UNIVERSITY OF CALIFORNIA, MERCED

RIBOSOMAL RNA BIOGENESIS AND NEURAL DIFFERENTIATION IN *DROSOPHILA*

A DISSERTATION SUBMITTED TO THE SCHOOL OF NATURAL SCIENCES AND THE DIVISION OF GRADUATE STUDIES OF UNIVERSITY OF CALIFORNIA, MERCED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

in

QUANTITATIVE AND SYSTEMS BIOLOGY

by

Jade Fee Quail
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Committee:
Professor Fred Wolf
Professor David Ardell
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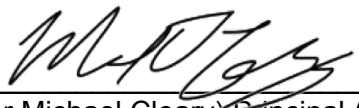
I certify that I have read this dissertation and that, in my opinion, it is fully adequate in scope and quality as a dissertation for the degree of Doctor of Philosophy.



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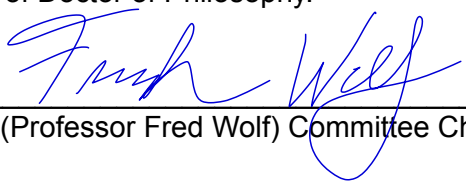

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University of California Merced 2022

Dedication

To my parents, Becky and Scott Fee, for always believing in me, and for teaching me to believe in myself.

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- **Fee, J**, Aboukilila, M, Cleary, MD. Progenitor-derived ribosomal RNA supports protein synthesis in *Drosophila* neurons. *Nat Sci*. 2022; 2:e20210032. https://doi.org/10.1002/ntls.20210032

- Burow DA, Martin S, **Quail JF**, Alhusaini N, Coller J, Cleary MD. Attenuated Codon Optimality Contributes to Neural-Specific mRNA Decay in *Drosophila*. *Cell Rep*. 2018 Aug 14;24(7):1704-1712. doi: 10.1016/j.celrep.2018.07.039

Presentations

- **Fee, J**, Aboukilila, M, Cleary, MD. Progenitor-derived ribosomal RNA supports protein synthesis in *Drosophila* neurons. *Neuro Joint Lab Meeting*. University of California Merced (oral presentation). 2021 Sep 10.

- Burow DA, **Quail JF**, Martin S, Alhusaini N, Coller J, Cleary MD. Attenuated Codon Optimality Establishes Neural-Specific mRNA Stability. *Bay Area RNA Club Meeting*. University of California San Francisco (poster presentation). 2017 Dec 04.

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Abstract

Cell growth and differentiation are highly regulated to allow organisms to develop properly. During differentiation, cells often switch entire gene transcription and translation programs in order to accommodate changing metabolic and functional needs. One way differentiation is regulated is through changes to the cell's ribosome concentration. Decreases in ribosome concentration tend to move cells away from proliferation and towards differentiation. This regulation of cell physiology at the level of ribosome concentration has important implications for human diseases, including cancer and neurodegenerative disorders.

This work describes a mechanism influencing cell differentiation in the developing *Drosophila* nervous system. While it is generally common that progeny cells have lower concentrations of mature ribosomes than their progenitors, the mechanisms behind this change are poorly understood in the nervous system. I demonstrate that decreased ribosome concentration in neurons compared to neuroblasts is driven by a significant decrease in ribosomal RNA transcription in these post-mitotic cells. My data also show that high levels of translation in newborn neurons is supported exclusively by ribosomal RNA that is inherited from their progenitors.

This work adds to a growing body of evidence linking changes in ribosome concentration to changes in the cell cycle and to the proteome. Additionally, it lays the groundwork for future studies of this kind to proceed with confidence using the developing brain of *Drosophila* as a model.

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Chapter 1

Introduction

The mechanisms that regulate gene expression are among the most critical in biology regardless of context. Tightly controlled gene expression is responsible for everything from the initial assignment of the body axis in embryos to the functioning of the nervous system. It is because of this universal influence that we continue to study and define the array of mechanisms and systems responsible for gene expression control, and ultimately, organism development and survival. One highly influential factor affecting the regulation of gene expression is the ribosome. Traditionally viewed simply as an invariant component of the translation machinery, it is now understood that ribosomes influence gene expression in a variety of ways beyond simply converting mRNA to protein. In this chapter, I review the essential events in ribosome biogenesis, the state of knowledge regarding differential ribosome biogenesis and translation activity, introduce the *Drosophila* nervous system as a useful model for studying ribosome biogenesis in progenitors versus differentiated cells, summarize potential mechanistic implications of altered ribosome concentration, and finally, place ribosome biogenesis in the context of human disease. This review and synthesis is meant to provide the background and rationale for the study described in Chapter 2.

1.1 Ribosome biogenesis and translation activity

Ribosomes are composed of protein (ribosomal protein, or “RP”) and rRNA (ribosomal RNA) components. In eukaryotes, ribosomes are composed of roughly 50% rRNA and 50% RP. Because of this hybrid nature, ribosomes therefore depend on pathways related to both protein synthesis and rRNA synthesis for formation. A change to either of these processes will likely disrupt ribosome creation and assembly. The amount of ribosomes present in a cell typically correlates with its proliferative state. In eukaryotes, an actively dividing cell may have up to 10 million ribosomes. Mature ribosomes exist as both free-floating complexes and as complexes tethered to the endoplasmic reticulum, where they synthesize proteins entering the secretory pathways. Ribosomes are composed of two subunits, commonly referred to as the large and small subunit. The subunits are classified by their sedimentation rates, which is measured using Svedberg units, abbreviated “S”. In both prokaryotes and eukaryotes, the subunits differ by 20S. In prokaryotes, the ribosome is made of the small 30S subunit and the large 50S subunit. For eukaryotes, subunits are slightly larger, at 40S and 60S. The 40S subunit is composed of 33 RPs and an 18S rRNA. The 60S subunit includes 47 RPs along with three separate rRNAs, named the 25S, 5S, and 5.8S rRNAs. Ribosome biogenesis - both rRNA transcription and assembly of RPs and rRNAs into large and small subunits - occurs in the nucleolus, a subcompartment of the nucleus (*Fox, 2010, Wilson, 2012, and Baßler and Hurt, 2018*). Correspondingly, a large nucleolus is a hallmark of cells undergoing high levels of ribosome biogenesis.

Ribosome biogenesis in eukaryotes occurs over multiple steps in a highly regulated process guided by a host of ribosome assembly factors (AFs) and small nucleolar RNAs (snoRNAs). Initially, pre-rRNAs are synthesized in a subcompartment of the nucleolus named the fibrillar compartment, which houses multiple “fibrillar centers.” These pre-rRNAs are named according to the same convention as their final ribosomal subunit

counterparts; they are the 90S, 66S, and 43S pre-rRNAs. These same 90S, 66S, and 43S pre-rRNAs are found in eukaryotes from yeast to humans, demonstrating that ribosome assembly is a highly conserved process (*Tamaoki, 1966, Warner and Soeiro, 1967, and Baßler and Hurt, 2018*). These pre-rRNAs undergo endo- and exonucleolytic cleavages facilitated by snoRNAs and are joined with relevant RPs (which are imported into the nucleus after translation) to create ribosomal "precursor particles." This process of precursor particle assembly takes place in the granular compartment of the nucleolus with the help of AFs. It is important to note that ribosomes are assembled in a hierarchical manner: both AFs and RPs are incorporated into the nascent ribosomal precursor particles in a specific order. The pre-60S and pre-40S ribosomal subunits are then exported from the nucleus to allow maturation and eventual enzymatic function in the cytosol (*Watkins, 2012 and Baßler and Hurt, 2018).*

The primary activity of the ribosome is to carry out translation: the process of "translating" the message encoded in messenger RNA (mRNA) to that of the amino acids of protein. Ribosomes complex with mRNA and transfer RNAs (tRNAs) to carry out this process. The ribosome facilitates connection of these two molecules by fitting each into different pockets, like most enzymes. Ribosomes have three pockets into which tRNAs can fit: the acceptor "A" site, the peptidyl "P" site, and the exit "E" site. mRNA is read in a triplicate pattern, and each nucleotide triplicate is known as a "codon." Each codon pairs with the anticodon loop of a distinct tRNA, which is loaded with the corresponding amino acid that codon encodes. For example, the start codon "AUG" on the mRNA is recognized by a tRNA with the reverse complement sequence"CAU" in its anticodon position. This same tRNA is charged with the amino acid methionine, so AUG in the mRNA "translates" to methionine in the nascent protein. tRNAs are named by the amino acids they are charged with, such as $tRNA^{Met}$.

The ribosome scans an mRNA until it finds the start codon, at which time it is able to begin translating with the assistance of $tRNA^{Met}$. As translation continues, tRNAs are shuffled through the A, P, and E sites as they enter and exit the translation process. The catalytic aspect of translation is the formation of a new peptide bond between amino acids on the growing peptide chain. This formation of this bond is catalyzed by rRNA in the ribosome, rather than by an RP. In any given snapshot of the middle of the translation process, tRNAs occupy all three pockets in the ribosome. The E site contains the empty tRNA which has already donated its amino acid to the growing chain and is about to exit the ribosome. The P site contains the tRNA with most recently added amino acid. The nascent polypeptide chain is attached to this tRNA. The A site contains the newest tRNA with the next required amino acid. The rRNA catalyzes peptide bond formation between the two newest amino acids (in the P site and A site). As the bond forms, the growing peptide chain is transferred to the tRNA located in the A site, via attachment to its amino acid. The large and small subunits of the ribosome then act as a ratchet, shifting all tRNAs forward one position. Eventually, when a stop codon is encountered in the mRNA, a termination factor with no amino acid attached will enter the ribosome, and translation is terminated, resulting in a free, nascent protein.

1.2 Differential ribosome abundance and cell fate determination

It is generally understood that ribosome biogenesis promotes cell proliferation and cell growth (*Zhang, 2014*). While all living cells must undergo some level of gene

transcription and translation, they are not equal in these abilities. For example, cancer cells may increase translation initiation to increase protein output and sustain proliferation (*Ruggero, 2013*). Additionally, changes to total translation can impair HSC (**h**ematopoietic **s**tem **c**ell) homeostasis (*Signer, 2014*). Just as ribosome biogenesis is associated with cell proliferation, decreases in ribosome biogenesis and concentration is increasingly being recognized as a mechanism driving cell fate decisions and differentiation. Changing the ribosome concentration of a cell consequently influences its translation output, though not always in a straightforward manner. Ribosome concentration can be changed in a cell via three mechanisms: by changes in rRNA transcription, by changes in RP transcription and/or translation, or by changes in ribosome degradation or assembly/disassembly and repair. Possibly the best documented example of these three mechanisms directing differentiation is a decrease in rRNA synthesis. rRNA transcription by RNA Pol I is a tightly controlled process in all organisms, requiring multiple factors for initiation, including TIF-IA (Transcription Initiation Factor IA) in both *Drosophila* and mammals. TIF-IA activity influences the rate of rRNA transcription and is regulated by phosphorylation as part of the TOR (**t**arget **o**f **r**apamycin) kinase pathway (*Grewal, 2007*). Often, reduction in rRNA transcription comes in response to a cell-external signal and simultaneously with cell cycle arrest. It has therefore been difficult to determine the extent to which cell cycle arrest versus rRNA transcription reduction directly promotes differentiation.

