a significant increase in complexity at 320mg/dl ($p<0.014$) compared to non-ethanol exposed knockdown cells.

Discussion

Fetal alcohol exposure causes developmental impairments that lead to neurological deficits and structural abnormalities in the central nervous system (Bertrand et al., 2005). Identification of cellular targets of ethanol during neuronal differentiation is important to advance our understanding of FASD. Central nervous system neurons arise from the generation of post mitotic cells from the germinal and ventricular zones followed by initial neuritogenesis and differentiation. These cells are representative of asynchronous phases of the cell cycle and give rise to different populations of cells spatiotemporally. The underlying molecular mechanisms responsible for ethanol's teratogenic effects are still poorly understood because of the complexity of molecular mechanisms involved in nervous system development. Amongst key features identified in fetal alcohol exposure is the fact that ethanol exposure promotes premature differentiation and aberrant migration of cortical neurons in neural stem cell populations (Santillano et al., 2005, Prock and Miranda, 2007, Camarillo and Miranda, 2008), suggesting ethanol is exerting its effect on genes necessary for growth and differentiation.

Ethanol exposure dysregulate production of nascent mRNA transcripts

In this study neural stem cells were exposed to different concentrations of alcohol during the period of neurogenesis, alcohol was then completely removed and cells allowed to differentiate. During neural development neural stem cells exist as heterogeneous populations, with distinct molecular expression patterns and varying

phases of transformation (Tingling et al., 2013). We show here that different subpopulations of cells remain distinct and persisted throughout the development period. We found that immediately following exposure, ethanol dysregulate global transcript production and the effect of this initial dysregulation was different for each subpopulation of cells. Ethanol is known to reorganize cell programing, we did not see an immediate effect in our NSC population, we however see that the long-term effect was very substantial with overall increase in production of nascent mRNA in our EDCs and LDCs. A global increase in transcript production has several implications for neurogenesis, which occurs in a spatio-temporal manner, aberrant overexpression of genes that are required only transiently poses the problem of disrupting regulatory machinery of the developing neurons. This underlying reprogramming at the stem cell state that persists into final fate not only corroborate fetal ethanol exposure as being deleterious to developing neurons but it also shows us that there is a distinct subpopulation of cells that are resistant to ethanol's effects. Future research into identifying other unique properties of each subpopulation may provide us with options to mitigate FAE effects.

Ethanol exposure reduces neuron size: Impact on brain growth and microcephaly

Neuronal functionality is dependent on its morphology. The ability to form synapses depends on efficient neuritogenesis and differentiation into functional mature neurons. It is recognized that one of the major effects of fetal alcohol exposure is microcephaly, a reduction in brain size (Archibald et al., 2001, Lipinski et al., 2012).

Morphological assessment of ethanol pre-exposed neural cells showed a consistent reduction in cell size irrespective of the differentiation state. A brain size reduction in fetal alcohol exposure is a predictor of neurocognitive performance (Coles et al., 2011, Fryer et al., 2012) and neuronal migration and differentiation (Zhou et al., 2005).

Ethanol exposure reverses the spatio-temporal decrease of Hu expression

Because Hu genes are important for neuron differentiation we sought to find out if ethanol was affecting Hu expression. Our data was consistent with known data that the Hu gene expression is highest in early neuritogenesis and significantly reduced following differentiation (Mansfield and Keene, 2012) we observed this in two of the three Hu genes, however following ethanol pre-exposure this reduction was completely reversed especially in the LDCs, interestingly it is the LDC nuclear area and nuclear perimeter that are enlarged in ethanol pre-exposed cells. Here we see for the first time that an increase in morphology of a specific stage of neural development correlate with an increase in transcript expression of Hu. Moreover we see that an early effect of ethanol exposure is to reduce cell size in early differentiating migrating cells.

Loss of HuB/D prevents ethanol induced morphology changes

Hu proteins are expressed very early in neurons and is required for neural lineage commitment (Kim et al., 1996). Hu proteins are also important in regulating gene expression during brain development (Bolognani et al., 2006) and enhancing neurite outgrowth (Anderson et al., 2003, Smith et al., 2004). Since we saw that ethanol was

reversing the spatiotemporal decrease of nELAVs transcripts and causing a modest reversal of protein expression and knowing the importance of Hu proteins in neuron growth and differentiation, we knocked down Hu with the rationale that the ablation of Hu would lead to a similar reduction in cell size similar to what was seen in our morphology study. Interestingly we discovered that when Hu was knocked down ethanol was no longer able to affect cell morphology. This observation suggests that that Hu was necessary for ethanol to affect cell morphology and is therefore a likely target of ethanol. Our Hu knockdown data showing reduction of dendritic processes was also consistent with previous studies showing that nELAVs are required for growth of dendritic processes and differentiation (Anderson et al., 2001).

CHAPTER III

HU FAMILY OF RNA BINDING PROTEINS INTERACTOME: TARGETS AND BINDING PARTNERS

Overview

The maternal fetal environment is critical for the development of the embryo. Ethanol exposure during development lead to an array of congenital malformations and neurological deficits termed Fetal Alcohol Spectrum disorders. Alcohol exposure during critical periods of embryonic development is considered to be targeting molecular factors responsible for stem cell maturation, however the exact molecular mechanism remains unknown. RNA binding proteins have been identified as critical post-transcriptional regulators governing neuronal development. A conserved family of RNA binding proteins, the neuronal ELAVs/Hu proteins play important roles in neuronal stem cell maturation. Developing neurons are susceptible to ethanol exposure; we demonstrated that this susceptibility is mediated via the Hu RNA binding protein. However, these proteins interact with other critical regulators of neuronal development such as other RNA binding proteins, other proteins, and microRNAs. We hypothesize that as a teratogen, ethanol is disrupting the dynamic relationship between the Hu protein interactome, gene targets and Hu repressors such as miRNAs leading to altered progression of fetal stem cell maturation.

Introduction

Fetal Alcohol Exposure continues to be the predominant cause of a preventable cluster of birth defects collectively termed Fetal Alcohol Syndrome Disorders (FASD) (Bertrand et al., 2005, Jones and Smith, 1973, May et al., 2013a). With a prevalence rate of FASD ranging from 0.2 - 7/1000 live births in the United States (Senturias and Asamoah, 2014, May et al., 2009) and between 68-89.2/100 live births in a South African population, the highest levels of occurrence recorded so far (May et al., 2007). Other studies have shown similar prevalence rates to the United States in Australia (Mutch et al., 2014), and in Italy (May et al., 2011). The total numbers of actual fetal alcohol exposure unknown. Globally FASD is proving to be a serious socioeconomic factor. In Canada it is estimated that the cost associated with FASD per individual is approximately $22,000 per annum (Stade et al., 2009). Children diagnosed with FAS in the United States incurred a mean annual medical cost of $16,782 (Amendah et al., 2011). The underlying molecular mechanism of ethanol on the developing fetal brain is unknown. Ethanol is believed to be disrupting biological programs regulating neural development. Hu proteins, neuronal homologs of drosophila's ELAV family of RNA binding proteins are important post-transcriptional regulators involved in neural development. RNA binding proteins modulate all aspects of RNA processing, from transcription, splicing, polyadenylation, RNA modification and translation. Hu proteins are mRNA stability factors that regulate genes required for both neural stem cell maintenance and neuritogenesis. Developing neurons are susceptible to ethanol; we postulate that this susceptibility is mediated via the Hu RNA binding protein.

Identification of Hu binding partners such as other proteins, mRNAs or miRNAs is important for defining temporal and spatial expression patterns of both Hu and their binding partners. Known mRNA targets of Hu proteins include GAP-43 and Musashi (MSI) (Sakakibara et al., 2001, Pascale et al., 2008, Okano et al., 2005, Nakamura et al., 1994). We know that Hu proteins are localized both in nuclear and cytoplasmic compartments, its specific role in the mammalian nucleus is less understood but it is thought to associate with newly made transcripts as a part of its transcript stability mechanisms. Hu proteins interact with 3'UTR of target transcripts and modulate transcript stability and translation. Hu also forms complexes with other proteins; Hu interacts with MSI and eukaryotic translation initiation factor 4A (eIF4A). In addition, a small class of non-coding RNAs called microRNAs regulates Hu expression. As we begin to evaluate sets of genes within interactomes and not on single gene outcomes, we may better understand the mechanisms by which teratogens such as alcohol alters neuronal development (Figure 10).

