UNIVERSITY OF CALIFORNIA, MERCED

N^6-methyladenosine (m^6A) dynamics during *Drosophila melanogaster* neural development

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Quantitative and Systems Biology

by

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2022

N^6-methyladenosine (m^6A) dynamics during *Drosophila melanogaster* neural development

The Dissertation is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, Merced 2022

Dedication

I would like to dedicate this work to my husband Sammy Villa, my family, especially my mom Jeya Sami, my father, Jerry Sami, my Amama, my friends, and my pups Pora, Penny, and Allie for their love and consistent support of me. I would not have been able to accomplish this without them, and I love them.

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Acknowledgement

This research was supported by funds from the Quantitative and Systems Biology department of UC Merced, and the NIH.

I would like to thank my advisor Mike Cleary for all the support, patience, and advice given throughout my PhD. I will never be able to repay you for your kindness, but I will pass on your mentorship and thoughtfulness to everyone I meet! None of this work would be possible without you.

I would also like to thank all of the members of the Cleary Lab who have helped me over the years: Mohamed Aboukilila, Jade Fee, Rhondene Wint. I would also like to thank the Wolf lab for their support, especially Dr. Fred Wolf and Sammy Villa.

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Curriculum Vitae

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Objective

Obtain a molecular biologist position where I will use my skills, experience as a wet lab RNA biologist, and hone my knowledge of bioinformatics to develop molecular therapeutics and solutions to complex problems.

Education

University of California, Merced CA
Ph.D. Candidate in Quantitative & Systems Biology **2022**
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California State University, Sacramento CA **2016**
B.S. in Cell & Molecular Biology

Research Interests

Neurodevelopment and neural cell differentiation
RNA post-transcriptional modifications
RNA metabolism and therapeutics

Research Experience

University of California, Merced CA **08/2017 – 08/2022**
Graduate Researcher
Examining the effects of a post-transcriptional RNA modification on different neural cells in D. melanogaster. Used immunoprecipitation and RNA decay measurements to look at RNA metabolism in these cell types. Confirming m^6A *methylation and decay measurements with confocal microscopy and rt-qPCR.*

California State University, Sacramento CA **08/2012 - 05/2016**
Undergraduate Researcher
Worked with Dr. Andrew Reams using the soil bacterium Acinetobacter baylyi, to optimize a model system to study the phenomenon of gene amplification. My project focused on determining if these mutants were induced by the stress of selective media or if they existed prior to selection, a mechanism seen in cancerous tumor formation.

University of Alabama, Samford, AL **05/2015 - 08/2015**
NSF REU Summer Researcher
Worked with Dr. David Johnson to evaluate the mutualistic relationship between mites and Passalid beetles. Also researched Nosema parasitic infections in Honeybees to study cases of mass extinction. Used a scanning electron microscope to picture and quantify mites. Used DNA purification kits and PCR to verify Nosema strains.

Teaching Experience

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Coordinated and managed teaching assistants (PhDs) and learning assistants (BS) to work for Bio 001 class. Organized discussion activities, conducted peer observations for TAs, and taught two main lectures about the nervous system for the Bio 001 class.

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PUBLICATIONS AND PAPERS

Josephine D. Sami, Whitney England, Robert C. Spitale, Michael D. Cleary. N^6-methyladenosine (m^6A) Dynamics During Drosophila Neural Development. In Draft.

Rhondene R Wint, Josephine D. Sami, Michael D. Cleary. Codon optimality and tRNA dynamics during neural differentiation. In Draft.

Mohamed Y. Aboukilila, Josephine D. Sami, Jingtian Wang, Whitney England, Robert C. Spitale, Michael D. Cleary. 2020. Identification of novel regulators of dendrite arborization using cell type-specific RNA metabolic labeling. PLOS ONE. Dec 15(12):e0240386. doi: 10.1371/journal.pone.024038

CONFERENCE ABSTRACTS

Sami, Josephine, Aboukilila, Mohamed and Cleary, Mike. The Effects of N^6-methyladenosine (m^6A) on RNA Metabolism in Neural Cells. Poster presented at: 26th Annual Meeting of the RNA Society. May 26, 2021; virtual.

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Wint, Rhondene, Sami, Josephine, Cleary, Mike. Codon optimality and tRNA dynamics during neural differentiation. Poster presented at: 62nd Annual Drosophila Research Conference by Genetics Society of America. March 23, 2021; virtual.

Sami, Josephine, Reis, Liz, Reams, Andrew. Optimizing and Categorizing the Selection for Gene Amplification Mutants in Acinetobacter baylyi. Poster presented at: CSU Program for Education & Research in Biotechnology (CSUPERB) Symposium. January 2017; Santa Clara, CA.

Sami, Josephine, Reis, Liz, Reams, Andrew. Optimizing and Verifying Selection for Gene Amplification Mutants in Acinetobacter baylyi. Poster presented at: CSUS Provost Poster Symposium. November 2016; Sacramento CA.

Sami, Josephine, Reis, Liz, Reams, Andrew. Optimizing and Verifying Selection for Gene Amplification Mutants in Acinetobacter baylyi. Poster presented at: Society for Advancement of Chicanos/Hispanics and Native Americans in Science (SACNAS). October 2016; Long Beach, CA.

Sami, Josephine, Reis, Liz, Reams, Andrew. Gene Amplification under Growth-limiting Conditions in the Soil Bacterium Acinetobacter baylyi. Poster presented at: CSU Program for Education & Research in Biotechnology (CSUPERB) Symposium. January 2016; Anaheim, CA.

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Sami, Josephine and Johnson, David. The Rate of Nosema Infections in Honeybee Hives in Relation to Temperature. Talk and Poster presented at: NSF Research Experiences for Undergraduates (NSF-REU) Presentation. July 2015; Samford, Al.

AWARDS AND GRANTS

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Abstract

N^6-methyladenosine (m^6A) dynamics during *Drosophila melanogaster* neural development

By

Josephine D Sami

Doctor of Philosophy in Quantitative and Systems Biology

University of California, Merced

Professor Mike Cleary, Dissertation Advisor

Stem cell proliferation and differentiation is tightly regulated, and this balance is incredibly important for the health of an organism, especially in development of the central nervous system. N^6-methyladenosine (m^6A) is the most prevalent post-transcriptional mRNA modification in eukaryotes and has especially elevated levels in the *Drosophila melanogaster* central nervous system. However, the extent to which the m^6A-modified transcriptome differs among cells of the nervous system and how m^6A contributes to the metabolism of RNA in different cells remains to be seen.

To address this gap in research, we have mapped the modification in neural progenitor cells and differentiated neurons using *Drosophila* larval brains. I used *Drosophila* genetics, cell type-specific mRNA decay measurements, m^6A immunoprecipitation, and immunofluorescence to map m^6A and determine its effects in each cell type. Here, I show that while m^6A is rarely cell-type specific, mRNA decay is differentially regulated in these cells. m^6A correlates with decreased mRNA stability in neuroblasts, but this cell type-specificity is likely due to m^6A-independent stabilization of target transcripts in neurons. I propose a model in which the relationship between m^6A and mRNA stability is not causal but rather is indicative of a compensatory mechanism in which m^6A enhances translation of low stability mRNAs. This model is supported by in vivo quantitative imaging that shows m^6A promotes target protein production in neuroblasts and neurons.

My thesis work provides a rare view of the cell type-specific distribution and function of m^6A. This work contributes to the general field of epitranscriptome research and further establishes the *Drosophila* larval brain as a useful model for answering fundamental questions about m^6A.

Key Words: RNA, N^6-methyladenosine (m^6A), mRNA decay, neurogenesis, neuroblasts, neurons

Chapter 1: Introduction

Studies have shown that a major regulator of gene expression are RNA molecules. Research in this area has uncovered several layers of RNA-mediated mechanisms of gene regulation not seen traditionally in the central dogma. While this process is tightly regulated and controlled to process the full functional protein for the organism, a new layer of regulation was uncovered: post-transcriptional RNA modifications. Known as the "epitranscriptome," literature in this field has shown that it greatly affects the genome. N^6-methyladenosine is one of the most prevalent RNA modifications, and early studies have shown it plays a significant role in early development and in the nervous system. I aim to research this modification within the context of neurogenesis, and in neural cell populations using the larval stage of the model organism *Drosophila melanogaster*. My aim is to identify if m^6A plays a distinct role in different cell types using the larval central nervous system. I also determine if m^6A has an effect on RNA metabolism in this cell population. Using molecular biology, biosynthetic tagging, immunofluorescence, and *Drosophila* genetics, I will aim to answer these questions by reviewing these events. This review provides the information and rationale meant for the study in Chapter 2.

1.1 *Drosophila melanogaster* as a model organism

Drosophila melanogaster is a model organism used frequently to study genetics, early development, nervous system regulation and neurogenesis. Years of establishing genetic tools for this organism enabled detailed study of these fields and understanding of complex gene regulatory processes. The regulation of neurogenesis is also conserved with other mammalian systems, which makes it an attractive model organism to study in the lab (*Mira H. & Morante J., 2020 and Gerstein et al., 2014*). In addition to the ease in studying different developmental stages, *Drosophila* study also comes with established genetic tools to study differential cell populations and different tissues of the organism.

Growing at 25°C normally takes *Drosophila* about ten days to mature to adulthood, and includes developmental stages such as embryogenesis, larval development, pupation, and adulthood (*Jeibmann et al., 2009*). While mammalian systems develop more slowly, *Drosophila* have a relatively short window from embryonic development to the mature adult fly. After egg fertilization, the first instar larvae are birthed around 24 hours later, and in 10 days, a fully functional adult fly is produced (*Crews, 2019*). Larvae use their central nervous system (CNS) to feed, forage, and grow. During brain development stem cells proliferate and divide in order to generate the different cells that drive these behaviors. This CNS consists of a brain and ventral nerve cord (VNC) that contain about 15,000 cells, (*Crews, 2019*) about 100 neuroblasts in each hemisphere, and about 10,000 differentiated neurons in total (*Homem & Knoblich, 2012 and Carney et al., 2012*).

1.1.1 Neurogenesis and Stem Cells in *Drosophila melanogaster*

Neuroblasts are formed during embryonic development and are generated from lateral inhibition, or lateral specification, when different cell types are established (*Appel B et al., 2001)*. This mechanism is activated when the ligand Delta activates its receptor, Notch, and drives cells to specific developmental pathways (*Weinmaster G et al., 1991*). Neurons arise from these neural progenitor cells through asymmetric division; one daughter stem cell self-renews, while the other cell differentiates into neurons and glial cells. Cell cycle

regulation is regulated to coordinate proper cell growth at specific developmental times in order to prevent dysfunction and tumorigenesis. The first wave of neurogenesis begins when neuroblasts are generated from the neuro-ectoderm by delamination (*Kim et al., 2009*). They then enter quiescence during late embryogenesis, but a second wave of neurogenesis occurs in the larval stage (*Homem & Knoblich, 2012)*. When the organism reaches the pupal stage, this ends the second wave of neurogenesis and neuroblasts do not appear in this stage, or in the adult stages. Neuroblasts from these waves eventually give rise to the majority of neurons that form the adult CNS.

