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frontiers

pumilio regulates sleep homeostasis in response to chronic sleep deprivation in *Drosophila melanogaster*

Luis A. De Jesús-Olmo[1], Norma Rodríguez[1], Marcelo Francia[1], Jonathan Alemán-Rios[1], Carlos J. Pacheco-Agosto[1], Joselyn Ortega-Torres[1], Richard Nieves[1], Nicolás Fuenzalida-Uribe[1], Alfredo Ghezzi[1], José L. Agosto[1]*

[1]Department of Biology, University of Puerto Rico, Rio Piedras, P.R., USA.

***Correspondence:**

Luis A. De Jesús-Olmo

luis.dejesus3@upr.edu

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Abstract

Recent studies have identified the *Drosophila* brain circuits involved in the sleep/wake switch and have pointed to the modulation of neuronal excitability as one of the underlying mechanisms triggering sleep need. In this study we aimed to explore the link between the homeostatic regulation of neuronal excitability and sleep behavior in the circadian circuit. For this purpose, we selected the neuronal homeostasis protein Pumilio (Pum), whose main function is to repress protein translation and has been linked to modulation of neuronal excitability during chronic patterns of altered neuronal activity. Here we explore the effects of Pum on sleep homeostasis in *Drosophila melanogaster*, which shares most of the major features of mammalian sleep homeostasis. Our evidence indicates that Pum is necessary for sleep rebound and that its effect is more pronounced during chronic sleep deprivation (84 hours) than acute deprivation (12 hours). Knockdown of *pum,* results in a reduction of sleep rebound during acute sleep deprivation and the complete abolishment of sleep rebound during chronic sleep deprivation. These behavioral changes were associated with accompanying changes in the expression of genes involved in the regulation of neuronal excitability. Interestingly, *pum* knockdown also increased baseline daytime sleep, suggesting that Pum differentially regulates rebound and normal sleep. Based on these findings, we propose that Pum is a critical regulator of sleep homeostasis through neural adaptations triggered during sleep deprivation and induces rebound sleep by altering neuronal excitability.

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35

36 ## 1 Introduction

37 It is well established, even by our own experience, that the urge to sleep increases as a function of
38 time awake. This urge, or sleep drive, triggers a prolonged compensatory sleep after the organism is
39 sleep deprived (Daan et al., 1984; Allada, et al., 2017). This compensatory sleep, which is also called
40 sleep rebound, is a key indicator of the homeostatic regulation of sleep (Vyazovskiy, et al., 2009). In
41 this process, deviations from a reference level of sleep are compensated, i.e. lack of sleep fosters
42 compensatory increase in the intensity and duration of sleep, whereas excessive sleep counteracts the
43 sleep need (Tobler and Ackermann, 2007). More than a century of sleep research has made important
44 progress in understanding the function of sleep and its regulatory circuitry, but the molecular basis of
45 sleep homeostasis remains elusive (Cirelli & Tononi, 2008; Siegel 2008; Sehgal et al., 2007; Donlea
46 2017). Understanding the molecular mechanisms involved in the regulation of sleep homeostasis is
47 key for the overall understanding the regulation of both the sleep circuit and the sleep function. To
48 achieve that level of understanding, we need to study the link between molecular markers, sleep brain
49 circuits and homeostatic sleep behavior.

50 The fruit fly *Drosophila melanogaster* is an ideal model to study the molecular markers impacting
51 sleep behavior. Sleep rebound is a stable phenotype in flies which shares most major features of
52 mammalian sleep homeostasis (Huber, et al., 2004). *Drosophila* shows easily measurable and
53 recognizable sleep patterns linked to reduced brain activity (Nitz et al., 2002; Van Swinderen et al.,
54 2004), limited sensory responsiveness during sleep and display a robust homeostatic sleep rebound
55 (Hendricks et al., 2000; Shaw, et al., 2000) as occurs in mammals. Moreover, it has been
56 demonstrated that humans and fruit flies have a common sleep control mechanism involving GABA
57 receptors in brain neurons linked to the circadian clock (Parisky, et al., 2009; Chung, et al., 2009). In
58 addition, fly genetics has been used as a tool to validate human sleep biomarkers affected by sleep
59 deprivation (Thimgan et al., 2013). Hence, we circumscribed our study of the molecular relationship
60 between homeostatic markers and sleep behavior to the fly model.

61 Recent studies have shown that two structures of *Drosophila*'s brain central complex, the Ellipsoid
62 Body (EB) and the fan body (FB), induce sleep when artificially activated, and produce insomnia,
63 when inhibited (Liu, et al., 2016; Donlea, et al., 2011). Other studies have shown that neuronal
64 microcircuits in the mushroom body (MB) drives rebound recovery after sleep deprivation
65 (Sitaraman, et al., 2015). Follow up studies have produced important progress by identifying
66 dopamine as the neuromodulator responsible for the homeostatic switch operation between
67 sleep/wake, which is mediated by potassium currents (Pimentel, et al., 2016). Homeostatic sleep
68 seems to be controlled by the dorsal FB neurons, which are electrically active during wake and
69 electrically silent during rest (Pimentel, et al., 2016). These studies point to the regulation of neuronal
70 excitability as an important effector of the sleep regulation. Nevertheless, the underlying molecular
71 framework that connects neuronal excitability with sleep behavior is a relatively unexplored area of
72 research.

73 Several genes have been identified to regulate normal sleep, but only a few genes have been linked to
74 the molecular regulation of homeostatic sleep compensation after sleep deprivation. A mutation in
75 the *Shaker* (*Sh*) gene, which encodes a voltage dependent potassium channel involved in membrane
76 repolarization, increases neuronal excitability and reduces normal sleep (Cirelli et al., 2005), but fails
77 to alter sleep rebound. Interestingly, the *Shaker* activator *sleepless* (*sss*), which encodes for a brain-
78 enriched glycosyl-phosphatidylinositol-anchored protein, impairs sleep rebound (Koh, et al., 2008),

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79 perhaps by a mechanism independent of *Shaker*. The gene *crossveinless* (*cv-c*), which codes for a
80 Rho-GTPase-activating protein, is necessary for dorsal FB neurons to transduce the excitability
81 produced by sleep pressure into homeostatic sleep (Donlea, et al., 2014). Knocking down the *Cullin 3*
82 (*Cul3*) ubiquitin ligase gene and its putative adaptor *insomniac (inc),* reduces sleep rebound after
83 sleep deprivation (Pfeiffenberger & Allada, 2012). Mutants of fragile X mental retardation gene
84 (*Fmr1*), a translational inhibitor that causes the most common form of inherited mental retardation in
85 humans, have also been reported to reduce sleep rebound (Bushey, et al., 2009). In addition, it was
86 reported that interfering with the expression of the genes *sandman* (*sand*) and *Sh* in the dorsal FB
87 neurons, increased or decreased sleep respectively as part of the sleep/wake switch (Pimentel, et al.,
88 2016). The regulatory picture presented by these genes and the other neuromodulators and proteins
89 known to affect homeostatic sleep compensation seems far from complete, although together, they
90 also point to neuronal excitability as a key component of sleep homeostatic regulation.

91 Unregulated neuronal excitability may lead to a potentially disruptive positive feedback. To cope
92 with this, neurons have evolved compensatory mechanisms to reduce excitability. The mechanisms
93 by which neurons stabilize firing activity have been collectively termed "homeostatic plasticity"
94 (Marder & Prinz, 2003; Turrigiano & Nelson, 2004; Turrigiano 2008; 2012; Davis 2006; Pozo &
95 Goda, 2010). Therefore, it is plausible that wake promoting neurons, after prolonged times of
96 wakefulness, would utilize one of the homeostatic plasticity mechanisms to regulate neuronal
97 excitability. In this study, we begin to explore the relationship between neuronal homeostasis
98 mechanisms and sleep regulation by testing the role of the neuronal homeostasis gene *pumilio* (*pum*)
99 on the regulation of compensatory sleep.