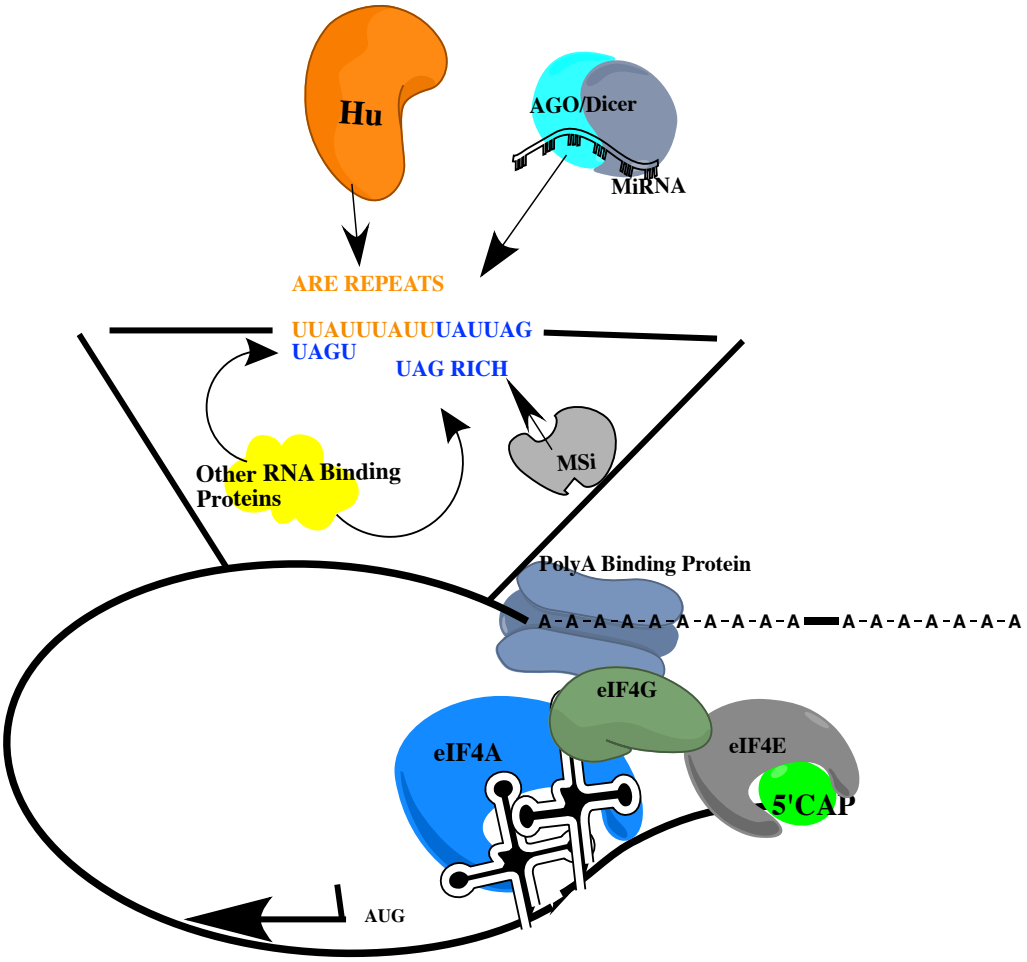


Figure 10. Schematic of the Hu interactome. Post transcriptional regulation and transcript processing of Hu and its binding partners.

Musashi is an RNA binding protein that is evolutionary conserved and regulates genes that are involved in stem cell maintenance and self-renewal (Nakamura et al., 1994). Musashi is highly expressed in the developing nervous system (Sakakibara et al., 1996) and is sometimes used as a marker for stemness (Kurihara et al., 1997, Good et al., 1998, Kaneko et al., 2000). Musashi's role in stem cell maintenance is thought to occur by translation repression of its target genes (MacNicol et al., 2008). Musashi possesses RNA recognition motifs (RRMs) similar to Hu, however Musashi only has two RRMs whereas Hu has three RRMs (Kurihara et al., 1997). Musashi binds to the 3'UTR of its target mRNAs, the exact binding mechanism is unknown but it has been shown that Msi preferentially bind to GU rich regions in the 3'UTR (Kurihara et al., 1997). More recently it was shown that Musashi binds to UAG, a three nucleotide motif in the 3'UTR (Zearfoss et al., 2014) Hu proteins share an interesting relationship with Musashi, Hu proteins target and stabilize Musashi mRNA (Ratti et al., 2006) in addition Hu protein and Musashi protein colocalize in neural stem and progenitor cells (Ratti et al., 2006) suggesting protein-protein interaction. It is theorized that as a part of the post-transcriptional regulatory interactome, both Hu and MSI RNA binding proteins act in conjunction to determine the spatio-temporal maturation of neural stem cells.

Neuritogenesis is important for neuron maturation and morphology. Proteins that promote growth of axons and dendrites and allow for synaptogenesis regulate neuron morphology and are important for neural function. Growth Associated Protein, GAP-43 is a phosphoprotein that regulates neuron growth; it is localized to the growth cones during neuronal development (Strittmatter et al., 1995, Beckel-Mitchener et al., 2002).

Because of its role in neuritogenesis and synaptogenesis, GAP-43 is important for the spatio-temporal organization of the brain during development and is important for neural circuitry. Hu has been shown to target and stabilize GAP-43 mRNA, hence its role in neuronal maturation.

Because Hu RNA binding proteins are significant post-transcriptional regulators acting as central nodes in the interactome, factors affecting Hu expression becomes important determinants of developmental processes, microRNAs are one such factor that target Hu proteins. MicroRNAs are 18-25 nucleotide small noncoding RNAs that act as translation repressors by binding to sequences in the 3' UTR of target mRNA (Cullen, 2004, Ruan et al., 2009, Sevli et al., 2010). MicroRNAs are an important class of post-transcriptional regulators that was first identified in *C. elegans,* but also exist in plants, animals and viruses (Lee et al., 1993, Lau et al., 2001, Lu et al., 2008). MicroRNAs are thought to affect gene expression post transcriptionally by formation of partial complementarity to their target mRNA, via their seed region, which is located between nucleotides 2-8 and thereby inhibiting translation (Wu et al., 2006, Treiber et al., 2012). Following synthesis in the nucleus, microRNAs are translocated to the cytoplasm where they are loaded onto the RNA Induced Silencing Complex (RISC) (Long and Lahiri, 2012). RISC is made up of the proteins Dicer and Argonaute (AGO) that are both necessary for post-transcriptional gene repression by microRNAs. Dicers role is to cleave the precursor microRNA to produce mature miRNAs (Lee et al., 2013). It is the action of the AGO protein that mediates the translation repression by its endonuclease activity (Huang and Li, 2014, Ender and Meister, 2010, Wei et al., 2012a). MicroRNAs

have been shown to be potent regulators of neural development (Yoo et al., 2011, Zhao et al., 2009, Sanuki et al., 2011). Ethanol affects the expression of several developmental associated mRNAs (Grummer and Zachman, 1995, Naassila and Daoust, 2002). Because microRNAs bind to the 3'UTR it is proposed that microRNAs work in conjunction with RNA binding proteins to regulate gene expression.

Post-transcriptional gene regulation inevitably serves the purpose of either enhancing or repressing of translation. Translation is a highly regulated process and requires continuous integration of multiple molecular mechanisms. Several post-transcriptional regulators such as Hu and MSI proteins along with microRNAs and the RISC complex actively partake in translational control (Pillai et al., 2004, Glorian et al., 2011). Translation initiation is the rate-limiting step of protein synthesis (Splan et al., 2008) and most translational control is exerted at the level of translation initiation. The eukaryotic initiation factor 4F (eIF4F) is a multiprotein complex responsible for cap dependent translation initiation at the 5'UTR. eIF4F acts as a scaffolding protein with binding domains for interaction with eIF4A, eIF4G and the poly A binding protein (Gingras et al., 1999, Andreou and Klostermeier, 2014). eIF4A is a DEAD-box protein that binds to the 5'UTR of mRNA, it is a RNA-dependent ATPase and RNA helicase (Gingras et al., 1999, Ozes et al., 2011, Rogers et al., 2002). eIF4G is a cap binding scaffolding protein with binding domains for both eIF4A, eIF4E, eIF3 and polyA binding protein (Gingras et al., 1999, Andreou and Klostermeier, 2014). Understanding ethanol's effect on the Hu family of RNA binding proteins and their binding partners not

only unravel the complexities involved in neural development, but also assist with designing therapeutic for FASD.

Methods

Ethics statement

Timed-pregnant C57Bl/6 mice (Harlan, Texas) were housed in an AAALAC-approved facility at TAMHSC. All protocols were conducted with the approval of the Institutional Animal Care and Use Committee at TAMHSC (Approval number AUP2010-197).

Neural precursor cultures

Neural precursor cells were obtained from the dorsal telencephalic vesicles of gestational day 12.5 C57BL/6 fetal mouse based on methods by Santillano et al., 2005 (Santillano et al., 2005). Isolated precursor cells were maintained as neurospheres in serum free mitogenic media, containing Dulbecco's Minimal Essential Medium DMEM/F12 (Lifetechnologies cat. no. 23017-015), 20 ng/ml Basic Fibroblast Growth Factor bFGF (BD Biosciences cat. no. 354060), 20ng/ml human Epidermal Growth factor hEGF (PreproTech cat. no. 100-15), 0.15 ng/ml leukemia inhibitory factor LIF (Alomone labs cat. no. L-200), Insulin-transferrin selenium-X ITS-X (Lifetechnologies cat. no. 51500-056), 5ug/ml Heparin (Sigma-Aldrich cat. no. H4784), 50uM

Progesterone (Sigma-Aldrich: cat. no. P7556), Penicillin/Streptomycin (Life technologies cat no. 15140-122).

In-vitro differentiation

To study the effects of ethanol exposure on early stem cell maturation, the experimental model was according to protocol by (Camarillo et al., 2007). Neurosphere cultures containing approximately 2 x 10^6 cells were assigned as either control or ethanol treated group with doses of 120mg/dl (26mM) ethanol or 320mg/dl (70mM) ethanol (Sigma Aldrich cat. no. E7148) for a total of 5 days with refreshment of media on day 3. For stem cell maturation, cells were later transferred to either of two ethanol-free mitogen withdrawal differentiation media (Camarillo et al., 2007). For differentiation flasks were prepared by coating with 1mg/ml laminin (Life technologies cat. no. 23017015) for two hours at room temperature prior to use. Immediately before use excess laminin was carefully removed, without disturbing the coating and the flask gently washed once with 1X PBS solution. Cells were prepared for differentiation by centrifuging at 1000 rpm for 5 minutes to remove ethanol containing mitogenic media. Cells were then resuspended in either a partial mitogenic media containing DMEM/F12, 5ug/ml Heparin, 1ml 100X ITS-x, 50uM progesterone, 1ml Penicillin/Streptomycin and 2mg/ml bFGF to model an early period of differentiation or to a mitogen free media containing DMEM/F12, 5mg/ml Heparin, 100X ITS-x, 50μM progesterone and Penicillin/Streptomycin) modeling a later period of neural differentiation. The resuspended cells were then transferred to the freshly prepared laminin coated culture

flasks and incubated for 72 hours in 5% CO_2 at 37°C. Cells plated in the early differentiation model are called Early Differentiated cells (EDCs) and cells plated in the later differentiation model are called (LDCs). Non-differentiated cells are called Neural Stem Cells (NSCs).