In the *Drosophila* CNS, there are two types of neuroblasts: Type I and Type II. The majority of the neuroblasts in the anterior and posterior consist of Type I neuroblasts, while Type II neuroblasts mostly reside in the posterior side of the brain (*Bowman et al., 2008 and Bello et al., 2008*). Type I neuroblasts differentiate into a ganglion mother cell that itself differentiates into neurons and/or glia (*Bello et al., 2008*). Type II neuroblasts divide to generate an intermediate progenitor cell, which then eventually divides into two neurons or glial cells (*Bello et al., 2008*). The two neuroblast types also differ molecularly; Type II do not contain the transcription factors Prospero (Pros) and Asense (Ase), while the Type I neuroblasts do (*Boone & Doe, 2008 and Carney et al., 2012*). Loss of these transcription factors may be used to generate mutations to target particular types of neural cells.

The process of differentiation and proliferation is incredibly important to balance gene expression, especially in the CNS. Dysregulation of these processes may lead to tumor formation, cancer, and severe defects in the organism. Defects in differentiation and cell fate commitment have been implicated to form neuroblast tumors in *Drosophila* (*Weng et al., 2010 and Zhu et al., 2011*). Neural cells need further study to see variations in neurogenesis and gene expression.

1.2 Cell type specificity and purification using *Drosophila* genetics

Understanding how gene regulation changes with various developmental pathways is critical, especially regarding cell-type specific variation and cellular diversity. Depending on the developmental stage and the molecular molecules being studied, many tools exist to further our understanding of gene expression. However, analyzing RNA specific cell populations, especially in *in vivo* conditions, remains challenging. One tool used to study cell types of interest is by expressing a transgene into the *Drosophila* genome; this is known as the Gal4/UAS system (*Brand & Perrimon, 1993 and Osterwalder et al., 2001*). The transcription factor Gal4 encodes one transgene under the control of a tissue or cell type-specific promoter. The transcription factor would activate the expression of another transgene by binding to the UAS, a specific binding site. This allows expression of potentially deleterious transgenes to be activated and studied in the fly line. Using biochemical tagging and purification of cell-type specific RNAs is also advantageous. This would allow the study of a particular developmental stage of RNAs to be analyzed while generating enough material for a thorough analysis. For my study, I used an established technique called EC-tagging to collect cell-type specific mRNA decay measurements. This labeling technique requires the fusion of cytosine deaminase (CD) and uracil phosphoribosyltransferase (UPRT). The activity of these enzymes converts 5-ethynylcytosine (5EC) to 5-ethynyluridine monophosphate that is incorporated into newly made RNAs as an ethynyl group (*Hida & Aboukilila et al., 2017*). This ethynyl group allows purification of cell-type specific nascent RNAs.

1.3 Post-Transcriptional regulation of gene expression

Gene expression starts with the genetic material of DNA transcribed into RNA and is eventually translated into protein. However, this process is not linear, and there are many steps where alterations to this pathway occur to properly regulate protein levels for the organism. Transcription is regulated by multiple transcription factors, epigenetic marks, and chromatin biology (*Buccitelli & Selbach, 2020*). mRNA processing is also a complex mechanism that includes splicing, transport, and degradation. On top of this regulation, before the mRNA becomes a protein, post-transcriptional modifications play a key role in regulating protein translation. Recent advancements in technology are now allowing researchers to study gene expression at these individual levels, including chromatin immunoprecipitation (ChIP-seq), RNA sequencing (RNA-seq), and at the protein level with mass spectrometry proteomics (*Buccitelli & Selbach, 2020*). In particular, RNA processing controls a wide variety of mechanisms that influence gene expression. Processing of mRNAs begin with the pre-mRNA, and includes capping, splicing, and polyadenylation (*Martins & Fåhraeus, 2017*). When the mature mRNA completes this processing, it is then transported into the cytoplasm where it continues the process to become a protein.

1.3.1 mRNA Decay and Translation

While gene expression studies have originally focused on just gene transcription, a large part of the equation was missed; namely, mRNA decay and turnover. mRNA turnover in the cytoplasm starts with poly(A) tail removal and is helped with the exosome complex to proceed with 3'-5' degradation (*Kilchert et al., 2016 and Łabno et al., 2016*). mRNA turnover responds to various cellular signals, such as AU-rich elements, RNA-binding proteins, and other factors (*Garneau et al., 2007*). As mRNAs undergo quality control in the cytoplasm, different mechanisms of degradation may be triggered as they may identify transcripts that may result in aberrant proteins or impair protein translation. There are different mechanisms of degradation, from nonsense mediated decay (NMD) when a premature stop codon is present in the transcript, to non-stop decay (NSD) when a transcript lacks a stop codon. There is also the no-go decay (NGD) pathway which is usually associated with translational stalling events (*Roy & Jacobson 2013*). mRNA decay can also be triggered by specific 3'UTR RNA binding proteins that recruit deadenylation complexes (*Tuck et al., 2020*). mRNA decay is an important function for the proper regulation of gene expression.

The initial round of translation starts with the newly synthesized transcript and its 5' cap binding to the cap-binding complex (*Isken & Maquat, 2008*). The cap then bids to the eukaryotic translation initiation factor 4E (eIF4E), which then supports m^6A-dependent translation (*Meyer et al., 2015 and Lin et al., 2016*). After the first round of translation, the cap-binding complex disassociates from the mRNA and the new eIF4E complex then recruits the pre-initiation complex and continues translation. eIF4E then interacts with the 3'-poly(A) tail binding protein to connect the translating mRNA in a closed loop during the next rounds of translation (*Wolfgang & Wollenhaupt, 2012*).

1.3.2 RNA Modifications

Epitranscriptomic modifications have added a new layer of gene expression, and play a prominent role, especially in the CNS. About 172 distinct types of modifications have been identified, and are present in the adenosine, cytidine, guanosine, uridine, and ribose nucleotides (*Chokkalla et al., 2022*). These are modified with a variety of chemical modifications. Functional chemical groups such as methyl, acyl, thioalkyl, and glycosyl groups can all be added onto RNA, and have been implicated in a variety of mechanisms changing gene expression (*Boccaletto et al., 2017*). With advancements to technology and new molecular biology applications, these modifications are associated with many functions that actively regulate the transcriptome; roles such as RNA stability and translation initiation, amongst others. While the most prevalent modified RNAs are tRNAs, mRNA modifications are also prevalent, especially by N^6-methyladenosine (m^6A) (*Shi et al., 2020*).

1.3.3 N^6-methyladenosine: Post-transcriptional Modification

m^6A is the most abundant modification in mRNA, which was first found in mammalian cell lines in the 1970s (*Perry & Kelley, 1974 and Desrosiers et al., 1974*). However, the mark is also found on noncoding RNAs, such as ribosomal RNA and small nuclear RNAs (Ishigami et al., 2021). However, initial studies were exceedingly rare, due to the difficulty in mapping this modification. This was in part due to expensive mass spectrometry assays, low abundances of isolating mRNAs, and the difficulty in differentiating m^6A from N6, 2′-O-dimethyladenosine (m^6Am). However, in 2012, the advent of a new mapping technique called m^6A-specific methylated RNA immunoprecipitation with next-generation sequencing (MeRIP-Seq) allowed researchers to map this modification throughout the transcriptome and discover the significance of the mark (*Meyer et al., 2012 and Dominissini et al., 2012*). This technique used anti-m^6A antibodies to immunoprecipitate RNAs containing m^6A after fragmenting it. After this technique was published, more followed, each trying to specify where the methylation occurred with the best accuracy. Another technique let researchers find m^6A at single nucleotide resolution: m^6A individual-nucleotide-resolution cross-linking and immunoprecipitation (miCLIP) (*Linder et al., 2015*). This technique used ultraviolet-induced antibody-RNA cross-linking and reverse transcription to induce mutational signatures that allowed full transcriptome-wide mapping of m^6A. Other mapping techniques (*Capitanchik et al., 2020*) include using an enzyme-based assay that recognized specific sites (*Garcia-Campos et al., 2019*), a fusion domain based assay that allows detection based on C → U mutations to identify m^6A (*Meyer, 2019*), and direct RNA sequencing by Nanopore (*Lorenz et al., 2019 and Leger et al., 2019*). While all techniques have limitations and difficulties, the renewed interest in m^6A has uncovered a large new layer of regulating gene expression.

The fundamental question of why some mRNAs are methylated and others are not is still being debated. Some key similarities in m^6A studies show that m^6A is selective, and that most mRNAs have only a single site in mammalian systems, but that some have multiple (*Zaccara et al., 2020*). The first paper to describe the functionality of m^6A showed that the presence of m^6A was correlated to mRNA degradation (Sommer et al., 1978). A study in *Arabidopsis thaliana* in 2008 deleted the main methyltransferase of m^6A (METTL3) and found that this led to developmental delays (*Zhong et al., 2008*). Many studies also found impairments in pluripotency and embryonic stem cell renewal in knockouts of m^6A in cell

lines (*Batista et al., 2014 and Geula et al., 2015*). Thus, transcripts that were m^{6}A modified were more likely to reside on genes that controlled the differentiation and self-renewal process. In some transcripts, m^{6}A has an effect on splicing and epigenetic silencing (*Wei et al., 2021 and Liu et al., 2021*). However, recent studies have found that the main function of m^{6}A is to degrade transcripts and promote mRNA decay, through three cytoplasmic m^{6}A binding proteins (*Zaccara and Jaffrey, 2020*). Early studies have stated that m^{6}A is a dynamic modification, however, these studies did not take the stoichiometry into account, and were analyzed with few replicates and flawed analysis (*Murakami & Jaffrey 2022)*. The idea that m^{6}A could be added and removed from the mRNA is highly unlikely, as the modification is added co-transcriptionally, and both the main RNA-binding proteins that add or remove the modification are also located in the nucleus. m^{6}A levels have been shown to rise and fall with various stressors to the organism studied, such as with DNA damage and oxidative stress (*Xiang et al., 2017*), but this would only affect nascent RNAs made after the stimulus. Overall, the functionally of this RNA modification has effect on the developing mRNA, but functionality needs further examination, and the exact stichometry of m^{6}A carefully needs to be examined.

