Homeostatic scaling is driven by a translation-dependent degradation axis that recruits miRISC remodelling

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Abstract

Homeostatic scaling in neurons has been majorly attributed to the individual contribution of either translation or degradation; however there remains limited insight towards understanding how the interplay between the two processes effectuates synaptic homeostasis. Here, we report that a co-dependence between the translation and degradation mechanisms drives synaptic homeostasis whereas abrogation of either prevents it. Coordination between the two processes is achieved through the formation of a tripartite complex between translation regulators, the 26S proteasome and the miRNA-induced-silencing-complex (miRISC) components such as MOV10 and Trim32 on actively translating transcripts or polysomes. Disruption of polysomes abolishes this ternary interaction, suggesting that translating RNAs facilitate the combinatorial action of the proteasome and the translational apparatus. We identify that synaptic downscaling involves miRISC remodelling which entails the mTOR-dependent translation of Trim32, an E3 ligase and the subsequent degradation of its target, MOV10. MOV10 degradation is sufficient to invoke downscaling by enhancing Arc expression and causing the subsequent removal of post-synaptic AMPA receptors. We propose a mechanism that exploits a translation-driven degradation paradigm to invoke miRISC remodelling and induce homeostatic scaling during chronic network activity.

Introduction:

Neurons employ a unique stratagem, known as synaptic scaling, to counter the run-away excitation and subsequent loss of input specificity that arise due to Hebbian changes; they rely on a compensatory remodelling of synapses throughout the network while maintaining differences in their synaptic weightage (Burrone & Murthy, 2003; Keck *et al*, 2017; Turrigiano, 2017; Turrigiano & Nelson, 2004; Vitureira & Goda, 2013; Pozo & Goda, 2010). A complex

42 interplay of sensors and effectors within neurons serve to oppose global fluctuations in a
43 network and establish synaptic homeostasis by modifying post-synaptic glutamatergic currents
44 in a cell-autonomous manner (Davis, 2006; Ibata *et al*, 2008; Wierenga *et al*, 2006). In the
45 context of homeostatic scaling, 'sensors' are classified as molecules that sense deviations in
46 the overall network activity and 'effectors' scale the neuronal output commensurately.

47 Few molecular sensors of scaling have been identified to date; the eukaryotic elongation factor
48 eEF2 and its dedicated kinase, eEF2 kinase or CamKIII are the two reported thus far (Sutton
49 *et al*, 2007). However, there remains a huge chasm in identifying the repertoire of molecular
50 cascades that serve to link events where neurons sense deviations in the network firing rate
51 and subsequently initiate the scaling process. One such cascade is the mTORC1 (mammalian
52 Target Of Rapamycin Complex-1) signalling pathway that regulates presynaptic compensation
53 by promoting BDNF synthesis in the post-synaptic compartment (Henry *et al*, 2012, 2018). In
54 contrast, AMPA-receptors (AMPARs) have been identified, by overwhelming consensus, to be
55 the predominant "end-point-effectors" in all paradigms of synaptic scaling (Gainey *et al*, 2009;
56 O'Brien *et al*, 1998; Tatavarty *et al*, 2013; Thiagarajan *et al*, 2005). Unlike NMDARs, AMPARs
57 undergo *de novo* translation during network destabilizations (Sutton *et al*, 2006) and chronic
58 changes in the post-synaptic response during scaling has been attributed to the abundance of
59 surface AMPARs (GluA1 and GluA2 subunits) (Lissin *et al*, 1998). Among the key modifiers of
60 AMPAR expression, miRNAs are known to play pivotal roles in synaptic scaling (Hou *et al*,
61 2015; Letellier *et al*, 2014; Rajman *et al*, 2017; Silva *et al*, 2019). Relief from translational
62 repression by miRNAs necessitates that mRNAs exit the functional miRISC, which entails that
63 the latter undergo dynamic changes in its composition (Banerjee *et al*, 2009; Kenny *et al*,

64 2014). What remains surprising however, is our lack of knowledge about how compositional
65 changes within the miRISC are achieved during the restoration of homeostasis.

66 The requirement for discrete sets of sensors and effectors is fulfilled within neurons through
67 varied mechanisms including translation and ubiquitin-mediated proteasomal (UPS)
68 degradation. An enhanced degradation of post-synaptic-density (PSD) proteins including
69 GluA1 and GluA2 has been observed in contexts of altered network excitability (Ehlers, 2003)
70 whereas complete inhibition of UPS activity was shown to occlude synaptic compensation
71 (Jakawich *et al*, 2010). The integral role of *de novo* translation in sculpting the neuronal
72 proteome was recently highlighted when proteomic analysis of neurons undergoing upscaling
73 and downscaling revealed a remarkable diversity of newly synthesized proteins. Of particular
74 interest was the significant enrichment in the expression of the proteasome core complex
75 during the downscaling of synaptic activity (Schanzenbächer *et al*, 2016, 2018). The demand
76 for the translation of proteasome complexes implies that proteasomes work alongside
77 translation mechanisms during downscaling. Reports documenting the co-localization of
78 ribosomes and the proteasome in neuronal dendrites (Bingol & Schuman, 2006; Ostroff *et al*,
79 2002) further emphasize the possibility that these two opposing machineries physically interact
80 within the post-synaptic microcosm. The remodelling of the proteome through the dynamic
81 regulation of protein biogenesis and degradation has been termed as cellular 'proteostasis'
82 (Hanus & Schuman, 2013). However, several questions remain unexplored in the context of
83 homeostatic scaling, such as a) what factor establishes the link between translation and
84 protein degradation machineries to shape the proteome during scaling? b) Which process
85 among translation and degradation takes precedence? c) What are the signalling mechanisms

86 that connect events of ‘sensing’ the bicuculline-mediated hyperactivity and the final down-
87 regulation of AMPARs?

88 Here, we demonstrate a defined mechanism of synaptic scaling accomplished through the
89 remodelling of miRISC *via* RNA-dependent coordination between translation and proteasome-
90 mediated degradation. We observe that isolated inhibition of either translation or proteasomal
91 activity offsets synaptic homeostasis and restoration of homeostasis necessitates the
92 combination of both processes. We provide empirical evidence demonstrating that a direct
93 interaction between translation and protein degradation machinaries is achieved when the two
94 apparatus are tethered to actively translating transcripts linked to miRISC. We find that in
95 contexts of chronic hyperactivity, mTORC1-dependent translation of the E3 ligase Trim32
96 promotes the degradation of MOV10, both members of the miRISC. Similar to hyperactivity-
97 driven downscaling, the knockdown of MOV10 is sufficient to decrease the synaptic strength
98 by reducing surface AMPARs. This occurs due to enhanced Arc expression following loss of
99 MOV10. Comprehensively, our study shows that mTORC1 is triggered during synaptic
100 downscaling to effectuate an RNA-dependent, translation-driven protein degradation axis that
101 regulates miRISC remodelling to adjust the synaptic strength *via* Arc-mediated removal of
102 surface AMPARs.

103

104 **Results**

105 **Co-dependence of protein synthesis and degradation drives synaptic homeostasis**

106 To test the existence of coordination between translation and degradation in the regulation of
107 synaptic homeostasis, we measured miniature EPSCs (mEPSCs) from cultured hippocampal

108 neurons (DIV 18-24) after pharmacological inhibition of protein synthesis (anisomycin, 40μM)
109 and proteasomal activity (lactacystin, 10μM) for 24 hours. Application of either lactacystin or
110 anisomycin increased (2.99± 1.23 pA, $p<0.02$) and decreased (5.76± 0.5 pA, $p<0.01$) mEPSC
111 amplitude respectively. Co-application of both inhibitors restored mEPSC amplitude to that of
112 vehicle treated neurons (Figure 1A-B). The frequency of mEPSCs remained unaltered upon
113 inhibition of translation and proteasome blockade either alone or in combination (Figure 1C),
114 suggesting that this could be a post-synaptic phenomenon. Our data implies that interfering
115 with either protein synthesis or degradation disturbs the balance of synaptic activity, while
116 blocking both synthesis and degradation altogether restores it. Next, we stimulated synaptic
117 downscaling using bicuculline (10μM, 24 hr) and observed that like previous reports , here too,
118 chronic application of bicuculline lead to a significant decrease in mEPSC amplitude (5.56 ±
119 0.31 pA, $p<0.01$) without any detectable change in frequency (Figure 1D-F). The extent of
120 decrease in mEPSC amplitude within bicuculline-treated neurons recapitulated the decrease
121 observed in neurons where translation was blocked (bicuculline treated neuron 5.56 ± 0.31 pA
122 decrease *vs.* anisomycin treated neuron 5.76 ± 0.5 pA decrease) (Figure 1B and 1E). We
123 measured the mEPSC amplitude and frequency from hippocampal neurons when bicuculline
124 was co-applied with anisomycin and lactacystin. The dual application of bicuculline and
125 anisomycin did not result in any significant change in mEPSC amplitude when compared to
126 neurons treated with bicuculline alone (Figure 1D-E). This confirms that, rather than inducing
127 an additive effect,

Figure:1

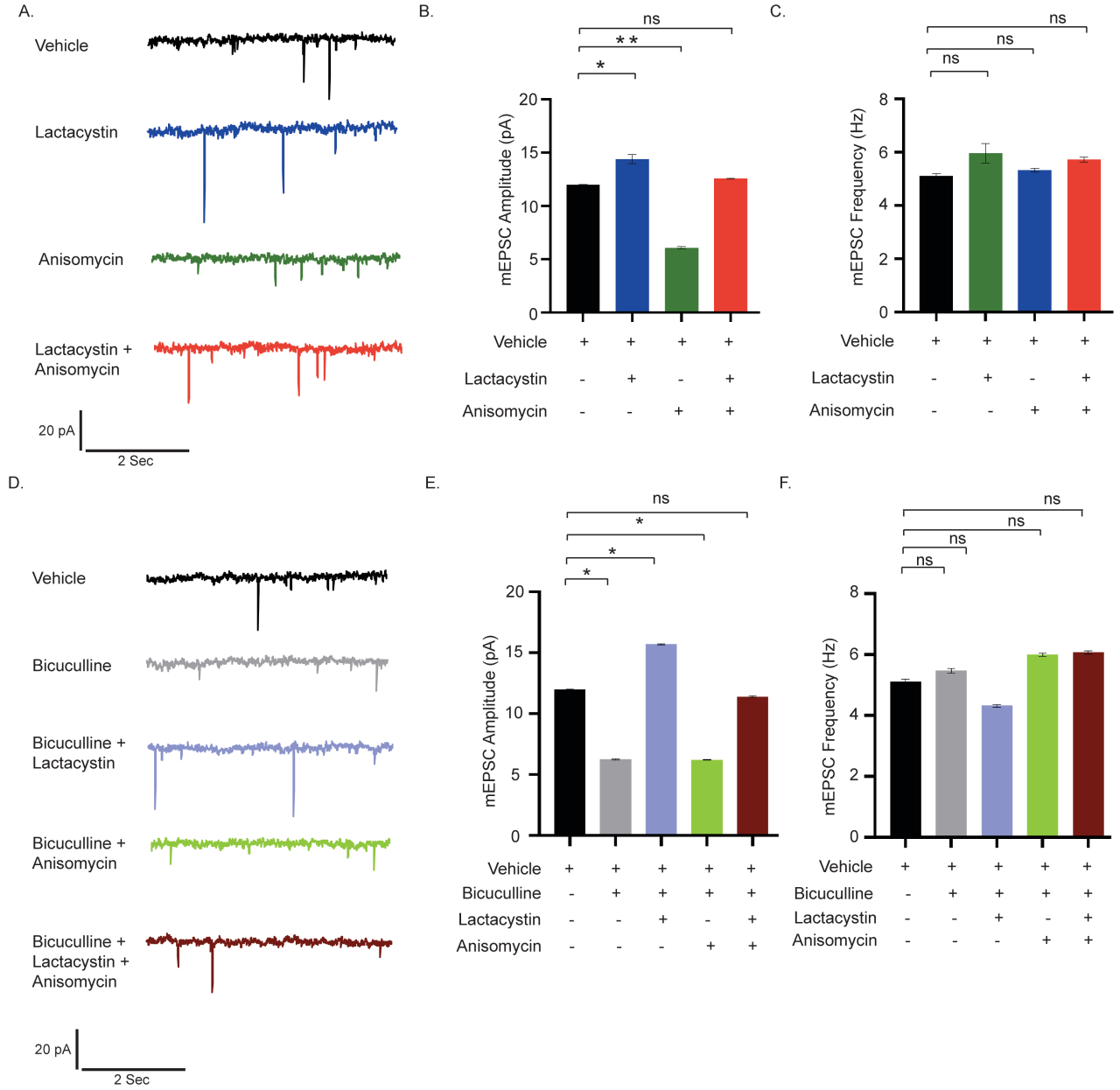

Figure 1: Synaptic scaling is co-regulated by protein synthesis and degradation.

(A-C) mEPSC traces from hippocampal neurons treated with vehicle, lactacystin, anisomycin and both (A). Mean mEPSC amplitude (B) and frequency (C). n=13-15. *p<0.024, **p<0.01. ns, not significant. Data shown as Mean ± SEM. One Way ANOVA and Fisher's LSD. Scale as indicated.

(D-F) mEPSC traces from neurons treated with vehicle, bicuculline alone or in combination with lactacystin, anisomycin (D). Mean mEPSC amplitude (E) and frequency (F). n=12–16. *p<0.01. ns, not significant. Data shown as Mean ± SEM. One Way ANOVA and Fisher's LSD. Scale as indicated.

