Translational regulation enhances distinction of cell types in the nervous system

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Author Contributions:

TI, SK, and HT conceptualized the project; TI performed ribosome profiling and RNA-seq with the help of YS, MM, and SI; MK performed immunohistochemistry and fluorescent in-situ hybridization, and managed fly care; SK constructed the transgenic reporter strain; YS, MM, and SI contributed to reagents, protocols, and analytic tools; TI analyzed all data with the help of YS and SI; TI and HT wrote the manuscript; SK, YS and SI edited the manuscript.

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The authors declare no competing interest.

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Abstract:

Multicellular organisms are composed of specialized cell types with distinct proteomes. While recent advances in single-cell transcriptome analyses have revealed differential expression of mRNAs, cellular diversity in translational profiles remains underinvestigated. By performing RNA-seq and Ribo-seq in genetically-defined cells in the *Drosophila* brain, we here revealed substantial posttranscriptional regulations that augment the cell-type distinctions at the level of protein expression. Specifically, we found that translational efficiency of proteins fundamental to neuronal functions, such as ion channels and neurotransmitter receptors, was maintained low in glia, leading to their preferential translation in neurons. Notably, distribution of ribosome footprints on these mRNAs exhibited a remarkable bias towards the 5′ leaders in glia. Using transgenic reporter strains, we provide evidence that the small upstream open reading frames (uORFs) in the 5' leader confer selective translational suppression in glia. Overall, these findings underscore the profound impact of translational regulation in shaping the proteomics for cell-type distinction and provide new insights into the molecular mechanisms driving cell-type diversity.

Introduction:

Gene expression is regulated both at the transcription and translation levels (Becker et al., 2018; Casas-Vila et al., 2017; Li et al., 2020; Liu et al., 2016; Schwanhäusser et al., 2011), and its heterogeneity defines the specialized morphologies and functions of cells. The *Drosophila* brain is a well-studied model tissue with a diverse array of cell types, classifiable by morphology, cell lineage, or gene expression (Scheffer et al., 2020; Zeng and Sanes, 2017). Recent advances in single-cell transcriptomics have identified groups of differentially expressed genes and provided an in-depth overview of transcriptional regulations (Croset et al., 2018; Davie et al., 2018; Li et al., 2022). While these inventories provided a powerful way to classify cell types, there have been cases falling short in explaining proteomic or morphological diversity (Lago-Baldaia et al., 2023; Li et al., 2020). Therefore, posttranscriptional regulations play pivotal roles in distinguishing cell-type specific proteomes.

Ribosome profiling or Ribo-seq, which is based on deep-sequencing of mRNA fragments protected by ribosomes from RNase treatment (ribosome footprints), has been a powerful approach to provide a genome-wide snapshot of protein synthesis ('translatome') (Ingolia et al., 2009). Application of this method, combined with transcriptome analysis, revealed multiple layers of translational regulation in cells. For example, this comparison allowed measurements

of translational efficiency (TE), which is quantified as the number of ribosome footprints on the coding sequence per mRNA, and discoveries of previously unannotated ORFs (Dunn et al., 2013; Ingolia et al., 2011, 2009; Zhang et al., 2018). While TE profiles have been reported to be variable among dissected animal tissues (Fujii et al., 2017; Wang et al., 2021; Zhang et al., 2018), differences in translational regulations among identified cell types remain unclear.

Applying ribosome profiling to *Drosophila* heads, we here examine the comprehensive landscape of translational profiles between neuronal and glial cells. Due to the size of the fly brain (~0.5 mm) and intricate intercellular adhesions among neurons and glia (Kremer et al., 2017), surgical separation is impractical. We thus biochemically purified ribosome-bound mRNAs through genetic tagging of ribosomes in target cells (Chen and Dickman, 2017; Sapkota et al., 2019; Scheckel et al., 2020; Thomas et al., 2012; You et al., 2021) and further performed Ribo-seq and RNA-seq. By this comparative transcriptome-translatome analyses, we suggest that differential translational programs enhance the distinction of protein synthesis between neuronal and glial cells.

Results:

Comparative transcriptome-translatome analyses reveal translational suppression of selective groups of proteins in the fly heads

To gain an overview of the translation status, we first applied conventional Ribo-seq in the whole fly head, and successfully monitored footprint distribution at a single-codon resolution (Figure 1A, see 'Materials and Methods' for technical details). The majority (96.2%) of ribosome footprints was mapped onto the annotated coding sequences (CDS), and its distribution displayed a clear 3-nt periodicity, reflecting the codon-wise movement (Figure 1B).

To compare transcriptome and translatome, we also performed RNA-seq from the same lysate (Figure 1A). As previously reported, the transcript level and the number of ribosome footprints did not always match, suggesting substantial posttranscriptional regulations (R^2 = 0.664; Figure 1C). For instance, while *Shaker* (*Sh*) and *Trehalase* (*Treh*), which encode a voltage-gated K^+ channel and an enzyme that hydrolyzes trehalose, respectively, were similar regarding transcript levels, far more ribosome footprints were detected on *Treh* (Figure 1D-E). We therefore measured translational efficiency (TE), ribosome footprints normalized by mRNA reads. TE was much higher for *Treh* than *Sh* (Figure 1F), and we found a striking genome-wide variability with more than 20-fold TE difference between the 5 and 95 percentiles (Figure 1G). Kyoto Encyclopedia of Genes and Genome (KEGG) pathway enrichment analysis

94 revealed that transcripts involved in fatty acid metabolism and proteasome are actively
95 translated (Figure 1H). In contrast, ribosome proteins, as previously reported (Chen and
96 Dickman, 2017; Cho et al., 2015), and proteins mediating neuronal ligand-receptor interactions
97 were significantly enriched in the transcripts with low TE, suggesting translational suppression
98 (Figure 1H). Indeed, many transcripts encoding ligand- or voltage-gated ion channels, G-
99 protein coupled receptors (GPCR) showed remarkably low TE (Figure 1C and 1I). These
100 results suggest translational regulations specific to neuronal transcripts in the fly head.

101 **Translational regulation enhances the difference in the gene expression profiles**
102 **between cell types**

103 Because the translatome/transcriptome status of the whole heads was a mixed average
104 of diverse cell types, such as neurons, glial cells, fat bodies, and muscles, we set up an
105 experimental approach to dissect cell-type specific translational regulations. By expressing
106 epitope-tagged RpL3 (uL3 in universal nomenclature) (Chen and Dickman, 2017) under the
107 control of UAS using the *nSyb*- or the *repo-GAL4* drivers, we immunopurified the tagged
108 ribosomes and associated mRNAs separately from neurons and glia, and performed Ribo-seq
109 (Figure 2A). By immunohistochemistry, we confirmed that *UAS-RpL3::FLAG* on the third
110 chromosome exhibited minimum leakage expression in the brain and did not display any
111 apparent morphological defects upon expression using either driver, compared to other
112 insertions or constructs (Chen and Dickman, 2017; Huang et al., 2019; Thomas et al., 2012)
113 (Figures 2A and Figure 2 – figure supplement 1A-B). The exogenously expressed RpL3::FLAG
114 was highly concentrated in cell bodies but also detectable in neurites, consistent with the
115 subcellular localization of the endogenous ribosome (Figure 2 – figure supplement 1C-D).