Immunofluorescence

Indirect immunofluorescence was carried out on cells grown on glass coverslips for 72 hours. Cells were incubated overnight with primary antibodies against HuB/D, GAP-43, and Musashi-1. Following incubation, antigenic sites were localized with goat anti-rabbit or anti-mouse FITC or TRITC conjugated secondary antibodies. Images were captured on

Western blot analysis: whole cell and nuclear and cytoplasmic components

Total proteins were extracted from neural stem cells and differentiated cells using cell extraction buffer containing 50mM Tris pH 7.4 (Life Technologies cat. no.15504-020), 150mM NaCl (Sigma-Aldrich cat. no. S-3014), 10% Glycerol (Fisher Scientific cat. no. BP229-1), 1mM EGTA (Fisher Scientific cat. No. 02783-100), 1mM Na-Orthovanadate, pH 10 (Sigma-Aldrich cat. no. S-6508), 5μM ZnCl2 (Sigma-Aldrich cat. No. Z-48750), 100mM NaF (Sigma-Aldrich cat. No. S-7920), 10ug/ml Aprotonin (Sigma-Aldrich cat. No. A-1153) 1ug/ml Leupeptin (Sigma-Aldrich cat. No. L-9783), 1%Triton X-100 (Fisher scientific cat.no. BP151-100) and distilled water. On day of use of extraction buffer 1mM Phenylmethylsulfonylfluoride was added to buffer. Samples

were centrifuged at 15000-18000 rpm for 10 minutes; the soluble protein in the extract the supernatant was collected and quantified using the Pierce® BCA protein Assay Kit; cat no. 232225 followed by spectrophotometric measurements on TECAN® plate reader or NanoDrop® 2000 spectrophotometer.

To obtain the subcellular distribution of the GAP-43 and Musashi, proteins were extracted from separate nuclear and cytoplasmic fractions according to manufactures protocol using the NE-PER Nuclear and Cytoplasmic extraction kit (Thermo Scientific cat. No 78833). Samples containing 15ug of protein was mixed with a 5X loading dye, 6.25 ml TRIS-HCl pH 6.8, Glycerol, β-mercaptoethanol, SDS, Bromophenol blue and heated at 95 °C for 7-8 minutes and then resolved using the NuPage Novex 4-12% Bis Tris Minigels (Life Technologies cat. no NP0336BOX) in a 1X NuPAGE® MOPS running buffer. Proteins were transferred to polyvinylidene (PVDF) membranes using the Life technologies iBlot® system. The membranes were washed 3 times in 1X TTBS (TBS-Tween) and blocked for 1 Hr. with 5% non-fat dry milk in 1X TTBS at room temperature. Membranes were each incubated with primary antibodies against Elavl2 (dilution 1:200) (Sigma-Aldrich cat.no. H1538), HuC (dilution 1:300) (Abcam cat. no. Ab78027), HuD (dilution 1:500) (Sigma-Aldrich H5789) and Musashi (1:200) (EMD Millipore cat. no. 04-1041), overnight at 4°C. Following incubation membranes were washed 3 times in 1X TTBS and incubated with horseradish peroxidase conjugated with (appropriate secondary antibodies) with goat anti-mouse IgG (BD Pharmingen 554002) or goat anti-rabbit IgG (Santa Cruz Sc-2004) dilution 1:2000 at room temperature for 1Hr. Bands were detected with ECL Western Lightening Western Blot Detection System

(Pierce cat. no. 32109). Blots were imaged on the FluorChemQ® imaging equipment and quantified using the alphaView® analysis software. The optical density of each band was corrected by α-tubulin or Anti-Histone core antibody (Abcam cat. no ab7832).

Real time qPCR

For mRNA expression analysis, total mRNA was isolated using mirVana miRNA isolation kit (Life technologies cat. no. AM1561) total RNA extraction protocol, mRNA was quantified on the NanoDrop 2000 spectrophotometer (Thermo Scientific) and then reverse transcribed into cDNA using qScript™ cDNA Supermix (Quanta Biosciences cat. no. 95048). Primers to Musashi and GAP43 were designed using NCBI PrimerBlast and USCSC Genome BLAT browsers. (List of forward and reverse primers: table 1). Real Time qPCR was carried out using the Applied Biosystems 7900HT Fast Real-Time PCR System and Quanta Biosciences Perfecta SYBR® Green Supermix (Quanta Biosciences cat. no. 95073-012).

Reversible crosslinking coupled with immunopreciptation followed by analysis of immunoprecipated RNA microarray analysis

To identify the cellular context within which RNA binding proteins interact with cognate mRNAs and to identify how RNA binding proteins associate with each other as a component of the ribonucleoprotein complex a combination of biochemical and genetic approach in the form of Reversible Cross-linking followed by Ribonucleoprotein Immunoprecipitation, reversal of crosslinking, RT-PCR and microarray will be

employed. A Ribonucleoprotein Immunoprecipitation assay will be carried out according to protocol by Niranjanakumari, (Niranjanakumari et al., 2002) with optimizations suitable for our experimental paradigm. Briefly, neural progenitors are cultured according to our laboratory protocol outlined above and cross-linked using paraformaldehyde. Following crosslinking, immunoprecipitation of HuB/D and Musashi along with associated molecules was carried out using Dynabeads® Protein G was carried out following manufacturers protocol. Following immunoprecipitation, characterization of the immunoprecipitated components was then analyzed by microarray analysis. For mRNA microarray analysis, 1.0µg of purified mRNA samples from either HuB/D or Musashi RIP from each treatment group were combined with 50pg RNA spike-in control. First strand cDNA synthesis was carried out at 42°C for 2hrs followed by second strand cDNA synthesis at 16°C for 2hrs. Following cDNA purification Biotin labeled cRNA samples were synthesized by a linear amplification method using Ambion's MessageAmp™ II-Biotin Enhanced Kit (Life Technologies, cat. no. Am1791). 10µg of biotin labeled cRNA samples were fragmented at 94° C for 20 minutes and then combined with hybridization buffer (Applied Microarray, Tempe AZ). Hybridization was done with the CodeLink™ expression bioarray (Applied Microarray, Tempe AZ) at 37°C for 18h. Post-hybridization processing and secondary-labeling with Alexa Fluor 647-Streptavidin was done according to the manufacturer's instructions (Applied Microarrays). Microarrays were scanned using a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA).

Statistical analyses

Standard statistical analysis (ANOVA) was applied to each experimental data set. $p < 0.05$, is considered statistically significant.

Results

Effect of ethanol MSI and GAP-43 transcripts and protein expression

We previously showed that ethanol was exerting its effect on developing neural stem cells through Hu RNA binding proteins, we next explored if this effect would disrupt Hu protein relationship with its binding partners which include MSI, GAP-43 and several presumptive microRNAs. When target transcript expression was analyzed we saw that the levels of MSI mRNA was affected by ethanol exposure. There was a significant interaction effect between differentiation state and ethanol exposure $F(4,24) = 3.57$, $p<0.02$. Our data indicated that MSI expression was highest in NSCs with a trend towards maturation dependent reduction transcript levels were highest in NSCs compared to EDM ($p<0.03$) and LDM ($p<0.008$). In NSCs ethanol exposure at 120mg/dl ($p<0.04$) and 320mg/dl ($p<0.04$) reduced MSI mRNA transcripts. In LDCs there was an attempt to compensate for the loss in NSCs that resulted in an increase of MSI transcript levels in the 120mg/dl exposed cells, this however was not significant compared to control (Figure 11**)**. GAP-43 transcript levels were not altered by differentiation state or ethanol exposure. The cellular localization of Hu and GAP-43 and Hu and Musashi are shown in Figures 24-25 in Appendix.

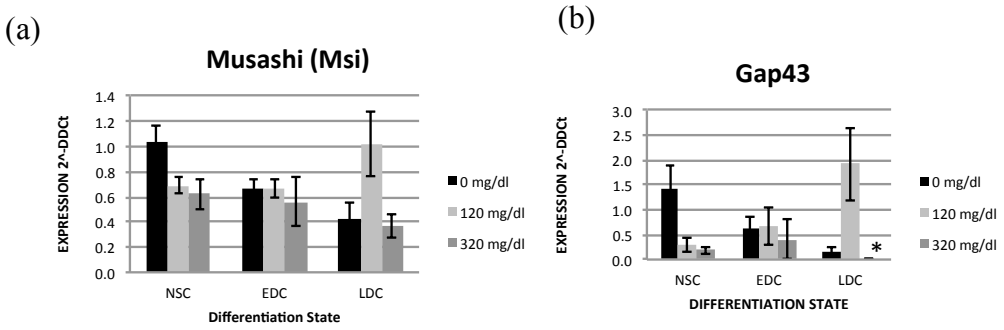
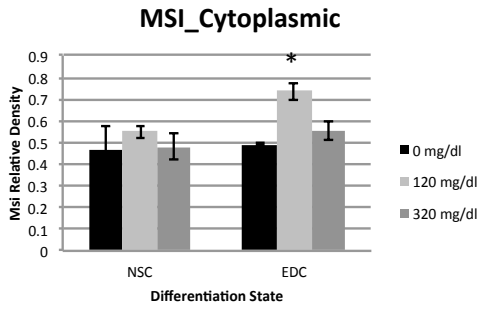
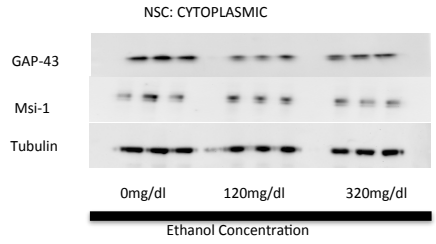


Figure 11. MRNA expression of GAP43 and MSI. (a) GAP-43 and (b) MSI in differentiating neurons