100 The protein encoded by *pum* is characterized by a highly conserved RNA-binding domain, which
101 acts as a post-transcriptional repressor of mRNA targets. Binding occurs through an RNA consensus
102 sequence in the 3'-UTR of target transcripts—the Pumilio Response Element (PRE), 5'-
103 UGUANAUA-3', that is related to the Nanos Response Element (NRE) (Wang et al., 2018). While it
104 was originally described in *Drosophila* for its critical role in embryonic development, Pum has an
105 important role in the development of the nervous system. Pum is known for controlling the
106 elaboration of dendritic branches (Ye, et al., 2014), and is also required for proper adaptive responses
107 and memory storage (Dubnau, et al., 2003). Evidence of its regulatory role if neuronal homeostatic
108 processes include Pum's repression of translation of the *Drosophila* voltage-gated sodium channel
109 (*paralytic*) in an activity dependent manner (Mee, et al., 2004; Murano, et al., 2008). Pum-mediated
110 repression of the voltage gated sodium channel plays a pivotal role in the regulation of neuronal
111 homeostasis, given the central role of the sodium channel in the regulation of membrane excitability
112 (Weston & Baines, 2007). Furthermore, *pum* was found to be necessary for the homeostatic
113 compensation of increased neuronal activity, or what is known as homeostatic synaptic depression
114 (Fiore, et al., 2014). In addition, Pum has been found to influence synaptic bouton size/number,
115 synaptic growth and function by regulating expression of eukaryotic initiation factor 4E (eIF4E),
116 which is the limiting factor for the initiation of the CAP dependent translation in Eukaryotes (Menon,
117 et al., 2004; Vessey, et al., 2006; Cao, et al., 2009). Pum was our first choice to study neuronal
118 homeostasis effects on compensatory sleep because microarray experiments show that *pum* is
119 expressed in PDF-expressing cells, which are key circadian cells known to promote wakefulness in
120 *Drosophila* (Kula-Eversole, et al., 2010; Parisky, et al. 2008). With over 1000 potential targets and
121 many others indirect targets through its eIF4E regulatory role, based on the cumulative evidence,
122 Pum could be considered a master regulator of neuronal homeostatic processes (Gerber, et al. 2006;
123 Chen, et al. 2008; Menon, et al. 2004).

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125 Our data shows that sleep rebound is reduced by knocking down *pum* in the circadian circuit. This
126 effect is more pronounced after chronic sleep deprivation in comparison with acute sleep deprivation.
127 Our behavioral and molecular data correlates with *pum's* differential involvement in regulating
128 compensatory sleep as a function of sleep need. This, in turn, suggests a mechanistic framework for
129 linking sleep function and regulation through neuronal homeostasis mechanisms.

130

131 **Results**

132 ***Pumilio* regulates sleep rebound differentially between acute and chronic mechanical sleep**
133 **deprivation**

134 Studies exploring the mechanisms of neuronal homeostasis often involve long-term manipulations of
135 neural activity, spanning from 48 hours to the entire life span (Davis, 2013; Turrigiano et al., 1998;
136 Turrigiano, 2012). Moreover, studies linking *pum* with neuronal homeostasis primarily use genetic
137 manipulations that alter neuronal activity throughout the lifetime of the organisms (Weston and
138 Baines, 2007; Mee et al., 2004; Muraro et al., 2008). Thus, in this study we decided to explore the
139 role of *pum* in the regulation of sleep homeostasis induced by chronic (long-term) sleep deprivation
140 as well as acute sleep deprivation (SD).

141 We knocked down the expression of *pum* using a transgenic fly containing a *pum* RNA interference
142 construct (*pum*^{RNAi}) under control of the upstream activating sequence (UAS) of the yeast
143 transcription factor Gal4. To activate the UAS-*pum*^{RNAi} we used a second transgenic construct that
144 expressed Gal4 under control of the *timeless* (*tim*) gene promoter (*tim*-Gal4). When both transgenes
145 are present in the same fly (*tim*-Gal4/UAS-*pum*^{RNAi}), the *pum*^{RNAi} construct is expressed
146 constitutively in *tim* expressing neurons. We selected the *tim*-Gal4 driver because it is a strong and
147 broadly expressed promoter targeting circadian cells found in several brain structures including the
148 wake promoting, PDF-expressing ventral lateral neurons and both the EB and FB neurons (Kaneko &
149 Hall 2000).

150 In our first set of experiments, we subjected the pum^{RNAi} (UAS-*pum*^{RNAi}/*tim*-Gal4) and their "sibling"
151 control flies (UAS-*pum*^{RNAi}/+), which carry the *pum*^{RNAi} construct by itself, to either chronic or acute
152 mechanical SD protocol. In both protocols, flies were placed in the Drosophila Activity Monitors to
153 be monitored for 6 days for baseline sleep. After the 6th day, flies were subjected to mechanical SD
154 using an apparent random shaking program (see methods). Both chronic and acute deprivation
155 protocols were identical in terms of stimulus intensity and pattern; the only difference was the
156 duration of the deprivation period. For chronic sleep deprivation, the SD protocol was active for the
157 first 84 hours starting at the beginning of the first dark period (Fig.1), while for acute sleep
158 deprivation, the SD protocol lasted only 12 hours, which encompassed the entirety of the dark period
159 preceding the sleep recovery period.

160 The results from the chronic SD showed a strong effectiveness of the sleep deprivation method
161 during the first 12 hours (Fig. 1A). However, as time progressed, we noticed a gradual increase in the
162 amount of sleep in all the sleep deprived genotypes during sustained mechanical deprivation.
163 However, this increase in sleep through time did not seem to affect the sleep rebound, as control flies
164 were able to produce a normal sleep rebound pattern that initiated at the 84th hour—immediately after
165 the SD protocol was terminated (Fig. 1 A-B). Surprisingly, we noticed that *pum*^{RNAi} flies did not
166 show any rebound (Fig. 1C). To determine if this lack of sleep rebound was related to an insufficient

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167 sleep deprivation, we quantified the sleep lost and used this value to normalize the sleep recovery
168 after deprivation. The quantification of cumulative sleep loss during the 84-hour deprivation period
169 showed a significant difference between the *pum*^{RNAi}/*tim*-Gal4 flies and the *tim*-Gal4/+ control flies,
170 but no difference between the *pum*^{RNAi}/*tim*-Gal4 flies and the UAS-*pum*^{RNAi}/+ controls (Fig 1D).
171 The fact that this difference was not significant between both controls and pum^{RNAi} flies, suggests the
172 difference in effectiveness could be due to the genetic background rather than the knockdown of
173 *pum*. The results for sleep recovery show a normal recovery pattern in both controls after sleep
174 deprivation as indicated by the increase in cumulative sleep recovered during the first hours after SD,
175 when compared to non-sleep deprived flies during the same time period (Fig. 1E). After normalizing
176 by the sleep lost, *pum*^{RNAi} flies showed a negative sleep recovery, which indicates *pum*^{RNAi} flies were
177 more active than the non-deprived controls after 84 hrs of continuous deprivation (Fig. 1E). This loss
178 of homeostatic regulation in the recovery of *pum*^{RNAi} flies was maintained up to 96 hours post-
179 deprivation (see supplementary figure S2). In our experiments, the UAS-*pum*^{RNAi}/+ control lines are
180 siblings of the UAS-*pum*^{RNAi}/*tim*-Gal4 flies. Meanwhile the *tim*-Gal4/+ controls were generated
181 directly by crossing the parental *tim*-Gal4 line with a non-transgenic wild-type (CS), which can
182 introduce differences in genetic background. Thus, our conclusions are based mostly on the results
183 from "sibling controls" because they have a greater genetic similarity, which results in a more similar
184 baseline sleep pattern than parental controls (Figs. 1 A-C). Hence, for the acute SD experiments,
185 parental controls were not used.