138 chronic inhibition of protein synthesis in itself is sufficient to induce downscaling and could
139 potentially override the effect observed due to bicuculline.
140 Disruption of proteasome function by lactacystin during bicuculline-treatment led to a
141 significant increase in mEPSC amplitude (9.45 ± 0.25 pA increase as compared to bicuculline
142 treated neurons, $p<0.01$) without altering frequency (Figure 1E-F). The increase was effectively
143 more than the basal activity of vehicle-treated neurons (3.88 ± 0.28 pA, $p<0.01$) and mimicked
144 the increase in mEPSC amplitude brought by lactacystin alone (Fig 1B and 1E). Although the
145 influence of lactacystin on mEPSC amplitude is opposite to that of anisomycin, their individual
146 effects override that of bicuculline in each condition. Co-application of both inhibitors during
147 bicuculline-induced hyperactivation produced mEPSC amplitudes comparable to vehicle
148 treated neurons (Figure 1E). Our data indicates that the co-inhibition of translation and
149 degradation restricts any molecular changes away from the basal level, thus, maintaining the
150 synaptic strength at the established physiological set point.
151
152 **Synchronized translation and degradation regulates AMPAR distribution during scaling**
153 Since adjustment of synaptic strengths is directly correlated to the surface distribution of
154 surface AMPARs (sAMPARs), we measured the surface expression of GluA1 and GluA2
155 (sGluA1/A2) to identify how concerted mechanisms of synthesis and degradation influence the
156 distribution of sAMPARs during scaling. Neurons (DIV 21-24) were live-labelled using N-
157 terminus specific antibodies against GluA1 and GluA2 following bicuculline treatment, either
158 alone or in presence of both anisomycin and lactacystin for 24 hours and synapses marked by
159 PSD95.

Figure: 2

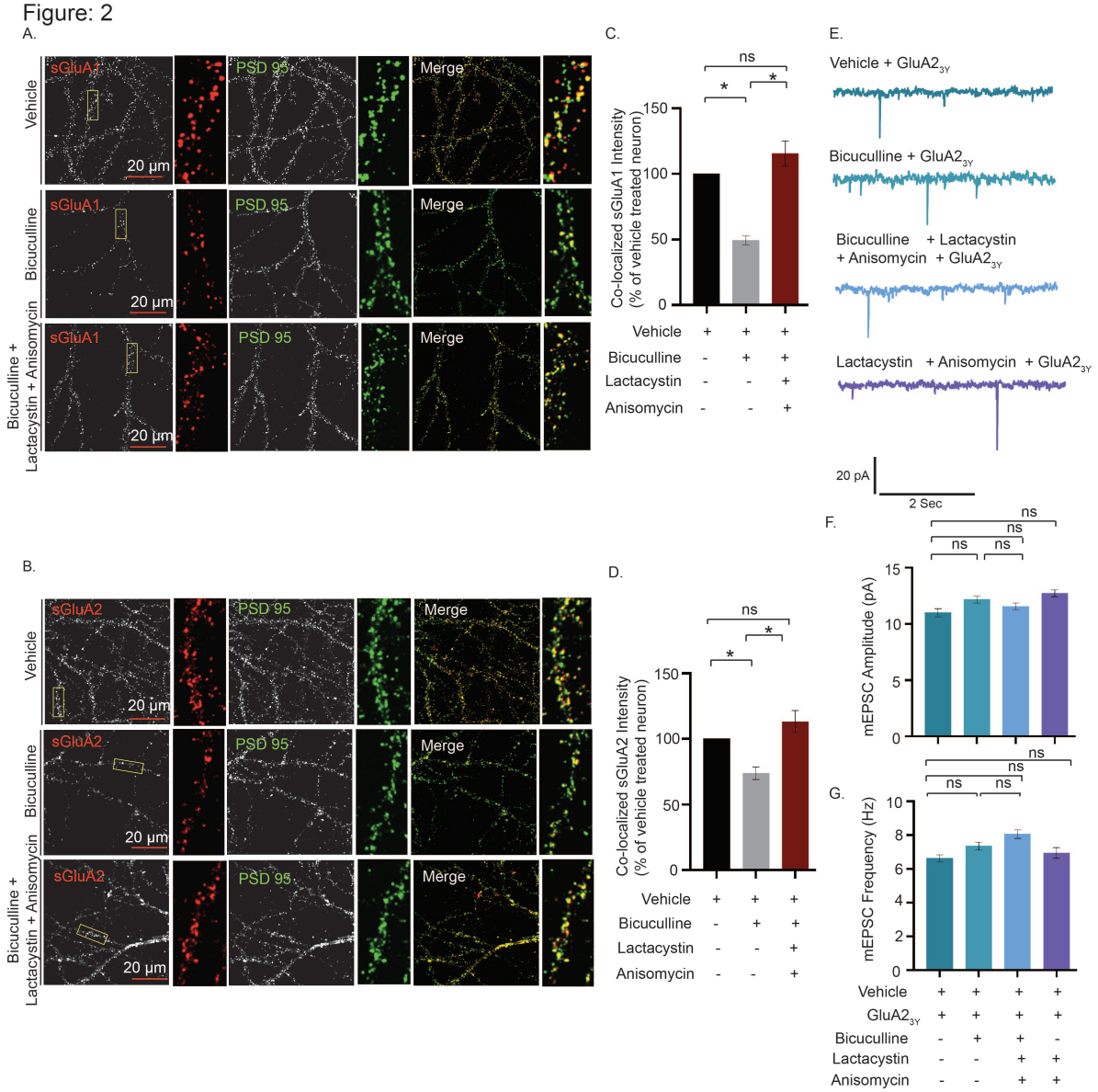

Figure 2: Co-inhibition of protein synthesis and degradation restores hyperactivity driven reduction of synaptic AMPAR expression

(A-D) High magnification images of sGluA1 or sGluA2 (red), PSD95 (green) and sGluA1/PSD95 (A) or sGluA2/PSD95 (B) (merged) images from neurons treated with vehicle, bicuculline alone or in combination with lactacystin and anisomycin. Normalized intensity of sGluA1 (C) or sGluA2 (D) co-localized with PSD95 particles. n=56-57, sGluA1 and n=31-63, sGluA2. *p<0.01. Dendrite marked in yellow box was digitally amplified.

(E-G) mEPSCs traces from hippocampal neurons treated with $GluA_{23y}$ either alone or in presence of bicuculline, lactacystin + anisomycin and bicuculline + lactacystin + anisomycin (E). Mean mEPSC amplitude (F) and

171 frequency (G). n=10 - 13. ns, not significant. Data shown as Mean ± SEM. One Way ANOVA and Fisher's LSD.
172 Scale as indicated. See also Figure S1.
173
174

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175 The surface expression of sGluA1/A2 in excitatory neurons was decreased following network
176 hyperactivity (50.6 ± 6.68%, $p<0.01$ for sGluA1 and 26.1 ± 6.62%, $p<0.01$ for sGluA2) (Figure
177 2A-D, S1 A-B). Consistent with our electrophysiological data, inhibition of both the translation
178 apparatus and the proteasome in bicuculline-treated neurons increased sGluA1/A2 levels
179 (133.95 ± 8.77 %, $p<0.01$ for sGluA1, 53.17 ± 6.44%, $p<0.0001$ for sGluA2) when compared to
180 neurons treated with bicuculline alone (Figure 2C-D, S1A-B). Thus our data indicates that a
181 dual inhibition of protein synthesis and degradation restores the synaptic sGluA1/A2 following
182 network hyperactivity.
183
184 To reaffirm whether they are indeed the end-point effectors of synaptic downscaling, we used
185 $GluA2_{3Y}$, a synthetic peptide derived from the GluA2 carboxy tail of AMPA receptors to block
186 the endocytosis of the AMPARs (Gainey *et al*, 2009), effectively ensuring the number of
187 AMPARs to remain unchanged throughout 24 hours. Consistent with previous studies, no
188 significant changes in mEPSC amplitude were detected upon inhibition of GluA2 endocytosis
189 during chronic application of bicuculline ($GluA2_{3Y}$ treated neuron 11.18 ± 1.06 pA *vs.* $GluA2_{3Y}$
190 + bicuculline treated neuron 12.25 ± 1.15 pA, $p<0.49$) (Figure 2E-F). Application of $GluA2_{3Y}$
191 did not alter mEPSC amplitude as compared to vehicle treated neurons ($GluA2_{3Y}$ treated
192 neuron 11.18 ± 1.06 *vs.* vehicle treated neurons 11.82 ± 0.24 pA) (Figure S1C-D), nor any
193 change observed between neurons treated with $GluA2_{3Y}$ and those treated with both
194 lactacystin and anisomycin in presence or absence of bicuculline (Figure 2F-G). mEPSC
195 frequency remained unaltered throughout while mEPSC amplitude in each condition was

196 similar to that of control neurons (Figure 2G, S1E). Collectively, these observations indicate
197 that changes in the abundance of surface-AMPARs during scaling is facilitated by proteomic
198 remodelling that exploits both translation and degradation processes.
199
200 **RNA-dependent tethering of the proteasome and translation regulators**
201
202 The co-localization of polyribosomes and proteasomes to sites of synaptic activity (Bingol and
203 Schuman, 2006; Ostroff et al., 2002) indicate that the proteasomal machinery could remain
204 physically associated with actively translating transcripts in order to make the necessary
205 proteomic changes. To evaluate this, we analysed polysomes from the hippocampus of 8-10
206 week old rat and assessed whether the sedimentation pattern of proteasomes matches those
207 of actively translating, polyribosome-associated mRNA fractions. We observed that several
208 components of the proteasomal machinery such as α7 subunit of 20S; Rpt1, Rpt3 and Rpt6
209 subunits of the 19S proteasome co-sedimented with translation initiation factors such as
210 eIF4E,p70S6 kinase and its phosphorylated form, the regulatory kinase of mTORC1-mediated
211 protein synthesis within actively translating polysomes (Figure 3A-B, S2A). We also detected
212 the polysomal distribution of MOV10, an RNA binding protein known to be poly-ubiquitinated
213 upon synaptic activation, and Trim32, an E3 ligase, both components of the miRISC
214 (Schwamborn *et al*, 2009) (Figure 3A-B).

Figure:3

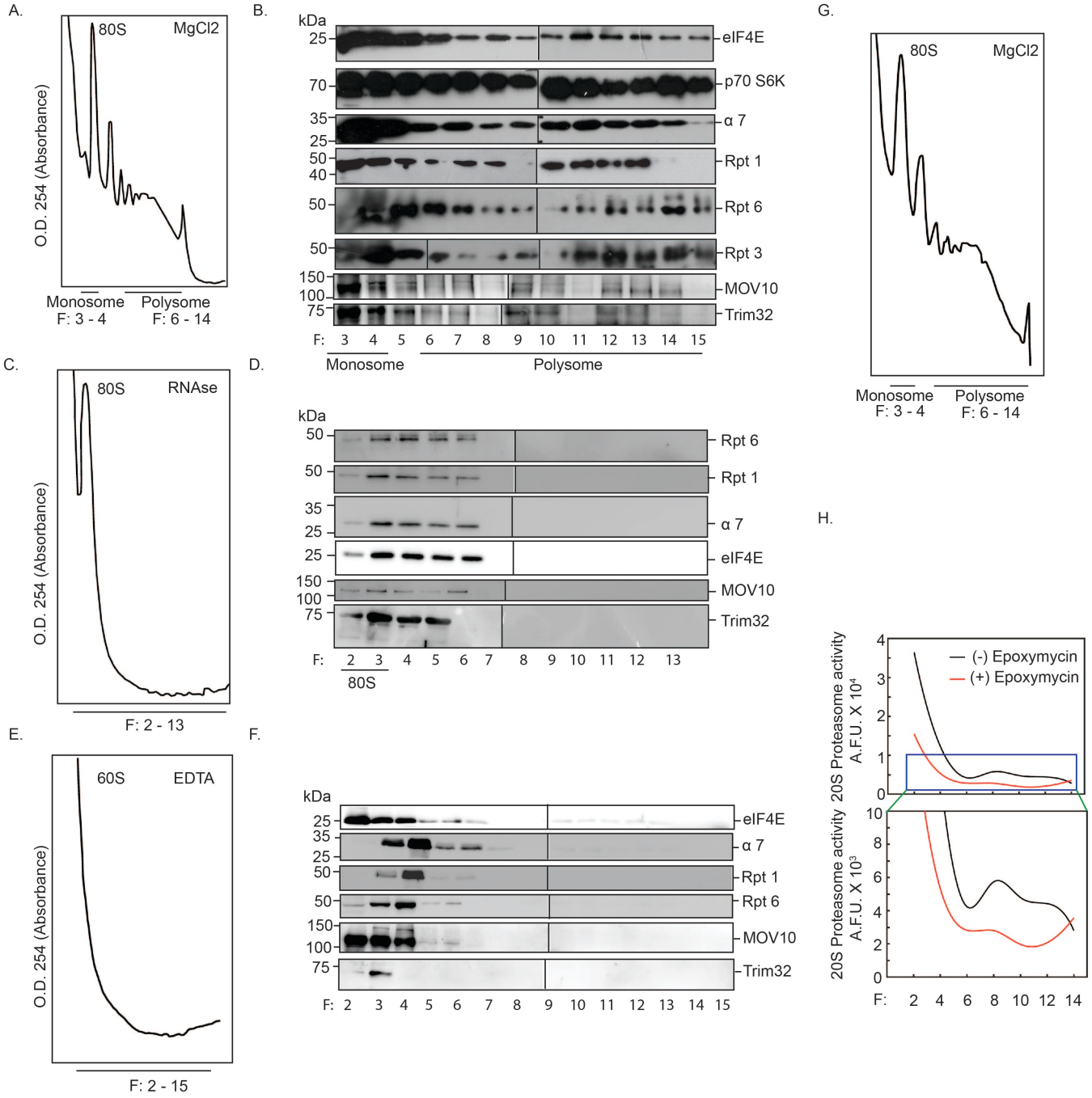

Figure 3: RNA-dependent association between active proteasomes and translating polyribosomes

(A-F) Absorbance profile at 254nm (A_{254}) of fractionated cytoplasmic extracts from hippocampal tissue incubated without (A) or with RNAse (C) or with EDTA (E). Monosome (80S) or 60S Ribosome or Polysome fractions as indicated. Western blot analysis of fractions without (B) or with RNAse (D) or with EDTA (F) treatment showing

221 distribution of translation regulators eIF4E and p70S6 Kinase; α7 subunit of 20S core and Rpt1, Rpt3, Rpt6 of 19S
222 cap; miRISC proteins MOV10 and Trim32.
223
224 (G-H) A_{254} profile of fractionated cytoplasmic extract (G) and quantitation of catalytic activity of proteasomes
225 present in alternate fractions from two polysome preparations (H). See also Figure S2.
226
227
228 RNAse or EDTA treatment of cytoplasmic lysates prior to density gradient fractionation led to a
229 complete collapse of the polysome profile, simultaneously shifting the sedimentation of the
230 proteasome subunits, E3 ligase, translation regulators and RNA binding proteins to the lighter
231 fractions (Figure 3C-F, S2B-C). The disruption of association between the translational and
232 proteasomal modules on RNAse and EDTA treatment suggests that translating transcripts act
233 as scaffolds to facilitate their tripartite interaction. These observations ruled out a possible
234 causality for the observed co-sedimentation due to similar densities of protein complexes
235 associated with translation and proteasome machineries. Furthermore, we observed that the
236 polysome-associated 26S proteasome is catalytically active as detected by its ability to cleave
237 a fluorogenic proteasome substrate that is blocked by proteasome inhibitor epoxymycin
238 (Figure 3G-H, S2D).
239
240 **Proteasome and the regulators of translation directly interact with each other within**
241 **excitatory neurons.**
242
243 Whole-cell patch clamp recordings demonstrating that the co-regulation of translation and
244 proteasome-mediated protein degradation is necessary for synaptic homeostasis, was
245 measured from excitatory hippocampal neurons.