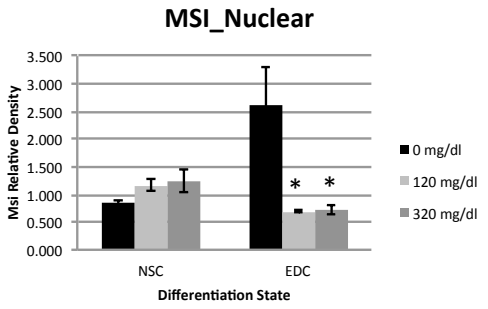
We next assessed if ethanol was having an effect on MSI protein levels (Figure 12**)**. In our cytoplasmic fraction ethanol exposure had an effect on MSI protein levels, $F(2,12) = 4.59$, $p<0.03$. Specifically in the EDCs ethanol exposure caused an increase in protein expression at exposure levels at 120mg/dl compared to controls ($p<0.007$). In the nuclear fraction ethanol exposure decreased MSI protein expression levels at 120mg/dl ($p<0.04$) and at 320mg/dl ($p<0.05$), compared to controls. There was no effect of ethanol exposure on protein expression of GAP-43 in cytoplasmic or nuclear fraction (Figure 13**).**

(a)





(b)



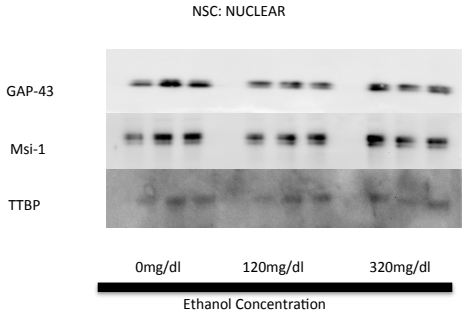


Figure 12. Ethanols effect on protein expression of Hu target Musashi. Protein quantification of Musashi MSI in (a) Cytoplasmic fraction, (b) Nuclear fraction by western blotting. Densitometry values normalized for loading with β–tubulin for cytoplasmic fractions and TATA binding protein (TTBP) for nuclear fractions..

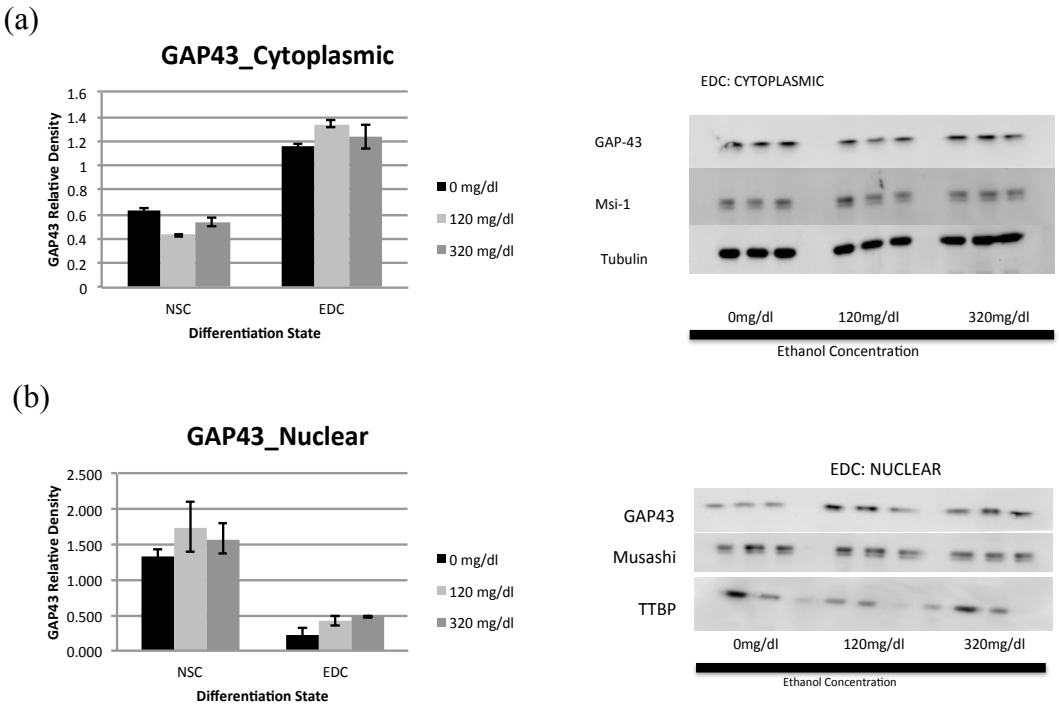


Figure 13. Ethanols effect on protein expression of Hu target GAP-43. Protein quantification of GAP-43 in (a) Cytoplasmic fraction, (b) Nuclear fraction by western blotting. Densitometry values normalized for loading with β–tubulin for cytoplasmic fractions and TATA binding protein (TTBP) for nuclear fractions.

Here we show that MSI protein is more susceptible to ethanol exposure than GAP-43, in addition A Pearsons correlation done to assess the relationship between Hu protein levels and MSI protein expression, there was a positive correlation $r = 0.94$, $p<0.05$.

Hu and MSI interact with eIF4A but not eIF4g during translation

To further explore the role of Hu in translation enhancement, we investigated protein-protein interaction of Hu and MSI with two key translation initiation factors, eIF4A and eIF4G. Our data shows that eIF4A and Hu are binding partners but not eIF4G. We also showed that MSI and eIF4A interacts but MSI did not associate with eIF4G (Figure 14**).** In addition these interactions were not altered by ethanol exposure. Overall eIF4A protein levels were also assessed in whole lysates, ethanol exposure did not affect eIF4A protein levels in whole lysates (Figure 15**).**

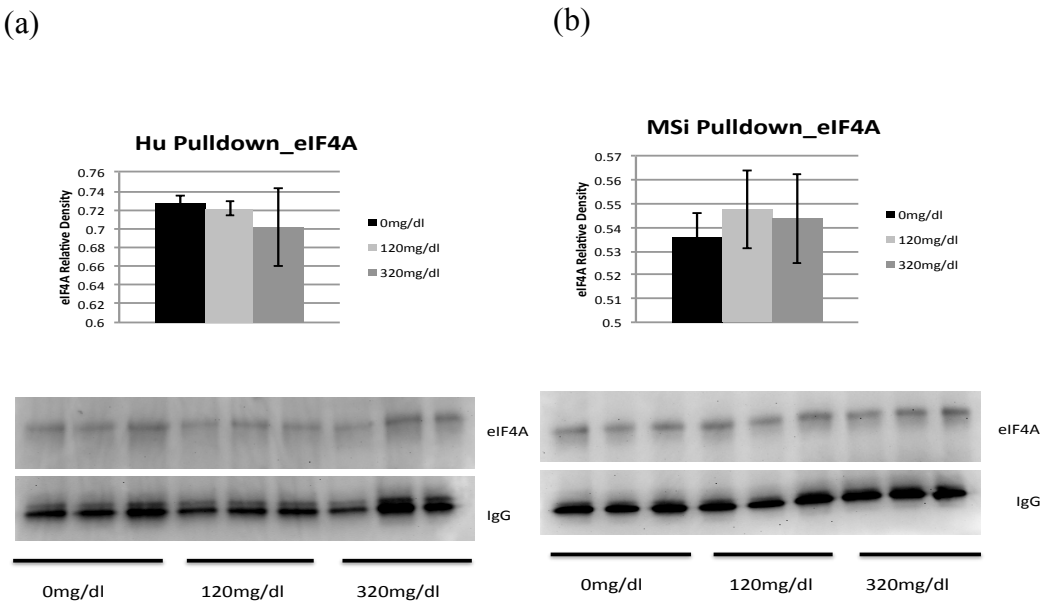


Figure 14. Interaction of Hu and MSI with eIF4A. Western blotting quantification of eIF4A expression following immunoprecipitation with (a) HuB/D and (b) Musashi. Densitometry values normalized for loading with IgG.

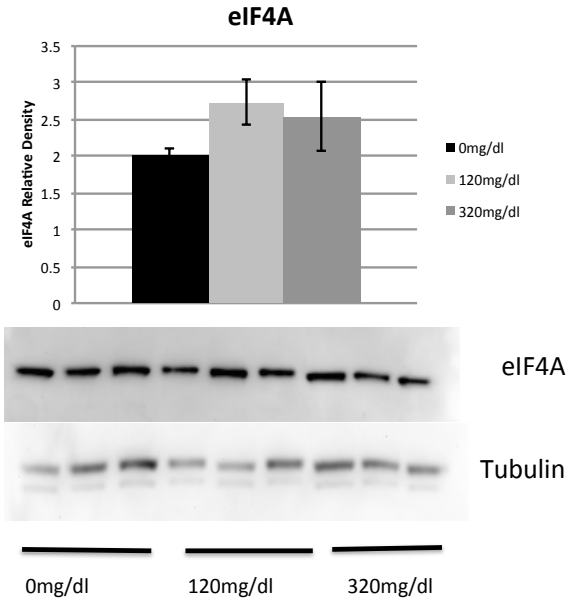


Figure 15. Protein Expression of eIF4A in whole lysates. Densitometry values normalized for loading with β–tubulin.

Identification of novel nELAVs gene interactions using RNA immunoprecipitation (RIP) and microarray analysis

Because of the strong correlation between HuB/D and MSI expression, we carried out RIP using antibodies for both Hu and MSI to identify genes targeted by each RNA binding protein. We hypothesized that as RNA binding proteins with opposing effects on differentiation and a strong correlation of between their protein expression levels the association may be synergistic or antagonistic in regulating the spatiotemporal

development of the nervous system and as such we may see a significant association of the same genes with both RBPs. To determine this we carried out RIP using antibodies for Hu and MSI followed by microarray analysis to identify associated genes. Furthermore RIP and microarray was done on both ethanol exposed and control NSC. By crosslinking prior to immunoprecipitation we ensure that the RNA-Protein interactions remain intact. Ontology analysis showed that several genes related to neural development and neurogenesis was associated with each RBP; moreover we also identified groups of genes commonly associated with both, (Figure 16**)**. Cluster analysis with average linkage and rows centering on genes showing ethanol exposed were different from controls (Figure 17**)**. Importantly we also saw that ethanol exposure changed these association patterns. Identifying novel binding interactions and identifying ethanol's disruption of these genes will not only provide information on ethanol exposure but also guide future studies into understanding FASD.