186 The results from the 12 hours acute SD showed sleep lost effectivity close to 100% for both *pum*^{RNAi}
187 and "sibling" controls (Fig. 2A-B). During the deprivation period (0 to 12 hours), the cumulative
188 sleep loss in deprived flies did not show a significant difference between the two genotypes (Fig. 2E)
189 Once again, controls showed an effective sleep rebound (Fig. 2A), while *pum*^{RNAi} flies showed a
190 reduction in sleep rebound (Fig. 2B). However, this time the rebound was not completely abolished
191 as we observed during chronic SD (Fig. 2B vs 2D). We included the chronic deprivation rebound
192 period as a point of comparison between acute vs chronic (Figs. 2C-D). The results from the acute
193 SD sleep recovery resembled the results from chronic SD with a normal rebound in "sibling" controls
194 and reduced sleep recovery in *pum*^{RNAi} flies. Nevertheless, the sleep recovery of *pum*^{RNAi} flies was not
195 negative as we observed during chronic SD (Fig. 2F). When acute vs chronic SD results are
196 compared (Fig 2G), we see significant differences, not only between the genotypes, but also within
197 *pum*^{RNAi} flies exposed to acute vs chronic SD, while the rebound difference of the "sibling" control
198 between acute vs chronic SD remains constant. These results suggest that *pum* differentially regulates
199 acute vs chronic SD. This interpretation is in fact reinforced by our molecular experiments
200 contrasting gene expression changes between acute and chronic SD as reported below and in the
201 supplementary material (supplementary Fig S3).

202 So far, our findings link the duration of sleep deprivation to *pum* regulation, which is consistent with
203 the expected role of neuronal homeostasis on sleep regulation. Since we observed greater
204 homeostatic changes during chronic SD, we continued throughout the study using chronic SD to
205 measure *pum*'s regulatory effects in compensatory sleep. The difference in sleep rebound between
206 *pum*^{RNAi} vs parental flies does not seem to be related to non-specific effects of the genetic
207 background affecting baseline sleep because daytime baseline sleep of *pum*^{RNAi} flies is higher than
208 both parental and "sibling" controls (supplementary Fig S1). If baseline sleep would have been a
209 contributing factor for the recovery results, we should have expected a higher sleep rebound. The fact
210 that we obtained a lower rebound indicates pum knockdown rather that genetic differences
211 influencing baseline sleep are the culprit of our results.

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212

213 ***Pumilio* differentially changes expression level of genes associated with neuronal excitability in**
214 **chronic vs acute SD**

215 To determine if the reduction in homeostatic sleep rebound observed in *pum*^{RNAi} flies can be
216 explained by changes in gene expression, we performed a quantitative reverse-transcription
217 polymerase chain reaction (qRT-PCR) for a selected group of genes encoding synaptic proteins,
218 synaptic translation modulators, neurotransmitter receptors and ion channels. In addition, we wanted
219 to assess if the behavioral differences observed between acute vs chronic SD correlated with gene
220 expression patterns. If Pum is necessary to reduce neuronal excitability caused by the high neural
221 activity induced by SD, then knocking down *pum* should increase gene expression of synaptic
222 proteins associated with neuronal excitability. In addition, if Pum recruitment is directly influenced
223 by sleep need, as suggested by the behavioral differences between acute vs chronic sleep, then the
224 increased sleep need during chronic SD would cause a differential expression of synaptic markers
225 between acute and chronic SD.

226 For our analysis, we selected the synaptic genes *bruchpilot* (*brp*), *disks large 1* (*dlg1*) and *Synapsin*
227 (S*yn*) as their protein products are known to increase after acute SD, as shown by western blots of
228 whole fly brains (Gilestro, et al., 2009). In addition, we selected three genes that encode translation
229 regulators —the *eukaryotic translation initiation factor 4E1* (*eIF4E1*), *Target of rapamycin* (*Tor*),
230 and the Protein Kinase B (*Akt1*) because, as previously stated, EIF4E is a direct Pum target and both
231 TOR and AKT are upstream regulators of EIF4E (Miron, et. al., 2003). We also included genes for
232 the voltage gated sodium channel *paralytic (para)*, the voltage gated potassium channel *Shaker*
233 *cognate l* (*Shal*) and *slowpoke* (*slo*), and the potassium channel modulator *sleepless* (*sss,* also known
234 as *quiver* (*qvr*)), due to their relation to neuronal excitability. To complete the qRT-PCR testing
235 panel, we also included the nicotinic Acetylcholine Receptor gene (*nAchRα1*), the $GABA_A$ receptor
236 gene *Resistant to dieldrin* (*Rdl*) and the *Glutamic acid decarboxylase 1* (*Gad1*), which synthetize for
237 the enzyme that synthesizes the inhibitory neurotransmitter GABA (Lee, et al., 2003), because they
238 also have been associated to regulations in neuronal excitability (see table S1 for references).

239 The RNA for the qRT-PCR study was extracted from whole heads, which were frozen two hours
240 after the completion of the SD protocol. We evaluated the gene expression for non-deprived
241 conditions against acute SD (12 hours) and chronic SD (84 hrs). The non-deprived results come from
242 flies of each of the phenotypes handled in parallel to the deprived flies during the same experimental
243 dates. First, we assesed the effects of *pum* knockdown within non-deprived flies on basal gene
244 expression of our gene panel. Results show that the expression of *Shal* and *Gad1* was significantly
245 increased in *pum*^{RNAi} flies as compared to the sibling controls (Fig. 3A). These results align with
246 previous studies characterizing *pum* effects in neuronal excitability, which have shown a significant
247 diminution of *Shal* mRNA when *pum* is overexpressed pan-neuronally (Murano, et. al., 2008). In
248 addition, the expression increase in the inhibitory neurotransmitter synthesis enzyme *Gad1* was
249 expected because Gad1 is a predicted target of Pum (Chen, et al., 2008). Furthermore, it has been
250 shown that GABA acts as a slow inhibitory neurotransmitter in circadian neurons (Hamasaka, et al.,
251 2005), promoting fly sleep (Parisky, et al., 2008). The fact that *pum*^{RNAi} flies showed increase levels
252 of *Shal* and *Gad1* in non-deprived flies, suggests that the presence of Pum is also necessary to
253 maintain normal sleep. This fact was corroborated by the increase in baseline sleep of *pum*^{RNAi} flies
254 (supplementary Fig. S1), which should be expected under increased GABAergic inhibition of wake
255 promoting neurons (Parisky, et al., 2008).

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256 Next, we assessed the changes in gene expression induced by acute and chronic SD, in both *pum*^{RNAi}
257 flies and sibling controls. The qRT-PCR results showed that four genes displayed significant
258 expression changes after acute SD but no change in response to chronic SD. These genes are:
259 *nAchRα1, Rdl*, *para* and *slo* (Fig. 3B-E). For *nAchRα1*, this change was exacerbated in the *pum*^{RNAi}
260 flies, whereas for *Rdl*, *para* and *slo*, the effect of acute SD in expression observed in control flies was
261 abolished by the knockdown in *pum.* In contrast, eight different genes displayed significant changes
262 between *pum*^{RNAi} flies and sibling controls in response to chronic SD, but no change in response to
263 acute SD (Fig. 3F-M). A *pum* knockdown-dependent increase was observed in *eIF4E1, Tor, Akt, brp,*
264 dl, and *Shal*; whereas a *pum* knockdown-dependent decreas was observed in *Syn* and *Gad1*. These
265 results showed a concordance between the selected markers overexpressed by *pum's* knockdown and
266 their association with increased neuronal excitability. We observed gene expression increases in
267 *pum*^{RNAi} flies but not in the "sibling" controls in synaptic translation genes like *eIF4E, Tor, Akt*
268 (Penney, et al., 2012; Lee, et al., 2011; Howlett, et al., 2008) (Fig 3F-H); and genes coding for
269 synaptic proteins like *brp* and *dlg* (Kittel, et al., 2006; Prange, et al., 2004) (Fig 3I-J). In addition, we
270 saw an expression increase the *Shal* potassium channel (Fig 3K), which has been associated with
271 neuronal excitability during repetitive locomotor activity (Ping, et al., 2011). We also saw an
272 expression decrease in the synaptic protein gene *Syn* (Fig. 3L). The silencing of *Syn* increases
273 intrinsic cell excitability associated with increased Ca^{2+} and Ca^{2+}-dependent BK currents (Brenes, et
274 al., 2015), which is also aligned with our expected results. In addition, *Gad1* was also less expressed
275 in the *pum*^{RNAi} flies than their respective controls. These results are expected because GABAergic
276 inhibition of wake promoting neurons has been shown to regulate sleep in *Drosophila* (Agosto, et al.,
277 2008; Chung, et al., 2009). These combined results confirmed our hypothesis that *pum*'s effects in
278 compensatory sleep behavior is correlated to changes in gene expression from selected neuronal
279 excitability genes, and that acute vs chronic SD exhibit differential gene expression patterns, which
280 points towards a differential regulation in acute vs chronic SD.