Figure: 4

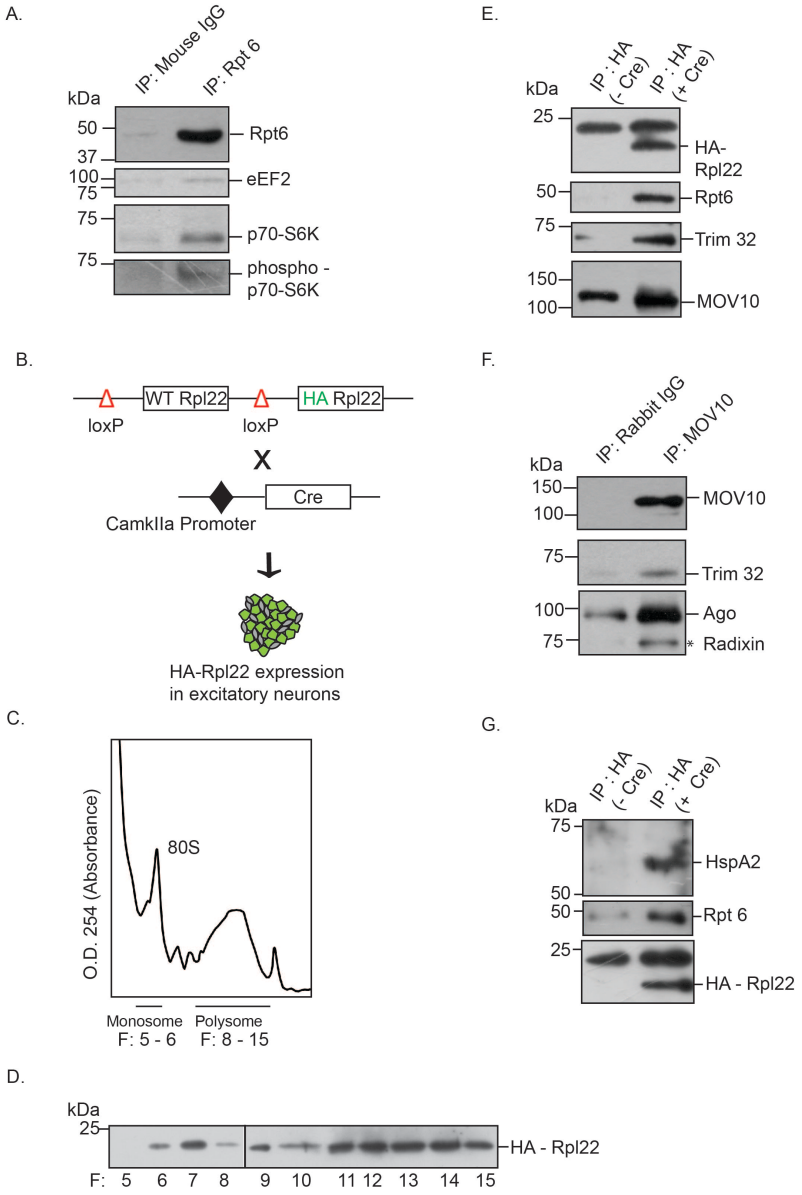

Figure 4: Interaction between proteasome and actively translating RNA-associated polyribosomes.

(A) Proteasome associated protein complex was immunoprecipitated from hippocampal lysate using antibody against Rpt6 and mouse IgG. Western blot of purified protein complex using antibodies against eEF2, p70S6 Kinase, phospho-p70S6 kinase. (B) RiboTag mouse crossed with CamkIIa promoter- driven Cre recombinase mouse results in deletion of wild-type Rpl22 ribosomal protein and replacement of HA tagged Rpl22 in forebrain excitatory neurons. (C) A_{254} profile showing indicated fractions of Monosome and Polysome. (D) Polysome fractions showing enrichement of HA-Rpl22 as detected by western blot using antibody against HA. (E) HA-

255 tagged Rpl22 containing polyribosome affinity purified using antibody against HA. Western blot analysis of affinity
256 purified complex using antibodies against HA, Rpt6, Trim32 and MOV10. (F) MOV10 was immunoprecipitated
257 from hippocampal lysate. Western blot analysis of MOV10-immunoprecipitated protein complex showed the co-
258 precipitation of Trim32 with miRISC components MOV10 and Ago. (G) Detection of HspA2 and Rpt6 in HA affinity
259 purified protein complex from HA-Rpl22 expressing neurons by western blot using antibody against HspA2 and
260 Rpt6 and HA.
261
262

263 Consistent with this observation, co-sedimentation of proteasome subunits along with
264 translating mRNA linked to protein synthesis regulators including miRISC led us to enquire
265 whether components of ternary complex directly interact with each other in excitatory neurons
266 of hippocampus. To evaluate this, we immunoprecipitated the 19S proteasomal complex using
267 Rpt6 antibody from hippocampal neurons. We observed the co-precipitation of eEF2, a
268 translation elongation factor that functions as a "sensor" of change in network activity (Figure
269 4A). We also found that p70S6 kinase as well as its phosphorylated form- a known regulator of
270 the mTORC1-dependent protein synthesis (Ma & Blenis, 2009) co-precipitated with the 19S
271 proteasome (Figure 4A). We further analyzed the proteins interacting with polysomes within
272 excitatory neurons by expressing Hemagglutinin (HA) tagged ribosomal protein Rpl22 (HA-
273 Rpl22) that gets incorporated into polysomes (Sanz *et al*, 2009; Shigeoka *et al*, 2016) (Figure
274 4B-D, S2E). We reasoned that the analysis of HA-Rpl22-affinity purified complexes would
275 confirm whether the polysome-associated translation and degradation machinaries directly
276 interact with each other. Our western blot analysis of HA-Rpl22 affinity-purified protein complex
277 showed that Rpt6 directly interacts with Trim32 and MOV10 (Figure 4E). The interaction of
278 MOV10 with ribosomes is crucial as it gives credence to the association of miRNAs with
279 polysomes, as per previous reports (Krichevsky *et al*, 2003). Immunoprecipitation of MOV10
280 from hippocampal neurons allowed us to detect the co-precipitation of both Argonaute (Ago)

281 and Trim32, confirming that the latter is an integral component of the Ago-containing miRISC
282 (Figure 4F). We also detected the chaperone protein HspA2 in the HA-affinity purified fraction
283 along with Rpt6 (Figure 4G), suggesting that HspA2 could tether proteasomes to actively
284 translating transcripts.
285
286 **Protein synthesis drives mTORC1-dependent proteasomal degradation to cause miRISC**
287 **remodelling during synaptic downscaling**
288
289 Association between MOV10 and Trim32, all members of the miRISC, and their direct
290 interaction with protein synthesis as well as degradation machineries led us to analyze whether
291 concerted translation and degradation during bicuculline induced chronic network hyperactivity
292 could influence miRISC remodelling. Bicuculline treatment of hippocampal neurons (DIV 18 -
293 21) enhanced (101.7 ± 10.06% increase, $p<0.0001$) Trim32 with a concomitant decrease
294 (65.94 ± 2.67% decrease, $p<0.001$) in MOV10 (Figure 5A-C). The increase in Trim32
295 expression post bicuculline treatment was blocked by anisomycin and surprisingly resulted in
296 the inhibition of MOV10 degradation (82.28 ± 12.90% protected MOV10, $p<0.03$) (Figure 5C).
297 This indicates that the degradation of MOV10 is dependent on enhanced Trim32 synthesis and
298 that Trim32 translation precedes the commencement of MOV10 degradation. Treatment with
299 lactacystin resulted in the expected protection of MOV10 from degradation (71.93 ± 5.74%
300 MOV10 protected, $p<0.01$) upon bicuculline-induced hyperactivity (Figure 5C); whereas there
301 remained no change in the Trim32 expression levels (Figure 5B). Moreover, co-application of
302 lactacystin and anisomycin during bicuculline-induced hyperactivity changed the expression of
303 MOV10 and Trim32 commensurate to basal levels (Figure 5B-C). We have also analyzed
304 Trim32 and MOV10 expression after anisomycin and lactacystin treatment, either alone or in

305 combination, to assess the necessity of chronic hyperactivity in the co-ordinated control of
306 miRISC remodelling. We observed that the chronic inhibition of protein synthesis led to a
307 modest but statistically significant decrease of both Trim32 (22.42 ± 0.70% decrease, $p<0.001$)
308 and MOV10 (28.14 ± 0.48% decrease, $p<0.0003$) (Figure S3A-C). However, chronic inhibition
309 of proteasome has no effect on Trim32 and MOV10 expression (Figure S3A-C). We reasoned
310 that the significant decrease of both proteins was observed due to combined effect of global
311 inhibition of translation and ongoing basal level of protein degradation. These observations
312 indicate that the miRISC remodelling occurs in conditions of chronic network hyperactivity
313 induced by bicuculline.
314
315 Reciprocal patterns between MOV10 and Trim32 expression levels led us to analyse whether
316 the latter is the E3-ligase responsible for the UPS-mediated degradation of MOV10. Consistent
317 with our hypothesis, knockdown of Trim32 (85.43 ± 4.04% knockdown, $p<0.01$) by shRNA-
318 mediated RNAi enhanced the expression of MOV10 (44.74 ± 14.33% increase, $p<0.02$)
319 (Figure 5D-F), suggesting that Trim32 translation drives the proteasome-mediated degradation
320 of MOV10 and that it may be sufficient for MOV10 ubiquitination and subsequent degradation.
321 These observations show that Trim32 translation drives the proteasome-mediated degradation
322 of MOV10 and suggests that Trim32 may be the only miRISC-associated E3 ligase required
323 for MOV10 ubiquitination.

Figure: 5

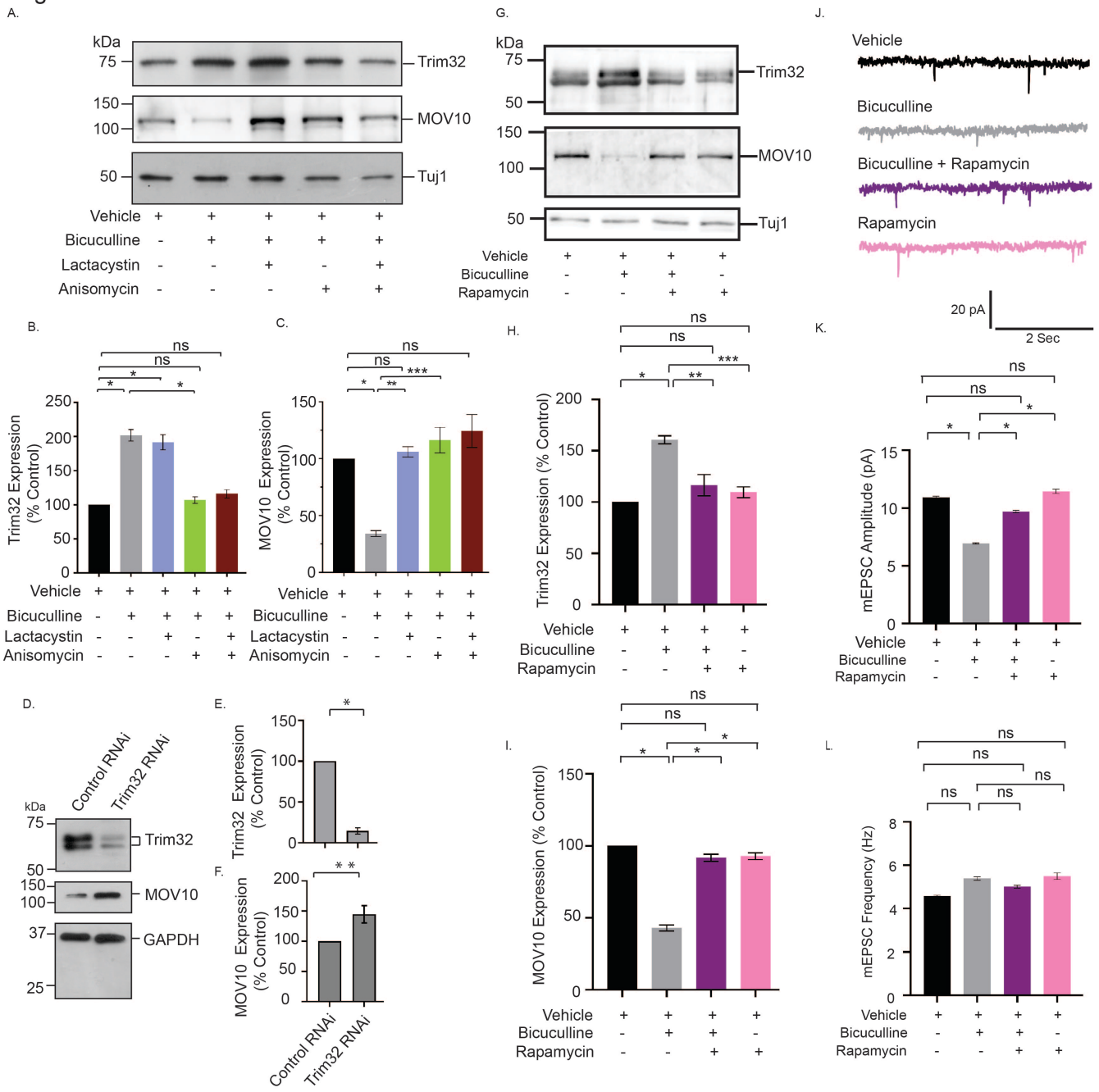

Figure 5: Synthesis of Trim32 facilitates MOV10 degradation and requires activation of mTORC1

(A-C) Western blot analysis showing the expression of Trim32, MOV10 and Tuj1 from neurons treated with bicuculline with or without lactacystin, anisomycin or both (A). Quantitation of Trim32 (B) and MOV10 (C) expression. n=3. Data shown as Mean ± SEM. *p<0.0001 (B) and *p<0.001, **p<0.006, ***p<0.02 (C). ns, not significant. One Way ANOVA and Fisher's LSD.