(a)

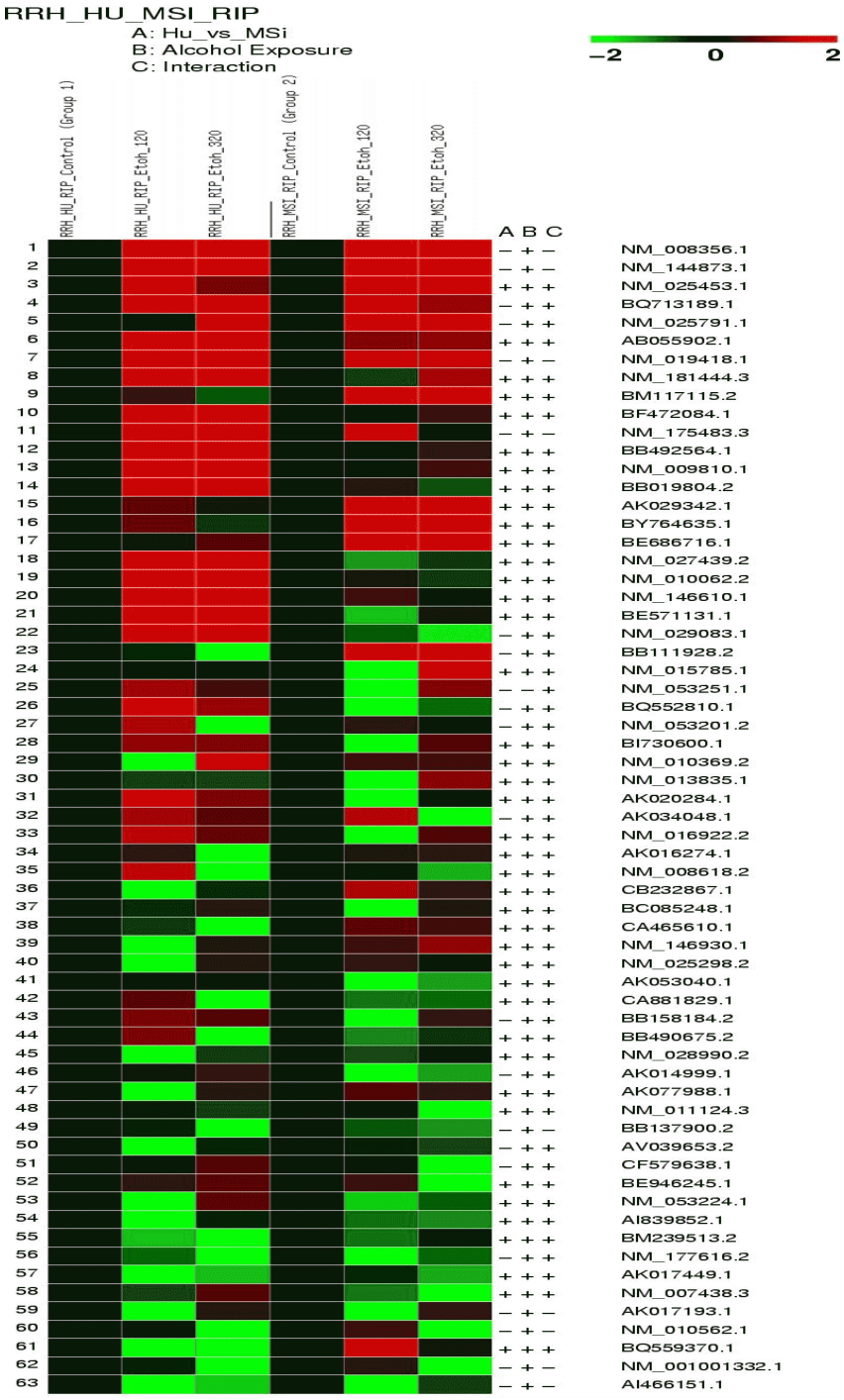


Figure 16. Comparison of gene expression following Hu and MSI RIP. (a) Heat map of replicates showing genes commonly unregulated and down regulated and differentially between in Hu and MSI pulldown (b) Boxplots of normalized samples.

(b)

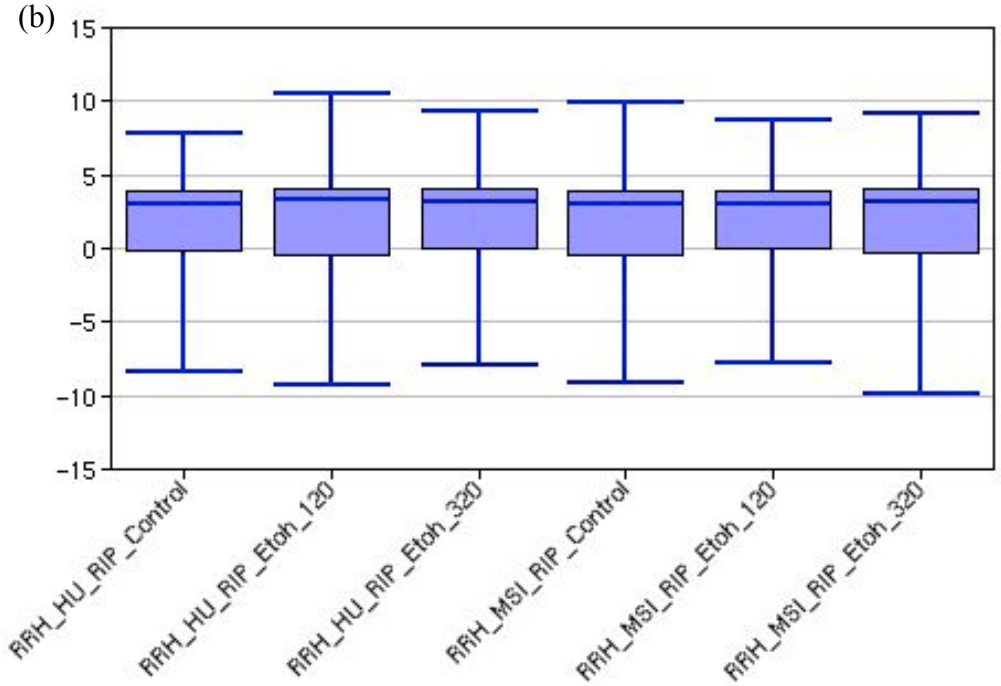


Figure 16 continued

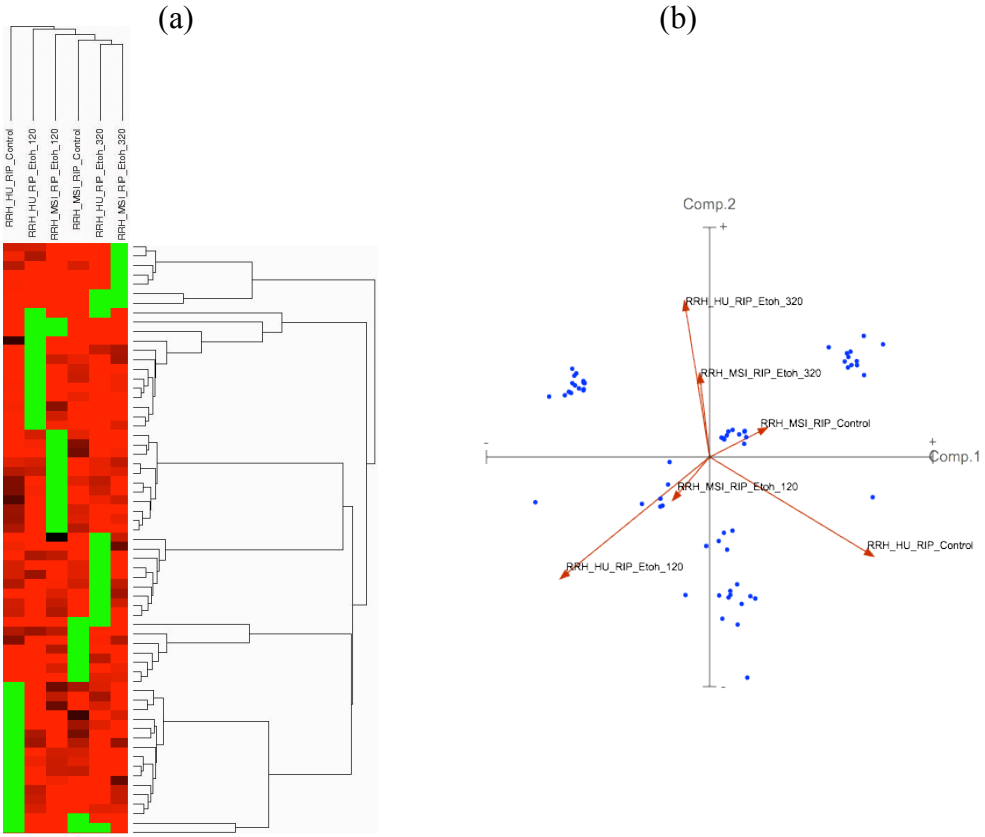
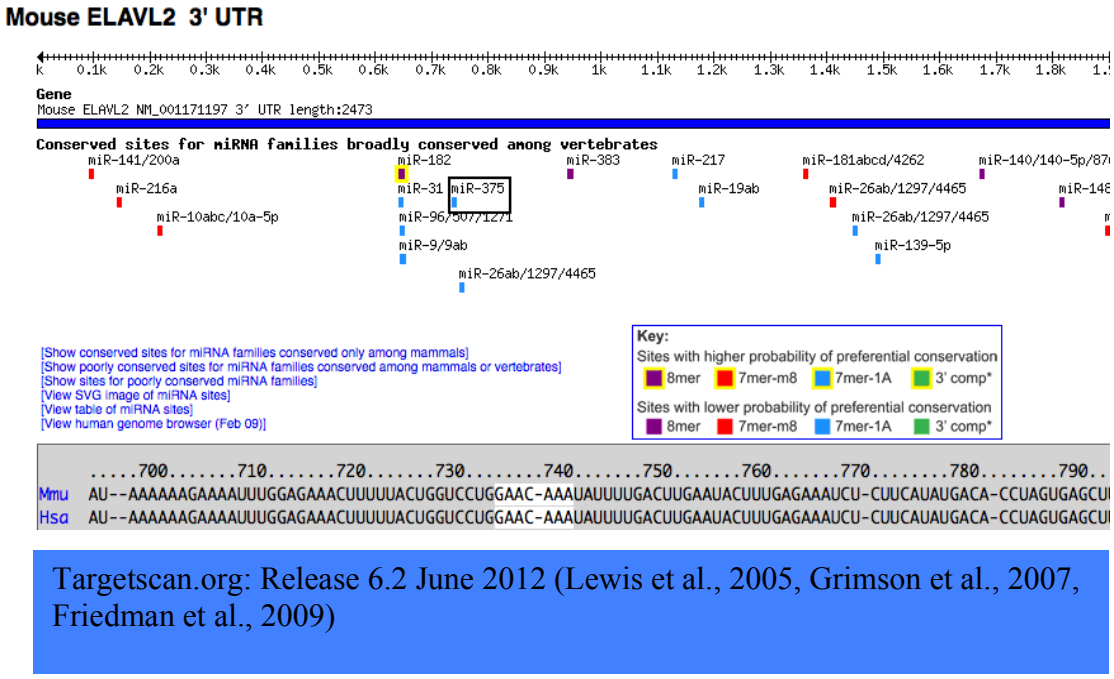