281 ***Pumilio* mutants show reduced sleep rebound**

282 Finally, we used mutant fly lines to further validate our results independently of transgenic flies. To
283 confirm the effects of *pum* knockdown in sleep homeostasis we selected the classical loss of function
284 allele *pum*^{13} (also known as *pum*^{680}). *Pum*^{13} is a dominant negative allele that bears a single amino
285 acid substitution, which not only knocks down *pum* function but also interferes with normal *pum*
286 function in heterozygotes (Wharton, et al., 1998). Thus, in addition to the semi-lethal *pum*^{13}
287 homozygous mutants, we used *pum*^{13}*/TM3* heterozygotes in our experiments.

288 The sleep deprivation produced similar sleep lost amounts in each of the lines tested. Fig 4A-C and
289 D). Nonetheless, the sleep recovery showed a significant difference between both wild type (+/+)
290 and *pum*^{13}/+ flies compared to *pum*^{13}/*pum*^{13} flies (Fig 4E). By the end of the recovery period, the
291 differences between *pum*^{13}/+ and the knockout *pum*^{13}/*pum*^{13} were still maintained. Moreover,
292 *pum*^{13}/*pum*^{13} escaper flies completely abolished rebound to chronic sleep deprivation for the first 12
293 hours of the recovery period (Fig. 4E). This suggests that differential *pum* levels between the
294 heterozygote and the *pum*^{13} homozygote, have correlative regulatory effects in sleep rebound.

295 Additionally, we used the p-element insertion *pum* allele, Milord-1, to confirm the mutant results
296 with another independent line. This line was generated by single transposon mutagenesis inserted in
297 the *pum* transcriptional unit (Dubnau, et al., 2003). We compared this line with controls obtained
298 from a wild type stock Canton S flies. As expected, Milord-1 flies showed a significant sleep rebound
299 reduction (Fig 5D). Although there was a significant sleep lost difference between the genotypes at
300 the end of the deprivation period (Fig. 5C), the ANOVA table results did not show a significant

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301 difference between the genotypes for the whole deprivation period. In addition, the sleep recovery
302 calculation normalizes by the sleep lost, therefore, any sleep lost differences affecting the results
303 have already been considered.

304

305 **Discussion:**

306 Through a combination of transgenic RNAi knockdown and mutant analysis, our results indicate that
307 *pum* is necessary for the compensatory sleep behavior displayed after sleep deprivation in
308 *Drosophila.* The *pum*-dependant regulation of sleep compensation, and its effects on synaptic gene
309 expression, increases as sleep needs increases. Compensatory sleep rebound after a 12-hour sleep
310 deprivation protocol (acute SD) was slightly reduced by knockdown of *pum* in *tim* neurons, but
311 completely abolished after 84-hour of sleep deprivation (chronic SD). These differential effects were
312 accompanied by a series of distinct changes in the expression of genes encoding synaptic proteins as
313 well as synaptic translation factors. Together our data suggests that neuronal homeostasis
314 mechanisms led by Pum differentially regulate compensatory sleep after acute and chronic SD, most
315 likely through the regulation of neuronal excitability.

316 Interestingly, we also observed that *pum*^{RNAi} flies have increased day-time sleep in non-deprived
317 conditions (Fig. 1, Fig. S1A), suggesting that other sleep behaviors are also regulated by *pum.* This
318 effect of *pum* could perhaps be explained by the increased expression levels of *Gad1* and *Shal* in
319 *pum*^{RNAi} non-deprived flies, as both genes are associated with a depression in overall neural activity.
320 Additionally, the role of *pum* on regulating baseline sleep seems to be disconnected from its role in
321 regulating sleep rebound. For instance, the daytime baseline sleep, in *pum*^{RNAi} flies is about two times
322 the baseline of both control flies (Fig. S1A), but the same flies showed no rebound sleep after SD,
323 suggesting that the homeostatic sleep rebound is independently regulated from baseline sleep. This
324 interpretation is supported by reports from other groups. Shaw, et al, (2002) previously reported that
325 *cycle* (*cyc01*) mutants showed an exaggerated response to sleep deprivation, which was 3 times as
326 high as baseline sleep. In a similar way, Seidner, et al., (2002) found evidence suggesting that
327 baseline sleep and homeostatic sleep can be regulated by distinct neural circuits.

328 Initial studies of chronic SD in other species have also pointed to a potential difference in the
329 regulatory mechanisms between acute vs chronic SD. Rats exposed to chronic SD do not seem to
330 regain the sleep lost even after a full 3-day recovery period, whereas in acute deprivation, most of the
331 sleep was regained (Kim, et al., 2007). Critics attributed these differences, between acute and chronic
332 SD, to the increase in sleep pressure, which force micro-sleep episodes or EEG artifacts during
333 chronic SD (Leemburg, et al., 2010). A recent study showed that chronically sleep deprived animals
334 no longer expressing the compensatory increases that characterize sleep homeostasis in daily sleep
335 time and sleep intensity (Kim, et al., 2013). The authors of the study suggested that this decoupling
336 of sleepiness from sleep time/intensity imply that there is one sleep regulation system mediating
337 sleepiness (homeostatic), and another regulatory system for sleep time/intensity (allostatic) (Kim, et
338 al., 2013). Whether the lack of sleep compensation observed during chronic SD is a real mechanistic
339 phenomenon or an artifact of the deprivation method remained controversial. In our study, we wanted
340 to test if the behavioral differences reported by the literature, between acute and chronic SD, were
341 regulated by the same mechanism under the *pum* gene. Our results point to the presence of a
342 differential homeostatic response between acute vs chronic SD in *pum* knockdowns, which suggests
343 that *pum* participation in sleep homeostatic regulation is proportional to sleep need. Our data
344 indicates that *pum* regulation of sleep rebound is done through the activation of different genes

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345 between acute and chronic SD. This difference seems to be aligned with fast action ion channel genes
346 for acute SD and translation related and/or genes in which we expect to require more time to become
347 active for chronic SD. Furthermore, we can hypothesize that individual neuroadaptations either
348 promote or inhibit sleep rebound, and the neuroadaptations that promote rebound accumulate with
349 sleep need. In this scenario, *pum* seems to be a key player among neuroadaptations promoting sleep
350 rebound, which can be confirmed by the fact that *pum*^{RNAi} flies continued with a lower sleep recovery
351 for a few days after SD was discontinued (Fig. 2S).