This mechanism of decreasing rRNA transcription to drive differentiation has been described during development of the female *Drosophila* germline (*Zhang, 2014*) and the mammalian forebrain (*Chau, 2018*). The phenomenon has also been demonstrated in mammalian tissue culture of HL-60 and THP-1 cell lines (lineages commonly used to study hematopoietic differentiation) and mouse hematopoietic stem cells (HSCs) (*Hayashi, 2014*). In this case, downregulation of rRNA transcription by addition of actinomycin D (Act D) and by siRNA (small interfering RNA) downregulation of TIF-IA expression both resulted in increased expression of the major pro-differentiation marker CD11b. TIF-IA knockdown in (non-cancerous) mouse HSCs similarly resulted in a higher proportion of differentiated cells in the culture. Possibly the most significant finding provided by this study is that the differentiation effect linked to decreases in rRNA transcription are not in fact a simple byproduct of cell cycle arrest. THP-1 cells were treated with Act D or roscovitine, a cell cycle inhibitor that functions through CDK (cyclin dependent kinase) inhibition. Inhibition of the cell cycle without repression of rRNA transcription was insufficient to induce CD11b expression in these cells, indicating that differentiation is a direct result of changes to rRNA transcription levels independent of cell cycle arrest (*Hayashi, 2014*).

Interestingly, although it seems logical that a global decrease in translation would be driven by a decrease in ribosome abundance, these two measures are often inversely correlated. In some cases, ribosome abundance decreases while global translation increases, specifically during cell differentiation. Ribosome concentration in mouse ESCs (**e**mbryonic **s**tem **c**ells) decreases during differentiation to embryoid bodies (*Ingolia, 2011*), despite an increase in translation. Similarly, rRNA and RP production decreases in the *Drosophila* female germline as differentiation occurs, but overall translation increases (*Zhang, 2014 and Sanchez, 2016*). The female *Drosophila* germline is composed of ovarian germline stem cells (GSCs). This stem cell population undergoes asymmetric divisions in order to maintain their stem cell population while also

producing cells that enter the differentiation pathway to become cystoblasts (*Fuller and Spradling*, 2007). Pol I activity (responsible for transcribing rRNA genes) is regulated in part by a three subunit SL1-like complex which includes the protein underdeveloped, or Udd (*Zhang, 2014*). When Udd is disrupted, the GSC population becomes less proliferative, and eventually dies out. Essentially, when Pol I activity is reduced, the cells are driven towards differentiation into cysts. Likewise, when Pol I activity is increased in newly born daughter cells on the differentiation pathway, maturation into cysts is delayed. This demonstrates that rRNA synthesis is a direct contributor to the differentiation status of these germline stem cells (*Zhang*, *2014*). The takeaway from these experiments is not necessarily that the two processes are unrelated, but rather that a decrease in ribosome abundance is sufficient to drive cell differentiation through selection for specific translation programs.

Neural differentiation appears to be strongly influenced by changes in ribosome abundance. Compared to progenitors, RP and rRNA production are decreased in postmitotic neurons (*Slomnicki, 2016 and Qian, 2006*), and it is thought that this decrease drives progenitors towards differentiation. Compartment-specific changes in ribosome concentration are also associated with neural differentiation. Mature neurons do not contain ribosomes in their axons, and the process of clearing these ribosomes is highly regulated via a ubiquitin-proteasome mechanism (*Kleiman, 1994 and Costa, 2019*). Additionally, there is evidence that ribosome inheritance is sufficient to sustain differentiated neurons (*Hetman, 2019*), implying that novel ribosome biogenesis is not a constant process in post-mitotic neurons.

1.3 *Drosophila melanogaster* as a model for understanding cell fate specification and asymmetric cell divisions during neurogenesis

While brain development presents an attractive system in which to address questions of ribosomal regulation of cell fate, multiple aspects of mammalian brain development can hinder research progress. Probably the most obvious example is that much of mammalian neurogenesis occurs *in utero*. Because of this, access to developing mice or other mammalian brains comes with the technical challenge and side-effect prone step of extracting embryos from the uterus. Mammals are also relatively expensive to keep in a lab and require complex protocols for husbandry and handling. Mammals also tend to develop slowly compared to other animals and take time to reach sexual maturity. It can be useful, therefore, to choose a more appropriate model organism for studying the early developing brain. *Drosophila* develop quickly, are relatively cost-effective, and, perhaps most significantly, develop external to their mothers. Their brains also have proven to be quite useful in studying brain development at large. They share many homologous brain structures, genes, and mechanisms with humans, making them an excellent candidate for research in neurodevelopment (*Fortini, 2000*). Additionally, larval *Drosophila* brains are easily extracted and manipulated in the lab. Studies of the developing *Drosophila* nervous system tend to focus largely on the larval stages of development, when the brain exists as an easily dissected and visualized organ.

The *Drosophila* brain consists of two hemispheres and a ventral nerve cord, with each hemisphere containing both a central brain region and an optic lobe. The larval brain contains roughly 100 neuroblasts per hemisphere. There are two types of neuroblasts in

the *Drosophila* brain: Type I and Type II (*Bowman, 2008, Bello, 2008, and Boone, 2008*). Type I neuroblasts divide asymmetrically to produce one neuroblast daughter and one ganglion mother cell, or GMC. A GMC only experiences one more division, resulting in two postmitotic neurons or glial cells (*Kohwi, 2013*). Type II neuroblasts also divide asymmetrically, giving rise to a neuroblast daughter and an intermediate neural progenitor, or INP. Unlike GMCs, INPs experience multiple rounds of asymmetric divisions, each time resulting in one INP and one GMC, which goes on to divide similarly to GMCs of Type I lineages. Identity is largely conferred to these intermediate progenitors through differential expression of the proteins Ase (asense), a transcription factor that promotes the progenitor fate, and Pros (prospero), a transcription factor that promotes differentiation (*Jarman, 1993, Hirata, 1995, and Li, 2014*). Of the 100 neuroblasts per hemisphere, only eight are Type II neuroblasts. However, due to their proliferative nature, most mature neurons are of Type II neuroblast descent. Type II neuroblast differentiation is also similar to that of mammalian neural stem cells, whose differentiation pathway includes intermediate progenitors called transit-amplifying cells (*Brand and Livesey, 2011, Henrique and Bally-Cuif, 2010, and Li, 2014*).

Neuroblasts grow and divide based on external, sometimes system-wide signals, such as changes in insulin levels. At the same time, neuroblasts experience some heterogeneity in their size and division rates. These characteristics seem to be controlled largely by cell autonomous factors, including two RNA binding proteins, Imp (IGF2BP) and Syp (Syncrip/hnRNPQ) (*Liu, 2015)*. These proteins work in an antagonistic system to ensure tight temporal control over neuroblast growth and proliferation (when Imp levels are high, Syp levels are low, and vice versa). In Type I neuroblasts, Imp has been shown to bind multiple RNAs, among the most impactful of which is the well studied transcription factor Myc (*Samuels, 2020*). Myc is considered an oncogene and is known for its prominent roles in cell proliferation and growth, partly through its activation of ribosomal protein transcription and RNA polymerase I component transcription (*Dang, 2013, Gallant, 2013, van Riggelen, 2010, and Grewal, 2005*). The number of myc mRNA transcripts in the cell increases as Imp increases. Myc is therefore likely stabilized by Imp binding, preserving the transcript for a longer period of time in the cell and allowing it to be translated more times before degradation. Increased Imp therefore results in correspondingly high Myc protein levels and stronger Myc-associated effects, including cell growth and proliferation. Type I neuroblasts with high levels of Imp are comparatively larger and experience higher rates of division than their wildtype counterparts. Syp appears to aid this system indirectly via its negative regulation of Imp. Importantly, while Myc protein is not found downstream of the Type I neuroblast in the lineage, myc transcript is present; translation of myc mRNA is restricted to neuroblasts. This implies that Myc translation is not dependent on the Imp/Syp system. Rather, Myc protein levels in Type I neuroblasts are elevated due to transcript stabilization provided by Imp binding. Translational regulation of Myc does occur in progeny of the Type II neuroblast lineage through the RNA binding protein Brat (Brain Tumor). However, Brat has not been implicated in Type I neuroblast lineage (*Samuels, 2020*). It is likely that similar translational controls exist in the Type I neuroblast lineage, though none have been revealed to date.

Neuroblast size must be tightly regulated during development to maintain the balance between proliferation and differentiation. Cells must maintain a large enough size to continue dividing; an otherwise healthy cell that is too small simply will not divide. Most

neuroblasts in the embryonic *Drosophila* brain shrink in size with each division until entering a quiescent state just prior to the larval stage. Upon exiting quiescence after larval hatching, the neuroblasts enter a cell growth period before resuming proliferation and maintain their size throughout this period until they eventually enter a smaller, permanently post-proliferative state at the end of larval neurogenesis (*Truman and Bate, 1988*). Mbm (Mushroom Body Miniature) is a nucleolar protein that has been shown to influence neuroblast size. Mbm is required for neuroblast proliferation and appears to affect this process via its regulation of cell size and growth (Mbm loss of function mutants are significantly smaller than wildtype cells). Mbm is also required for proper RNA processing and small ribosomal subunit biogenesis, which in turn affects whole ribosome biogenesis and total protein output within the cell. Mbm is known to be transcriptionally activated by the growth-promoting transcription factor Myc. While much of a neuroblast's decision between whether to proliferate or not depends on Mbm activity, cell fate specification functions independently of Mbm (*Hovhanyan, 2014*) and relies more on the asymmetric cell division mechanisms.

Asymmetric cell division (ACD) is essential to maintaining a stem cell population (in the brain, neuroblasts) while also sending cells down the differentiation path (ganglion mother cells, or GMCs). This process is tightly regulated by a sizable network of proteins. Disruption to this network tends to result in tumor formation, due largely to the fact that stem cell populations are primed to maintain their proliferative abilities with every division in order to prevent depletion of the stem cell reserve. The self-renewing capabilities of these cells easily turn tumorous when dysregulated. The fate of a neuroblast's daughter cells is determined by two independent protein networks, each localized to either the apical or basal side of the cell (*Li, 2014*). The apical proteins ensure that the apical daughter cell remains a neuroblast, while the basal proteins ensure the daughter adopts GMC fate. The apical protein network depends largely on the cooperative efforts of three proteins known collectively as the Par complex: Baz (Bazooka), Par6 (partitioning-defective 6), and aPKC (protein kinase C) (*Smith, 2007, Wirtz-Peitz, 2008, Atwood, 2009, Li, 2014*). There are two basal networks: one protein complex includes Brat (Brain Tumor), Mira (Miranda), and Pros (Prospero), while the other consists of Numb and Pon (Partner of Numb) (*Betschinger, 2006, Hirata, 1995, Knoblich, 1995, Spana, 1995, Ikeshima-Kataoka, 1997, Fuerstenberg, 1998, Lee, 2006, Lu, 1998, Li, 2014*). Together, the disparate factors localized at the apical and basal sides of the cell function to establish essentially opposite cell fates between neuroblast daughter cells (*Li, 2014*).

