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Context Fear Memory Formation Is Regulated By Hippocampal Lncrna-Mediated Histone Methylation Changes

Anderson Alan Butler
University of Alabama at Birmingham

CONTEXT FEAR MEMORY FORMATION IS REGULATED BY HIPPOCAMPAL LNCRNA-MEDIATED HISTONE METHYLATION CHANGES

by

ANDERSON ALAN BUTLER II

FARAH D LUBIN, CHAIR
LORI MACMAHON
KAZUTOSHI NAKAZAWA
NICOLE RIDDLE
LIOU SUN

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CONTEXT FEAR MEMORY FORMATION IS REGULATED BY HIPPOCAMPAL LNCRNA-MEDIATED HISTONE METHYLATION CHANGES

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CELL, MOLECULAR, AND DEVELOPMENTAL BIOLOGY

ABSTRACT

The post-translational modification of histones regulates gene expression and is critical for the formation and maintenance of hippocampus-dependent long-term memories. Changes in gene-specific expression of various epigenetic marks during the aging process are sufficiently consistent as to be used as an aging landmark or epigenetic clock in both humans and other species; however, the molecular mechanisms which govern the application of these marks aging are poorly explored. Recently, long noncoding RNAs (lncRNAs) have been implicated as regulators of histone methyltransferases and other chromatin-modifying enzymes (CMEs). Despite the relevance of such mechanisms to both aging and memory formation, the behavioral relevance of lncRNA-mediated histone methylation remains obscure in the hippocampus. In my dissertation research, I have examined the aging hippocampus for evidence of disrupted epigenetic marks that might explain the commonly observed age-related impairment in hippocampus-dependent memory formation. I observed dysregulation of the histone 3 lysine 9 dimethylation mark (H3K9me2) and uncovered a role for the aging-regulated lncRNA *Neat1* in regulating H3K9me2 in the context of hippocampus-dependent long-term memory formation. Using an informatics approach, I have identified neuronal genes under the regulatory control of the age-related lncRNA *Neat1*, including the immediate early gene (IEG) c-*Fos*. Suppression of the lncRNA *Neat1* in cultured neuronal cells revealed widespread changes in gene expression as well as perturbations of histone 3 lysine 9 dimethylation (H3K9me2), a re-

pressive histone modification that I observed to be dysregulated in the aging hippocampus. Focusing on the epigenetic landscape proximal to *c-Fos*, I observed site-specific H3K9me2 changes corresponding with observed changes in *c-Fos* transcript abundance. In addition to epigenetic regulation of the memory-permissive gene *c-Fos*, we observed that *Neat1* expression bidirectionally controls rodent performance in contextual fear conditioning, where mimicking age-related increases in hippocampal *Neat1* expression was sufficient to impair memory, while suppression of *Neat1* in both young and old mice was sufficient to improve memory. These results imply that the aging-induced lncRNA Neat1 is a potent molecular brake on hippocampus-dependent long-term memory formation, and suggest that epigenetic dysregulation in the hippocampus may be driven by chronic alterations in the transcription of noncoding RNAs.

Keywords: Chromatin, Epigenetics, aging, Neat1, CRISPRa, c-Fos

DEDICATION

To my mother and father, it is impossible to thank you adequately for everything you've done. I could not have asked for better parents.

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INTRODUCTION

Epigenetic Regulation of Gene Expression

Epigenetic mechanisms have emerged as critical components of many cellular processes. As related to the nervous system, the term epigenetics refers to the study of long-term, potentially heritable changes in gene expression patterns that do not result directly from mutations within DNA. Epigenetic modifications, including modifications to both DNA residues themselves as well as to other components of chromatin such as histone proteins and chromatin associated RNAs, play a principal role in nuclear organization and the transcriptional activity of genes.

The application, erasure, and interpretation of epigenetic marks are carried out by a complex and frequently interconnected network of regulatory enzymes, with the stability of any particular mark depending both on the metabolic activity of chromatin modifying enzymes (CMEs), the existence of self-perpetuating feedback loops, and the rate of turnover of the target molecules themselves (Chory et al., 2019). For many marks, these factors result in a long-lived, if not necessarily permanent change to the state of the target gene. The semi-permanent nature of many epigenetic modifications allows for multitudes of genetically identical cells to differentiate into phenotypically distinct lineages, each expressing unique genes and performing unique functions, and current evidence indicates that these same modifications are crucial for maintaining cellular

identities, with disruption of epigenetic processes resulting in phenotypic plasticity and potential cellular transformation (Wutz, 2013).

DNA Modifications

In addition to the primary residues of nucleic acids, more than 40 naturally occurring modified residues have been observed on DNA (Sood et al., 2016). While these modifications have the potential to greatly enhance the informational content of the genome, few such modifications have been extensively studied and, to date, only three of these have demonstrated functional significance in the adult brain: 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC), and recently N(6)-methyladenine.

The methylation of DNA cytosine residues on the 5 position of the pyramidal ring is catalyzed by a number of DNA methyltransferase enzymes (DNMTs) and occurs primarily at cytosine residues located immediately 5' to guanine residues (CpG). Due to the location on the 5' As a result of Watson-crick base pairing, CpGs occur symmetrically on both strands of the DNA, and these CpG dyads may be unmethylated, hemimethylated on one or the other strand, or fully methylated on both CpGs of the dyad. Of the ~28 million CpG sites in the human genome, 60–80% are methylated as 5mC (Smith and Meissner, 2013). CpG sites within active gene promoters are generally unmethylated; however, methylated CpG sites tend to correspond to transcriptionally silent gene promoters. Recent technological advancements have provided the tools necessary to demonstrate the sufficiency of promoter 5mC methylation to repress gene expression (Liu et al., 2016); however, the mechanism of 5mC transcriptional repression is complex and relies upon interactions with transcription factors or other epigenetic

reader proteins, rather than by directly interfering with Watson-Crick base pairing or inhibiting Pol II transcription (Xu et al., 2015). Transcription factor binding may either be inhibited (e.g. human papillomavirus E2) or facilitated (e.g. Kaiso) at sites of 5mC methylation (Thain et al., 1996; Yin et al., 2017). DNA 5mC methylation is selectively bound by a number of well characterized epigenetic reader proteins containing a methyl binding domain (MBD) including the methyl CpG binding protein 2 (Mecp2), which then mediates transcriptional repression via induction of repressive histone methylation (Fuks et al., 2003). Thus, DNA 5mC methylation functions as a potent transcription repression mechanism both by direct blocking of transcription factors as well as via indirect mechanisms.

The oxidation of 5mC to 5hmC by Tet family proteins was long thought to be simply the initial reaction in Tet-mediated DNA demethylation, a step on the way towards further oxidation into 5-formylcytosine (5fC), 5-carboxylcytosine (5caC), and demethylation via the (Ito et al 2011); however, recent profiling studies in cells and rodents have indicated that 5hmC is predominantly stable (Bachman et al., 2014). This finding, in addition to recent observations that localization of Tet enzymes is sufficient to ablate local 5mC and relieve 5mC-mediated transcriptional repression (Liu et al., 2016), suggests a role for 5hmC as an epigenetic mark which both alleviates 5mC repression in a semi-permanent fashion. Large quantities of 5hmC expression are observed in several cell types and organs (Sun et al., 2014), with the highest levels of levels of mammalian 5hmC expression are found in the central nervous system, with approximately 5hmC methylation approximately 40% as abundant as 5mC in Purkinje neurons (Kriaucionis and Heintz, 2009). While still relatively unexplored compared to

5mC, these findings suggest that 5hmC is stable epigenetic mark with significant relevance for neuronal and cognitive function.

Methylation of adenine residues, while a predominant epigenetic mark in many prokaryotes (Greer et al., 2015), has only recently been characterized in mammals. Nonetheless, the enzymes which catalyzes the methylation of adenine bases are evolutionarily conserved, and recent technological advances have established that high levels of N6-methyladenine occur on DNA in mammalian embryonic stem cells (Wu et al., 2016) and several mammalian tissues, including cortical regions of the brain (Xiang Li et al., 2019). Similar to m6A in *Caenorhabditis elegans* and *Drosophila melanogaster*, m6A methylation in the mammals seems to be largely permissive to transcription. While the functional relevance of m6A is largely unknown, recent findings suggest that CNS m6A is a critical regulator of neuronal transcription and cognitive processes (Xiang Li et al., 2019).

Several rarer epigenetic modifications to DNA are likely have equally important, if perhaps less permanent effects on gene expression. Recent experiments have demonstrated that the poorly characterized marks 5fC and 5caC, results of continued TET-mediated oxidation of 5hmC, may disrupt Watson-Crick base pairing and cause local disruption in chromatin structure (Szulik et al., 2015). Both 5fC and 5caC are targets of thymine DNA glycosylase (TDG) and subject to glycosylase-mediated base excision repair (Szulik et al., 2015); however, while, one might anticipate a short life-span due to base excision repair, recent work has demonstrated that 5fC may be stable within mammalian cells (Bachman et al., 2015). Much work remains to be done to

achieve an understanding of the impact DNA modifications may have on gene expression.

Histone Modifications

In contrast to epigenetic modifications on DNA itself, post-translational modifications (PTMs) on histones are inherently more transient. Histones may be either displaced (Kujirai et al., 2018; Workman, 2006) and/or functionally modified (Yang et al., 2016) by the processes of transcription. Nonetheless, such displacements are often transient, and mechanisms of self-perpetuation such as bookmarking (Deluz et al., 2016; Festuccia et al., 2016) allow the persistence of histone modifications across dramatic perturbations to chromatin structure. In contrast to DNA modifications, a relatively large number of histone PTMs have been characterized, although much greater complexity is possible given the nature of proteins and the large number of potential post-translational modifications. Well-studied marks include histone acetylation, phosphorylation, methylation, among others; however, the function of each mark may be distinct depending on the location of the mark within the histone sequence and the locus at which the target nucleosome resides.

A number of single histone modifications have been functionally characterized, and correlate strongly with transcriptional regulation. Among marks which correspond to active transcription are H3K4me3, H3K36me3, and all histone acetylation, with H3K4me3 and H3K36me3 corresponding to promoters and gene bodies actively transcribed by Pol II, respectively. In contrast, H3K9me3, and H3K27me3 are distributed in transcriptionally repressed regions. Stretches of the genome which have epigenetic

marks rendering them transcriptionally active are often referred to as euchromatin, while those regions which possess repressive marks are (relatively) silent are considered to be heterochromatic. Some marks are mutually exclusive, for example, histone 3 may monomethylated, dimethylated, trimethylated, or acetylated at lysine 9 (H3K9me1, H3K9me2, H3K9me3, and H3K9ac), but the marks inherently cannot coexist simultaneously on the same lysine residue. Still, this might not preclude simultaneous coexistence of two different marks on two H3 proteins within the same nucleosome), and moreover, there are often incidences of crosstalk between different histone marks or even DNA modifications, and even extremely similar marks may precipitate hugely different effects. For example, while arginine dimethylation at H3R8 is transcriptionally permissive, lysine dimethylation at the adjacent amino acid (H3K9) is transcriptionally repressive. Moreover, while lysine dimethylation and trimethylation at H3K9 are repressive, monomethylation on this residue is transcriptionally permissive. Thus, the overall effect of histone methylation state may rely on the placement of a single methyl group at one of two adjacent amino acid residues, and such a choice may shift the epigenetic state from transcriptionally active (H3K9me1) to repressive (H3K9me2/3).

A number of cross-talk mechanisms exist between various epigenetic marks, while DNA methylation commonly facilitates the application of repressive H3K9 methylation, a number of DNA methylation-associated proteins also interfere with H3K27me3 application, resulting in a low degree of overlap between these marks. Thus, the combinatorial nature of histone PTMs and other epigenetic marks allows a large amount of information to be encoded per nucleosome. A number of ongoing large-scale initiatives are underway to gather sufficient data to unravel some aspects of combinatorial

histone PTMs and other epigenetic mechanisms in model organisms (modENCODE Consortium et al., 2010); however, the immense scope of the problem makes exhaustive solutions unlikely with current technical approaches.

Of particular interest to this work in the transcriptionally repressive mark H3K9me2. While the presence of H3K9me2 in gene promoters tends to correspond with gene repression (Barski et al., 2007), this correlation with transcriptional regulation is somewhat weak. Writer enzymes of H3K9me2 include several methyltransferases, including the , Y, and Z. These methyltransferases are often capable of mediating mono-, di-, and trimethylation *in vitro*. While capable of mediating H3K9me3 *in vitro*, the heterodimeric G9a/GLP complex preferentially meditates dimethylation, and is believed to be the primary *in vivo* source of H3K9me2 (Tachibana et al., 2005). Interestingly, recent evidence suggests that H3K9me2 might mediate recruitment of nuclear argonaut proteins and co-transcriptional silencing via RNAi, and that this process is integral for mediating further transcriptional silencing via recruitment of HP1 and H3K9 tri-methyltransferases (Jih et al., 2017). As neither G9a nor GLP possess DNA binding domains, recruitment of the G9a/GLP complex is dependent upon interactions cofactors including both proteins and RNAs. A number of protein transcription factors recruit G9a, the G9a/GLP complex has been observed to bind to the lncRNAs AIRN (Nagano et al., 2008) and *NEAT1* (Li and Cheng, 2018), among others. Interestingly, direct interaction with the DNA 5mC methyltransferase Dnmt1 has been observed to direct H3K9me2 during development by recruiting G9a (as well as SUV39H1) to DNA (Estève et al., 2006), and in turn, G9a has been observed to be critical for *de novo* DNA methylation (Epsztejn-Litman et al., 2008).

In conclusion, understanding gene expression regulation by epigenetic mechanisms remains a challenge, in part to the inherently cell-type and temporally specific nature of epigenetic changes, and in part due to the complex nature of signaling through epigenetic mechanisms.

Neurocognitive Epigenetics

Given the extensive role of the epigenome in the control of development and lineage commitment, it is not surprising that epigenetic mechanisms have been shown to play critical roles in the development of the nervous system (Juliandi et al., 2010; Maze et al., 2013; Rudenko and Tsai, 2014; Yu et al., 2010). Yet, in the past decade it has become increasingly clear that the mechanisms of epigenetic regulation continue to play a critical role in controlling neuronal transcription into old age (Morse et al., 2015) and despite the postmitotic state of most neurons. This is especially evident in the context of transcription-dependent cognitive processes such as long-term memory formation, where global manipulation of varied epigenetic processes can either impair or improve performance in memory tasks (Table 1).

Within the brain, one particularly well-studied structure that is critically involved in long-term spatial and emotional memory tasks is the hippocampus. The hippocampus is a distinctive structure with a high degree of spatial organization, consisting of several unidirectional loops which give rise to hippocampal network function. The hippocampus may be divided into visually identifiable subfields, including the cornu ammonis areas (CA1-CA4), the dentate gyrus, and the subiculum. Information flows through the hippocampal trisynaptic circuit in a largely linear fashion, with the dentate gyrus

receiving input from layer 2 of the entorhinal cortex via the perforant pathway, then relaying information to the CA3 pyramidal layer. CA3 pyramidal cells then project onto the pyramidal layer of CA1, the highly studied Schaffer collateral synapse. CA1 cells then form the primary output of the hippocampus, sending axons to the subiculum and layer 5 of the EC, completing the trisynaptic circuit. While the trisynaptic circuit may be considered the primary pathway of information flow through the hippocampus, additional well-known pathways contribute to the complexity of the circuitry. For example, layer 3 of the entorhinal cortex also projects directly to area CA1 in a connection known as the temporoammonic circuit (Figure 1). Moreover, a large number of recurrent connections and long-range projections to and from distinct cell populations are yet being discovered, along with distinctive behavioral and information processing roles for particular subregions, for example, CA2 is critical for social memory (Barker et al., 2017), and sends long range axonal projections to the supramammilary nucleus of the hypothalamus, a region critical for arousal (Cui et al., 2013; Dudek et al., 2016).

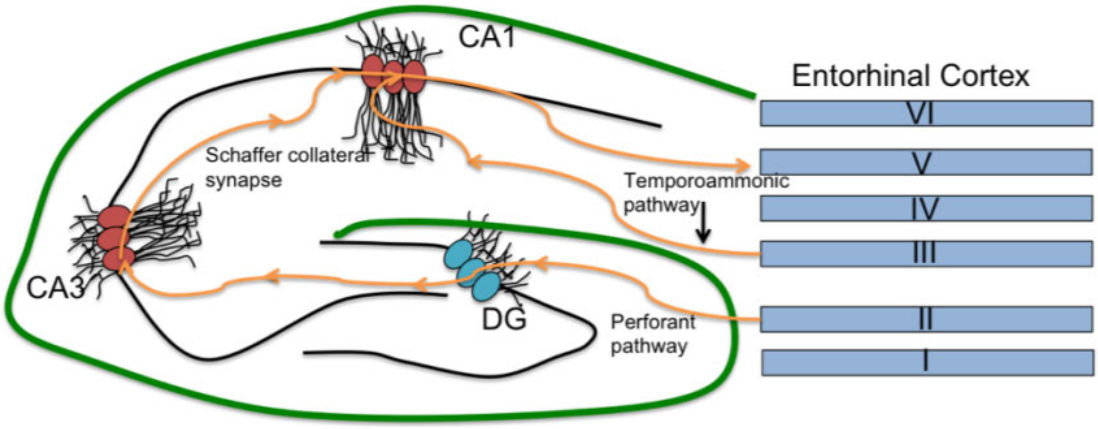

Figure 1. Projections of the trisynaptic circuit and temporoammonic pathway through the hippocampus to and from the entorhinal cortex (Parrish 2015).

While the importance of epigenetic regulation for cognitive function has been well established, most studies to date have assayed either global changes in the levels of epigenetic marks within whole brain regions such as the hippocampus. As a result of these studies, we have recognized that the epigenetic landscape differs across brain regions (Rizzardi et al., 2019) and that dysregulation of CMEs can have profound effects on brain function (Jarome and Lubin, 2014). Indeed, current evidence from genetic association studies suggests that dysfunction of epigenetic processes play a role in several cognitive disorders, including schizophrenia, bipolar disorder, and major depressive

Structure	Task	Histone/DNA modification	Gene(s)	Manipulation	Time of Injection	Effect on Memory	Citations
Hippocampus (CA1)	CFC	↑ H3 phosphorylation	–	–	–	–	Chwang, O'Riordan, Levenson, and Sweatt (2006)
Hippocampus (CA1)	CFC	↑ H3 acetylation	–	HDAC inhibitor	Pre-Training	Enhanced	Levenson et al. (2004)
Hippocampus (CA1)	CFC	↑ H3K4 trimethylation	↑ zif268, BDNF	MLL Knockout	Pre-Training	Impaired	Gupta et al. (2010)
Hippocampus (CA1)	CFC	↑ H3K9 dimethylation	↓ DNMT3a, cFOS, G9a	G9a inhibitor	Pre-Training	Impaired	Gupta-Agarwal et al. (2012)
Hippocampus	CFC	↑ H3 acetylation	↑ Homer1	HDAC inhibitor	Post-Training	Enhanced	Mahan et al. (2012)
Hippocampus (CA1)	CFC	↓ DNA methylation	↑ ↓ BDNF	DNMT inhibitor	Pre-Training	Impaired	Lubin et al. (2008)
Hippocampus (CA1)	CFC	↓ DNA methylation	↑ Reelin ↓ PP1	DNMT inhibitor	Post-Training	Impaired	Miller and Sweatt (2007)
Hippocampus (CA1)	CFC	↓ DNA demethylation	↓ Zif268, NPAS4, cFOS	Tet1 knockdown	Pre-Training	Impaired	Kaas et al. (2013)
Entorhinal cortex	CFC	↑ H3K9 dimethylation	↓ G9a	G9a inhibitor	Pre-Training	Enhanced	Gupta-Agarwal et al. (2012)
Amygdala (LA)	DFC	↑ H3 acetylation	–	HDAC inhibitor	Post-Training	Enhanced	Monsey et al. (2011)
Amygdala (LA)	DFC	DNA methylation	–	DNMT inhibitor	Post-Training	Impaired	Monsey et al. (2011)
Amygdala	DFC	H3K9 methylation	↑ Homer1a	HDAC inhibitor	Post-Training	No effect	Mahan et al. (2012)
Amygdala (LA)	DFC	Histone acetylation	–	HAT inhibitor	Post-Training	Impaired	Maddox, Watts, Doyere, et al. (2013), Maddox, Watts and Schafe (2013)
Amygdala (LA)	DFC	H3K9 dimethylation	↓ G9a	G9a inhibitor	Pre-Training	Impaired	Gupta-Agarwal et al. (2014)
mPFC (ACC, PLC)	CFC	↑ DNA methylation	↓ CaN	DNMT inhibitor	Post-Training (29 days)	Impaired	Miller et al. (2010)
mPFC	TFC	Histone acetylation	–	HDAC/HAT inhibitor	Post-Training	Enhanced/ Impaired	Sui et al. (2012)
mPFC	TFC	DNA 5mC	–	DNMT inhibitor	Post-Training	Impaired	Sui et al. (2012)
ILPFC	EL	↑ DNA m6A	↑ BDNF	–	–	Enhanced	Li et al. (2019)

Table 1. Epigenetic mechanisms of memory consolidation and extinction (Adapted from (Jarome and Lubin, 2014))

disorder (Network and Pathway Analysis Subgroup of Psychiatric Genomics Consortium, 2015). Moreover, recent studies from our lab indicate a role for specific epigenetic mechanisms, including H3K9me2, in cognitive decline with normal aging (Morse et al., 2015).

Studies such as those described above have advanced our knowledge of epigenetic regulation in specific brain regions and given rise to the nascent field of neuroepigenetics. While many marks have been implicated in neuronal function, as yet, the questions of which cofactors direct CMEs to their neuronal target gene regions remains largely unsolved, especially in the context of activity-dependent changes. Yet, we are aware that site-specific changes may follow from global neuroepigenetic changes, and that precise, often exon-specific changes in the epigenetic landscape are reproducibly observed during processes such as memory formation.

The precise abundance of euchromatin and heterochromatin within the genome vary by species; however, studies of human cells indicate that >85% of the genome is actively transcribed (Hangauer et al., 2013; International Human Genome Sequencing Consortium, 2004), with a large number of transcripts consisting of noncoding RNAs (Derrien et al., 2012; Harrow et al., 2012). While lncRNAs in general exhibit a high degree of tissue-specificity, the mammalian hippocampus seems to be a hotbed of ncRNA expression, with thousands of detectable ncRNAs expressed at baseline (Kadakkuzha et al., 2015). As lncRNAs have been observed to mediate epigenetic regulation in other tissues and other cognitive processes (Spadaro et al., 2015), for my thesis work, I chose to focus on lncRNA-mediated epigenetic signaling as a promising

mechanism for explaining targeted epigenetic changes in the context of memory formation.

Neuroepigenetic Changes in Aging

In spite of numerous feedback loops that seem to prevent runaway signaling via epigenetic mechanisms in young animals (Qian et al., 2017), a number of epigenetic marks tend to precipitate across the lifespan. Age-related epigenetic changes may be either global, site-specific, or both, and may vary by tissue or cell type.

A number of studies have generated so-called "epigenetic clocks" from epigenomics data using machine learning methods, groups of genes whose epigenetic states may be strongly predictive of either health- or lifespan, as well as cognitive fitness, depending on the particular set of genes considered (Horvath and Raj, 2018; Levine et al., 2015; Marioni et al., 2015; Quach et al., 2017). While such clocks are generally reliable and precise in their predictions, predictive clocks may be assembled *de novo* from the epigenetic states of relatively few genes, indicating that aging-related epigenetic changes are widespread – a general phenomenon observed throughout the genome, rather than a unique property of a few highly predictive genes. Likewise, while many epigenetic clocks have focused primarily upon 5mC methylation, numerous studies now show that predictive clocks may be assembled using other epigenetic marks, including 5hmC levels (Field et al., 2018).

In the brain, a number of studies have attempted to generate epigenetic clocks tuned to the cognitive health span, rather than other phenotypic markers (Levine et al., 2015). The success of these studies indicates that, like the more general health span, a

large number of epigenetic changes in the brain are highly correlated with aging. While it is unlikely that all such observed correlations play a causal role with regard to cognitive decline, the predictive capacity of epigenetic changes suggests involvement at some level.

As epigenetic mechanisms play critical roles in cognition as described previously, it is logical to hypothesize that age-related epigenetic changes might mediate cognitive dysfunction in the aging brain. Within the rodent hippocampus, studies from our lab and others have characterized large-scale changes in gene expression as well as epigenetic changes with aging in the hippocampus, including increased H3K9 methylation (Morse et al., 2015; Snigdha et al., 2016). While our own experiments have indicated upregulation of H3K9me2 in the hippocampus (Morse et al., 2015), there are opposing studies which report depletion of hippocampal H3K9 methylation (Elsner et al., 2013). These insights from our lab and others agree that this particular aspect of epigenetic regulation is perturbed within the aging brain but differ as to the precise nature of these changes. As these experiments were carried out using differing rodent strains and experimental procedures, it remains unclear at this point whether this heterogeneity is due to differences in the epigenome across rodent strains, tissue subregions, or other variables.

In accordance with the view that age-related epigenetic changes might mediate cognitive impairments, therapies designed to alleviate age-related epigenetic changes at a global scale are sometimes sufficient to partially restore normal neuronal and learning function in the aged brain (Fischer, 2014; Singewald et al., 2017; Stilling and Fischer, 2011), although some caution must be taken in the interpretation of such results, as little data exists on the long-term effects of such treatment, or the effects of such treatments on

recall of existing memories. However, the ultimate causes which steer epigenetic mechanism into dysfunction in age-related cognitive impairments remain a mystery.

Together, the sufficiency of therapeutic targeting of epigenetic mechanisms to improve cognition, along with the capability of epigenetic clocks to robustly predict cognitive age imply the existence of a programmatic function of regulatory processes across the lifespan to epigenetically modify target genes within brain regions important for cognitive function. And importantly, these observations suggest that such signaling might be delayed or halted through intervention to extend the cognitive healthspan; however, the identity of such aging-associated master regulators of epigenetic mechanisms has yet to be demonstrated.

Noncoding RNAs

Although poorly described relative to protein-coding genes, noncoding RNAs (ncRNAs) nonetheless comprise a major portion of the mammalian transcriptome (Derrien et al., 2012; Harrow et al., 2012). While competing estimates as to the abundance of ncRNAs persist, the consensus of the field is that ncRNAs are quite plentiful, particularly in the brain (Harrow et al., 2012, 2006; Lunnon et al., 2014; Mercer et al., 2008). A number of regulatory functions have been attributed to species of ncRNAs, which will be described in more detail below. However, it bears mentioning that, to date, only a small fraction of the total ncRNAs genes have been examined with regards to biological function. Moreover, the regulatory functions described below are not limited inherently to noncoding RNAs. Although to date, most of regulatory RNA functions have been examined solely in ncRNAs due to the inherent experimental

confounds of mRNA's protein-coding potential, it is likely that many mRNAs also behave similarly to ncRNAs in a biological context, and have both translation-independent functions and protein-coding potential. Lending some credence to this hypothesis, translation-independent functions have been identified in heavily-studied pathways such as p53 signaling (Candeias et al., 2008; Gajjar et al., 2012; Naski et al., 2009). Moreover, some annotated ncRNA genes have been discovered to possess protein coding potential, further complicating precise annotation. Nonetheless, both coding and noncoding transcriptomes hold many mysteries yet to be explored, particularly in the context of the brain.

It is a common practice in the field to classify ncRNAs as either long or short, with the division being set at a length of 200 nucleotides. While arbitrary, this division allows for the useful separation of the many characterized classes of small functional RNAs, including miRNAs (micro-RNAs), piRNAs (PIWI-interacting RNAs), siRNAs (small interfering RNA), snoRNAs (small nucleolar RNAs), and tRNAs (transfer RNAs) from the majority of the less well characterized lncRNAs (long non-coding RNAs) (Mattick and Rinn, 2015). Among other roles, both short and long ncRNAs have been shown to regulate the neuronal epigenome (Cam, 2010; Schaukowitch and Kim, 2014) -a finding with exciting implications for the cognitive sciences. In the next sections, I will highlight key findings that are beginning to elucidate a role for ncRNAs in the control of neuronal and cognitive function via epigenetic mechanisms, as well as cognitive disorders.

Short Noncoding RNAs

Canonical mechanisms of miRNAs. When Lee and colleagues (Lee et al., 1993) first showed that the small (22-nucleotide), ncRNA dubbed *lin-4* represses the translation of several developmental genes in *C. elegans*, the scientific community failed to recognize this discovery as anything more than a curious feature of the invertebrate model's genetics. As a consequence, few of these ncRNAs were discovered or characterized until the discovery of RNA interference (RNAi), a post-transcriptional regulatory process which is outlined below.

Beginning with small, regulatory RNAs that were shown to be conserved in plants and animals in the early 2000s (Lagos-Quintana et al., 2001) and continuing to the present, the known roles of miRNAs have expanded to encompass the view that as many as 60% of coding transcripts are regulated by miRNA activity (Lagos-Quintana et al., 2001; Lewis et al., 2005). Since the days of Ambrose and Lee, tens of thousands of miRNAs have been annotated (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006; Kozomara and Griffiths-Jones, 2014), and miRNAs have been shown to regulate such diverse biological processes as developmental pattern formation (Lagos-Quintana et al., 2001), pluripotency (Leonardo et al., 2012), cell signaling (Ichimura et al., 2011), cardiovascular disease (Ono et al., 2011), cancer (Lu et al., 2005), diabetes (Fernandez-Valverde et al., 2011), neural plasticity (Smalheiser, 2014), and memory (Bredy et al., 2011; Konopka et al., 2010), among others (Park et al., 2010).

In the canonical pathway (reviewed in (Krol et al., 2010)), a nascent miRNA begins as a transcript of intronic or intergenic DNA, a miRNA precursor molecule known as a primary miRNA (pri-miRNA). While still in the nucleus, this pri-miRNA is bound

and cleaved by a microprocessor complex composed of Drosha and Dgcr8. Processing of the pri-miRNA by this complex leads the formation of a hairpin-like structure called pre-miRNA (Bartel, 2004; Krol et al., 2010). The pre-miRNA is then exported into the cytoplasm via Exportin 5 where it undergoes further cleavage by the RNAase enzyme Dicer, thereby forming a complementary duplex of two miRNA strands. Unwinding of this duplex releases one of the RNA strands, while the mature miRNA is bound to an Ago protein in the RNA-induced silencing complex (RISC). The mature miRNA, coupled with RISC (now called miRISC), then functions to detect complementary sequences inside messenger RNAs, usually found in the 3'-untranslated region of the target mRNA (Bartel, 2004; Pillai et al., 2007). The binding of the miRISC complex to the target mRNA results in silencing of the mRNA. This may occur either by degrading the target transcript via the endonuclease activity of Ago2, or by simply preventing translation of the target transcript in cases of less perfect complementarity.

Although studies have generally focused on the regulation of mRNA by the canonical RNAi pathway, there is considerable evidence that interaction between canonical RNAi and other ncRNA signaling pathways occurs and may have broad ramifications in neuroplasticity and cognition (reviewed in (Barry, 2014)). Specifically, a number of long noncoding RNAs (discussed in more detail below) have been observed to competitively bind miRNAs, thus allowing miRNA target genes to escape silencing. These competing endogenous RNAs (ceRNA) have been observed to regulate numerous aspects of neuronal function, including cell survival. Moreover, such mechanisms may intersect with epigenetic mechanisms, as is the case for the miR-101, which may be competitively bound by numerous lncRNAs including Malat1, to regulate autophagy and

cell death in the epileptic hippocampus (Wu and Yi, 2018), but which also regulates translation of the H3K27 methyltransferase Ezh2 (Qian et al., 2017).

Canonical mechanisms of siRNAs. There are significant functional similarities between miRNA and siRNA mediated RNAi. In this section we will highlight some of the more unique aspects of siRNA generation and regulation. Similar to miRNAs, siRNAs are short (~21 nucleotide), non-coding transcripts that are canonically generated from exogenous dsRNAs. When siRNAs were first discovered in plants by David Baulcombe and colleagues (Hamilton and Baulcombe, 1999), they appeared to function as part of a natural, antiviral immune response, described as follows. Upon exposure, exogenous, double-stranded RNAs (dsRNAs) from viruses are digested by Dicer in the cytoplasm, generating short RNA duplexes. These RNA duplexes are bound by Argonaut as part of the RISC complex, and guide the complex to a complementary target, in this case a copy of the viral RNA. Once bound, the endonuclease "slicer" activity of Ago2 is activated by the complementation of the siRNA-target interaction, mediated target destruction (Valencia-Sanchez et al., 2006). In this fashion, canonical siRNA-mediated RNAi initiation turns viral RNA against itself for destruction.

While only recently discovered, non-canonical endogenous mechanisms of siRNA (endo-siRNA) generation and function are beginning to be characterized in mammalian systems. Below, we discuss emerging roles for siRNA-directed epigenetic regulation of gene expression changes.