The function of m^{6}A may be found where the RNA is methylated. In mammalian systems, m^{6}A is enriched along the 3′ untranslated region (UTR) and near stop codons, although this may be due to the terminal exon-exon junction rather than the stop codon itself. (*Meyer et al., 2012 and Dominissini et al., 2012*). There may also be a link between this methylation and the structural architecture of a gene. The length and distribution of exon and introns within a gene were found to influence this methylation (*Murakami & Jaffrey 2022).* Studies have found that m^{6}A is correlated to reside on long internal exons (>140bp) (*Ke et al., 2015*), although this only explains a subset of transcripts. Early studies showed m^{6}A favoring specific consensus sequences. However, due to the rarity of m^{6}A methylation (about 1 in1000 nucleotides), this was shown to be incorrect (*Murakami & Jaffrey 2022).* Studies have also implicated that m^{6}A is tissue or cell-type specific, however more studies need to be completed to ensure that these effects are not the results of changes in mere mRNA expression, limited replication of experiments, or artifacts of bioinformatic analyses.

1.3.4 m^{6}A Methylation by methyltransferase complex (writers/erasers)

Methylation and demethylation of an mRNA begins with the writing and erasing of this mark, occurring in the nucleus. The writing of m^{6}A is accomplished by a multi-subunit methyltransferase complex composing of the catalytically active subunit METTL3, and its adaptors, which include METTL14, WTAP, VIRMA, RBM15/15B, HAKAI and others (*Śledź & Jinek 2016, Zaccara et al., 2020 and Wang et al., 2016*). Other methyltransferases methylate rRNAs and other non-coding RNAs (*Ma et al., 2019 and van Tran et al., 2019*). While DRACH is a historically enriched consensus sequence known for m^{6}A (D = A, G, or U; R = G or A; H = A, C or U), not every sequence is methylated. Only specific transcripts are marked with m^{6}A, but more research is needed to understand why that is (*Murakami & Jaffrey 2022 and Zaccara et al., 2020)*. Since methylation occurs co-transcriptionally, there is evidence that RNA polymerase II may be bound by the writer complex, and may induce this methylation (*Salditt-Georgieff, M. et al. 1976 and Slobodin et al., 2017*). RNA-binding components of the writer complex may direct methylation by binding the writer to mRNA, since some m^{6}A sites are near to RNA-binding domains (*Zaccara et al., 2020).* m^{6}A erasers seem to influence this landscape, although to what extent remains unknown.

While implicated as an m^{6}A eraser in early studies (*Gerken et al., 2007*) of m^{6}A, the fat mass and obesity-associated protein (FTO), does not seem to deplete the modification in recent transcriptome-wide studies, instead showing preference to demethylate m^{6}Am (*Garcia-Campos et al., 2019).* An m^{6}A demethylase known as ALKBH5 showed reduced levels of m^{6}A in cells and is implicated to be important for sperm development and in certain cancers (*Zhang, S et al., 2017 and Zhang, C et al., 2016*). Since there is relatively little time between the processing of the mRNA in the nucleus until its export to the cytoplasm, the question of how dynamically regulated m^{6}A is remains to be seen and needs more study to determine.

1.3.5 m^{6}A Reader Proteins

mRNA regulation is shaped largely due to various m^{6}A-binding proteins, or readers that recognize the mark. m^{6}A is recognized by these three readers in distinct ways: by directly binding to m^{6}A, by inducing structural changes after binding, or by indirectly binding to m^{6}A-binding proteins (*Zaccara et al., 2020).* The first category is comprised of mainly YTH domain proteins, which are localized in the cytoplasm as well as the nucleus and affect mRNA regulation in many ways. YTHDC1 studies show that the protein may impact splicing, and may recruit splicing factors (*Xiao et al., 2016*). YTHDC2 studies have shown that m6A promotes translation when bound to a reporter RNA (*Dhote et al., 2012*). YTHDF proteins include YTHDF1, YTHDF2 and YTHDF3 and have been shown to differentially influence m^{6}A mRNAs by promoting translation, degradation of transcripts, and both functions respectively (*Shi et al., 2017, Wang et al., 2014 and Wang et al., 2015*). However, a paper published in 2020 suggested that the reader roles are largely redundant; the true effect of the protein on RNA was to promote mRNA degradation (*Zaccara & Jaffrey, 2020*). While the effects of m^{6}A are correlated with various readers, much work needs to be performed to elucidate the true roles each are playing in the mRNA landscape.

1.3.6 m^{6}A Studies in *Drosophila melanogaster*

While m^{6}A is abundant in the mammalian system, it is also prevalent in the developing *Drosophila* nervous system (*Kan et al., 2017*). In flies, m^{6}A is highly prevalent in the developing larval brain and decreases as the organism matures to adulthood (*Lence et al., 2016*). Drosophila has direct orthologs of the mammalian m^{6}A writers, readers, and erasers. However, while vertebrates have five members of the YTH domain proteins, only two exist in Drosophila: the nuclear YT521-B and the cytoplasmic CG6422 (*Dezi et al., 2016 and Lence et al., 2016*). Another advantage to studying this RNA modification in flies is that there is no m^{6}Am in flies, which is infrequently mischaracterized as an m6A modification, since the antibody in mammals will not differentiate the two. Moreover, flies with complete loss of function for m6A are viable and able to be characterized (*Kan et al., 2017*). The loss of the catalytically active subunit METTL3 is much more documented in adult flies, leading to defects in locomotion, flight, learning and memory and behavior (*Kan et al., 2021)*. Studies have found that m^{6}A directly affects sex determination (*Guo et al., 2018, Lence et al., 2015*) and splicing (*Kan et al., 2017).* m^{6}A was also found to regulate axonal growth through its interactions with the cytoplasmic m^{6}A reader (*Worpenberb et al., 2021*). In what may be a fly specific role, a recent study done in Drosophila S2 cells showed that m^{6}A releases pausing on the RNA pol II through chromatin recruitment (*Akhtar et al., 2021*). In this study, removing the methyltransferase complex results in more RNA pol II pausing, and affects transcription. In *Drosophila*, m^{6}A has been mapped

throughout the transcriptome using Me-RIP seq, miCLIP, LC-MS, and for RNA-binding proteins by using TRIBE (*Worpenberb et al., 2021*). While researchers have studied the effects of m^{6}A in flies as a whole, there is very little information of whether m^{6}A has cell-type specific effects, especially in the central nervous system.

1.4 The Role of N^6-methyladenosine (m^{6}A) in Disease

Levels of m^{6}A seem to be the high in the nervous system. The modification plays important roles in stem cell differentiation, regulating RNA turnover, and may influence a number of disorders. Loss of m^{6}A has a conserved effect across organisms, affecting stem cell differentiation and embryonic neurogenesis. The loss of m^{6}A in *Drosophila* leads to reduced lifespan, altered neural gene expression and severe behavioral defects. In mice models, m^{6}A regulates brain function, and the loss of m^{6}A in early development is lethal for embryonic mice. In other studies, the loss of one of the methyltransferase subunits is enough to disrupt embryogenesis, even if METTL3, the catalytic subunit, is preserved. Researchers generated a conditional knockout of the m^{6}A writer subunit METTL14 in the mice forebrain and found that neural progenitors were delayed in progressing through the cell cycle (*Yoon et al., 2017*). Additionally, when the m^{6}A reader YTHDF2 was knocked out in mice, researchers found that cortical neurogenesis was affected and that mice die at late embryonic developmental stages (*Li et al., 2018*). There is also growing research on how m^{6}A also influences cancer cell metastasis and growth (*Yang et al., 2020*). In one study, METTL3 is shown to be expressed in prominent levels in acute myeloid leukemia (AML), a cancer that effects hematopoietic progenitor cells (*Chen et al., 2018*). The levels of m^{6}A in specific transcripts are increased and this inhibits cell differentiation. There is also growing evidence that m^{6}A levels affect psychiatric disorders. The fragile C mental retardation protein (FMRP) was recently found to be an m^{6}A reader that regulated neural differentiation and facilitated the nuclear export of m^{6}A- modified mRNAs (*Edens et al., 2019*). Many of the same transcripts that were modified by m^{6}A were related to disorders such as autism and schizophrenia (Angelova et al., 2018).

1.5 Objective & Aims of My Dissertation Research

My dissertation work investigates the hypothesis that m^{6}A has different targets and different metabolic effects in progenitors and neurons in the developing *Drosophila* nervous system by focusing on the following aims:

Aim 1: Map m^{6}A targets in neural progenitors and differentiated neurons in the *Drosophila* larval brain.

Aim 2: Measure cell type-specific mRNA decay and identify links between m^{6}A modifications and mRNA metabolism within neural progenitors and differentiated neurons.

Experimental approaches used to investigate these aims are presented in Chapter 2. My thesis work looks at the role of m^{6}A in mRNA turnover in specific neural cells using the developing *Drosophila* nervous system. Chapter 3 examines potential future directions for this work.

Chapter 2: N^6-methyladenosine (m^6A) dynamics during *Drosophila melanogaster* neural development

This chapter is composed of an original research manuscript draft. The tentative title and authors are:

Josephine D. Sami, Whitney England, Robert C. Spitale, Michael D. Cleary. N^6-methyladenosine (m^6A) Dynamics During Drosophila Neural Development.

I am the sole first author on this work and performed all of the experiments described here. Whitney England and Robert Spitale at U.C. Irvine provided computational analysis guidance and support. Michael Cleary is the sole corresponding author.

Introduction

N^6-methyladenosine or "m6A" is the most common nucleotide modification within eukaryotic mRNAs. This epitranscriptome mark is recognized by "reader" proteins that affect multiple mRNA metabolic processes, including splicing, decay, and translation (He and He, 2021). m6A is highly enriched in the nervous system of multiple organisms, including mammals, and has been implicated in events ranging from neural stem cell differentiation (Wang et al., 2018) to synaptic plasticity (Merkurjev et al., 2018). While multiple lines of evidence support the importance of m6A in neural development, a comprehensive understanding of the neurodevelopmental processes affected by m6A is still being acquired. In particular, whether or not m6A-modified mRNAs and the metabolic effects of m6A vary by neural cell type or neurodevelopmental stage is largely unknown. This information is important for determining the degree to which m6A influences the diversity of cellular fates and functions in the nervous system.

In mammals, cytoplasmic m6A is primarily found in the 3' UTR or at stop codons and is recognized by three readers: YTHDF1, YTHDF2, and YTHDF3. Early work assigned distinct roles to each reader (YTHDF1 and 3 promote translation, YTHDF2 promotes mRNA degradation) and suggested that each "DF" protein bound distinct target mRNAs (Murakami and Jaffrey, 2022). However, recent studies strongly suggest that all DF proteins target the same set of mRNAs and act redundantly via a single mechanism to induce mRNA decay (Zaccara and Jaffrey, 2020). There may be exceptions to this m6A rule in mammals: for example, rare 5'UTR m6A promotes translation by directly recruiting the initiation factor eIF3 (Meyer et al., 2015). Dynamic regulation of m6A target metabolism could conceivably occur via variation in m6A stoichiometry (the fraction of transcripts that contain the modification), but quantitative analyses of m6A across cell types mainly supports a model in which m6A targeting and frequency is uniform regardless of cell type or physiology (Murakami and Jaffrey, 2022).