Cell cycle regulators also play a role in monitoring ACD in neuroblasts. Two kinases, Aur-A and Polo, are both required for the proper orientation of neuroblast divisions and for localization of asymmetric proteins (*Wirtz-Peitz, 2008 and Wang, 2007, Li, 2014*). Aur-A drives asymmetry through interaction with apical destined proteins, while Polo acts predominantly on basal localization. Aur-A phosphorylates Par6 during interphase. This phosphorylation is required for formation of the Par complex (*Wirtz-Peitz, 2008*). Mud is also suspected to be regulated by Aur-A, since studies in Aur-A mutants show delocalization of Mud in neuroblasts (*Wang, 2006*). Similarly, Polo phosphorylates Pon which in turn drives basal localization of both Pon and Numb (*Wang, 2007*). Both Aur-A and Polo are required for proper spindle orientation and both help to localize aPKC. These kinase reactions are modulated by a series of phosphatases, including PP2A (protein phosphatase 2A) and PP4 (protein phosphatase 4). Loss of either of these

phosphatases results in irregular neuroblast maintenance (*Sousa-Nunes, 2009, Chabu, 2009, Ogawa 2009, and Li, 2014*).

Another equally essential component of successful ACD is spindle orientation. The spindle fiber orientation determines the axis of impending cleavage between nascent daughter cells. Because the apical and basal regions of the mother cell are distinct, it is crucial that an ACD occurs along the axis perpendicular to the apical-basal axis. Insc (Inscuteable) is a primary regulator of spindle positioning in the mitotic neuroblast. Insc binds with Pins (Partner of Inscuteable) to then associate with the proteins Mud (Mushroom Body Defect; a homolog of mammalian NuMA (nuclear mitotic apparatus), which is important in microtubule stability) and Dlg (Discs Large) and with the Gαi-Loco complex (*Kraut, 1996, Schaefer, 2000, Yu, 2000, Du, 2001, Nipper, 2007, Siller, 2006, Li, 2014*). These proteins drive spindle formation. Insc acts as a bridge between these spindle-forming proteins and the apical-determining Par complex, imparting orientation to the development of the spindle fibers (*Li, 2014*).

The centrioles are also critically important to ACD. A mitotic neuroblast duplicates its centrioles during interphase, and the resulting two centrioles have distinct features: the daughter centriole becomes a MTOC (microtubule- organizing center) by associating with the characteristic group of centrosome proteins known as pericentriolar material, or PCM (*Rieder, 2001 and Li, 2014*). The mother centriole, by comparison, loses the PCM and is unable to to serve as a MTOC. The daughter centriole segregates to the apical side of the neuroblast, while the mother centriole moves to the basal side. This segregation is critical to maintaining the site of GMC budding in future rounds of neuroblast ACD (*Rebollo, 2007, Rusan, 2007, Conduit, 2010, Li, 2014*). Defects in spindle orientation tend to result in symmetric cell divisions, where both apical and basal proteins are distributed to both daughters. This can in turn lead to an excess of self-renewing neuroblasts (*Lee, 2006, Li, 2014*).

1.4 Mechanistic implications of differential ribosome abundance

The well-defined mechanisms controlling asymmetric cell divisions and cell fate decisions in *Drosophila* neuroblast lineages make this an attractive model for studying how differential ribosome abundance might be established between a progenitor cell and its differentiated progeny. For example, are pre-rRNAs or ribosome components asymmetrically localized and does ribosome biogenesis activity differ between progenitors and differentiated progeny? The tools available for genetic manipulation in *Drosophila* also make this a promising system for investigating the mechanistic implications of elevated or decreased ribosome abundance. There are several predicted outcomes, at the molecular and cellular level, of changes to ribosome concentration, summarized below.

Groups of mRNAs respond differently to changes in ribosome concentrations. In general, transcripts with inefficient translation initiation are functionally deprioritized by a limited ribosome pool. Similarly, studies in the brain have demonstrated that deficiencies in a neuronal specific tRNA can result in phenotypic defects (*Ishimura, 2014 and Mills 2017*). Together, this indicates a link between translation efficiency and the transcriptome of a cell. The more *efficiently* a transcript can be translated, the more likely it *will* be translated when ribosome supply is limited.

A complex relationship between ribosome concentration and specific mRNA translation capacity was long suspected due to the nonlinear relationship between ribosome abundance and translation initiation across transcripts (*Lodish, 1974*). This phenomenon is linked to translation initiation efficiency differences between transcripts. Each transcript has an associated maximum capacity with which it can be loaded with ribosomes. Once this maximum is reached, translation actually decreases with increased ribosome concentration. This is due to "crowding" of the transcript by elongating ribosomes, which essentially cause a traffic jam and slow translation efficiency. Under normal conditions, transcripts tend to not be loaded to their full capacity with ribosomes to allow more efficient translation (*Lackner, 2007*). Changes to ribosome concentration influence protein content of the cell according to the translation initiation kinetics of individual transcripts. Translation of transcripts with short, highly efficient translation initiation times increases with decreasing ribosome concentration initially, due to the avoidance of overcrowding. Conversely, transcripts with slow translation initiation kinetics experience reduced protein output. This is likely due to somewhat stable secondary structures in the 5'UTR and/or other obstacles to translation initiation, including upstream open reading frames (uORFs) and internal ribosome entry sites (IRES). When many ribosomes are present, these 5'UTR secondary structures are kept in a relaxed state due to near constant occupation of the site by new ribosomes. Similarly, other obstacles are subdued due to heavy occupation by multiple ribosomes. However, in the absence of this constant flow, these structures can easily reform between incidents of ribosome occupation, creating a lag while this structure is unfolded to allow another round of translation. Absence of constant occupation also removes the "shortcut" to translation provided by the presence of other ribosomes (*Zarai, 2016 and Mills and Green, 2017*). It is clear to see how cells could use this system of translation efficiency to rapidly switch transcriptome programs. By clustering translation initiation kinetics of transcripts needed for a certain program (i.e. differentiation vs proliferation), the cell can turn an entire program of translation on or off simply by changing its ribosome concentration.

One question surrounding the ribosome's role in lineage commitment is whether the composition of the ribosomes has more, less, or equal influence over translation than the number of functional ribosomes in the cell. Recent evidence shows that, when one RP is mutated, the ribosome composition may change (*Shi, 2017*). Importantly, it has been shown that it is the *quantity* of ribosomes that affect lineage commitment in HSCs, as opposed to ribosome composition (*Khajuria, 2018*), likely due to the mechanisms described above.

In addition to the relationship between ribosome concentration and efficient translation initiation, the rate at which ribosomes translocate along their mRNA template (the elongation rate) is another important factor that determines translation output.. Elongation rate depends on how quickly a ribosome can decode a particular codon. Recent work has shown that inefficiently decoded codons can promote degradation of the mRNA and this mechanism is a widespread determinant of mRNA stability in cells. The term "codon optimality" refers to the correlation between usage of a given codon and the availability of the corresponding, charged tRNA needed to "decode" that codon (*Presnyak 2015*). Importantly, an abundance of charged tRNAs corresponds to a faster decoding time by the ribosome. Conversely, a deficit of relevant tRNAs leads the

ribosome to stall on the transcript at “non-optimal” codons. This stalling plays a direct role in recruiting the mRNA decapping machinery and in subsequent decay (*Radhakrishnan 2016*).

Our lab determined that this role of the ribosome in regulating mRNA decay is attenuated in the developing *Drosophila* nervous system (*Burow 2018*). Although it is clear that codon optimality is a main driver of mRNA decay in the whole embryo, this relationship falls apart in the nervous system. I demonstrated that this discrepancy is not, in fact, due to a difference in the relative abundance of charged tRNAs (those tRNAs which are loaded with their corresponding amino acid) in neuroblasts versus the whole embryo. Charged tRNA levels between these tissues are essentially equivalent. Therefore, a separate mechanism likely influences the relationship between codon content and mRNA half life in the developing nervous system. It is possible that the codon optimality – mRNA decay link is sensitive to the combined effects of ribosome density along a transcript (determined by initiation rates) and the stalling induced collision of ribosomes “backed up” at non-optimal codons.

Finally, increasing evidence suggests that deficiencies in ribosome concentrations trigger “ribosome stress” signals in cells, which together activate the p53 signaling pathway. This pathway leads to cell cycle arrest and eventual apoptosis (*Gentilella, 2017, Lindström, 2022*). The fact that abnormal ribosome levels can trigger this pathway demonstrates the need for the levels of ribosomes in cells to be tightly controlled (*Mills, 2017*). The Larp protein (Larp1 in humans) physically associates with both the large ribosomal subunit and the 5’ end of certain RNAs. These RNAs are known as “5’-TOP” RNAs, and include most RP transcripts in most model systems. The association of Larp with both the transcript and the large subunit acts to stabilize the transcript in normal situations. Loss of Larp causes corresponding loss of transcript stability. In these mutants, 5’-TOP RNAs, including RP transcripts, are destabilized and degraded more rapidly. The result is a decrease in the overall ribosome concentration in the cell, since there is a deficit of RP components. These cells are therefore under ribosome stress. The situation leads to activation of the p53 pathway and eventual cell cycle arrest, demonstrating again the relationship between active/functional ribosome level and critical cell fate decisions (*Gentilella, 2017*).

1.5 Defects in ribosome abundance are relevant to human disease

Neurogenesis is clearly a complex and, therefore, tightly regulated process. The influence of ribosomes in directing and maintaining neurogenesis has only recently started to come to light. A variety of both neurodegenerative and neurodevelopmental syndromes, including autism, have now been shown to have ties to disruption of ribosomes at some level. Some external factors known to harm neurodevelopment (for example, the Zika virus), have also been linked to disruption of ribosome biogenesis. While the precise mechanisms underlying each malady in this group of diseases may be unique, the results of each form a constellation of pathologies; aberrant ribosome biogenesis and/or function is directly linked to neurological defects (*Hetman, 2019*).

Ribosomopathies are diseases affecting specific cell or tissue types and caused primarily by either RP haploinsufficiency or defect in ribosome biogenesis. The unequal distribution of defects throughout the body in these cases demonstrates the need to

tightly and differentially control the production of ribosomes across cell types (*Mills, 2017*). As described above, certain RNAs are given translational preference when the ribosome supply is limited, influencing which genes are expressed and ultimately deciding the fate of the progenitor cells (*Mills, 2017 and Khajuria, 2018*). Cell types with differential ribosome concentrations are therefore primed to express different cohorts of genes to establish cell type specific gene expression patterns.