Emerging mechanisms of siRNA directed epigenetic regulation. siRNAs have been shown to participate in epigenetic regulation of genes through DNA methylation as well as by histone modification (Chen et al., 2012; Palanichamy et al., 2010). The precise mechanism of siRNA generation differs depending on the organism involved. In *Schizosaccaromyces pombe,* endo-siRNAs are generated by an RNA-directed RNA polymerase complex (RDRC), and epigenetic regulation is carried out by the RNA-induced transcriptional silencing (RITS) complex, with the latter being dependent on siRNAs generated by the former (for review see (Verdel et al., 2009)). In *Arabidopsis thaliana*, this process involves two plant-specific RNA polymerase II-related RNA polymerase enzymes: Pol IV and Pol V (Matzke and Mosher, 2014). First, transcripts from Pol IV are used as templates by the RNA-dependent RNA polymerase RDR2 to form dsRNA which is reduced into 24-nucleotide duplexes by the Dicer protein DCL3. From the cytoplasm, one strand of these duplexes are then loaded onto Ago4, where they translocate into the nucleus (Ye et al., 2012) and bind to complementary, nascent transcripts created by Pol V (He et al., 2009; Wierzbicki et al., 2008). Once stabilized to a target transcript by Pol V and the Pol V transcript binding protein KTF1, Ago4 associates with the DRM2 DNA methyltransferase, a writer of the 5mC epigenetic mark at CHH sites (Gao et al., 2010). RDM1, a subunit of the final complex responsible for linking Ago4 to Pol V and DRM2, has itself an affinity for methylated DNA, a finding that suggests a predilection of the Pol V-Ago4 complex for pre-existing sites of methylated DNA. While still speculative, these studies suggest a parallel between siRNA-directed

histone modification and siRNA-directed DNA methylation insofar as both may be mediated as part of a self-perpetuating feedback loop (Zhong et al., 2014). Although endo-siRNA generation and function is well-characterized in *Schizosaccaromyces pombe* and *Arabidopsis thaliana,* studies of siRNAs and their epigenetic function in human cells (Tam et al., 2008) are more recent. Mammalian endo-siRNAs are known to be generated from hybridized mRNAs and antisense transcripts (Watanabe et al., 2008) which may then regulate the epigenome. An alternative pathway for the generation of such endo-siRNAs has also been identified in which a complex composed of human TERT (hTERT), Brahma-related gene 1 (BRG1), and nucleostemin (NS) –together referred to as the TBN complex. This complex produces dsRNAs, which are then processed into siRNAs that facilitate the formation of heterochromatic regions (Maida et al., 2014).

Promisingly, several studies have demonstrated endo-siRNA-mediated histone methylation and DNA methylation in cultured cells (Babiarz et al., 2008; Chen et al., 2012; Palanichamy et al., 2010). Of particular relevance to this work is the finding that H3K9me2-mediated epigenetic repression may rely on crosstalk with RNAi pathways and Argonaut proteins. Further, as described previously, there is some evidence that long noncoding RNAs might also crosstalk with RNAi pathways by binding and titrating sncRNAs, generating sncRNAs, or binding and directing Ago proteins. Still, the mechanistic actions of mammalian endo-siRNA remain poorly characterized, and a neurological role for these endo-siRNAs remains to be established. Importantly, targeted sequencing studies have shown large numbers of these RNAs in human somatic cells (Castellano and Stebbing, 2013), and recent studies have identified putative endo-siRNA populations in hippocampal tissues (Smalheiser, 2012; Smalheiser et al., 2011).

Epigenetic regulation via piRNAs. In exploring the role for ncRNAs in cognition and cognitive sciences, piRNAs have become a topic of some intriguing investigations. piRNAs are distinguished from siRNAs by their size (they are slightly longer at 26–31 nt rather than 20–24 nt), and association with Piwi proteins, a clade of the Ago family (Le Thomas et al., 2014). Unlike miRNAs and siRNAs, piRNAs are generated from single stranded RNA species in a *Dicer*-independent manner (Carone et al., 2010; Han and Zamore, 2014). piRNAs are preeminently expressed and best characterized in the context of germ cell development (Cox et al., 1998; Kuramochi-Miyagawa et al., 2004; Lin and Spradling, 1997). Indeed, the name "PIWI" has its origin owing to the discovery of the "P-element induced wimpy testis" in the gonadal cells of *Drosophila*. PIWI proteins translocate into the nucleus in an RNA-dependent manner, guided by piRNAs (Cox et al., 1998; Grimaud et al., 2006). Here they serve to silence transposons in the nuclei of germ cells (Girard et al., 2006), for the purpose of genome protection in the vulnerable germline DNA. However, this functionality is not exclusive, as protein-coding genes may also code for piRNAs (Robine et al., 2009). Moreover, in recent studies numerous piRNAs have been discovered to be expressed in adult tissues (including in brain tissues), suggesting additional roles and new possibilities for epigenome regulation (Rajasethupathy et al., 2012; Ross et al., 2014).

With regard to epigenetic regulation, piRNAs have been shown to target heterochromatic regions with the help of bound heterochromatin protein 1a (HP1a) as part of a Piwi-piRNA complex (Brower-Toland et al., 2007), a complex typically associated with repressive histone lysine methylation marks, but that may also facilitate

transcription (Piacentini et al., 2009). This discovery suggests that piRNA can form an initiator complex on chromatin that recruits other chromatin modifying agents (Yin and Lin, 2007). An additional study that highlights chromatin regulation by piRNAs is demonstrated by Carmell and colleagues, showing that loss of a murine Piwi resulted in increased transposon expression due to a loss of inhibitory DNA methylation at transposon sites (Carmell et al., 2007). Further elucidation of this mechanism by Aravin and colleagues revealed that piRNA-mediated silencing of transposons by Piwi orthologs plays a significant role maintaining the genome integrity of the mouse testis (Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008). Interestingly, some transposable elements have been identified as sources of dsRNAs, which feed into the endo-siRNA pathway suggesting a degree of redundancy between endo-siRNA and piRNA pathways (Han and Zamore, 2014). While still largely unexplored in mammalian systems, one population of piRNAs has been identified in the murine hippocampus via next generation sequencing (Lee et al., 2011).

With regard to neuroepigenetic functionality of piRNAs, recent studies have revealed a population of serotonin-induced piRNAs in the CNS of *Aplysia.* Moreover, these studies demonstrated that knockout studies have demonstrated the necessary of Piwi for serotonin induced long term facilitation *(Rajasethupathy et al., 2012)* –a synaptic correlate for memory formation. piRNA has also been demonstrated to silence CREB2 – a suppressor of memory formation- in an activity-dependent manner in *Aplysia (Rajasethupathy et al., 2012)*, further supporting the idea that piRNA signaling is necessary for memory formation. Collectively, these results are suggestive of a broader

role for piRNAs in epigenetic regulation than was previously expected and future studies will likely uncover additional piRNAs mediating neuroepigenetic regulation.

Long Noncoding RNAs

Discovery and characterization of long noncoding RNAs. If sncRNAs can usually be separated into distinct classes by clearly defined homologies of structure and function, lncRNAs might be considered the opposite, with the term representing a conglomerate of heterogeneous and often modular sets of transcripts (Guttman and Rinn, 2012; Tsai et al., 2010; Wright, 2014). While lncRNA genes tend to utilize alternative splicing to a lesser degree than protein coding genes, there are nonetheless many gene and transcript isoforms of lncRNAs which may contribute further to the complexity of the system. Moreover, like mRNAs, the primary sequence of lncRNA residues may be edited at a single-nucleotide level post-transcriptionally (Picardi et al., 2014). This large degree of variability in genomic structure is complemented by extensive variability in lncRNA functionality. While itself heterogenous, the most common working nomenclature of lncRNA structure tends to derive transcript names on the basis of their genomic location relative to nearby protein coding genes (Mattick and Rinn, 2015; Wright, 2014). Among these subcategories of lncRNAs are antisense, bidirectional, intergenic, intronic, and overlapping transcripts (Figure 2).

Although the first functional role for lncRNAs were described prior to sncRNAs (Pachnis et al., 1988), it is only recently that the abundance of lncRNAs in the mammalian transcriptome has been recognized. Recent studies have identified several thousands of lncRNA genes in the human transcriptome (Harrow et al., 2012; Sanli et al., 2013). While these studies have expanded our knowledge of the transcriptome, most observations are still limited in scope to cultured cells and resting state expression within tissues. Given the highly specific expression profiles of many known lncRNAs and their lower expression levels (10-fold lower than protein coding genes, on average) (Cabili et

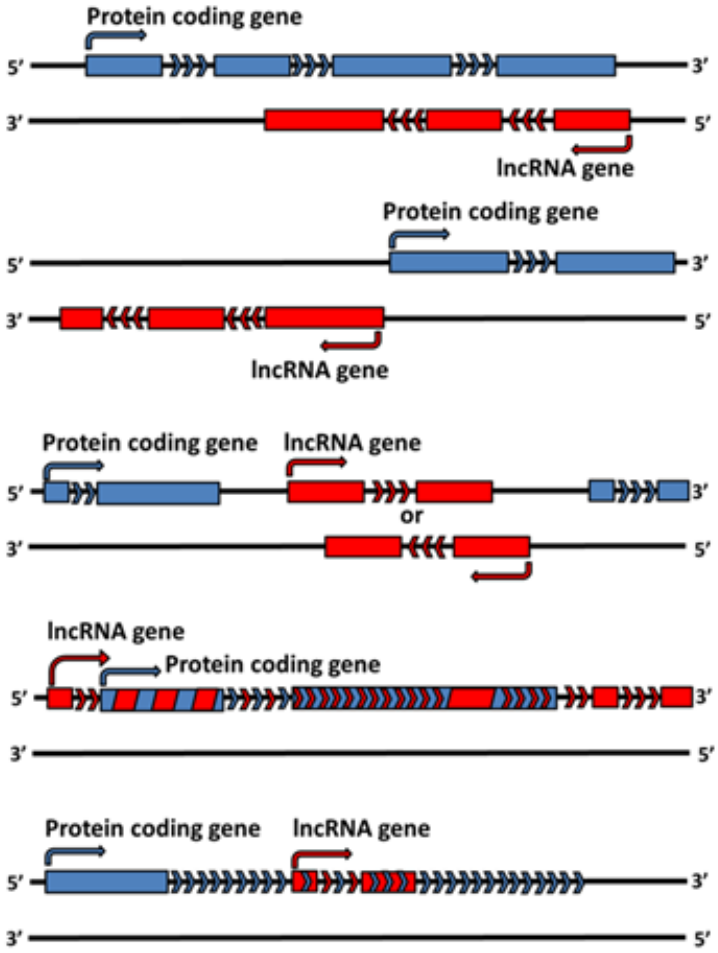

Figure 2. Genomic origins of lncRNAs various lncRNA genes (Adapted from (Butler et al., 2016))

al., 2011; Pauli et al., 2012), it is likely that many functional lncRNA transcripts are expressed below the power of detection for such studies. Indeed, novel deep-sequencing methodologies have demonstrated that the full transcriptome is much larger than established, lower depth sequencing studies have revealed (Fu et al., 2014; Mercer et al., 2014, 2012). Thus, a thorough investigation of lncRNA abundance will likely require the targeted deep transcriptional profiling of specific tissues and cell types, or perturbations.

Many mammalian protein coding loci also express some form of antisense transcript, with some estimates being as high as 80% of protein coding genes (Katayama et al., 2005; Klevebring et al., 2010; Morris, 2009). Often, such transcripts have regulatory impacts on associated protein coding genes (Pelechano and Steinmetz, 2013), though this is not a necessity, nor does it preclude additional *in trans* effects (Mattick and Rinn, 2015). While inherently difficult to target genetically, such antisense transcripts have been observed to play a critical role in directing the epigenetic regulation of target genes (Morris, 2009)

Many lncRNAs demonstrate properties typically associated with protein coding genes, such as chromatin structure typical of RNA polymerase II (Pol II) transcription, alternative splicing sites, and regulation by transcription factors (Morris and Mattick, 2014). Furthermore, many lncRNAs are polyadenylated and capped with 5'-methylguanisine (Sanli et al., 2013), and there have even been reports of lncRNAs associating with ribosomes—although ribosome profiling experiments have shown that such associations are usually inactive (Chew et al., 2013; Guttman et al., 2013). Surprisingly, some lncRNAs have been observed to produce small protein products, and more recent studies suggest that global translation of all ncRNAs may occur in a manner

similar to pervasive transcription (Ingolia et al., 2014), though the biological importance of these mechanisms remain largely undefined.

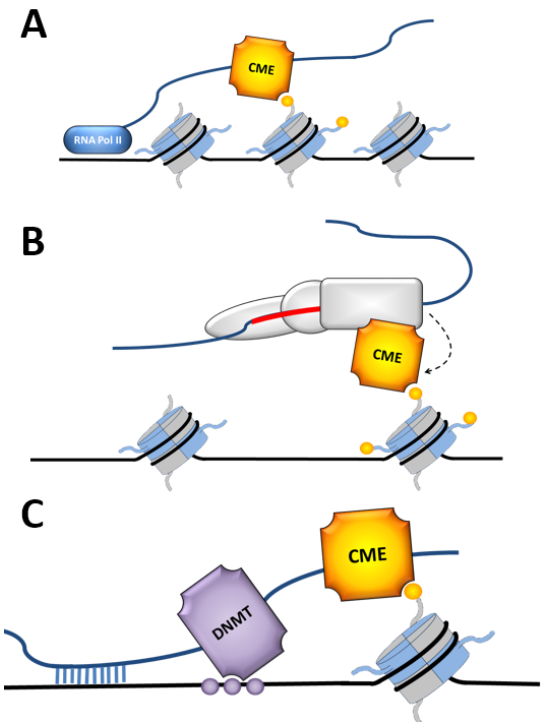

Figure 3. Established mechanisms of lncRNA-mediated epigenetic regulation. (Adapted from (Butler et al., 2016))

A number of molecular mechanisms have been attributed to lncRNA function. These include the regulation of transcription (Jiao and Slack, 2014; Modarresi et al., 2012; Zhang et al., 2012), epigenetic regulation (Schaukowitch and Kim, 2014), scaffolding of protein complexes (Froberg et al., 2013; Zappulla and Cech, 2006), guiding of regulatory complexes (Froberg et al., 2013), acting as decoys to regulatory complexes (Di Ruscio et al., 2013), or simply being transcribed (Kornienko et al., 2013). These mechanisms of action often rely on the ability of RNAs to bind both proteins and nucleic acids in a targeted manner. An RNA molecule's primary structure—that is, the linear sequence of nucleotides—allows RNA transcripts to bind homologous DNA

regions via canonical or non-canonical base pairing. Recently, tools have been developed for the computational prediction of lncRNA DNA-binding motifs and binding sites (He et al., 2015). Such hybridization also allows single stranded RNA fold into complex secondary and tertiary structures, or to pair with other RNA molecules. It is these structural arrangements, in addition to sequence specificity, that often underlie interactions with RNA binding proteins (RBPs) (Li et al., 2014, 2010). Many currently known mechanisms of lncRNA activity largely rely on interaction with RBPs and alterations in localization, activity, or association with other proteins. RBPs are a functionally and structurally diverse class of molecules, and recent studies have estimated that 40% of RBPs (out of a cohort of 1,542 RBPs) are involved in ncRNA related processes (Gerstberger et al., 2014). Additionally, lncRNAs have been observed to bind and regulate other small RNA molecules such as miRNAs (Bosia et al., 2013; Kartha and Subramanian, 2014), and extensive noncoding interactomes have been proposed (Jalali et al., 2013).

In the nucleus, lncRNAs have been demonstrated to modulate gene expression via regulation of transcription and the epigenetic landscape (Figure 3) (Morris and Mattick, 2014; Nakagawa and Kageyama, 2014). Studies have shown that lncRNAs bind to a number of CMEs, usually writers of epigenetic marks (Nakagawa and Kageyama, 2014). The extent of such a phenomenon was established in 2009, when it was shown that some 20% of lncRNAs (out of a cohort of 3300) associate with the Polycomb repressive complex 2 (PRC2), a histone methyltransferase that catalyzes the addition of repressive H3K27 methylation (Khalil et al., 2009). Additionally, binding of lncRNAs to CMEs can prevent or restrict CME activity, as was recently demonstrated to occur at the CEBPA

locus, where an overlapping lncRNA (sometimes described as an extracoding RNA or ecRNA) preferentially binds the DNA methyltransferase DNMT1 (Di Ruscio et al., 2013), ultimately leading to decreased local DNA methylation. Interestingly, this phenomenon is not restricted to the CEBPA locus, but occurs at multiple methylation sites across the epigenome (Di Ruscio et al., 2013).

Long noncoding RNAs in cognitive disorders.

The importance of epigenetic regulation to neuronal and cognitive function has become increasingly clear. A recent GWAS study of common cognitive disorders found that epigenetic—specifically, histone methylation—pathways were strongly associated with impaired cognition (Network and Pathway Analysis Subgroup of Psychiatric Genomics Consortium, 2015), and a number of screening studies suggest that lncRNA dysregulation is associated with neurodevelopmental and cognitive disorders (Zhao et al., 2015), including Rett syndrome (Petazzi et al., 2013), autism (Ziats and Rennert, 2013), and Fragile X syndrome (Spadaro et al., 2015). While the widespread mechanisms of ncRNA mediated regulation have been established for some time, only in very recent years have these mechanisms been investigated in a neurological or cognitive context. lncRNAs have been found to be co-expressed with genes that are critical for neuronal activity, including *c-Fos*, *Arc*, and *Bdnf*, suggesting a coordinated regulatory network of protein coding and non-coding genes involved in neuronal plasticity (Lipovich et al., 2012; Spadaro et al., 2015). Additionally, lncRNAs are known to play a role in normal brain development (Sauvageau et al., 2013). While the majority of lncRNA transcripts have been characterized in either cell culture or during development, efforts to examine

the functional roles of neuronal lncRNAs in cognition are ongoing. In the section below, I will recount some established examples of lncRNAs functioning in the context of the adult brain, and their impact, if known, on cognition or cognitive disorders.

Malat1. This highly conserved nuclear lncRNA, also known as *Neat2*, is expressed in numerous tissues, exhibiting a high degree of expression in neurons (Bernard et al., 2010; Carithers et al., 2015). Knockdown studies of Malat1 have resulted in decreased synaptic density in cultured hippocampal neurons (Bernard et al., 2010). Post-mortem studies have demonstrated that Malat1 is upregulated in multiple brain regions in both human alcoholism as well as rodent models of alcoholism (Kryger et al., 2012). Malat1 has been demonstrated to regulate gene expression *in cis,* controlling the expression of proximally located genes which are involved in nuclear function (Zhang et al., 2012). It has also been shown to bind hundreds of sites *in trans,* where it preferentially binds the gene body of active genes in a transcription dependent fashion (West et al., 2014). Epigenetically, Malat1 has been shown to associate *in vivo* with EZH2, a subunit of the Polycomb repressive complex 2 (PRC2) (Guil et al., 2012). Interestingly, and despite many functional associations, Malat1 knockout in mice does not affect viability or normal development (Eißmann et al., 2012; Zhang et al., 2012).

Gomafu. The lncRNA Gomafu has also been shown to play multiple roles in the adult brain. Gomafu has been observed to govern SZ-related alternative splicing by acting as a splicing factor scaffold for QK1 and SRSF1, and it is known to be dysregulated in postmortem studies of schizophrenia patients (Barry et al., 2014).

Recently, additional studies have suggested that Gomafu functions *in cis* to mediate epigenetic regulation of gene expression via the PRC1 complex, and that knockdown of Gomafu in adult mice results in abnormal behavioral phenotypes and increased anxiety (Spadaro et al., 2015).

BACE1-AS. In another clinical application, the antisense lncRNA BACE1-AS has been implicated in Alzheimer's disease (AD). AD is a progressive neurodegenerative disorder which has been previously associated with epigenetic dysregulation, particularly in histone acetylation (Lu et al., 2014; Stilling and Fischer, 2011). A characteristic marker of AD pathology is the accumulation of beta amyloid plaques consisting of oligomerized amyloid beta peptides. These plaques form as a result of processing of amyloid precursor protiens (APP), the rate limiting step of which is the cleavage of APP by the Beta-secretase enzyme (BACE1) (Sathya et al., 2012). Dysregulation of BACE1 has been shown to contribute to AD pathology via the overproduction of Aβ (Sathya et al., 2012). Recent studies have identified an antisense lncRNA at the BACE1 locus (BACE1-AS) which physically associates with and stabilizes BACE1 mRNA, increasing BACE1 expression both *in vitro* and *in vivo*, and ultimately resulting in increased generation of Aβ (Liu et al., 2014). BACE1 mRNA is targeted by the miR-485-5p, which normally results in BACE1 repression; however, BACE1-AS prevents this repression by competitively binding the miRNA target site (Faghihi et al., 2010). Both the BACE1-AS lncRNA and BACE1 mRNA have been shown to be overexpressed in the parietal lobe and in the cerebellum of postmortem AD patients, suggesting a relevant mechanistic link between the BACE1-AS lncRNA and the pathophysiology of AD (Faghihi et al., 2010).

Interestingly, knockdown of BACE1-AS in senile plaque SH-SY5Y cells results in attenuated cleavage of APP and reduced abundance of Aβ1–42 oligomers (Liu et al., 2014), and reduced Alzheimer's pathology in an APP mouse model of AD (Modarresi et al., 2011). Of particular relevance to the alleviation of AD morbidities, knockdown of the BACE1-AS transcript in rodent models of AD has been observed to improve performance on the Morris water maze memory task in the senescence accelerated SAMP8 mouse model (Zhang et al., 2018). While the dysregulation of lncRNAs has been implicated in cognitive disorders, the task of exploring the role of lncRNA mediated epigenetic regulation in normal cognitive function remains incomplete.

Transgenerational impacts of ncRNA mediated epigenetic regulation

Since the discovery of epigenetics, there has been much curiosity and speculation as to the transgenerational heritability of epigenetic marks. In mammals, much of the epigenome is erased during the processes of fertilization and generation of primary germ cells (reviewed in (Morgan et al., 2005)); nonetheless, evidence of a transgenerationally-heritable epigenome has steadily accumulated, including heritable cognitive changes and behavioral phenotypes (Carone et al., 2010; Dias and Ressler, 2014; Dias et al., 2015; Gapp et al., 2014; Pembrey et al., 2006; Skinner et al., 2012). A simple explanation for this phenomenon would be incomplete erasure of DNA and histone modifications. While there is some evidence in support of this hypothesis (reviewed in (Migicovsky and Kovalchuk, 2011)), other studies have demonstrated the existence of an indirect mechanism of chromatin regulation via generational transfer of ncRNAs (Benito et al., 2018).

Recently developed mammalian epimutation models –which demonstrate phenotypes derived from a heritable change in gene expression, as opposed to an altered genome- have demonstrated the sufficiency of parental RNA to alter the epigenome of treated progeny (Yuan et al., 2015). Additionally, in a rodent stress model, treatment of fertilized mouse oocytes with ncRNAs from the sperm of stressed males is sufficient to recapitulate heritable stress-related behavioral and metabolic phenotypes (Gapp et al., 2014), indicating that an altered transcriptome is sufficient for the transfer of epigenetic information.

The most direct evidence for a neuronal role in transgenerational epigenetic phenomenon comes from *C. elegans*, where neuronally expressed RNA species are transported to the cells of the germline. These RNAs then initiate the transgenerational epigenetic silencing of particular genomic loci, thereby impacting gene expression in the germ line and potentially in any progeny (Devanapally et al., 2015). It is tempting to speculate that an analogous mechanism could exist in mammals, by which somatic tissues such those of the brain may regulate the epigenome of cells distant in both space and time. Clearly, such a finding would have far-reaching consequences for cognitive science.

Neat1

The lncRNA *Neat1*, which is the primary focus of this research, is the nuclear enriched abundant transcript 1 (*Neat1*). While the lncRNA field is only beginning to expand into the brain and the investigation of cognitive phenomenon, as described above, few lncRNAs are as well studied in the context of cancer biology as the lncRNA *Neat1*

(S. Li et al., 2018; Yang et al., 2017). A host of recent studies have investigated the role of *Neat1* in breast cancer (W. Li et al., 2017; X. Li et al., 2017; Qian et al., 2017; Zhao et al., 2017), cervical cancer (Wang and Zhu, 2018), colorectal cancer (Li et al., 2015; Peng et al., 2017; Wu et al., 2015), gastric cancer (Fu et al., 2016; H. Wang et al., 2018), lung cancer (Jen et al., 2017; Qi et al., 2018; You et al., 2014), ovarian cancer (Chai et al., 2016; Y. Liu et al., 2018), prostate cancer (Chakravarty et al., 2014; Xiong et al., 2018). These recent studies in the context of tumors or cancer cell lines have characterized a number of molecular pathways by which *Neat1* regulates numerous aspects of cell signaling, including both paraspeckle-dependent processes such as sequestration of transcription factors (Choudhry et al., 2015; Hirose et al., 2014; Imamura et al., 2014) or splicing factors (Cooper et al., 2014), as well as paraspeckle-independent roles for *Neat1* via scaffolding of chromatin modifying enzymes (Qian et al., 2017).

Multiple transcript isoforms of *Neat1* exist in both rodents and in humans, with the longer of the major isoforms proving essential for the formation of nuclear paraspeckle (Clemson et al., 2009; Sasaki et al., 2009; Yamazaki and Hirose, 2015). Nuclear paraspeckles are irregularly shaped, non-membrane bound bodies found within the nuclear interchromatin space. Paraspeckles allow the sequestration of a number of important functions within a phase-separated nuclear space (Yamazaki et al., 2018), and have been shown to be important for control of diverse molecular pathways, including mRNA editing , miRNA maturation, and transcription factor sequestration. As a critical component of the paraspeckles, the long isoform of *Neat1* may thus be implicated in numerous global processes of RNA biology as well as epigenetic regulation; however, while the short isoform of *Neat1* localizes to the exterior of paraspeckles as well, it is not

critical for paraspeckle formation (R. Li et al., 2017). Furthermore, extensive binding of *Neat1* to chromatin has been documented experimentally (R. Li et al., 2017), and *Neat1*-dependent epigenetic regulation has been demonstrated experimentally at specific loci, suggesting that there may be distinct functions of paraspeckle-associated and chromatin-associated *Neat1*. Interestingly, while the lncRNA *Neat1* is evolutionarily conserved across the mammalian lineage, conservation of *Neat1* is not uniform across long and short isoforms, with the 5' region, which is shared between both long and short isoforms, constituting the region with the highest degree of sequence conservation.

While the mechanisms of *Neat1* activity have been robustly characterized in the context of cancer biology due to its known role as a potent, multi-cancer oncogene, recent expression profiling studies have linked tissue-specific overexpression of *Neat1* in normal aging, as well as neurodegenerative and cognitive disorders, including amyotrophic lateral sclerosis (ALS) (Shelkovnikova et al., 2018), Alzheimer's disease, Huntington's disease, Parkinson's Disease, epilepsy, and schizophrenia. While expression of *Neat1* and/or induction of paraspeckle assembly has been observed in numerous neurodegenerative disorders, reports differ as to whether *Neat1* upregulation is ultimately protective or destructive, with (Shelkovnikova et al., 2018) and (Soreq et al., 2014) reporting a neuroprotective effect of *Neat1* in ALS and Parkinson's disease, while (Liu and Lu, 2018) and (Barry et al., 2017) implying harmful roles for *Neat1* expression in Parkinson's disease and epilepsy, respectively.

Additionally, recent evidence from the study of cultured neurons and rodent models of epilepsy suggests that *Neat1* may play a role in neuroplasticity (Barry et al., 2017). Specifically, that *Neat1* knockdown enhances neuronal excitability. Importantly,

this study also reported a loss of activity-dependent *Neat1* down-regulation in rat model systems of temporal lobe epilepsy, a disorder with well-documented deficits in hippocampus dependent memory formation. Despite extensive health relevance to cognitive science, the role of *Neat1* and *Neat1*-mediated epigenetic regulation within a neurobiological and behavioral context remain unexplored. In this work, I have aimed to investigate the functional role of the lncRNA *Neat1* in the context of the epigenetic regulation of gene expression within the hippocampus, and the role that age-related changes in *Neat1* expression might play in the epigenetic control of memory deficits in the aging brain.

ENVIRONMENTAL ENRICHMENT REVERSES HISTONE METHYLATION CHANGES IN THE AGED HIPPOCAMPUS AND RESTORES AGE-RELATED MEMORY DEFICITS

by

SARAH J. MORSE†, ANDERSON A. BUTLER†, ROBIN L. DAVIS, IAN J. SOLLER
FARAH D. LUBIN

† These authors contributed equally to this work.

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ABSTRACT

A decline in long-term memory (LTM) formation is a common feature of the normal aging process, which corresponds with abnormal expression of memory-related genes in the aged hippocampus. Epigenetic modulation of chromatin structure is required for proper transcriptional control of genes, such as the brain-derived neurotrophic factor (Bdnf) and Zif268 in the hippocampus during the consolidation of new memories. Recently, the view has emerged that aberrant transcriptional regulation of memory-related genes may be reflective of an altered epigenetic landscape within the aged hippocampus, resulting in memory deficits with aging. Here, we found that baseline resting levels for tri-methylation of histone H3 at lysine 4 (H3K4me3) and acetylation of histone H3 at lysine 9 and 14 (H3K9,K14ac) were altered in the aged hippocampus as compared to levels in the hippocampus of young adult rats. Interestingly, object learning failed to increase activity-dependent H3K4me3 and di-methylation of histone H3 at lysine 9 (H3K9me2) levels in the hippocampus of aged adults as compared to young adults. Treatment with the LSD-1 histone demethylase inhibitor, t-PCP, increased baseline resting H3K4me3 and H3K9,K14ac levels in the young adult hippocampus, while young adult rats exhibited similar memory deficits as observed in aged rats. After environmental enrichment (EE), we found that object learning induced increases in H3K4me3 levels around the Bdnf, but not the Zif268, gene region in the aged

hippocampus and rescued memory deficits in aged adults. Collectively, these results suggest that histone lysine methylation levels are abnormally regulated in the aged hippocampus and identify histone lysine methylation as a transcriptional mechanism by which EE may serve to restore memory formation with aging.

INTRODUCTION

Hippocampus-dependent long term memory (LTM) deficits are well documented as part of the normal aging process in both humans and rodent animal models (Bizon et al., 2009; Gallagher and Rapp, 1997). These age-related memory deficits occur without significant alterations in gross hippocampal morphology (Shamy et al., 2006). Instead, such memory deficits are reflective of a general dysregulation of memory-permissive genes within the aged hippocampus that are crucial to the process of LTM formation, including deviations in immediate-early genes like brain-derived neurotrophic factor (Bdnf) and Zif268 (also known as Egr1) (Blalock et al., 2003; Bramham, 2007; Burger, 2010; Gupta et al., 2010; Hall et al., 2000; Kadish et al., 2009; Lubin et al., 2008; Poirier et al., 2007; Rowe et al., 2007). A growing literature suggests that epigenetic modulation of chromatin structure around gene regions is a transcriptional mechanism necessary for the formation and maintenance of LTM (Gupta et al., 2010; Levenson et al., 2004; Lubin et al., 2008). Therefore, alterations in the epigenetic landscape within the aged hippocampus may result in activity-dependent perturbations of gene transcription changes necessary for proper LTM formation (Castellano et al., 2012; Fischer et al., 2007; Peleg et al., 2010; Penner et al., 2011). Histone lysine methylation is a unique epigenetic transcriptional regulator whose function relies on the recruitment of proteins to

regulate chromatin structure and subsequent cellular transcriptional activity (for review, see (Jarome and Lubin, 2013; Lubin, 2011)). During the process of memory consolidation, tri-methylation of histone H3 at lysine 4 (H3K4me3) and di-methylation of histone H3 lysine 9 (H3K9me2) are involved in the upregulation or downregulation of genes, respectively, within the hippocampus and disruption of these epigenetic mechanisms produces memory deficits (Gupta et al., 2010; Gupta-Agarwal et al., 2012). With respect to memory deficits in normal aging, the role of histone lysine methylation remains to be explored. Additionally, environmental enrichment (EE) reverses age-related cognitive decline (Bouet et al., 2011; Freret et al., 2012; Frick and Fernandez, 2003; Leal-Galicia et al., 2008; Mora-Gallegos et al., 2015), yet the effect of EE on histone lysine methylation changes in the aged hippocampus in response to learning is currently unknown. This prompted us to explore the possibility that EE interacts with histone lysine methylation transcriptional mechanisms to restore proper memory formation with aging.

RESULTS

Baseline Resting Hippocampal Histone Lysine Methylation Levels in Young and Aged Adults

To begin exploring the potential role of histone lysine methylation in age-associated LTM dysfunction, we first assessed baseline resting levels of two distinct H3 lysine methylation modifications (H3K9me2 and H3K4me3) within the hippocampus of aged adult rats as compared to young adult rats. Using Western blotting analysis, we observed a strong trend towards elevation, but non-significant increase in baseline resting

H3K9me2 (Figure 1A; $t(9) = 1.863$, $p = 0.0954$) and a significant increase in H3K4me3 levels (Figure 1A; $t(17) = 2.881$, $p = 0.0104$) in area CA1 of the hippocampus from aged adults compared to young adults. As previously described (Lubin, 2011; Penner et al., 2011), we found that baseline resting H3K9,K14ac levels were markedly reduced in area CA1 of aged adult rats (Figure 1A; $t(9) = 3.012$, $p < 0.05$). In area CA3b of the hippocampus, baseline resting H3K4me3 levels were significantly elevated with aging compared to young adults (Figure 1B; $t(8) = 2.843$, $p < 0.05$), while baseline resting H3K4me3 levels in the dentate gyrus (DG) region remained unchanged (Figure 1C). Interestingly, no age-related alterations were observed in H3K9me2 or H3K9,K14ac levels in CA3b or DG, suggesting that age-related widespread disruption of histone-mediated gene transcription primarily occurred in area CA1 of the hippocampus. Furthermore, these results indicate that aberrant histone methylation levels, specifically H3K4me3, may contribute to transcriptional dysregulation in the aged hippocampus.