352 The qRT-PCR results support the hypothesis that *pum*^{RNAi} flies are in a higher excitable state than
353 "sibling" controls. The significant expression increase observed in *nAchRα1* (Fig. 3B) during acute
354 SD aligns with an increase excitability in *pum*^{RNAi} flies as acetylcholine is a major excitatory
355 neurotransmitter. Furthermore, in mammals, acetylcholine has been shown to control the excitability
356 of the circadian Suprachiasmatic nucleus (SCN) (Yang, et al., 2010). Also, *pum*^{RNAi} flies showed
357 significantly less expression of the GABA receptor gene *rdl* compared to the "sibling" control (Fig.
358 3C). Previous studies have shown that reduced expression of *rdl* in PDF wake promoting neurons
359 reduces sleep (Chung, et al., 2009), which could also explain the reduced sleep rebound of *pum*^{RNAi}
360 flies. Additionally, the potassium channel *slo* also showed an increased expression in the "sibling"
361 control compared to *pum*^{RNAi} flies. *slo* has been found to both increase or decrease neuronal
362 excitability depending on the circuit where it was manipulated (Jepson, et al., 2013), therefore, we
363 need to view this result in the context of the other gene expression changes.

364 The expression increases in *eif4e, Tor, Akt, brp, dlg,* and *Shal,* in *pum*^{RNAi} flies during chronic SD,
365 are aligned with an expected increase in neuronal excitability induced by prolonged wakefulness and
366 the knockdown of *pum* in the circadian circuit. Studies have shown that down-regulation of the Pum
367 target eIF4E, reduced dendritic spine branching, thus affecting spine morphogenesis and synaptic
368 function (Vessey, et al., 2010). Other studies have shown that TOR promotes retrograde
369 compensatory enhancement in neurotransmitter release key to the homeostatic response in the
370 *Drosophila* NMJ (Penney, et al., 2012). In addition, the levels of p-Akt increases strongly after
371 glutamate application in *Drosophila* larvae (Howlett, et al., 2008). The *brp* mutants have shown
372 impaired vesicle release and reduced Ca+ channels density in *Drosophila* neuro muscular junction
373 (NMJ) (Kittel; et. Al., 2006), thus increased levels of BRP are important for efficient
374 neurotransmitter release. In mice, the overexpression of Pum target Dlg (also known as PSD-95),
375 resulted in enhanced excitatory synapse size and miniature frequency and a reduced the number of
376 inhibitory synaptic contacts (Prange, et al., 2004). Moreover, blocking the potassium channel *Shal* in
377 wake promoting neurons, delays sleep onset (Feng, et al., 2018), suggesting neuronal excitability of
378 wake promoting neurons regulates sleep. Furthermore, *Syn*, which is associated with reserve vesicle
379 release (Gitler, et al., 2008), showed a reduced expression in our qRT-PCR results. These results are
380 also correlated to neuronal excitability. A study in mice reported increases in spontaneous and
381 evoked activities in *Syn* knockouts (Chiappalone, et al., 2008). In sum, the expression changes of all
382 these targets in sleep deprived UAS-*pum*^{RNAi}/tim-Gal4 knockdown compared to the control flies
383 demonstrates that the observed *pum* effects in chronic compensatory sleep can be associated with
384 significant molecular changes aligned with changes into structural synaptic homeostasis that underlie
385 an increased neuronal excitability in whole brain.
386

387 Out of the fourteen genes tested, only *para*, a direct Pum target, was contrary to our expectation
388 during acute SD. Although *tim*-Gal4 is strongly expressed in glial cells (Kaneko & Hall, 2000), the
389 circadian neurons expressing *tim*-Gal4 represent a relatively small number of cells in the fly brain,

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390 therefore, gene expression effects of *pum* knockdown over its direct molecular targets will be
391 confounded with gene expression from the rest of brain cells. Nevertheless, it is reasonable to expect
392 an indirect over-expression in a significant number of genes associated with neuronal excitability.
393 Some of the relatively small number of circadian neurons in the fly brain have an important wake
394 promoting role (Parisky, et al 2008), therefore they project widely into the brain and regulate a
395 significant proportion of it. We hypothesize that knocking down *pum* in the circadian circuit avoids
396 brain processes to "shut down" the neuronal excitability generated during chronic SD, hence the
397 markers for increased neuronal excitability appear to be brain-wide over-expressed. It seems that
398 prolonged sleep deprivation induces brain-wide changes in the expression of synaptic proteins and
399 other neuromodulators, which trigger neuronal homeostatic processes to reduce neural activity. Our
400 data supports the hypothesis that knocking down *pum* would disrupt this regulation allowing both the
401 molecular expression and the behavioral activity of these flies to reflect a prolonged state of neuronal
402 excitability.

403 The decrease in sleep rebound observed in *pum* knockdown is aligned with an increase in neuronal
404 excitability, which was expected based on our hypothesis, by reducing the expression of the neuronal
405 homeostasis gene *pum*. Pum is known to regulate sodium currents (Ina) and excitability in
406 *Drosophila* motor neurons through translational repression and binding with *para*-RNA (Baines, et
407 al., 2003), therefore reducing the number of available sodium channels. Reducing *pum* expression
408 means there could be more sodium channels available and consequently, more neurons excited.
409 Those excited neurons would have a diminished homeostatic mechanism to couple with the increased
410 in excitability, resulting in prolonged wakefulness even after sleep deprivation stimulus was
411 discontinued. Additional evidence in the literature supports the notion of a direct correlation between
412 ion channels availability and wakefulness. Parisky, et al (2008), expressed the EKO potassium
413 channel to hyperpolarize Ventral Lateral neurons (LNv) to reduce their excitability. In addition, they
414 knocked down the *Shaw* potassium channel gene or expressed a dominant-negative Na+/K+-ATPase
415 α subunit in the pdf LNv neurons in order to increase neuronal excitability. The results showed that
416 suppressed LNvs increased sleep whereas hyperactive LNvs increased wake. Furthermore, studies in
417 rats have shown increases in cortical neurons firing with increase in time awake (Vyazovskiy, et al.,
418 2009). Moreover, Donlea, et al, (2014) found that the *crossveinless (cv-c)* mutants show decreased
419 electrical activity in sleep promoting dorsal fan neurons. Additionally, the same study found that
420 sleep pressure increases electrical excitability of sleep promoting neurons and this mechanism was
421 blunted in *cv-c* mutants. This further strengthen our argument that *pum* regulates sleep homeostasis
422 through the regulation of neuronal excitability. Identifying that a neuronal homeostasis gene, with a
423 characterized mechanism of action, regulates sleep homeostasis, adds an important piece of
424 information to further understand sleep homeostatic regulation.

425 Although this is the first time the neuronal homeostasis gene *pum* is linked to sleep homeostasis,
426 there is additional evidence in the literature supporting the concept of neuronal homeostasis as a sleep
427 regulatory mechanism. The neuronal homeostasis protein Homer mediates homeostatic scaling by
428 evoking agonist-independent signaling of glutamate receptors (mGluRs) which scales down the
429 expression of synaptic AMPA receptors (Hu, et al., 2010). Deletion of Homer in *Drosophila*
430 produces fragmented sleep and failure to sustain long sleep bouts during sleep deprivation (Naidoo,
431 et al., 2012). In addition, experiments where flies had a mutated shaker potassium (K+) channels
432 exhibit reduced sleep (Cirelli, et al., 2005). The close functional relationship between neuronal
433 sodium and potassium channels suggests the expression of sodium channels could also be associated
434 with changes in the sleep phenotype. This was corroborated in experiments where a mutation in the

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435 sodium Na(v)1.6 channel gene, which *pum* regulates (Driscoll, et al., 2013), caused an increase in
436 non-rapid eye movement (non-REM) sleep in rodents (Papale, et al., 2010).

437 Further studies characterizing additional Pum targets as well as other genes involved in neuronal
438 homeostasis warrant exciting findings about the molecular control of sleep. Moreover, identifying the
439 specific circuits where Pum is required for sleep regulation in both flies and mammals could provide
440 a better picture of the mechanistic relationship between sleep function and molecular sleep
441 regulation.