Here we investigate m6A dynamics within the developing *Drosophila melanogaster* central nervous system. *Drosophila* provides multiple advantages for m6A research: m6A is present at elevated levels in the embryonic, larval, and adult nervous system; deletion of the *Mettl3* methyltransferase gene is not lethal, thus allowing molecular and phenotypic analyses in m6A-null animals; and the *Drosophila* genome encodes a single cytoplasmic reader, Ythdf, thus simplifying experiments aimed at manipulating the m6A system. The m6A methyltranscriptome has previously been mapped in *Drosophila* cell lines (Worpenberg et al., 2021), embryos (Kan et al., 2017), and adult heads (Kan et al., 2021).

Multiple genetic approaches have demonstrated that m6A is involved in *Drosophila* sex determination (Haussmann et al., 2016), locomotion (Lence et al., 2016), learning and memory (Kan et al., 2021), and axon growth (Worpenberg et al., 2021). As in mammals, multiple molecular mechanisms have been assigned to m6A in *Drosophila*. In the nucleus, m6A regulates splicing (Haussmann et al., 2016) and m6A at the 5' end of nascent transcripts relieves RNA polymerase II (RNAP II) pausing to promote transcription (Akhtar et al., 2021). In mature cytoplasmic transcripts, m6A is almost exclusively found in the 5' UTR (in contrast to the 3' UTR and stop codon localization found in mammals). *Drosophila* 5' UTR m6A is thought to affect translation in one of two ways. First, m6A *decreases* translation of a subset of targets that are bound, in a Ythdf-dependent manner, by the translation repressor Fmr1 (Worpenberg et al., 2021). Second, 5' UTR m6A has been shown to *increase* translation based on reporter assays and the observation that *Mettl3* loss-of-function causes a widespread decrease in nascent protein production (Kan et al., 2021), suggesting enhancement of translation by m6A. 5' UTR m6A is enriched among transcripts with low translation efficiency and Kan et al. proposed a model in which an m6A-dependent mechanism counteracts inefficient translation to augment target protein production (Kan et al., 2021).

While previous work in *Drosophila* identified m6A targets and molecular mechanisms, several knowledge gaps remain, especially with respect to neural development. First, it is unclear to what degree prior m6A mapping efforts identified targets relevant to neural progenitors; mapping in embryos included all cell types (of which neural progenitors are a tiny fraction) and adult heads lack neural progenitors. Second, while prior work ruled out a correlation between m6A and mRNA decay (Kan et al, 2021), this was based on comparison of adult head m6A targets and embryonic central nervous system mRNA decay; m6A targets and mRNA half-lives have yet to be compared in equivalent neural cell populations. Finally, experiments aimed at measuring the effects of m6A on target protein output *in vivo* in the nervous system are lacking and could help identify mechanisms relevant to specific neural cell types.

This work addresses the above knowledge gaps by obtaining methyltranscriptome maps that are representative of the neural progenitor and neuron populations in the *Drosophila* larval brain. The larval brain contains a well-defined population of neural stem cells, called neuroblasts, that undergo multiple rounds of asymmetric self-renewing divisions to produce neurons and glia. Using combinations of genetic manipulation and RNA profiling techniques, we obtained neurodevelopmental m6A maps that allowed comparisons of m6A stoichiometry between neuroblasts and neurons as well as investigation of how m6A influences mRNA stability in neuroblasts and neurons. We found extensive m6A targeting of neurodevelopmental regulators, including m6A modification of progenitor-specific transcripts. However, among transcripts expressed in both neuroblasts and neurons, we did not find any evidence of differential m6A stoichiometry. We confirmed the previously described correlation between m6A and translation efficiency and found a neuroblast-specific correlation between m6A and mRNA decay. Finally, we used *in vivo* imaging to demonstrate that m6A enhances target protein output in neuroblasts and neurons.

Results

Transcripts encoding neurodevelopmental regulators are m6A modified in neuroblasts and neurons

Near the end of *Drosophila* larval neurogenesis, the combined brain lobes contain approximately 10,000 neurons, approximately 500 glia, and only 200 neuroblasts (Carney et al., 2012)(Pereanu et al., 2005). To increase representation of the neuroblast methyltranscriptome, we used a genetic modification that causes neuroblasts to undergo symmetric self-renewing divisions, thus generating larval brains with abundant ectopic neuroblasts and relatively few neurons (Carney et al., 2012). In these experiments, we used *insc-Gal4* to drive expression of *UAS-aPKC^{CAAX}* in neuroblasts and harvested larval brains at 96 - 102 hours after larval hatching (ALH) as a source of "neuroblast-biased RNA". In contrast, we used wildtype larval brains at 96 - 102 hours ALH as a source of "neuron-biased RNA" since neurons are vastly more abundant than any other cell type at this stage. In addition to collecting RNA samples that cover the neuron and neuroblast methyltranscriptomes, we collected RNA from stage-matched brains of *Mettl3* null larvae to obtain negative control "m6A null RNA". Brain RNA from each genotype was split in two; half was used for quantification of total mRNA abundance (input RNA-seq) and half was used for methyltranscriptome purification using anti-m6A immunoprecipitation (meRIP-seq) (Meyer et al., 2012). This experimental design is summarized in **Figure 1A**. As a first step, we used input RNA to test for differential abundance of known neuroblast or neuron-specific mRNAs in the neuron-biased and neuroblast-biased samples. We confirmed that *insc-Gal4 > UAS-aPKC^{CAAX}* samples are enriched in neuroblast-specific transcripts and depleted of neuron-specific transcripts (**Figure 1B**).

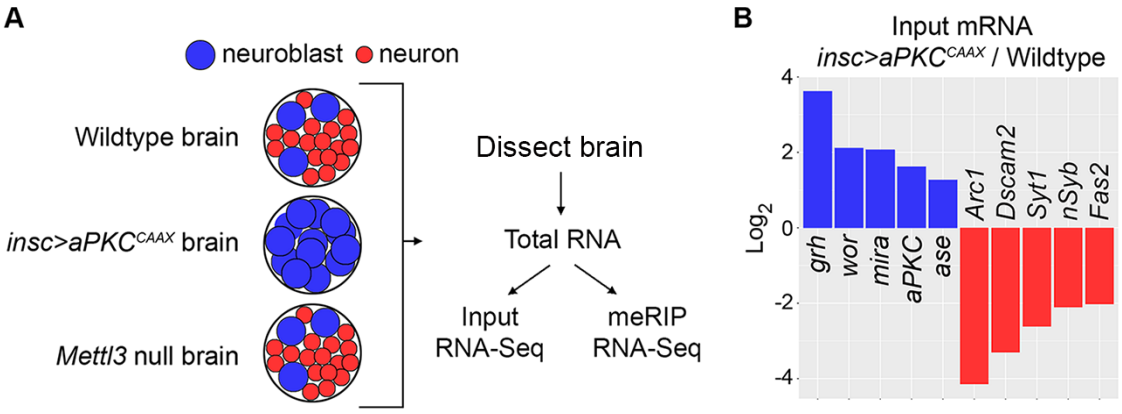

Figure 1. Confirmation of neuroblast-biased and neuron-biased transcriptomes. (A) Summary of experimental design. (B) Relative abundance of known neuroblast-specific mRNAs (blue) and known neuron-specific mRNAs (red) in *insc>aPKC^{CAAX}* vs. wildtype brains. Average fold-change is shown, based on biological replicate measurements.

Subsequent meRIP-seq analysis of neuroblast-biased, neuron-biased, and m6A-null RNA samples identified 867 m6A targets in the larval brain (**Figure 2A** and **SUPPLEMENTAL TABLE 1**). 634 of these targets (73%) were also identified in adult *Drosophila* heads by Kan et al., revealing a high degree of m6A conservation across life cycle stages. As previously described, the m6A-null meRIP-seq data were useful for identifying "background" signal. This allowed high-confidence target identification and more accurate mapping of m6A peaks along a transcript: only peaks that were significantly enriched compared to m6A-null meRIP were included. Using this approach, we found that the vast majority of m6A peaks in the neuroblast-biased and neuron-biased transcriptomes are in the 5' UTR (**Figure 2B**). We used sequences from the combined neuron-biased and neuroblast-biased datasets to search for motifs associated with m6A and found significant enrichment of an AAACV motif. This motif contains the invariant AAAC core identified in other *Drosophila* m6A mapping studies (Kan et al., 2021) (Worpenberg et al., 2021), further supporting the accuracy of our m6A mapping.

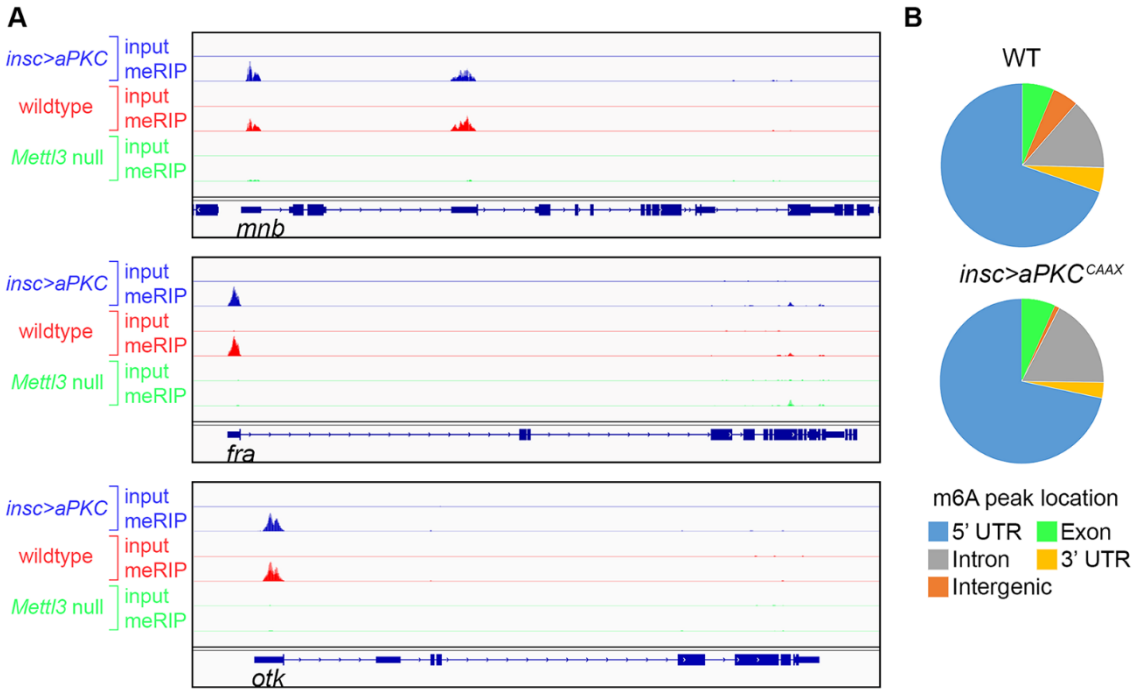

Figure 2. m^{6}A peaks map to 5' UTRs in neuroblast-biased and neuron-biased brains.
(A) IGV plots of representative meRIP-seq data. Note that 5'UTR peaks are missing or significantly reduced in *Mettl3* null brains while other peaks, for example in the downstream exons of *fra*, are independent of *Mettl3*. Such *Mettl3*-independent peaks were excluded from target identification and m^{6}A position mapping. (B) Fraction of m^{6}A peaks within different gene regions according to neuroblast-biased and neuron-biased meRIP.