Learning induced Histone Lysine Methyl ation and Gene Expression Changes in the Young and Aged Hippocampus

We next determined whether learning triggers histone lysine methylation changes in the hippocampus of aged adult rats as compared to young adult rats. Because we found that posttranslational modifications of histone proteins were primarily dysregulated in area CA1 of aged adult rats, we focused our remaining experiments in this region of the hippocampus. Using Western blotting analysis, we observed significant elevation in H3K4me3 (Figure 2A) and H3K9me2 (F igure 2B) levels in area CA1 of the hippocampus from young adult rats, but not aged rats at one hour after object learning.

Next we investigated whether expression changes in the memory-related genes, Zif268 (also known as Egr1) and Bdnf exon IX (a coding exon shared between all rat Bdnf transcript variants) correlated with histone lysine methylation changes in the hippocampus of aged adult rats as compared to young adult rats. We found that Zif268 mRNA levels were significantly increased in area CA1 of the hippocampus from aged adult rats as compared to young adult rats (Figure 3A) and were observed to further increase following training in the novel object recognition (NOR) memory task (Figure 3A). We observed no significant changes in Bdnf exon IX mRNA levels (Figure 3B).

Inhibition of the LSD1 Histone Demethylase Mimics Age-Related Histone Lysine Methylation Changes and Memory Impairments in Young Adults

Based on the finding that baseline resting histone lysine methylation levels were significantly altered in the hippocampus of aged adults compared to young adults, we hypothesized that manipulating histone lysine methylation levels in young adults may produce similar effects on memory formation observed in aged adults. Therefore, we next sought to determine the effect of manipulating histone lysine methylation levels in young adult rats using the Lysine Specific Demethylase 1A (LSD1) inhibitor t-PCP (Neelamegam et al., 2012). We measured the effect of t-PCP on both baseline re sting and behaviorally-induced H3K9me2, H3K4me3, and H3K9,14ac levels in the hippocampus of young adult rats.

First, we assessed baseline resting histone modification levels in area CA1 from saline-vehicle or t-PCP-treated animals not exposed to the NOR training arena or homecaged animals (Naïve). Western blotting analysis revealed that t-PCP treatment

significantly increased baseline resting H3K4me3 and H3K9,K14ac levels in area CA1 from naïve adults, confirming that LSD1 inhibition successfully elevated histone methylation levels while simultaneously increasing histone acetylation levels in the hippocampus (Figure 4). Conversely, resting H3K9me2 levels were significantly reduced in area CA1 from t-PCP-treated adults compared to vehicle-treated controls (Figure 4). Together, these results may indicate a global shift towards more transcriptionally active chromatin (H3K4me3 and H3K9,K14ac) in area CA1 of t-PCP treated animals.

We next determined the effects of LSD1 inhibition with t-PCP on the formation of hippocampus-dependent memory. A schematic of our experimental design is outlined in Figure 5A. We assessed the impact of LSD1 blockade on LTM formation using two hippocampus-dependent novelty discrimination memory tasks: NOR and object location (OL). The discrimination index percentage (Figure 5B) and the time spent exploring each object was recorded (Figure 5C,D). We found that LSD1 inhibition significantly blocked memory formation in both the NOR and OL (Figure 5C,D) memory tasks relative to saline-vehicle treated controls. These results suggest that disruption of histone lysine methylation levels is sufficient to impair the formation of hippocampus-dependent memory in young adults.

Having established that alterations in H3K4me3 methylation were associated with age-related memory deficits and that mimicry of these age-related changes im paired LTM formation in young adults, we next explored the possibility of restoring appropriate H3K4me3 levels and rescuing memory deficits in aged animals through EE behavioral therapy. We first confirmed that age-associated memory impairments could be restored by a modified EE. The modified EE protocol used in the following experiments lacked

the characteristic exercise components (i.e., running wheel), as motor stimulation induces neurogenesis, which alone can result in gene expression changes, such as Bdnf within the DG region of the hippocampus (Boehme et al., 2011; Farmer et al., 2004; Yu et al., 2014).

Aged adults were exposed to the modified EE protocol consisting of a variety of toys and social interaction for 1 h each day for a period of 5 weeks prior to exposure to the NOR and OL memory tasks (Figure 6A). Analysis of the discrimination index revealed that aged controls that did not receive EE demonstrated no preference for the novel object or novel location, which is characteristic of memory impairments (Figure 6B). Aged adults exposed to the EE showed a significant improvement over nonenriched controls in both NOR and OL memory tasks (Figure 6C,D).

No exploratory preference was detected during the acquisition phase and both non-enriched aged animals exhibited sufficient object interaction during the acquisition and retrieval phases thus ruling out the possibility that poor performance by the aged control adults was due to exploratory preferences or insufficient exploration during training (Figure 6C). EE aged adults exhibited a significant preference for the NO/NL during the retrieval phase (Figure 6D; $t(10) = 3.283$, $p < 0.01$), confirming that EE restored LTM in aged adults. Furthermore, analysis of object interaction times for the aged-enriched group ensured that enhanced LTM formation was not due to increased motor activity during the 5-week EE protocol (Figure 6D). These results confirm that an exercise-free EE protocol improves LTM formation in aged adults.

We next assessed the effect of EE on expression of the memory permissive genes Bdnf and Zif268 in laser capture microdissection (LCM) captured pyramidal neurons

from area CA1. Prior to object learning aged animals were divided into two separate groups, one group experienced the 5-week EE protocol and the other group served as non-enriched aged controls. At 1 h after object training, we found that EE significantly increased Bdnf , but not Zif268, mRNA levels in LCM-captured pyramidal neurons from area CA1 during memory formation (Figure 7A; $t(7) = 2.923$, $p < 0.05$).

Chromatin immunoprecipitation (ChIP) analysis revealed that H3K4me3 levels significantly increased at the Bdnf exon 4 promoter (Figure 7B). We did not observe any significant activity dependent changes in H3K4me3 levels at the Zif268 promoter region (Figure 7B), which is in agreement with our finding that EE did not alter activity dependent Zif268 mRNA levels (Figure 7A). Together, these results strongly support histone lysine methylation mechanisms as molecular targets in the restoration of age-related memory by EE.

METHODS

Animals. Young adult (3 months) and aged adult (19–22 months) male Fischer-344 rats (National Institute on Aging at Harlan) were used in these experiments. Animals were singly housed under light/dark 12 h/12 h and allowed access to food and water *ad libitum*. Animals were handled 3–5 min each and allowed to acclimate to laboratory conditions for 5 days prior to experiments. All procedures were performed in accordance with the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham and national guidelines and policies.

Environmental enrichment: Rats were transported to the laboratory 30 min before enrichment or handling. Aged rats were introduced pair-wise to one another in the empty enrichment cage for 5 min per session for 2 days to reduce stress and possible aggression with a maximum of 3 sessions per animal per day. All animals were subsequently exposed to EE for 1 h per day for 5 weeks. EE consisted of a large cage (76.2 cm × 76.2 cm × 25.4 cm) in which the animals were able to interact socially and explore tunnels, plastic blocks and balls, ladders, and other toys. To avoid behavioral habituation, additional new toys were introduced throughout the 5 week-period and objects were rearranged daily. Running wheels were specifically excluded to avoid neurogenesis effects in the dentate gyrus and the associated effects on gene expression arising from exercise-mediated alterations of synaptic plasticity (van Praag et al., 1999; Vaynman et al., 2004, 2003). Control animals remained in standard housing conditions and were transported alongside the EE animals followed by placement in a separate room where they received similar amounts of handling.

Novel object recognition: In the novel object recognition (NOR) task rats were individually placed in a 31 cm × 31 cm box, which was covered with a white sheet and cleaned with 50% isopropanol before and after each trial. On day 1 of NOR, rats were exposed to the empty arena for 5 min for habituation to the environment to reduce stress during subsequent trial phases. After 24 h, animals were exposed to a 10 min training session with two identical sample objects. For biochemistry studies, one set of animals was sacrificed 1 h after participation in this training session for tissue harvesting, during the consolidation phase of memory formation in which de novo protein synthesis occurs.

During the testing phase, animals' memory performances were assessed via their ability to discriminate between a novel and familiar object (Ennaceur et al., 1989). This testing phase occurred 24 h after the training phase and lasted for a 5 min duration. Sample and novel objects were cleaned after each trial and positions were randomly exchanged throughout trials. All objects were weighted and adhered to the floor to avoid movement out of the scoring area. Trials were recorded using the Noldus Ethovision software, using the Phenotyper camera box (Noldus) placed directly above the arena. Trials were scored by a researcher blind to the animal's identity. Contact with objects was defined as previously described (Bevins and Besheer, 2006). Drug—Animals were intraperitoneally (IP) injected with either saline (0.9% NaCl, pH 7.4) or trans-2-Phenylcyclopropylamine hydrochloride (3 mg/kg, Sigma Chemical).

Object Location: In the Object Location (OL) task rats were individually placed in a 31 cm × 31 cm box, which was covered with a white sheet and cleaned with 50% isopropanol before and after each trial. On day 1 of NOR, rats were exposed to the empty arena for 5 min for habituation to the environment to reduce stress during subsequent trial phases. After 24 h, animals were exposed to a 10 min training session with two identical sample objects in predetermined locations. All objects were weighted and adhered to the floor to avoid movement out of the scoring area. During the testing phase, animals' memory performances were assessed via their ability to discriminate between objects in novel or familiar locations (NL or FL, respectively). This testing phase occurred 24 h after the training phase and lasted for a total of 5 min. Objects were cleaned after each trial and the object in the novel location was randomly exchanged throughout trials.

Trials were as described above. Trials were scored by a researcher blind to the animal's identity. Contact with objects was defined as previously described (Bevins and Besheer, 2006).

Tissue collection: After decapitation, brains were removed and hemisected. One half of the brain was cut at the optic chiasm and 5 mm posterior to the brain, placed in cassettes with freezing medium on dry ice and flash-frozen at − 80 °C. Slices were made using a LEICA cryostat cleaned with RNase inhibitor and UV-sterilized prior to use. Four 10 μm single-hemisphere slices were mounted onto PEN membrane glass slides (Applied Biosystems) for use in laser-capture microdissection. The remaining hemisphere was immersed in oxygenated (95%/5% O2/CO2) ice-cold cutting saline (containing the following (in mM): 110 sucrose, 60 NaCl, 3 KCl, 1.25 NaH2PO4, 28 NaHCO3, 0.5 CaCl2, 7 MgCl2, 5 glucose, 0.6 ascorbate) prior to removal of the whole hippocampus and subdissection under a dissection microscope for isolation of areas CA1, CA3, and dentate gyrus. These samples were frozen on dry ice and stored at − 80 °C.

Laser-capture microdissection. Hippocampal region CA1 pyramidal layer tissue samples were collected via laser-capture microdissection. Hippocampal slices were dried with 70% EtOH followed by rehydration with Milli-Q H2O (Millipore, Billerica, MA, USA). Next, tissues were stained with 1% cresyl violet staining solution (Sigma-Aldrich, St. Louis, MO, USA) with 5% SUPERase-IN RNase inhibitor (Ambion (now Life Technologies), Grand Island, NY, USA) followed by additional application of 1% cresyl violet solution without RNase inhibitor. Excess cresyl violet was washed with H2O

followed by 70% EtOH, 30 s, 95% EtOH, 30 s, and 2 washes of 10 0% EtOH, 30 s. Xylene was added to tissues and allowed to dry prior to loading into the LCM machine. Laser settings used for microdissection were power: 75–85 mW and pulse: 1600–3000. Cells were captured onto CapSure HS LCM Caps (Arcturus (now Life Technologies), Grand Island, NY, USA), incubated for 30 min at 42 °C in extraction buffer from the AllPrep DNA/RNA mini kit (Qiagen, Ve nlo, Netherlands). Total RNA was extracted immediately after this incubation according to the manufacturer's instructions. RNA was stored at − 80 °C.

Real-time RT-PCR: Total RNA extracted was quantified spectrophotometrically using a Thermo-Scientific Nano-Drop. mRNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. PCR amplifications were performed either in an iQ5 real-time PCR system (Bio-Rad), or in a CFX96 real-time PCR system (Bio-Rad), using Biorad iQ SYBR mastermix, SsoAdvanced SYBR mastermix according to manufacturer's instructions.

Histone extraction: Histone extractions were performed as previously described (Gupta et al., 2010; Gupta-Agarwal et al., 2012; Ryley Parrish et al., 2013). Briefly, all procedures were performed on ice with solutions chilled to 4 °C and all centrifugation steps were performed at 4°C. Tissue from each hippocampal subfield was Dounce homogenized using no more than 6 strokes of a glass pestle (Kontes Glass) in ice-cold homogenization buffer containing the following (in mM): 250 sucrose, 50 Tris, pH 7.5, 25 KCL, 0.5 phenymethylsulfonyl fluoride, 1% protease inhibitor mixture (Sigma, St.

Lous, MO, USA), and 0.9 Na+butyrate. Tissue homogenates were centrifuged at 7700× g for 1 min. The pellet was resuspended in 0.5 mL of 0.4N H2SO4, incubated for 30 min, and centrifuged at 14,000× g for 10 min. Proteins were precipitated from the supernatant by the addition of 250 µ L of 100% trichloroacetic acid containing 4 mg/m L deoxycholic acid (Na+ salt, Sigma) for 30 min. Histone proteins were collected by centrifugation at 14,000× g for 30 min and the resulting pellet was washed with 1 mL acidified acetone (0.1% HCl) followed by 1 mL acetone for 5 min each. Between washes, protein precipitates were collected by centrifugation at 14,000× g for 5 min. Finally, the acid purified histone proteins were resuspended in 10 mM Tris, pH 8 and stored at − 80 °C until Western blotting.

Western blotting: Protein concentrations were determined using a DC protein assay (Bio-Rad) and aliquots of samples were normalized to 0.2 µ g/ µ L. Laemmli sample buffer (final concentration: 6.25 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 1.25% 2-mercaptoethanol, 0.1% bromophenol blue) was added to samples prior to performing SDS-PAGE on a 12% acrylamide resolving gel with a 4% acrylamide stacking gel. Histone proteins were transferred to Immobilon-FL polyviny lidene difluoride (PVDF) membranes (Millipore) for immunobloting, during which PVDF membranes were incubated in primary antibodies for 1 h at room temperature or overnight at 4 °C followed by incubation in secondary antibodies for 1 h at room temperature. Immunostained proteins were detected via the Odyssey Infrared Imaging System (LI-COR). Primary antibodies were obtained from Millipore Biotechnology and diluted in 1:1 PBST:Odyssey Blocking Buffer (LI-COR) as follows : anti-H3K4me3 (1:500), anti-H3K9me2 (1:500),

and anti-H3 (1:1000). In all cases, the primary anti body host was rabbit. The secondary antibodies were goat anti-rabbit IRDye 800CW and IRDye 700DX fluorescent antibodies (LI-COR) diluted 1:20,000 in 1:1 PBS:Odyssey Blocking Buffer (LI-COR).

Chromatin immunoprecipitation: ChIP analysis was performed as previously described (Gupta et al., 2010; Gupta-Agarwal et al., 2012). Briefly, area CA1 of the hippocampus was microdissected and placed in ice-cold PBS solution containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/mL protease inhibitor cocktail (Sigma) and phosphatase inhibitors (1 mM Na3VO4 and 10 mM NaF)). Tissue was incubated in 1% formaldehyde in PBS at 37,°C for 10 min prior to homogenization in SDS lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS). Chromatin was sheared using a Branson Sonifier 250 at 1.5 power and constant duty cycle. Lysates were centrifuged to remove debris and then diluted 1:10 in ChIP dilution buffer (16.7 mM Tris, pH 8.1, 0.01% SDS, 1.1% Triton X-100, 167 mM NaCl , 1.2 mM EDTA). Immunoprecipitations were carried out at 4 °C overnight with the primary antibody (anti-H3 K4me3) or no antibody (control). Immune complexes were collected with a protein Aagarose bead/salmon sperm slurry and then washed with low salt buffer (20 mM Tris, pH 8.0, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl), high salt buffer, (20 mM Tris, pH 8.1, 0.1% SDS, 1% Triton X-100, 500 mM NaCl, 1 mM EDTA), LiCl immune complex buffer (0.25 M Li Cl, 10 mM Tris, pH 8.1, 1% deoxycholicacid, 1% IGEPAL-CA630, 500 mM NaCl, 2 mM EDTA), and TE buffer. 1× TE c ontaining 1% SDS was used to extract immune complexes. Protein-DNA cross-links were reverted by heating at 65 °C overnight and proteins were digested by proteinase K (100 μg, 2 h at

37°C). DNA was extracted by phenol/chlorophorm/isoamyl alcohol and ethanol-precipitated. Immunoprecipitated DNA was analyzed via quantitative real-time PCR using primers specific for 150–200 bp segments corresponding to promoters upstream of the rat Bdnf or Zif268 transcription start site.

DISCUSSION

Post-translational modifications of histone proteins, including histone phosphorylation, acetylation and methylation, have emerged as crucial regulators of transcripti onal activity during LTM formation (reviewed in (Lubin, 2011; Lubin et al., 2011)). Interestingly, histone lysine methylation marks have been shown to cause downstream effects on mechanisms via recruitment of specific chromatin-modifying enzymes (Reviewed in (Jarome and Lubin, 2014, 2013; Lubin, 2011; Lubin et al., 2011; Rudenko and Tsai, 2014)). Such studies suggest a critical role for histone lysine methylation in the development of age-associated cognitive deficits relative to histone acetylation; however, the role of hippocampal histone lysine methylation changes in the context of age-related cognitive decline had not been previously explored. In the present study, we investigated the role of hippocampal histone lysine methylation levels changes in age-associated LTM impairments and made several important findings. We found that advanced age corresponds strongly with elevated resting H3K4me3 levels in multiple regions of the hippocampus. Additionally, we found that age-related memory impairment was strongly associated with alterations in histone lysine methylation levels in the aged hippocampus. Inhibition of the LSD1 histone demethylase in young adults resulted in increased H3K4me3 and H3K9,K14ac levels and decreased H3K9me2 levels in the

hippocampus. We further observed that manipulating histone lysine methylation levels via inhibition of the LSD1 histone demethylase in young adults reproduced the age-associated increases in baseline resting H3K4me3 levels in area CA1 of the hippocampus, and similarly impaired performances in multiple hippocampus-dependent memory tasks. While we cannot discount the possibility of off-target effects resulting from systemic t-PCP treatment, previous work has demonstrated the ability of t-PCP and derived LSD1 inhibitors to be brain penetrant and impact hippocampus-dependent memory formation (van Praag et al., 1999). Given that LSD1 can target H3K9me2 for demethylation (Sun et al., 2010), one would expect increases in H3K9me2 levels with LSD1 inhibition; however, we observed significant decreases in H3K9me2. These results suggest a global shift towards more transcriptionally active chromatin, or alternatively a loss of gene silencing. Intriguingly, a similar effect is known to occur during the aging process; loss of heterochromatin (Wood et al., 2010). Collectively, our results and these findings are suggestive of a shift from a transcriptionally repressed to a transcriptionally active epigenetic landscape with aging, which is consistent with observed changes in histone acetylation, yet produced memory impairments in young adults. Next, we examined the effects of EE on histone me thylation levels in the aged hippocampus and LTM formation impairments with age. We demonstrated that EE improves performance in hippocampus-dependent memory tasks and found that EE reversed learning-induced Bdnf gene expression concomitant with changes in H3K4me3 levels at the Bdnf exon IV promoter in the aged hippocampus. This suggests that histone lysine methylation changes in the aged-hippocampus are reversed with EE and associated with rescue of age-related memory impairments with EE therapy. Interestingly, previous studies have demonstrated

that baseline histone lysine methylation marks including the repressive H3K9me3 and H3K27me3 marks are decreased at the Bdnf gene locus after three to four weeks EE in a mouse model (Kuzumaki et al., 2011). While not directly comparable due to differences in study design, our present results add to these prior findings by demons trating that learning induced differences in histone methylation in the aged hippocampus, which is in strong correlation with normalization of learning induced BDNF gene expression after EE. Together, these results suggest that EE regulates Bdnf expression through both baseline and learning induced changes in histone lysine methylation. Given the complex nature of the EE protocol, and its significant impact on animal health, it is quite likely that other potentially significant genes are also regulated. While our data suggest that these critical memory genes may be involved, recent studies suggest that histone lysine methylation pathways themselves are in fact a principal contributor to cognitive disorders (Network and Pathway Analysis Subgroup of Psychiatric Genomics Consortium, 2015). In conclusion, we observed increased baseline resting histone lysine methylation levels (H3K4me3, H3K9me2) in the aged hippocampus. We found that manipulating baseline resting histone lysine methylation levels in area CA1 of young adults with previously in tact memory led to the dysregulation of both histone methylation and acetylation levels in the hippocampus of young adults during memory formation, which resulted in memory impairments. EE reversed age-associated memory impairments and increased Bdnf transcription in association with increases in H3K4me3 levels at Bdnf promoter 4 in the aged hippocampus in response to object learning. These findings provide insights into histone lysine methylation-mediated transcriptional changes in the aged hippocampus, and implicate histone lysine methylation as a newly identified molecular mechanism

affected by EE, involved in the restorative effect of EE on age-related memory deficits. Future studies should focus on the interrogati on of EE-induced histone methyltransferases and histone demethylase activity to better identify potential therapeutic targets for the treatment of age-associated memory deficits.

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FIGURES

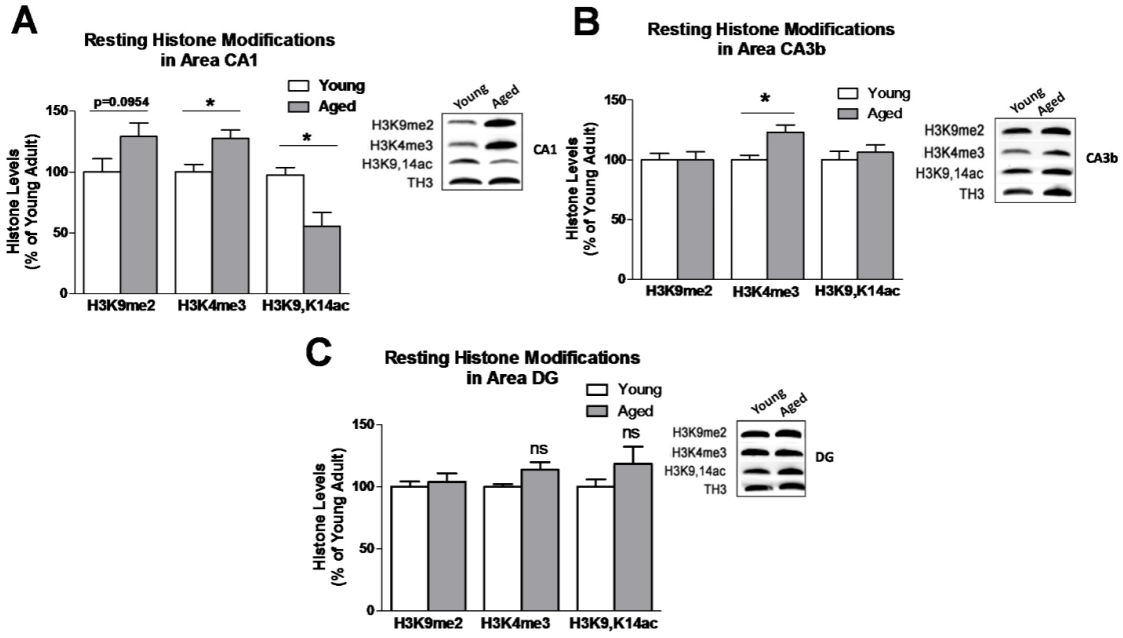

Figure 1. Resting histone modification levels in the aging hippocampus. Animals were sacrificed directly from their home cages and histone modification levels were assessed in aged adults (19-22 mo) compared to young adults (3 mo). In area CA1 (A), we observed significantly different resting levels of H3K4me3 and H3K9,K14ac between young and aged animals (young H3K9me2, n = 5; aged H3K9me2, n = 6; young H3K4me3, n = 9; aged H3K4me3, n = 10; young H3K9,K14ac, n = 6; aged H3K9,K14ac, n = 6); In region CA3b (B), we observed increased H3K4me3 (young H3K9me2, n = 6;

aged H3K9me2, n = 6; young H3K4me3, n = 4; aged H3K4me3, n = 6; young H3K9,K14ac, n = 6; aged H3K9,K14ac, n = 6). In the dentate gyrus (DG) region (C), we observed no differences in resting state levels of examined histone modifications between age groups (young, n = 6; aged, n = 6). Histone levels are presented as a percentage of the young adult group. Student's t-test; * $p < 0.05$ compared to young adults. Data are shown ± SEM.

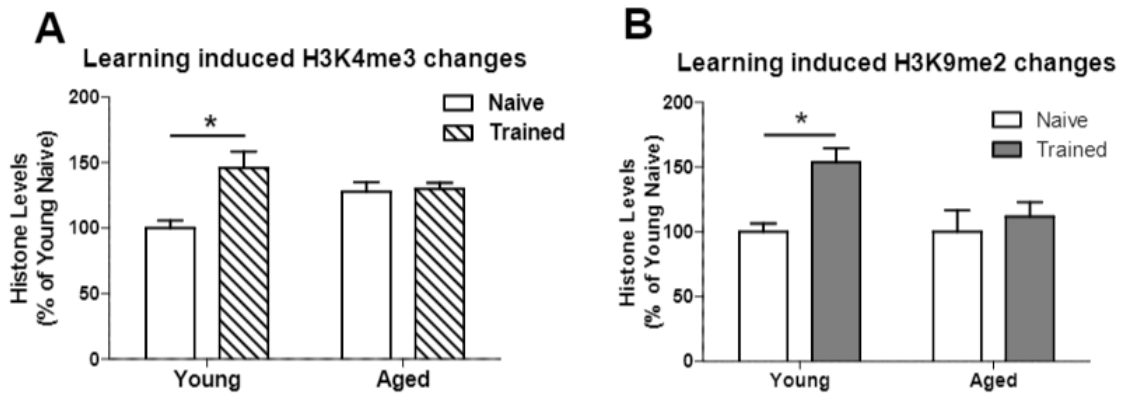

Figure 2. Learning-induced histone H3 methylation levels are altered in the aged hippocampus. Young and aged adults were sacrificed from their home cages (naïve) or at 1 h after training in NOR paradigm (Trained). Histone modification levels were detected by Western blotting and quantified by optical densitometry. H3K4me3 and H3K9me2 levels were assessed in area CA1 of the hippocampus. (A) Significant increases in H3 K4me3 were detected in young-trained adults relative to young-naïve adults. A significant interaction between aging and learning (training) was detected, and learning-dependent increases in H3K9me2 levels seen in young adults were disrupted in aged adults; (B) Significant increases in H3K9me2 levels were detected in young-trained adults relative to young-naïve adults. No significant interaction was detected between aging and training; however, learning (training) induced increases in H3K9me2 observed in young animal s were disrupted in aged animals. Results are presented as a percentage of young adult naïve. Group sizes: young naïve, n = 3; young trained, n = 5; Aged naïve, n = 4, Aged trained, n = 6. Two-Way ANOVA; * $p < 0.05$. Data are shown ± SEM.

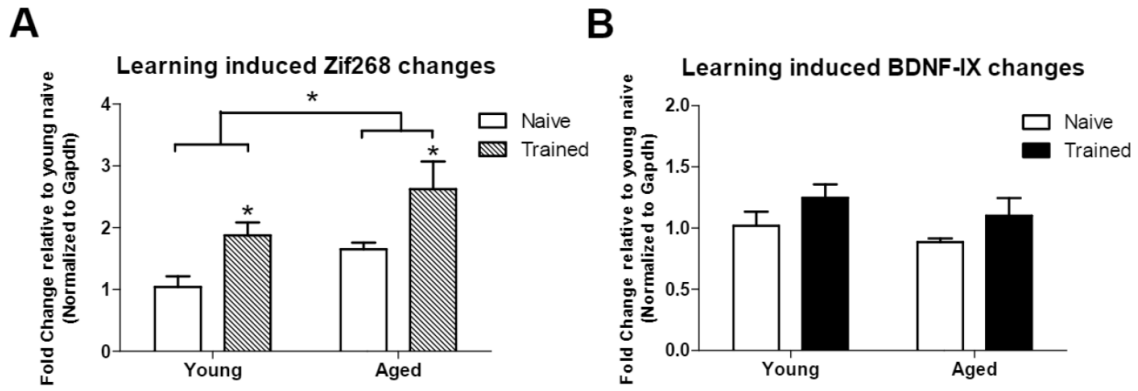

Figure 3. Learning induced changes in memory relate d gene expression in area CA1 of aged adults. Young and aged adults were sacrificed from their home cages (Naïve) or 1 h after training in NOR (Trained). H3K4me3 and H3K9me2 were assessed in hippocampal regions CA1. (A) Zif268 mRNA levels are significantly increased after NOR training; (B) Bdnf mRNA levels were not significantly altered with training. Results are presented relative to young-naïve adults. Group sizes: young naïve, n = 4; young trained, n = 6; aged naïve, n = 4; aged trained, n = 6. Two-Way ANOVA; * $p < 0.05$. Data are shown ± SEM.

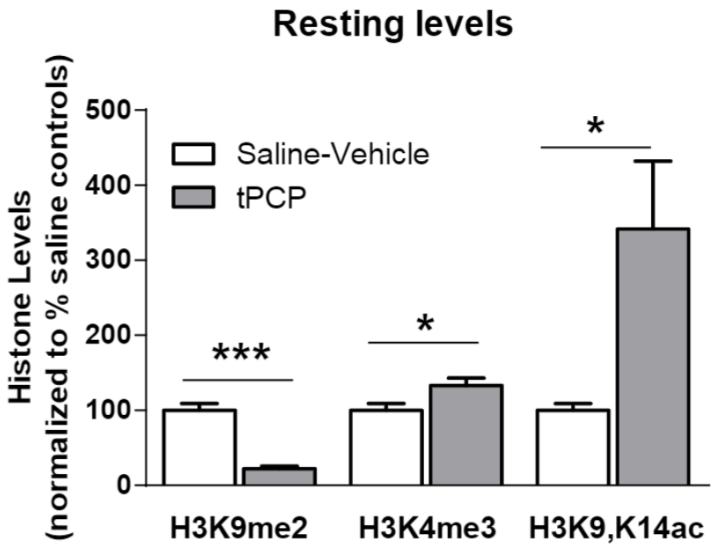

Figure 4. LSD1 inhibition alters baseline resting histone modification levels in area CA1 of young adults. Animals were given IP injections of 3 mg/kg t-PCP or saline-vehicle and the effect of LSD1 inhibition was assessed on H3K9me2, H3K4me3, and H3K9,K14ac levels in area CA1. Histone H3 modifications were normalized to total histone H3 protein levels. Histone modification levels are presented relative to the percentage of saline-vehicle controls. Group sizes: saline H3K9me2, n = 4; tPCP H3K9me2, n = 4; saline H3K4me3, n = 4; saline H3K9,K14ac, n = 4, tPCP H3K9,K14ac, n = 3. Student's t-test; * $p < 0.05$, error bars represent the SEM.

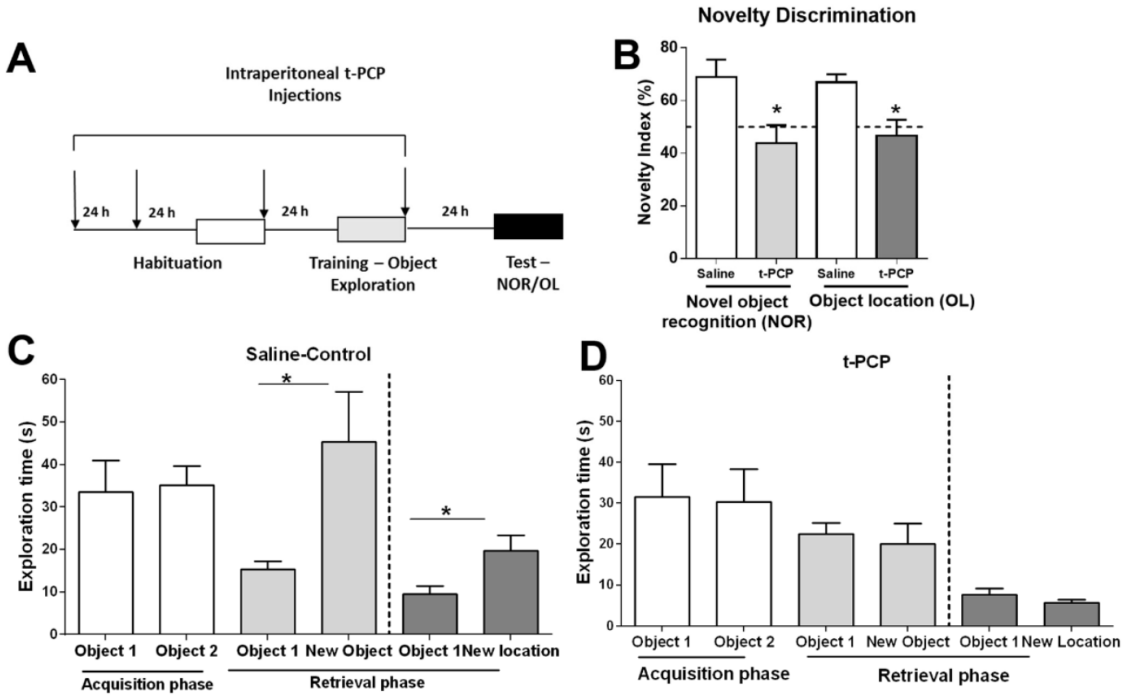

Figure 5. LSD1 inhibition impairs memory formation in young adults. (A) Diagram of the experimental design for the novel object recognition (NOR) and object location (OL) learning paradigms. Animals were IP injected with t-PCP or saline-vehicle as indicated; (B – D) LSD1 inhibition impaired performance in NOR and OL learning tasks; (B) Discrimination index [calculated as NS/(FS+NS)*100; FS = familiar stimulus; NS = novel stimulus], with an index ≥ 50% indicating levels of memory retention. Novelty exploration data for saline-vehicle controls (C) and t-PCP-treated animals (D) are presented as the total duration, in seconds, of exploration per object. During the training or acquisition phase, objects 1 and 2 represented two identical sample objects. During the testing or retrieval phase, object 1 represented the familiar object or location, and the new object or location are represented as such. Group sizes NOR: Saline, n = 6; tPCP, n = 4; OL: Saline, n = 5; tPCP, n = 5. Student's t-test; ns = not significant, * $p < 0.05$, Error bars represent the SEM.