442

443 **Materials and methods:**

444 **Fly Stocks:** Drosophila stocks were raised on standard Drosophila medium in a 12/12 h light/dark
445 cycle. The following stocks were used in this study: The UAS-*pum*^{RNAi} (stock #26725: y[1] v[1];
446 P{y[+t7.7] v[+t1.8]=TRiP.JF02267}attP2) fly line was obtained from Bloomington Stock Center;
447 The *tim*-Gal4 transgenic line: *yw; cyo/tim*-Gal4 was obtained from Dr. Leslie Griffith's and Dr.
448 Michael Rosbash's labs at Brandeis University. These two lines were crossed to obtain both UAS-
449 *pum*^{RNAi}/*tim*-Gal4 experimental flies and the "sibling" control flies UAS-*pum*^{RNAi}/+. The Milord-1
450 P{lacZ}$^{pummilord-1}$ was obtained from Dr. Josh Dubnau. The mutant *pum*^{13} (*pum*^{680}) and Canton S wild
451 type flies were also obtained from Bloomington Stock Center and crossed to obtain both *pum*^{13}/+ and
452 *pum*^{13}/*pum*^{13} flies used in Figure 4.

453 **Sleep assays:** Sleep assays used 1-2 days old female flies. The individuals were collected, separated
454 by phenotype and placed into controlled temperature for 6-7 days under 12h:12h light dark cycles for
455 entrainment. The individuals were then anesthetized with CO_2 and placed in individual tubes
456 containing fly food (5% sucrose, 2% agar). Tubes were then placed in *Drosophila* Activity Monitors
457 (DAM) within an environmentally controlled incubator (26°C, 80% humidity, light intensity of 800
458 lux) and connected to the monitoring system (TriKinetics, Waltham, MA) under 12h:12h light dark
459 cycles. After 4-5 days of baseline recordings, after changing the fly food to avoid dryness and microbial
460 growth, the different groups of flies were sleep deprived with the methods described below. The genetic
461 controls ("siblings") were handled and tested side by side to the experimental flies. Flies with less than
462 80% deprivation within the first 12 hrs were excluded from the analysis. Number of individuals tested
463 and number of experiment replications depicted are stated in figure legends. A cumulative sleep lost
464 plot was calculated for each individual by comparing the percentage of sleep lost during sleep
465 deprivation to the average sleep of the non-deprived flies. The individual sleep recovery (rebound) was
466 calculated by dividing the cumulative amount of sleep regained by the total amount of sleep lost during
467 deprivation.

468

469 **Mechanical sleep deprivation**: Mechanical deprivation was performed using a commercially
470 available *Drosophila* sleep deprivation apparatus (Trikinetics Inc., VMP Vortexer Mounting Plate).
471 The apparatus was controlled by the Trikinetics software, shaking the monitors for 30 seconds on
472 alternate settings of 4, 5 and 8 minutes to create an apparently random shaking pattern. The same
473 pattern was used for all experiments. This set-up continued for 84 consecutive hours at the start of the
474 first night for all chronic SD. For the acute SD experiment, the same set up was used but for only 12
475 hours of the deprivation night. Although this protocol results in partial sleep deprivation, rather than
476 total deprivation, it induces significant sleep lost, normally around 80%, and allows the flies to survive
477 through the chronic sleep deprivation period. Due to the long SD time of 84 hours and the baseline

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478 period, we perform a fly food change the day before SD to avoid microbial growth and food dryness.
479 This change is coordinated with the morning peak and performed simultaneously for all experimental
480 groups.

481

482 **Statistical methods:** All statistical comparisons for significance between control and experimental
483 groups was calculated using a significance cut off $p < 0.05$. All statistical analyses were performed
484 using Graphpad Prism 8 software. Statistical analyses performed are included in the figure legends.

485

486 **Measurement of gene expression by qRT-PCR:** RNA was extracted from heads of adult flies using
487 the Qiagen RNeasy Mini kit (Qiagen, Crawley, UK). Five heads were pooled to make one sample and
488 homogenized with a plastic mortar in 100ul of lysis buffer containing 0.1 M-mercaptoethanol, then
489 250 ul of lysis buffer was added and centrifuged. 350 ul of 70% ethanol was added and passed through
490 a RNeasy column. After washing in buffer, immobilized nucleic acids were then treated with 190 U of
491 DNase I for 15 min, washed again in stages according to manufacturer's protocol, and then eluted in
492 20 ul of RNase-free water. Quantification of RNA concentration was made using a ND-1000 Nanodrop
493 spectrophotometer (Nanodrop, Wilmington, DE). All extracted RNA samples were analyzed to assure
494 quality using the Agilent Bioanalyzer, any samples showing signs of degradation were discarded. After
495 adjusting for concentration, synthesis of cDNA was performed with the iScript Reverse transcription
496 Supermix (Bio-Rad) as per manufacturer protocol. The mix was incubated at 25 ºC for 5 min, then at
497 42 ºC for 30 min followed by 85°C for 5 min to inactivate reverse transcription. From the total reaction
498 volume of 20ul, 1 ul of cDNA was used for each PCR sample. All primers were obtained from
499 Integrated DNA Technologies. An Eppendorf Mastercycler Thermal Cycler was used for the relative
500 quantification of target mRNAs. Reactions contained 5 ul of Syber green (SYBR) (Invitrogen), 0.5 ul
501 of each forward and reverse primer (both 10 mM), 3 ul of water, and 1 ul of cDNA. Cycling was as
502 follows: initial denaturation of 15 sec at 95°C, then 40 cycles of annealing for 60 sec. for all primer
503 pairs used, extension at 65°C for 1:20 min and melting curve generation at 95°C. Each group of 7
504 samples were tested in triplicate. Final mRNA levels were expressed as relative fold change normalized
505 against *rp49* mRNA. The comparative cycle threshold (Ct) method (User Bulletin 2, 1997; Applied
506 Biosystems, Foster City, CA) was used to analyze the data.

507

508 **Conflicts of interest:**

509 The authors declare that the research was conducted in the absence of any commercial or financial
510 relationships that could be construed as a potential conflict of interest.

511

512 **Authors Contributions:**

513 J.L.A., N.R. and L.A.D. designed the study. J.A., N.R., C.J.P, J.O., R.N., M.F. and L.A.D. performed
514 the experiments and data analysis. J.L.A, A.G.,N.F. and L.A.D. wrote/reviewed the manuscript.

515

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526

527 **Supplementary Information:**

528 **Fig S1:** Transgenic flies showed increased baseline sleep.

529 **Fig S2:** *Pum* knockdown shows reduced sleep recovery up to 96 hours after chronic sleep deprivation.

530 **Fig S3:** *Pum*^{RNAi} acute and chronic SD time course from qRT-PCR flies confirmed acute SD
531 differences in sleep rebound.

532 **Table S1**: Summary of PR-PCR results in relation to each marker's effect in neuronal excitability.