Figure 17. Microarray analysis. (a) Microarray screen of Hu and MSI bound mRNAs. Euclidean cluster analysis with average linkage and rows centering on genes showing ethanol exposed were different from controls. (b) Principal Component Analysis

MiRNA expression and Microarray of polyA transcripts

Targetscan prediction, (Figure 18), (Lewis et al., 2005, Grimson et al., 2007, Friedman et al., 2009), of miRNAs targeting nELAVs revealed three potential candidates for our assessment, mir-375, mir-410 and mir-4661-3p (list of primers: Table 2**)**. Preliminary qPCR analysis of expression of mir-375, mir-410 and mir-4661-3p in in both neural stem cells and early and later differentiated cell populations, showed that

mir-375 was most stably expressed, Figure 19. In addition, several studies have shown that mir-375 is involved in cell growth and proliferation (Liu et al., 2010, Xu et al., 2014). As a result we focused the remainder of our assessment using only mir-375.



Targetscan.org: Release 6.2 June 2012 (Lewis et al., 2005, Grimson et al., 2007, Friedman et al., 2009)

Figure 18. Targetscan prediction of ELAVL2 (HuB) as target of miR-375.

Table 2. List of miRNA primer sequences.

Gene	Forward primer	Reverse primer
miR-375	5'-ACACAGCCAATGGTCCAACC -3'	5'- GGTGAGGAGCAGTTGTTGTTTA-3'
miR-410	5'- AATCCTGCAAGTTGGTTCGGG-3'	5'-GAGTAGTTCACAAACCCATAGCC-3'
miR-4661	5'-GCCTCAGGTGTCAAATGGACC-3'	5'-CCATACCCTAAACTCTGTCCTGT -3'
U6 snRNA	5'- TCCGGTGAGGTCCGTTAGG-3'	5'-CAGACTCATCGGGTCGTCC-3'

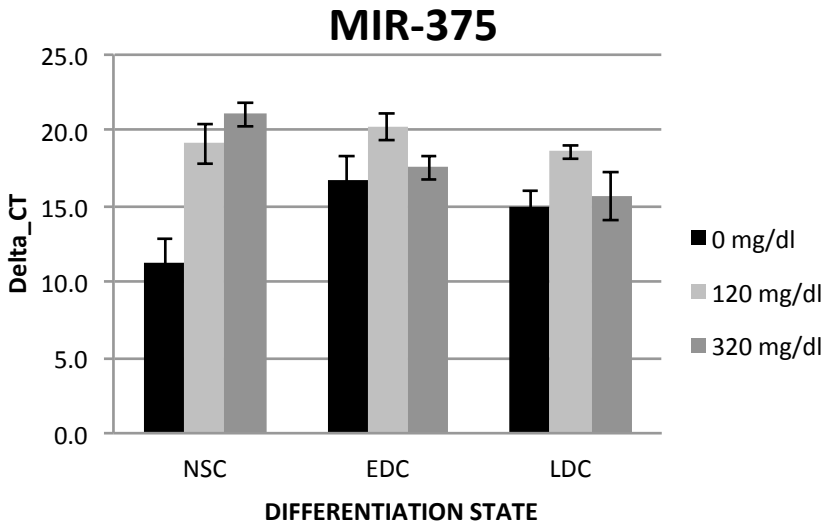
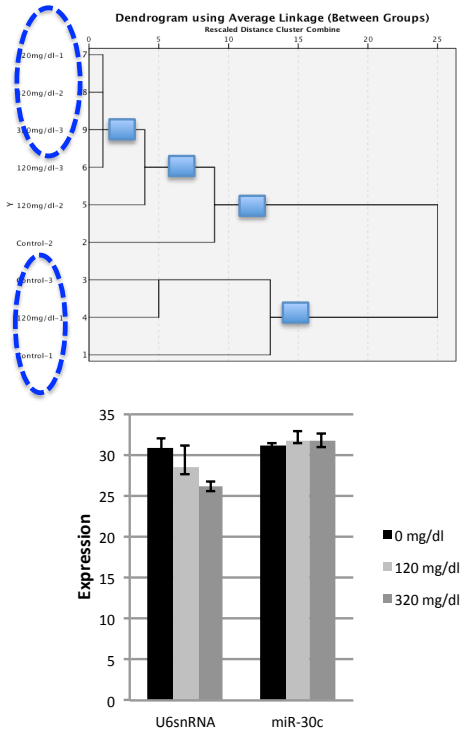


Figure 19. Mir-375 expression in neural stem cells

We saw that there was an interaction effect of ethanol exposure and differentiation state on mir-375 expression $F(4,43) = 4.46$, ($p<0.004$). Ethanol exposure caused overexpression of mir-375 expression in NSCs at 120mg/dl exposure ($p<0.003$) and at 320mg/dl ($p<0.002$) and in LDCs at 120mg/dl ($p<0.008$). Furthermore we wanted to see if there are novel ethanol sensitive miRNAs. To ensure that were not evaluating free circulating miRNAs that would give false amplification results, we carried out a polyA pull-down prior to CDNA synthesis. This ensures that only miRNAs that are associated with mature RNAs will be assessed. Exiqon miRCURY LNA Universal RT miRNA PCR panels (Exiqon miRNA Ready-to-use PCR Human Panels I+II V2.M/R; Exiqon, Vedbaek, Denmark) of prealiquoted LNA PCR primer sets on 384 well plates arrays were used for high throughput miRNA expression analysis. Combined Ct values

for panel 1 and panel 2 were used for data analysis (Figure 20). DIANA-mirPath (Papadopoulos et al., 2009) analyses of relevant signaling pathways are shown in figures 21-23.



5 microRNAs showed significant p-values for involvement axon guidance and neurotrophin signaling pathways

miRNA	Target Gene
106a	Mitogen activated protein kinase 1 **(MAPK1)**
	Synapse associated protein 1 **(SYAP1)**
26a	Neurofilament light chain **(NEFL)**
	Doublecortin domain containing 2 **(DCDC2)**
	eukaryotic Initiation facgor 4G **(eIF4G)**
30c	Neuregulin 3 **(NRG3)**
	Synaptogyrin **(SYNGR3)**
	Semaphorin 3A **(SEMA 3A)**
30b	**SOX 9**
	Neuregulin 3 **(NRG3)**
	Defects in morphology 1 **(DEM1)**
	Semaphorin 3A **(SEMA 3A)**
93	eukaryotic translation initiation factor 5A **(eIF5A)**
	Mitogen activated protein kinase kinase kinase 2 **(MAP3k2)**
	Ephrin receptor 4A and 4B **(EPHA/B)**
	Semaphorin 3A **(SEMA 4A)**

Figure 20. Identification of novel ethanol sensitive microRNAs. PolyA pull down for identification of miRNAs that are ethanol sensitive and are involved in growth and development.