331 (D-F) Western blot analysis of neurons infected with lentivirus expressing Trim32 or non-targetting control shRNA
332 showing expression of Trim32, MOV10 and Tuj1 (D). Quantitation of Trim32 (E) and MOV10 (F). n=5. Data shown
333 as Mean ± SEM. *p<0.01, **p<0.02. Unpaired t-test with Welch's correction.
334
335 (G-I) Western blot analysis from neurons treated with bicuculline, Rapamycin or both. showing expression levels
336 of Trim32, MOV10 and Tuj1 (G). Quantitation of Trim32 (H) MOV10 (I) expression. Data shown as Mean ± SEM.
337 n=5. *p<0.0001, **p<0.0007, ***p<0.0001 (H) and *p<0.001 (I). ns, not significant. One Way ANOVA and
338 Bonferroni's correction. See also Figure S2.
339
340 (J-L) mEPSC traces from neurons treated with vehicle, bicuculline, rapamycin or both (J). Mean mEPSC
341 amplitude (K) and frequency (L). n=8-9. *p<0.01. ns, not significant. Data shown as Mean ± SEM. One Way
342 ANOVA and Fisher's LSD.

343
344 Having observed the co-precipitation of the downstream effectors of the mTORC1 signalling
345 cascade with the 26S proteosomal subunit Rpt6, we focused on identifying whether mTORC1
346 signalling plays a role in causing synaptic downscaling in response to chronic hyperactivity.
347 Bicuculline-treatment of hippocampal neurons in the presence of rapamycin (100nM, 24 hr), a
348 selective inhibitor of mTORC1, completely abolished chronic hyperactivity-driven Trim32
349 synthesis (16.48 ± 8.6% increase as compared to control, p=0.99) and consecutive MOV10
350 degradation (8.19 ± 2.81% decrease as compared to control, p=0.06) (Figure 5G-I).
351 Rapamycin treatment alone did not alter the expression patterns of Trim32 and MOV10 (Figure
352 5G-I). This led us to hypothesize that mTORC1 pathway acts upstream of Trim32, serving to
353 regulate its synthesis in response to bicuculline. Chronic bicuculline treatment lead to a
354 significant enhancement of p70S6 kinase phosphorylation (92.07 ± 20.22 % increase,
355 p<0.001) which was blocked by rapamycin (Figure S3D-E), indicating that the mTORC1
356 signalling cascade is key in effectuating bicuculline-induced synaptic downscaling. Consistent
357 with our biochemical data, we observed that co-incubation of rapamycin and bicuculline
358 prevented the decrease in mEPSC amplitude (2.47 ± 0.26 pA increase as compared to

359 bicuculline treated neurons, $p<0.01$) but not frequency (Figure 5J-L). Just as above, rapamycin
360 treatment alone has no effect, indicating that chronic hyperactivity acts as a triggering point for
361 mTORC1 activation (Figure 5K-L) and this subsequently plays a role in driving TRIM32
362 translation.
363
364 **MOV10 degradation is sufficient to invoke downscaling of AMPARs**
365
366 MOV10 is an integral component of miRISC and its removal from the protein complex disrupts
367 miRISC function. MOV10 degradation in response to chronic bicuculline treatment, made us
368 question whether its loss alone was sufficient to cause pervasive changes in the miRISC and
369 bring about synaptic downscaling. We mimicked hyperactivity-driven MOV10 degradation by
370 lentivirus-mediated RNAi of MOV10. Intensity of sGluA1/A2 puncta that co-localized with
371 PSD95 was analyzed following MOV10 knockdown (DIV21-24). We observed that loss of
372 MOV10 reduced the expression of sGluA1 (35.03 ± 9.35 % for shRNA#1, $p<0.01$ and 58.38 ±
373 10.27 % for shRNA#2, $p<0.01$) and sGluA2 (49.4 ± 12.9% for shRNA#1, $p<0.01$) at the
374 synapses (Figure 6A-D, S4), that recapitulated the re-distribution of sGluA1/sGluA2 in neurons
375 under chronic bicuculline treatment (Figure 2C-D). The knockdown of MOV10 reduced mEPSC
376 amplitude (3.48 ± 0.24 pA for shRNA#1 and 4.05 ± 0.23 pA for shRNA#2, $p<0.01$) but not
377 frequency (Figure 6E-G), an observation that mirrors synaptic downscaling following
378 bicuculline treatment (Figure 1E). We have used two shRNAs against MOV10 for its effective
379 knockdown and also a non-targeting shRNA to eliminate the possibility of an off-target effect.

Figure: 6

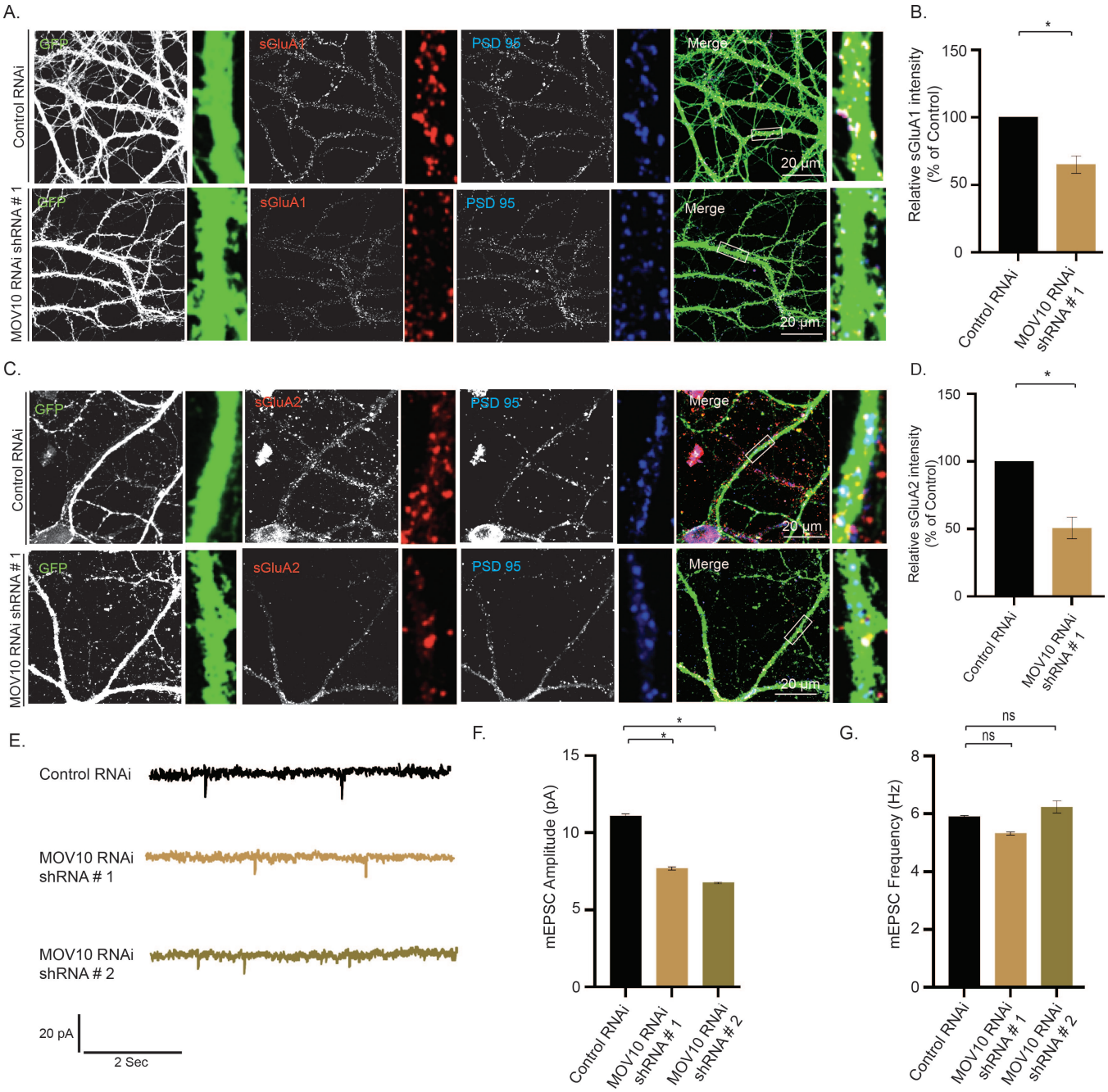

Figure 6: MOV10 regulates synaptic activity by modulating abundance of sAMPAR

(A-D) High magnification images of neurons transduced with lentivirus co-expressing EGFP and MOV10 or non-targetting shRNA showing expression of sGluA1 (A) or sGluA2 (C) (red), PSD95 (blue), GFP (green) and GFP/sGluA1/PSD95 or GFP/sGluA2/PSD95 (merged). Quantitation of normalized intensity of synaptic sGluA1 (B)

386 or sGluA2 (D). n=26 - 30, GluA1; n=12 – 15, GluA2. Data shown as Mean ± SEM. *p<0.01. One Way ANOVA and
387 Fisher's LSD. Dendrite marked in yellow box was digitally amplified. See also Figure S3.
388
389 (E-G) mEPSC traces from transduced neurons (E). Mean mEPSC amplitude (F) and frequency (G). n=12 – 13.
390 *p<0.01. ns, not significant. Data shown as Mean ± SEM. One Way ANOVA.
391
392

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393 How MOV10 degradation leads to the removal of sAMPARs to regulate synaptic downscaling?
394 Arc/Arg3.1, an immediate early gene, has been shown to be dynamically regulated by chronic
395 changes in synaptic activity, and evokes synaptic scaling (Shepherd *et al*, 2006).
396 Overexpression of Arc decreases sAMPARs *via* endocytosis whereas its knockdown increases
397 them (Chowdhury *et al*, 2006). Arc expression has been shown to be regulated by diverse
398 mechanisms including translational control that involves miRNAs (Wibrand *et al*, 2012;
399 Paolantoni *et al*, 2018). These observation prompted us to analyze the Arc expression
400 following MOV10 knockdown. We observed that the loss of MOV10 enhanced Arc (106.4 ±
401 11.92% increase, p<0.003 for shRNA # 1 and 176.1 ± 20.24% increase, p<0.003 for shRNA #
402 2) (Figure 7A-B). The extent of increase in Arc protein was commensurate with the efficacy of
403 two shRNAs against MOV10 (Figure 7A-B). We also observed that this differential
404 enhancement of Arc is reflected in the proportionate removal of sAMPARs and concomitant
405 decrease in mEPSC amplitude (Figure 6E). Our data showed that bicuculline-induced chronic
406 hyperactivity, which degrades MOV10, also enhanced Arc expression (132.1 ± 27.45%
407 increase, p<0.04) (Figure 7C-D). This activity-driven enhancement of Arc is blocked by the
408 inhibition of mTORC1 by rapamycin (100nM, 24 hr). We observed that rapamycin treatment
409 alone has no effect (Figure 7C-D).