116 Through the purification of FLAG-tagged ribosomes, we successfully profiled translatome
117 from neurons and glial cells in the fly heads: Footprints were found on 10,821 (78.4 % of all
118 the annotated genes) and 10,994 (79.7 %) genes in neurons and glia, respectively, with decent
119 reproducibility among the biological replicates (R^2 > 0.9, Figure 2 – figure supplement 2A). The
120 FLAG-tagged RpL3 in the corresponding cells far exceeded the endogenous RpL3, as RpL3
121 reads were 7.8 and 42.7 times higher in neurons and glia, respectively, compared to the wild-
122 type whole-head samples (Figure 2 – figure supplement 2B). The known marker genes were
123 strongly enriched while non-target markers were depleted (Figures 2B and Figure 2 – figure
124 supplement 2C-D) (Croset et al., 2018; Davie et al., 2018; Li et al., 2022), and the KEGG
125 enrichment analysis showed significant enrichment of footprints on genes associated with the
126 known functions of these cell types (Figure 2 – figure supplement 2E). Interestingly, the KEGG

127 analysis also revealed that neurons exhibit a greater extent of protein synthesis related to
128 oxidative phosphorylation and mitochondrial ribosome proteins, while glial cells show higher
129 expression of proteins associated with glycolysis (Figure 2 – figure supplement 2E-F). These
130 findings support the glia-neuron lactate shuttle hypothesis, a recently proposed concept of
131 metabolic specialization (Mason, 2017; Volkenhoff et al., 2015). Furthermore, apart from the
132 annotated CDS, we detected clustered ribosome footprints on *Hsr-ω*, previously annotated as
133 a long non-coding RNA, strongly suggesting the synthesis of hitherto undescribed
134 polypeptides (Figure 2 – figure supplement 2G) (Singh, 2022). Altogether, the combination of
135 genetic labelling of ribosomes in selective cell types and Ribo-seq revealed the differential
136 translatome profiles in the fly heads.

137 To further examine translational regulation by calculating TE, we performed RNA-seq
138 from the same immunoprecipitated complexes, similar to Translating Ribosome Affinity
139 Purification (TRAP) (Heiman et al., 2008) (Figures 2A and Figure 2 – figure supplement 3A-
140 B). Because this approach relies on the 80S-ribosome-mRNA complex, we may miss mRNA
141 with little or no translation. Nevertheless, our transcriptome was similar to the sn-transcriptome
142 data (Li et al., 2022) (Figure 2 – figure supplement 3C). We identified groups of genes
143 undergoing neuron- or glia- specific translational regulations compared to the whole heads
144 (Figure 2 – figure supplement 4A). Genes mediating fatty acid metabolism and degradation,
145 for example, were actively translated in the whole head, but showed lower TE in neurons or in
146 glia (Figures 1H and 2C). Because many of these genes are highly expressed in the fat bodies
147 (Dobson et al., 2018), these results suggest selective translational enhancement in the fat
148 body. Strikingly, TE of genes involved in neuroactive ligand-receptor interaction was
149 significantly higher in neurons but lower in glia (Figure 2C-D), suggesting cell-type specific
150 translational regulation of these genes.

151 This differential translational regulation was highlighted in the weak TE correlation
152 between neurons and glia (R^2 = 0.534, Figure 2E). We found a genome-wide tendency that
153 genes transcribed less in glia are further suppressed at translation (Figure 2F). Specifically,
154 many functionally-characterized neuronal genes, such as voltage- or ligand- gated ion
155 channels, G-protein coupled receptors, neuropeptides and proteins for visual perception,
156 showed particularly lower TE in glia (Figures 2E, 2G-H and Figure 2 – figure supplement 4C).
157 For these genes, the distinction between neuronal and glial cells was much exaggerated at
158 the level of translation than at transcription (Figure 2H). Consistently on the genome-wide
159 scale, the inter-cell type correlation became weaker in Ribo-seq data compared to in RNA-seq
160 (R^2 = 0.59 vs. 0.81, Figure 2 – figure supplement 3B). Altogether, these data indicate

161 substantial contributions of translational regulation to shaping the cell-type specific protein
162 expression.

163 **Biased distribution of ribosomes toward upstream open reading frames of neural genes**
164 **in glial cells**

165 We next analyzed the distribution of ribosome footprints on the differentially translated
166 transcripts (DTT). Fat-body related genes showed lower TE in neurons compared to the whole
167 head (Figure 2C). Among these genes, we found a remarkable ribosomal accumulation on the
168 start codon specifically in neurons (Figure 3 – figure supplement 1A-B), as if the first round of
169 the elongation cycle was arrested in neurons. Through the reanalysis of the published RNA-
170 seq data (Dobson et al., 2018), we found that mRNAs showing strong ribosomal accumulation
171 on the start codons are highly abundant in the fat bodies (Figure 3 – figure supplement 1C).
172 On the other hand, DTTs suppressed in glial cells compared to neurons (defined as genes
173 with more than 10 times higher TE in neurons than in glia, n = 161), we noticed that glial
174 ribosome footprints were remarkably biased towards 5′ leaders (Figure 3A-B). Notably, this
175 pattern was not obvious on the genome-wide scale (Figure 3A-B). The high 5′ leader/CDS
176 ratio of ribosome footprints in glia was commonly observed on many transcripts with known
177 neuronal functions, such as *Rab3, Syt4, Arr1* and *Syn* (Figure 3D-E). Conversely, we observed
178 accumulated ribosome footprints on the 5′ leaders of several glial marker genes specifically in
179 neurons (Figure 3 – figure supplement 2). Altogether, these results suggest that the translation
180 of 5' leaders in selective mRNAs differentiates protein synthesis among cell types.

181 We reasoned translational downregulation via upstream open reading frames (uORFs) in
182 the 5′ leaders in glia, as the translation of uORFs was reported to suppress that of the
183 downstream main ORF (Ferreira et al., 2013; Zhang et al., 2019, 2018). Consistent with this
184 idea, metagene plot around the AUG codons on 5′ leaders revealed strong accumulation of
185 footprints on the upstream AUG codons, similar to those observed on the initiation codon of
186 CDSs (Figures 4A-B). We calculated the footprint accumulation score on each codon (defined
187 as the ratio of footprints on each codon with surrounding -50/+50 nt), and found that upstream
188 AUG and the near cognate codons (NUG or AUN) showed relatively high accumulation (Figure
189 4C). On the other hand, inside the annotated CDS, none of the codons exhibited such
190 significant accumulation (Figures 4D). Consistently, we found that transcripts related to
191 neuronal functions typically contain long 5′ leaders and many upstream AUG (Figure 4 – figure
192 supplement 1). We thus propose that glial cells suppress the translation of neuronal transcripts
193 by stalling ribosomes on 5′ leader via uORF.

194 **uORFs in *Rh1* confer translational suppression in glia**

195 We next asked if the 5′ leader sequences of neuronal genes cause cell-type differences
196 in translation. To this end, we focused on *Rh1* (*Rhodopsin 1*, also known as *ninaE*). *Rh1*
197 encodes the opsin with a broadband spectral sensitivity, also detecting stimuli of other sensory
198 modalities (Leung et al., 2020; O'Tousa et al., 1985; Shen et al., 2011; Zuker et al., 1985).
199 Therefore, the Rh1 protein is heavily expressed in the photoreceptor neurons, but the mRNA
200 is clearly detectable also in glia (Li et al., 2022) (Figure 5A, Figure 5A – figure supplement 1A).
201 Consistent with the protein expression, we found that TE of *Rh1* in neurons was 13.5 times as
202 high as that in glial cells (Figure 2 – figure supplement 4C). Ribosome footprints on the *Rh1*
203 transcript were heavily biased to 5′ leader in glia, with the striking accumulation on the putative
204 uORFs composed only of the start and stop codons (Figure 5B).

205 To address the function of these sequences, we constructed a transgenic reporter strain
206 using the *Rh1* UTR sequences under the control of UAS (Figure 5C), and directed gene
207 expression ubiquitously using *Tub-GAL4*. The reporter mRNA was detected both in neurons
208 and glial cells (Figure 5D; Figure 5 – figure supplement 1B). On the other hand, the protein
209 levels were much more heterogeneous and strikingly weak in glia than in neurons, in contrast
210 to the ubiquitous expression observed in the control reporter strain (DeLuca and Spradling,
211 2018) (Figure 5D). Driving the reporter expression using the *nSyb-* or *repo-GAL4* further
212 corroborated cell-type specific suppression in glia (Figure 5 – figure supplement 1C-D).
213 Strikingly, when the 6-base putative uORFs were mutated, the in-vivo protein-to-mRNA ratio
214 of the reporter was significantly increased in glia but not in neurons (Figure 5E-G). Based on
215 these results, we propose that glial cells selectively suppress the protein synthesis of neuronal
216 genes through uORF and thereby enhance the translatome distinction from neurons.