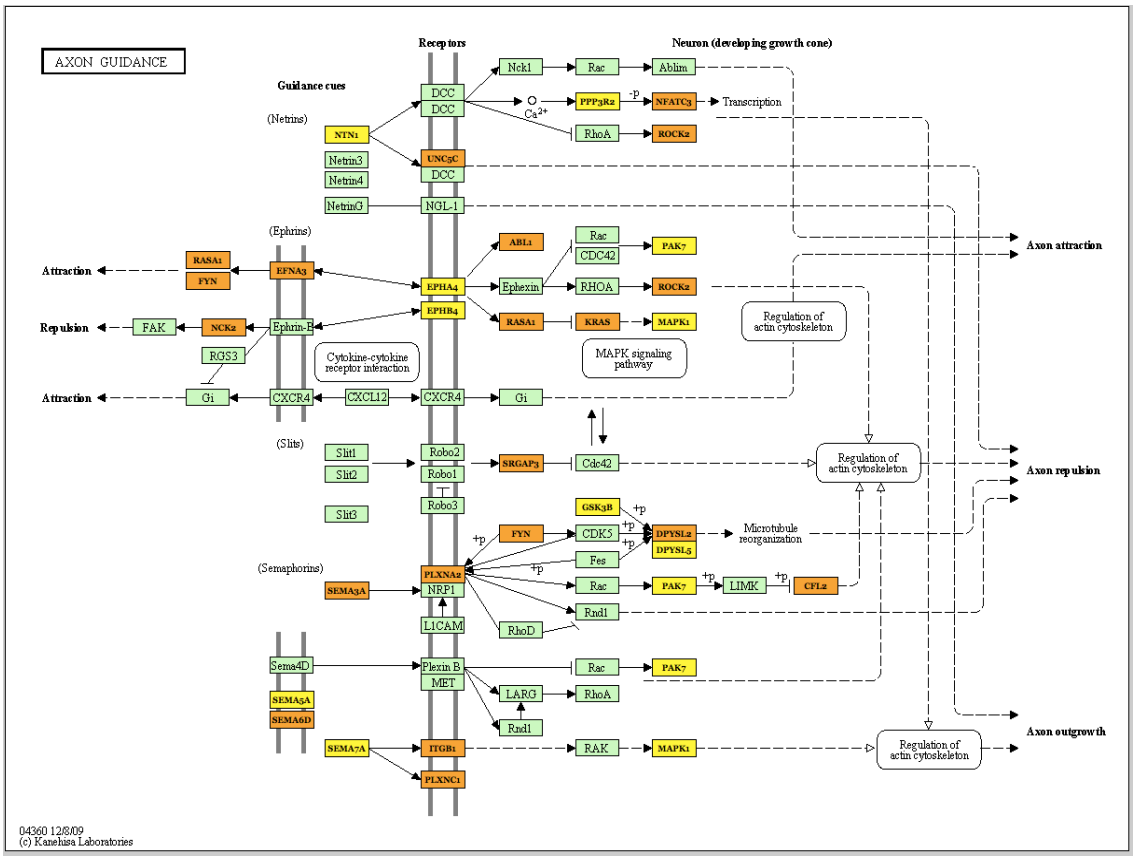


Figure 21. Microarray analysis signaling pathway involved in axon guidance. DIANA-mirPath; (Papadopoulos et al., 2009).

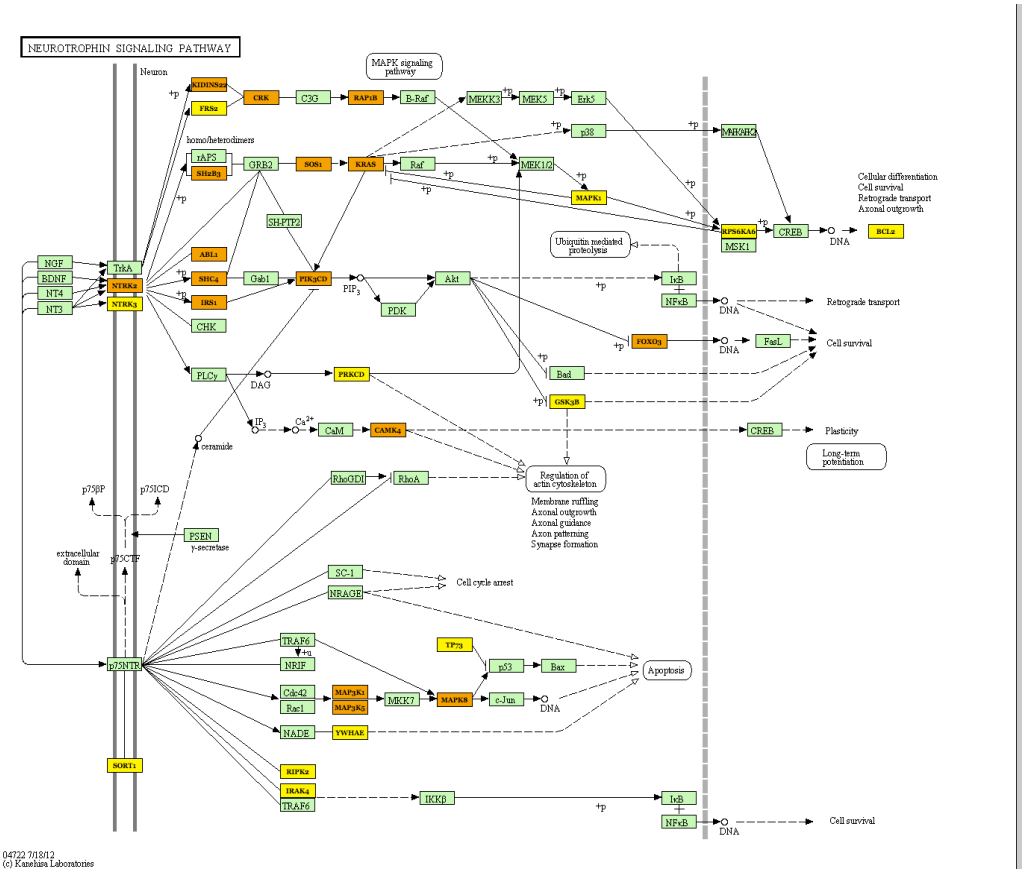


Figure 22. Microarray analysis: signaling pathways involved in Neurotrophin signaling. DIANA-mirPath; (Papadopoulos et al., 2009).

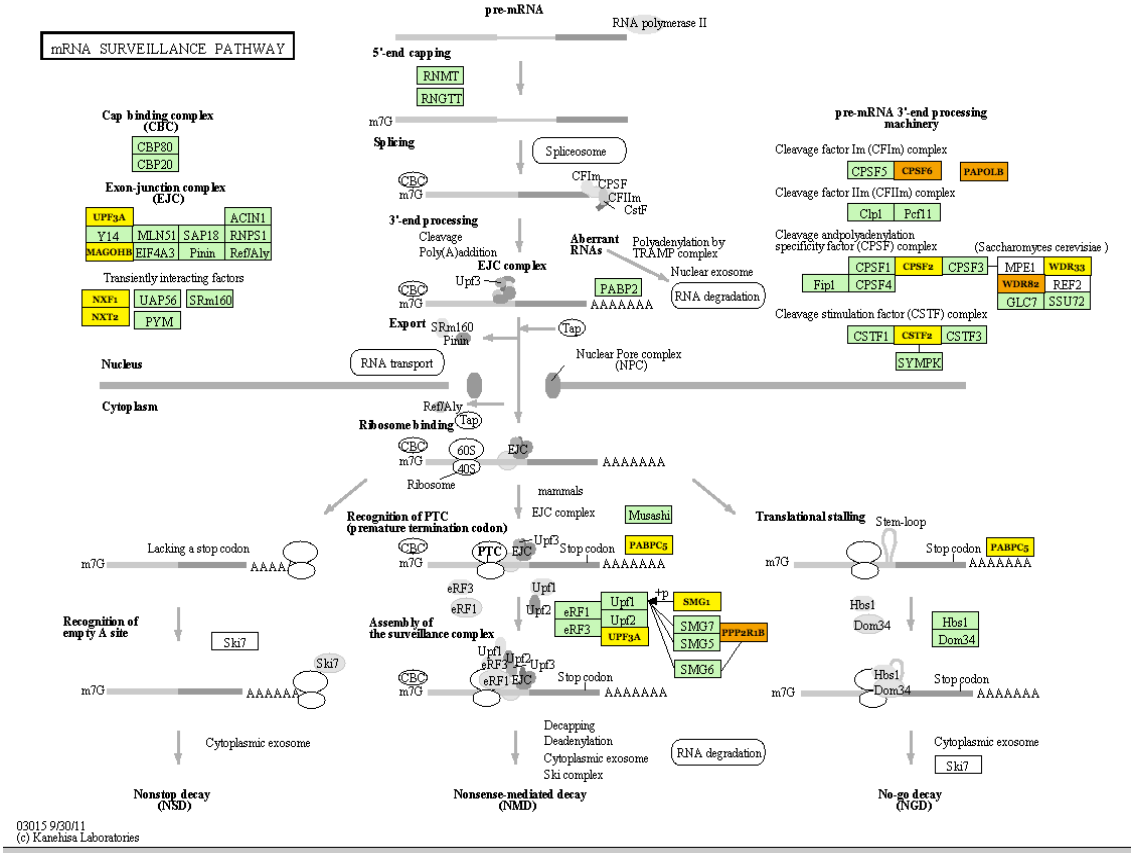


Figure 23. Microarray analysis signaling pathway involved the mRNA surveillance pathway. DIANA-mirPath; (Papadopoulos et al., 2009)

Discussion

Because the relationship between RNA binding proteins and their targets are complex any factor that causes change regardless of the directionality will have an effect on the interaction. The challenge is pinpointing how these changes contribute to modulating downstream processes. An added complexity is that RNA binding protein

genes are also vulnerable targets of post-transcriptional regulators such as microRNAs and the dynamic interaction does influence RNA binding proteins functions. Here we show that of known targets of nELAVs MSI does seem to have more of an association with Hu in our developmental model and is also targeted by ethanol. Our data shows that ethanol targets MSI expression just at the interface where stemness is lost, that is at the neural progenitor stage triggering the assumption that there was a considerable effect on the stem cells that caused reprogramming to be noticed at early differentiation when MSI expression is less significant in maintaining stemness but become important for other molecular events. As we see from the array data Musashi and several other genes interact but there is paucity of information on these interactions and their overall role in neural development.