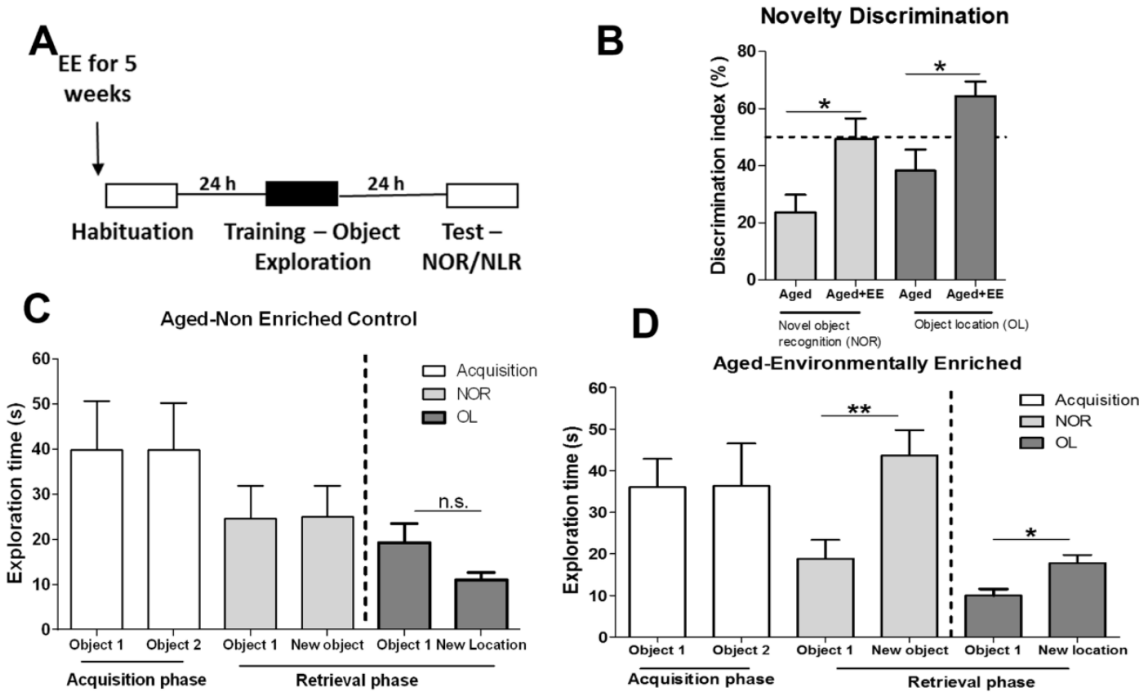

Figure 6. Environmental enrichment restores memory formation in aged adults. (A) Aged adults experience a 5-week EE protocol prior to behavioral experiments. Age-matched non-enriched controls received similar handling procedures; (B) A novelty discrimination index indicate that memory impairment in aged adults was rescued with EE. Object exploration during training and testing phases (NOR and OL) for non-enriched aged controls (C) and aged-enriched adults (D) are presented as the total duration, in seconds, of exploration. Objects 1 and 2 represent the sample objects during acquisition. During the retrieval phase, object 1 represents the familiar object or familiar object location. Group sizes NOR: Aged, n = 5; Aged + EE, n = 6; OL: Aged, n = 4; Aged + EE, n = 5. Student's t-test; * $p < 0.05$ between indicated groups. * $p < 0.05$, ** $p < 0.01$ between indicated groups. Error bars represent SEM.

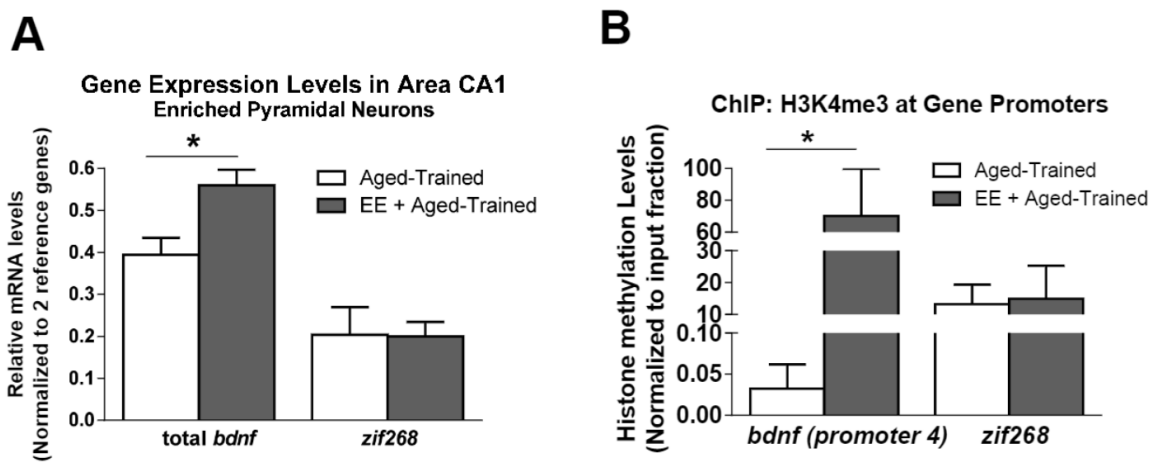

Figure 7. Environmental enrichment elevates H3K4me3 methylation levels at the Bdnf gene in the aged hippocampus. (A) Object Learning increases Bdnf mRNA level in the hippocampus of EE aged adults (n = 4) relative to non-enriched aged adults (n = 5); (B) ChIP analysis revealed EE dependent increases in H3K4me3 levels at the Bdnf promoter 4 during memory formation; Group sizes for (B): Aged-Trained, n = 4; EE + Aged-trained, n = 3. Student's t-test; * $p < 0.05$ compared to non-enriched aged-trained adults. Data are shown ± SEM.

LNCRNA NEAT1 DRIVES NEURONAL HISTONE METHYLATION AND AGE-RELATED MEMORY IMPAIRMENTS

by

Anderson A. Butler, Daniel R. Johnston, Simranjit Kaur, and Farah D. Lubin*

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ABSTRACT

Histone methylation is critical for the formation and maintenance of long-term memories. Long noncoding RNAs (lncRNAs) are regulators of histone methyltransferases and other chromatin modifying enzymes (CMEs). We investigated how lncRNA *Neat1*-mediated histone methylation contributes to hippocampus-dependent long-term memory formation, using a combination of transcriptomics, RNA binding protein immunoprecipitation, CRISPR mediated gene activation, and behavioral approaches. Suppression of the lncRNA *Neat1* revealed widespread changes in gene transcription as well as perturbations of histone 3 lysine 9 dimethylation (H3K9me2), a repressive histone modification mark that is dysregulated in the aging hippocampus. We identified a Neat1-dependent mechanism of transcriptional repression via H3K9me2 at the *c-Fos* promoter corresponding with observed changes in *c-Fos* mRNA levels. Overexpression of hippocampal *Neat1* via CRISPRa is sufficient to impair memory formation in young adults, recapitulating observed memory deficits in old adults, while *Neat1* suppression in both young and old adult mice improves memory. These results suggest that lncRNA *Neat1* is a potent epigenetic regulator of hippocampus-dependent long-term memory formation.

INTRODUCTION

While recent efforts have characterized thousands of lncRNAs in the human and mammalian genome, few lncRNAs are as well-studied, as the human Nuclear-Enriched Abundant Transcript 1 (*NEAT1*). *NEAT1* is evolutionarily conserved between rodents and humans, particularly within the 5' region of the transcript(Hutchinson et al., 2007). Multiple isoforms of *NEAT1* exist in rodents and in humans, with the longer of the major isoforms proving essential for phase separation and induction of nuclear paraspeckle assembly(Clemson et al., 2009; Yamazaki et al., 2018), while the shorter *NEAT1* transcripts do not appear to be a major regulator of paraspeckle formation(Li et al., 2017). Recent studies have characterized a number of molecular pathways by which *NEAT1* regulates the epigenome, including both paraspeckle-dependent sequestration of transcription factors, as well as paraspeckle-independent roles for *NEAT1* in transcriptional regulation via scaffolding of CMEs(Chen et al., 2018; Imamura et al., 2014; Li and Cheng, 2018). Additionally, *NEAT1* itself has been observed to bind numerous genomic loci and to effect regulation of transcription(Chakravarty et al., 2014; West et al., 2014).

Research on the human *NEAT1* has been largely focused on its role as an oncogene in various cancers (as reviewed previously(Yu et al., 2017)), which occurs largely through its regulation of epigenetic mechanisms. However, the rodent homolog *Neat1* is also upregulated in the hippocampus of aging mice(Stilling et al., 2014) and has recently been linked to multiple cognitive and neurodegenerative disorders, including schizophrenia(J. Li et al., 2018), Huntington's Disease(Sunwoo et al., 2017), Parkinson's Disease(Liu and Lu, 2018; Yan et al., 2018), Alzheimer's Disease(Puthiyedth et al.,

2016), and epilepsy(Barry et al., 2017; Lipovich et al., 2012). Furthermore, recent evidence suggests that *Neat1* may play a role in neuroplasticity(Barry et al., 2017); however, despite such extensive health relevance, the role of *Neat1* in epigenetic regulation of genes within hippocampal neurons, particularly during long-term memory formation. We used RNA-sequencing, CRISPR mediated gene activation (CRISPRa), and memory tests to investigate the functional role of lncRNA *Neat1* in gene expression dynamics and the role that age-related changes in *Neat1* expression might play in memory deficits in older adults.

RESULTS

Expression of the Long Noncoding RNA NEAT1 is Restricted in Human CNS Tissues

Expression of *NEAT1* is abundant in many cultured cell lines including those characterized in the ENCODE project(ENCODE Project Consortium, 2012) (Fig. 1A). However, we observed that in contrast to the abundant expression of *NEAT1* observed in most tissues, the human central nervous system (CNS) as a whole, as well as the hippocampus (outlined in red, Figs. 1B-C) express minimal quantities of *NEAT1(Carithers et al., 2015; GTEx Consortium, 2013)*. Unsupervised hierarchical clustering based on tissue expression of *NEAT1* supports this observation, as CNS tissues segregate cleanly when sorted based on *NEAT1* transcript expression (Fig. 1D, Supplemental Figs. 1A-B).

Examination of single-cell RNA-seq data from resected human CNS tissue and glioblastoma(Darmanis et al., 2017) further suggests that expression of *NEAT1* within CNS cells is restricted in neurons, while other cell types including astrocytes,

oligodendrocytes, and vascular cells express *NEAT1* at higher levels. (Supplemental Figs. 1C-D). This is in contrast to the neighboring lncRNA transcript MALAT1 which appears to be ubiquitously expressed at high levels in all CNS cell types (Supplemental Fig. 1E). Given the growing body of literature that has noted overexpression of *NEAT1* in the aging brain(Barry et al., 2015; Pereira Fernandes et al., 2018), as well as the established role of *NEAT1* as a regulator of epigenetic mechanisms, and the recently-described role of *NEAT1* in cognitive disorders such as schizophrenia(J. Li et al., 2018), we sought to further investigate the role of the lncRNA *NEAT1* on the neuroepigenetic mechanisms of cognition.

NEAT1 Regulates the Immediate Early Gene c-Fos Involved in Synaptic Plasticity

To investigate the role of *NEAT1* at the transcriptomic level, we analyzed a publicly available RNA-seq dataset from iPSC-derived human neurons. Antisense oligo (ASO) knockdown of *NEAT1* in KCl-treated human neurons revealed an extensive cohort of differentially expressed mRNAs. Knockdown alone was not sufficient to perturb the transcriptome in resting iPSC-derived human neurons, as evidenced by an imperfect separation via unsupervised hierarchical clustering prior to KCl stimulation (Fig. 2A). In contrast, *NEAT1*-knockdown appears to dramatically potentiate KCl-driven differential expression of many genes (Fig. 2A-B).

To gain some insight into the health relevance for observed *NEAT1*-mediated changes in gene expression in human neurons, we queried the annotated disease classes from the Genetic Association Database via the Database for Annotation, Visualization and Integrated Discovery (DAVID)(Huang et al., 2009), and observed significant enrichment

for three disease classes: cancer, renal, and aging. (Supplemental File 2). *NEAT1*-regulated genes appear to be non-randomly distributed among annotated biological processes (Fig. 2C), molecular functions (Supplemental File 2) and cellular components (Fig. 2D). Significant gene ontology (GO) term enrichment was partially consistent with previous observations of the *NEAT1* regulatory axis, as we observed significant regulation of GO terms associated with viral gene expression; however, we also observed significant enrichment of GO terms important for hippocampal function, including the transcription factor AP-1 complex (GO:0035976, Fig. 2C-D).

The human Fos proto-oncogene (*FOS*, also known as *C-FOS*), a critical component of the AP-1 transcription factor subunit appeared to be overexpressed in human neurons after knocking down *NEAT1* both in quiescent and KCl-stimulated neurons and has a known relevance for hippocampus-dependent memory formation(Fleischmann et al., 2003) (Figs. 2E-F). Thus, we selected the murine homolog *c-Fos* as a candidate gene for further studies of *Neat1*'s regulatory potential

Neat1 is Regulated by Neuronal Excitability and Controls c-Fos Gene Expression

As modeling *Neat1* expression changes in response to *in vivo* neuronal activity and behavioral experience required the mammalian model organisms, we next sought to examine the regulatory capacity of *Neat1* in rodent neurons. For this purpose, we knocked down murine *Neat1* in the mouse Neuro-2a (N2a) cell line using small interfering RNAs (siRNAs). We observed that 24h after treatment with *Neat1*-targeting siRNAs (Fig. 3A), expression of the *c-Fos* mRNA was significantly upregulated (Fig. 3B).

Interestingly, while our observations of c-*Fos* transcript expression in murine neurons recapitulated observations from human neurons, we observed that expression of the immediate early genes Egr1 and Btg2 were not overexpressed in mouse (Supplemental Fig. 2A-B) as they were in human neurons (Supplemental File 2), suggesting that there are species-specific regulatory differences in the *Neat1* regulatory axis.

As mice with the *c-Fos* gene knocked out in the CNS show a specific loss of hippocampus-dependent spatial and associative learning tasks(Fleischmann et al., 2003), we next sought to investigate the relevance of *Neat1* expression during memory consolidation after a hippocampus-dependent learning task. One hour after training in contextual fear conditioning we observed a significant reduction in the expression of *Neat1* in the dorsal hippocampus coinciding with previously reported increases in expression of the *c-Fos* mRNA (Figs. 3C-D). As baseline expression of *Neat1* in neurons is expected to be quite restricted compared to other cell types, we next stimulated neurons with KCl to ascertain the effect of activity on the expression of *Neat1*. Consistent with recent reports(Barry et al., 2017), we observed that KCl stimulation drives a rapid reduction in *Neat1* expression in both N2a cells (Supplementary Fig. 2E) and primary hippocampal pyramidal neurons, as recently reported (Figs. 3E-F), and consistent with the effects of context fear conditioning *in vivo*.

Neat1 Regulates H3K9me2 Globally and Controls c-Fos Promoter H3K9me2 and Gene Expression

We next sought to investigate the *c-Fos*-relevant mechanisms of *Neat1*-orchestrated transcriptional control. To accomplish this, we used publicly available data

assaying *Neat1* chromatin binding via capture hybridization analysis of RNA targets and high-throughput sequencing (CHART-seq) in human MCF7 cells(West et al., 2014). After mapping *Neat1*-bound peaks to the nearest transcription start sites, we observed that only a small subset of genes directly bound by *Neat1* are differentially expressed either after *Neat1* knockdown or in the context of neuronal activation. However, we observed significant enrichment of *Neat1* binding near genes associated with histone methyltransferase activity, including the H3K9 dimethyltransferase Ehmt1 (also known as GLP) (Figs. 4A-B; Supplemental File 3).

As *c-Fos* has previously been observed to be regulated by the Ehmt1/2 complex in the context of hippocampus-dependent memory formation(Gupta-Agarwal et al., 2012), we next sought to investigate the role of *Neat1* in the regulation of histone methylation and H3K9me2 specifically. After knockdown of *Neat1* in neuronal cells, we observed that H3K9me2 is reduced at a global scale while the expression of other histone modifications are unchanged (Fig. 4C-F). To ascertain whether the lncRNA *Neat1* physically associates with the H3K9me2 methyltransferase complex in neurons, we performed RNA binding protein immunoprecipitations against the Ehmt2 subunit of the obligatory Ehmt1/2 heterodimer(Gupta-Agarwal et al., 2012; Sharma et al., 2017; Tachibana et al., 2008). Consistent with recently published results(Li and Cheng, 2018), we observed interaction between *Neat1* and the H3K9me2 methyltransferase Ehmt2, suggesting multiple possible modes of action for *Neat1*-mediated regulation of H3K9me2.

To assess the functional relevance of the *Neat1*-H3K9me2 regulatory axis on the expression of *c-Fos* mRNA, we performed ChIP in conjunction with qPCR at the *c-Fos* promoter. We observed that after *Neat1* knockdown with siRNAs, H3K9me2 at the *c-Fos*

promoter was significantly depleted (Fig. 4G), consistent with observed changes in gene expression (Fig. 3B), while H3K9me2 within the *c-Fos* gene body were not significantly changed (Supplementary Fig. 2F).

Neat1 Knockdown Regulates Hippocampal Memory Formation and the Epigenetic Landscape at the c-Fos Promoter *in vivo*

Having demonstrated that *Neat1* represses the epigenetic landscape and neuronal expression of the memory-critical *c-Fos* gene, we next sought to investigate the functional role of *Neat1* expression on *c-Fos* promoter methylation and memory formation *in vivo*.

To ask whether *Neat1* expression impacts hippocampus-dependent memory formation, we knocked down expression of *Neat1* in hippocampal area CA1 by directly infusing *Neat1*-targeting siRNAs or non-targeted siRNAs and assayed long term memory function using contextual fear conditioning, a hippocampus-dependent memory task (Fig. 5A). We observed that five days after intra-CA1 injection of *Neat1*-targeting siRNAs, a time when we observe significant reduction in expression of *Neat1* (Supplemental Fig. 3A), mice had no differences in freezing behavior during the training phase of contextual fear conditioning, either before or after delivery of the foot shock (Fig. 5B). However, when returned to the training context 24h later, mice that received *Neat1*-targeting siRNAs displayed significant increases in freezing behavior relative to mice that received non-targeting siRNAs (Fig. 5C).

To determine whether *Neat1* expression impacts *c-Fos* promoter methylation *in vivo*, we sacrificed an additional cohort of behaviorally naïve animals five days after injection of siRNAs and performed ChIP-qPCR assays on one hemisphere of dorsal CA1

tissue collected from around the injection site. Consistent with our results in cultured neurons (Fig. 4G), we observed that concurrently with *Neat1* knockdown five days after infusion with siRNAs, *Neat1* knockdown significantly reduced H3K9me2 at the *c-Fos* promoter in dorsal area CA1 (Fig. 5E). Thus, we hypothesized a model in which *Neat1* expression might be regulating memory formation via epigenetic repression of *c-Fos*.

Mimicking Age-Related Upregulation of Neat1 is Sufficient to Respectively Restore or Impair Hippocampus-Dependent Memory Formation

Numerous studies have reported overexpression of *Neat1* in senescing cells, as well as in aging CNS tissues in both humans and mice(Akbarian, 2010; Stilling et al., 2014). Upon comparing publicly available hippocampus RNA-seq datasets from 3 month-old young mice versus 24 month-old aged mice we observed upregulation of *Neat1* relative to young animals, consistent with previously reported results(Stilling et al., 2014) (Fig. 6A), as well as downregulation of *c-Fos* gene expression (Fig. 6B), consistent with previously reported age-associated hippocampus-dependent memory impairments(Stilling et al., 2014).

We next tested whether hippocampus-dependent memory formation might be improved in aged mice by knockdown of *Neat1*. To this end, we knocked down expression of *Neat1* in the hippocampal area CA1 of 18-19 month-old mice, an age at which we have previously observed significant upregulation of H3K9me2 in the aging rat hippocampus(Morse et al., 2015) (Supplemental Fig. 4), by directly infusing *Neat1*-targeting siRNAs or non-targeted siRNAs and assayed long term memory function using contextual fear conditioning, with three pairings of the shock to the novel context (Fig.

6C). We observed that knockdown of *Neat1* in the dorsal hippocampus of aged mice resulted in significant improvements in freezing after 24h (Figs. 6D-E), but not during training, similar to results seen in young mice (Fig. 5C).

We next sought to test the sufficiency of *Neat1* overexpression to regulate performance in memory tasks, we designed a single guide RNA (sgRNA) targeting *Neat1* for overexpression from the endogenous locus (Fig. 7A, Supplemental Fig. 3B-C), and delivered the CRISPRa system *in vivo* into dorsal CA1 via *in vivo* transfection (Fig. 7B). Mice were then trained in contextual fear conditioning with three pairings of the shock to the novel context (Fig. 6D). Animals overexpressing *Neat1* from the endogenous locus (*Neat1*-OE) had no significant differences in freezing during the training period, either before or after exposure to the unconditioned stimulus (Fig. 6E); however, when returned to the training context 24 h after training, *Neat1*-OE animals froze significantly less than control animals which received only the sgRNA plasmid (Fig. 6F), suggesting that elevated *Neat1* in area CA1 is sufficient to impair hippocampus-dependent memory formation.

METHODS

Animal housing. Naïve 3-7 month-old or 18 month-old C57BL/6 mice were group housed (2-7 animals/cage) in plastic cages with *ad libitum* access to food and water and were maintained on a 12-h light/dark cycle. All behavioral tests were conducted during the light cycle, and all procedures were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee and done in accordance with the National Institute of Health ethical guidelines.

Cell culture. N2A cells were maintained in DMEM supplemented with 10% FBS. After thawing, the cells were passaged a minimum of two times prior to use in experiments. The cells were kept at 37°C in a 5% CO_2 incubator. Dissociated cultures of hippocampal pyramidal cells were obtained from embryonic day 18 rat embryos as described previously (Meadows et al., 2015). Briefly, timed-pregnancy female Sprague-Dawley rats were terminally anesthetized and embryos were removed from the uterus, then transferred to Hank's balanced salt solution (HBSS, Gibco) for dissection. Primary rat hippocampal neurons were dissociated via incubation with papain for 20 min at 37°C, rinsed in HBSS, then resuspended in Neurobasal medium (Gibco) and further mechanically dissociated by passing through a series of progressively smaller fire-polished glass Pasteur pipettes. The resulting suspension was passed through a 70-µm cell strainer and plated on poly-L-lysine coated 24-well plates (~$7.5x10^4$ cells per well). Cells were maintained for 2 weeks in Neurobasal supplemented with B-27 and Glutamax (Thermo Fisher Scientific) at 37°C and 5% CO_2. For KCl stimulation, 6.25µL 1M KCl (Sigma) was added to two-week in vitro cultures, for a final concentration of 12.5mM KCl.

siRNA Delivery. Young, 3-7 month-old mice were anesthetized with an intraperitoneal injection of ketamine-dexmedetomidine and received bilateral intra-CA1 injections of Lincode SMARTpool siRNAs (Dharmacon) targeting the murine *Neat1* (#R-160022-00-0005) or a negative control (#D-001320-10-05), conjugated with *in vivo* JetPEI (PolyPlus Transfection), an *in vivo* transfection reagent, at the stereotaxic coordinates (AP -2.0mm, ML ±1.5 mm, DV−1.7 mm) with respect to bregma. Aliquots of siRNA stocks (100µM) were diluted to a concentration of ~2.5µM and conjugated with *in vivo* JetPEI on

the day of surgery. Infusions were given over a 10 min period (0.1μL per min) for a total volume of 1μL per hemisphere. After a 48 h recovery period, mice were handled daily for >3min and trained in contextual fear conditioning at five days post-surgery. Aged (18-19 month old) mice were treated similarly but were anesthetized with vaporized isoflurane (3% induction, 2% maintenance). Mice were sacrificed at ten days post-surgery and dorsal area CA1 was harvested from each hemisphere.

CRISPRa Delivery. Mice were anesthetized with an intraperitoneal injection of ketamine-dexmedetomidine and received bilateral intra-CA1 injections of a guide RNA expression vector driven by the murine U6 promoter and targeting the murine *Neat1* promoter region (Addgene #44248) either alone or in conjunction with an expression vector coding for the S. pyogenes dCas9 fused to two copies of the VP64 transactivator domain (Addgene #59791). Endotoxin-free plasmids were purified using an endotoxin-free plasmid DNA purification kit (Machery-Nagel) and aliquoted to minimize freeze-thaw cycles. Endotoxin-free plasmid stocks were diluted to a final concentration of ~500ng/uL in sterile 10% glucose and incubated with in vivo JetPEI for 15 min at room temperature on the day of surgery. The resulting transfection complex was delivered via direct infusion at the stereotaxic coordinates (AP -2.0mm, ML ±1.5 mm, DV−1.4 mm) with respect to bregma. Infusions were given over a 10 min period (0.1μL per min) for a total volume of 1μL (~500ng plasmid DNA) per hemisphere.

Contextual fear conditioning. Mice were trained to either a weak or strong contextual fear conditioning (CFC) paradigm in a novel context, and long term memory

was assessed upon returning the animals to the training context 24h after training. The weak CFC paradigm consisted of a 118s baseline followed by a single shock (0.5mA, 2 sec) pairing in the novel context, while the strong CFC paradigm consisted of a 119-sec baseline followed by three shock pairings (0.5mA, 1s) with interleaved rest periods of 59 sec each. Twenty-four h after training, animals were placed back into the training context for five min to test retention. Freezing behavior was scored by Med Associates software.

Collection of whole area CA1. One hour after training, the whole brain was removed by gross dissection and placed in oxygenated (95%/5% O_2/CO_2) ice-cold cutting solution (110mM sucrose, 60mM NaCl, 3mM KCl, 1.25mM NaH_2PO_4, 28mM $NaHCO_3$, 0.5mM $CaCl_2$, 7mM $MgCl_2$, 5mM glucose, and 0.6mM ascorbate). The CA1 region of the hippocampus was then microdissected from each hemisphere and flash frozen on dry ice.

Collection of dorsal area CA1. Animals were sacrificed by cervical dislocation after overdosing with isoflurane at experiment-specific time points, and the whole brain was rapidly removed and immediately frozen on dry ice. The CA1 region of the dorsal hippocampus was then dissected out with the aid of a mouse brain matrix (Harvard Apparatus) to collect the area of CA1 targeted by siRNA or CRISPRa infusions. All tissue was stored at -80°C prior to processing.

Western blotting. Normalized proteins (2-10µg) were separated via electrophoresis on either 10% or 20% polyacrylamide gels, transferred onto an Immobilon-FL membrane using a turbo transfer system (Biorad). Membranes were blocked in Licor blocking buffer

and probed with the following primary antibodies for histone H3 (1:1000; Abcam #ab1791), H3K9me2 (1:1000; Millipore #07-441), H3K27me3 (1:1000; Millipore #07-449), H3K4me3 (1:1000; Millipore #04-745). Secondary goat anti-rabbit 700CW antibody (1:20,000; Licor Biosciences) was used for detection of proteins using the Licor Odyssey system. All protein quantification was done using ImageStudio Lite software (Licor).

Reverse transcription qPCR (RT-qPCR). Section text RNA was extracted from isolated CA1 or cultured cells using Trizol reagent according to the manufacturer's recommended protocol (Fisher). RNA yield was quantified spectrophotometrically (Nanodrop 2000c), and ~200ng of RNA was DNAse treated (Amplification grade DNAse I, Sigma), converted to cDNA (iScript cDNA synthesis kit; Biorad), and PCR amplified on the CFX1000 real-time PCR system (BioRad), with primer annealing temperatures of 60°C. See supplemental table for full descriptions of primers used. All data were analyzed using the delta delta Ct method.

Cell culture. ChIP was performed as described previously(Jarome et al., 2015; Morse et al., 2015). Briefly, samples were fixed in PBS with 1% formaldehyde for ten minutes at room temperature, chromatin was sheared using a Bioruptor XL on high power, lysates cleared by centrifugation and diluted in TE and RIPA buffer. Extracts were mixed with MagnaChIP protein A/G beads and immunoprecipitations were carried out at 4°C overnight with 5µg primary antibody (anti-H, Abcam #ab40542; anti-Ezh2, #ab3748) or no antibody (control). Immune complexes were sequentially washed with low salt buffer (20 mM Tris, pH 8.0, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl), high

salt buffer (20 mM Tris, pH 8.1, 0.1% SDS, 1% Triton X-100, 500 mM NaCl, 1 mM EDTA), LiCl immune complex buffer (0.25 M LiCl, 10 mM Tris, pH 8.1, 1% deoxycholic acid, 1% IGEPAL-CA630, 500 mM NaCl, 2 mM EDTA), and TE buffer, and eluted into 1xTE containing 1% SDS. Protein-DNA crosslinks were reversed by heating at 65°C overnight. After proteinase K digestion (100µg; 2h at 37°C), DNA was purified by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. Immunoprecipitated DNA was quantified via spectrophotometry (Nanodrop 2000c) and ~15ng of DNA from each sample was assayed via quantitative real-time PCR using primers specific to mouse genes of interest. See supplemental table for full descriptions of primers used.

RNA binding protein immunoprecipitation (RIP). RIP was performed as described previously(Rinn et al., 2007). Briefly, ~5ug of primary antibody against Ehmt2 (Abcam #ab40542), Ezh2(Abcam #ab3748) or normal rabbit IgG (Cell signaling) were conjugated with 25uL MagnaChIP protein A/G beads (EMD Millipore). Freshly harvested nuclear pellets from at least 10^6 N2a cells were sheared via Dounce homogenization (15-20 strokes) in RIP buffer (150 mM KCl, 25 mM Tris pH 7.4, 5 mM EDTA, 0.5 mM DTT, 0.5% NP40, 1x Protease Inhibitor Cocktail (Sigma), 100 U/ml SUPERASin (Ambion), cleared via centrifugation at 13,000 RPM to remove nuclear membrane and debris, and split into fractions for IP. Sheared nuclear extracts were mixed with antibody conjugated MagnaChIP protein A/G beads and immunoprecipitations were carried out at 4°C for four hours. Beads were then immobilized on a magnetic tube rack, and immune complexes were sequentially washed three times with RIP buffer. Beads were then resuspended in 1mL of

Trizol (Thermo Fisher), and coprecipitated RNAs were isolated according to the manufacturer's recommended protocol. RT-qPCR for *Neat1* was then performed as described above.

Statistical analyses. Data from all experiments were analyzed using Analysis of Variance (ANOVA) with Fisher LSD post hoc test or with Student's t-test unless otherwise noted in the figure legend. Values reported in the text and error bars are the mean ± SEM unless otherwise noted. All datasets were screened for outliers prior to analysis via Grubb's test (α=0.05) and outliers were subsequently excluded. Statistical tests were performed in R or Prism 7 (GraphPad). Nonparametric tests were used where appropriate and tests were 2-tailed unless otherwise noted. For all experiments, *n* indicates the number of biological replicates. For cell culture experiments, this indicates the number of independently growing flasks or wells. For experiments involving animal behavior, this indicates the number of animals used. For experiments involving tissue collection from animals, this indicates the number of animals we collected the tissue from.

GTEx data. Data from the GTEx Analysis Release V7 (dbGaP Accession phs000424.v7.p2) were obtained via the GTEx portal web tool. Expression values plotted are in transcripts per million (TPM), using the GENCODE annotated transcript for isoforms or a gene level model based on the GENCODE model with isoforms collapsed to single genes. Isoform expression values were hierarchically clustered using Euclidean distance and average linkage; dendrogram scale shows cluster distance.