217

218 **Discussion:**

219 In this study, the comparative translatome-transcriptome analyses in the whole heads,
220 neurons and glial cells revealed the significant diversity of translational regulations across
221 different cell types. Particularly noteworthy was the differential translation of transcripts
222 encoding neuronal proteins, including ion channels and neurotransmitter receptors (Figure 2).
223 These neuronal transcripts exhibited preferential translation in neurons (Figure 2), and the
224 relatively long 5′ UTR of these transcripts strongly stalled ribosomes in glia (Figures 3, 4 and
225 Figure 4 – figure supplement 1). This characteristic feature of long 5′ leaders containing
226 numerous uORFs is also observed in neuronal transcripts in mammals (Glock et al., 2021).

227 While the 5′ leader-mediated translational regulations are known to be critical for quick
228 response to environmental changes, such as starvation or oxidative stress (Harding et al.,
229 2003; Mueller and Hinnebusch, 1986; Young and Wek, 2016), our study sheds light on its roles
230 in contrasting protein expression among cell types. Furthermore, considering a pivotal role of
231 *de novo* protein synthesis for long-lasting adaptation (Flexner et al., 1963; Tully et al., 1994),
232 it is plausible that similar mechanisms are employed for neuronal plasticity as well.

233 The next obvious question would be how the translation of neuronal transcripts is
234 differentiated among neuronal and glial cells. Ribosomes can initiate translation at uORFs
235 more frequently in glia. Alternatively, the post-termination 40S subunits reinitiate translation of
236 the coding sequence more often in neurons. These two possibilities can be distinguished by
237 profiling the rates of initiation or reinitiation, achievable through sequencing the footprints of
238 the 40S subunits, known as translation complex profile sequencing, coupled with conventional
239 Ribo-seq (Archer et al., 2016; Bohlen et al., 2020; Wagner et al., 2020). Although there are
240 various technical challenges, the application of this technique to specific cells within the brain
241 would elucidate these possibilities. Furthermore, previous studies have identified eIF1, eIF2α
242 kinases, and DENR/MCT1, as facilitators of translation of main ORF whose 5′ leaders harbor
243 uORFs (Ivanov et al., 2010; Schleich et al., 2014; Sonenberg and Hinnebusch, 2009; Zhou et
244 al., 2020). Interestingly, all these proteins are expressed more in neurons than in glia in our
245 dataset (supplementary table 1). Selective activation of these molecular machineries might
246 underlie the cell type specific translation.

247 Our cell-type specific translatome analysis further revealed translational regulations
248 beyond 5′ leaders. We found a remarkable ribosomal stall at the initiation codon in several
249 transcripts, a phenomenon observed in neurons but not in the entire heads (Figure 3 – figure
250 supplement 1). These transcripts are known to be massively expressed in the fat bodies but
251 less in the nervous system (Figure 3 – figure supplement 1C) (Dobson et al., 2018), and the
252 translation was further suppressed in neurons (Figure 3 – figure supplement 1). Therefore,
253 transition from initiation to elongation may serve as another regulatory checkpoint of protein
254 synthesis (Harnett et al., 2022; Wang et al., 2019), which enhances cell-type distinctions.
255 Furthermore, we found ribosome footprints also on the 3′ UTR of certain transcripts, such as
256 *Synapsin* (Figure 3C). Stop-codon readthrough has been reported to be more frequent in
257 neurons than in other cell types (Hudson et al., 2021; Karki et al., 2022; Prieto-Godino et al.,
258 2016). Because the readthrough events extend the protein C-terminus, its regulation can add
259 yet another layer of cell-type diversity (Dunn et al., 2013; Jungreis et al., 2011; Klagges et al.,

260 1996). Altogether, we here propose that translational regulations further differentiate
261 transcriptome distinctions, thereby shaping the cellular identity.

262 Due to the specialized functions of neuronal and glial cells, they express distinct sets of
263 proteins. Neurons allocate more ribosomes to proteins related to neurotransmission, visual
264 sensing and oxidative phosphorylation, while glial cells synthesize transporters and enzymes
265 for metabolism of amino acid, fatty acid or carbohydrates (Figure 2 – figure supplement 1F).
266 Despite these clear differences and specialization, a significant amount of neuronal and glial
267 cells has a common developmental origin. They originate from a stem cell lineage known as
268 neuro-glioblasts (Lai and Lee, 2006; Viktorin et al., 2011), and the fate of these cells can be
269 altered by the expression of a single gene, *glial cells missing* (*gcm*) (Hartenstein, 2011; Hosoya
270 et al., 1995). Therefore, translational regulations, in addition to transcriptional diversity, may
271 play a particularly important role in these sister cell types with distinct physiological roles.

272 In the *Drosophila* brain, approximately 100 stem cell lineages diverge into more than
273 5,000 morphologically distinct cell types (Ito et al., 2013; Scheffer et al., 2020; Yu et al., 2013).
274 Hence, translational regulations similar to those described in this study, or other possible
275 regulations, may play significant roles in further differentiating neuronal or glial subtypes.
276 Consistent with this idea, our *UAS-Rh1-Venus* reporter showed heterogeneous expression
277 even among neurons, contrasting with the more uniform expression observed in the control
278 *UASz-GFP* reporter (Figures 5D and Figure 5 – figure supplement 1C-D). In accordance,
279 choline acetyltransferase (ChAT), an enzyme needed to synthesize acetylcholine, and
280 vesicular acetylcholine transporter (VAChT) are transcribed in many glutamatergic and
281 GABAergic neurons but its protein synthesis is inhibited (Chen et al., 2023; Lacin et al., 2019).
282 Substantial post-transcriptional regulations are also implicated during development (Li et al.,
283 2020; Zhang et al., 2016). Taken together, multiple layers of transcriptional and
284 posttranscriptional regulations should shape the proteome diversity of cell types in the nervous
285 system. Further comparative transcriptome-translatome analyses using more specific GAL4
286 drivers should highlight the diversity of translational regulations leveraged in the brain.

287

288 **Limitation of the study:**

289 Because our cell-type specific Ribo-seq and RNA-seq are based on immunoprecipitation of
290 genetically tagged RpL3 (Chen et al., 2017), the read counts could contain biases, such as
291 underestimation of mRNA level with little or no translational activity, or over- and under-
292 representation of certain cell types originating from the heterogeneous expression of the

drivers.

295 **Materials and Methods:**

296 **Fly culture and genetics**

297 The flies were reared in a mass culture at 24 ºC under the 12-12 hour light-dark cycles on
298 the standard cornmeal food. The *Canton-S* strain was used as the wild-type. We utilized the
299 following transgenic strains: *w^{1118};;GMR57C10-GAL4* (*nSyb-GAL4*; BDSC #39171),
300 *w^{1118};;repo-GAL4* (BDSC #7415), *y^1w^{1118};;tublin-GAL4* (BDSC #5138), *w^{1118};MB010B* (BDSC
301 #68293), *w^{1118};;UAS-RpL3::FLAG* (BDSC #77132) (Chen and Dickman, 2017), *y^1v^1;UAS-Rh1-*
302 *Venus* (made in this study; see below), *y^1v^1;UAS-m-Rh1-Venus* (made in this study), *w;UASz-*
303 *GFP* (a kind gift from Dr. Steven DeLuca) (DeLuca and Spradling, 2018). Females of the GAL4
304 drivers were crossed to males of the UAS effectors, and the F1 progenies were used for the
305 experiments. Of note, although *UAS-EGFP::RpL10Ab* (Thomas et al., 2012) has been used to
306 isolate ribosomes from specific cells, its expression using the *repo-GAL4* caused lethality in
307 our hands.