TABLE 1. Top 20 GO categories enriched among m^6A targets, ranked by p-value.

GO Term	Definition	*P* value	Count	Fold Enrichment
GO:0022008	neurogenesis	2×10^{-112}	233	5.5
GO:0007154	cell communication	1.17E-92	291	3.6
GO:0048812	neuron projection morphogenesis	1.03817E-60	125	6.1
GO:0006355	regulation of transcription, DNA-templated	1.22591E-46	171	3.5
GO:0007611	learning or memory	3.0573E-16	41	5.5
GO:0050808	synapse organization	4.08262E-15	54	4.0
GO:0016358	dendrite development	4.85611E-14	41	4.9
GO:0008219	cell death	6.01438E-13	59	3.3
GO:0007010	cytoskeleton organization	6.47879E-13	79	2.7
GO:0042063	gliogenesis	9.82272E-13	28	6.7
GO:0008039	synaptic target recognition	1.37172E-11	20	9.3
GO:0008356	asymmetric cell division	2.66858E-11	28	6.0
GO:0007405	neuroblast proliferation	8.02033E-11	26	6.2
GO:0016055	Wnt signaling pathway	1.59515E-10	30	5.2
GO:0050795	regulation of behavior	1.5915E-09	26	5.5
GO:0007268	chemical synaptic transmission	1.08744E-08	45	3.2
GO:0030509	BMP signaling pathway	1.28894E-07	18	6.9
GO:0048665	neuron fate specification	2.56373E-07	13	10.1
GO:0000165	MAPK cascade	3.60313E-06	26	4.0
GO:0055057	neuroblast division	6.36049E-05	13	6.9

Table 1. Top 20 GO categories enriched among m6A targets, ranked by p-value. Count is the number of m6A targets in that category. Enrichment is the observed frequency of targets in that category (count / 867 total m6A targets) divided by the expected frequency (all genes in that category / total *Drosophila* genes).

To gain insight into the potential roles of m6A in larval brain development, we used gene ontology analysis to identify functional categories overrepresented among m6A targets. This revealed significant enrichment of transcripts encoding regulators of essential neurodevelopmental processes, such as “synapse organization,” “dendrite development,” “neuroblast proliferation” and “neuron fate specification” in addition to processes known to be broadly important for development, such as “cell death,” “cytoskeleton organization,” and “Wnt signaling pathway” (**TABLE 1**). As expected, the combined profiling of neuroblast-biased and neuron-biased brains allowed identification of a large number of m6A targets (233 genes) that were not identified by previous m6A mapping in adult heads (Kan et al., 2021). This novel set of m6A targets includes many genes known to regulate neuroblast proliferation, asymmetric cell division, neuron fate specification and axon pathfinding (**Figure 3A**).

Comparing neuron-biased and neuroblast-biased meRIP-seq data revealed several genes with higher m6A peaks in one genotype or the other, potentially indicating cell type-specific differences in m6A stoichiometry (**Figure 3B**). To test this possibility, we normalized meRIP-seq ratios (neuron-biased / neuroblast-biased) to input ratios (neuron-biased / neuroblast-biased). This identified cases where differential m6A peaks could be explained by differences in total transcript abundance. Following normalization for input reads and filtering for genes with statistically significant differences, we did not identify any evidence of differential m6A stoichiometry (**Figure 3C**). 135 genes had approximately equal input expression levels (fold-change ≤ 1.5 and no statistically significant difference between neuroblast-biased and neuron-biased input mRNA abundance), but none of these “uniformly” expressed transcripts showed evidence of elevated m6A frequency in neuroblast-biased or neuron-biased brains.

This suggests that elevated m6A peaks in neuroblast-biased brains, as shown for *Sp1* and *run* in Figure 3B, are due to elevated expression of the corresponding transcripts in neuroblasts. The converse is true for elevated m6A counts in neuron-biased brains. We further tested this conclusion using m6A immunoprecipitation and RT-qPCR of *Sp1* and *run* (**Figure 3D**). 5S rRNA served as a negative control in these experiments as it was not identified as a m6A target in our experiments and is known to lack methyladenosine in metazoans (Dannfald et al., 2021). meRIP and RT-qPCR confirmed *Sp1* and *run* as m6A targets and ruled out differential m6A between neuroblast-biased and neuron-biased brains. Overall, our m6A mapping indicates that m6A is selectively targeted to neurodevelopmental genes in neuroblasts and neurons and that for transcripts present in both cell types, the degree of m6A modification is largely constant.

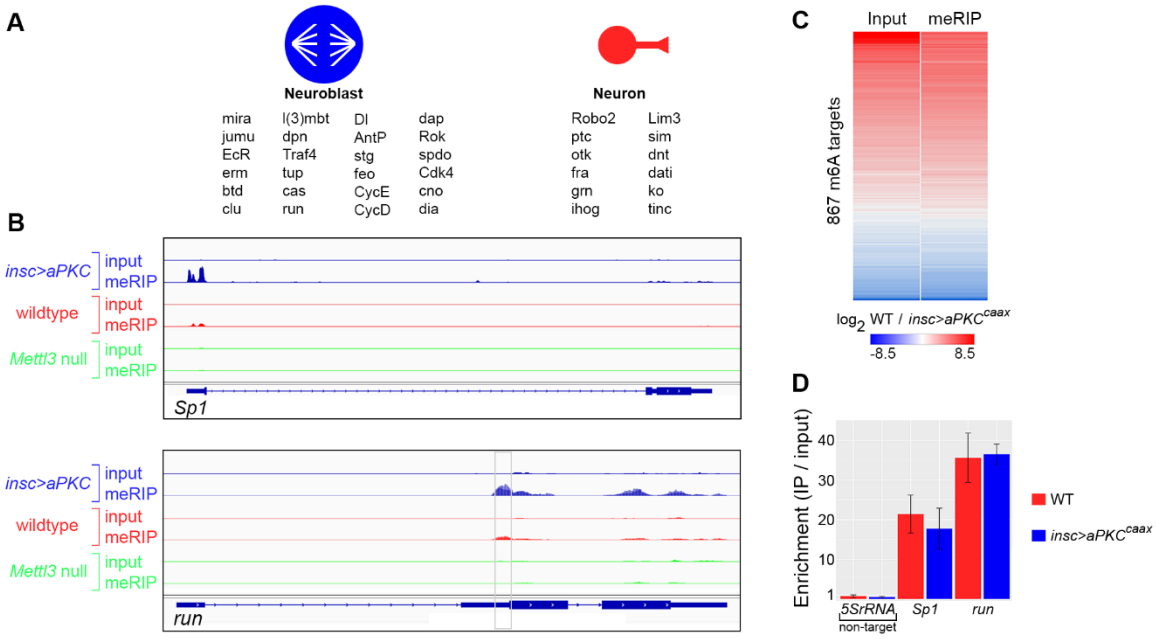

Figure 3. Novel m^{6}A targets and evidence of uniform m^{6}A **stoichiometry between neuroblast-biased and neuron-biased brains.**
(A) Partial list of novel m^{6}A targets identified in this study. Genes are listed below the cell type they are most associated with, e.g., cell cycle and fate determination genes are associated with neuroblasts, neuron identity and axon pathfinding genes are associated with neurons. (B) Representative IGV examples of genes with apparent increased m^{6}A frequency in neuroblast-biased brains. A single Mettl3-dependent peak in the 5'UTR of run is outlined in gray. (C) Heat map comparing neuron-biased / neuroblast-biased (WT / *insc > aPKC^{caax}*) ratios for all m^{6}A targets based on input RNA-seq and meRIP-RNA-seq. (D) RT-qPCR of transcripts in meRIP-enriched versus input RNA. 5S rRNA is a negative control (no m^{6}A). Data are average ± SEM for three independent input and meRIP samples and duplicate RT-qPCR reactions per sample.

m^{6}A correlates with low translation efficiency and low mRNA stability

Given that m6A has been implicated in a range of mRNA metabolic processes, we next sought clues to the molecular function of m6A during larval brain development. Akhtar et al. identified a role for m6A and the nuclear m6A reader in enhancing transcription by relieving RNAP II pausing at target genes. This was demonstrated in *Drosophila* S2 cells, and the phenomena has not been described *in vivo* or in a developmental context. To test this possible function, we used RNA-seq measurements of total mRNA abundance from wildtype larval brains and *Mettl3* null brains. We reasoned that if m6A significantly enhances transcription in larval brains, the absence of m6A would result in decreased target abundance due to increased RNAP II pausing. As previously shown for adult *Drosophila* heads [Kan et al, 2020], this analysis failed to identify a strong directional relationship between m6A and transcript abundance (**Figure 4A**). We also tested for a relationship between m6A and translation efficiency (TE). Using the adult head ribosome profiling data analyzed by Kan et al., we found a similar significant enrichment of m6A in mRNAs with low translation efficiency (**Figure 4B**).

Next, we tested for any relationship between m6A and mRNA stability. We obtained mRNA half-life measurements for neural progenitors and neurons using EC-tagging pulse-chase (Hida et al., 2017). Briefly, this approach uses targeted expression of a cytosine deaminase-uracil phosphoribosyltransferase (CD:UPRT) fusion enzyme to convert 5-ethynylcytosine (EC) into 5-ethynyluridine (EU)-monophosphate in specific cell types. EU is incorporated into nascent mRNAs of target cells and the tagged RNAs can be purified after “pulse” feeding 5EC and at subsequent “chase” timepoints in which excess uridine is provided to ensure no new tagged transcripts are made. We used *insc-Gal4* to express *UAS-CD:UPRT* in neural progenitors and *nSyb-Gal4* to express *UAS-CD:UPRT* in neurons. Globally, neural progenitor and neuron transcriptomes had similar half-life distributions (**Figure 4C and SUPPLEMENTAL TABLE 2**), indicating that transcriptome-wide mRNA decay kinetics do not significantly differ between neural progenitors and neurons. However, differences were revealed when we analyzed the half-lives of m6A targets: there was no relationship between m6A and mRNA stability in neurons (**Figure 4D**), while m6A targets were significantly less stable in neuroblasts (**Figure 4E**).