This regulatory role of ribosomes in determining cell lineage decisions was illuminated by studying the human genetic blood disorder Diamond-Blackfan anemia (DBA). In this disease, the erythroid lineage is severely reduced; progenitors and precursors of this lineage are lacking, where all other HSC lineages remain unaffected. Most mutations in this disease disrupt ribosome production through haploinsufficiency of one of the RP genes. This limits the supply of functional ribosomes in the patient's HSCs (*Khajuria, 2018*). One notable find from this study is that, in these RP mutants, transcription and translation efficiency of the RP transcripts appear to be unrelated. Translation of the affected transcripts was decreased, while transcription remained largely unaffected. This suggests that the deficiency of ribosomes in these mutants is realized at the level of translation, not transcription (*Khajuria, 2018*). In a DBA background, the group of transcripts most sensitive to ribosome insufficiencies includes ribosomal proteins themselves, as well as transcripts that are normally upregulated in early erythropoiesis, including the key transcription factor GATA1. Interestingly, these transcripts tended to be relatively short and efficiently translated under normal conditions (*Khajuria, 2018*).

1.6 Aims of my dissertation research

Given the multiple lines of evidence suggesting that differential rRNA synthesis contributes to cell fate decisions in the nervous system and the technical limitations of studying this process in mammalian model systems, my dissertation work focused on the following aims:

Aim 1: Identify patterns of differential rRNA synthesis among progenitors and neurons in the *Drosophila* larval brain

Aim 2: Determine if rRNA inheritance contributes to protein synthesis in *Drosophila* neurons

The experimental approaches used to address these aims and the conclusions supported by my research findings are presented in Chapter 2. My dissertation work establishes the developing *Drosophila* nervous system as a powerful model for investigating the role of differential ribosome abundance in cell fate determination. Potential future directions for this work are briefly summarized in Chapter 3.

Chapter 2

Progenitor-derived ribosomal RNA supports protein synthesis in *Drosophila* neurons

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Abstract: Global mRNA translation may differ dramatically between progenitor cells and their differentiated progeny. One way cell type-specific translation is established is through ribosome concentration. In addition to addressing unique metabolic needs, changes in ribosome concentration may influence cell fate. The mechanisms that determine ribosome abundance in progenitors versus differentiated progeny are not fully understood. Here we investigated this process by focusing on ribosomal RNA (rRNA) synthesis in *Drosophila* neural progenitors and neurons. We found that rRNA synthesis is robust in neural progenitors but is limited in post-mitotic neurons. Newly born neurons inherit rRNA from their progenitor parent and this inherited rRNA is sufficient for protein synthesis in neurons. Our findings support a model in which neuron-specific translation programs are established by rRNA inheritance.

Key Points:

- This work links changes in ribosomal RNA (rRNA) synthesis, often assumed to be an invariant feature of cells, to neural differentiation.
- We describe a process of rRNA inheritance, where rRNA made in neural progenitors is passed to their progeny.
- We show that rRNA inheritance is sufficient for neural development and protein synthesis in neurons.

Introduction

All cells require efficient mRNA translation, but protein synthesis capacity varies by cell type (*Buszczak, 2014*). Cell type-specific translation kinetics determine proliferation potential and maintain unique cellular properties. Cancer cells may alter translation initiation to globally increase protein synthesis and sustain proliferation (*Ruggero, 2013*). A delicate balance of translation activity is also critical for hematopoietic stem cell (HSC) homeostasis, with increased or decreased global translation impairing HSC function (*Signer, 2014*). Variation in ribosome concentration is one mechanism cells use to control translation output (*Mills and Green, 2017*). In addition to influencing growth and proliferative capacity, ribosome concentration can affect cell fate through biased translation of certain mRNAs (*Mills and Green, 2017 and Khajuria, 2018*). Calibration of ribosome abundance and altered protein synthesis has been described in several developmental contexts. Ribosomal protein levels decrease when mouse embryonic stem cells differentiate to embryoid bodies while global translation efficiency increases (*Ingolia, 2011*). Similarly, in the *Drosophila* female germline production of ribosomal RNA (rRNA) and ribosome assembly factors decreases along the differentiation pathway (*Zhang, 2014 and Sanchez, 2016*) yet germline stem cell differentiation is associated with increased global protein synthesis (*Sanchez, 2016*). These studies suggest that the

relationship between ribosome abundance and cell fate is more complex than simply meeting the metabolic needs of progenitors versus differentiated progeny.

Regulation of ribosomal RNA synthesis is one way of tuning global translation capacity. Decreased rRNA synthesis can affect developmental transitions in the *Drosophila* female germline (*Zhang, 2014*) and mammalian tissue culture cells (*Hayashi, 2014*). In eukaryotes, rRNA is transcribed by RNA polymerase I from tandem repeats of a gene encoding precursor rRNA (pre-rRNA). Pre-rRNA is processed into individual 28S, 18S, and 5.8S rRNAs while a fourth rRNA, 5S rRNA, is transcribed by RNA polymerase III. Ribosomal RNA transcription occurs at a specialized site in the nucleus called the nucleolus. Differences in rRNA transcription can be recognized via changes in nucleolus size (a larger nucleolus usually indicates more rRNA transcription) and via direct detection of nascent rRNA. For example, *Drosophila* female germline stem cells have large nucleoli and high rates of rRNA transcription but their differentiated progeny have smaller nucleoli and reduced rRNA synthesis (*Zhang, 2014*). Downregulation of rRNA synthesis also occurs during differentiation in the mammalian forebrain (*Chau, 2018*). Decreased ribosome abundance appears to be a common feature of neural differentiation: additional studies have shown that ribosomal protein production (*Slomnicki, 2016*) and rRNA synthesis (*Qian, 2006*) decreases in post-mitotic neurons. Compartment-specific changes, such as loss of ribosomes from mature axons, also occurs during neural development (*Kleiman, 1994 and Costa, 2019*). These findings raise interesting questions regarding how ribosome biogenesis is regulated to meet the mRNA translation needs of neurons. One possibility is that components of the translation machinery, including rRNAs, are primarily synthesized in progenitors then passed to neurons during differentiating divisions. The absence of cytokinesis in neurons and the long half-life of ribosomes (days to weeks) could establish a pool of ribosomes sufficient to meet the protein synthesis needs of neurons in the absence of any autonomous ribosome production (*Hetman, 2019*).

Drosophila neural stem cells, called neuroblasts, undergo multiple rounds of asymmetric self-renewing divisions to ultimately produce neurons and glia. There are two main types of neuroblast in the larval brain: type I neuroblasts produce a transient progenitor, the ganglion mother cell (GMC), at each division while type II neuroblasts produce intermediate neural progenitors (INPs) that undergo multiple rounds of asymmetric divisions, self-renewing and producing a GMC (*Homem, 2012*). In both lineages the GMC divides once to produce post-mitotic progeny. Previous work has shown that the nucleolus is smaller in differentiated cells (INPs, GMCs, neurons) compared to neuroblasts (*Betschinger, 2006 and Song, 2011*), suggesting that rRNA synthesis is restricted upon differentiation. The transcription factor Myc is likely a crucial regulator of this restriction. Myc is expressed at high levels in neuroblasts but is absent from INPs, GMCs and neurons (*Song, 2011*). Myc promotes cell growth and proliferation via several pathways, including transcriptional activation of genes encoding RNA polymerase I subunits (*Grewal, 2005*).

While decreased nucleolus size suggests rRNA synthesis is restricted upon neural differentiation in *Drosophila*, multiple questions remain. First, does direct measurement of rRNA synthesis confirm this prediction? Second, to what degree is rRNA synthesis restricted along the differentiation pathway? Third, since the absence of Myc is predicted to limit rRNA synthesis, how do differentiated progenitors and neurons obtain

the necessary amount of rRNA to support their translation needs? Here we show that high levels of nascent rRNAs are present in neuroblasts, INPs and GMCs but rRNA synthesis is severely restricted in neurons. Our data reveal that neural progenitors pass rRNA to their progeny during cytokinesis and suggest that the rRNA in INPs and GMCs is at least partly derived from their neuroblast parent. Ultimately, the rRNA in GMCs is inherited by neurons at cytokinesis. Finally, we show that progenitor-derived rRNAs are sufficient to support brain development and normal protein synthesis in neurons. This work supports a model in which rRNA inheritance establishes cell type-specific translation programs along the neural differentiation pathway.

Results

RNA-tagging reveals differential rRNA synthesis in neural progenitors and neurons.
We recently described a cell type-specific biosynthetic RNA tagging method called EC-tagging (*Hida, 2017*). EC-tagging allows cell type-specific labeling of nascent RNAs via conversion of 5-ethynylcytosine (EC) to 5-ethynyluridine (EU) monophosphate in cells that express a cytosine deaminase – uracil phosphoribosyltransferase fusion (CD:UPRT). The labeled RNA can be subsequently interrogated using “click chemistry”. One possible application is visualization of RNA via attachment of an azide-coupled fluorophore. This approach has been used for general RNA imaging (not cell type-specific) via direct application of EU (*Jao, 2008*). To test EC-tagging-based RNA imaging, we used the Gal4-UAS transgene expression system (*Brand, 1993*) to express CD:UPRT in progenitors or neurons. We used *insc-Gal4* for progenitor tagging since this line expresses Gal4 in type I neuroblasts, type II neuroblasts, and INPs (*Neumüller, 2011*). In addition, some amount of Gal4 and CD:UPRT likely perdures in GMCs and, to a lesser extent, neurons of *insc-Gal4* x *UAS-CD:UPRT* brains (as previously described for UPRT expression in neuroblasts and TU-tagging larval brains (*Lai, 2012*)). We used *nSyb-Gal4* for neuron tagging since this line expresses Gal4 only in neurons (*Pauli, 2008*). For all EC-tagging experiments, larvae were fed EC for 24 hours beginning at early L3-stage. We detected fluorescent RNA when tagging was targeted to neural progenitors but never detected fluorescent RNA when tagging was targeted to neurons (**Figure 1A**).

Since related EU-labeling experiments have shown EU incorporation into rRNA and mRNA (*Jao, 2008*) and rRNA accounts for at least 80% of the RNA in eukaryotic cells, we predicted that the tagged RNA in *Drosophila* brains is predominately rRNA. To measure relative rRNA synthesis in neuroblasts and neurons using an imaging-independent approach, we purified EC-tagged RNA from *insc > CD:UPRT* and *nSyb > CD:UPRT* brains following a 24-hour EC feeding. Nascent rRNA was quantified by RT-qPCR using primers specific for precursor rRNA (*Larson, 2012*). We also measured levels of a neuron-specific transcript, *Synaptotagmin 1* (*Syt1*), and confirmed that *Syt1* was enriched by *nSyb > CD:UPRT* tagging even though some neuronal tagging occurs in *insc > CD:UPRT* brains due to CD:UPRT perdurance (**Figure 1B**). Similar to the EC-tagging RNA imaging results, pre-rRNA levels were highly reduced in neurons compared to neuroblasts (**Figure 1B**).