308 **Library preparation for ribosome profiling**

309 *Tissue collection and lysate preparation*

310 Four to eight days old flies with mixed gender were flash-frozen with liquid nitrogen,
311 thoroughly vortexed, and the heads were isolated from the bodies with metal mesh in a similar
312 manner reported previously (Sun et al., 2020). Approximately 500 frozen heads were mixed
313 with 400 µl of frozen droplets of lysis buffer [20 mM Tris-HCl pH7.5, 150mM NaCl, 5 mM $MgCl_2$,
314 1 mM dithiothreitol, 1 % Triton X-100, 100 µg/ml chloramphenicol, and 100 µg/ml
315 cycloheximide] in a pre-chilled container, then pulverized with grinding at 3,000 rpm for 15
316 seconds using a Multi-beads Shocker (YASUI KIKAI). Cycloheximide and chroramphenicol
317 were added to the lysis buffer to prevent possible elongation and run-off of cytosolic and
318 mitochondrial ribosomes, respectively. The lysate was slowly thawed at 4 °C and the
319 supernatant was recovered after spinning down by a table top micro centrifuge. The lysate
320 was treated with 10 U of Turbo DNase (ThermoFisher Scientific) on ice for 10 min to digest
321 the genome DNA. The supernatant was further clarified by spinning at 20,000 g for 10 minutes.

322 *Immunoprecipitation*

323 Anti-FLAG M2 antibody (F1804, Sigma Aldrich) and Dynabeads M-280 bound to anti-
324 mouse IgG antibody (11201D, Invitrogen) were used for immunoprecipitation. 25 µl of the
325 beads solution, washed twice with the aforementioned lysis buffer, was mixed with 2.5 µl of
326 the M2 antibody, and incubated at 4 °C for 1 hour with rotation. Beads were incubated with the

327 lysate at 4 °C for 1 hour with rotation and washed for four times with the lysis buffer. The
328 ribosome-bound mRNA was eluted with 50 µl of 100 µg/ml 3× FLAG peptide (GEN-3XFLAG-
329 25, Protein Ark) dissolved in the lysis buffer.

330 *RNase digestion and library preparation*

331 Ribosome profiling was performed as described previously (McGlincy and Ingolia, 2017;
332 Mito et al., 2020) with modifications. We used RNase I from *E. coli* (N6901K, Epicentre) to
333 digest the crude (Figures 1, Figure 2 – figure supplement 1C-D) or the immunoprecipitated
334 (Figure 2) lysate. Concentration of RNA in lysate was measured with Qubit RNA HS kit
335 (Q32852, Thermo Fisher Scientific). RNase I was added at a dose of 0.25 U per 1 µg RNA in
336 a 50 µl reaction mixture, which was incubated at 25 °C for 45 minutes. We used 1.36 µg and
337 0.5 µg RNA to prepare the whole head libraries (Figures 1, Figure 2 – figure supplement 1C-
338 D) and the cell-type specific libraries (Figure 2-3), respectively. The RNase digestion was
339 stopped by adding 20 U of SUPERase•In (AM2694, ThermoFisher Scientific). Ribosomes
340 were isolated by MicroSpin S-400 HR columns (27-5140-01, GE Healthcare). Subsequently,
341 we purified RNA using the TRIzol-LS (10296010, ThermoFisher Scientific) and Direct-zol RNA
342 Microprep kit (R2062, Zymo Research), and isolated the RNA fragment ranging 17 to 34 nt by
343 polyacrylamide gel electrophoresis.

344 The isolated RNA fragments were ligated to custom-made preadenylated linkers
345 containing unique molecular identifiers and barcodes for library pooling, using T4 RNA ligase
346 2, truncated KQ (M0373L, New England Biolabs) (Mito et al., 2020). Ribosomal RNA was
347 depleted by hybridizing to the custom-made biotinylated 2′-*O*-methyl oligonucleotides with
348 complementary sequences to the *Drosophila* rRNA (see *Supplementary Method* for the
349 sequences), which can be pulled-down using the streptavidin-coated beads (65001,
350 ThermoFisher Scientific). The rRNA-depleted samples were reverse-transcribed with
351 ProtoScript II (M0368L, New England Biolabs), circularized with CircLigase II (CL9025K,
352 Epicentre), and PCR-amplified using Phusion polymerase (M0530S, New England Biolabs)
353 (Mito et al., 2020). The libraries were sequenced with the Illumina HiSeq 4000 system
354 (Illumina) with single-end reads of 50 bases.

355 **Library preparation for transcriptome analysis**

356 The crude (Figure 1) or the immunoprecipitated (Figure 2) lysate was prepared using the
357 same protocol as described above, but without RNase digestion. RNA was purified using
358 TRIzol-LS. The libraries were constructed in Azenta Japan Corporation, using the NEBNext
359 Poly(A) mRNA Magnetic Isolation Module (E7760, New England Biolabs) and MGIEasy RNA

360 Directional Library Prep kit (1000006386, MGI tech). Briefly, poly-A tailed mRNAs were
361 enriched with the oligo dT beads, fragmented, and reverse-transcribed using random primers.
362 After the second strand cDNA was synthesized, an adapter sequence was added. DNA library
363 was PCR-amplified. The libraries were sequenced with DNB-seq (MGI tech) with an option of
364 paired end reads for 150 bases.

365 **Data analysis**

366 Adaptor sequences were removed using Fastp (Chen et al., 2018), and the reads that
367 matched to the non-coding RNA were discarded. The remaining reads were mapped onto the
368 *Drosophila melanogaster* release 6 genome. Mapping was performed using STAR (Dobin et
369 al., 2013). PCR-duplicated reads were removed by referring to the unique molecular identifiers.
370 The number of uniquely mapped reads are as follows:

371 Ribo-seq:

Sample	#Reads
Canton-S, whole heads	1,427,090
nSyb-GAL4/UAS-RpL3::FLAG, after IP, replicate 1	2,443,467
nSyb-GAL4/UAS-RpL3::FLAG, after IP, replicate 2	1,135,284
repo-GAL4/UAS-RpL3::FLAG, after IP, replicate 1	2,698,259
repo-GAL4/UAS-RpL3::FLAG, after IP, replicate 2	2,133,920
nSyb-GAL4/UAS-RpL3::FLAG, whole heads, replicate 1	2,147,092
nSyb-GAL4/UAS-RpL3::FLAG, whole heads, replicate 2	2,361,588
repo-GAL4/UAS-RpL3::FLAG, whole heads, replicate 1	1,372,502
repo-GAL4/UAS-RpL3::FLAG, whole heads, replicate 2	1,846,122

372

373 RNA-seq:

Sample	#Reads
Canton-S, whole heads	29,132,939
nSyb-GAL4/UAS-RpL3::FLAG, after IP, replicate 1	40,638,850
nSyb-GAL4/UAS-RpL3::FLAG, after IP, replicate 2	34,247,848
repo-GAL4/UAS-RpL3::FLAG, after IP, replicate 1	30,906,375

repo-GAL4/UAS-RpL3::FLAG, after IP, replicate 2 31,928,337

374

375 For Ribo-seq analysis, fragments ranging from 20 to 34 nt for whole head samples and
376 21 to 36 nt for immunoprecipitated samples were used. For the whole head samples, the
377 position of the P site was estimated as 12 or 13 nt downstream from the 5′ end, for the 20 to
378 31 nt or 32-34 nt fragments, respectively (Ingolia et al., 2009). For the immunoprecipitated
379 samples, it was estimated as 12 or 13 nt downstream for the 21 nt or 22-36 nt fragments,
380 respectively. Footprints were considered to be on the CDS if the estimated P site was between
381 the annotated start and stop codons. RNA-seq analysis included all fragments greater than 30
382 nt in length. For genes with alternatively spliced transcripts, the isoform with the highest TPM
383 in the wild type RNA-seq sample (Figure 1) was selected as the 'representative' isoform. If not
384 specified, only the representative isoforms were considered. TE was calculated as TPM of
385 ribosome footprints on CDS divided by TPM of RNA-seq.