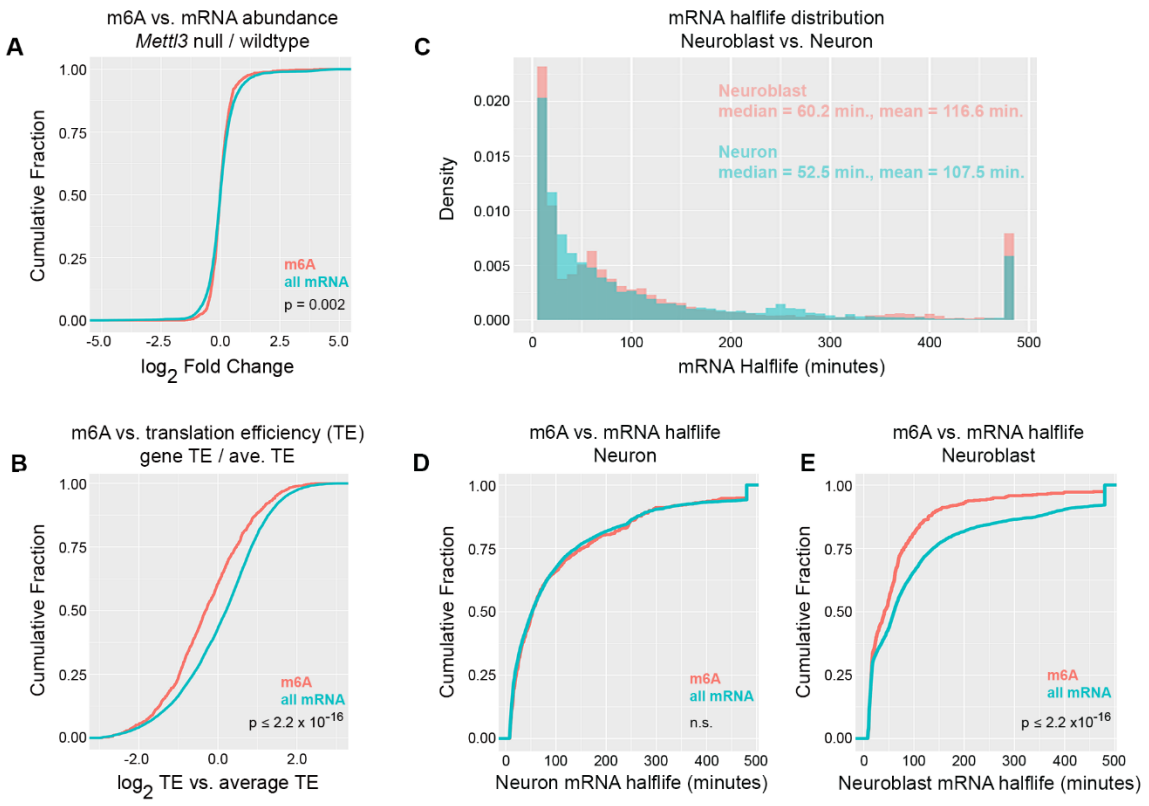

Figure 4. m^{6}A correlates with low translation efficiency generally and low mRNA stability specifically in neuroblasts.
(A) Loss of m^{6}A does not significantly affect target mRNA steady state abundance. Log2 fold-change in transcript abundance in *Mettl3* null brains versus wildtype brains, plotted as the cumulative distribution of m^{6}A targets compared to all larval brain transcripts. (B) m^{6}A correlates with low translation efficiency (TE). Log2 TE efficiency (transcript-specific TE / average TE), plotted as the cumulative distribution of m^{6}A targets compared to all larval brain transcripts with matching adult head TE data. (C) Distribution of mRNA half-lives in neuroblasts and neurons, as determined by EC-tagging pulse-chase. Half-life values greater than 480 minutes were rounded down to 480 minutes since accurate decay curve fitting was difficult for these very long-lived transcripts. (D) and (E) m^{6}A correlates with low mRNA half-life in neuroblasts but not neurons. mRNA half-life plotted as the cumulative distribution of m^{6}A targets compared to all mRNAs as measured in neurons (D) or neuroblasts (E). P-values for all cumulative distribution comparisons were determined by two sided Kolmogorov-Smirnov tests.

To further investigate the different relationships between m6A and stability in neuroblasts and neurons, we directly compared the half-lives of m6A targets in each cell type and found that 185 m6A targets are at least 1.5-fold more stable in neurons (**Figure 5A**). If one assumes m6A directly affects mRNA stability, this differential decay is surprising given that our data suggest m6A is constant between neuroblasts and neurons. Differential stability could be caused by varied Ythdf expression, however; our EC-tagging data and prior transcriptome profiling of purified neuroblasts and neurons (Yang et al., 2016) show that Ythdf mRNA is present at equally high levels in progenitors and neurons. Alternatively, these data agree with a model in which m6A does not affect mRNA stability and the difference between neuroblasts and neurons is due to m6A-independent stabilization of targets in neurons. GO analysis of the neuron-stabilized m6A targets revealed enrichment of transcripts involved in neuron-specific functions such as “synapse assembly”, “dendrite development” and “axon guidance” (**Figure 5B**), supporting the model that these transcripts are likely selectively stabilized to support the needs of mature neurons. We conclude that neuron-specific stabilization of m6A targets explains the lack of correlation between m6A and half-life in neurons.

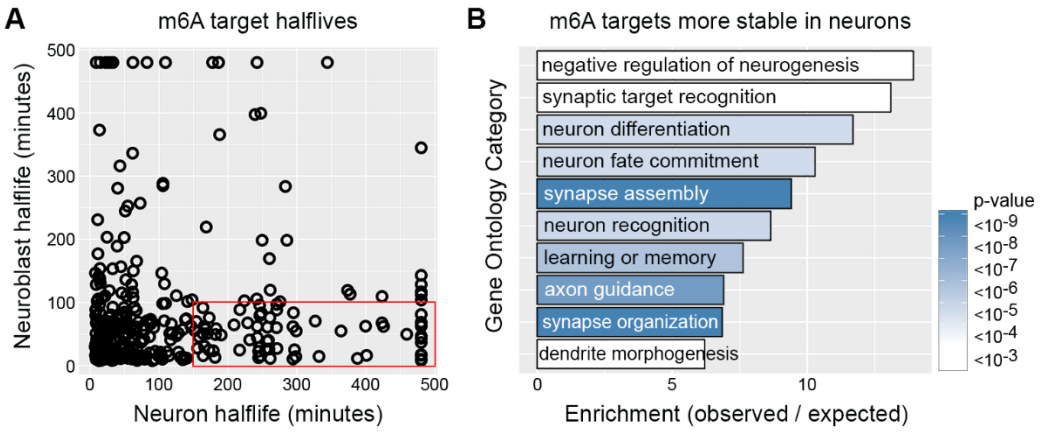

Figure 5. m^{6}A **targets encoding regulators of neuron-biased functions are stabilized in neurons.** (A) m^{6}A target half-life in neuroblasts compared to neurons. An example of neuron-stabilized transcripts (half-life ≤ 100 minutes in neuroblasts and ≥ 150 minutes in neurons) are outlined by a red box. (B) Gene ontology categories significantly enriched among m^{6}A targets that are ≥ 1.5-fold more stable in neurons.

m^{6}A and Ythdf enhance target protein expression in larval brains

The analyses described above reveal correlations between m6A, mRNA translation and mRNA decay, but these findings do not reveal underlying mechanisms. With respect to translation, two mechanisms have been described in *Drosophila*: translation inhibition that requires Fmr1 (Worpenberg et al., 2021) and Ythdf-dependent enhancement of translation (Kan et al., 2021). Comparing our m6A targets with previously identified m6A-dependent Fmr1 targets in the larval central nervous system revealed an overlap of only 5.8% (50 genes). Since the majority of our targets are not predicted to be regulated by Fmr1, we conclude that the translation enhancing effect is more relevant. With respect to mRNA stability, 3' UTR m6A in mammalian transcripts induces decay via DF proteins recruiting the CCR4-NOT deadenylase complex (Zaccara and Jaffrey, 2020) but a decay pathway triggered by 5'UTR m6A has not been described in any species. Instead, we predict that the relationship between m6A and mRNA is indicative of a compensatory mechanism, similar to that described for translation efficiency. In this case, we predict that 5'UTR m6A enhances translation of low stability transcripts whose decay is regulated by m6A-independent mechanisms.

According to the translation enhancement model, *Mettl3* deletion should decrease target protein production and *Ythdf* overexpression should increase target protein production. To test this model in the developing larval brain, we preformed quantitative immunofluorescent imaging of proteins encoded by m6A targets in wildtype brains, *Mettl3* null brains and *Ythdf* overexpressing brains (overexpressing *Ythdf* in neural progenitors using *insc-Gal4 > UAS-Ythdf*). We measured immunofluorescent signal for two m6A targets, the transcription factor Runt (Run) and the cell cycle regulator Cyclin D (CycD), in addition to one non-target, the transcription factor Asense (Ase). Translation efficiency data are not available for *run* and *ase*, likely because these genes are not expressed or are only expressed at low levels in adult brains, but the TE value for *CycD* in adult heads is 1.17 compared to an average value of 1.37 (Zhang et al., 2018). In contrast to the TE data, mRNA stability data are available for each of these genes. In neural progenitors *run* decays very rapidly (half-life of 5.1 minutes) and is more stable in neurons (half-life of 17.6 minutes). *CycD* and *ase* expression is primarily restricted to neural progenitors and we therefore only obtained progenitor-specific decay measurements for these transcripts: *CycD* has a half-life of 136.3 minutes and *ase* has a half-life of 16.1 minutes.

Runt expression in neuroblasts changed in a manner corresponding to the translation enhancement model: Runt signal decreased in *Mettl3* null neuroblasts and increased in *Ythdf* overexpressing neuroblasts (**Figure 6A**). In contrast, Runt signal was unaffected in *Mettl3* null neurons but increased in neurons of *Ythdf* overexpressing brains. Similar to Runt, CycD protein levels decreased in *Mettl3* null neuroblasts, but *Ythdf* overexpression did not alter CycD abundance (**Figure 6B**). Finally, as expected, neither *Mettl3* loss-of-function or *Ythdf* overexpression altered the abundance of the non-target Asense (**Figure 6C**). The *run* and *CycD* data support our prediction that m6A does not induce mRNA decay; if this were the case, *Mettl3* deletion would most likely increase protein levels (we observe the opposite effect) and *Ythdf* overexpression would decrease protein levels (again, we see the opposite). Instead, these results support the model that 5'UTR m6A enhances translation of target mRNAs in the developing nervous system.