Analysis of bulk RNAseq and ChIPseq data. Single or paired-end RNAseq data was imported into the public Galaxy server at usegalaxy.org directly from the European Nucleotide Archive (study accession numbers PRJEB9006 and PRJNA262674) in FASTQ format and run through a standardized workflow consisting of quality trimming via Trim Galore!(Krueger, 2015) (Galaxy Version 0.4.2), read alignment to via HISAT(Kim et al., 2015) (Galaxy Version 2.0.3), and feature counting via featureCounts (Galaxy Version 1.4.6.p5). Individual count files were grouped by treatment (Animal age) and differential expression testing was performed using DESeq2(Love et al., 2014) (Galaxy Version 2.11.39). All reference genomes and annotations were obtained from Gencode releases current at the time of analysis, including the Genome Reference Consortium Mouse Build 38 patch release 5 (GRCm38.p5) and evidence-based annotation of the mouse genome (GRCm38), version M16 (Ensembl 91), human build GRCh38 and the human annotation Release 25 (GRCh38.p7). Gene ontology (GO) enrichment was assessed using a PANTHER Overrepresentation Test web tool provided by the Gene Ontology Consortium(Ashburner et al., 2000; The Gene Ontology Consortium, 2017) (release date 2017-11-28). DAVID functional annotation was used to assess gene set enrichment for GAD_DISEASE_CLASS using default settings (DAVID 6.8).

CHART-seq data was accessed via NIH SRA Toolkit from accession PRJNA252626 and analyzed using similar read quality control and alignment tools as described above. CHART-seq peaks were called using the MACS2 algorithm(Chalei et al., 2014; Feng et al., 2012). Overlapping peaks were combined into a single peak, as recommended for input into ChIP-Enrich package. Using the ChIP-Enrich R package(Welch et al., 2014) (version 2.4.0), CHART-seq peaks from MACS2 were

assigned to the nearest transcription start site and GO Enrichment was assessed for Biological Processes and Molecular Functions.

scRNA-seq analysis. Data were obtained via the European Bioinformatics Institute's Single-cell Expression Atlas. T-distributed Stochastic Neighbor Embedding (t-SNE) plots were constructed using transcript per million (TPM) values from the transcriptomes of 3,589 single cells biopsied from four glioblastoma patients(Darmanis et al., 2017). Unbiased clusters were generated using a t-SNE perplexity of 10; plots were colored via biased inferred cell type, as reported by the authors of the dataset. Biopsied tissue included cells from the tumor core as well as peripheral tissue; however, all cells inferred to be neurons were collected from noncancerous tissue adjacent to the glioblastoma.

DISCUSSION

While previous studies have observed regulatory roles for the lncRNA *NEAT1*, including that *NEAT1* localizes to chromatin and governs chromatin modification(Chakravarty et al., 2014; West et al., 2014), little work has been done to resolve this regulatory role of *NEAT1* in the context of long-term memory formation. RNA sequencing analysis revealed that the human lncRNA *NEAT1* binds to the *EHMT1* locus and that *NEAT1* knockdown regulates neuronal *EHMT1* expression (Supplemental Data S1). We observed that murine *Neat1* acts as a potent regulator of H3K9me2 both in cultured cells and *in vivo* (see Fig. 4). Due to recent observations that *NEAT1* interacts directly with Ehmt2(Li and Cheng, 2018), an observation which we ourselves have reproduced via RIP

(see Fig. 4), we cannot yet ascertain whether transcriptional control of *EHMT1* or direct interaction with the repressor complex is the rate-limiting factor for H3K9me2 abundance. This intricate multipoint interaction is perhaps illustrative of the intricate systems of regulatory feedback which are thought to control epigenetic mechanisms. Nonetheless, knockdown of *Neat1* was sufficient to perturb this system and to result in both bulk and site-specific changes in H3K9me2 in neurons.

Previous investigations as to the epigenetic regulatory role of *Neat1* have resulted in paradoxical observations to the effect that *Neat1* binds to genomic loci and mediates activation of transcription(Chakravarty et al., 2014), but that suppression of *Neat1* expression results primarily in increased neuronal gene expression(Barry et al., 2017). We show here that *Neat1* induces widespread regulation of neuronal H3K9me2, potentially resolving this dilemma and further explaining age-related increases in H3K9me2 previously observed in the hippocampus. Moreover, we observed that *Neat1* expression is correlated with H3K9me2 globally as well as at the promoter of the aging-repressed memory-related gene *c-Fos*. While *Neat1* has been observed to act on and via numerous epigenetic mechanisms, to our knowledge this is the first observation that *Neat1*-mediated epigenetic mechanisms are sufficient to govern cognitive function.

Studies of the neuronal impact of *Neat1* expression have thus far been limited to the context of neurological disorders, and in many cases to cultured neuronal cells. Our observations suggest that *Neat1* plays a regulatory role in neuronal H3K9me2 both in cultured neurons and *in vivo*, and that increases in *Neat1* might play a significant role in the age-related decline of hippocampus-dependent memory formation. In humans, expression of *NEAT1* is generally limited in the CNS, and overexpression is a common

hallmark of several neurological disorders. While experimental reduction of *Neat1* has very recently been shown to have therapeutic potential in the context of such disorders, the impact of age-related changes in expression has remained unexplored until now. Interestingly, while our experiments were designed to investigate the age-related impact of *Neat1*, we note that recent experiments have described neuroinflammation-mediated increases in *Neat1* expression(Z. Li et al., 2018), and that the findings described in this manuscript implicate *Neat1* as a potential mechanism by which neuroinflammation might impact memory.

While the experiments described here are largely sufficient to explain prior observations of elevated H3K9me2 in the aging hippocampus (Supplementary Fig. 4;(Morse et al., 2015), our experiments indicate that increased expression of *Neat1* is not sufficient to explain all of the aging-related neuroepigenetic changes observed in this region. It is likely that many hippocampal lncRNAs have distinct or overlapping roles in the regulation of the neuroepigenetic aging process. Indeed, human *NEAT1* itself has been observed to associate with multiple chromatin modifying enzymes (Hirose et al., 2014; Li and Cheng, 2018; Murthy and Rangarajan, 2010; Spiniello et al., 2018). Although we did not detect significant regulation of histone modifications other than H3K9me2 at the global level after knockdown of *Neat1*, the absence of such observations does not preclude the existence of biologically or behaviorally meaningful epigenetic regulation that is more limited in scope and might be uncovered in future studies with a large-scale sequencing approach.

In this work, we demonstrate that *Neat1* regulates a critical transcriptional pathway for hippocampus-dependent memory in rodent neurons *in vitro, in vivo*, and

likewise in iPSC-derived human neurons. While attempts to establish the functionality of the evolutionarily conserved lncRNA *Neat1* have met with limited success, little has yet been done to functionally characterize *Neat1* in the context of cognition. Here, we observed that the lncRNA *Neat1* may serve as an endogenous molecular brake on the formation of hippocampus-dependent spatial memories.

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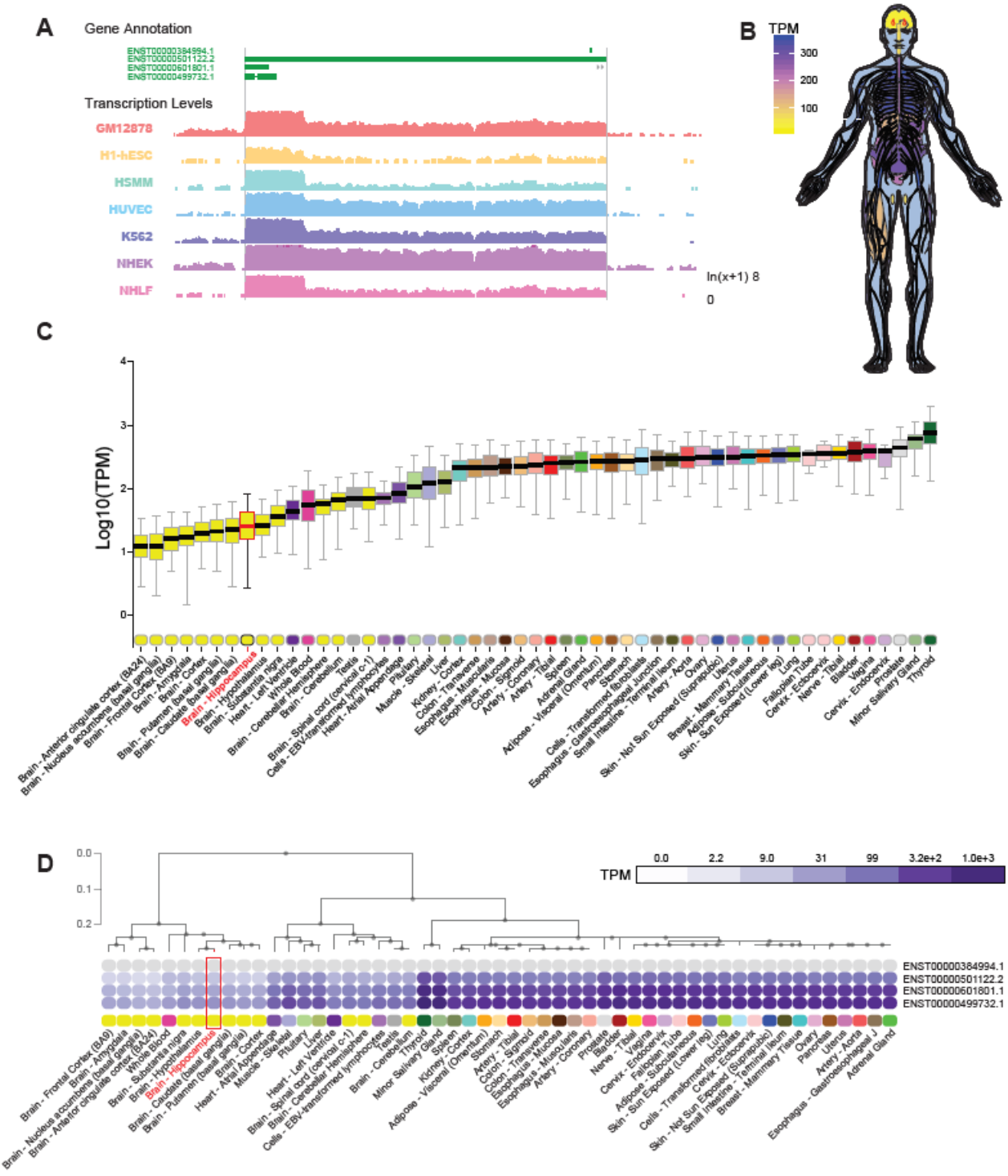
FIGURES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HSMM
HUVEC
K562
NHEK
NHLF
ln(x+1) 8
0
B
TPM
300
200
100
C
Log10(TPM)
4
3
2
1
0
Brain - Anterior cingulate cortex (BA24)
Brain - Nucleus accumbens (basal ganglia)
Brain - Frontal Cortex (BA9)
Brain - Amygdala
Brain - Cortex
Brain - Putamen (basal ganglia)
Brain - Caudate (basal ganglia)
Brain - Hippocampus
Brain - Hypothalamus
Brain - Substantia nigra
Heart - Left Ventricle
Whole Blood
Brain - Cerebellar Hemisphere
Brain - Cerebellum
Testis
Brain - Spinal cord (cervical c-1)
Cells - EBV-transformed lymphocytes
Pituitary
Heart - Atrial Appendage
Muscle - Skeletal
Liver
Kidney - Cortex
Colon - Transverse
Esophagus - Mucosa
Esophagus - Muscularis
Colon - Sigmoid
Artery - Coronary
Artery - Tibial
Spleen
Adrenal Gland
Pancreas
Stomach
Adipose - Visceral (Omentum)
Cells - Transformed fibroblasts
Esophagus - Gastroesophageal Junction
Small Intestine - Terminal Ileum
Artery - Aorta
Ovary
Uterus
Skin - Not Sun Exposed (Suprapubic)
Breast - Mammary Tissue
Adipose - Subcutaneous
Skin - Sun Exposed (Lower leg)
Lung
Fallopian Tube
Cervix - Ectocervix
Nerve - Tibial
Bladder
Vagina
Cervix - Endocervix
Prostate
Minor Salivary Gland
Thyroid
D
0.0
0.1
0.2
TPM
0.0
2.2
9.0
31
99
3.2e+2
1.0e+3
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Brain - Frontal Cortex (BA9)
Brain - Amygdala
Brain - Nucleus accumbens (basal ganglia)
Brain - Anterior cingulate cortex (BA24)
Whole Blood
Brain - Substantia nigra
Brain - Hypothalamus
Brain - Hippocampus
Brain - Caudate (basal ganglia)
Brain - Putamen (basal ganglia)
Heart - Atrial Appendage
Brain - Cortex
Muscle - Skeletal
Pituitary
Liver
Heart - Left Ventricle
Brain - Spinal cord (cervical c-1)
Brain - Cerebellar Hemisphere
Cells - EBV-transformed lymphocytes
Testis
Brain - Cerebellum
Thyroid
Minor Salivary Gland
Spleen
Kidney - Cortex
Adipose - Visceral (Omentum)
Stomach
Artery - Tibial
Colon - Sigmoid
Colon - Transverse
Esophagus - Mucosa
Esophagus - Muscularis
Artery - Coronary
Prostate
Bladder
Nerve - Tibial
Vagina
Cervix - Endocervix
Fallopian Tube
Adipose - Subcutaneous
Skin - Sun Exposed (Lower leg)
Lung
Cells - Transformed fibroblasts
Cervix - Ectocervix
Skin - Not Sun Exposed (Suprapubic)
Small Intestine - Terminal Ileum
Breast - Mammary Tissue
Ovary
Pancreas
Uterus
Artery - Aorta
Esophagus - Gastroesophageal J
Adrenal Gland

Figure 1. Restricted expression of lncRNA *NEAT1* in human CNS tissues. (A) University of California, Santa Cruz (UCSC) Genome browser track export showing expression of *NEAT1* in seven cell types from ENCODE. (B) Human body plot illustrating the expression of *NEAT1* in 53 human tissues from the GTEx project, values shown are the median transcripts per million (TPM) values by tissue, hippocampus outlined in red. (C) Bar plots showing median, upper quartile, and lower quartile expression of the *NEAT1* gene (ENSG00000245532.4) in 53 human tissues from the GTEx project; hippocampal expression outlined in red. (D) Hierarchical clustering of *NEAT1* based on transcript isoform level expression in 53 human tissues from the GTEx project. Dendrogram scale shows cluster distance. Expression values displayed in the heatmap are the median expression values in TPM for each isoform in each tissue.

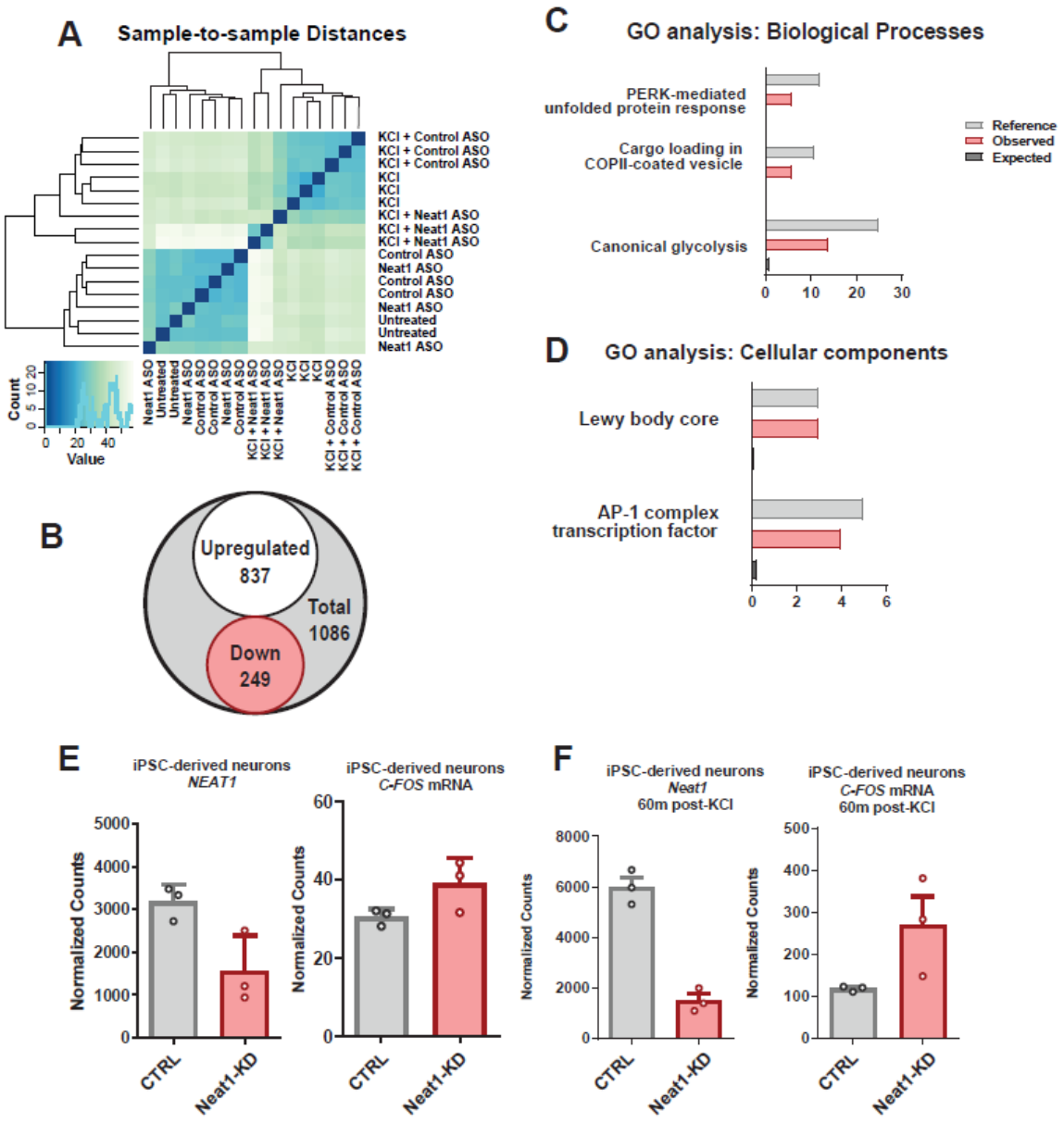


Figure 2. *NEAT1* regulates expression of *C-FOS* mRNA and the AP-1 complex in iPSC-derived human neurons. (A) Unsupervised hierarchical clustering transcriptomes from *Neat1* and KCl treated iPSC-derived human neurons, based on DESeq2-normalized counts (B) Venn diagram depicting the total number of differentially expressed genes detected between KCl+Control_antisense oligonucleotide (ASO) and KCl+*Neat1*_ASO groups via DESeq2. (C-D) Gene Ontology (GO) term enrichment for DE genes depicted in panel B.

All GO terms shown showed statistically significant enrichment (BH corrected $p < 0.05$) (E-F) Normalized count values for lncRNA *NEAT1* and *C-FOS* mRNA either prior to (E) or after (F) KCl treatment of iPSC-derived neurons.

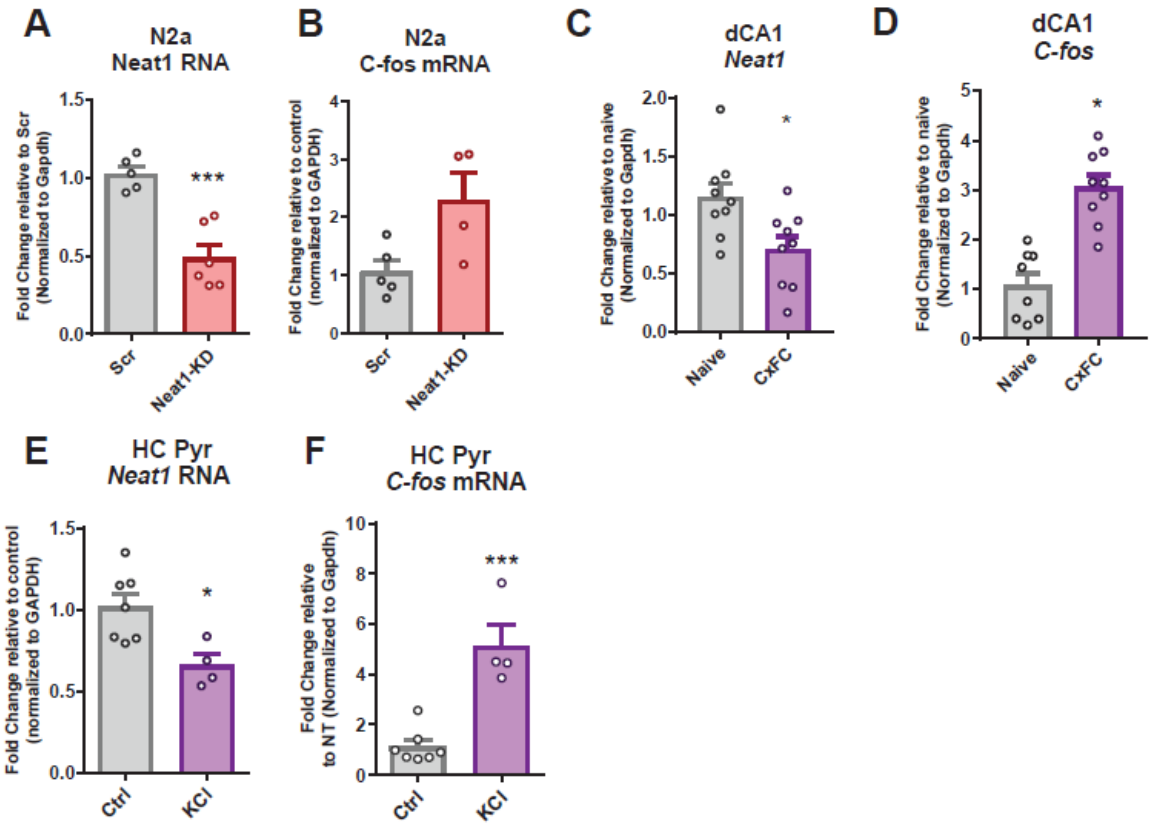
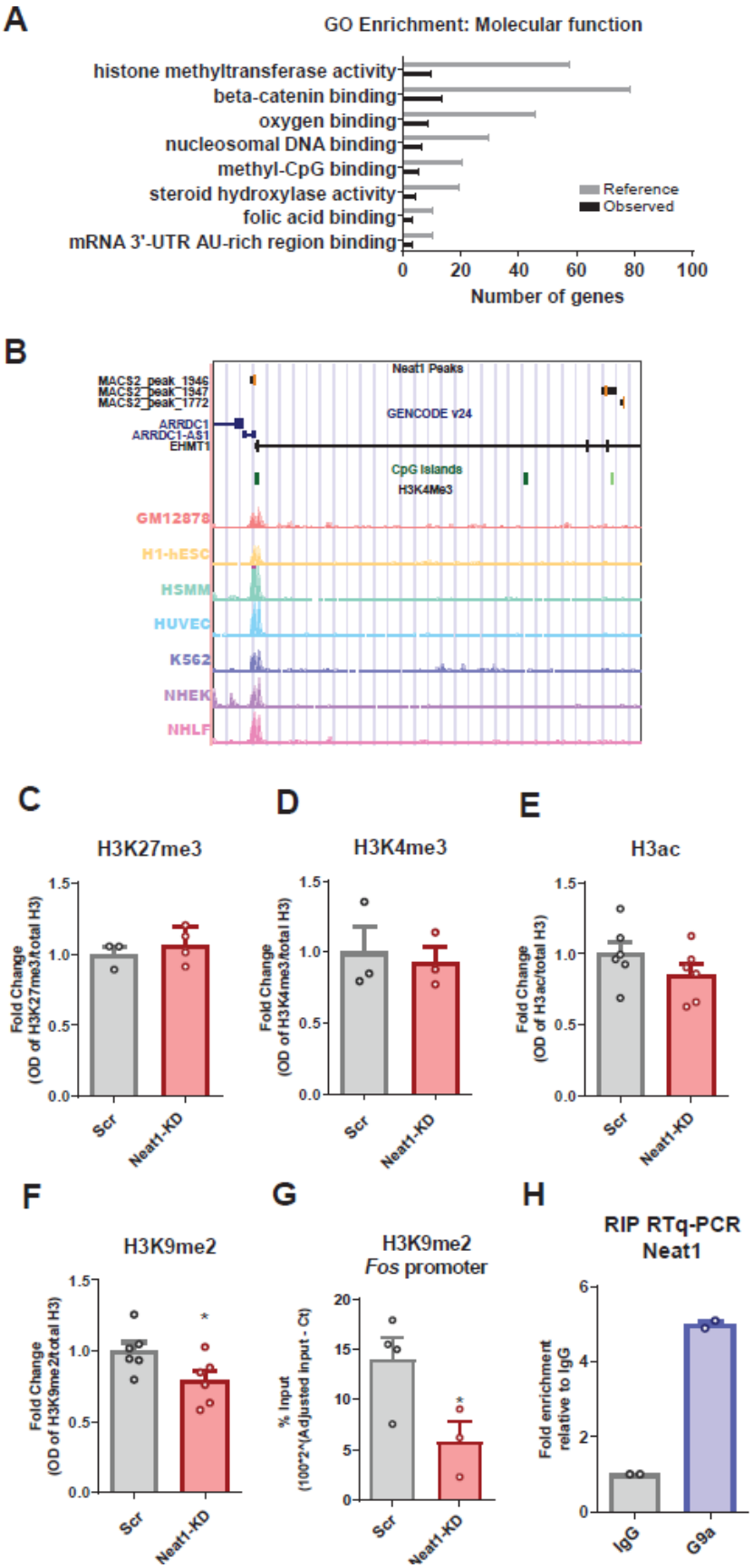


Figure 3. *Neat1* regulates expression of *c-Fos* mRNA in murine neuronal cells (A) siRNA treated murine N2a cells show significantly reduced abundance of *Neat1* transcript. (n = 5,6; $p < 0.0005$) (B) Expression of *c-Fos* mRNA after treatment with *Neat1*-targeting siRNAs (n = 5,4; $p = 0.0335$). (C-D) *Neat1* expression is decreased (C; $n = 9,9$; $p < 0.0148$) and *c-Fos* expression is increased (D; n = 8,9; $p < 0.0001$) *in vivo* in dorsal CA1 1h after training in contextual fear conditioning (E-F) Depolarization of rodent primary pyramidal neurons with KCl is sufficient to significantly reduce expression of *Neat1* (E; n = 7,4; p = 0.0147) and reproduce commonly observed increases in *c-Fos* transcription (F; n = 7,4; p = 0.0003).


A
GO Enrichment: Molecular function
histone methyltransferase activity
beta-catenin binding
oxygen binding
nucleosomal DNA binding
methyl-CpG binding
steroid hydroxylase activity
folic acid binding
mRNA 3'-UTR AU-rich region binding
Reference
Observed
0
20
40
60
80
100
Number of genes
B
Neat1 Peaks
MACS2_peak_1946
MACS2_peak_1947
MACS2_peak_1772
GENCODE v24
ARRDC1
ARRDC1-AS1
EHMT1
CpG Islands
H3K4Me3
GM12878
H1-hESC
HSMM
HUVEC
K562
NHEK
NHLF
C
H3K27me3
Fold Change (OD of H3K27me3/total H3)
Scr
Neat1-KD
D
H3K4me3
Fold Change (OD of H3K4me3/total H3)
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Neat1-KD
E
H3ac
Fold Change (OD of H3ac/total H3)
Scr
Neat1-KD
F
H3K9me2
Fold Change (OD of H3K9me2/total H3)
Scr
Neat1-KD
G
H3K9me2
Fos promoter
% Input (100*2^(Adjusted Input - Ct)
Scr
Neat1-KD
H
RIP RTq-PCR
Neat1
Fold enrichment relative to IgG
IgG
G9a

Figure 4. *Neat1* modulates neuronal H3K9me2. (A) *NEAT1* CHART-seq peaks were mapped to the nearest gene transcription start site, and functional enrichment was assessed using ChIP-ENRICH, with histone methyltransferase activity being noted as a significantly enriched GO term (BH corrected $p < 0.05$ for all terms shown). (B) UCSC genome browser plot showing *NEAT1*-binding peaks overlapping the human *EHMT1* gene. (C-F) Graphs depicting changes in histone modifications in N2a cells after siRNA knockdown of *Neat1* (C) H3K27me3 (n = 3; p = 0.4716) (D)H3K4me3 ($n = 3$; $p = 0.7548$) (E)H3ac (n = 6; p = 0.2377) (F) H3K9me2 ($n = 6$; $p = 0.0456$) (G) ChIP-qPCR assay indicating a loss of H3K9me2 at the *c-Fos* gene promoter in N2a cells (n = 4,3; p = 0.0472) (H) RNA binding protein immunoprecipitation for Ehmt2/*Neat1* interaction (n = 2)

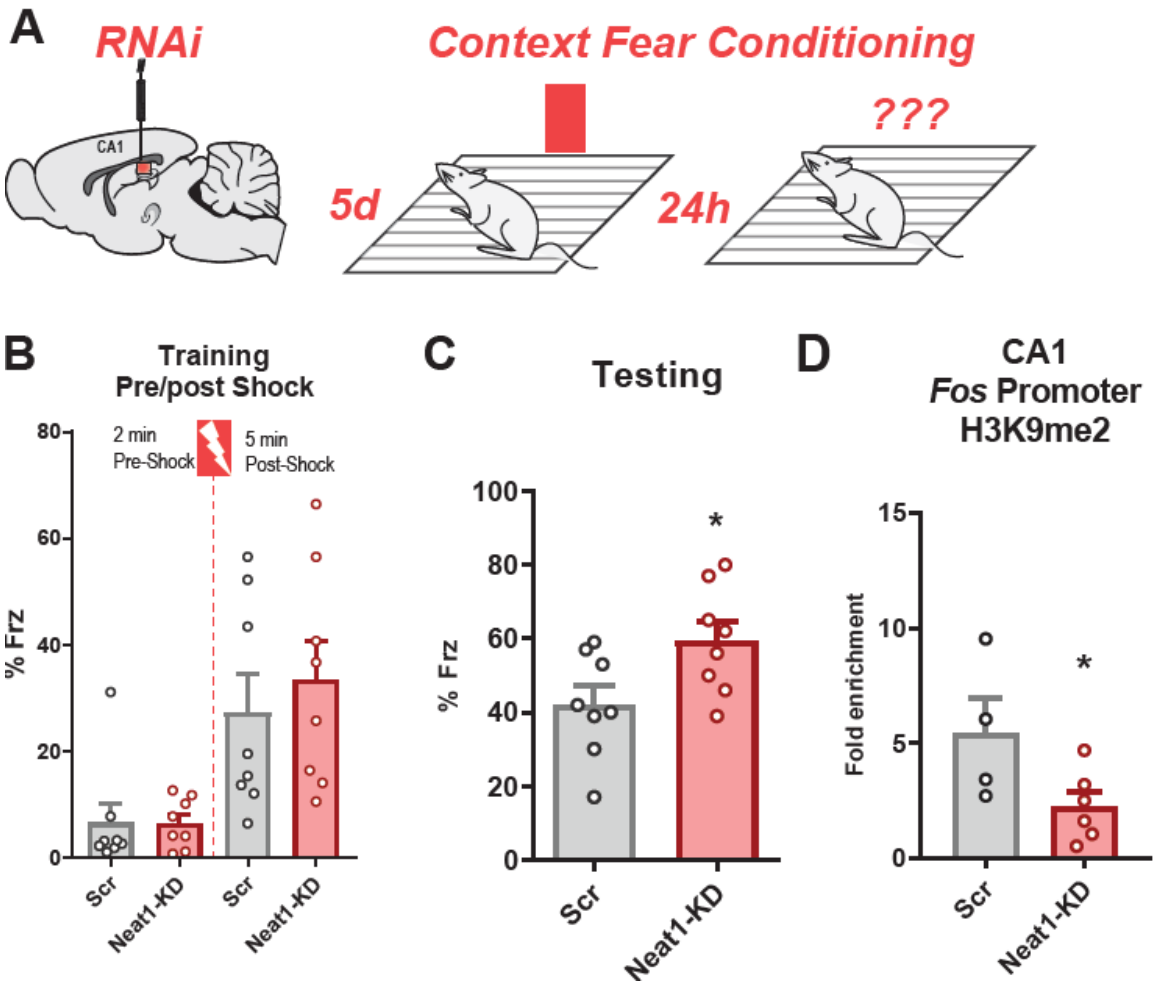


Figure 5. *Neat1* knockdown regulates *c-Fos* promoter methylation *in vivo* and improves long-term memory (A) Graphic depiction of siRNA infusion into hippocampal area CA1 and single-pairing contextual fear conditioning paradigm. Briefly, male C57BL/6 mice were trained 5d after bilateral infusion of siRNAs and tested 24h after training. (B) Freezing behavior as a percent of epoch during training phases of the contextual fear conditioning paradigm. No significant difference detected for either the Pre-shock (n = 8; p = 0.9826) or Post-shock (n = 8; p = 0.5626) epochs. (C) Freezing behavior as a percent of total time during the 5-min test trial (n = 8; p = 0.0307). (D) *c-Fos* promoter H3K9me2

remained depleted after *Neat1*-knockdown 5d after the conclusion of behaviour experiments (n = 18,18; p = 0.0450)

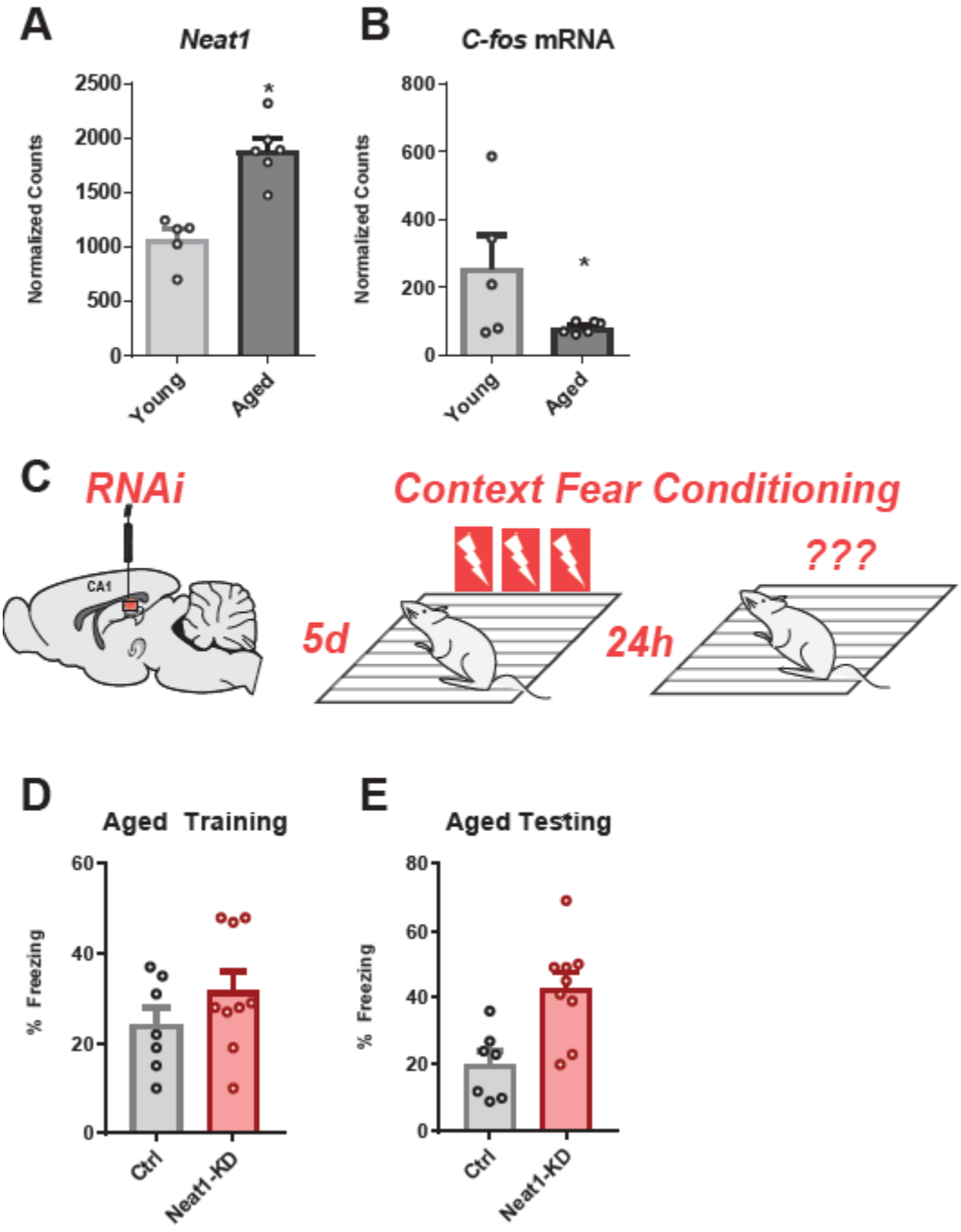


Figure 6. *Neat1*-knockdown improves long-term memory in aged animals (A-B) DESeq2-generated normalized counts of RNAseq data from 3mo and 24mo C57/B6 mice. *Neat1* expression (A) was significantly elevated and *c-Fos* mRNA (B) was significantly repressed in aged hippocampi relative to the hippocampi of young mice. (C) Graphic depiction of

siRNA infusion into hippocampal area CA1 and single-pairing contextual fear conditioning paradigm. Briefly, 18mo old male C57/B6 mice were trained 5d after bilateral infusion of siRNAs and tested 24h after training. (D-E). Aged mice (18 month-old) were trained with three pairings of shock with a novel context after knockdown of *Neat1* and demonstrated no significant difference during training (D) (n = 7,9; p = 0.2496), but significantly enhanced freezing 24h after testing (E) (n = 7,9; p = 0.0039).