386 The KEGG-enrichment analyses were performed using iPAGE (Figures 1H and 2D)
387 (Goodarzi et al., 2009) or DAVID (Figure 2 – figure supplement 2E and Figure 2 – figure
388 supplement 4A) (Dennis et al., 2003). Statistical tests were performed with GraphPad Prism
389 9.

390 The Fly Cell Atlas data were downloaded (https://www.ebi.ac.uk/gxa/sc/experiment/E-
391 MTAB-10519/download/zip?fileType=normalised&accessKey=), and the mean RPKM in
392 neuronal or glial cells was calculated for each gene according to the annotation (Li et al., 2022).

393 **Reporter construct and the transgenic strain**

394 DNA fragments containing a minimal hsp70 promoter (hsp70Bb) (DeLuca and Spradling,
395 2018), the 5′ leader or the mutated 5' leader of *Rh1-RA*, the first 15 bases of the CDS of *Rh1-*
396 *RA*, the Venus yellow fluorescent protein gene, and the 3′ UTR of *Rh1-RA* were synthesized
397 and cloned into the pBFv-UAS3 plasmid (Addgene #138399). The sequence of the resultant
398 plasmid is provided in the *Supporting Information Text*. The plasmid was then injected into y^1
399 v^1 *P{nos-phiC31}; P{CaryP}attP40,* and their progenies were screened for a *v*+ phenotype. A
400 single transformant was crossed to y^1 cho^2 v^1*; Sp/CyO* balancer to establish a transgenic line.

401 **Immunohistochemistry and fluorescent in-situ hybridization**

402 Immunohistochemistry (Figures 2A and Figure 2 – figure supplement 1) was performed
403 as previously described with minor modifications (Kanno et al., 2021). Briefly, dissected male
404 fly brains were fixed in 2 % paraformaldehyde in PBS for 1 hour at room temperature, washed

405 three times with PBST (0.1 % Triton X-100 in PBS), blocked with 3 % goat serum in PBST for
406 30 minutes, then incubated with the primary antibody solution at 4 ºC overnight (mouse anti-
407 FLAG (1:1000; Sigma-Aldrich; F1804), mouse anti-RpS6 (1:200; Cell Signaling; 54D2), and
408 rat anti-elav (1:20; DSHB; 7E8A10)). Subsequently, the brains were washed three times with
409 PBST, incubated with the secondary antibody solution at 4 ºC overnight (anti-mouse Alexa
410 Fluor 488 (1:400; Invitrogen; A11001), anti-mouse Cy3 (1:1000; Jackson ImmunoResearch;
411 115-166-003), and anti-rat Cy3 (1:200; Jackson ImmunoResearch; 112-166-003)), washed
412 three times with PBST and mounted with 86 % glycerol in PBS.

413 Fluorescent in-situ hybridization, combined with immunohistochemistry, was performed in
414 a similar manner to (Yang et al., 2017) with several modifications (Figure 5D-G). Dissected
415 male fly brains were fixed in PBS containing 3 % formaldehyde, 1 % glyoxal and 0.1 %
416 methanol for 30 min at room temperature, followed by three quick washes with PBT (0.5 %
417 Triton X-100 in PBS). Consistent with the previous study, the addition of glyoxal to the fixative
418 improved the FISH signal (Yao et al., 2021). The buffer was then exchanged to the wash
419 solution (10 % Hi-Di™ Formamide (Thermofisher scientific; 4311320) in 2x saline sodium
420 citrate (SSC)) and was incubated at 37 ºC for 5 minutes. Subsequently, the brains were
421 incubated with the custom-made Stellaris *Venus* or *GFP* probes (100 nM; see *Supporting*
422 *Information Text* for the sequences; LGC BioSearch Technologies) and the primary antibody
423 (mouse anti-Repo (1:100; DSHB; 8D12)) in the hybridization buffer (10% Hi-Di™ Formamide
424 in hybridization buffer (Stellaris RNA FISH Hybridization Buffer, SMF-HB1-10)) at 37 ºC for 16
425 hours. The probes and the antibody were then removed by washing the samples quickly three
426 times with preheated wash solution at 37 ºC, followed by three washes for 10 minutes at room
427 temperature. Blocking was performed with 3 % normal goat serum in PBT for 30 min at room
428 temperature. The secondary antibody (Cy3 goat anti-mouse (1:2000; Jackson
429 ImmunoResearch; 115-166-003)) was then added and was incubated at 4 ºC overnight. The
430 samples were washed once quickly, three times for 20 minutes, and once for 60 minutes with
431 PBT, and then mounted in 86 % glycerol in 1x Tris-HCl buffer (pH7.4).

432 Tissues to detect native GFP or Venus signals (Figure 5 – figure supplement 1) were
433 prepared as follows: dissected brains were fixed in PBS containing 3 % formaldehyde, 1 %
434 glyoxal and 0.1 % methanol for 30 min at room temperature, followed by one quick wash and
435 three washes for 10 minutes with PBT. The samples were then mounted in 86 % glycerol in
436 1x Tris-HCl buffer (pH7.4).

437 **Imaging and microscopes**

438 Imaging was done on the Olympus FV1200 confocal microscope with GaAsP sensors. A
439 100x/1.35 silicone immersion objective (UPLSAPO100XS, Olympus) or 30x/1.05 silicone
440 immersion objective (UPLSAPO30XS) was used. Scan settings were kept constant across
441 specimens to be compared.

442 **Data availability**

443 The raw sequence data were deposited in the National Center for Biotechnology
444 Information (NCBI) database with the project code (PRJNA992629). The custom scripts are
445 available in Zenodo (https://zenodo.org/records/10637789).

446

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466

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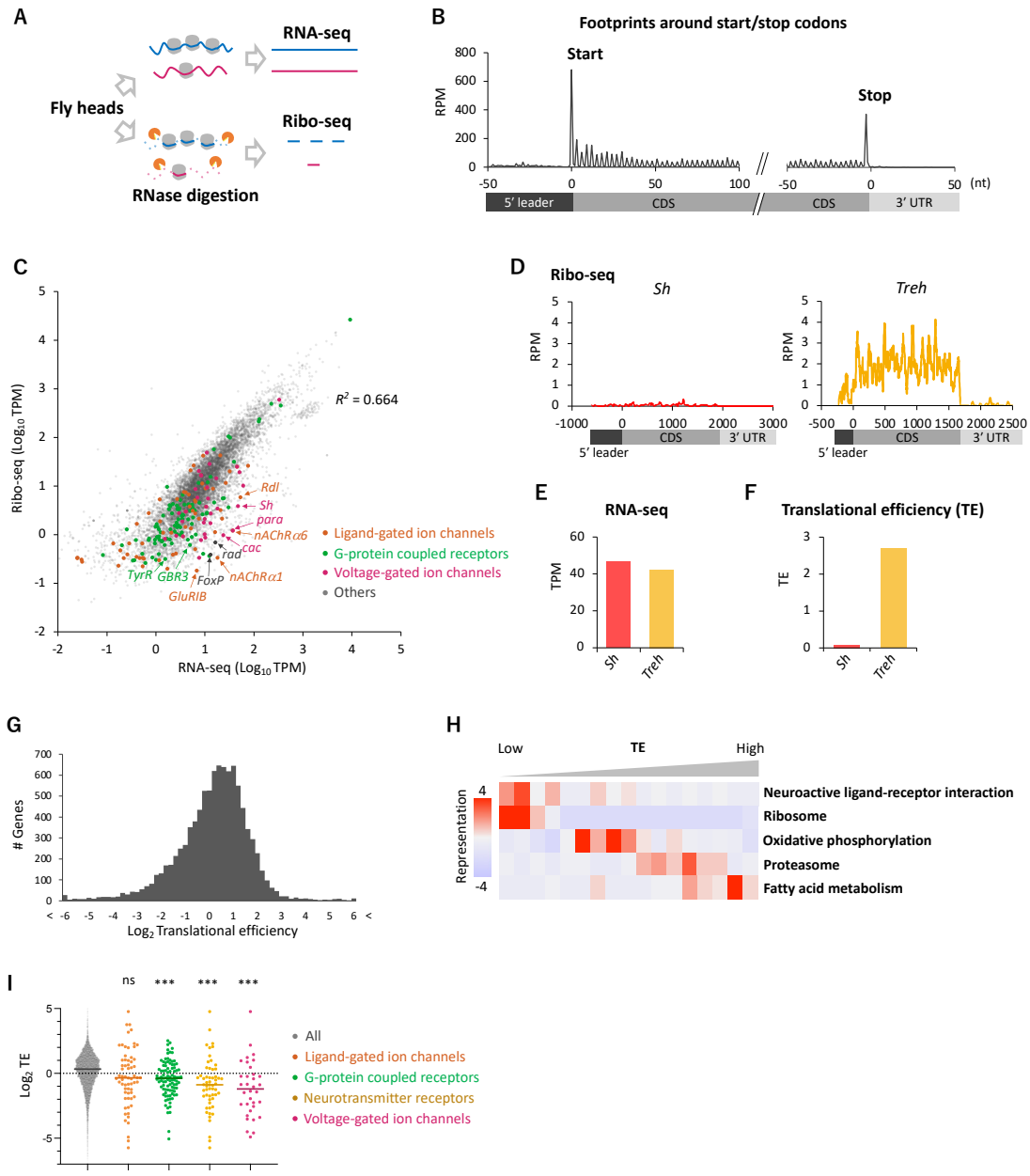