To determine if the fluorescent signal detected by RNA tagging is primarily rRNA, mRNA, or both, we performed EU-based RNA tagging of dissected brains in the presence or absence of pharmacologic inhibitors of RNA polymerases: triptolide to inhibit RNA

polymerase II (blocking mRNA synthesis) (*Henriques, 2013*) and actinomycin D to inhibit RNA polymerase I and II (blocking rRNA and mRNA synthesis) (*Jao, 2008*). Triptolide treated brains were indistinguishable from controls, while actinomycin D abolished the EU-tagged RNA signal (**Figure 1C**). These results confirm that the tagged RNA is predominately ribosomal RNA. We also imaged EU-tagged RNA in combination with antibody staining for Udd (a nucleolus protein) (*Zhang, 2014*) and PCNA (a marker of proliferating cells). As expected for rRNA, the EU-RNA signal is localized to the nucleolus and as predicted by our EC-tagging data, the signal is restricted to proliferating neuroblasts (**Figure 1D**). Altogether, these EC-tagging and EU-tagging data suggest that rRNA synthesis is limited in neurons.

Biosynthetically tagged rRNAs are restricted to recently born neurons.
We previously found that EU feeding results in more robust RNA tagging than EC feeding (*Hida, 2017*) and therefore sought to test if this approach might identify neuronal rRNA synthesis that is below the detection limit for EC-tagging. We initially attempted short EU feedings and found that feeding for a minimum of four hours was necessary to reliably detect tagged rRNA. This constraint likely reflects the time it takes for ingested EU to accumulate within the nucleoside pool of brain cells and for a visible threshold of tagged rRNA molecules to be produced. Following four hours of EU feeding, tagged rRNA was consistently detected in neuroblasts and differentiated progenitors (INPs and GMCs). In these experiments, tagged rRNA was never detected in neurons (**Figure 2A and B**).

The absence of tagged rRNA in neurons could be due to a low rate of rRNA synthesis and a signal below the limit of detection. We therefore tested longer EU feeding periods of 6 and 24 hours. After 6 hours, we detected tagged rRNA in neurons but only in a small number of recently born neurons (located near GMCs) and never mature neurons located further from progenitors (**Figures 2B and 3C**). After 24 hours the number of neurons containing tagged rRNA increased but the signal was still restricted to recently born neurons (**Figures 2C and D**). One interpretation of these results is that recently born neurons have a low rate of rRNA synthesis, revealed only by the longer labeling periods, while mature neurons produce little or no rRNA and tagged RNAs remain below the detection limit. Another possibility is that a period of 6 hours allows time for GMC divisions to generate neurons containing inherited rRNA. The average GMC cell cycle is 4.2 hours (*Homem, 2013*). Given this timing and the lag between initiation of EU feeding and detection of tagged rRNA, very few GMCs are expected to divide and pass tagged rRNA to neuronal progeny during a 4-hour feeding. However, during a 6-hour feeding and even more so a 24-hour feeding, there is time for multiple GMCs to divide and generate neurons with inherited rRNA. The results of these experiments do not rule out either explanation (low rate of rRNA synthesis or rRNA inheritance) and both mechanisms may contribute to the rRNA population in neurons. Neuron-specific measurement of pre-rRNA by EC-tagging and RT-qPCR (**Figure 1B**) already revealed that some low level rRNA transcription occurs in neurons. We next sought to investigate the possibility that rRNA synthesized in progenitors is passed to neurons.

Ribosomal RNA associates with mitotic chromosomes and is passed to progeny.
Previous work in HeLa cells revealed that pre-rRNA associates with chromosomes during mitosis and segregates to each daughter cell during cytokinesis (*Sirri, 2016*). We reasoned that a similar mechanism could mediate rRNA transfer from *Drosophila* neural

progenitors to their progeny. To test this possibility, we fed EU for 16 hours and imaged tagged rRNA along with phosphorylated histone H3, a marker of mitotic chromosomes, and Miranda, an asymmetrically localized protein that is briefly present in newly formed GMCs (*Ikeshima-Kataoka, 1997*). As described for HeLa cells, tagged rRNA overlapped closely with mitotic chromosomes, including chromosomes at the metaphase plate (**Figure 3A**) and chromosomes inherited by newly formed GMCs (**Figure 3B**).

To further test if rRNA is passed from progenitors to neurons, we performed a 6-hour EU "pulse" followed by an 18 hour "chase" with a twenty-fold excess of unmodified uridine to block any further production of tagged RNA, as previously described (*Burow, 2015*). As described above, the majority of tagged rRNA was concentrated in progenitor cells at the end of the 6-hour feeding, but some newly born neurons (adjacent to progenitors) were positively labeled (**Figure 3C**). Following the 18-hour chase, the rRNA signal was transferred from progenitors to recently born neurons. The transferred rRNA signal was strong throughout the cell, indicating localization to the nucleus (site of initial ribosome assembly) and cytoplasm (site of final ribosome maturation and mature ribosomes). These results support the rRNA inheritance model. Very little, if any, rRNA decay is expected during the 18-hour chase. We used larval neuroblast-specific EC-tagging pulse-chase experiments to measure RNA half-lives and detected no rRNA decay during a 12-hour chase (Supporting Information Figure 1). Previous work has also shown that *Drosophila* rRNA is extremely stable; rRNA produced in embryos lasts into larval stages with a half-life between 48 and 115 hours depending on growth conditions (*Winkles, 1985*). The high stability of rRNA supports our conclusion that the tagged RNA detected in neurons at the end of the chase is intact inherited rRNA.

<u>rRNA inheritance in neurons is sufficient for neurodevelopment and protein synthesis.</u>
We next asked if inhibition of rRNA synthesis would differentially affect neural progenitors and neurons. To achieve cell type-specific inhibition of rRNA synthesis, we used a previously characterized RNA interference line (*Zhang, 2014*) to knockdown *RNA polymerase I subunit B* (*Polr1B*) in progenitors (*insc-Gal4 x UAS-Polr1B{RNAi}*) or neurons (*nSyb-Gal4 x UAS- Polr1B{RNAi})*. *Polr1B* knockdown using *insc-Gal4* limits rRNA inheritance in neurons but does not affect rRNA synthesis in neurons (**Figure 4A**). *Polr1B* knockdown using *nSyb-Gal4* limits rRNA synthesis in neurons but does not affect rRNA inheritance (**Figure 4A**). Polr1B knockdown in neuroblasts has previously been shown to limit neurogenesis (*Neumüller, 2011*) and we found that *Polr1B* knockdown driven by *insc-Gal4* causes nearly 100% failure of adult flies to eclose from their pupal case (**Figure 4B**). In contrast, *Polr1B* knockdown in neurons had no effect on development: flies eclosed 100% of the time (**Figure 4B**).

Next we tested if inherited rRNA is sufficient to support bulk protein synthesis in neurons. We used a fluorophore-"clickable" amino acid analog (homopropargylglycine (HPG)) (*Hovhanyan, 2014*) to quantify protein synthesis in neurons of wildtype, *insc-Gal4 x UAS- Polr1B{RNAi},* and *nSyb-Gal4 x UAS- Polr1B{RNAi}* flies. Dissected brains were soaked in HPG for three hours to allow sufficient labeling while limiting the likelihood of proteins passing from GMCs to neurons at cytokinesis (based on the 4.2-hour average GMC cell cycle). As previously shown (*Neumüller, 2011*), *Polr1B* knockdown using *insc-Gal4* decreases progenitor proliferation, resulting in smaller brain size, but neurons are still produced (**Figure 4C**). These neurons, with intact autonomous rRNA synthesis but limited rRNA inheritance, had significantly reduced levels of protein synthesis

(**Figures 4C and D**). Neurons with intact rRNA inheritance but limited autonomous rRNA synthesis had wildtype levels of protein synthesis (**Figures 4C and D**). We interpret these data as evidence that inherited rRNAs significantly contribute to protein synthesis in neurons.

Discussion

We set out to investigate the possibility that ribosomal rRNA inheritance is a major contributing factor to establishing neuron-specific translation capacity. Using cell type-specific EC-tagging and standard EU-tagging, we found that nascent rRNA synthesis is very limited in neurons. We also found that progenitor rRNA, most likely in pre-rRNA form, associates with mitotic chromosomes and is passed to progeny at cytokinesis. The functional importance of rRNA inheritance was revealed by RNAi experiments targeting RNA polymerase I in either progenitors or neurons. Knockdown of RNA polymerase I in neurons had no effect on neurodevelopment or protein synthesis while knockdown in progenitors caused a severe neurodevelopment defect and significantly reduced protein synthesis in neurons. Our results support a model in which neurons utilize inherited rRNA to meet their protein synthesis needs.

While we primarily focused on rRNA inheritance in neurons, our data also suggest that rRNA is passed from neuroblasts to INPs and GMCs. In 4-hour EU-labeling experiments, tagged rRNA is strongly detected in these differentiated progenitors and this signal may reflect both nascent and inherited rRNA. The average neuroblast cell cycle is about 1.4 hours (*Homem, 2013*) so multiple INPs and GMCs are produced during a 4-hour EU feeding. Since we could not reliably detect tagged RNA using shorter labeling times, it is unlikely that the strong signal in INPs and GMCs is solely derived from rRNA synthesized in neuroblasts during interphase (no rRNA synthesis occurs during mitosis (*Sirri, 2016*)). Based on this relationship between labeling time and neuroblast cell cycle length, we predict that at least some nascent rRNA synthesis occurs in INPs and GMCs. This raises the question of how rRNA transcription is activated in progenitors that do not express Myc. One possibility is that sufficient rRNA polymerase I components, cofactors, and rRNA processing enzymes are inherited from the neuroblast. These inherited factors may support nucleolus assembly and rRNA synthesis in INPs and GMCs. Nucleolar proteins and pre-rRNA associate with mitotic chromosomes in mammalian cells and are thought to direct nucleolus formation in daughter cells (*Sirri, 2016*). A similar mechanism could direct nucleolus formation and activity in neuroblast progeny. In this model, the absence of Myc in GMCs would limit production of nucleolar factors and RNA polymerase I components so that little if any of the machinery necessary to trigger rRNA synthesis is inherited by neurons.

This work contributes to a growing body of evidence that the protein synthesis landscape of neurons is distinct from neural progenitors. While proliferating cells have a high demand for growth-supporting translation, the ribosomal properties of neurons might differ for reasons beyond their post-mitotic status. One possibility is that decreased ribosome concentration establishes neuron-specific translation programs. For example, a decrease in ribosome concentration can selectively limit translation of mRNAs whose translation is difficult to initiate (*Mills and Green, 2017*). Our findings may also relate to changes in ribosome requirements that occur throughout the lifetime of a neuron. Immature neurons require ribosomes in their axon growth cones to support pathfinding

and synapse formation, but ribosomes are essentially absent from the axons of mature neurons. A ubiquitin-proteosome mechanism removes ribosomes from mature axon terminals (*Costa, 2019*) but this system could be overwhelmed if mature neurons produced large amounts of ribosomes. Inherited rRNA in newly born neurons may help establish ribosome levels appropriate for growth and synapse formation while weak rRNA synthesis in mature neurons may help ensure ribosomes do not accumulate outside of dendrites and the soma. Similar evidence of dynamic changes in rRNA levels comes from EU imaging in cultured hippocampal neurons and larval zebrafish brains (*Akbalik, 2017*). This study identified a general decrease in RNA synthesis upon neuronal depolarization and a restriction of RNA synthesis to the neurogenic regions of the brain. Finally, reduced ribosome abundance may serve a protective function: lower levels of protein synthesis decrease the likelihood of generating misfolded proteins (*Buszczak, 2014*) that may be toxic to neurons.