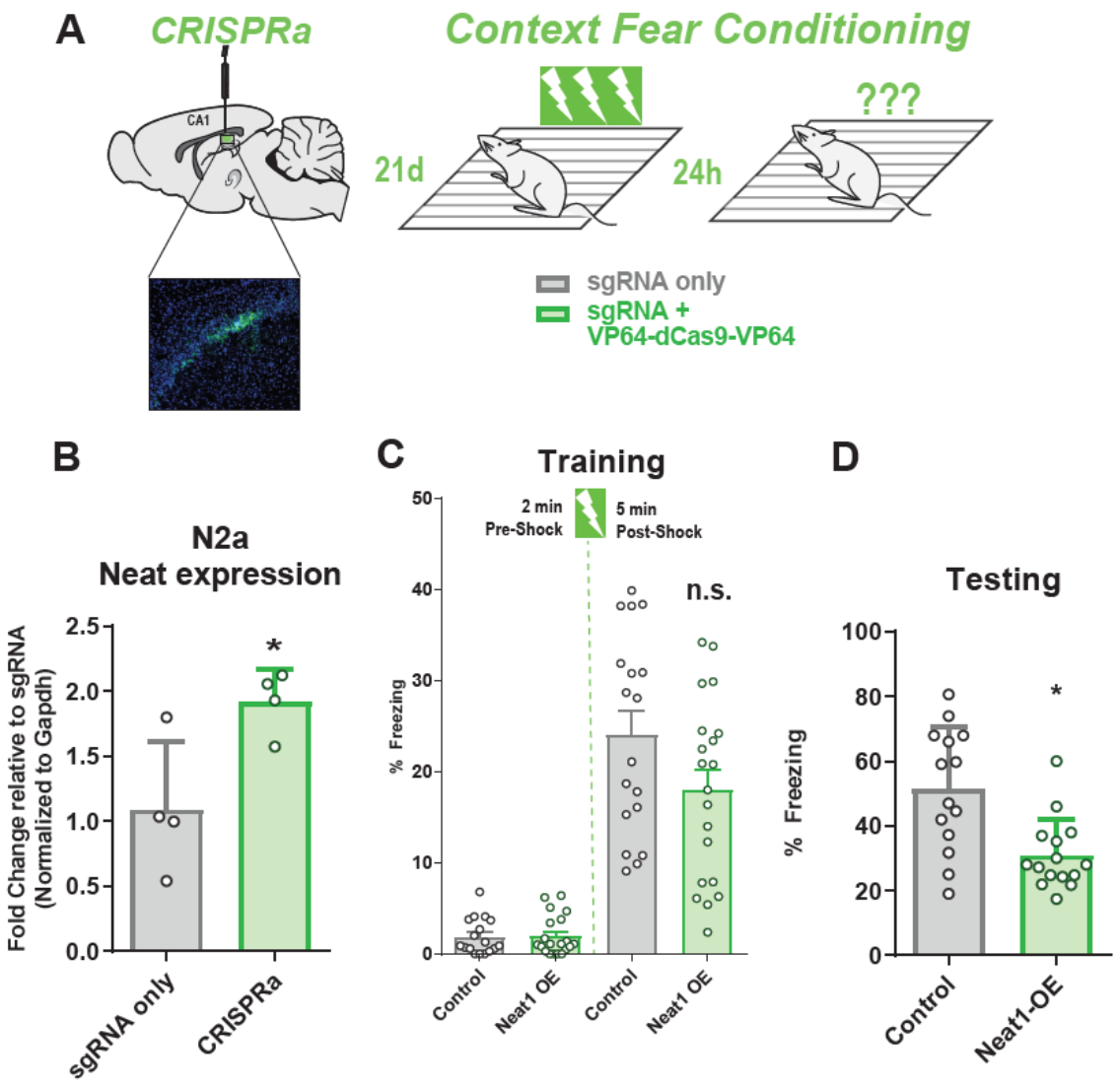
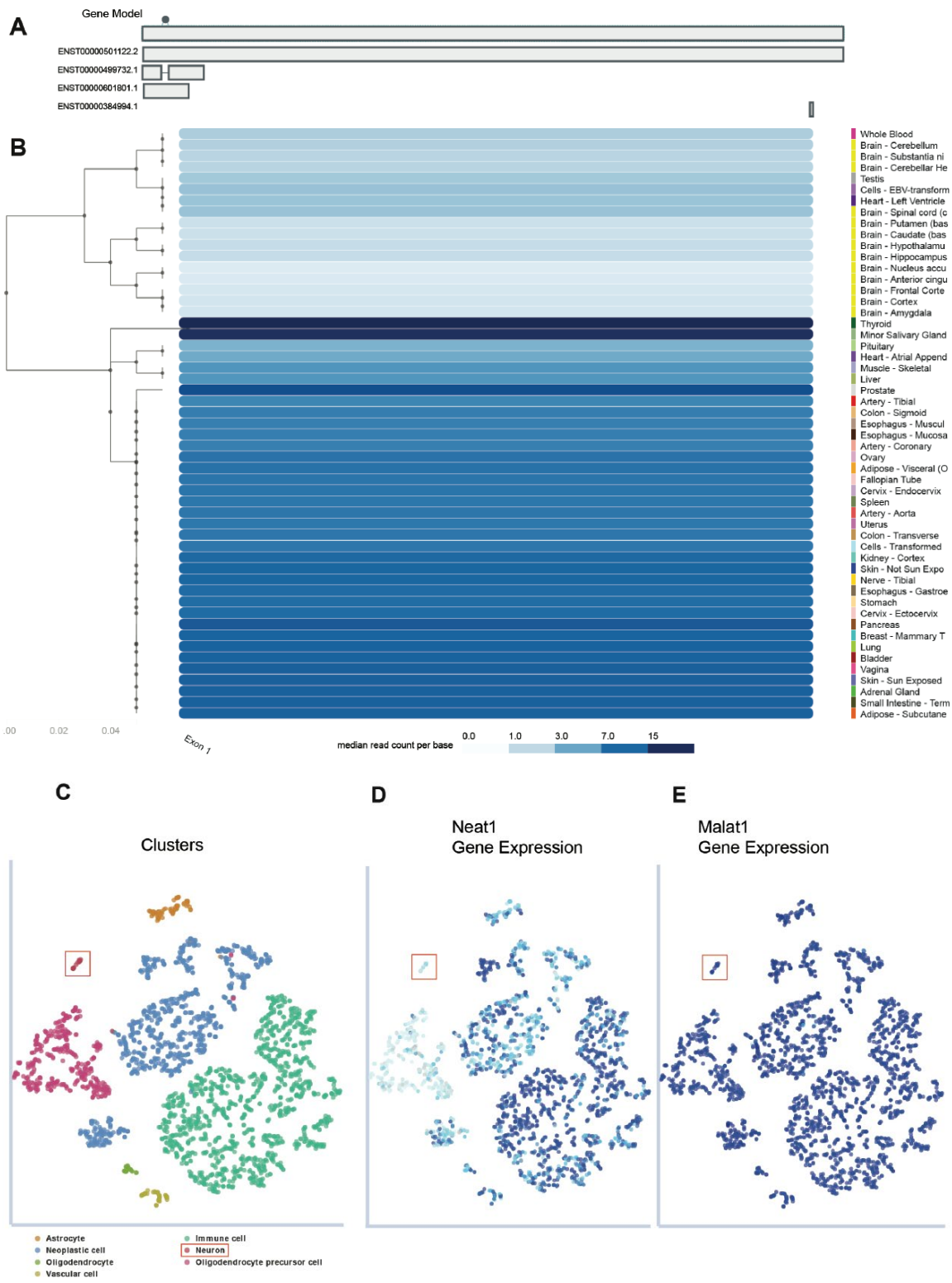


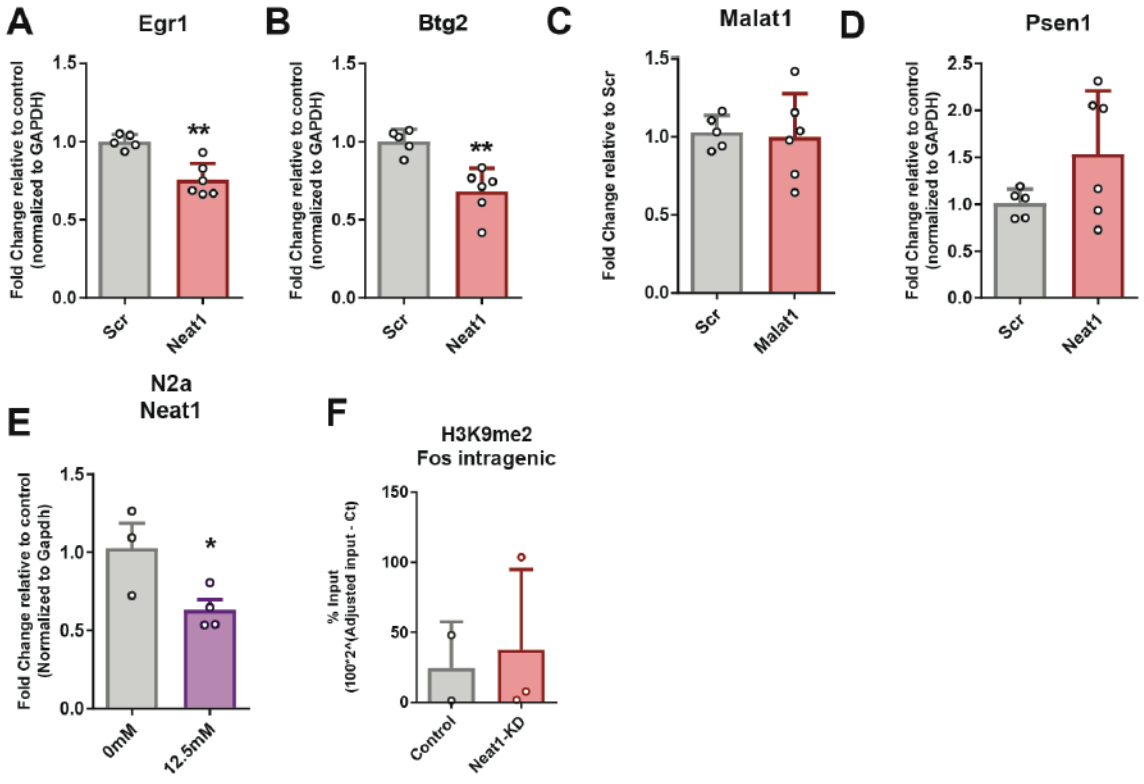
Figure 7. Mimicking age-related *Neat1* overexpression via CRISPRa impairs hippocampal memory formation (A) Graphic depiction CRISPRa system infusion into hippocampal area CA1, with visualization of hippocampal expression of EGFP fluorescent marker, and three-pairing contextual fear conditioning paradigm. Briefly, male C57BL/6 mice (3-7 month-old) were trained 21d after bilateral infusion of either sgRNA plasmid alone or co-delivered with a transcription-activating dCas9-effector protein and tested 24h after training. (B) Confirmation of efficacy of CRISPRa system to upregulate *Neat1* expression in murine neurons (C). Freezing behavior as a percent of epoch during training phases of the

contextual fear conditioning paradigm. No significant difference detected for either the Pre-shock ($n = 18$; $p = 0.3476$) or Post-first shock epochs ($n = 18$; $p = 0.0665$). (D) Freezing behavior as a percent of total time during the 5 min test trial ($n = 18$; $p = 0.0450$).

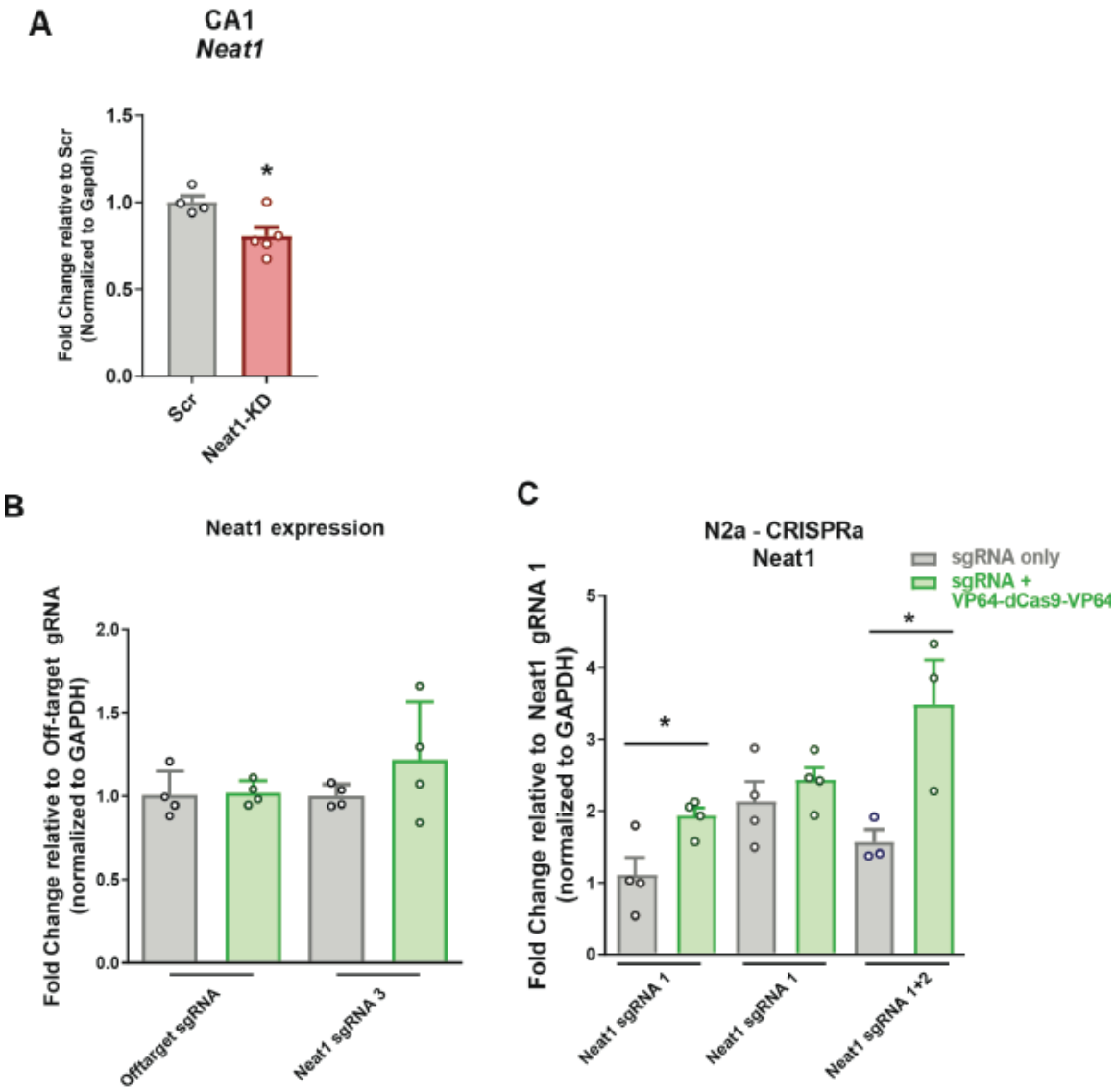


Supplemental Figure 1. *NEAT1* expression is uniquely reduced in the human CNS, and baseline expression is low in human neurons relative to other cell types. (A) Diagram of

NEAT1 gene structure used in hierarchical clustering of *NEAT1* (B) Hierarchical clustering of *NEAT1* based on exon level expression in 53 human tissues from the GTEx project. Dendrogram scale shows cluster distance. Expression values displayed in the heatmap are the median expression values in TPM for each exon in each tissue. (C-E) t-SNE plots constructed using transcript per million (TPM) values from the transcriptomes of 3,589 biopsied human single cells. (C) Inferred cell types in unique colors, with clustered neurons outlined in red. (D) Expression of *NEAT1* in single cells heatmap, from 0 to 1200 TPM. (E) Expression of MALAT1 (NEAT2) in single cells, from 0 to 12000 TPM.

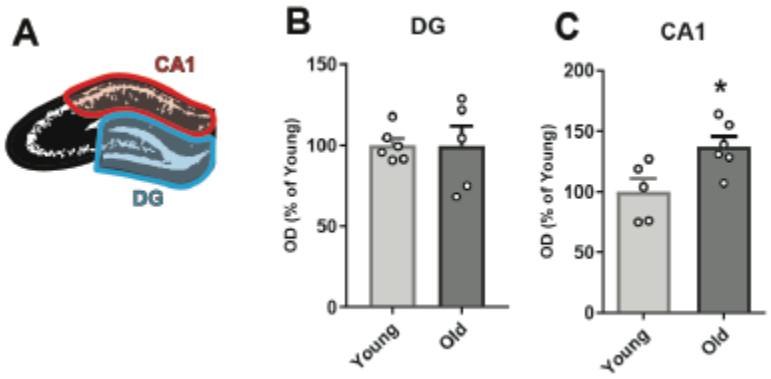


Supplemental Figure 2. Neuronal regulation of immediate early genes after *Neat1* knockdown (A-D) Differential expression of the immediate early genes (A) Egr1 (n = 5,6; p = 0.0010), (B) Btg2 (n = 5,6; p = 0.0018), (C) MALAT1 (n = 5,6; p = 0.8133), and (D) Psen1 (n = 5,6; p = 0.1228) after knockdown of *Neat1* in cultured neuronal cells. (E) Expression of the lncRNA *Neat1* in N2a cells after treatment with KCl (n = 3,4; p = 0.0495) (F) H3K9me2 at an intragenic region of the *c-Fos* gene is unchanged after *Neat1* knockdown in N2a cells (n = 2,3; p = 0.7939).

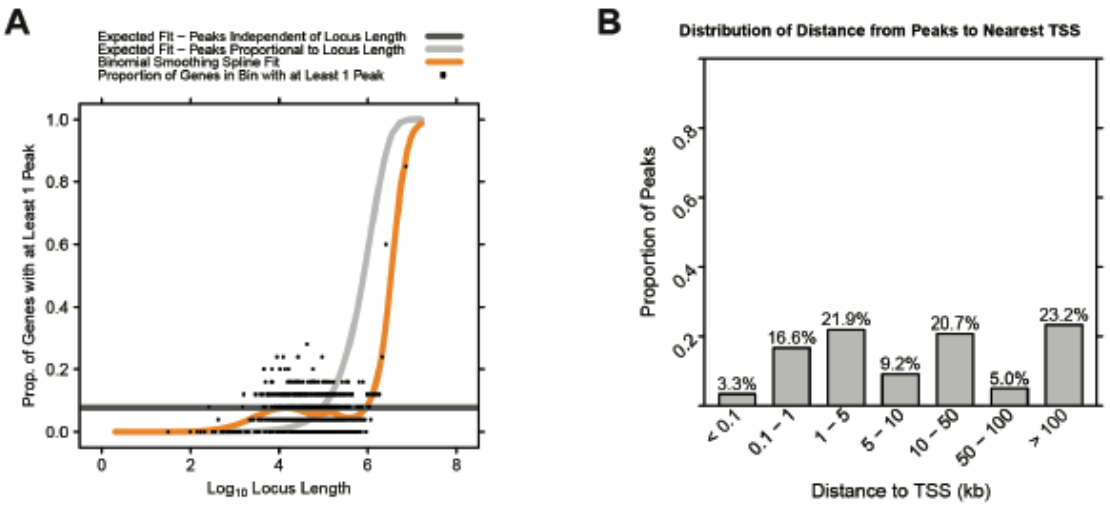


Supplemental Figure 3. Validation of *Neat1* expression manipulation via RNAi and CRISPRa (A) RT-qPCR quantification of *Neat1* expression in dCA1 of naïve mice 5d after *in vivo* transfection. (n = 4,5; p = 0.0242) (B-C) RT-qPCR quantification of *Neat1* in response to three different sgRNAs for CRISPRa-mediated upregulation of *Neat1*. (B) Transfection of an off-target sgRNA plasmid alone, *Neat1*-targeting sgRNA 3 plasmid alone, or off-target sgRNA plasmid and ddCas9-2xVP64, while sgRNA 3 in conjunction with dCas9-2xVP64 had a modest effect on expression (not statistically significant; p >

0.05). (C) Transfection of *Neat1* sgRNA plasmids #1 or #1/#2 in conjunction with dCas9-2xVP64 results in more robust transcriptional and additive upregulation of *Neat1* relative to cells receiving only the respective sgRNAs *(n* = 4,4,4,4,3,3; **p* < 0.05).



Supplemental Figure 4. Age-related elevation of H3K9me2 in dCA1 (A) Cartoon of dissection of CA1 from DG in *Rattus norvegicus*. (B) DG expression of H3K9me2 is unchanged between young and aged rats (n = 6,6; p = 0.9762), while expression of H3K9me2 in CA1 (C) is elevated with aging (n = 5,6; p = 0.0465).



Supplemental Figure 5. Quality control plots from Chip-Enrich. (A) Curves comparing *NEAT1*-bound peaks to locus length. (B) Distribution of distance from *NEAT1* bound peaks to the nearest gene TSS

DISCUSSION

Summary and Main Implications of Findings

While numerous studies have found associations between ncRNAs and cognitive disorders, few have investigated or characterized mechanisms involved with lncRNA signaling *in vivo* due partly to the young age of the lncRNA field as well as the complex and often interconnected regulatory nature of lncRNAs. Recent efforts to annotate noncoding elements within the genome have revealed a massive population of noncoding RNA genes, and a particularly large number of lncRNA genes -with some 13,000 mouse lncRNAs and 16,000 human lncRNAs having been annotated to date (GENCODE versions M20 and 29 respectively). Initial profiling studies have estimated that approximately 70% of mouse lncRNAs are expressed in the hippocampus (Kadakkuzha et al., 2015). However, including this study, approximately 0.2% of lncRNAs have been directly manipulated in any fashion in the context of the hippocampus, in any capacity (See Table 2 for descriptions of these reports), indicative of the vast gap in knowledge in this area of biology. Recently, the lncRNA *Neat1* has been observed to be regulated by and regulate neuronal activity. Also, *Neat1* has been strongly associated with progression of Alzheimer's disease and posited as a potential biomarker, given its strong correlation

with Alzheimer's disease in multiple tissue types, including the hippocampus(Wu et al., 2019).

lncRNA	Mechanism	Disease (Model)	Effect	Reference
Anril	NF-κB inactivation	Diabetes (streptozotocin)	Improves memory; Reduces apoptosis	(Wen et al., 2018)
CCAT1	miR-155 inhibiton	Neuropathic pain (bilateral sciatic nerve chronic constriction injury)	Reduces pain thresholds	(Dou et al., 2017)
EBF3-AS	EBF3 regulation	Alzheimer's (APP/PS1 mice)	Increases apoptosis	(Gu et al., 2018)
FTX	miR-21-5p inhibition	Epilepsy (Kainic acid)	Reduces apoptosis	(Xiangdan Li et al., 2019)
GAS5	miR-23a inhibition	Hypothermic circulatory arrest	Increases apoptosis	(Gao et al., 2019)
GAS5	Mir-23a inhibition	Hypoxia/ischemia-induced neonatal injury	Increases infarct size	(Zhao et al., 2018)
GM12371	Transcriptional regulation		Synapse structure and function	(Raveendra et al., 2018)
H19	Inhibits 5mC at IGF2	Diabetes (streptozotocin)	Reduces apoptosis	(Yu et al., 2019)
H19	Inhibits let-7b	Epilepsy (lithium-pilocarpine)	Synapse structure and function	(Han et al., 2018a)
H19	Induces proinflammatory cytokine release	Epilepsy (Kainic acid)	Glial cell activation	(Han et al., 2018b)
HOTAIR	Transcriptional regulation	Sevoflurane anesthesia	Impaires cognition	(J.-Y. Wang et al., 2018)
LINC00470	miR-101 inhibition, Transcriptional regulation	Glioblastoma	Increases autophagy	(C. Liu et al., 2018)
LOC103690121	PI3K/Akt	Diabetes (streptozotocin)	Induces apoptosis	(Hao et al., 2019)
LoNA	Elevated ribosomal synthesis and function	Alzheimer's (APP/PS1 mice)	Improves long term memory	(D. Li et al., 2018)
Malat1		Ischemia and reperfusion	Reduces apoptosis	(Shang et al., 2018)
Malat1	miR-101 inhibition	Epilepsy (lithium-pilocarpine)	Decreases apoptosis, autophagy	(Wu and Yi, 2018)
Neat1	miR-15/107 regulation, Transcriptional regulation	Alzheimer's disease	CDK5R1 activation	(Spreafico et al., 2018)

lncRNA	Mechanism	Disease (Model)	Effect	Reference
Neat1	Transcriptional regulation	Aging	Suppresses long-term memory	(Butler et al., 2019)
NKILA	NF-κB inactivation	Oxygen deprivation and Reoxygenation	Increases apoptosis, necrosis	(M. Wang et al., 2018)
PVT1				(Li et al., 2016)
PVT1	Wnt	Epilepsy (lithium-pilocarpine)	Improves memory and reduces apoptosis	(Zhao et al., 2019)
SCN1ANAT	SCN1A upregulation	Dravet syndrome	Upregulation of silenced allele improved seizures	(Hsiao et al., 2016)
SNHG12	miR-199a inhibition	Ischemia and reperfusion	Reduces apoptosis	(Yin et al., 2019)
Tsx			Suppresses short term hippocampal memory	(Anguera et al., 2011)
TUG1	SnoN-Ccd1 activation	Fragile X (FMRP knockdown cells)	Decreases axon length	(Guo et al., 2018)
Uc.173	miR-291a-2p inhibition	Lead toxicity	Reduces apoptosis	(Nan et al., 2016)

Table 2. Experimental manipulated hippocampal lncRNAs

Previous studies have observed epigenetic regulatory roles for the lncRNA *Neat1*, including both the direct and indirect regulation of chromatin modifying enzymes, chromatin binding, and mediation of site-specific epigenetic regulation. Here, we have attempted to integrate our experimental observations of *Neat1* in neurons and *in vivo* with previous observations from other biological systems with long-standing observations of the neuroepigenetic mechanisms of memory formation.

Recent studies have profiled the expression of hippocampal *Neat1* in various experimental models, and these studies have found *Neat1* upregulation to be strongly linked to multiple different cognitive disorders. In contrast to this, *Neat1* is also robustly transcriptionally repressed in response to neuronal activation, outside of the context of epilepsy, and in this dissertation, I have undertaken to establish the functional relevance of both *Neat1* suppression as well as *Neat1* upregulation for cognitive disorders. Our

observations indicate that *Neat1* expression constitutes an active repressor signal that is modulated during memory formation to allow for the activity driven expression of immediate early genes such as *c-Fos*, and we have demonstrated the relevance of this mechanism both in cultured neurons and in vivo in the rodent hippocampus.

Previous experiments from the Lubin lab and many others have clearly demonstrated the importance of the epigenetic regulation of gene expression for control over numerous cognitive processes, including long term memory formation. Over the course of the experiments conducted here and in previous studies we have observed the critical importance of H3K9me2 during both the processes of normal aging as well as memory formation. Epigenetic changes, including H3K9me2, within the hippocampus or in connected structures such as the entorhinal cortex, occur dynamically during memory formation, yet the direction of observed changes often differ in neighboring structures, subregion, or in a cell type specific fashion. Here we have demonstrated a role for *Neat1* in the activity dependent regulation of epigenetic signaling within both the CA1 sub region of the hippocampus and specifically within cultured neuronal cells.

Previous studies examining the regulatory function of *Neat1* Have demonstrated a preference for this lncRNA to bind to actively transcribed regions of the genome. Thus, we had initially hypothesized that *Neat1* knockdown would result primarily in repression of gene transcription. However, analysis of RNAseq after *Neat1* knockdown in human neurons revealed primarily upregulation of transcription. While previous studies have observed a wide-ranging impact of the lncRNA *Neat1* on the epigenome, and binding to a diverse set of chromatin modifying enzymes, here we observed a specific global regulation of H3K9me2.

While it is likely that multiple epigenetic marks are being regulated by *Neat1* in a more restricted, site specific fashion, The direct interaction of *Neat1* with the EHMT2 protein, which has been reported by us and other groups, as well as direct binding of the EHMT1 locus together suggest a mechanism by which *Neat1* may exercise multi-point control over H3K9me2, resulting in both global changes as well as site-specific regulatory activity.

Expression of lncRNAs tends to occur in a more site-specific fashion then expression of protein coding genes. Consistent with this trend, studies profiling expression of the lncRNA *Neat1* in human tissues have shown relatively limited expression in tissues of the CNS, while expression is quite abundant in many other human tissues. Moreover, examination of single cells reveals highly restricted expression of *Neat1*. However, in contrast to these observations, in the context of the aging hippocampus we have observed up regulation of *Neat1*. Previous insights from our lab and others have indicated that epigenetic regulation is often perturbed within the aging brain; however, there are disagreements within the literature as to the precise nature of these changes. It remains unclear at this point whether the heterogeneity in these reports might be due in part to differences in age-induced epigenetic changes across animal models, tissues, or tissue subregions.