Figure 1. Comparative transcriptome-translatome analyses in the *Drosophila* head.

(A) Schematics. Fly head lysate is digested with RNase I for Ribo-seq, while not for RNA-seq. Resultant short fragments or the whole mRNA are reverse-transcribed and sequenced. (B) Meta-genome ribosome distribution (estimated P-sites of the 21-nt fragments), relative to the annotated start and stop codons. RPM: reads per million. (C) Scatter plots of mRNA reads (x-axis, TPM: transcripts per million) and ribosome footprints on CDS (y-axis, TPM). Several neuron-related genes are highlighted with colors and arrows. The squared Pearson's correlation coefficient (R^2) is indicated. (D-F) Ribosome footprints (D), mRNA level (E), and

769 translational efficiency (F) of *Shaker-RB* (*Sh*) and *Trehalase-RA* (*Treh*). TE: translational
770 efficiency. TE is calculated as ribosome footprints on CDS (TPM) divided by the mRNA level
771 (TPM). (G) Histogram of TE. The bin size is 0.2 in the unit of log 2. Total 9,611 genes with at
772 least one read in both Ribo-seq and RNA-seq are plotted. (H) Kyoto Encyclopedia of Genes
773 and Genomes (KEGG) pathways enrichment analysis, visualized by iPAGE (Goodarzi et al.,
774 2009), based on TE. The 9,611 genes are ranked and binned according to TE (left to right: low
775 to high), and over- and under- representation is tested. The presented KEGG pathways show
776 P values smaller than 0.0005. (I) TE of transcripts in the denoted gene ontology terms. Bars
777 represent the median. ns: $P > 0.05$; ***: $P < 0.001$; in the Dunn's multiple comparisons test,
778 compared to the "all" group.

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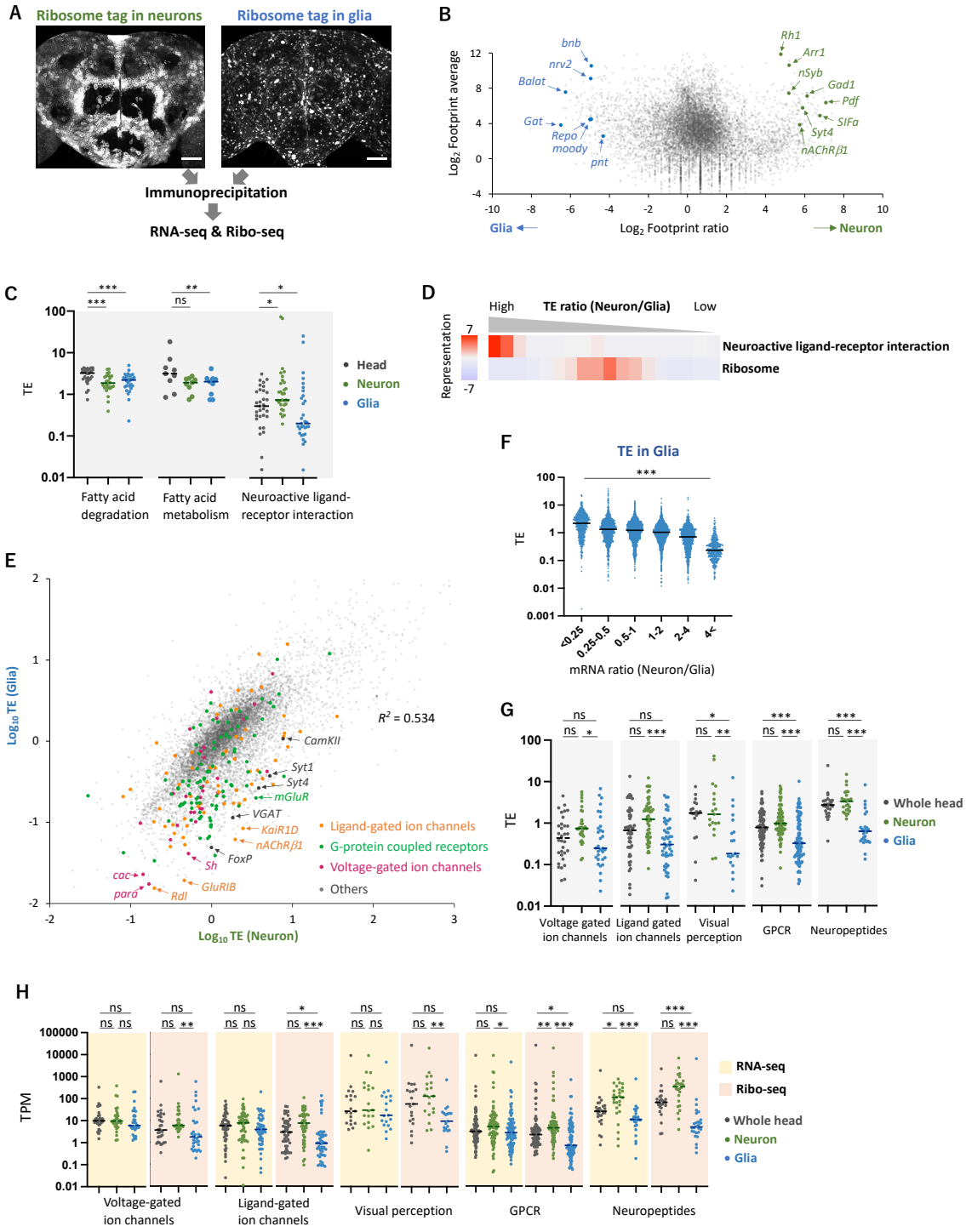

Figure 2. Cell-type specific Ribo-seq and RNA-seq reveal differential translational regulations.