533

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722

723 ***Figure 1: Pum*** **knockdown eliminates sleep recovery after chronic mechanical sleep deprivation.**
724 Sleep comparison of UAS-*pum*^{RNAi}/*tim*-Gal4 (experimental) vs *tim*-Gal4/+ (parental) flies and UAS-
725 *pum*^{RNAi}/+ (“sibling” controls) during chronic SD. The X axis indicates time after start of sleep
726 deprivation. **(A-C)** Depiction of sleep activity during the sleep deprivation and sleep rebound period
727 for all genotypes. **(D)** Cumulative sleep lost during deprivation expressed as a percentage of total sleep
728 in non-deprived flies of the same genotype. Two-way ANOVA using “genotype” as a factor and “time”
729 as a repeated measure showed significant differences in genotypes ($F(2, 132) = 11.24$ $P<0.0001$), time
730 ($F(167, 22044) = 1033$ $P<0.0001$) and interaction ($F(334, 22044) = 3.066$, $P<0.0001$). Post-hoc

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731 analysis using Dunnett's multiple comparisons test showed significant differences between *UAS-*
732 *pum*^{RNAi}/*tim*-Gal4 *vs* *tim*-Gal4/+ flies (P<0.0001). **(E)** Percent sleep recovery after SD. Two-Way
733 ANOVA with repeat measures indicated significant differences in genotypes (F (2, 132) = 18.58
734 P<0.0001) and interaction (F (94, 6204) = 13.73 P<0.0001). Post-hoc analysis using Sidak's multiple
735 comparisons test comparing both control genotypes against experimental flies, revealed significant
736 differences (P<0.0001) between UAS-*pum*^{RNAi}/*tim*-Gal4 *vs* *tim*-Gal4/+ flies and UAS-*pum*^{RNAi}/+
737 throughout the recovery period. The data shown represents two experiments with the following sample
738 sizes (N): *tim*-Gal4/+ Non-Deprived (N=56) and Deprived (N=53); UAS-*pum*^{RNAi}/+ Non-Deprived
739 (N=60) and Deprived (N=35); UAS-*pum*^{RNAi}/+ Non-Deprived (N=63) and Deprived (N=39). Because
740 the calculations of sleep lost and sleep recovery involve both the Non-Deprived and Deprived groups
741 (see methods), the N for panels A and B is equal to the N of the Deprived group. SD. Data points and
742 error bars represent means ± SEM. Stars indicate significance level (* denotes p<0.05; ** p< 0.01; ***
743 p< 0.001; **** p< 0.0001).

744 **Figure 2: *Pum* knockdown differentially reduces sleep recovery in acute vs chronic SD.** Sleep
745 comparison of UAS-*pum*^{RNAi}/*tim*-Gal4 (experimental) vs UAS-*pum*^{RNAi}/+ ("sibling" controls) during
746 acute (12 hours) mechanical SD. The X axis indicates time after sleep deprivation. **(A-B)** Depiction of
747 sleep activity during the sleep deprivation and sleep rebound period for both genotypes during acute
748 SD. **(C-D)** Depiction of the sleep activity during sleep deprivation and sleep rebound period for both
749 genotypes during hours 72 to 96 of chronic mechanical SD included for ease of comparison. The y-
750 axis shows the number of minutes that flies slept in intervals of 30 min. **(E)** Cumulative sleep lost
751 during deprivation expressed as a percentage of total sleep in non-deprived flies of the same genotype.
752 Two-way ANOVA, using "genotype" as a factor and "time" as a repeated measure, did not showed
753 significant differences between the genotypes (P=0.8664). **(F)** Percent sleep recovery after SD. Two-
754 Way ANOVA with repeat measures indicated significant difference in genotypes (F (1, 58) = 7.114,
755 P<0.0099) and interaction (F (23, 1334) = 3.054, P<0.0001). **(G)** Percent difference in rebound
756 between deprived and non-deprived flies after acute and chronic sleep deprivation protocols of UAS-
757 *pum*^{RNAi}/+ and UAS-*pum*^{RNAi}/*tim*-Gal4 flies. Two-way ANOVA with repeated measures showed a
758 significant difference in genotype (F (1, 91) = 13.72, P=0.0004) and time vs genotype interaction (F
759 (2, 106) = 13.97, P<0.0001). Post-hoc analysis using Tukey's multiple comparisons test revealed
760 significant differences between UAS-*pum*^{RNAi}/+ and UAS-*pum*^{RNAi}/*tim*-Gal4 at 84 hours of deprivation
761 (P<0.0001) no difference was observed at 12 hours (acute SD) (P=0.0735). The data shown represents
762 one experiment with the following sample sizes (N): UAS-*pum*^{RNAi}/+ Non-Deprived (N=31) and
763 Deprived (N=32); UAS-*pum*^{RNAi}/*tim*-Gal4 Non-Deprived (N=31) and Deprived (N=28). Because the
764 calculations of sleep lost and sleep recovery involve both the Non-Deprived and Deprived groups (see
765 methods), the N for panels A and B is equal to the N of the Deprived group. Data points and error bars
766 represent means ± SEM. Stars indicate significance level (* denotes p<0.05; ** p< 0.01; *** p< 0.001;
767 **** p< 0.0001).

768

769 **Figure 3: Pum knockdown results in differential expression patterns between acute (12 hours)**
770 **and chronic (84 hours) sleep deprivation**. Gene expression comparison of UAS-*pum*^{RNAi}/+
771 ("sibling" controls) vs UAS-*pum*^{RNAi}/*tim*-Gal4 (experimental) subjected to acute (12 hours) mechanical
772 SD vs chronic SD. **(A)** Baseline gene expression in non-deprived flies from both genotypes. **(B-E)**
773 Time-course plots for non-deprived, acutely deprived and chronically deprived flies showing
774 expression changes during acute deprivation. The fold change is expressed in log scale. **(F-J)** Time-
775 course plots for non-deprived, acutely deprived and chronically deprived flies showing expression

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776 changes during chronic SD. The fold change is expressed in log scale. Data points and error bars
777 represent means ± SEM. Two-way Analysis of variance (ANOVA) with repeated measures revealed
778 significant effects due to *pum*, Time (T=0, T=12, T=84 hrs SD) and interactions between the
779 parameters in some of the groups (see graphs for results). Stars indicate significance level (* denotes
780 p<0.05; ** p< 0.01; *** p< 0.001; **** p< 0.0001).

781

782 **Figure 4: *Pum^{13}* mutant show reduced sleep rebound after chronic SD.** Sleep comparison of wild
783 type, heterozygous and homozygous flies for the *pum^{13}* allele. **(A-C)** Depiction of sleep activity during
784 the sleep deprivation and sleep rebound period for all genotypes. The X axis indicates time after the
785 start of the sleep deprivation protocol. The y-axis shows the number of minutes that flies slept in
786 intervals of 30 min. **(D)** Cumulative sleep lost during deprivation expressed as a percentage of total
787 sleep in non-deprived flies of the same genotype. Two-way ANOVA, using "genotype" as a factor and
788 "time" as a repeated measure, did not show significant differences between the genotypes F (2, 63) =
789 0.3635, P=0.6967). **(E)** Percent sleep recovery after SD. Two-Way ANOVA with repeat measures
790 indicated significant difference in genotypes (F (2, 63) = 11.29 P<0.0001) and interaction (F (46, 1449)
791 = 5.667 P<0.0001). Post-hoc analysis using Uncorrected Fisher's LSD comparisons test comparing all
792 genotypes against *pum^{13}/pum^{13}* flies revealed significant differences with *pum^{13}*/+ flies (P=0.0319) and
793 with *pum^{13}/pum^{13}* (P<0.0001). The comparison between *pum^{13}*/+ and *pum^{13}/pum^{13}* show no difference
794 (P=0.0728). The data shown represents one experiment with the following sample sizes (N): 1) Canton-
795 S (+/+), Non-Deprived (N=30) and Deprived (N=17); *pum^{13}*/+, Non-Deprived (N=28) and Deprived
796 (N=28); *pum^{13}/pum^{13}*, Non-Deprived (N=30) and Deprived (N=22). Because the calculations of sleep
797 lost and sleep recovery involve both the Non-Deprived and Deprived groups (see methods), the N for
798 panels A and B is equal to the N of the Deprived group. Data points and error bars represent means ±
799 SEM. Stars indicate significance level (* denotes p<0.05; ** p< 0.01; *** p< 0.001; **** p< 0.0001).