Here we show that rRNA synthesis is restricted along the differentiation axis and that inherited rRNAs are sufficient for neurodevelopment and protein synthesis. Inheritance of ribosomal proteins remains to be investigated. We have previously shown that ribosomal protein mRNAs have decreased stability in neurons compared to other somatic cells (*Burow, 2015*). Ribosomal protein abundance in progenitors and neurons may also be regulated via mRNA decay and coordination of rRNA and ribosomal protein levels during differentiation is an important avenue for future studies. It will also be interesting to investigate differences in ribosome production among individual neuroblast lineages. A recent study on the effects of nucleolar stress found that neuroblasts that produce mushroom body neurons are less sensitive to nucleolar stress and have greater reserves of nucleolar proteins than other neuroblasts (*Baral, 2020*). Studies of ribosome synthesis and inheritance in *Drosophila* will help identify conserved mechanisms of neural translation and may contribute to our understanding of ribosomopathies that cause multiple human diseases (*Farley-Barnes, 2019*).

Materials and Methods

Drosophila genetics
The following lines were obtained from the Bloomington *Drosophila* Stock Center: Oregon-R-P2 (wildtype) (stock # 2376), *insc-Gal4* (stock # 8751), *nSyb-Gal4* (stock #51635), *wor-Gal4* ; *Dr / TM3, Ubx-lacZ* (stock #56553), and *UAS-myr::tdTomato* (stock #3222). For EC-tagging, Gal4 lines were crossed with *UAS-CD:UPRT* on the 3rd chromosome (stock # 77120). The *UAS-Polr1B{RNAi}* flies, constructed using the VALIUM20 vector (*Ni, 2011*), were a gift from Michael Buszczak.

EC-tagging for RNA purification and RT-qPCR
5-ethynylcytosine was synthesized as previously described (*Hida, 2017*). Larvae were staged using a 1-hour embryo collection. Newly hatched larvae were reared at 25°C and fed 1 mM 5EC beginning at 48 – 49 hours after larval hatching (ALH) and ending at 72 – 73 hours ALH. Total RNA was extracted from crudely dissected central nervous system tissue using Trizol. 10 µg of RNA was biotinylated using Click-iT Nascent RNA Capture reagents (ThermoFisher) and purified on Dynabeads MyOne Streptavidin T1 magnetic beads (ThermoFisher) as previously described (*Hida, 2017*). After the final wash, beads containing captured RNA were used to make first-strand cDNA with the SuperScript VILO cDNA Synthesis Kit (Invitrogen), as previously described (*Hida, 2017*).

Real-time PCR quantitation was performed on a Rotor-Gene Q (Qiagen) in 20 µL reactions using SYBR green detection. Pre-designed QuantiTect primers (Qiagen) were used for *Syt1* PCR. RNA polymerase II subunit B (*Polr2B*) primers, used for normalization, were Forward primer: TCAGCGTCTTAAGCACATGG and Reverse primer: TCGGAGACCTCGAATAAACG. Previously described sequences were used for pre-rRNA specific primers (*Larson, 2012*), synthesized by Integrated DNA Technologies. Pre-rRNA and *Syt1* Ct values were normalized to *Polr2B* and relative abundance calculated by the equation, fold change = $2^{-\Delta(\Delta Ct)}$. Duplicate RT-qPCR reactions (starting at the reverse transcription step) were performed for all target transcripts using two biological replicates of each genotype (n = 4 independent RT-qPCR measurements for each transcript).

EC-tagging and EU-tagging for RNA imaging

Larvae were fed 1 mM EC or 0.5 mM EU for the indicated times (time and developmental stage indicated in figure legends). For pulse-chase experiments, larvae were fed 0.5 mM EU during the 6-hour pulse then transferred to media containing 10 mM unmodified uridine for the 18-hour chase. For EU-tagging in the presence of RNA polymerase inhibitors, dissected middle L3 stage brains were incubated in D22 media containing 0.5 mM EU for four hours. Drug treated brains were pre-incubated in the presence of the inhibitor for two hours prior to addition of EU, control brains were pre-incubated in media alone. Triptolide (ThermoFisher) was used at a final concentration of 100 µM, 10-fold higher than the concentration known to inhibit RNA polymerase II in *Drosophila* tissue culture cells (*Henriques, 2013*). Actinomycin D (Millipore Sigma) was used at a final concentration of 700 µM, a concentration that is expected to affect RNA polymerase I and II (*Jao, 2008*). For all imaging experiments, brains were fixed in 4% paraformaldehyde prior to Alexa Fluor 488 addition using the Click-iT RNA Imaging Kit (ThermoFisher) as previously described for Click-iT kit-based detection of DNA labeled with 5-ethynyl-2'-deoxyuridine in *Drosophila* larval brains (*Daul, 2010*). The Click-iT reaction was followed by antibody staining according to standard methods (*Wu, 2006*). Imaging was performed using a Zeiss LSM 880 confocal microscope.

Antibodies

The following antibodies were used in combination with EU-tagging or HPG protein labeling: rat anti-Elav (Developmental Studies Hybridoma Bank (DSHB)) at 1:10, mouse anti-Pros (DSHB) at 1:20, guinea pig anti-Miranda (gift of C.Q. Doe) at 1:400, guinea pig anti-Udd (gift of M. Buszczak) at 1:1000, mouse anti-PCNA (Santa Cruz Biotechnology) at 1:500, and rabbit anti-phosphorylated histone H3 (Millipore Sigma) at 1:1000. Alexa-fluor conjugated secondary antibodies (ThermoFisher) were used.

HPG biosynthetic protein labeling and quantitation

Dissected larval brains were incubated in PBS containing 50 µM HPG for three hours. The Click-iT HPG Alexa Fluor 488 Protein Synthesis Assay Kit (ThermoFisher) was used to fluorescently label HPG-containing proteins. The HPG labeling reaction was followed by standard antibody staining to detect Elav protein. Pixel intensity measurements of the HPG-Alexa Fluor 488 signal were made using ImageJ and the ‘‘measure’’ tool applied to an identical size area encompassing multiple neurons per brain, with all staining procedures and confocal settings identical across all samples.

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The authors declare no competing financial interests.

Chapter 2 Figures and Figure Legends

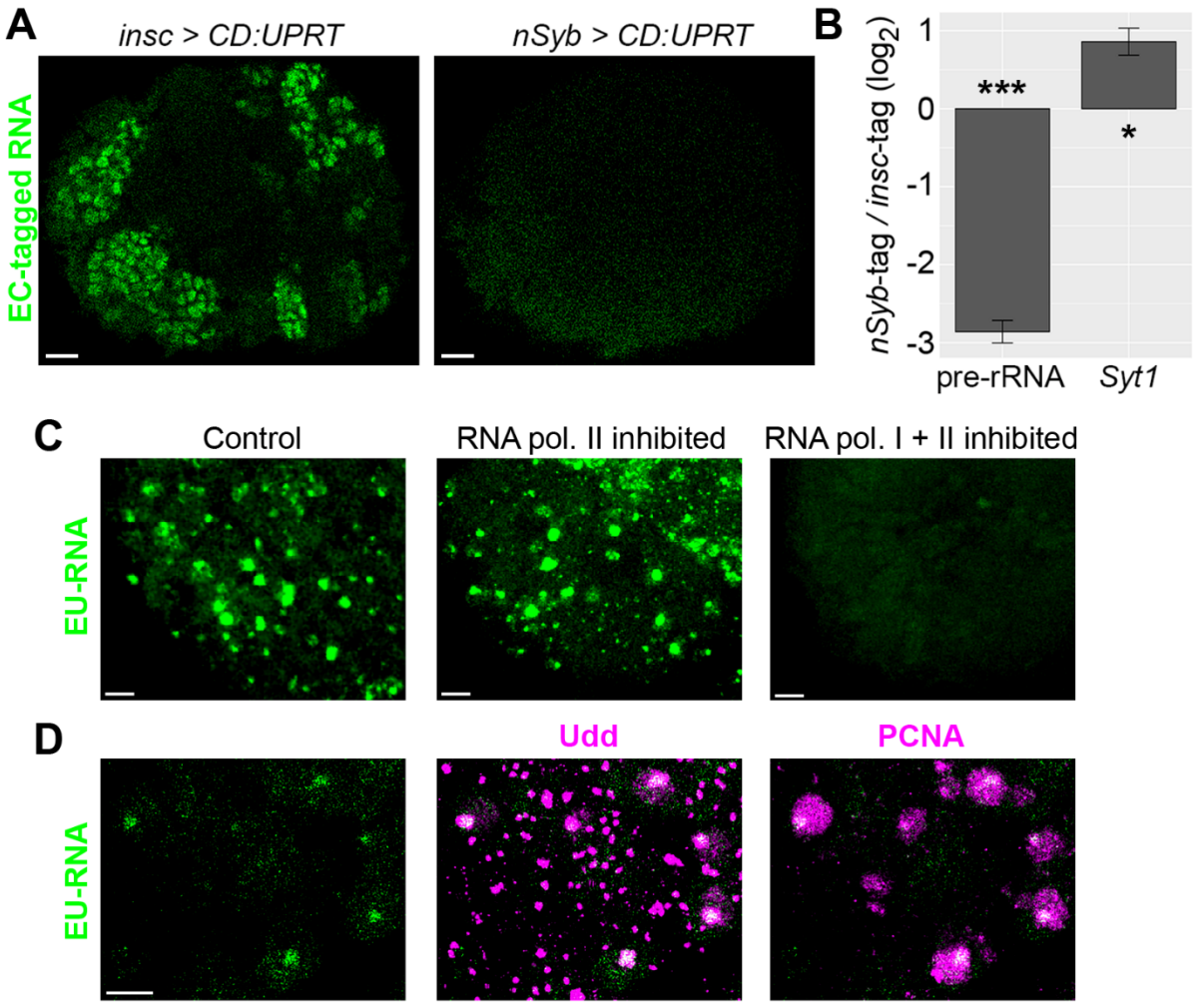

Figure 1. Cell type-specific biosynthetic RNA tagging reveals limited rRNA synthesis in neurons. A. Fluorescent RNA signal (green) in a single L3-stage brain lobe following 24 hours of EC-tagging targeted to neural progenitors (*insc* > *CD:UPRT*) or neurons (*nSyb* > *CD:UPRT*). Scale bar is 10 μm. A single confocal slice encompassing the entire brain lobe is shown. Images are representative of replicate experiments in which a minimum of 8 brain lobes were analyzed per condition. **B.** Relative precursor rRNA (pre-rRNA) and *Syt1* transcript abundance following 24 hours of EC-tagging (starting at 48 – 49 hours after larval hatching (ALH) and ending at 72 – 73 hours ALH) in neurons (*nSyb*-tag) or neural progenitors (*insc*-tag), as measured by RT-qPCR. Fold-change in relative abundance (*nSyb*-tag / *insc*-tag), after normalization to a RNA polymerase II subunit, is shown. Data are the mean and standard deviation from duplicate RT-qPCR reactions performed on biological replicate EC-tagging experiments. * = p-value < 0.05, *** = p-value < 0.001, Student's t-test. **C.** The fluorescent signal is predominately ribosomal RNA. Brains dissected from middle L3-stage larvae were soaked in EU alone (left panel), EU plus the mRNA synthesis inhibitor triptolide (middle panel), or EU plus the mRNA and rRNA synthesis inhibitor

actinomycin D (right panel). An equivalent region of central brain (excluding the optic lobe) is shown in each confocal stack. Scale bar is 10 μm**.** Images are representative of replicate experiments in which a minimum of 8 brain lobes were analyzed per condition. **D.** The EU-RNA signal localizes to the nucleolus of neuroblasts. EU-RNA signal alone (left panel), overlay of EU-RNA and the nucleolus marker Udd (middle panel), overlay of EU-RNA and the proliferating cell marker PCNA (right panel). All panels are from the same confocal stack. The large PCNA positive cells are neuroblasts. A confocal projection from the dorsal region of a single brain lobe is shown. Scale bar is 10 μm**.** The image is representative of results obtained from replicate experiments in which 8 brain lobes were analyzed.