(A) Schematics. FLAG-tagged ribosome protein L3 (RpL3::FLAG) is expressed in neurons (*nSyb-GAL4*) or in glial cells (*repo-GAL4*). RNA-seq and Ribo-seq are performed following

785 immunoprecipitation. Whole brain images of the exogenously expressed RpL3::FLAG are
786 shown. Scale bars: 50 μm. (B) The MA-plot of ribosome footprints on CDS among neurons
787 and glia. Each gene is plotted according to the fold change (x-axis) and the average (y-axis)
788 in the unit of $\log_2$. Several marker genes are highlighted with green (neuron) or blue (glia). (C)
789 TE of genes in the denoted KEGG pathways in the whole head (black), neurons (green), or in
790 glia (blue). Genes with TPM > 1 in the RNA-seq dataset are plotted. Bars represent the
791 median. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, the Dunn's multiple comparisons test. (D)
792 KEGG pathway enrichment analysis based on the ratio of TE in neurons to in glia. All genes
793 with at least one read in both cell types (total 9,732 genes) are ranked and binned according
794 to the neuron-to-glia ratio (left to right: high to low), and over- and under- representation is
795 tested. The presented KEGG pathways show *P* values smaller than 0.0005. (E) Scatter plot of
796 TE in neurons (x-axis) and in glia (y-axis). The squared Pearson's correlation coefficient (R^2)
797 is indicated. (F) TE in glia plotted according to the ratio of mRNA expression in neurons
798 compared to glia. ***: $P < 0.001$, Kruskal-Wallis test. All the 7,933 genes showing TPM > 1 in
799 RNA-seq are analyzed. (G) TE of transcripts, showing at least one read, in the indicated gene
800 ontology (GO) terms. Bars represent the median. **: $P < 0.01$, ***: $P < 0.001$, Dunn's multiple
801 comparisons test. (H) Read counts of genes (TPM) in the indicated GO terms in RNA-seq
802 (yellow) and in Ribo-seq (pink). The grey, green and blue dots indicate the read counts in the
803 whole head, neurons and glial cells, respectively. ns: $P > 0.05$, **: $P < 0.01$, ***: $P < 0.001$,
804 Dunn's multiple comparisons test.
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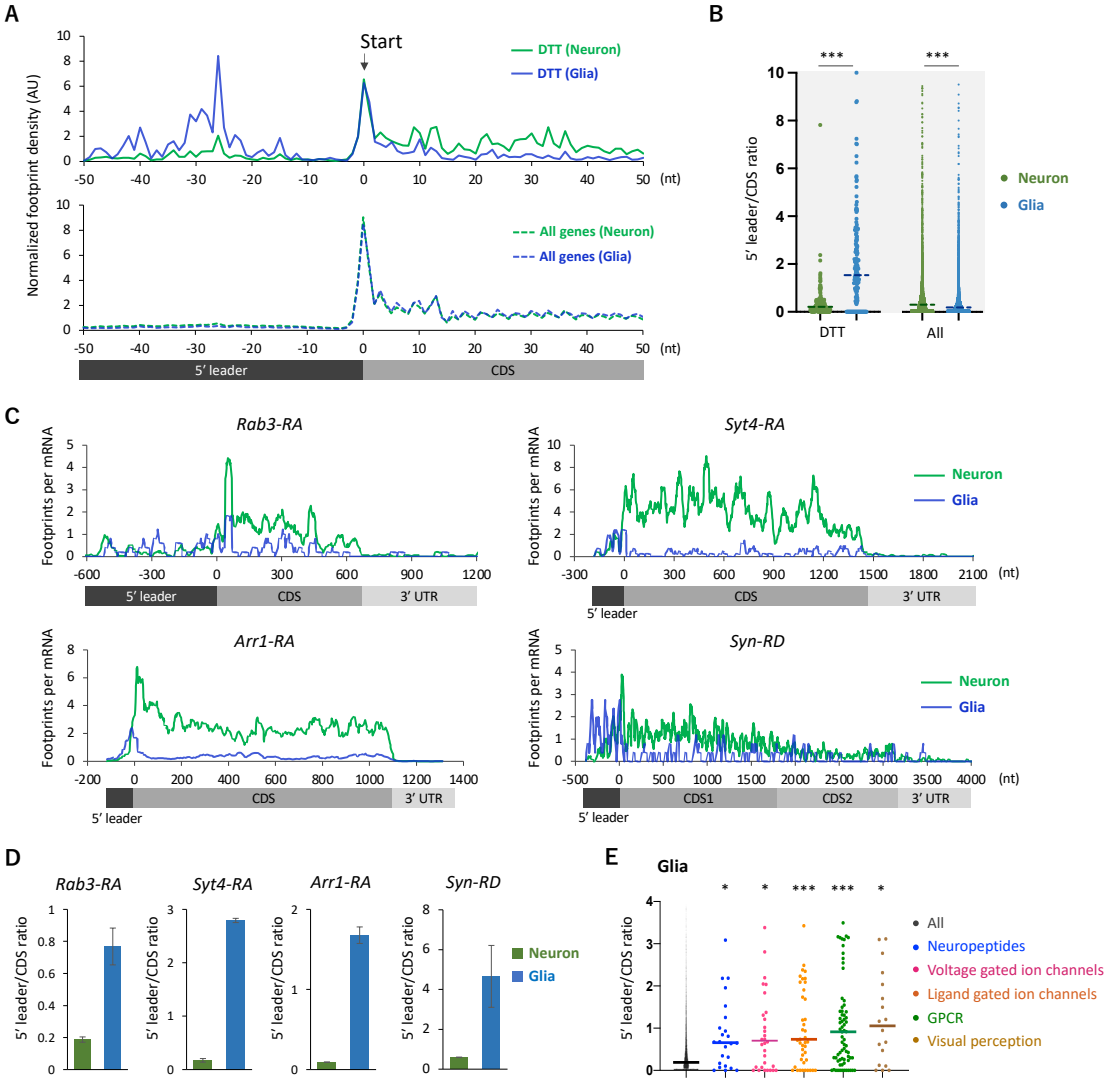

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807 **Figure 3. Ribosome stalling on the 5′ leaders of DTTs in glia.**

808 (A) Ribosome distribution (estimated P-sites) on the 161 DTTs around the start codons (solid
809 lines; start +/- 50 nt). These DTTs are defined as transcripts showing more than 10 times
810 higher TE in neurons compared to glia. The dotted lines in the bottom graph indicate the
811 genome-wide distribution. All the transcripts showing TPM > 1 in RNA-seq both in neurons
812 and glia are considered (7,933 genes in total), and the height is normalized by the total reads
813 on this region. (B) Ratio of ribosome density on 5′ leader (TPM) to CDS (TPM) of the 161 DTTs
814 or of all transcripts in neurons (green) or in glia (blue). The bars represent the median. ***: P
815 < 0.001, Mann-Whitney test of ranks. (C) Distribution of ribosome footprints on the
816 representative neuronal transcripts. Ribosome footprints (RPM) normalized by the mRNA level

817 (TPM) are shown. Note that *Syn-RD* harbors a stop codon in the CDS but a fraction of
818 ribosomes skip it, generating two annotated ORFs (CDS1 and CDS2) (Klagges et al., 1996).
819 (D) Ratio of ribosome density on 5′ leader to CDS (mean ± standard error of mean of the
820 biological replicates). (E) Ratio of ribosome density on 5′ leader to CDS on transcripts in the
821 indicated GO terms in glia. *: $P < 0.05$, ***: $P < 0.001$, Dunn's multiple comparisons test
822 compared to the "all" group.
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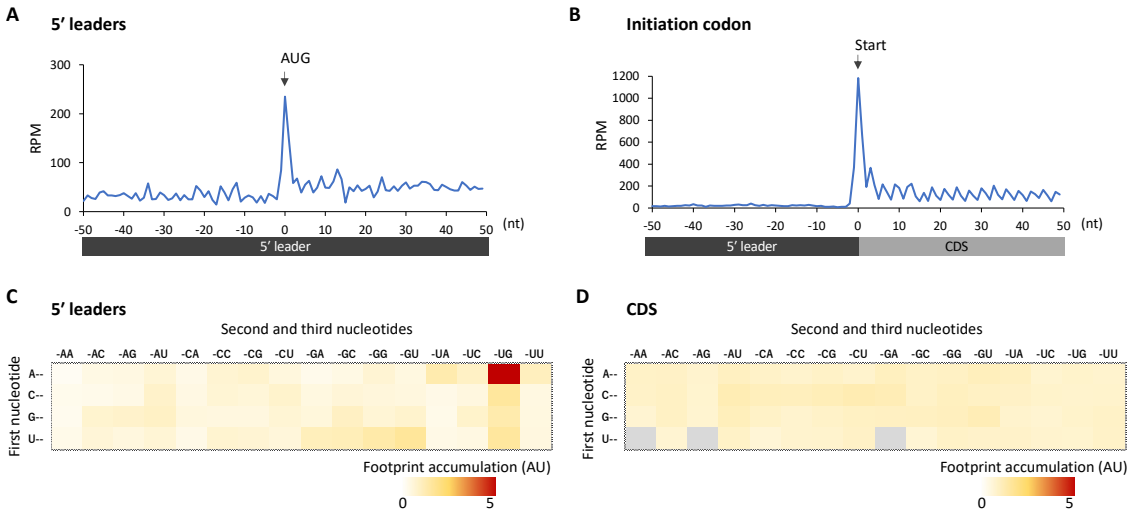

Figure 4. Footprint accumulation on upstream AUG in glia.