As predicted by our model of *Neat1* as an inducer of H3K9 dimethylation, we observe elevated expression of this epigenetic mark in the CA1 subregion of aged rodents. To expand upon this further, we examined expression of the memory related immediate early gene *c-Fos*, which has previously been reported as a memory-induced gene regulated by H3K9me2 in the hippocampus. When examining *c-Fos* expression via

RNAseq, we observed repression of *c-Fos* within the aging hippocampus, consistent with our model of age-related immediate early gene repression via *Neat1* and H3K9me2. Interestingly, both similar and opposite findings regarding the global levels of H3K9 methylation in the aging hippocampus, - gain and loss of H3K9 methylation, respectively- have been reported previously in the literature (Maleszewska et al., 2016; Snigdha et al., 2016). Building off our previous observations, which included subregion specific increases in H3K9me2 in the rodent hippocampus (Morse et al., 2015), we have observed age-dependent induction of the murine lncRNA *Neat1* and described regulatory roles for *Neat1* in the governance of both global and site-specific changes in H3K9me2 which seem to support the hypothesis that there is an age-induced accumulation of H3K9me2 suppressing hippocampal memory formation.

As mentioned previously, analysis of *Neat1*-capturing CHART-seq data revealed direct binding of the EHMT1 gene. Further RNA-seq analysis of iPSC-derived human neurons after *Neat1* knockdown revealed that the human *NEAT1* RNA governs expression of the EHMT1 mRNA. Indeed, at least at the EHMT1 gene, *NEAT1* seems to be mediating transcriptional activation. Rather than being the exception, this seems to be the case for many loci where *NEAT1* capture is sufficient to capture chromatin (West et al., 2014). However, transcriptional upregulation via direct *Neat1* binding is not sufficient to explain the general transcriptional upregulation observed after *Neat1* knockdown in iPSC-derived human neurons. Thus, as *Neat1* expression directly correlates with both expression of EHMT1 and global levels of H3K9me2, we propose a more nuanced model whereby *Neat1* control over H3K9m2 effects a large degree of regulatory control over memory-permissive genes, as this model is sufficient to explain the observed global

upregulation of the H3K9me2 during aging as well as the global loss of H3K9me2 after *Neat1* knockdown.

Interestingly, recent observations have been made in this dissertation and other recent publications have described a direct interaction between *Neat1* and the Ehmt2 protein, a second H3K9me2 protein, which together with Ehmt1 comprise an obligately heterodimeric H3K9me2 methyltransferase complex. This interaction has been observed to mediate H3K9me2 *in vivo* and to control site-specific histone methylation (Li and Cheng, 2018), and thus we hypothesize that *Neat1* might govern both Ehtm1 expression to regulate global H3K9me2, while also binding to the H3K9me2 proteins themselves to regulate Ehmt1/2 function in a site-specific fashion. Moreover, this double interaction comprises a potential regulatory feedback loop which might be further explored in future experiments. While we cannot definitively conclude at this time which, if either, of these two mechanisms might comprise the principal means of restraining memory formation, given the consistency of the observed inverse relationship between *Neat1* expression and the expression of memory permissive genes, as well as the correlation of *Neat1*-dependent H3K9me2 at the promoters of these genes, we expect that the repressive actions of *Neat1* via H3K9me2 will continue to prove of great importance for these processes.

In addition to study of the epigenetic mechanism of *Neat1*, I have also experimentally validated the hypothesis that *Neat1* serves to regulate hippocampus-dependent memory formation. Using contextual fear conditioning, we have conducted several experiments to mimic the effects of *Neat1*-upregulation within the hippocampus using CRISPRa and RNAi. As mentioned previously, endogenous upregulation of *Neat1*

occurs in numerous diseases including Alzheimer's disease and normal aging, conditions which are typified by impairments in hippocampal memory. Interestingly, overexpression of *Neat1* from the endogenous locus was sufficient to impair memory performance in contextual fear conditioning. While many comorbidities exist in aged animals, these experiments demonstrate the sufficiency of *Neat1* manipulation alone to alter cognitive performance and mimic impairments observed in aging and Alzheimer's disease. Multiple studies have proposed *Neat1* as a potential biomarker for Alzheimer's disease based solely on the predictive power of *Neat1* transcript abundance (Prinz et al., 2019; Wu et al., 2019); however, these findings suggest validate the use of *Neat1* as a biomarker for cognitive impairment, suggesting that expression of the lncRNA *Neat1* relative to controls might be directly predictive of hippocampal function.

Future Directions

While we have attempted to highlight the biomedical relevance of the *Neat1*-H3K9me2 regulatory mechanism, future investigations would allow for experimental designs which more carefully establish the mechanisms of *Neat1* action and the translational impact of *Neat1* manipulation.

By design, the hypotheses tested in this thesis work have largely been constructed from observations of the intact *Neat1* locus. While this has allowed us to assume the preservation of any *in cis* effects of *Neat1* overexpression, we have observed both *cis* and *trans* actions of *Neat1* regulation. Thus, future inquiries seeking to resolve the necessity of such *in cis* effects might directly test the necessity of the *Neat1* locus itself for the various functions we have described here. We expect that systematic deletions of sections

of the *Neat1* locus might make it possible to functionally dissect the transcription-activating functions of *Neat1* from regions that enable binding by the Ehmt1/2 complex. Previous studies using a partial knockout approach indicate that neither global knockouts of *Neat1* nor deletions of subregions of the gene are lethal to rodents under standard housing conditions. It would be of great interest to ascertain which regions of the *Neat1* locus might be most strongly associated with cognitive function, and whether these domains are localized within the evolutionarily conserved 5' region of the gene or more within the more species-specific 3' region of the gene.

Of major interest as a line of future inquiry would be an investigation of *Neat1* expression at the level of single-cells. While we have thus far investigated expression in a limited fashion in the context of human single-cell expression, a larger cohort of cells collected in both resting as well as an activated state might yield some insights into the cell-type specific regulation of the *Neat1* transcript. One might hypothesize that as the cohort of available nuclear cofactors would change in a cell-type dependent fashion, the behavior of scaffolding lncRNAs such as *Neat1* might also be indirectly altered. Recent studies characterizing the behavior of RNA binding proteins support this view, and suggest that RNA binding domains are largely promiscuous with regards to sequence and may thus depend in large part upon the stoichiometry of both the available pool of RNAs and the RNA binding proteins themselves. Given the large-scale changes to transcription and gene expression that occur during neuronal activity, such phenomenon could account for dramatic changes lncRNA binding and function. Specifically, it is unknown at present whether the effects of *Neat1* on memory will be shown to be cell-type specific. It has been observed that cell-type specific stimulation of other CNS cell types, such as

astrocytes, results in *c-Fos* induction and is sufficient to alter performance in hippocampus dependent memory tasks. While we have made many observations in neuronal cells, these observations do not preclude additional, and perhaps enlightening, effects of *Neat1* in other cell types. Thus, the development of cell-type specific manipulations and assays would constitute a major future direction for studying *Neat1*.

As cell-type specific resolution might increase the likelihood of capturing cell-type specific effects, further increasing the spatial resolution of techniques might also yield additional insights. While expression of *Neat1* is enriched in the nucleus, due to the high level of *Neat1* expression, a portion of the transcript likely escapes to the cytoplasm, and indeed has been reported to be involved in mRNA transport. Thus, a compartment-specific approach to assaying the function of *Neat1* might yield insights into yet-unexplored questions, for example, whether or not cytoplasmic *Neat1* expression drives phase separation. A number of highly impactful recent studies have characterized the importance of phase separation to nuclear chromatin dynamics. While testing the necessity of paraspeckle-localized *Neat1* for cognition or H3K9me2 would comprise an exciting future direction, much technical advancement yet remains to be accomplished before phase-specific manipulations are feasible, especially *in vivo*. Nonetheless, given the known role that *Neat1* plays in the assembly of nuclear paraspeckles, a thorough examination of the role of these structures during memory formation would be quite insightful.

While previous experiments conducted on the role of *c-Fos* in cognitive function in neurons have established its importance for memory formation, and we have confirmed that *Neat1* regulates *c-Fos* in both murine and human neurons, there are many

concerns regarding *in vivo* effects on multiple cell types within the experimental design which might be addressed in future studies. While we utilize surgically targeted overexpression and knockdown of *Neat1*, RNAi manipulations used in this work act in a non-specific manner, and our approach for CRISPRa manipulations acts in neuron-biased but non-specific fashion. These approaches include greater signal-to-noise ratio in biochemical assays, as using manual dissection we collect and assay all cell types within the targeted hippocampal subregion. Moreover, given the virus-inducible nature of the endogenous *Neat1* gene, the strategies utilized herein allow the avoidance of problematic viral infection protocols. However, technological advances in gene-targeted manipulation, labeling, and capturing of cell populations will likely allow the utilization of more refined techniques to ask relevant biological questions in animal models. Thus, while we have established surgical models of targeted *Neat1* knockdown and overexpression, design of appropriate genetic animal models to these effects might prove advantageous for the targeted manipulation or capture of specific developmental time-points and cell types.

While the lncRNA *Neat1* is evolutionarily conserved, this conservation is not perfect, especially at the more 3' region of the gene. Thus, a mouse model engineered to replace the murine *Neat1* with the human *NEAT1* gene may provide additional insight to questions of human biology. Advancing CRISPR technology might also make feasible the expression of the human *NEAT1* from the endogenous murine locus, as well as exogenous transgene-derived expression. Such a system could be controlled by an inducible switch to test the *in vivo* impact of *cis* and *trans* lncRNA expression upon cognition or molecular biology. Similarly, we expect that systematic mutations replacing

the murine with the human *Neat1* gene might make it possible to functionally dissect any species-specific effects or interactions. As described previously, such approaches are likely feasible, given previous studies which indicate that neither knockouts of *Neat1* are not lethal to rodents under standard housing conditions. It would be of great interest to ascertain which regions of the *Neat1* locus might be most strongly associated with cognitive function, and whether these functions are domains are localized within the evolutionarily conserved 5' region of the gene or more within the more species-specific 3' region of the gene. Likewise, as described previously, while we have established surgical models of targeted *Neat1* knockdown and overexpression, design of appropriate genetic animal models might prove advantageous in many ways.

Our own observations and observations from other labs indicate that downregulation of *Neat1* is an extremely rapid process, as is the consequent upregulation of the *c-Fos* mRNAs. However, many of the experiments here characterize the association of *Neat1*, both with genomic loci and with chromatin modifying enzymes, in resting cells, as opposed to in the context of recently-active neurons, or *in vivo*. In future studies, it is hoped that greater temporal resolution will be achieved for both the timecourse of manipulations and assays.

Although many direct gene targets of *Neat1* have been observed, we chose to focus here on regulation of the transcription factor *c-Fos*. While the *c-Fos* locus is not a direct binding target of the *NEAT1* lncRNA in MCF7 cells, modulation of *c-Fos* mRNA expression by *Neat1* has been described here in multiple experimental paradigms and systems, including both human and rodent neurons. The experiments conducted in this thesis expand upon previously published reports of H3K9me2 changes in the *c-Fos*

promoter after training in hippocampus-dependent memory paradigms, and establish an upstream signal for this epigenetic change as well as some insight into age-related dysfunction of *c-Fos* regulation. However, in future studies, we hope that more precise manipulations and live-cell assays will allow for a real-time observation of the effects of *Neat1* manipulation on neuronal activity and gene expression. The further devleopment of light-inducible CRISPRa systems will allow for the targeted manipulation of *Neat1* in living cells, while novel live-cell FISH probes might allow for the simultaneous assay (and localization) of *Neat1* and *c-Fos* during regulation.

While the experiments described here are largely sufficient to explain prior observations of H3K9me2 during aging, some questions are raised regarding the intersection of *Neat1* signaling with other epigenetic changes, especially in the context of aging. While we did not here observe global regulation of H3K27me3 or DNA methylation, association of *Neat1* (and indeed the Ehmt1/2 complex itself) with these epigenetic mechanisms has been previously reported, and indeed observations from our own lab include direct association *Neat1* with the H3K27 methyltransferase Ezh2 in neuronal cells. Thus, it is possible that, due to either crosstalk with H3K9me2 or direct regulation, *Neat1* may contribute to previously reported age-related dysfunction in these or other processes.

In this work, we provide insight into future directions of experimental exploration through the examination of the lncRNA *Neat1* and establish a clear relationship between *Neat1* and age-associated epigenetic regulation as well as cognitive function. We present here the idea that lncRNAs might act as tissue-specific, activity-dependent sensors which allow for the fine-tuned control epigenetic mechanisms involved in the processes of

neural function and cognition, and that the pathological expression, depletion, or function of lncRNAs might be implicated in the still poorly- understood etiologies of neurological diseases and disorders.

To date, only a few regulatory RNAs have been discovered to have both epigenetic and cognitive relevance. However, these few examples underscore the extent to which the numerous and heterogeneous ncRNAs are still unexplored in the context of the brain. Anatomically, these ncRNAs are often most highly expressed within the cognitive centers of the brain; however, only a vanishingly small portion of these genes have been explored in any context. Although the canonical functions of ncRNAs involve diverse transcriptional and translational mechanisms, new insights suggest that several classes of ncRNAs impact the epigenome, where both protein and RNA species converge to regulate cellular function. The observations contained herein and contemporary studies have shown that epigenetic regulation by ncRNAs is -to a largely unmeasured extent- actively influencing neuronal and cognitive function. It is likely that future studies will further confirm the impact of ncRNAs mediating epigenetic regulation on well-characterized cognitive functions, such as memory formation. We expect that continued investigation into the role of regulatory RNAs such as *Neat1* in the nervous system will further reveal novel epigenetic roles for this versatile class of molecules.

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APPENDIX A

REGULATORY RNAS AND CONTROL OF EPIGENETIC MECHANISMS: EXPECTATIONS FOR COGNITION AND COGNITIVE DYSFUNCTION

by

ANDERSON A BUTLER, WILLIAM M WEBB, FARAH D LUBIN*

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ABSTRACT

The diverse functions of noncoding RNAs (ncRNAs) can influence virtually every aspect of the transcriptional process including epigenetic regulation of genes. In the CNS, regulatory RNA networks and epigenetic mechanisms have broad relevance to gene transcription changes involved in long-term memory formation and cognition. Thus, it is becoming increasingly clear that multiple classes of ncRNAs impact neuronal development, neuroplasticity, and cognition. Currently, a large gap exists in our knowledge of how ncRNAs facilitate epigenetic processes, and how this phenomenon affects cognitive function. In this review, we discuss recent findings highlighting a provocative role for ncRNAs including lncRNAs and piRNAs in the control of epigenetic mechanisms involved in cognitive function. Furthermore, we discuss the putative roles for these ncRNAs in cognitive disorders such as schizophrenia and Alzheimer's disease.

INTRODUCTION

Epigenetic mechanisms have emerged as critical components of the memory formation process and are involved in cognition and cognitive disorders. As related to the nervous system, the term epigenetics refers to long-term, potentially heritable changes in gene expression patterns that do not result from mutations in the DNA sequence. This

broad definition encompasses a number of chemical modifications to DNA residues as well as alterations to closely associated molecules such as histone proteins or chromatin-associated RNAs (Bonasio et al., 2010). These epigenetic modifications (also referred to as epigenetic marks) are involved in numerous cellular and molecular functions, ultimately influencing nuclear organization and transcriptional activity at chromatin regions (Bonasio et al., 2010). The semipermanent nature of these epigenetic modifications thus allows genetically identical cells to differentiate into distinct lineages, express unique gene patterns, and perform unique functions in a lineage-dependent fashion (Gan et al., 2007; Hemberger et al., 2009).

As the epigenome plays an important role in driving cellular development, it is not surprising that epigenetic mechanisms critically control the development of the nervous system (Juliandi et al., 2010; Maze et al., 2013; Rudenko and Tsai, 2014; Yu et al., 2010). However, in the past decade it has become increasingly clear that despite the postmitotic state of most neurons, epigenetic mechanisms continue to play a critical role in controlling transcription into adulthood (reviewed in (Lubin et al., 2011). This is especially evident in the context of transcription-dependent cognitive processes such as long-term memory formation, where altered epigenetic processes can either impair or improve performance in memory tasks.

While the importance of epigenetic regulation in cognition has been well established, the mechanisms by which epigenetic marks are targeted to particular genes or loci remain relatively unexplored. Instead, most studies to date have examined either global changes in the levels of epigenetic marks within specific brain regions or the presence of epigenetic marks at particular genes and promoters with known cognitive

function. As a result of these studies, it is becoming increasingly clear that the neuro-epigenetic landscape can differ significantly within and across brain regions, and that dysregulation of chromatin-modifying enzymes (CMEs) can have profound effects on brain function, and thereby, cognition and cognitive disorders (Gupta-Agarwal et al., 2012; Jarome and Lubin, 2014; Network and Pathway Analysis Subgroup of Psychiatric Genomics Consortium, 2015). Indeed, evidence suggests that dysfunction of epigenetic processes is involved in the etiology of many cognitive disorders, including schizophrenia, bipolar disorder and major depressive disorder (Network and Pathway Analysis Subgroup of Psychiatric Genomics Consortium, 2015). Such studies have advanced our knowledge of the role of epigenetic mechanisms in specific brain regions, and have given rise to the rapidly expanding field of cognitive epigenetics. However, as very few CMEs have demonstrated sequence-specific DNA binding, the question of how CMEs are directed to their target gene regions remains largely unsolved. Some observations have indicated that DNA binding proteins, such as the transcription factors including nuclear factor-κB, Nanog, and Oct4, interact with CMEs to direct site-specific epigenetic gene regulation (Liang et al., 2008; Vento-Tormo et al., 2014; Zhong et al., 2002). Additionally, recent studies suggest that multiple families of regulatory RNAs also mediate epigenetic targeting.

To date, a majority of the research studies have described regulatory RNAs with little to no protein-coding potential, thus defined as noncoding RNAs (ncRNAs). Although poorly described relative to protein-coding genes, ncRNAs comprise a major portion of the mammalian transcriptome (Harrow et al., 2006). While estimates differ as to the abundance of ncRNAs, the general consensus of the field is that ncRNAs are quite

plentiful, particularly in brain tissues (Harrow et al., 2012, 2006; Lunnon et al., 2014; Mercer et al., 2008; Ravasi et al., 2006). It remains plausible that regulatory RNAs have both translation-independent functions and protein-coding potential, and indeed, translation-independent activity of mRNAs has been identified in well-studied pathways such as p53 signaling (Candeias et al., 2008; Gajjar et al., 2012; Naski et al., 2009). However, the field of epigenetics has largely focused on characterizing such functions in ncRNAs, in part as a precaution against experimental confounds.

Common practice in the epigenetics field describes ncRNAs as either long or short, with the division being set at a length of 200 nucleotides. While seemingly arbitrary, this division allows for the useful separation of the many characterized classes of small functional RNAs, including miRNAs, piRNAs (PIWI-interacting RNAs), siRNAs (small interfering RNA), snoRNAs (small nucleolar RNAs) and tRNAs (transfer RNAs) from the less well characterized long noncoding RNAs (lncRNAs) (Mattick and Rinn, 2015). Among other functions, both short and lncRNAs have attributable roles in neuronal epigenetic mechanisms (Cam, 2010; Schaukowitch and Kim, 2014) – a finding with exciting implications for cognitive sciences. In this review, we will highlight key findings that are beginning to elucidate a role for ncRNAs in the control of neuronal and cognitive function via epigenetic mechanisms. Our goal for this review is to draw attention to this phenomenon and encourage new investigations into ncRNA-mediated control of the epigenome in cognition and cognitive disorders. Further, while miRNAs are well studied with regard to cognitive disorders, they are poorly studied in the context of epigenetic function. In contrast, while piRNAs and lncRNAs are better characterized in the context of epigenetic function, they are poorly studied with regard to cognitive

disorders – even though many such disorders involve dysregulation of the epigenome. Nonetheless, emerging studies are beginning to explore the interesting idea that ncRNAs are involved in the epigenetic process underlying cognition and may be altered in cognitive disorders.

SHORT NCRNA

Mechanisms of Canonical RNA Interference

When Lee and colleagues (Lee et al., 1993) first demonstrated that the small (22-nucleotide) ncRNA dubbed lin–4 represses the translation of several developmental genes in Caenorhabditis elegans, the scientific community failed to recognize this discovery as anything more than a curious feature of the invertebrate model's genetics. As a consequence, few of these ncRNAs were identified or characterized until the discovery of RNA interference (RNAi), a post-transcriptional regulatory process that will be reviewed and discussed in later sections. However, we begin our discussion with small regulatory RNAs initially shown to be highly conserved in plants and animals in the early 2000s (Lagos-Quintana et al., 2001). Today, the known roles of miRNAs and their related structures have expanded to encompass the view that as many as 60% of protein-coding RNAs are regulated by miRNA activity (Friedman et al., 2009; Lewis et al., 2005). Since the days of Ambrose and Lee, tens of thousands of miRNAs have been annotated (Griffiths-Jones, 2004; Griffiths-Jones et al., 2008, 2006; Kozomara and Griffiths-Jones, 2014, 2011), and miRNAs have been shown to regulate such diverse biological processes as developmental pattern formation (Lagos-Quintana et al., 2001), pluripotency (Leonardo et al., 2012), cell signaling (Ichimura et al., 2011), cardiovascular disease

(Ono et al., 2011), cancer (Lu et al., 2005), diabetes (Fernandez-Valverde et al., 2011), neural plasticity and memory (Bredy et al., 2011), among others (Park et al., 2010). This review will largely describe miRNAs as they relate to the epigenome; however, in order to better understand how miRNAs have come to be associated with epigenetic activity, it is first necessary to understand the canonical post-transcriptional pathway by which these miRNAs regulate gene expression.

In the canonical pathway (reviewed in (Krol et al., 2010)), the nascent miRNA begins as a transcript of intronic or intergenic DNA, a miRNA precursor molecule known as a primary miRNA (pri-miRNA). While still in the nucleus, this pri-miRNA is bound and cleaved by a microprocessor complex composed of Drosha and Dgcr8. Processing of the pri-miRNA by this complex leads to the formation of a shorter hairpin-like structure defined as a precursor miRNA (pre-miRNA) (Bartel, 2004; Krol et al., 2010). The pre-miRNA is then exported into the cytoplasm via Exportin 5 where it undergoes further cleavage by the RNAase enzyme Dicer, thereby forming a complementary duplex of two miRNA strands. Unwinding of this duplex releases one of the RNA strands, while the remaining strand is bound to an AGO protein in the RNA-induced silencing complex (RISC). The mature miRNA, coupled with RISC (now referred to as miRISC), then functions to detect complementary sequences inside messenger RNAs, usually found in the 3′-untranslated region of the target mRNA (Bartel, 2004; Pillai et al., 2007). The binding of this miRISC complex to a target mRNA results in silencing of the mRNA, which may occur either by degrading the target transcript via the endonuclease activity of AGO2, or by simply preventing translation of the target transcript in cases of less perfect complementarity.

Although studies have generally focused on the regulation of mRNA by the canonical RNAi pathway, there is considerable evidence that interaction between canonical RNAi and other ncRNA signaling pathways occurs and may have broad ramifications in neuroplasticity and cognition (reviewed in (Barry, 2014)). Many miRNAs are expressed in the brain, and a number of studies have examined their role in cognitive disorders (Table 1) (Kocerha et al., 2015). Indeed, roles for miRNA have been attributed to molecular signaling in neurodevelopment (De Pietri Tonelli et al., 2008), neural stem cell differentiation (Huang et al., 2010; Kawase-Koga et al., 2010) and cortical neurodegeneration (Davis et al., 2008; Hébert et al., 2010), where knockout of the enzyme Dicer results in the dysregulation of these processes. Intriguingly, studies using an inducible Dicer1 knockout mouse model demonstrate improved performance in several memory tasks, suggesting that miRNA signaling may also act to restrict memory formation in some cases (Konopka et al., 2010).

Canonical miRNA Signalling in Schizophrenia

Among miRNA-related cognitive disorders, schizophrenia is perhaps the most extensively studied. Patients suffering from DiGeorge Syndrome often experience microdeletions affecting the miRNA microprocessing gene Dgcr8, and are 30-times more likely to suffer from schizophrenia or schizoaffective disorders (Stark et al., 2008). This observation suggests a critical importance for miRNA signaling in schizophrenia. Postmortem studies in the brains of schizophrenic patients have identified dysregulation of two miRNAs, miR-132 and miR-219, both of which have been linked to schizophrenia-associated cognitive or behavioral impairments (Kocerha et al., 2009;

Miller et al., 2012; Moreau et al., 2011; Santarelli et al., 2011). Both miRNAs are dysregulated in response to NMDA-receptor blockade (Krol et al., 2010), an interesting finding in light of the hypothesis that hypofunction/downregulation of the NMDA receptor plays a critical role in schizophrenia pathophysiology. A third candidate, miR-195, is increased in the brains of patients with schizophrenia, where it regulates several schizophrenia-related genes, including Bdnf (Mellios et al., 2008), Reln, Vsnl1, Htr2a and Grin3 (Beveridge et al., 2010). Indeed, in silico studies of miRNAs associated with transcription factors suggest that miR-195 plays a central role within a regulatory network of schizophrenia-related genes (Guo et al., 2010). Because schizophrenia is a heterogeneous and complex disorder that involves several brain regions and cell types, the role miRNAs in the control of gene transcription must be considered in this context as well. For example, patients with schizophrenia are known to exhibit a wide range of up-regulated miRNAs in the superior temporal gyrus and prefrontal cortex, including miR-107, miR-15a, miR-15b, miR-16, miR-195, miR181b, let-7e, miR-20a, miR-26b, miR-19a (Beveridge et al., 2010). Interestingly, none of the miRNAs described above are specific to neurons: miR-219 regulates oligodendrocyte maturation (Dugas et al., 2010), while miR-195 and miR-132 are expressed in astrocytes (Mor et al., 2011; Moser and Fritzler, 2010; Numakawa et al., 2011). Comprehensive studies should further elucidate the role of miRNAs in the nervous system, as well as test the possibility that global changes in miRNA processing may result in the disease's pathogenesis (Beveridge et al., 2010). While the miRNAs described above target schizophrenia related genes directly, there is also evidence for miRNA-mediated epigenetic dysfunction in schizophrenia, and some schizophrenia-related miRNAs are known to be more directly involved in

epigenetic regulation via the targeting of CMEs (Figure 1). Such regulatory miRNAs include miR-132, miR-212 and miR-195 (Beveridge et al., 2010; Li et al., 2011; Tognini et al., 2011; Zhang et al., 2011). miR-132, which was described above, is downregulated in the prefrontal cortex (PFC) of patients with schizophrenia, and regulates expression of DNA methyltransferase 3 α (DNMT3-α), thereby regulating DNA methylation (Miller et al., 2012). This is especially interesting, considering that DNMT3-α is known to be upregulated some brain regions in schizophrenia – including the PFC (see (Grayson and Guidotti, 2013) for review). Another promising candidate is miR-137, an miRNA identified as having a SNP strongly associated with schizophrenia in a meta-analysis of genome-wide association studies (Schizophrenia Psychiatric Genome-Wide Association Study (GWAS) Consortium, 2011). miR-137 targets multiple chromatin-modifying genes (Akbarian, 2010), including the histone-lysine N -methyl-transferase EZH2 (Szulwach et al., 2010), as well as the lysine-specific histone demethylase 1A (KDM1A), which governs neuron differentiation and migration (Sun et al., 2011). While such epigenetic regulation may occur, future studies are required to determine the exact nature and extent of miRNA-mediated epigenetic regulation in the development and pathophysiology of cognitive disorders such as schizophrenia.

Canonical Function of siRNA-Mediated RNA Interference

There are significant functional similarities between miRNA- and siRNA-mediated RNAi. In this section we will highlight some of the more unique aspects of siRNA generation and regulation. Similar to miRNAs, siRNAs are short (~21 nucleotide), noncoding transcripts that are canonically generated from exogenous

dsRNAs. When siRNAs were first discovered in plants by David Baulcombe and colleagues (Hamilton and Baulcombe, 1999), they appeared to function as part of a natural, antiviral immune response. Upon exposure, exogenous, double-stranded RNAs (dsRNAs) – often from viruses – are digested by Dicer in the cytoplasm, generating short RNA duplexes. These RNA duplexes are bound by AGO as part of the RISC complex and guide the complex to a complementary target, in this case, a copy of the viral RNA. Once bound, the endonuclease 'slicer' activity of AGO2 is further activated by the complementation of the siRNA-target interaction, thus mediating target destruction (Valencia-Sanchez et al., 2006). In this fashion, canonical siRNA-mediated RNAi initiation turns viral RNA against itself for destruction. Interestingly, newly discovered noncanonical mechanisms of endogenous siRNA (endo-siRNA) generation and function are gaining increased relevance in cognitive neuroscience. Below, we discuss emerging roles for siRNA-directed epigenetic regulation of gene expression changes.

Emerging Mechanisms of siRNA-Directed Epigenetic Regulation

siRNAs participate in epigenetic regulation of genes through DNA methylation as well as by histone modification (Figure 2) (Chen et al., 2012). The precise mechanism of siRNA generation depends on the organism involved. In Schizosaccaromyces pombe, endo-siRNAs are generated by an RNA-directed RNA polymerase complex (RDRC), and epigenetic regulation is carried out by the RNA-induced transcriptional silencing (RITS) complex, with the latter being dependent on siRNAs gener ated by the former (for review see (Verdel et al., 2009)). In Arabidopsis thaliana, this process involves two plant-specific RNA polymerase II related RNA polymerase enzymes: Pol IV and Pol V

(Matzke and Mosher, 2014). First, transcripts from Pol IV are used as templates by the RNA-dependent RNA polymerase RDR2 to form dsRNA which is reduced into 24-nucleotide duplexes by the Dicer protein DCL3. From the cytoplasm, one strand of a duplex is then loaded onto AGO4, which translocate into the nucleus (Ye et al., 2012) and binds to complementary, nascent transcripts created by Pol V (He et al., 2009; Wierzbicki et al., 2008). Once stabilized to a target transcript by Pol V and the Pol V transcript binding protein KTF1, AGO4 associates with the DRM2 DNA methyltransferase, a writer of the 5 mC epigenetic mark at CHH sites (Gao et al., 2010). RDM1, a subunit of the final complex responsible for linking AGO4 to Pol V and DRM2, has itself an affinity for methylated DNA, a finding that suggests a predilection of the Pol V-AGO4 complex for pre-existing sites of methylated DNA. While still speculative, these studies suggest a parallel between siRNA-directed histone modification and siRNA-directed DNA methylation insofar as both may be mediated as part of a self-perpetuating feedback loop (Zhong et al., 2014).

Although endo-siRNA generation and epigenetic function is well-characterized in S. pombe and A. thaliana, other studies speculate on a potential role in endogenous production of siRNAs and their epigenetic function in human cells (Tam et al., 2008; Watanabe et al., 2008). Mammalian endo-siRNAs are known to be generated from hybridized mRNAs and antisense transcripts (Watanabe et al., 2008), which may then regulate the epigenome. An alternative pathway for the generation of such endo-siRNAs has also been identified in which a complex composed of human TERT (hTERT), Brahma-related gene 1 (BRG1) and nucleostemin (NS) – together referred to as the TBN

complex-produces dsRNAs. These dsRNAs can then be processed into siRNAs that facilitate the formation of heterochromatic regions (Maida et al., 2014).

Promisingly, several studies demonstrate endo-siRNA-mediated histone methylation and DNA methylation in cultured mammalian cells (Babiarz et al., 2008; Chen et al., 2012; Palanichamy et al., 2010). The mechanistic actions of mammalian endo-siRNA are poorly characterized; moreover, a neurological role for these endo-siRNAs also remains to be established, as little neuroepigenetic research has focused on these mechanisms. Importantly, targeted sequencing studies demonstrate a large number of these RNAs in human somatic cells (Castellano and Stebbing, 2013), and recent studies have identified putative endo-siRNA populations in hippocampal tissues (Smalheiser, 2012; Smalheiser et al., 2011). Thus, indicating a potential epigenetic role for endo-siRNAs in neuronal alterations.

piRNAs: Regulators of the Epigenome and Neuroplasticity

In exploring the role for ncRNAs in cognition and cognitive sciences, piRNAs have become a topic of some intriguing investigations. piRNAs are distinguished from siRNAs by their size (they are slightly longer at 26–31 nt rather than 20–24 nt), and association with PIWI proteins, a clade of the AGO family (Le Thomas et al., 2014). Unlike miRNAs and siRNAs, piRNAs are generated from single-stranded RNA species in a Dicer-independent manner (Han and Zamore, 2014). piRNAs are preeminently expressed and best characterized in the context of germ cell development (Kuramochi-Miyagawa et al., 2004). Indeed, the name ‘PIWI’ has its humorous origin owing to the discovery of the ‘P-element induced wimpy testis’ in the gonadal cells of Drosophila.

PIWI proteins translocate into the nucleus in an RNA-dependent manner, guided by piRNAs (Cox et al., 1998; Grimaud et al., 2006) where they serve to silence transposons in the nuclei of germ cells (Girard et al., 2006), ostensibly for the purpose of genome protection in the vulnerable germline DNA. However, this functionality is not exclusive, as protein-coding genes may also code for piRNAs (Robine et al., 2009). Moreover, recent studies provide evidence for numerous piRNAs expressed in adult organ tissues, including in the brain, suggesting new possibilities for epigenome regulation in neurons (Rajasethupathy et al., 2012).