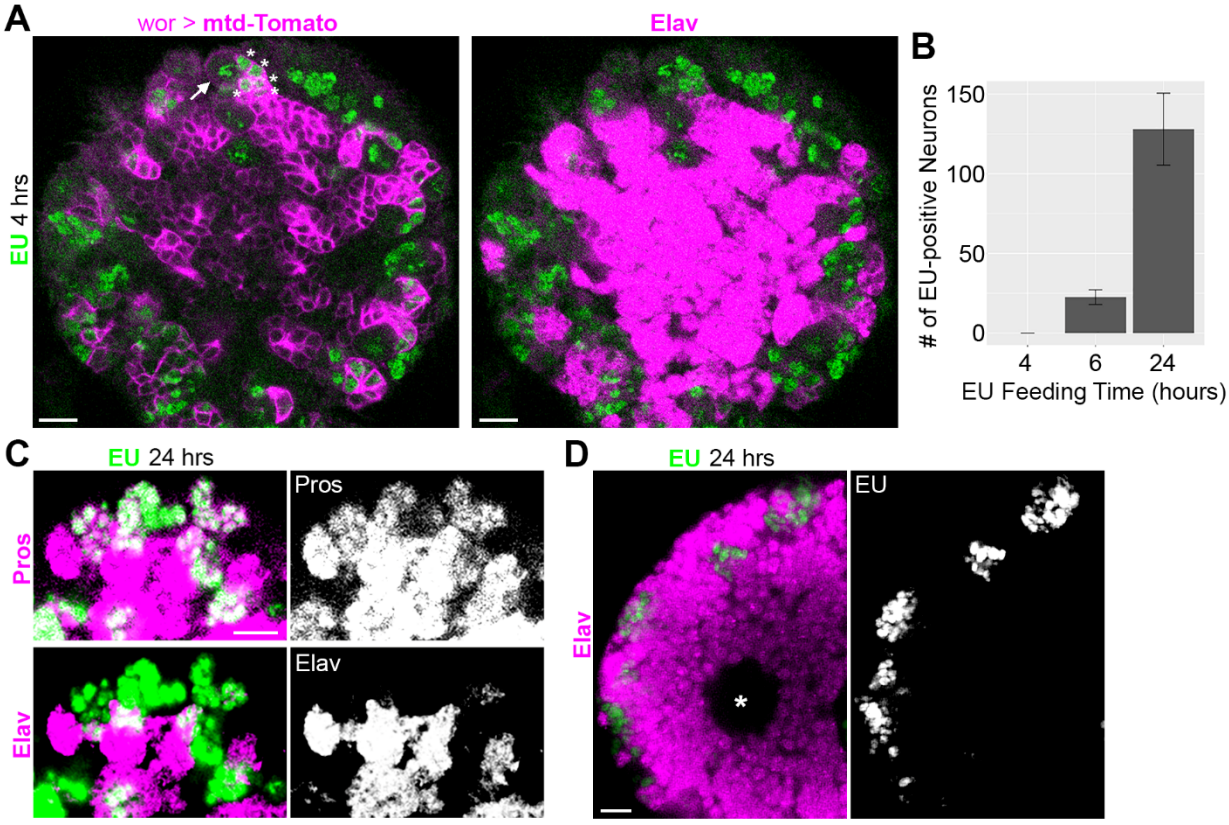

Figure 2. Biosynthetically tagged rRNA is only detected in newly born neurons. **A.** Fluorescent RNA signal (green) in a single middle L3-stage brain lobe following 4-hours of EU feeding. *Wor-gal4* driven expression of *UAS-mtd-Tomato* (*wor > mtd-Tomato*) results in Tomato signal in the plasma membrane of neuroblasts and their progeny, including neurons made before the 4-hour EU feeding (mtd-Tomato in magenta, left panel). Antibody stain for the neuron-specific protein Elav is shown in magenta in the right panel (the two panels are from the same single confocal image). In the left panel, a single EU-positive neuroblast is indicated by an arrow and associated EU-positive GMCs are indicated by asterisks. There are no EU-positive neurons in this image. Scale bar is 10 μm. The image is representative of replicate experiments in which 8 brain lobes were analyzed. **B.** Plot showing the relationship between EU feeding time and the total number of EU-positive neurons per brain lobe. Data are the mean and standard deviation for multiple brain lobes (4-hour feeding n = 8, 6-hour feeding n = 6, 24-hour feeding n = 6). **C.** Fluorescent RNA signal (green) in progenitors and neurons following 24 hours of EU feeding (spanning 48 to 72 hours ALH). A single confocal image is shown, with Pros and Elav signal separated. Pros is expressed in INPs, GMCs and neurons while Elav is only expressed in neurons. Multiple EU-positive neurons are visible and all are located adjacent to progenitors (progenitors are Pros-positive, Elav-negative). Scale bar is 10 μm. **D.** Fluorescent RNA signal (green) in a cross section of larval central brain following 24 hours of EU feeding. Recently born neurons are located near the periphery while older neurons are located deeper, near the neuropil (asterisk). Scale bar is 10 μm. Images in C and D are representative of replicate experiments in which 6 brain lobes were analyzed.

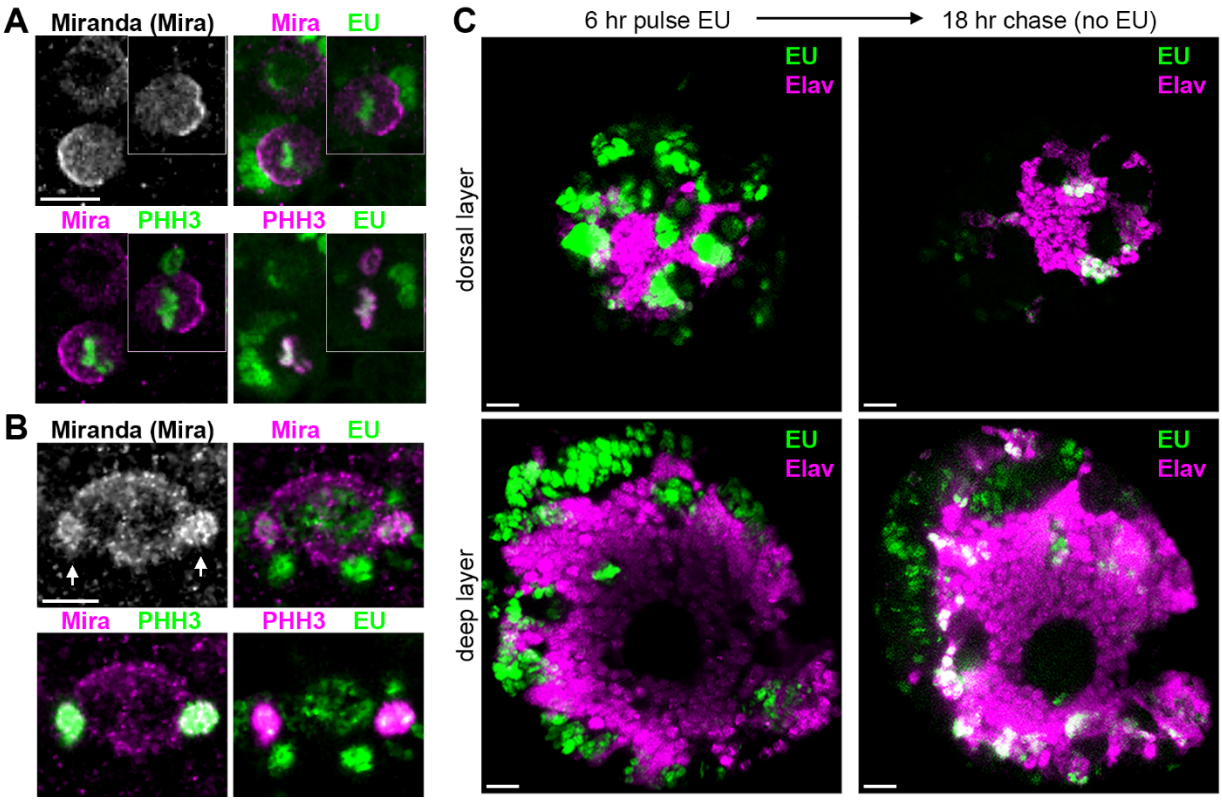

Figure 3. Progenitor rRNA associates with mitotic chromosomes and is passed to progeny cells. A. Fluorescent RNA signal (green) following a 16-hour EU feeding beginning in early L3-stage, co-stained with antibodies for PHH3 (mitotic chromosomes) and Miranda (cortex of mitotic neuroblasts, INPs, and newly formed GMCs). A single confocal image is shown, with one neuroblast (outlined by white line) repositioned for clarity. Two mitotic neuroblasts with a basal Miranda crescent and chromosomes at the metaphase plate are shown. Scale bar is 10 μm. **B.** Same experimental conditions as part A. Two adjacent neuroblasts and recently produced GMCs (indicated by arrows) are shown. Scale bar is 10 μm. Images in A and B are representative of replicate experiments in which 10 brain lobes were analyzed. **C.** Fluorescent RNA signal (green) following a 6-hour EU feeding (pulse) and a 6-hour EU feeding followed by 18 hours of feeding in the absence of EU and an excess of unmodified uridine (chase). Pulse and pulse-chase experiments were started at 64 - 65 hours ALH. Top images show dorsal brain lobe regions following the pulse (left panel) and chase (right panel). Bottom images show deeper brain lobe regions following the pulse (left panel) and chase (right panel). Neurons were identified by antibody staining for Elav (magenta). Scale bar is 10 μm. Images are representative of replicate experiments in which 8 brain lobes were analyzed per condition (pulse and chase).