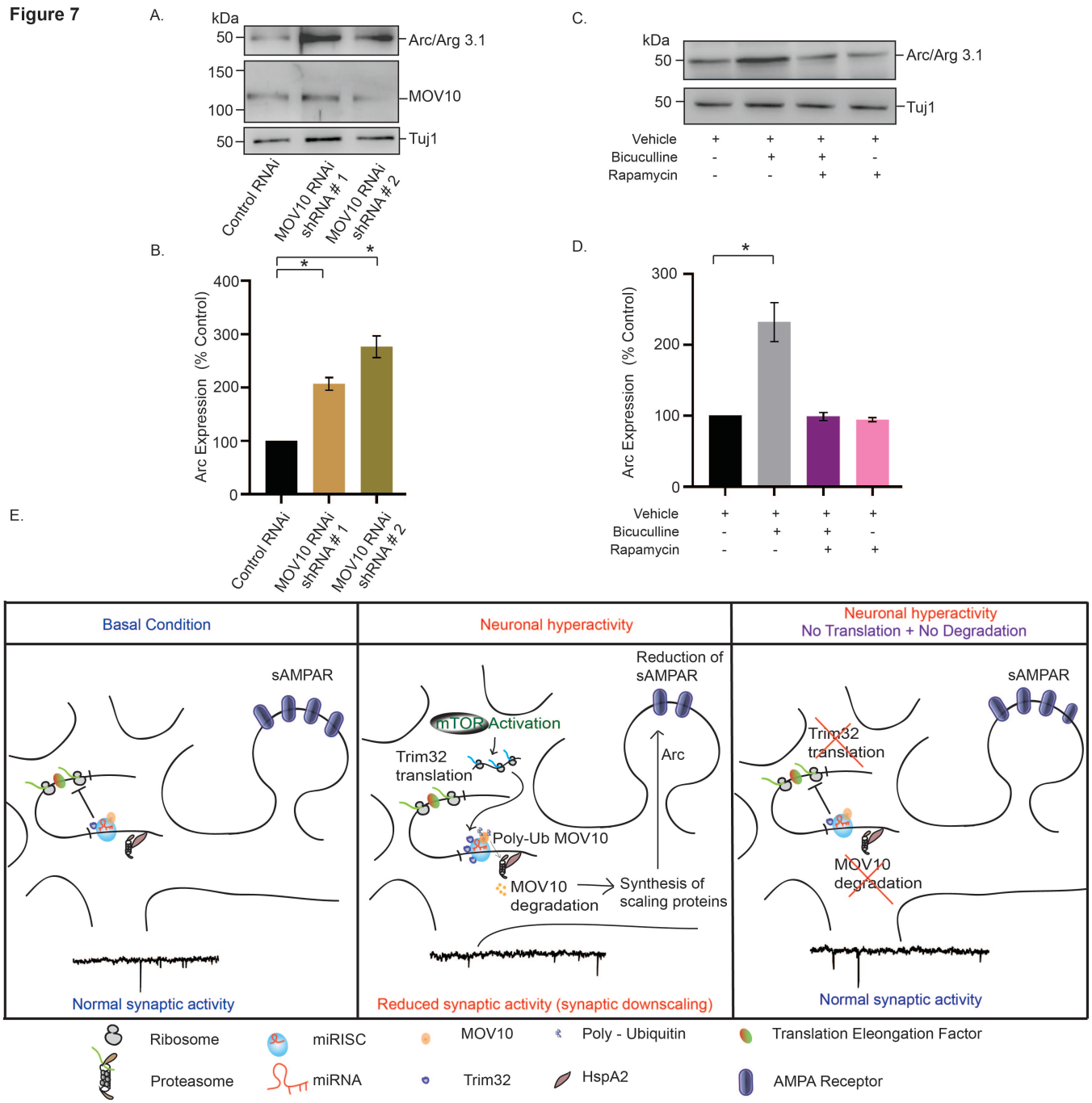

Figure 7: mTORC1-mediated regulation of Arc expression upon chronic hyperactivity involves MOV10

(A-B) Western blot analysis showing the Arc protein level after MOV10 knockdown in neurons infected with lentivirus expressing two different shRNAs against MOV10 (A). Quantitation of Arc expression (B). n=4.* $p<0.003$. Data shown as Mean ± SEM. One Way ANOVA and Fisher's LSD.

418 (C-D) Western blot analysis of neurons treated with bicuculline in presence or absence of rapamycin showing the
419 expression of Arc protein (C). Quantitation of Arc expression (D). n=3. *p<0.04. Data shown as Mean ± SEM.
420 One Way ANOVA and Fisher's LSD.
421
422 (E) Schematic representation showing maintenance of homeostatic synaptic activity by coordinated control of
423 protein synthesis and degradation that modulates composition of miRISC.

424
425 Taken together, our data demonstrates that the bicuculline-induced downscaling of synaptic
426 strength occurs *via* an mTORC1-mediated translation-dependent proteasomal degradation of
427 MOV10 involving removal sAMPARs *via* Arc (Figure 7E).
428

429 ## Discussion

430
431 Here we provide empirical evidence emphasizing that synchrony between protein synthesis
432 and proteasomal activity is critical to establish homeostasis at synapses. We used a paradigm
433 of chronic network hyperactivity to invoke downscaling and determined a) translation and
434 degradation apparatuses remain linked by RNA scaffolds; b) it is the translation of Trim32 that
435 drives the degradation of MOV10 to cause miRISC remodelling, thus the current paradigm is
436 an example of translation preceding degradation; c) miRISC is a key node in the translation-
437 degradation axis, with mTORC1 being the upstream signalling component which is a part of
438 the 'sensor' machinery, and Arc-induced removal of sAMPARs being the final effectors of
439 downscaling.

440 **Co-regulation of protein synthesis and degradation drives AMPAR-mediated synaptic**
441 **downscaling**

442 We find that chronic perturbation of either translation or proteasomal activity occludes synaptic
443 homeostasis, while homeostasis remains unperturbed when there is simultaneous inhibition of

444 both. Chronic application of bicuculline along with either lactacystin or anisomycin leads to
445 alterations of mEPSC amplitude that exactly mirror observations where bicuculline is absent
446 (Figure 1B *vs* Figure 1E). Thus, the effects of bicuculline-induced changes to the existing
447 proteome are overshadowed by those accomplished by the individual action of the proteasome
448 or the translation machinery (Figures 1). mEPSC frequency remain unaltered in all conditions.
449 The importance of these observations is multi-faceted; it establishes that, i) congruent protein
450 synthesis and degradation pathways regulate synaptic scaling; ii) the constancy of the
451 proteomic pool in the presence of lactacystin and anisomycin renders the effect of any network
452 destabilizing stimuli like bicuculline to be redundant, and iii) long-term changes in the proteome
453 predominantly affects the physiology of the post-synaptic compartment.
454 Our observations echo previous findings in Hebbian plasticity; wherein, protein synthesis
455 during LTP/LTD was required to counter the changes in the proteomic pool triggered by protein
456 degradation. The blockade of L-LTP accomplished by inhibiting protein synthesis was revoked
457 on the simultaneous application of proteasomal blockers and translational inhibitors (Fonseca
458 *et al*, 2006). Abrogation of proteasomal activity allowed mGluR-dependent LTD to proceed
459 even in the absence of protein synthesis (Klein *et al*, 2015). These observations emphatically
460 suggest the existence of a proteostasis network that enable compositional changes to the
461 proteome in contexts of acute or chronic changes in synaptic function (Jayaraj *et al*, 2020;
462 Cajigas *et al*, 2010; Hanus & Schuman, 2013). As LTP and LTD modify the cellular proteome
463 through the simultaneous recruitment of protein synthesis and degradation; it stands to reason
464 that homeostatic scaling mechanisms may also employ a functional synergy of the two to
465 recompense for the changes brought about by unconstrained Hebbian processes.

466 AMPAR-mediated currents decrease more than NMDAR currents during chronic network
467 hyperactivity (O'Brien *et al*, 1998; Lissin *et al*, 1998) and unlike NMDARs, the turnover of
468 AMPARs is translation-dependent (Goold & Nicoll, 2010). The surface distribution of AMPARs,
469 therefore remain accurate readouts of synaptic output. We wanted to confirm whether the
470 combined action of translation and degradation affect post-synaptic scaling through any other
471 effectors and reasoned that if synaptic strength is dominated by changes in AMPAR currents,
472 restricting changes to the sAMPAR abundance should prevent the scaling of mEPSCs even
473 under chronic hyperactivity. Similar to the observations in synaptic upscaling (Gainey *et al*,
474 2009), the inhibition of GluA2-endocytosis by $GluA2_{3Y}$ peptide also blocked synaptic down
475 scaling (Figure 2); reinforcing that AMPARs indeed remain the end-point effectors despite
476 changes to the proteome.
477

478 **RNA-dependent association of the translation and degradation apparatus underlie their**
479 **functional coherence**

480 The co-localization of polyribosomes and proteasomes in neuronal subcompartments suggest
481 that for translation and proteasomal degradation to work in tandem, physical proximity between
482 the two modules cannot be ruled out (Bingol & Schuman, 2006; Ostroff *et al*, 2002). Polysome
483 analysis showed the co-sedimentation of members of the 19S proteasome (Rpt1, Rpt3 and
484 Rpt6 subunits) and the 20S proteasome (α7 subunit) along with translation initiation factors
485 such as eIF4E and p70S6 kinase, a downstream effector of mTORC1. Abrogation of the
486 sedimentation pattern in the presence of RNAse and EDTA, is indicative of an RNA-dependent
487 direct interaction between translation and protein degradation (Figure 3). Affinity purification of
488 polyribosomes containing HA-Rpl22 confirmed that there is direct interaction between

489 members of the two modules (Figure 4). Such existence of direct interaction between
490 polyribosome and catalytically active proteasomes allows close temporal coordination between
491 translation and protein degradation. How do protein degradation machineries remain tethered
492 to actively translating mRNAs? We have identified that HspA2 (Hsp70 family), a chaperone
493 protein, remains tethered to proteasomes (Figure 4). Hsp70 family of proteins is known to
494 influence both the synthesis and degradation of proteins by their association with 26S
495 proteasomal subunits (Tai *et al*, 2010) and translation initiation factors (Shalgi *et al*, 2013).
496 HspA2 therefore, is a component of proteostasis coordinators which includes proteasome,
497 translation regulators and chaperon proteins.
498

499 **mTORC1-mediated Trim32 synthesis precedes MOV10 degradation during downscaling**

500 The co-incident detection of MOV10 and miRNAs from polysome fractions purified from
501 neurons give credence to the existence of a tripartite (translating RNA associated miRISC –
502 proteasome complex – translation apparatus) regulatory axis underlying scaling. Under the
503 chronic influence of bicuculline, synthesis of the E3 ligase Trim32 precedes the degradation of
504 MOV10, the alternative possibility that MOV10 degradation leads to increased *de novo*
505 translation of Trim32, is not supported, since protein synthesis inhibition by anisomycin leads
506 to MOV10 rescue (Figure 5). Loss of the E3-ligase Trim32 elevated the basal level of MOV10,
507 indicating a ubiquitin-dependent degradation by the proteasome (Figure 5). Although MOV10
508 has been shown to also regulate miRISC-independent function to modulate RNA modification
509 (Warkocki *et al*, 2018) and stability (Gregersen *et al*, 2014), association of MOV10 with
510 Argonaute indeed emphasize the remodelling of miRISC during synaptic downscaling. What
511 post-synaptic signalling cascade triggers Trim32 translation? We find that chronic bicuculline

512 induction triggers the mTORC1-dependent synthesis of Trim32 that is abrogated on rapamycin
513 treatment (Figure 5). Identification of the mTOR downstream effector p70S6 kinase within the
514 tripartite complex further suggests that the mTOR signalling is crucial for driving proteostasis.
515
516 A recent study has demonstrated that a slow turnover of plasticity proteins (measured at 1,3
517 and 7 days in cultured neurons) is essential to create long-term changes to the neuronal
518 proteome during both up and down-scaling (Dörrbaum *et al*, 2020).The authors have argued
519 that the slow turnover rate is more energy-saving and therefore a preferred cellular
520 mechanism. However, this study also identifies a very small fraction of previously reported
521 scaling factors with fast turnover rates specifically influencing up- and down- scaling. Our
522 reports support the latter findings, where we observe that both the increase in Trim32
523 synthesis and the resulted degradation of MOV10 happen within 24 hours during synaptic
524 downscaling, suggesting a fast turnover. As both MOV10 and Trim32 are part of the miRISC,
525 their fast turnover rates seems plausible, considering that participation of the miRISC to relieve
526 the translational depression of several transcripts encoding plasticity proteins needs to happen
527 fast in order to boost changes to the proteome. Although in terms of energy expenditure the
528 coordinated regulation of translation and degradation is expensive, this cellular trade-off may
529 be necessary to trigger the remodelling of a very limited number of master regulators of the
530 neuronal proteome, such as miRISC, during synaptic downscaling.
531
532 **Degradative control of miRISC remodelling underlies homeostatic scaling**
533 Most studies have focused on the influence of single miRNAs in regulating AMPAR distribution
534 during scaling, however, they have been inadequate in providing a holistic view of the miRNA-

535 mediated control of synaptic scaling (Hou *et al*, 2015; Letellier *et al*, 2014; Rajman *et al*, 2017;
536 Silva *et al*, 2019). We found that loss of MOV10 function, single-handedly accounted for the
537 loss of sGluA1/A2, accompanied by commensurate decrease in mEPSC amplitude, thus
538 effectively recapitulating the post-synaptic events during downscaling (Figure 6). How
539 AMPARs are downregulated post chronic bicuculline treatment? Similar to previous
540 observation (Shepherd *et al*, 2006), our study showed that bicuculline-induced hyperactivity
541 enhances Arc protein, a key regulator of AMPAR removal from synapses. The enhancement of
542 Arc expression and reduction of sAMPARs after loss of MOV10 demonstrates Arc translation
543 to be a crucial intermediate between miRISC remodelling and synaptic downscaling. The
544 expression of Arc has been shown to be regulated by a set of miRNAs (Wibrand *et al*, 2012),
545 thus reinforcing our hypothesis that in context of chronic hyperactivity miRISC remodelling will
546 take place prior to the Arc translation.
547 In contrast to chronic hyperactivity driven loss of MOV10, its polyubiquitination and subsequent
548 localized degradation at active synapses has been shown to occur within minutes upon
549 glutamate stimulation of hippocampal neurons in culture or during fear memory formation in
550 amygdala (Banerjee *et al*, 2009; Jarome *et al*, 2011).These observations indicate that MOV10
551 degradation is a common player involved in both Hebbian and homeostatic forms of plasticity.
552 Hebbian plasticity paradigms triggers homeostatic scaling in neurons as a compensatory
553 mechanism (Vitureira & Goda, 2013); these two opposing forms of plasticity must involve a
554 combination of overlapping and distinct molecular players. Our data demonstrates the
555 requirement of a rapamycin-sensitive, MOV10 degradation-dependent Arc translation in
556 homeostatic scaling that is distinct from the rapamycin-insensitive dendritic translation of Arc

557 ocurring during Hebbian plasticity (Na *et al*, 2016). We speculate that homeostatic and
558 Hebbian plasticity engages distinct signalling pathways that converge at miRISC remodelling.
559 Though most homeostatic scaling studies including ours used hippocamapal neurons in culture
560 to investigate the mechanistic details, the use of this model leaves a lacuna to evaluate how
561 input-specific gene expression control at selective synapses during Hebbian plasticity
562 influences compensatory changes across all synaptic inputs to achieve network homeostasis.
563 Therefore, physiological relevance of homeostatic scaling needs to be studied in association
564 with Hebbian plasticity in order to delineate factors contributing to proteostasis involving cell
565 intrinsic and extrinsic variables within a circuit. In this context, the study of synaptic scaling
566 during sleep poses distinct advantages. Homeostatic downscaling is a key attribute observed
567 in excitatory neurons during sleep, where, in order to aid the consolidation of contextual
568 memory, synapses undergo pervasive remodelling by a protein kinase A-dependent
569 dephosphorylation and removal of sAMPARs (Diering *et al*, 2017). Sleep promotes mTORC1-
570 mediated Arc translation that is necessary for consolidation of occular domaince plasticity in
571 the cat visual cortex (Seibt *et al*, 2012). Therefore, impact of sleep in memory consolidation is
572 an effective paradigm to analyse how proteostasis at the synapses drive the homeostatic
573 scaling on a larger scale. The kinetics of miRISC-dependent proteome remodelling during
574 sleep and its correlation with synaptic events may be explored further in order to understand
575 the extent to which syncretic translation and degradation processes influence the temporal
576 resolution of scaling during such physiological functions.
577
578
579
580

581 **Author Contributions**

582 S.B and S.S designed the study. S.S., and B.S. performed all experiments. S.S., B.S., and
583 S.B. analyzed the data. J.P.C. provided critical comments on electrophysiology experiments
584 and manuscript. S.V.S.M gave comments on manuscript. S.S. and S.B wrote the manuscript.