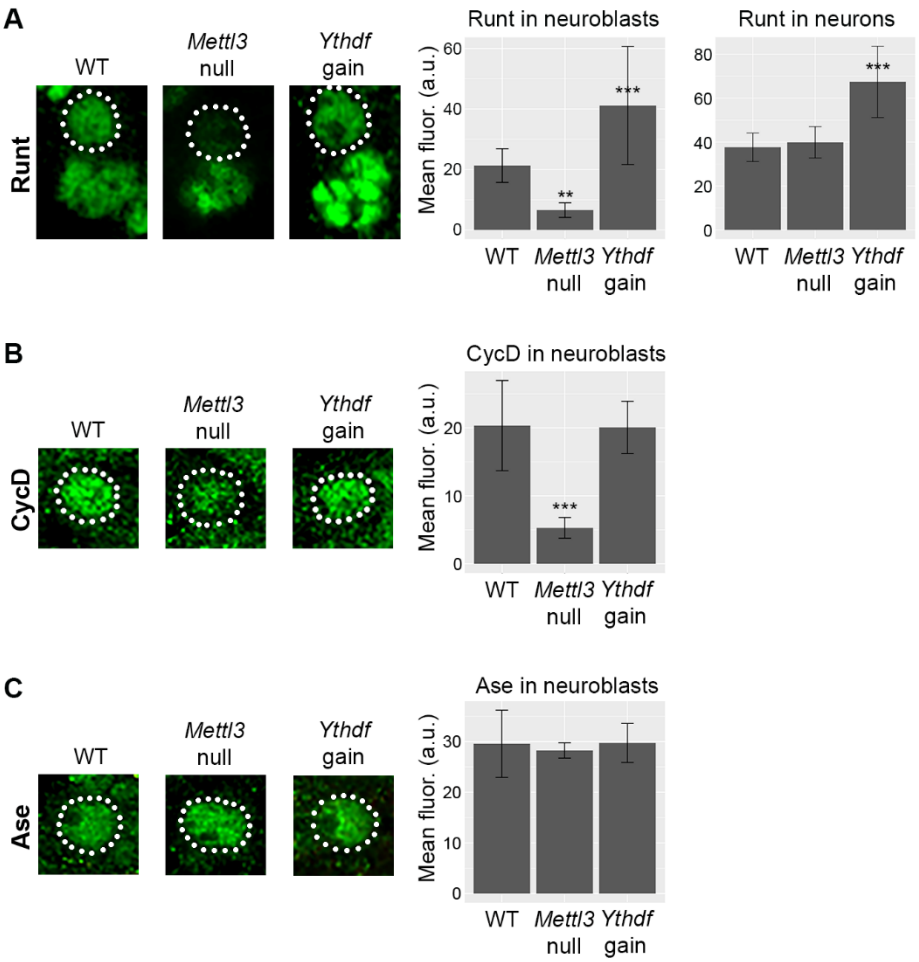

Figure 6. m^{6}A **and Ythdf increase target protein abundance in neuroblasts and neurons.**
(A) Representative images of Runt in neuroblasts (outlined by white dotted line) and neurons (clustered below neuroblast) in wildtype, *Mettl3* null and *Ythdf* overexpressing brains. The fluorescent signal intensity (mean and standard deviation) for Runt in each genotype and cell type is shown at right. (B) Representative images of CycD in neuroblasts (outlined by white dotted line) in wildtype, *Mettl3* null and *Ythdf* overexpressing brains. CycD is not expressed in neurons. The fluorescent signal intensity (mean and standard deviation) for CycD in each genotype and cell type is shown at right. (C) Representative images of Ase in neuroblasts (outlined by white dotted line) in wildtype, *Mettl3* null and *Ythdf* overexpressing brains. Ase is not expressed in neurons. The fluorescent signal intensity (mean and standard deviation) for Ase in each genotype and cell type is shown at right. All fluorescent intensity measurements are derived from analysis of a minimum of 20 cells from at least 6 different brain lobes. Statistical significance determined by one way ANOVA followed by Tukey post-test. P-values: ** = $1x10^{-4}$, *** ≤ $1x10^{-7}$.

DISCUSSION

Precise deployment of genetic information during neurogenesis requires multiple layers of post-transcriptional control. Epitranscriptome regulation by m6A is one such layer, but the full diversity of cell types and pathways affected by m6A, and the degree to which m6A modification and target metabolism are dynamically regulated, is not fully understood. We investigated these questions of m6A dynamics in the context of *Drosophila* larval brain development. The m6A profiles we obtained from neuroblast-biased and neuron-biased brains expand the list of known m6A targets in the *Drosophila* nervous system, contributing to a deeper understanding of m6A targeting during neurodevelopment. Importantly, our results lend support to the model that m6A stoichiometry of individual transcripts is largely uniform and does not vary according to cell type. In spite of this uniformity, we show that m6A targets may be metabolized in a cell type-specific manner, particularly if mRNA processing pathways vary by cell type. Finally, we provide neural-specific *in vivo* evidence to support the translation enhancement model proposed by Kan et al. Altogether our results point to m6A as an important modifier of protein output from key neurodevelopmental transcripts.

While *insc>aPKC*^{CAAX} brains are not exclusively composed of neuroblasts and wildtype brains are not exclusively composed of neurons, the transcriptomes of each are heavily biased toward one cell type or the other and have a high likelihood of revealing differential m6A stoichiometry. However, no significant differential m6A targeting was indicated by our analyses. This outcome agrees with the theory that differential m6A stoichiometry is rare (Murakami and Jaffrey, 2022). Part of this theory is based on the mechanics of m6A deposition and removal; the enzymes that write and erase m6A appear to be ubiquitous and it is unclear how their activity might be conditionally modified to alter only a subset of targets. In the context of *Drosophila* neural differentiation, dynamic m6A targeting would require selective alteration of methyltransferase activity between neuroblasts and neurons in a way that targeted specific genes, or transcript-specific demethylase activity in one cell type versus the other. While such mechanisms may exist and could involve differences in RNAP II pausing at target genes, we interpret our results as supporting the “non-dynamic m6A” model, at least along the neural differentiation axis in *Drosophila*.

In addition to identifying novel m6A targets, we also obtained transcriptome-wide mRNA decay measurements in neural progenitors and neurons. A link between m6A and mRNA decay in *Drosophila* was previously ruled out by comparing adult head m6A targets and embryonic central nervous system mRNA half-lives. A major limitation of this prior analysis is that the embryo data are mainly derived from decay measurements in neurons; neural progenitor-specific measurements were missing. Our cell type-specific mRNA half-life data revealed a correlation between m6A and short half-life in neuroblasts but no correlation between m6A and half-life in neurons. It is important to recognize that our m6A – mRNA decay results demonstrate a correlation (or lack thereof) and not causation. Given that m6A stoichiometry is constant between neuroblasts and neurons, that the Ythdf reader is expressed at equal levels in both cell types (Yang et al., 2016), and that a molecular pathway linking 5’ UTR m6A to mRNA decay is not known, we interpret these results as evidence of m6A-independent stabilization of target transcripts in neurons. Neuron-specific stabilization of m6A targets could occur via various mechanisms and act synergistically with the translation enhancing effect of m6A to boost protein production in neurons relative to neuroblasts. Such a mechanism supports the concept that m6A is a

modifier of protein output from target transcripts but not the main driver of target mRNA metabolism. This model also partly explains why loss of m6A in *Mettl3* null animals does not result in severe neurodevelopmental defects; underlying transcriptional and post-transcriptional regulatory pathways allow grossly normal neurodevelopment to occur in the absence of m6A.

A major question in developmental biology is how varying rates of transcription, decay and translation combine to determine gene expression dynamics. Short mRNA half-life and inefficient translation favor low protein output, but the m6A pathway may have evolved to fine-tune protein levels of targets with these properties. For example, rapid decay of *run* in neuroblasts is expected to result in very low protein levels. m6A-dependent enhancement of *run* translation could increase the output of each transcript prior to degradation and may help achieve expression levels appropriate for Runt activity in neuroblasts. Our quantitative imaging of Runt in neuroblasts supports this model: Runt levels decrease in *Mettl3* null brains and increase in *Ythdf* overexpressing brains. *Runt* mRNA half-life increases threefold in neurons and there is a corresponding increase in Runt signal in neurons compared to neuroblasts. Loss of *Mettl3* in neurons does not result in a quantifiable decrease in Runt levels, perhaps because neuron-specific stabilization of *run* mRNA compensates for the loss of m6A. Surprisingly, *Ythdf* overexpression in neural progenitors significantly increased Runt signal in neurons. This may be due to elevated Runt production in progenitors and excess Runt being actively or passively inherited by neurons. Alternatively, Ythdf itself may be inherited by neurons where it is sufficient to increase Runt production. While *Mettl3* loss-of-function decreased CycD signal in neuroblasts, *Ythdf* overexpression had no effect. This may indicate a role for m6A position in affecting translation: the largest *Mettl3*-dependent peak in *run* is concentrated near the start codon, while *CycD* has two Mettl3-dependent peaks distributed more broadly over the 5' UTR (data not shown). Whether m6A position along a transcript determines the degree to which Ythdf enhances translation remains to be determined.

Our finding that m6A is targeted to neurodevelopmental regulatory genes in neuroblasts and neurons raises the question of how target specificity is achieved. A recently described targeting mechanism in *Drosophila* provides an intriguing answer that could also explain the relationship between m6A, mRNA half-life and translation efficiency. In *Drosophila*, the m6A methyltransferase complex (MTC) is selectively recruited to promoters where RNAP II is bound in a paused, non-elongating state (Akhtar et al., 2021). Importantly, it is well established that genes involved in developmental transitions and dynamic cellular processes have high levels of paused RNAP II in *Drosophila* (Akhtar et al., 2021) (Lagha et al., 2013) (Zeitlinger et al., 2007). Additionally, we and others have shown that transcripts involved in developmental transitions and dynamic cellular processes tend to have short half-lives (Burow et al., 2015) (Thomsen et al., 2010), and in many instances those transcripts become more stable in neurons (Burow et al., 2018). Finally, transcripts encoding developmental regulators are also known to contain sequence features like uORFs (Zhang et al., 2018) or secondary structures (Jackson et al., 2010) that influence translation efficiency. The fact that genes encoding developmental regulators are transcriptionally-regulated by paused RNAP II (the signal for m6A methylation) and are post-transcriptionally regulated via dynamic mRNA decay and translation provides a parsimonious explanation for the m6A – mRNA decay – TE correlations we identified.