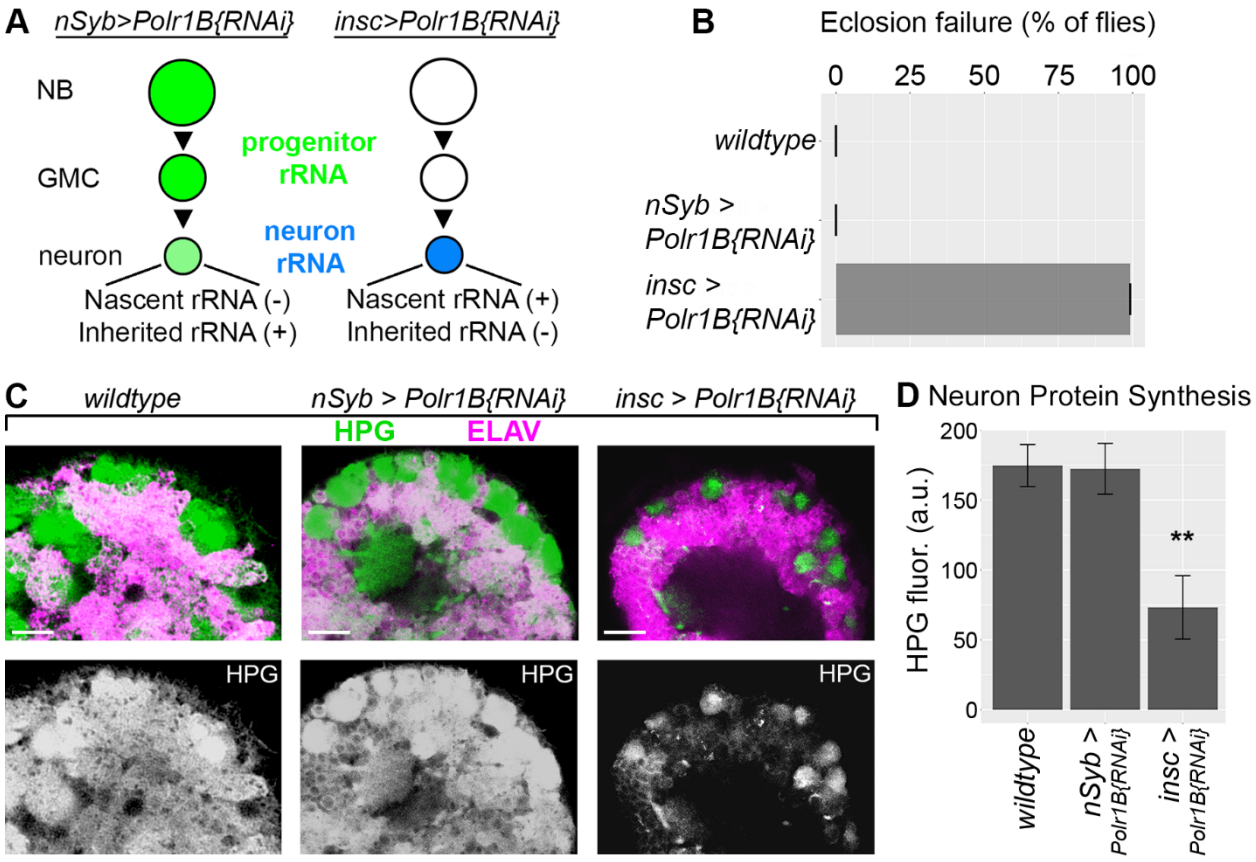

Figure 4. Inhibition of rRNA inheritance causes neurodevelopment and protein synthesis defects. A. Summary of rRNA sources in RNA Polymerase I knockdown experiments (progenitor-derived = green, neuron-derived = blue). **B.** Eclosion failure in control (*UAS-Polr1B{RNAi}* with no Gal4 activation) and *Polr1B* knockdown flies. Each condition was tested in triplicate. All pupae and adults were counted fourteen days ALH and ranged from 70 – 85 individuals per experiment. **C.** HPG-based imaging of protein synthesis in control and *Polr1B* knockdown middle L3-stage larval brain lobes. HPG-tagged proteins are labeled green, Elav is labeled magenta. Scale bar is 10 μm. Images are representative of replicate experiments in which 6 brain lobes were analyzed per genotype. **D.** Quantification of protein synthesis: fluorescence intensity (arbitrary units (a.u.)) was measured in a fixed-size area of neurons (Elav-positive) across all three genotypes. Three regions were measured per brain lobe and six brain lobes were analyzed per genotype. ** = p-value < 1×10^{-5}, Student's t-test compared to wildtype.

Chapter 3

Conclusions and Future Directions

Cellular differentiation is one of the most universal processes in biology. All metazoans begin as a single cell and require a precise program of differentiation and fate specification to develop to their adult form. Understanding the processes of cellular differentiation that drive development of the nervous system is necessary to support advances in medicine (with the promise of treating neurodevelopmental and neurodegenerative disorders) in addition to elevating our collective understanding of fundamental biological processes.

My dissertation work adds to a growing body of evidence that ribosome levels play a direct role in controlling cell differentiation. Using the *Drosophila* larval nervous system as a model, I demonstrated that ribosomal RNA (rRNA) is actively synthesized in neuroblasts but is weakly, if at all, synthesized in post-mitotic neurons. I was able to trace this nascent rRNA through cell division into newly born neurons, showing that nascent rRNA is inherited by neuroblast progeny. I obtained important clues to underlying mechanisms of inheritance by showing that nascent rRNA associates with mitotic chromosomes at the metaphase plate. Finally, I demonstrated that inherited rRNA is sufficient to support normal levels of protein synthesis in neurons.

My dissertation work establishes the developing *Drosophila* nervous system as a promising model for investigating differential ribosome biogenesis along a well-defined cellular differentiation axis. While there are several potential future directions, I summarize two of the most promising below.

1. Identify mechanisms that coordinate rRNA and ribosomal protein levels during neural differentiation.

It will be interesting to investigate whether ribosomal proteins (RPs) follow the same pattern of inheritance as their rRNA counterparts. Our lab demonstrated that 5'TOP (Terminal Oligo-Pyrimidine sequence) mRNAs are destabilized in the *Drosophila* nervous system when compared to their stability in the whole embryo (*Burow 2018*). All *Drosophila* RP transcripts fall into this 5'TOP category. It is not yet known whether these transcripts and/or proteins are synthesized in *Drosophila* neuroblasts and inherited by their progeny or if they are independently synthesized in the progeny.

The mRNA binding protein LARP1 ("La-related protein"; homolog of *Drosophila* Larp) has been shown to influence stability of 5'TOP mRNAs. LARP1 physically associates with both the 40S ribosome and the 5'end of these 5'TOP mRNAs to increase the halflives of these transcripts. Loss of LARP1 causes corresponding loss of transcript stability. In LARP1 mutants, 5'-TOP mRNAs, including RP transcripts, are destabilized and degraded more rapidly. The result is a decrease in the overall ribosome concentration in the cell, since there is a deficit of RP components. These cells are therefore under ribosome stress. Cells under ribosome stress will not progress through the cell cycle due to cell cycle arrest as a result of the IRBS (Impaired Ribosome Biogenesis) checkpoint. Recently, LARP1 has been shown to be involved in mediating the IRBS checkpoint in cells. This checkpoint senses the levels of available ribosomes

in the cell through a ribosomal precursor complex consisting of RPL5, RPL11, and 5S rRNA. In normal growth conditions, this complex becomes incorporated in the mature 60S ribosome. Under conditions of insufficient ribosome biogenesis, the complex instead binds and inhibits the p53-E3-ligase Hdm2. p53 binding by Hdm2 promotes degradation of p53 which in turn allows for progression through the cell cycle (*Bursac, 2012 and Donati, 2013*). It was demonstrated that the IRBS checkpoint is activated in cells deficient in either LARP1 or the 40S ribosome. p53 is stabilized in the absence (or inhibition) of Hdm2, which leads to p53 accumulation in the cell and cell cycle arrest (*Gentilella*, 2017). Larp has been shown to be present at high levels in the cytoplasm *Drosophila* neuroblasts. However, it is nearly completely absent from the surrounding ganglion cells (*Blagden, 2009*).

It seems reasonable that Larp is largely responsible for the unexpectedly unstable halflives of 5'TOP mRNAs our lab found in *Drosophila* (*Burow, 2018*) and is likely involved in changing the proliferative state of neuroblasts through the differentiation pathway. As ribosome biogenesis decreases in neuroblast progeny, decreases in 40S availability may lead to destabilization of 5'TOP transcripts and activation of the IRBS checkpoint, leading to the eventual exit from the cell cycle that comes with differentiation.

2. Identify neuroblast- and neuron-specific transcripts whose translation is sensitive to ribosome concentration.

My work suggests that neuroblast versus neuron fates may be determined by transcripts that are differentially affected, in a cell type-specific manner, by ribosome concentration. This hypothesis is based on the model that a cell can efficiently alter its proteome by biasing translation programs toward cohorts of mRNAs with efficient or inefficient translation initiation kinetics. Translation initiation kinetics are a key determinant of protein synthesis levels. As the number of ribosomes on a transcript approaches the maximum, translation efficiency decreases; models predict that optimum translation occurs at about 50% transcript occupancy by ribosomes (*Zarai, 2016*). Transcripts that have inefficient translation initiation kinetics share similarities in that they tend to contain obstacles to ribosome occupancy, including structured 5'UTRs, upstream open reading frames (uORFs), or internal ribosome entry sites (IRES) (*Marshall, 2014, De Vos, 2011, and Raveh, 2016*). When ribosome concentration is relatively high, these transcripts experience steady ribosome occupancy, which is beneficial to protein output. However, when the ribosome concentration decreases, these obstacles become significant barriers to translation initiation (*Mills and Green, 2017*). My work suggests that neuroblasts have a higher concentration of ribosomes than their differentiated progeny. One possibility is that differentiation-promoting transcripts have highly efficient translation initiation rates; this would decrease protein synthesis in neuroblasts and increase protein synthesis as ribosome levels decline in differentiating progeny. Conversely, transcripts that encode proteins supporting cell proliferation and the multipotent state might have relatively poor translation initiation kinetics. Such transcripts would be translated at high levels in neuroblasts but poorly translated in differentiating progeny. Testing these hypotheses will require bioinformatic interrogation of the neural transcriptome (such as prediction of relevant 5'UTR elements) as well as quantitative measurements of mRNA translation in neuroblasts and neurons, potentially through ribosome profiling targeted to each cell type.

Understanding how differential ribosome biogenesis affects translation programs will certainly help advance the field of neural development and has potential implications for treating ribosomopathies as well as neurodevelopmental disorders. My dissertation work provides an important foundation for the future directions summarized above. I look forward to seeing the discoveries that build upon my work.

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