585

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593

594 Authors declare no conflict of interest

595

596 **References:**

597

598

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Supplementary Information

Synchrony between translation and proteasome-dependent degradation drives homeostatic scaling in excitatory synapses.

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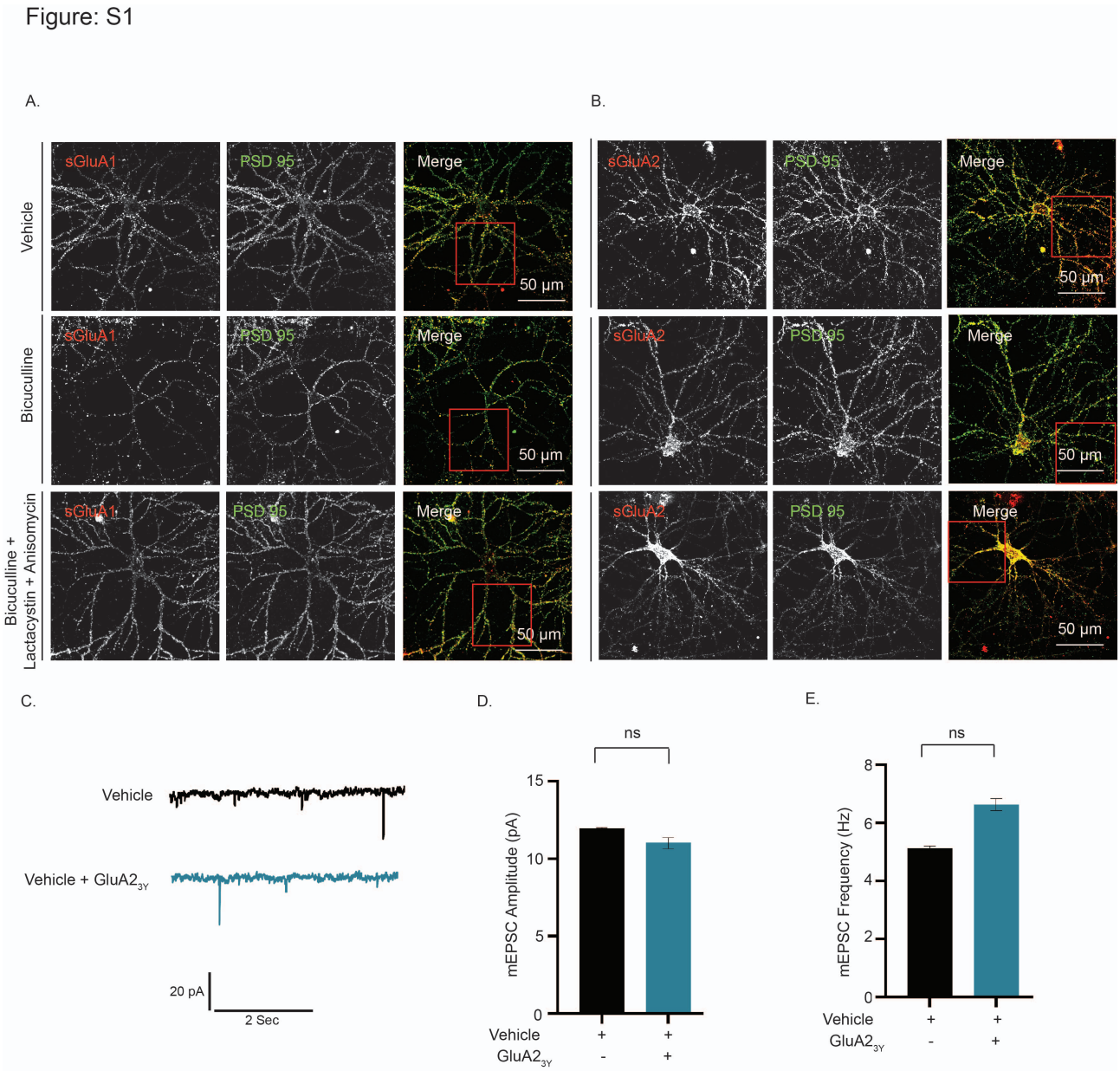
Supplementary Figure



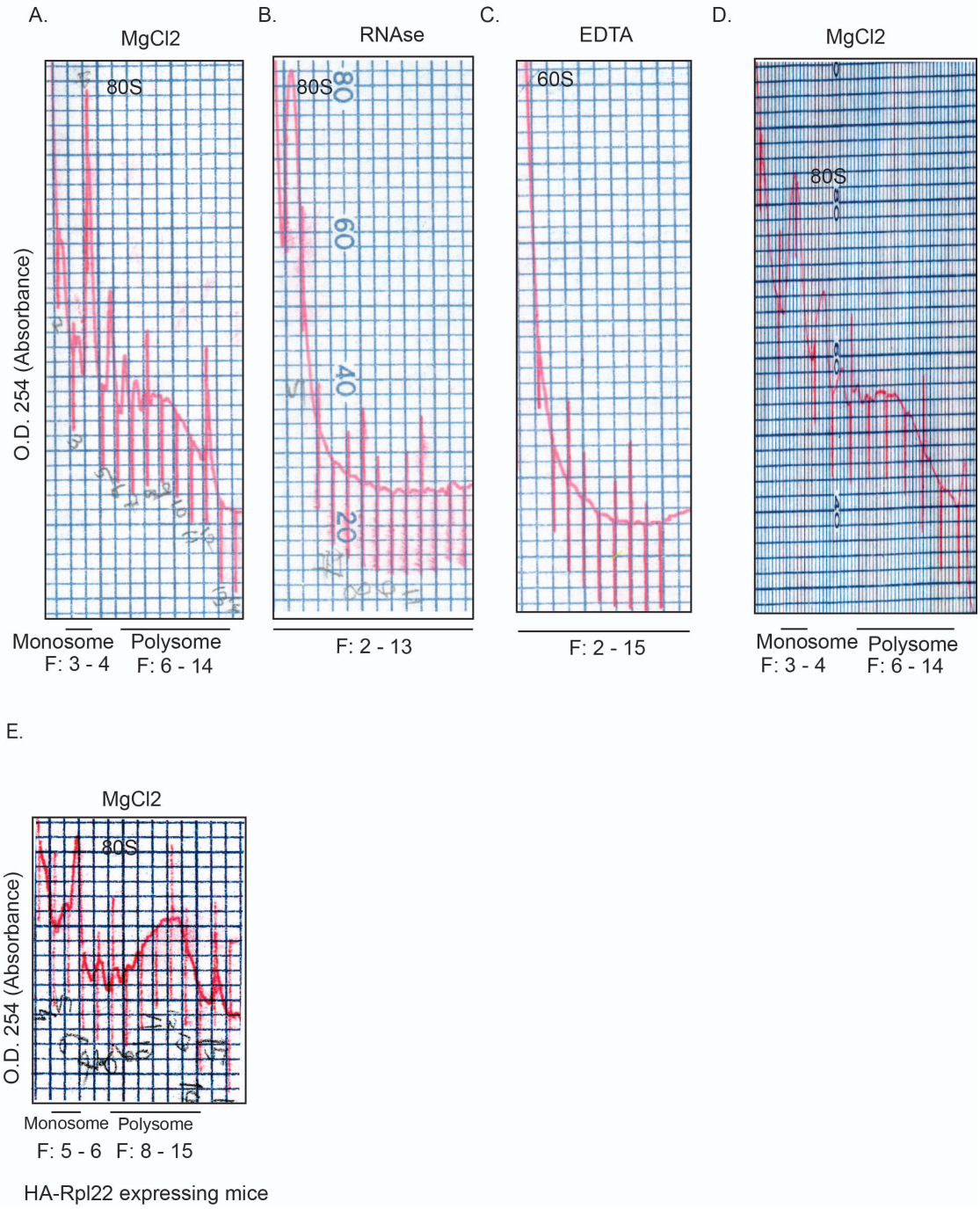
Supplementary Figure 1: Synaptic downscaling by coordinated control of protein synthesis and degradation involves AMPARs.

(A-B) Hippocampal neurons were immunostained with GluA1 (A) or GluA2 (B) and PSD95 as described in Figure 2A-B. Photomicrograph showing images for surface GluA1 or GluA2 (red) and PSD95 (green) and sGluA1/PSD95 or sGluA2/PSD95 (merged). High magnification images of dendrites shown in Figure 2 marked in red square. Scale bar as indicated. Quantitation shown in Figure 2C-D.

(C-E) mEPSCs traces from hippocampal neurons (DIV18-24) treated with vehicle or GluA23y for 24 hours (C) as described in Figure 2E. Scale as indicated. Mean mEPSC amplitudes (D) and

38 frequencies (E) in neurons treated as indicated. n=12. Data shown as Mean ± SEM. One Way
39 ANOVA and Fisher's LSD.
40

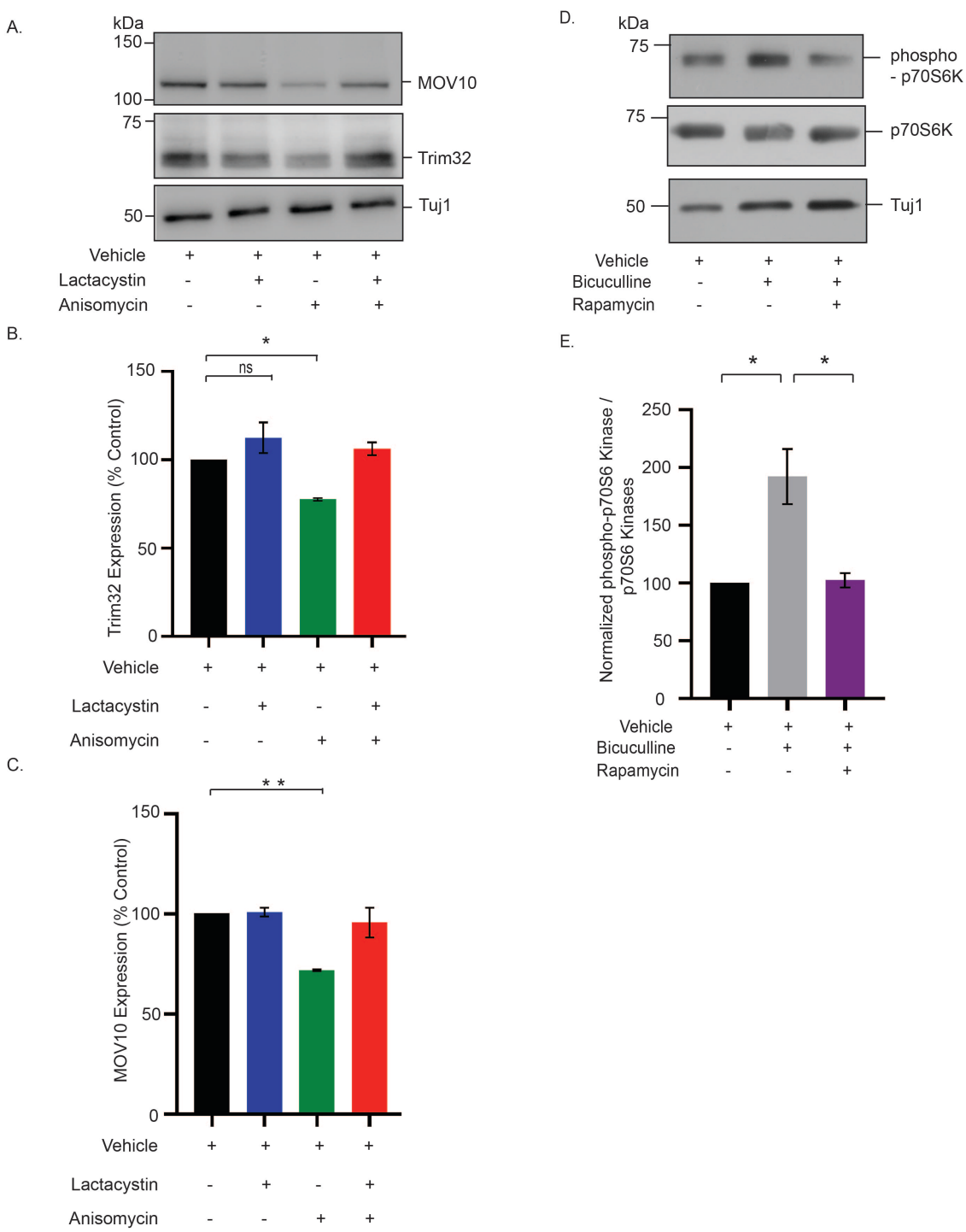
Figure: S2



41
42
43 **Supplementary Figure 2: O. D_{254} profile of polysome fractionation**
44 (A-E) A_{254} profile obtained from spectrophotometer attached gradient fractionator shown in Figure 3
45 and 4. Traces were drawn from original A_{254} profile obtained from cytoplasmic extract treated with