Impact of Hu proteins on translation

Translation regulation mandates the process of embryonic development and is a highly complex process involving several proteins chief of which are the translation initiation factors. Translation initiation is widely studied and is shown to be the most highly regulated area of translation. It is known that Hu proteins increase stability of several RNAs that are specifically expressed during neural development. Hu proteins have been shown to both enhance and suppress translation (Galban et al., 2008, Kullmann et al., 2002), but are more recognized as enhancers. It is established that Hu proteins bind to and stabilize their target transcripts but their roles go beyond transcript stability. In our crosslinking and immunoprecipitation study to evaluate protein-protein

interactions, we saw that nELAVs specifically interacted with eIF4A but not eIF4G. This is consistent with previous studies showing an interaction with eIF4A but not with eIF4G (Fukao et al., 2009). Our data suggests that Hu the interaction between eIF4A and Hu is for further anchoring of the Hu protein within the transcription machinery to allow for prevention of other RNA binding proteins or miRNAs from interacting with and destabilizing the transcript. This is a conceivable concept, as the transcript has to be efficiently and stably incorporated into the ribonucleoprotein complex for efficient translation. Therefore Hu proteins are not just for translocation and delivery but are essential for translation. Ethanol exposure did not affect the interaction between Hu and eIF4A in our experimental model. MSI also interacted with eIF4A, and because interestingly MSI and Hu share common transcripts this data suggests that Hu, which are known translation enhancers, and MSI, which is known to repress translation, are acting antagonistically in regulating neural development.

Impact of MicroRNAs

Because we saw that ethanol increased mir-375 we went back to our nELAVs protein expression data and there was no observed correlation between the expression of mir-375 and HuB/D protein levels. MiRNAs work by primarily inhibiting translation of their targets by effectively blocking the translation machinery and hence final protein. The expected result would have been that overexpression or decrease of mir-375 would lead to the opposing effect in protein levels. Conceptually we cannot conclude the lack of such association because we did not do a direct expression study (explain better by

saying which method could better answer the question). Since mir-375 expression was induced by ethanol exposure making it an ethanol sensitive miRNA. A uniquely relevant study of mir-375 effect on HuD by Abdelmohsen (Abdelmohsen et al., 2010) showed that overexpression of mir-375 inhibited neurite differentiation (Abdelmohsen et al., 2010). Since we saw that ethanol exposure induced expression of mir-375, would knocking down mir-375 in ethanol pre-exposed cells rescue them from growth and differentiation inhibition seen in our morphology data? Mir-375 inhibitor was introduced in-vitro according to transfection protocol. Sholl analysis data showed that there was a decrease in cell complexity following ethanol exposure. Interestingly for dendritic length we saw that ethanol exposure and non-exposed were not statistically different.

CHAPTER IV

SUMMARY

In summary we observe key neural development factors such as heterogeneous subcellular populations. Suggesting that cohorts of cells act as functional niches that are differentially targeted by ethanol. When one carries out genome-wide studies it is usually assumed that variations taken collectively will explain the complexities involved in the interactome. Here we show that it is important to identify population cohorts as functional units. This is very difficult to undertake but will contribute more to our understanding of complex cell biology and regulation of biological processes. From our global transcription analyses the data identified multiple population subtypes in all three differentiation states, with ethanol differentially regulating transcription levels in some groups but not others. Our morphology data is also consistent with our findings of population heterogeneity where we saw very low MAP2 expression in cells taken from the ventricular zone. Normally MAP2 expression is seen later in development, as it is necessary for neuritogenesis (Huang et al., 2013). This observation we theorized, is an explanation for the spectrum of variations observed in FAS. It is known that development period of exposure explains some of the variations (Maier et al., 1999) in the outcome of FAS.

Future identification of all unique factors within these subpopulations of developing neurons will significantly assist in development of targeted therapeutics in mitigating the effects of fetal alcohol exposure.

We also found that ethanol is specifically targeting expression levels of RNA binding proteins that possesses RRMs that bind to AREs. From our evaluations Hu and MSI were significantly affected by ethanol compared to GAP-43, eIF4A and eIF4G. Further structural analyses and binding site studies between Hu, Musashi and ethanol would shed light on how the structure of RNA binding proteins possessing RRMs that binds to AREs are targeted by alcohol. From our knockdown evaluation of HuB and HuD it is seems that ethanol's effect on neuron morphology is through a direct interaction with Hu.

RNA binding proteins act as central nodes post-transcriptionally therefore factors that changes Hu expression during critical development time points will be deleterious and may account for some structural and neurological deficits seen in FAS. It has been shown that Hu proteins increases amount of and stability of GAP-43 which is necessary for neuritogenesis. We saw that even though ethanol exposure did not significantly alter GAP-43 expression, RIP array showed that several other genes are interacting with Hu and are important in neuron growth and development whose expressions are changed following exposure. In addition ethanol sensitivity is not necessarily dose dependent as we observed in several instances where exposure at 120mg/dl seemed to have a more significant aberration of morphology and expression levels from the control compared to exposure at 320mg/dl.

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APPENDIX

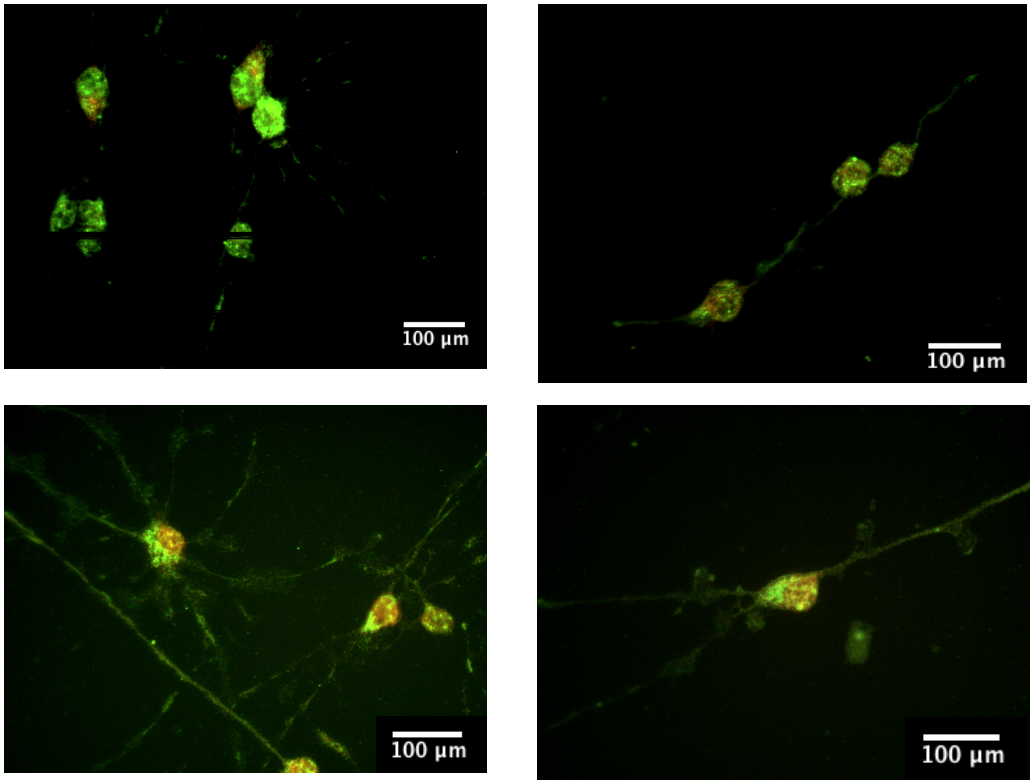

Figure 24. Representative immunofluorescence images showing cellular localization of Hu and GAP-43. HuB/D: red, GAP-43-green.

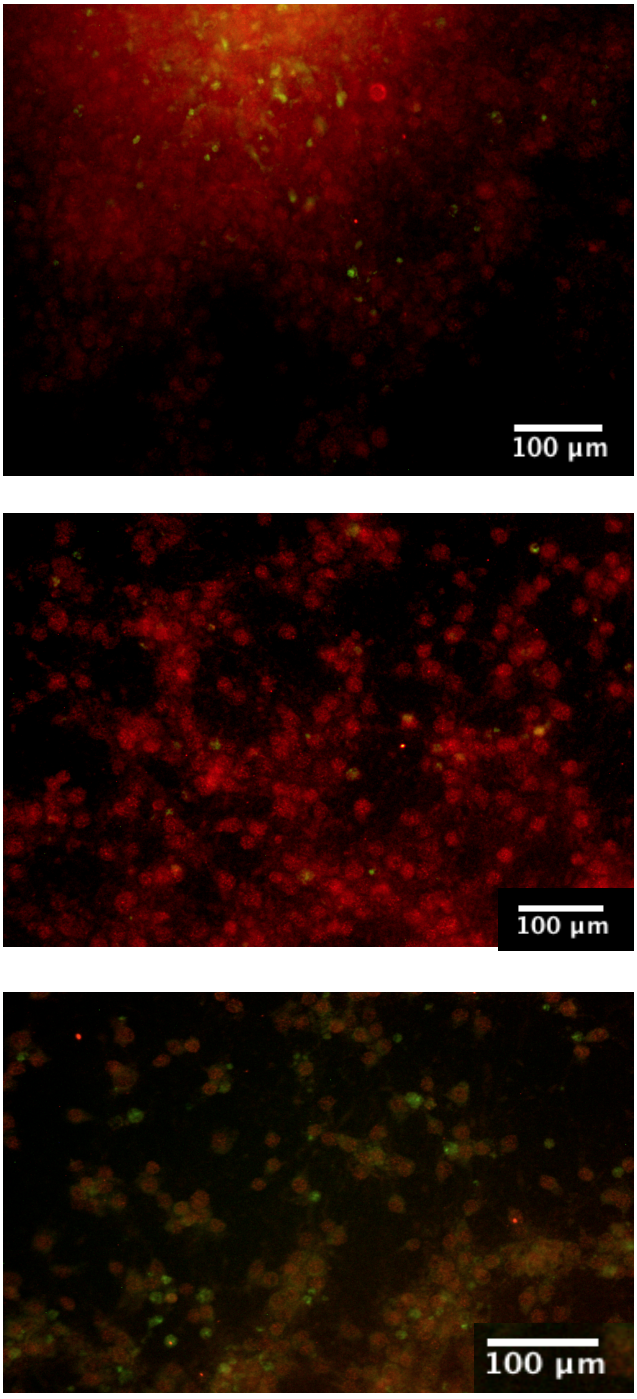

Figure 25. Representative immunofluorescence images showing expression of Hu and Musashi in NSC. HuB/D: red, Musashi-green