With regard to epigenetic initiation and targeting, piRNAs have been shown to target heterochromatic regions with the help of bound heterochromatin protein 1a (HP1a) as part of a PIWI-piRNA complex (Brower-Toland et al., 2007). This complex typically associates with repressive histone lysine methylation marks, but may also facilitate transcription (Piacentini et al., 2009), and some evidence suggests that piRNA could form an initiator complex on chromatin that recruits other chromatin-modifying agents (Yin and Lin, 2007). Additionally, Carmell and colleagues provide an interesting set of studies that suggests chromatin regulation by piRNAs. Specifically, Carmell and colleagues demonstrate that knockout of a murine PIWI results in increased transposon expression due to a loss of inhibitory DNA methylation at transposon sites (Carmell et al., 2007). Further elucidation of this mechanism by Aravin and colleagues revealed that piRNA-mediated silencing of transposons by PIWI orthologs plays a significant role in maintaining the genome integrity of the mouse testis (Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008). Interestingly, some transposable elements have been identified as sources of dsRNAs, which feed into the endo-siRNA pathway, suggesting a degree of

redundancy between endo-siRNA and piRNA pathways (Han and Zamore, 2014). While still largely unexplored in mammalian systems, at least one population of piRNAs has been identified in the murine hippocampus via next-generation sequencing (Lee et al., 2011).

With regard to the neuroepigenetic function of piRNAs, recent studies suggest an epigeneticallyactive population of serotonin-induced piRNAs in the CNS of Aplysia. These studies demonstrate (via knockout of PIWI) the necessity of PIWI for serotonin induced long-term facilitation (Rajasethupathy et al., 2012) – a synaptic correlate for memory formation. piRNAs have also been demonstrated to silence CREB2 – a suppressor of memory formation- in an activity-dependent manner in Aplysia (Rajasethupathy et al., 2012), further supporting the idea that piRNA signaling is necessary for memory formation. Collectively, these results are suggestive of a broader role for piRNAs in epigenetic regulation than was previously expected and future studies will likely uncover additional piRNAs mediating neuroepigenetic regulation.

LNCRNAS

Discovery and Characterization of lncRNAs

Typically, sncRNAs can be separated into distinct classes by clearly defined homologies of structure and function, while lncRNAs represent a heterogeneous and often modular set of transcripts (Guttman and Rinn, 2012; Tsai et al., 2010; Wright, 2014). As a result, the most common working nomenclature of lncRNA structure describes transcripts on the basis of their genomic location relative to nearby proteincoding genes (Mattick and Rinn, 2015; Wright, 2014). Among these subcategories

of lncRNAs are antisense, bidirectional, intergenic, intronic and overlapping transcripts (Figure 3).

Although the first functional role attributed to a lncRNA was described prior to the discovery of sncRNAs (Pachnis et al., 1988), it is only recently that the abundance of lncRNAs in the mammalian transcriptome has been fully recognized. Recent studies have identified thousands of lncRNA genes in the human transcriptome (Harrow et al., 2012; Sanli et al., 2013). While these studies have expanded our knowledge of the transcriptome, most observations are still limited in scope to cultured cells and resting state expression profiles within tissues. Given the highly specific expression profiles of many known lncRNAs and their comparatively lower expression levels (ten-fold lower than protein coding genes, on average) (Cabili et al., 2011; Pauli et al., 2012), it is likely that many functional lncRNA transcripts are expressed below the power of detection for such studies. Indeed, novel deep-sequencing methodologies demonstrate that the full transcriptome is much larger than current sequencing studies have revealed (Fu et al., 2014; Mercer et al., 2014, 2012). A thorough investigation of lncRNA abundance will likely require the targeted transcriptional profiling of specific tissues, cell types or perturbations. Moreover, as many as 80% of mammalian protein coding loci express some form of antisense transcript along with their respective mRNAs (Katayama et al., 2005; Klevebring et al., 2010; Morris, 2009). Antisense transcripts can often impact the regulation of associated protein coding genes (Pelechano and Steinmetz, 2013), though this is not a necessity, nor does it preclude additional in trans effects (Mattick and Rinn, 2015).

In addition to shared genomic loci, lncRNAs and mRNAs share characteristics that distinguish them from other small RNA species. Similar to mRNAs, lncRNAs demonstrate properties such as chromatin structure typical of RNA polymerase II (Pol II) transcription, alternative splicing sites and regulation by transcription factors (Morris and Mattick, 2014). Furthermore, many lncRNAs are polyadenylated and capped with 5′-methylguanisine (Sanli et al., 2013). There have even been reports of lncRNAs associating with ribosomes – although ribosome profiling experiments suggest that such associations are usually inactive (Chew et al., 2013; Guttman et al., 2013). Surprisingly, some translation of lncRNAs has been observed, resulting in the expression of small proteins products, though recent studies suggest that global translation of all ncRNAs may occur in a manner resembling pervasive gene transcription (Ingolia et al., 2014), though the importance of this mechanism remains to be defined in any cell system.

lncRNAs impact cellular processes via a number of molecular mechanisms. These include regulation of transcription (Jiao and Slack, 2014; Modarresi et al., 2012; Zhang et al., 2012), epigenetic regulation (Schaukowitch and Kim, 2014), scaffolding of protein complexes (Froberg et al., 2013; Zappulla and Cech, 2006), guiding of regulatory complexes (Froberg et al., 2013), acting as decoys to regulatory complexes (Di Ruscio et al., 2013) or simply being transcribed (Kornienko et al., 2013). These mechanisms of action often rely on the ability of RNAs to bind both proteins and nucleic acids in a targeted manner. An RNA molecule's primary structure – that is, the linear sequence of nucleotides – allows RNA transcripts to bind homologous DNA regions via canonical or noncanonical base pairing. Recently, tools have been developed for the computational prediction of lncRNA DNA-binding motifs and binding sites (He et al., 2015). Similar

hybridization also allows single stranded RNA to fold into complex secondary and tertiary structures, and to bind with other RNA molecules. It is these structural arrangements, in addition to sequence specificity, that often underlie interactions with RNA-binding proteins (RBPs) (Li et al., 2014, 2010). Many currently known mechanisms of lncRNA activity rely largely on interaction with RBPs and alterations in localization, activity or association with other proteins. RBPs are a functionally and structurally diverse class of molecules, and recent studies have estimated that 40% of RBPs (out of a cohort of 1542 RBPs) are involved in ncRNArelated processes (Gerstberger et al., 2014). Additionally, lncRNAs have been observed to bind and regulate other small RNA molecules such as miRNAs (Bosia et al., 2012; Kartha and Subramanian, 2014), and extensive noncoding interactomes have been proposed (Jalali et al., 2013).

In the nucleus, lncRNAs modulate gene expression via regulation of transcription and the epigenetic landscape (Figure 4) (Morris and Mattick, 2014; Nakagawa and Kageyama, 2014). Indeed, lncRNAs can bind to a number of CMEs, usually 'writers' of epigenetic marks (Nakagawa and Kageyama, 2014). The extent of such a phenomenon was established in 2009, when it was shown that some 20% of lncRNAs (out of a cohort of 3300) associate with the PRC2, a histone methyltransferase that catalyzes the addition of repressive H3K27 methylation (Khalil et al., 2009). Additionally, binding of lncRNAs to CMEs can prevent or restrict CME activity, as was recently demonstrated to occur at the CEBPA locus, where an overlapping lncRNA (sometimes described as an extracoding RNA or ecRNA) preferentially binds the DNA methyltransferase DNMT1 (Di Ruscio et al., 2013), ultimately leading to decreased local DNA methylation. Interestingly, this

phenomenon is not restricted to the CEBPA locus but occurs at multiple methylation sites across the epigenome (Di Ruscio et al., 2013).

lncRNAs in Cognitive Disorders

The importance of epigenetics is become increasingly recognized in neuronal alterations and cognitive function. A recent GWAS study of common cognitive disorders found that epigenetic – specifically, histone methylation – pathways were strongly associated with impaired cognition (Network and Pathway Analysis Subgroup of Psychiatric Genomics Consortium, 2015), and a number of screening studies suggest that lncRNA dysregulation is associated with neurodevelopmental and cognitive disorders (Zhao et al., 2015), including Rett syndrome (Petazzi et al., 2013), autism (Ziats and Rennert, 2013) and Fragile X syndrome (Spadaro et al., 2015). While the widespread mechanisms of ncRNA-mediated regulation have been established for some time, only in very recent years have these mechanisms been investigated in a neurological or cognitive context. lncRNAs have been found to be co-expressed with genes that are critical for neuronal activity, including C-fos, Arc and BDNF, suggesting an extensive network of protein coding and noncoding genes involved in neuronal plasticity (Lipovich et al., 2012; Spadaro et al., 2015). Add it iona l ly, lncRNAs are known to play a role in normal brain development (Sauvageau et al., 2013). While the majority of lncRNAs transcripts have been characterized either in cell culture or during development, efforts to examine the functional roles of neuronal lncRNAs in cognition are still continuing. Here, we will recount some of the better-characterized examples of lncRNA functioning in the context of the adult brain, and their impact on cognition or cognitive disorders.

Malat1. One lncRNA that has been observed to regulate neuronal activity is Malat1 (also known as Neat2). This highly conserved nuclear lncRNA is expressed in numerous tissues, with a high degree of expression in neurons (Bernard et al., 2010). Knockdown studies of Malat1 have resulted in decreased synaptic density in cultured hippocampal neurons (Bernard et al., 2010), and postmortem studies have demonstrated that Malat1 is upregulated in multiple brain regions in both human alcoholism as well as rodent models of alcoholism (Kryger et al., 2012). Malat1 can regulate gene expression in cis, thus controlling the expression of proximally located genes which are involved in nuclear function (Zhang et al., 2012). Conversely, Malat1 can associate with hundreds of sites in trans, where it preferentially binds the gene body of active genes in a transcription-dependent fashion (West et al., 2014). Epigenetically, Malat1 has been shown to associate in vivo with EZH2, a subunit of the PRC2 (Guil et al., 2012). Interestingly, and despite many functional associations, Malat1 knockout in mice does not affect viability or normal development (Eißmann et al., 2012; Zhang et al., 2012).

Gomafu. The lncRNA Gomafu has also been shown to play multiple roles in the adult brain. Gomafu has been observed to govern SZ-related alternative splicing by acting as a splicing factor scaffold for QK1 and SRSF1, and it is known to be dysregulated in postmortem studies of schizophrenia patients (Barry et al., 2014). Recently, an additional study has suggested that Gomafu functions in cis to mediate epigenetic regulation of gene expression via the PRC1 complex, and that knockdown of

Gomafu in adult mice results in abnormal behavioral phenotypes and increased anxiety (Spadaro et al., 2015).

BACE1-AS. Another example of lncRNAs involved in neuronal disorders, is the antisense lncRNA, BACE1-AS that has been implicated in Alzheimer's disease (AD). AD is a progressive neuro-degenerative disorder which has been previously associated with epigenetic dysregulation, particularly in histone acetylation (Lu et al., 2014; Stilling and Fischer, 2011). A characteristic marker of AD pathology is the accumulation of β amyloid plaques consisting of oligomerized amyloid β peptides. These plaques form as a result of the processing of amyloid precursor protiens (APP), the rate limiting step of which is the cleavage of APP by the Beta-secretase enzyme (BACE1) (Sathya et al., 2012). Dysregulation of BACE1 contributes to AD pathology via the overproduction of Aβ (Sathya et al., 2012). Recent studies have identified an antisense lncRNA at the BACE1 locus (BACE1-AS) which physically associates with and stabilizes BACE1 mRNA, increasing BACE1 expression both in vitro and in vivo, and ultimately resulting in increased generation of Aβ (Liu et al., 2014). BACE1 mRNA is targeted by the miR-485–5p, which normally results in BACE1 repression; however, BACE1-AS prevents this repression by competitively binding the miRNA target site (Faghihi et al., 2010). Both the BACE1-AS lncRNA and BACE1 mRNA are overexpressed in the parietal lobe and in the cerebellum of postmortem AD patients, suggesting a relevant mechanistic link between the BACE1-AS lncRNA and the pathophysiology of AD (Faghihi et al., 2010). Interestingly, knockdown of BACE1 or BACE1-AS results in reduction of Alzheimer's pathology in an APP mouse model of AD (Modarresi et al., 2011). While the

dysregulation of lncRNAs has been implicated in cognitive disorders, the task of exploring the role of lncRNA-mediated epigenetic regulation in normal cognitive function remains incomplete.

TRANSGENERATIONAL IMPACTS OF NCRNA-MEDIATED EPIGENETIC REGULATION

Since the discovery of epigenetics, there has been much curiosity and speculation as to the transgenerational heritability of epigenetic marks. In mammals, much of the epigenome is erased during the processes of fertilization and generation of primary germ cells (reviewed in (Morgan et al., 2005)); nonetheless, evidence of a transgenerationally altered epigenome has steadily accumulated, including heritable cognitive changes and behavioural phenotypes (Carone et al., 2010; Dias and Ressler, 2014; Gapp et al., 2014; Pembrey et al., 2006; Skinner et al., 2012). A simple explanation for this phenomenon would be incomplete erasure of DNA and histone modifications. While there is some evidence in support of this hypothesis (reviewed in (Migicovsky and Kovalchuk, 2011)), other studies have demonstrated the existence of an indirect mechanism of chromatin regulation via generational transfer of ncRNAs..

Recently developed mammalian epimutation models – in which phenotypes are derived from a heritable change in gene expression, as opposed to an altered genome – have demonstrated the sufficiency of parental RNA to alter the epigenome of treated progeny (Yuan et al., 2015). Additionally, in a rodent stress model, treatment of fertilized mouse oocytes with ncRNAs from the sperm of stressed males is sufficient to recapitulate heritable stress-related behavioural and metabolic phenotypes (Gapp et al., 2014). These

results indicate that alterations in the transcriptome are sufficient for the transfer of epigenetic information across generations, and play a critical role in cognitive function.

The most direct evidence for a neuronal role in transgenerational epigenetic phenomenon comes from C. elegans, where neuronally expressed RNA species are transported to the cells of the germline. These RNAs then initiate the transgenerational epigenetic silencing of particular genomic loci, thereby impacting gene expression in the germ line and, potentially, in any progeny (Devanapally et al., 2015). It is tempting to speculate that an analogous mechanism could exist in mammals, by which somatic tissues such those of the brain may regulate the epigenome of cells distant in both space and time. Clearly, such a finding would have far-reaching consequences for cognitive science.

CONCLUSIONS AND FUTURE PERSPECTIVE

While numerous studies have found associations between ncRNAs, cognition and cognitive disorders, few have fully investigated and characterized the diverse mechanisms that can be attributed to ncRNAs. In this review, we provide insights for future direction in the investigation of different classes of ncRNAs and discuss regulatory RNAs that have both established roles in cellular and molecular processes and a defined relationship to the epigenome. Thus, we present the provocative research idea that ncRNAs might serve to control epigenetic mechanisms involved in cognition by illustrating the few cases of such phenomena that have been described in the literature.

To date, only a few regulatory RNAs have been discovered to have both epigenetic and cognitive relevance. However, these few examples underscore the extent

to which the numerous and heterogeneous classes of regulatory RNAs are still unexplored. Anatomically, these species are expressed primarily within the cognitive centers of the brain, and indeed, their relevance to cognition is well established. However, emerging studies are beginning to explore beyond the canonical pathways of regulatory RNA function established in previous decades. The studies we have reviewed here demonstrate the long-term impact of regulatory RNAs on the epigenome and thereby cognition. Although the canonical functions of ncRNAs involve diverse mechanisms, new insights suggest that several classes of ncRNAs impact the epigenome, a common ground where both protein and RNA species converge to regulate cellular function. Ground-breaking studies are beginning to demonstrate that epigenetic regulation by ncRNAs is – to a yet poorly explored extent – actively influencing neuronal and cognitive function. Therefore, it is likely that future studies will focus on increasing knowledge of ncRNA-mediated epigenetic regulation on well-characterized cognitive functions, such as memory formation. Moreover, we fully expect that further investigations into the role of regulatory RNAs will reveal novel epigenetic roles for this versatile class of molecules in cognitive disorders.

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TABLES

Table 1 Examples of noncoding RNAs in epigenetically-linked cognitive disorders.

Name	Type	Putative epigenetic MOA	Disorder	Ref.
17A	lncRNA	None indicated	AD	[53]
1810014B01Rik	lncRNA	None indicated	AD, PD	[53]
BACE1-AS	lncRNA	None indicated	AD	[54]
GDNFOS	lncRNA	None indicated	AD	[53]
Gomafu	lncRNA	Binds to the polycomb repressor complex, PRC1	Scz, Anx	[55]
Malat–1	lncRNA	Associates with the EZH2 subunit of the polycomb repressor complex	Alc	[56]
NAT-Rad18	lncRNA	None indicated	AD	[53]
Sox2OT	lncRNA	None indicated	AD, PD	[57]
miR-106b	miRNA	None indicated	Scz	[58]
miR-125a	miRNA	–	MDD	[59]
miR-132	miRNA	Targets the DNA methyltransferase DNMT3-α	Scz, MDD	[60,61]
miR-137	miRNA	Targets the EZH2 subunit of the polycomb repressor complex; targets the histone demethylase KDM1A	Scz	[62]
miR-16	miRNA	None indicated	Scz	[58]
miR-182	miRNA	None indicated	MDD	[59,61]
miR-195	miRNA	None indicated	Scz	[63]
miR-212	miRNA	None indicated	Scz	[64]
miR-219-3p	miRNA	None indicated	Scz	[58]
miR-298	miRNA	None indicated	MDD	[59]
miR-29c	miRNA	None indicated	AD	[65]

AD: Alzheimers disease; Alc: Alcoholism; Anx: Anxiety; MDD: Major depressive disorder; MOA: Mechanism of Action; PD: Parkinsons disease; Scz: Schizophrenia.

Table 1. Examples of noncoding RNAs in epigenetically-linked cognitive disorders.

FIGURES

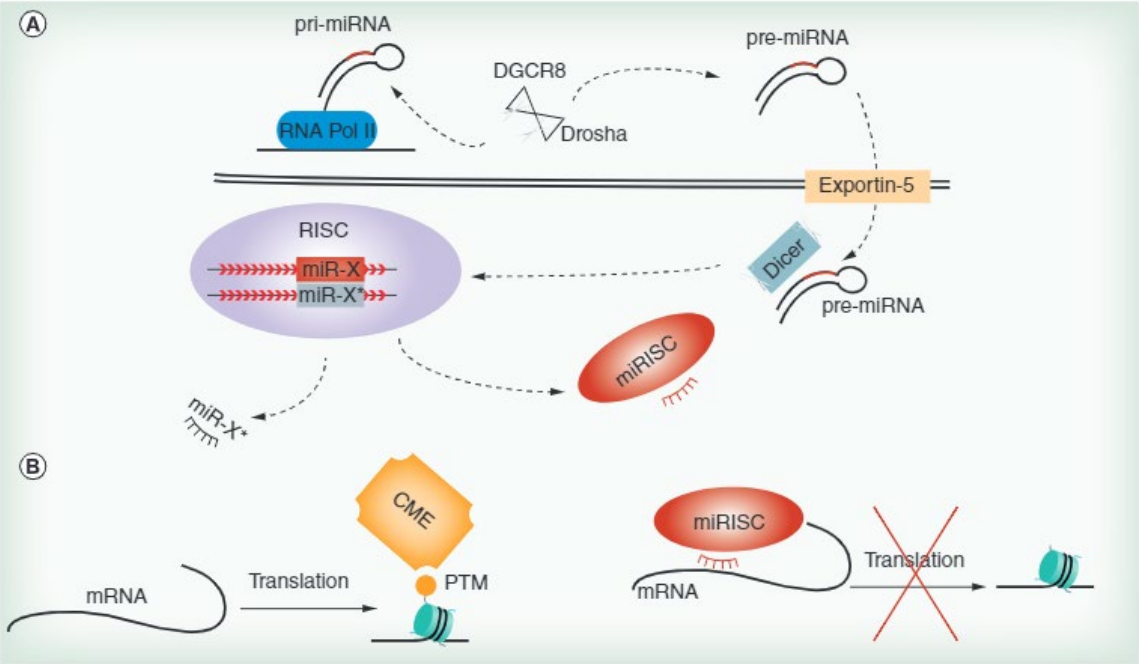

Figure 1. Canonical mechanism of miRNA generation and epigenetic regulation. (A) Schematic of canonical miRNA biogenesis. Pri-miRNA are transcribed by RNA Pol II, and stem-loop regions are processed by Drosha and DGCR8. The resulting pre-miRNA is exported through the nuclear membrane into the cytoplasm, where Dicer further cleaves the pre-miRNA into a short double-stranded RNA region. A guide strand is selected and bound by AGO within the RISC complex, while the passenger strand is cleaved and degraded. (B) miRNA and siRNA in conjunction with the cytoplasmic RISC complex target proteins involved in epigenetic regulation at the mRNA level. This posttranscriptional silencing, ultimately results in global alterations in the epigenome and cellular function. AGO: Argonaute; CME: Chromatin-modifying enzyme; miRISC:

miRNA in complex with RISC; PTM: Posttranslational modifications; Pri-miRNA: Primary miRNA; Pre-miRNA: Precursor miRNA; RISC: RNA-induced silencing complex.

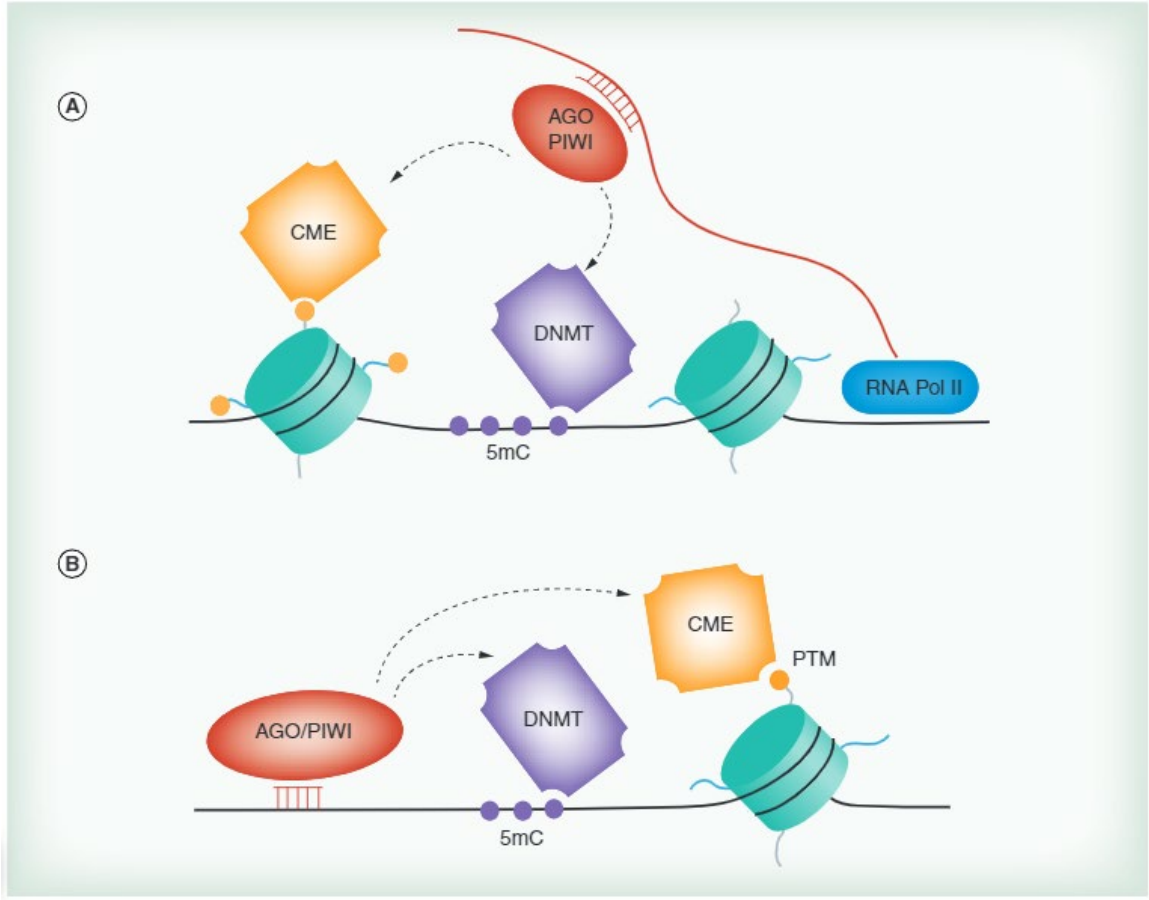

Figure 2. Epigenetic regulation by nuclear short noncoding RNA. Proposed mechanisms of sncRNA-mediated epigenetic regulation (A) Studies have demonstrated short non-coding RNA (sncRNA)-mediated targeting of transcripts and may result in the recruitment of CMEs and epigenetic regulation via DNA methylation or the post-translational modification of associated proteins such as histones. (B) sncRNAs in complex with AGO/PIWI also associate with DNA. This results in the recruitment of CMEs and epigenetic regulation. AGO/PIWI indicates a member of either the argonaute or PIWI family of proteins. CME: Chromatin-modifying enzyme; DNMT: DNA

methyltransferase; PTM: Post-translational modifications; RNA Pol II: RNA polymerase II.

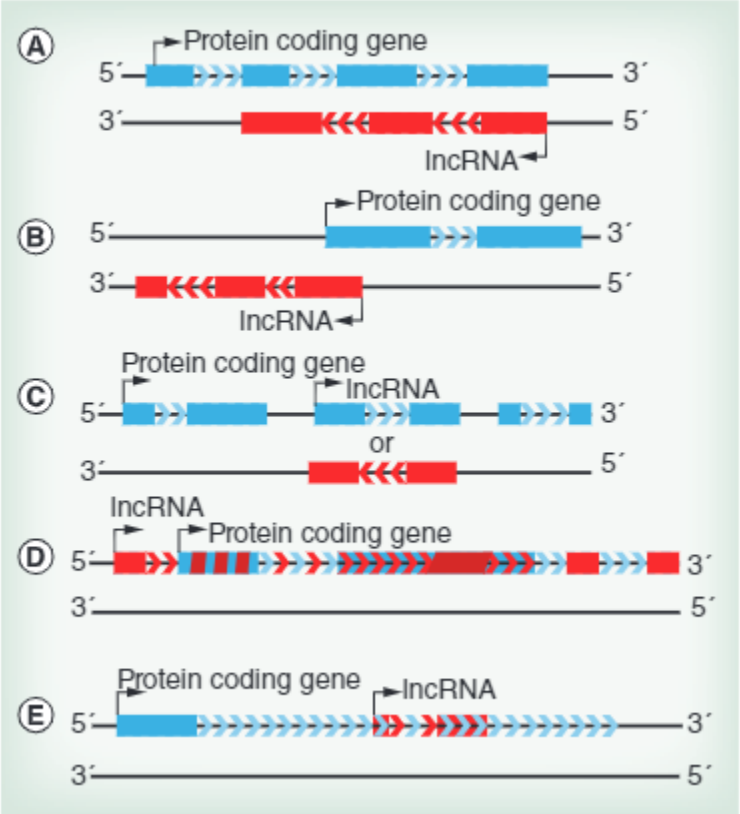

Figure 3. Origins of long noncoding RNAs. (A–E) Many lncRNA genomic loci are colocalized with protein coding genes, and they are often described in relation to these genes. A number of common naming conventions have come into general use to describe the various protein coding gene associated lncRNAs. (A) Antisense transcripts overlap protein coding genes, but are transcribed from the antisense strand. (B) Bidirectional transcripts share transcription start sites with protein coding genes, but are transcribed in the opposite direction. (C) Intergenic transcripts do not overlap with protein coding genes. (D) Overlapping transcripts overlap significantly with or encompass protein coding genes on the sense strand. (E) Intronic transcripts are located within a sense-strand intron of a protein coding gene. Solid bars indicate exons of mRNAs (blue),

lncRNAs (red). Diagonal stripes indicate overlapping exons. Chevron arrows indicate introns of mRNAs (blue), lncRNAs (red) or overlapping transcripts (alternating red and blue). Curved arrows indicate transcription start sites.

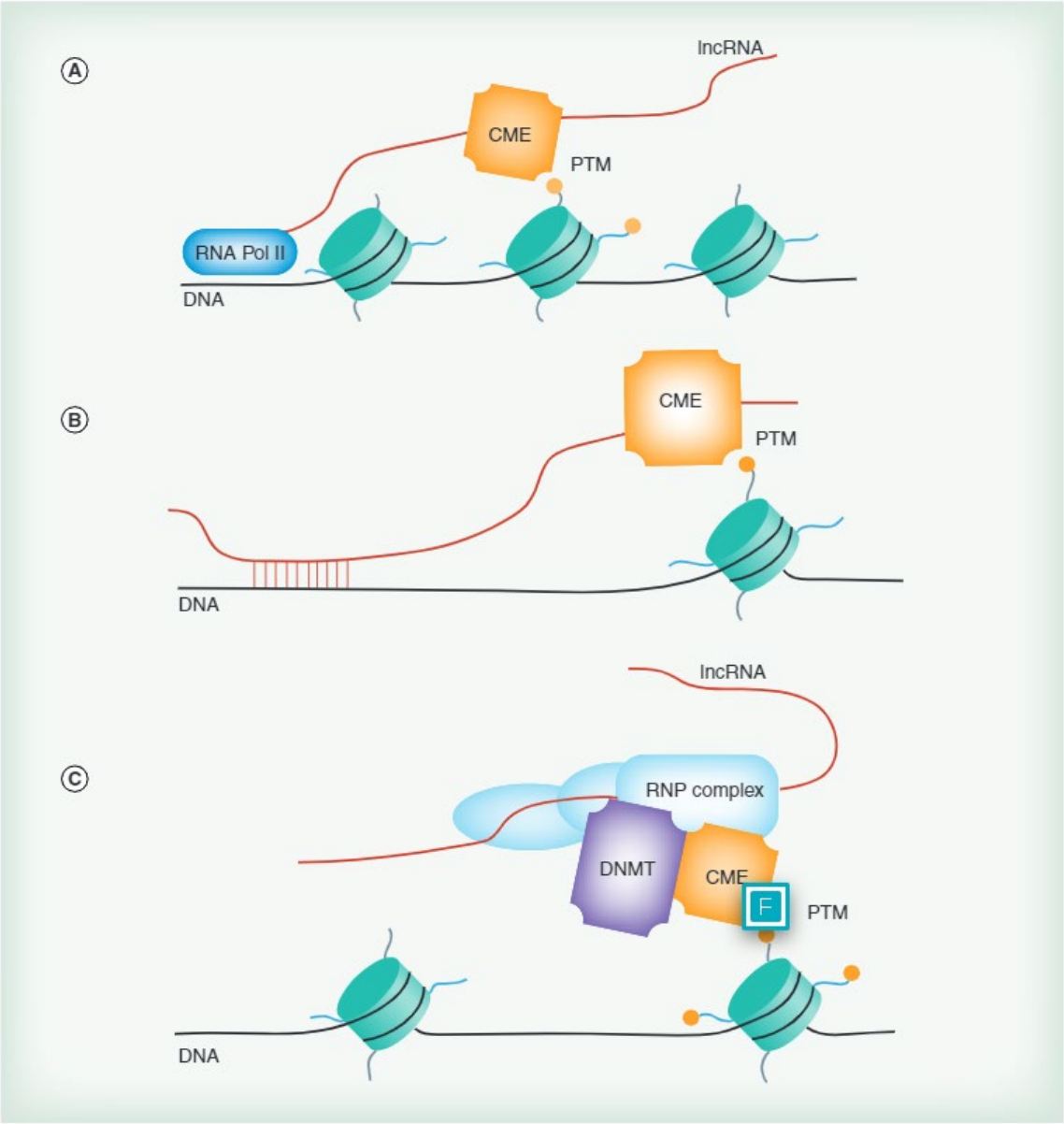

Figure 4. lncRNA-mediated epigenetic regulation. lncRNAs possess a number of mechanisms by which they initiate or facilitate epigenetic regulation, and multiple archetypal functions are often utilized within a single lncRNA transcript. (A) lncRNAs often recruit chromatin-modifying enzymes in cis, thereby mediating epigenetic regulation of nearby genes (dimitrova, zhang, redrup). (B) lncRNAs may also act as guides, targeting associated CMEs to target loci in trans, potentially through direct

interaction with target regions. (C) lncRNAs may act as scaffolding factors, and mediate the assembly of ribonucleoprotein complexes with multiple regulatory functions. This may occur either in cis, as occurs with the direct CMEs to target loci in trans. CME: Chromatin-modifying enzyme; DNMT: DNA methyltransferase; PTM: Post-translational modifications; RNA Pol II: RNA polymerase II; RNP complex: Ribonucleoprotein complex.

APPENDIX B

IACUC APPROVAL FORMS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 21-Apr-2016

TO: Lubin, Farah D

FROM: Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 21-Apr-2016.

Protocol PI: Lubin, Farah D

Title: Chromatin Remodeling Mechanisms of Gene Transcription in Memory

Sponsor: National Institute of Mental Health/NIH/DHHS

Animal Project Number (APN): IACUC-09652

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Institutional Animal Care and Use Committee (IACUC) | Mailing Address:
CH19 Suite 403 | CH19 Suite 403
933 19th Street South | 1530 3rd Ave S
(205) 934-7692 | Birmingham, AL 35294-0019
FAX (205) 934-1188 |