46 $MgCl_2$ (A), RNAse (B), EDTA (C), $MgCl_2$ (D) shown in Figure 3 and $MgCl_2$ treated extract from
47 mouse expressing HA-Rpl22 in excitatory neurons (E) shown in Figure 4.
48

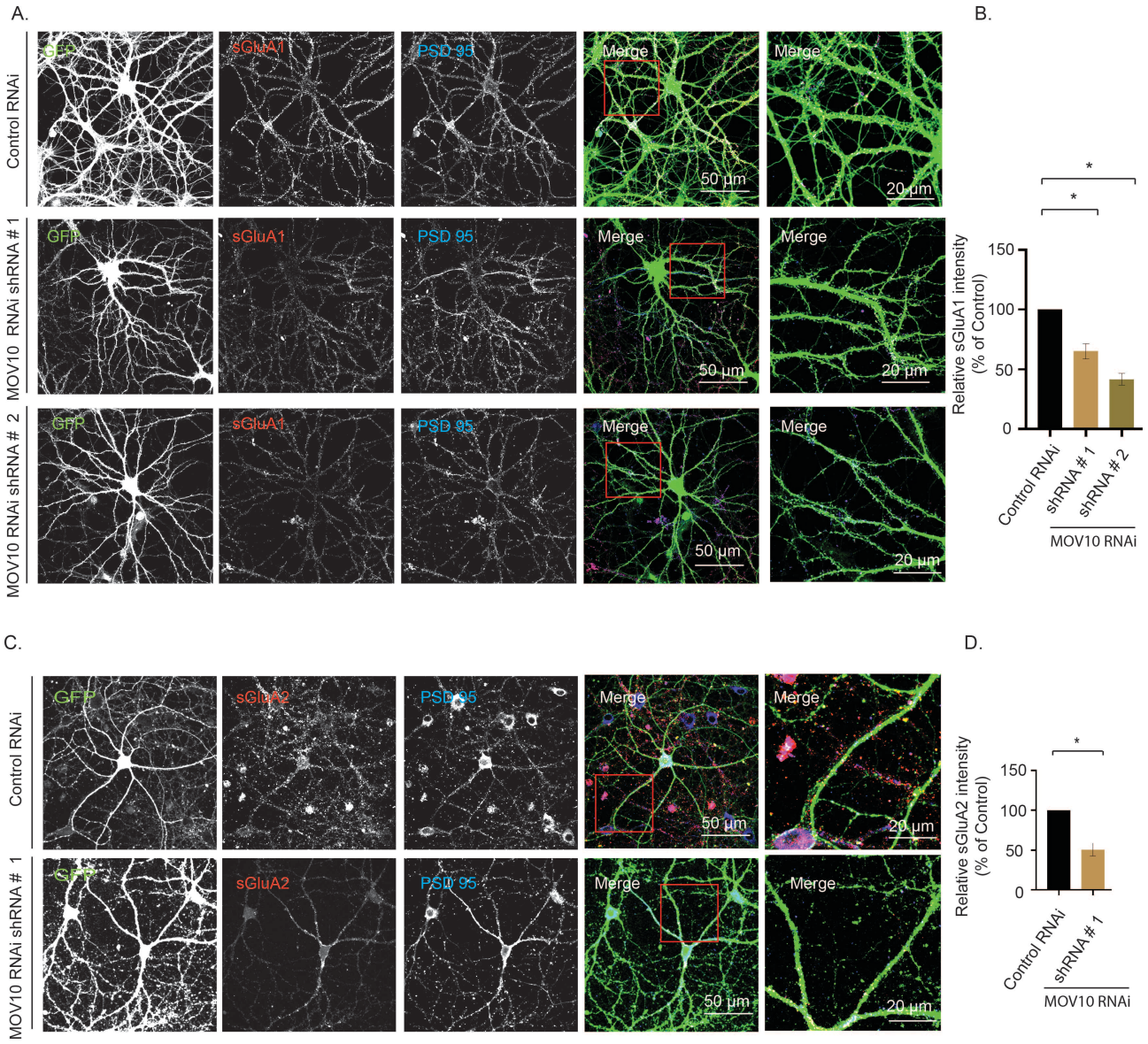
Figure: S3:



49
50
51 **Supplementary Figure 3: Expression profile of miRISC members and translation regulators**
52 **under basal and activity-dependent conditions**
53 (A-C) Hippocampal neurons (DIV21) treated with lactacystin, anisomycin and both for 24
54 hours. Photomicrograph showing the expression of Trim32 and MOV10 as detected by

55 western blot ananlysis (A). Quantitation of Trim32 (B) and MOV10 (C). Data shown as Mean
56 ± SEM, n=3, *p<0.001 and ** p<0.0003. One Way ANOVA and Fisher's LSD. See also Figure 5.
57
58 (D-E) Photomicrograph showing bicuculline treatment of hippocampal neurons (DIV21-22)
59 enhanced phosphorylation of p70S6 Kinase (D). Quantitation of p70 S6 Kinase phosphorylation
60 (E). n=4. Data shown as Mean ± SEM, *p<0.001.One Way ANOVA and Fisher's LSD. See also
61 Figure 5.
62

Figure: S4



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Supplementary Figure 4:

Surface AMPARs expression following MOV10 knckdown

(A-B) Hippocampal neurons (DIV14-15) transduced with lentiviruses expressing two shRNAs against MOV10 (#1 or # 2) along with GFP. Transduced neurons (DIV21-24) were immunostained for surface GluA1 and co-immunostained for PSD95. Photomicrograph showing confocal images of GFP (green), sGluA1 (red), PSD95 (blue) and GFP/sGluA1/PSD95 (merged) (A). High magnification images of dendrites shown in Figure 6 marked in red square. Relative intensity of surface GluA1 particles at the synapse (overlap with PSD95 particles onto GFP expressing dendrites) (B). Normalized intensity of surface GluA1 relative to control was plotted. Data shown as Mean ± SEM. *p<0.01. One Way ANOVA and Fisher's LSD.

(C-D) Hippocampal neurons (DIV14-15) transduced with lentivirus expressing shRNA against MOV10 (#1) along with GFP. Transduced neurons (DIV21-24) were immunostained for surface GluA2 and PSD95. Photomicrograph showing confocal images of GFP (green), sGluA2 (red), PSD95 (blue) and GFP/sGluA2/PSD95 (merged). High magnification images of dendrites shown in Figure 6 marked in red square. Scale as indicated. Relative intensity of surface GluA2 particles at the synapse (overlap with PSD95 particles onto GFP expressing dendrites). Normalized intensity of surface GluA2 relative to control was plotted. Data shown as Mean ± SEM. *p<0.01. One Way ANOVA and Fisher's LSD.

97 **Supplementary Methods**

98

99 **Primary neuronal culture**

100 Hippocampal neuronal cultures from rat (Sprague-Dawley) were prepared and maintained
101 as described previously (Kaech & Banker, 2006). Briefly, hippocampi from embryonic day
102 18 (E18) pups were dissected, treated with trypsin (0.25%), dissociated by trituration to
103 make single cell suspension and plated onto poly-L-lysine (1mg/ml) coated glass coverslip
104 (160 – 250 cells / mm^2). 160 - 170 cells /mm^2 were used for electrophysiology and surface
105 labeling experiments. 200 - 250 cells /mm^2 cells were used for all biochemical
106 experiments. Neurons were maintained in Neurobasal medium (Gibco) containing B27
107 supplements (Gibco) at 5% CO_2 / 37°C up to 25 days prior to commencement of
108 experiments. Animal experiments were performed with the approval of the Institutional
109 Animal Ethics (IAEC) committee of National Brain Research Centre.

110

111 **Lentivirus production and transduction**

112 Lentivirus preparations and transduction into hippocampal neuronal cultures were
113 performed as described previously (Banerjee *et al*, 2009). Validated shRNA against
114 Trim32 (TATACCTTGCCTGAAGATC) (Schwamborn *et al*, 2009) was cloned into MluI and
115 ClaI sites of pLVTHM vector (Addgene) and verified by sequencing. pLVTHM vectors
116 containing MOV10 shRNA cassettes (sh#1:TTATACAAGGAGTTGTAGGTG) or (sh#2:
117 ACTTAGCTCTAGTTCATAACC) (Banerjee *et al*, 2009) and non-targetting control
118 (ATCTCGCTTGGGCGAGAGTAAG) were used for lentivirus preparation. *E. coli* Stbl3
119 strain was used to propagate pLVTHM plasmid and DH5α was used to propagate psPAX2
120 packaging plasmid (Addgene) and pMD2.G envelop plasmid (Addgene). Purified plasmids
121 were prepared by Endo Free Maxiprep kit (Qiagen). Lentiviruses were produced by co-

122 transfection of 20µg transfer vector (EGFP cassette under EF1α promoter and shRNA
123 cassette against MOV10 or Trim32 or non-targeting control under H1 promoter in pLVTHM
124 plasmid), 15µg psPAX2 and 6µg pMD2.G into HEK293T cells. The cells were grown in low
125 glucose DMEM media (Gibco) with 10% Fetal Bovine Serum (Gibco) and maintained at
126 5% CO_2 / 37°C. HEK293T (2×10^6 cells) were transfected by calcium phosphate method.
127 Following transfections media containing transfection mixture was replaced with fresh
128 media after 8 hours. Culture supernatant containing lentivirus particles were collected 72
129 hours post-transfection and concentrated virus stock was prepared by ultracentifugation.
130 Viral titers were determined by infecting HEK293T cells followed by FACS analysis.
131 Typically, titer of concentrated viral stock was 1-2 × 10^7 TU/ml.
132 To perform RNAi, hippocampal neurons at Days *In Vitro* (DIV) 14-15 were infected with
133 lentivirus expressing shRNAs against MOV10, Trim32 and non-targeting control
134 respectively. We have used two shRNAs against MOV10 for its effective knockdown (data
135 not shown) and also a non-targeting shRNA to eliminate the possibility of an off-target
136 effect. Viral infections were performed at MOI of 1 for 6 hours and following infection
137 lentivirus containing media was replaced with fresh Neurobasal media with B27
138 supplements. Neurons were incubated up to DIV25 prior to surface labeling and
139 biochemical experiments. Viral infected neurons were tracked by EGFP expression for
140 electrophysiology and imaging experiments.
141
142 **Surface labeling of GluA1/A2**
143 Surface expression of AMPAR subunits (GluA1 or GluA2) was analyzed by live-labeling of
144 hippocampal neurons with primary antibodies against surface epitopes of GluA1 (Millipore)
145 or GluA2 (Millipore). Neurons (DIV 21-24) were immunostained as described previously
146 (Schwarz *et al*, 2010). Prior to immunostaining, neurons were treated with vehicle

147 (DMSO), bicuculline (10μM) alone or in combination with lactacystin (10μM) and
148 anisomycin (40μM) for 24 hours and transduced with lentivirus for effective knockdown of
149 MOV10 expression. Live neurons were incubated for 15 minutes at 5% CO_2 / 37°C with N-
150 terminus specific mouse GluA1 (1:25) or mouse GluA2 (1:10) antibodies diluted in
151 Neurobasal media containing B27 supplements. Following incubation, the cells were
152 washed twice with phosphate buffered saline containing Mg^{2+} and Ca^{2+} (PBS-MC; 137mM
153 NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2mM KH_2PO_4, 1 mM Mg_2Cl_2 and 0.1 mM $CaCl_2$).
154 Cells were then fixed in PBS-MC containing 2% paraformaldehyde and 2% sucrose for 20
155 minutes at 37°C, washed three times in PBS-MC at room temperature and blocked with
156 PBS-MC containing 2% BSA for 30 minutes at room temperature. Cells were incubated
157 with Alexa-546 conjugated goat-anti-mouse secondary antibody (1:200, Invitrogen) at
158 room temperature for 60 minutes in blocking solution. Cells were permeabilized with PBS-
159 MC containing 0.1% Triton-X-100 at room temperature for 5 minutes. Cells were further
160 incubated with blocking solution for 60 minutes and then with goat PSD95 antibody (1:200,
161 Abcam) for 8 hours at 4°C. Cells were incubated with Alexa-633 or Alexa-488 conjugated
162 donkey-anti-goat secondary antibody (1:200, Invitrogen) at room temperature for 60
163 minutes. Cells were washed three times with PBS-MC at room temperature and mounted
164 on Vectashield mounting media with DAPI (Vector Laboratories).
165

166 **Confocal Imaging and Image Analysis**

167 Hippocampal neurons were imaged using a Leica TCS SP8 point scanning confocal
168 microscope with a Leica Plan Apochromat 63X NA = 1.4 oil immersion objective at 1024 ×
169 1024 pixel resolution. High magnification images were captured using 2X optical zoom.
170 We have obtained 4-6 optical sections with 0.5μM step size. GFP and Alexa 488 were
171 excited by 488 nM Argon laser. Alexa 546 and Alexa 633 were excited by solid state and

