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.

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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.

Introduction

All cells require efficient mRNA translation, but protein synthesis capacity varies by cell type [1]. 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 [2]. A delicate balance of translation activity is also critical for hematopoietic stem cell (HSC) homeostasis, with increased or decreased global translation impairing HSC function [3]. Variation in ribosome concentration is

one mechanism cells use to control translation output [4]. In addition to influencing growth and proliferative capacity, ribosome concentration can affect cell fate through biased translation of certain mRNAs [4, 5]. 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 [6]. Similarly, in the *Drosophila* female germline production of ribosomal RNA (rRNA) and ribosome assembly factors decreases along the differentiation pathway [7, 8] yet germline stem cell differentiation is associated with increased global protein synthesis [8]. 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 [7] and mammalian tissue culture cells [9]. 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 [7]. Downregulation of rRNA synthesis also occurs during differentiation in the mammalian forebrain [10]. Decreased ribosome abundance appears to be a common feature of neural differentiation: additional studies have shown that ribosomal protein production [11] and rRNA synthesis [12] decreases in post-mitotic neurons. Compartment-specific changes, such as loss of ribosomes from mature axons, also occurs during neural development [13, 14]. 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 [15].

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 [16]. 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 [17, 18], 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 [18]. Myc promotes cell growth and proliferation via several pathways, including transcriptional activation of genes encoding RNA polymerase I subunits [19].

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 [20]. 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 cytidine 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 [21]. To test EC-tagging-based RNA imaging, we used the Gal4-UAS transgene expression system [22] 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 [23]. In addition, Gal4 and CD:UPRT activities likely perdure in GMCs of *insc-Gal4* x *UAS-CD:UPRT* brains. We used*nSyb-Gal4* for neuron tagging since this line expresses Gal4 only in neurons [24]. For all EC-tagging experiments, larvae were fed EC for 24 hours. 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 [21] 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 test this prediction with an imaging-independent approach, we purified EC-tagged RNA from neuroblasts and neurons following a 24-hour EC feeding. Nascent rRNA was quantified by RT-qPCR using primers specific for precursor rRNA [26]. We also measured levels of a neuron-specific transcript, *Synaptotagmin 1*(*Syt1*). *Syt1* was enriched by neuron-specific EC-tagging, confirming the cell type-specificity of the labeling (**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) [25] and actinomycin D to inhibit RNA polymerase I and II (blocking rRNA and mRNA synthesis) [21]. 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) [7] 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 [20] 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 [27]. 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 [28]. 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 [29]. 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 unmodified uridine. 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 (the longest chase timepoint tested, data not shown). 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 [30]. 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.

rRNA inheritance in neurons is sufficient for neurodevelopment and protein synthesis.

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 [7] 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**). As previously described for a RNAi screen in neuroblasts [23], we found that *Polr1B* knockdown driven by *insc-Gal4* limits neurogenesis and causes nearly 100% failure of adult flies to eclose from their pupal case (**Figure 4B**). *Insc-Gal4 x UAS-Polr1B*{*RNAi*} flies manually dissected from their pupal case were alive but unable to walk or fly, indicative of neurologic defects. In contrast, *Polr1B* knockdown in neurons had no effect on development: flies eclosed 100% of the time (**Figure 4B**) and exhibited normal motor function.

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)) [31] 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 [23], *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 [27] 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 [28]). 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 [28]. 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 [4]. 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 [14] 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 [32]. 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 [1] 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 somatic cells [33]. 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 [34]. 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 [35].

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 [36], were a gift from Michael Buszczak.

EC-tagging for RNA purification and RT-qPCR

5-ethynylcytosine was synthesized as previously described [20]. Larvae were reared at 25°C and fed 1 mM 5EC from 48 – 72 hours after hatching. 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 [20]. 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 [20]. 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 [26], synthesized by Integrated DNA Technologies. Pre-rRNA and*Syt1 Ct* values were normalized to *Polr2B* and relative abundance calculated by the equation, fold change = $2^{-\Delta(\Delta^{\curvearrowright}\tau)}$. RT-qPCR was performed on biological replicates of each genotype.

EC-tagging and EU-tagging for RNA imaging

Larvae were fed 1 mM EC or 0.5 mM EU for the indicated times. For EU-tagging in the presence of RNA polymerase inhibitors, dissected 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 [25]. We confirmed this concentration blocks production of mRNA in cultured brains using a dot blot (data not shown). 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 [21]. 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 [37]. The Click-iT reaction was followed by antibody staining according to standard methods [38]. 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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Figure Legends

Figure 1. Cell type-specific biosynthetic RNA tagging reveals limited rRNA synthesis in neurons. A. Fluorescent RNA signal (green) in a single 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.**B.** Relative precursor rRNA (pre-rRNA) and *Syt1*transcript abundance following 24 hours of EC-tagging 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 two biological replicates. **C.** The fluorescent signal is predominately ribosomal RNA. Dissected brains 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 is shown in each confocal stack. Scale bar is 10 μm**. 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. Scale bar is 10 μm**.**

Figure 2. Biosynthetically tagged rRNA is only detected in newly born neurons. A. Fluorescent RNA signal (green) in a single 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. **B.** Plot showing the relationship between EU feeding time and the number of EU-positive neurons. 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. 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.

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, 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.**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). 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.

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. **C.** HPG-based imaging of protein synthesis in control and *Polr1B* knockdown flies. HPG-tagged proteins are labeled green, Elav is labeled magenta. Scale bar is 10 μm. **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 $< 1 \times 10^{-5}$, Student's t-test compared to wildtype.