Conclusions

This work expands our understanding of the role of m6A in neural development by providing a detailed view of m6A targeting and target metabolism in neural progenitors and neurons. The use of neuroblast-biased brains allowed identification of m6A targets missed by prior profiling efforts and allowed comparison of m6A stoichiometry between neuroblast-biased and neuron-biased transcriptomes. We found that there is little variation in the m6A stoichiometry between these transcriptomes. Our neuroblast and neuron mRNA half-life data revealed a strong correlation between m6A and low mRNA stability in neuroblasts but not neurons. We conclude that the lack of correlation in neurons is due to m6A-independent stabilization of those targets upon differentiation, in accordance with evidence that 5'UTR m6A in *Drosophila* affects translation and not stability. Finally, we provide neural-specific *in vivo* evidence to support the translation enhancement model. Overall, our findings contribute to the view that m6A is important for fine-tuning gene expression during neural development and that dynamic changes in m6A stoichiometry are rare.

Methods

Drosophila genetics

The following lines were obtained from the Bloomington *Drosophila* Stock Center: Oregon-R-P2 (wildtype) (stock # 2376), *insc-Gal4* (stock # 8751), and *nSyb-Gal4* (stock #51635). *UAS-aPKC*^{CAAX} was a gift from C.Y. Lee. *UAS-Ythdf* and *Mettl3* null flies were gifts from E. Lai. For EC-tagging, Gal4 lines were crossed with *UAS-CD:UPRT* on the 3rd chromosome (stock # 77120).

meRIP

m6A-RNA immunoprecipitation was performed as previously described (Meyer et al., 2012). Biological replicate experiments (from dissection to library generation) were performed for all three genotypes (wildtype, *insc > aPKC*^{CAAX}*,* and *Mettl3* null). Purified m6A-RNA was used to make sequencing libraries using the NuGen Ovation Universal RNA-Seq protocol, including adapter ligation and ribosomal RNA depletion using a *Drosophila*-specific AnyDeplete rRNA primer mixture. Libraries were amplified and purified according to the NuGen protocol and quality was assessed using an Agilent Bioanalyzer DNA high-sensitivity chip.

EC-tagging pulse-chase

5-ethynylcytosine was synthesized as previously described (Hida et al., 2017). Biological triplicates were prepared by carrying out 5EC feeding and RNA processing independently. Larvae were reared at 25°C and fed 1 mM 5EC from 72 – 84 hours after hatching prior to RNA extraction (pulse samples) or transferred to media with 10 mM uridine for 3, 6, or 12 hours prior to RNA extraction (chase samples). Crudely dissected central nervous system RNA was extracted using Trizol. For each genotype and timepoint, duplicate 20 mg RNA samples were biotinylated using Click-iT Nascent RNA Capture reagents (ThermoFisher), purified on Dynabeads MyOne Streptavidin T1 magnetic beads (ThermoFisher), and used for "on bead" RNA-seq library synthesis, as previously described (Aboukilila et al., 2020).

RNA-seq Sequencing and bioinformatics

Sequencing was performed on a HiSeq 2500. Sequence data were pre-processed with *FastQC and* used with default parameters. Reads were trimmed using Trimmomatic to discard any reads with adaptor contamination and low-quality bases. We used *STAR* to map reads to the Ensembl gene annotation for *Drosophila melanogaster* (BDGP6). Peaks were found by running MACS2 (Zhang et al., 2008) with default parameters. For input RNA-seq and pulse-chase RNA-seq, reads were mapped using *kallisto*. meRIP-seq data were quantified and mapped using *featureCounts* and those data were used in differential expression analysis with *limma-voom* (Law et al., 2014). *Limma-voom* was used to identify genes with significantly higher meRIP-seq counts in wildtype brains compared to *Mettl3* null brains. All candidates that lacked significant counts above *Mettl3* null were visually inspected in IGV to determine if the gene should be considered a m6A target. *PeakAnnotator* was used to annotate m6A position, as previously described (Dominissini et al., 2013). We tested for significant differences in meRIP-seq counts from wildtype and *insc > aPKC*^{CAAX} brains using *limma-voom*. Gene ontology analysis was performed using GO TermFinder (Boyle et al., 2004) with the full *Drosophila melanogaster* gene set as background and default settings for all other parameters.

RT-qPCR

First strand cDNA was made using the SuperScript VILO cDNA Synthesis Kit (Invitrogen). Real-time PCR quantitation was performed on a Rotor-Gene Q (Qiagen) in 20 mL reactions using SYBR green detection. Custom PCR oligonucleotides (Integrated DNA Technologies) were used for all targets: *run* forward (TAGGACAAAGGACCCCAATC), *run* reverse (TCGTCGCACGATTTTATGAG), *Sp1* forward (TTGAAGCTATCTTGCGGTTG), *Sp1* reverse (ATAGAGCGGGCGTTTCTTTC), *5S rRNA* forward (GCCAACGACCATACCACGCT), *5S rRNA* reverse (AGGCCAACAACACGCGGTATTCCCA) . Triplicate RT-qPCR experiments (starting at the m6A immunoprecipitation step) were performed for all target transcripts.

Imaging and quantification of target proteins

The following antibodies were used: guinea pig anti-Runt (gift of C. Desplan) at 1:400, rabbit anti-CycD (Santa Cruz Biotech, sc-25765) at 1:250, and rabbit anti-Ase (gift of Y.N. Jan) at 1:1,000. Alexa-fluor conjugated secondary antibodies (ThermoFisher) were used. Brain imaging was performed using a Zeiss LSM 880 confocal microscope. Immunostaining was performed in parallel for all targets and genotypes, to minimize batch effects and confocal settings were kept constant. Pixel intensity measurements were made using ImageJ and the ‘‘measure’’ tool applied to an identical size area of interphase nuclei of neuroblasts, individual neurons, and multiple brain regions lacking expression of the protein of interest to calculate background signal.

Chapter 3: Conclusions and Future Directions

In the nervous system, stem cell renewal and differentiation are both crucial for proper development. RNA metabolism, particularly turnover and translation, is required for the appropriate gene regulation underlying stem cell renewal and differentiation. N^6-methyladenosine (m^6A), an RNA post-transcriptional modification that has elevated levels in the central nervous system, has an essential role in RNA metabolism and neural development, although the exact molecular and cellular functions of m^6A may vary by species, cell type and target mRNA. Understanding the complexities of these processes will be valuable for advances in RNA biology and developmental biology and could potentially contribute to advances in treating diseases caused by defective RNA metabolism or aid in the development of RNA-based therapeutics.

My dissertation displays the importance of m^6A in regulating RNA metabolism in the nervous system. I used the *Drosophila* larval nervous system to map m^6A in both neural progenitor stem cells and post-mitotic neurons. While I found that m^6A levels are not cell-type specific, one surprising finding is that m^6A correlates with decreased mRNA stability in neuroblasts but not in neurons. I integrate this result with other findings regarding m^6A mechanisms in *Drosophila* to conclude that m^6A does not directly affect stability and that m^6A targets are stabilized in neurons via an independent mechanism. This supports the general view that m^6A fine-tunes gene expression and in *Drosophila* this is mainly achieved through enhancing translation. I generated evidence to support this model through *in vivo* imaging; loss of m^6A decreased target protein abundance while Ythdf overexpression increased target protein abundance.

My dissertation shows a unique look at m^6A through cell type-specific methods. This work establishes the validity and usefulness of the *Drosophila* larval brain to understand post-transcriptional modifications like m^6A. I summarize promising future directions below.

Test the hypothesis that m^6A does not directly affect mRNA stability

Our model that m^6A does not directly affect mRNA stability but is deposited on low stability mRNAs due to the nature of the targeting mechanism (genes with paused RNAP II tend to encode low stability mRNAs) and likely acts to "boost" translation of those mRNAs, is consistent with our data and the current understanding of m^6A molecular mechanisms. However, to completely rule out a direct effect on stability, it will be necessary to measure mRNA stability (via EC-tagging) in neuroblasts and neurons with lacking m^6A (*Mettl3* null background), lacking Ythdf, and overexpressing Ythdf. The genetics of such experiments are somewhat complicated to establish but obtaining these mRNA stability measurements should be possible.

Identify Ythdf interactors in neuroblasts versus neurons

My proposed model assumes that Ythdf functions in an identical manner, via the same interacting partners, in neuroblasts and neurons. However, this might not be true and different interactions could explain different mRNA metabolism effects and cell type specific functions. Ythdf interactions, including coordinated targeting with other RNA-binding proteins, could be further investigated using techniques such as TRIBE (McMahon et al., 2016), PAR-CLIP-seq (Shi et al., 2017) or proximity-induced

biotinylation (Youn et al., 2018). Zaccara & Jaffrey have shown that all three DF proteins in mammalian cell lines interact with RNA degradation pathways proteins (*Zaccara et al., 2020)*. This type of interaction makes sense for 3' UTR m^{6}A in mammalian mRNAs, with the first step being recruitment of deadenylation complexes, but does not obviously conform to any mechanism for the 5'' UTR m^{6}A that predominates in Drosophila mRNAs. Instead, I would anticipate interactions with translation regulators. Regarding intersection with other RNA-binding proteins, it would be particularly informative to test for neuron-specific targeting of m^{6}A-modified transcripts by proteins that act to stabilize those transcripts.

Obtain higher resolution m^{6}A maps and integrate additional transcript features

For various technical reasons, my work employed MeRIP-seq for mapping m^{6}A peaks. While this is an effective method for identifying the general location of m^{6}A and allowed us to confirm the previously described 5'UTR bias in *Drosophila*, other methods, particularly miCLIP, provide individual nucleotide resolution and could reveal aspects of differential m^{6}A stoichiometry overlooked in my work. There are many examples in the literature that show where the m^{6}A mark lies affects translation or degradation rates. As described for run and CycD, I found some preliminary evidence that m^{6}A location within the 5'UTR or proximity to start codons may influence the translation enhancing effects of Ythdf. It would be interesting to quantify this in reporter lines, to add m^{6}A to specific areas and remove it, and to quantify reporter output in different cell types. Bioinformatically analyzing the structure of the transcripts with m^{6}A could yield information as well; do neuroblast transcripts with m^{6}A have different secondary structure from those transcripts in neurons? In yeast and mammals, methylation is more likely to occur in unstructured regions, due to accessibility of the methyltransferase complex (*Garcia-Campos et al., 2019; Schwartz et al., 2013; and Spitale et al., 2015*). Additionally, bioinformatic approaches could investigate the relationship between codon content, m^{6}A, and mRNA metabolism. This may be relevant to translation enhancement and the relationship between translation and decay previously described in *Drosophila* (*Burow et al., 2018*).

My thesis work provides a solid foundation for these future directions. I anticipate that the results and approaches described in my thesis will help advance m^{6}A research broadly and guide important future research.

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