(A) Meta-genome ribosome distribution (estimated P-sites of the 32-nt fragments) around the upstream AUG codons in glia. (B) Meta-genome ribosome distribution (estimated P-sites of the 32-nt fragments) around the annotated start codons in glia. (C) Footprint accumulation on 5′ leader in glia, defined as the number of ribosome footprints (estimated P-sites) on each codon normalized by the average on the surrounding (-50 to +50) regions. (D) Footprint accumulation inside the annotated CDS in glia. Annotated in-frame codons except the start and the stop codons are considered. AU: arbitrary unit.

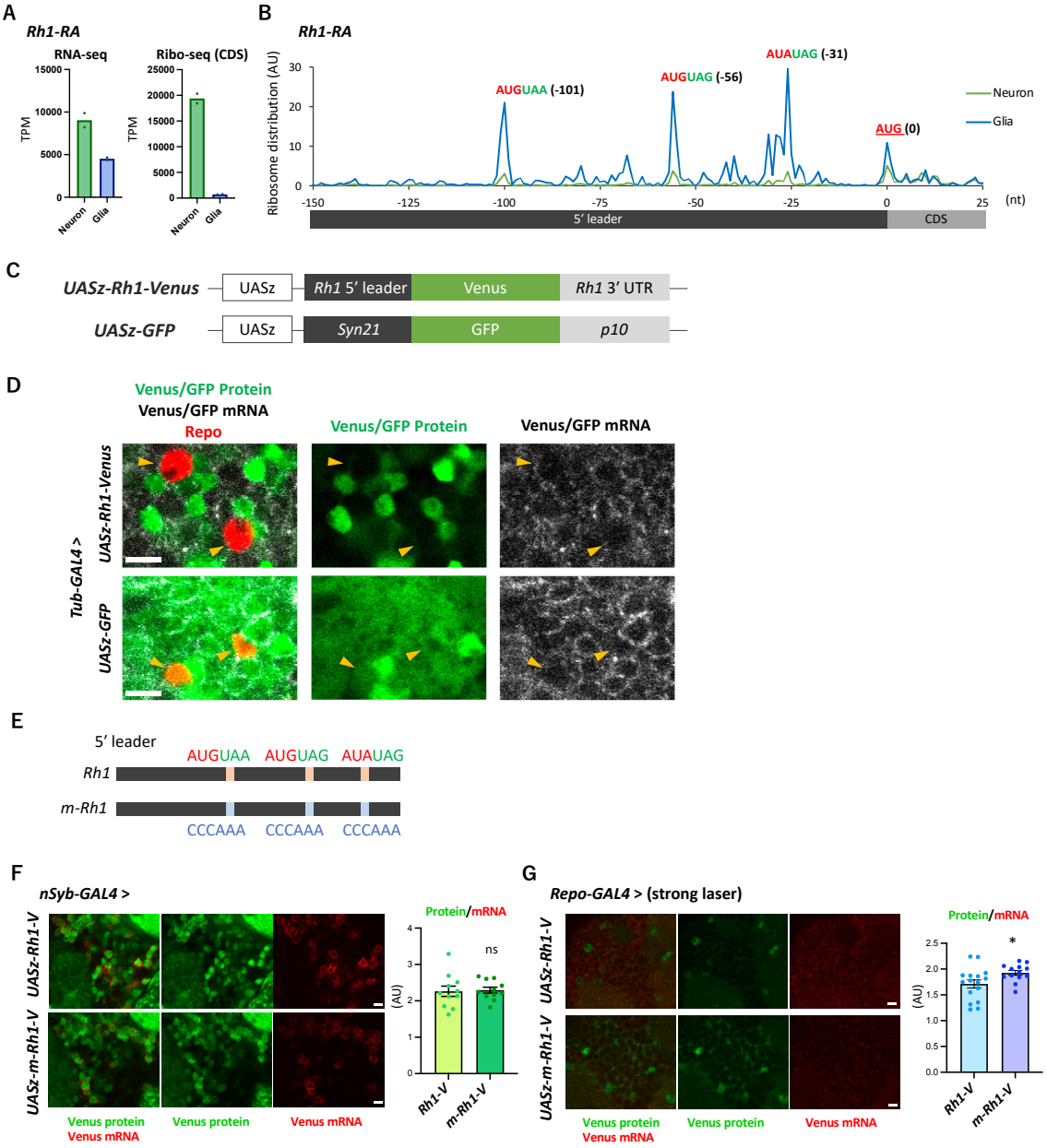

Figure 5. The transgenic *Rh1-Venus* reporter reveals differential translation in neuronal and glial cells.

(A) Reads on *Rh1-RA* in RNA- or Ribo- seq. (B) Ribosome distribution (estimated P-sites) on *Rh1-RA* in neurons (green) and in glia (blue), with 0 on the x-axis indicating the start codon of the CDS. 6-base upstream ORFs, consisting of consecutive start (or the near-cognate) and stop codons, are highlighted. Note that footprints are normalized by the total read counts on the transcript in each cell type to see the relative distribution of ribosomes. (C) Schematics of the *Rh1* (*UASz-Rh1-Venus*) or the control (*UASz-GFP*) reporter. For the *Rh1* reporter, 5′

843 leader and 3′ UTR sequences of *Rh1-RA* are fused to CDS of the *Venus* fluorescent protein.
844 For the control reporter, synthetic 5′ leader sequences (*syn21*) and viral *p10* terminator are
845 fused to GFP (DeLuca and Spradling, 2018). Note that both reporters contain the same
846 promoter (*UASz*) (DeLuca and Spradling, 2018) and are inserted onto the identical genomic
847 locus (*attP40*). (D) Expression of the *Rh1*- or the control reporters driven by *Tubulin-GAL4*.
848 Sliced confocal images of the cortical regions next to the antennal lobe are shown. We made
849 consistent observation in 4 brains in each genotype. Green: EGFP or Venus fluorescent signal.
850 Red: Immunohistochemical signal of repo protein as a glial marker. Grey: EGFP or Venus
851 mRNA. Orange arrowheads indicate glial cells marked by the repo expression. Scale bars: 5
852 μm. (E) Schematics of the mutated *Rh1* reporter (*m-Rh1*). The minimal uORF is replaced with
853 CCCAAA. (F) The expression of the *Rh1*- or *m-Rh1*- reporters, driven by the *nSyb*- or the *repo*-
854 GAL4. Sliced confocal images of the cortical regions next to the antennal lobe are shown.
855 Green: Venus fluorescent signal. Red: Venus mRNA signal. The total protein signal was
856 normalized by the total mRNA signal for each brain. N = 8 (*nSub* > *Rh1*), 8 (*nSyb* > *m-Rh1*),
857 16 (*repo* > *Rh1*), 13 (*repo* > *m-Rh1*). ns: $P > 0.05$, *: $P < 0.05$, Man-Whitney test of ranks.