172 Helium-Neon lasers respectively. GFP, Alexa 488 and Alexa 546 signals were detected by
173 hybrid detectors and Alexa 633 was detected by PMT. All images (8 bit) were acquired
174 with identical settings for laser power, detector gain and pinhole diameter for each
175 experiment and between experiments.
176 High magnification images, captured from confocal microscopy, were analyzed to observe
177 the intensity of GluA1/A2 expression colocalizing with PSD95 (and GFP for MOV10 RNAi
178 experiments). Images from the different channels were stacked and projected at maximum
179 intensity using ImageJ (NIH). These images were then analyzed using custom written
180 Matlab (Mathworks) programs. First, PSD95 and GFP image signals were thresholded to
181 identify the pixels expressing PSD95 and GFP. Then, the pixels of GluA1/A2, colocalizing
182 with PSD-95 and/or GFP were filtered and the average global intensity of these
183 colocalizing GluA1 pixels were collected, plotted and further analyzed for statistics.
184
185 **Polysome fractionation and TCA precipitation of polysome fractions:**
186 Polysomes from the hippocampi of 8-10 week old SD rats were analyzed following
187 previous protocol (Stefani *et al*, 2004). Following decapitation, the brains were removed
188 and placed in ice-cold HEPES HBSS (HHBSS: 1× Hank's basal salt solution, 2.5 mM
189 HEPES-KOH pH 7.4, 35 mM glucose, and 4 mM NaHCO3) containing 100 µg/ml of
190 cycloheximide. From this point on, all procedures were done at 4°C. Hippocampi were
191 dissected, pooled and homgenised in homgenization buffer (10 mM HEPES-KOH pH 7.4,
192 150 mM KCl, 5 mM MgCl2, and 0.5 mM DTT) containing EDTA-free protease and RNase
193 inhibitors (Roche). 1.2mL of homogenization buffer per four hippocampi were used.
194 Tissues were homogenised manually with a Dounce homogeniser and the homogenate
195 was spun at 2000 × g, 10 min at 4°C to discard nucleus. The supernatant (S1) was
196 collected and NP-40 was added to a final concentration of 1% v/v. After 5 min of

197 incubation on ice, S1 was spun at 20,000 g for 10 min, the resultant supernatant (S2) was
198 loaded onto a 20-50% w/w linear density gradient of sucrose (Sucrose buffer: 10 mM
199 HEPES-KOH pH 7.4, 150 mM KCl, 5 mM $MgCl_2$). In the indicated conditions, EDTA
200 (30mM) or a combination of RnaseT1 (Ambion,1000U/mL) and RnaseA (Ambion,40U/mL),
201 was added to S2 and incubated for 10mins at room temperature before loading it onto the
202 gradient. The gradients were centrifuged at 40,000 g, 2 hr at 4°C in a Beckman
203 Instruments (Fullerton, CA) SW 41 rotor. Fractions of 0.75 ml volume were collected with
204 continuous monitoring at 254 nm using an ISCO UA-6 UV detector. For HA-Rpl22
205 transgenic mice, exact protocols as above were followed. Hippocampi from 8-10 week old
206 mice were homogenized and loaded onto the sucrose gradient.
207 Tri-chloroacetic acid (TCA) was added to polysome fractions at 25% of their volume. All
208 the fractions were incubated on ice for 30 mins post TCA addition followed by
209 centrifugation at 13,000g for 30 mins at 4^0C. The pellets were washed with ice-cold
210 acetone (Merck) twice and dried. Acetone residues were allowed to evaporate and the
211 pellets were resuspended in Laemmli buffer.
212
213 **Proteasome activity assay**
214 Proteasome activity present in monosome and polysome fractions were analyzed by 20S
215 Proteasome Assay Kit (Enzo Lifesciences) as per manufacturer's protocol. Briefly, 20S
216 proteasome chymotrypsin-like activity was tested by incubating 80µl of each fraction with
217 Suc-LLVY-AMC fluoregenic peptide substrate with or without epoxymycin (500nM) for 15
218 minutes at 30ºC. Fluorescence was detected by fluoremeter (Tecan).
219
220
221

222 **Immunoprecipitation from HA-Rpl22 mice:**

223 HA-tagged ribosomes from adult male mice were immunoprecipitated following previous
224 protocol (Sanz *et al*, 2009) with minor modifications. RiboTag mice were crossed with
225 CamKII-Cre mice and CamKII-Cre:RiboTag offspring expressing HA-epitope-tagged-Rpl22
226 were selected by genotyping. Prior to beginning the experiment, anti-HA-tagged beads
227 (200µl) were washed twice with citrate-phosphate buffer pH-5, (24mM citric acid, 52mM
228 dibasic sodium phosphate) and allowed to equilibrate twice for 5 minutes each in
229 immunoprecipitation buffer (50mM Tris pH-7.5, 100mM KCl, 12mM $MgCl_2$, 1% Nonidet P-
230 40). Hippocampi from three adult (8-10 week old) HA-Rpl22 male mice were taken for
231 preparing homogenates, along with the same number of age-matched RiboTag mice who
232 do not express epitope-tagged Rpl22. Hippocampi were rapidly removed and weighed
233 before homogenization in (10% w/vol) polysome buffer (50mM Tris pH-7.5, 100mM KCl,
234 12mM $MgCl_2$, 1% Nonidet P-40(NP-40), 1mM DTT, 100µg/ml cycloheximide, EDTA free
235 Roche Protease inhibitor cocktail, 200U/ml RNAse Inhibitor) using a Dounce homogenizer.
236 Homogenates were then pelleted at 5000g, 10 minutes at 4ºC followed by collection of
237 supernatant and re-centrifugation of the supernatant at 10,000g for 10 minutes at 4ºC to
238 create a post-mitochondrial supernatant. The supernatant was pre-cleared with protein-G
239 agarose beads (Invitrogen) for 1 hour, followed by centrifugation at 8000g, 4ºC, for 10
240 minutes to remove the beads. 2% of the total volume was kept aside as total input. The
241 supernatant (250µl) was then incubated with the equilibrated anti-HA tagged affinity matrix
242 for 6 hours with continuous mixing. The matrix was recovered by centrifugation at 8000g,
243 4ºC for 15 minutes followed by two washes with high salt buffer HS-150 (Tris 50mM pH-
244 7.5, KCl 150mM, $MgCl_2$ 12mM, 1% NP-40, DTT 1mM, 100µg/ml cycloheximide, protease
245 and RNAse inhibitors as above) for 5 minutes and two washes with high salt buffer HS-300
246 (Tris 50mM pH-7.5, KCl 300mM, $MgCl_2$ 12mM, 1% NP-40, DTT 1mM, 100µg/ml

247 cycloheximide, protease and RNAse inhibitors as above) for 5 minutes. All procedures
248 were done at 4°C. The pellets were boiled in Laemmli buffer and supernatant was used for
249 Western Blot analysis.
250
251 **Immunoprecipitation of 26S proteosome subunits and MOV10 from rodent**
252 **hippocampus:**
253 Hippocampi of four adult (8-10 week old) male Sprague Dawley rats were collected and
254 homogenized in tissue lysis buffer (50mM Tris pH-7.5, 150mM NaCl, 1% NP-40, 2mM
255 EDTA, Roche protease inhibitor cocktail, 200U/ml Invitrogen RNAse inhibitor, and
256 phosphatase inhibitor cocktail (Sigma)) (10% w/vol) using a Dounce homogenizer. Prior to
257 this, recombinant protein G-agarose beads (Invitrogen) were equilibrated in wash buffer
258 WB-150 (10mM Tris pH8, 150mM NaCl, and 0.1% NP-40) twice for 5 mins each and
259 centrifuged at 5000g for 2 minutes at 4ºC to recover. The homogenates were centrifuged
260 at 2000g, 4ºC for 10 minutes followed by collection of supernatant and re-centrifugation at
261 10,000g at 4ºC for 15 minutes to get a post-mitochondrial supernatant. Protein content of
262 the supernatant was measured using the BCA protein estimation method (Pierce). 2% of
263 the total protein content was kept aside as total input and the remaining was divided into
264 two parts having equal protein content (~250µl each); one to be used for isotype control
265 and the other for experiment purposes. Protein-G agarose beads were added (20µg) to
266 each part and allowed to incubate with continuous mixing at 4^0C for 1 hour. The pre-
267 cleared supernatants were collected by centrifugation at 5000g for 10 minutes at 4^0C. To
268 the control fraction, 5µg of IgG isotype control was added (Mouse IgG in case of Rpt6 and
269 Rabbit IgG in case of MOV10 immunoprecipitation). To the experimental fractions, 5µg of
270 Rpt6 or MOV10 antibody was added and both fractions were allowed to incubate for 4
271 hours with continuous mixing. 40µg of proteinG agarose beads were added to the fractions

272 and further incubated for 2 hours. The beads were recovered by centrifugation and
273 washed twice with wash buffer IPP-150 (50mM Tris pH7.5, 150mM NaCl, 12mM $MgCl_2$,
274 1% NP-40 and 0.5 mMDTT along with RNAse, protease and phosphatase inhibitors, see
275 reagent list) followed by twice with IPP-300 (same constituents as IPP-150 except NaCl
276 concentration is 300mM). In case of Rpt6, a further stringent wash with IPP-450 (450mM
277 NaCl, rest same as IPP-150) was required. All procedures were done at 4^0C. The total
278 input, control and the immunoprecipitated samples were boiled in Laemmli buffer and
279 stored for further analysis.
280
281 **Western Blot for immunoprecipitated samples and polysomes**:
282 Immunoprecipitated samples were analyzed as per previous protocols (Banerjee *et al*,
283 2009). Briefly, samples were boiled in Laemmli buffer and equal volumes resolved on a 8-
284 10% SDS-PAGE. Post transfer of proteins on nitrocellulose membrane (Millipore), blots
285 were blocked with 5% BSA for 1 hour and probed with primary antibodies overnight. In
286 case of MOV10 IP samples, immunoblotting was done against itself (Bethyl Lab), Trim32
287 (Abcam) and Ago (Millipore). In case of Rpt6 IP samples, blots were probed for eEF2
288 (CST) p70S6 kinase (CST),phospho-P70S6 kinase and Rpt6 itself (Enzo) overnight at
289 4ºC. See Reagent details for more information. Following extensive washing with Tris-
290 Buffer-Saline containing 0.1% Tween-20 (0.1%TBST), secondary antibody supplied with
291 the CleanBlot HRP detection kit (Thermo Scientific) was used to detect the proteins using
292 standard chemiluminescence detection on X-ray films. Band intensities were quantified by
293 densitometry using ImageJ software.
294 Equal volumes of TCA-precipitated polysome fractions were resolved on 8-10% SDS-
295 PAGE and transferred onto PVDF membranes. Following blocking with 5% BSA, blots
296 were probed with Rpt6, Rpt1, Rpt3 and 20Sα core subunit (Enzo), eIF4E and p70 S6

297 kinase (CST) overnight at 4ºC. Post incubation, blots were washed with 0.1% TBST and
298 probed with appropriate secondary antibodies. Blots were detected using standard
299 chemiluminescence (Millipore) detection.
300
301 **Western Blot for primary neuron cultures:**
302 Cultured rat hippocampal neurons (DIV 21-24) were incubated with bicuculline (10μM),
303 anisomycin (40μM), lactacystin (10μM), rapamycin (100nM) alone or in combination for 24
304 hours. Post incubation, cells were washed twice in pre-warmed phosphate buffer saline
305 and collected in Laemmli buffer . Equal volumes of lysates were resolved on 8-10% SDS-
306 PAGE, transferred onto nitrocellulose membrane, blocked with 5% BSA and probed with
307 antibodies against MOV10 and Trim32. For each lane, immunoblotting was also performed
308 with Tuj1 (Sigma) or as the internal control to normalize protein levels. Blots were detected
309 using standard ECL chemiluminescence detection (Millipore) and band intensity
310 determined by ImageJ. MOV10 and Trim32 RNAi samples were also detected similarly,
311 but using GAPDH (Sigma) as the internal control.
312
313 **Electrophysiology**
314 Whole cell patch clamp experiments were performed using primary hippocampal neurons
315 (DIV18-25). Neurons were incubated with bicuculline (10μM), anisomycin (40μM),
316 lactacystin (10μM), rapamycin (100nM) and GluA23y (10μM) for 24 hours. Neurons were
317 patched with glass micro-electrodes with an open-tip resistance of 3-8MΩ. Cells with
318 series resistance >30MΩ were excluded from the analysis. To measure the excitatory
319 currents, the following composition of internal solution was used: 100mM Cesium
320 gluconate, 0.2mM EGTA, 5mM MgCl2, 2mM ATP, 0.3mM GTP, 40mM HEPES, pH 7.2
321 (285-290 mOsm). Miniature EPSCs (mEPSCs) were recorded by holding the cells at -

322 70mV in a recording solution consisting of: 119mM NaCl, 5mM Kcl, 2mM CaCl2, 2mM
323 MgCl2, 30mM glucose, 10mM HEPES, pH7.4 (310-320 mOsm) in the presence of 1μM
324 tetrodotoxin and 10μM Bicuculline.
325 Average of mEPSC events for 300s from each neuron was analyzed and only the events
326 with <-4pA of peak amplitudes, >0.3pA/ms of rise rates, and 1-12ms of decay time
327 constants were selected for the analysis.
328 All recorded signals were amplified by Multiclamp 700B (Molecular devices), filtered at 10
329 Khz and digitised at 10-50 KHz. Analog to digital conversion was performed using Digidata
330 1440A (Molecular Devices). All data were acquired and analysed using pClamp10.5
331 software (Molecular Devices) and custom Matlab filtering algorithms. Cells with holding
332 currents greater than -100pA were excluded from the analysis, as well as any cell which
333 was unstable during the recording.
334
335 **Statistical Analysis:**
336 Statistical Analyses were performed for all experiments. Whole cell patch clamp
337 amplitudes and frequencies were analyzed using one-way ANOVA with post-hoc Fisher's
338 LSD test to test pairwise differences across the groups. Imaging and western blot data
339 were analyzed for statistical significance using one-way ANOVA with post-hoc Fisher's
340 LSD test. Western blot data related to RNAi experiment was analyzed using unpaired t-
341 test with Welch's correction. Data is reported as absolute differences in mean ± SEM for
342 electrophysiology data or percent differences in mean ± SEM for imaging and western blot
343 data between groups.
344
345
346

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348

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