800 **Figure 5: The Milord-1 fly line shows reduced sleep rebound after chronic SD.** Sleep comparison
801 of wild type and Milord-1 flies. **(A-B)** Depiction of sleep activity during the sleep deprivation and sleep
802 rebound period for all genotypes. The X axis indicates time after the start of the sleep deprivation
803 protocol. The y-axis shows the number of minutes that flies slept in intervals of 30 min. **(C)** Cumulative
804 sleep lost during deprivation expressed as a percentage of total sleep in non-deprived flies of the same
805 genotype. Two-way ANOVA using "genotype" as a factor and "time" as a repeated measure showed
806 no significant differences between the genotypes (F (1, 58) = 3.712, P=0.0589). **(D)** Percent sleep
807 recovery after SD. Two-Way ANOVA with repeat measures indicated significant difference in
808 genotypes (F (1, 58) = 5.193 P=0.0264) and interaction (F (23, 1334) = 1.695 P<0.0213). The data
809 shown represents two experiments with the following sample sizes (N): Canton-S (+/+) Non-Deprived
810 (N=30) and Deprived (N=17); Milord-1 Non-Deprived (N=62) and Deprived (N=45). Because the
811 calculations of sleep lost and sleep recovery involve both the Non-Deprived and Deprived groups (see
812 methods), the N for panels A and B is equal to the N of the Deprived group. Data points and error bars
813 represent means ± SEM. Stars indicate significance level (* denotes p<0.05; ** p< 0.01; *** p< 0.001;
814 **** p< a 0.0001).

815

816 **Figure S1: Transgenic flies showed increased baseline sleep. (A)** Graphs showing the average sleep
817 activity for UAS-*pum*^{RNAi}/+ ("sibling" control) and UAS-*pum*^{RNAi}/*tim*-Gal4 under baseline sleep
818 conditions compared to parental *tim*-Gal4/+ baseline. The y-axis shows the number of minutes that
819 flies slept in intervals of 30 min. **(B)** Graph showing baseline sleep for all *pum^{13}* lines. **(C)** Graph

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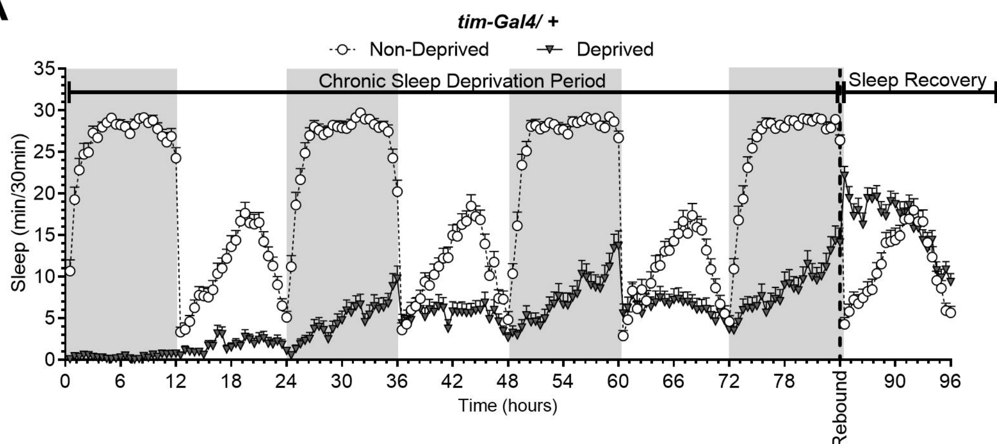
820 showing baseline sleep for Milord-1 line. Stars indicate significance level (* denotes p<0.05; ** p<
821 0.01; *** p< 0.001; **** p< 0.0001).

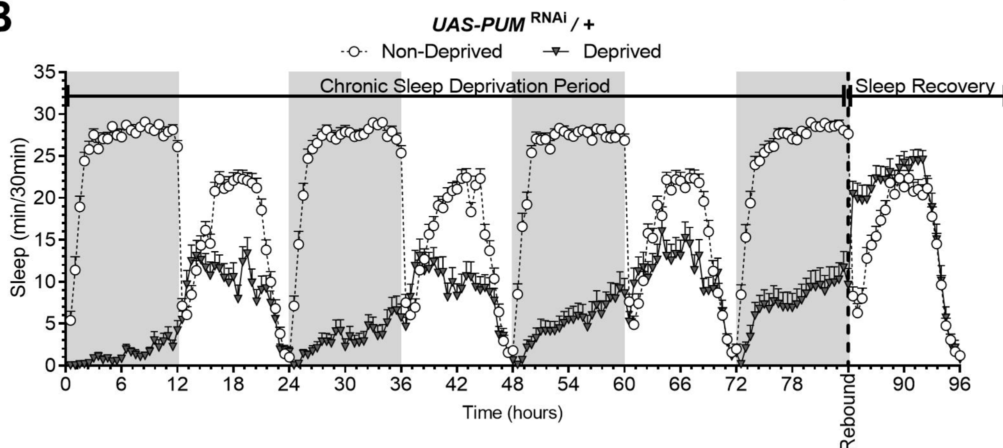
822

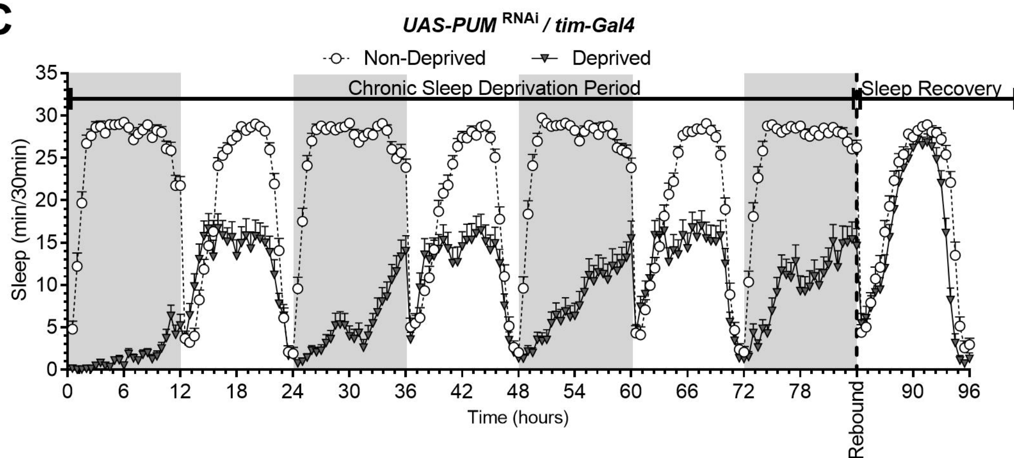
823 **Figure S2: *Pum* knockdown shows reduced sleep recovery up to 96 hours after chronic sleep**
824 **deprivation.** Sleep comparison of UAS-*pum*^{RNAi}/*tim*-Gal4 (experimental) vs UAS-*pum*^{RNAi}/+
825 ("sibling" controls) for the period following chronic SD. **(A-B)** Depiction of sleep activity during the
826 recovery period for both genotypes after chronic mechanical SD. **(C)** Extended percent sleep recovery
827 after SD. Graph depicting up to 108 hours of sleep recovery after chronic SD. Two-Way ANOVA with
828 repeat measures indicated significant differences between the genotypes (F (1, 80) = 18.1 P<0.0001)
829 and interaction (F (167, 13360) = 8.362 P<0.0001). Post-hoc analysis using Tukey's multiple
830 comparisons test revealed significant differences between UAS-*pum*^{RNAi}/*tim*-Gal4 throughout the
831 recovery period. The y-axis shows the number of minutes that flies slept in intervals of 30min. The
832 data shown represents two experiments with the following sample sizes (N): UAS-*pum*^{RNAi}/+ Non-
833 Deprived (N=60) and Deprived (N=39); UAS-*pum*^{RNAi}/*tim*-Gal4 Non-Deprived (N=63) and Deprived
834 (N=43). Because the calculations of sleep lost and sleep recovery involve both the Non-Deprived and
835 Deprived groups (see methods), the N for panels A and B is equal to the N of the Deprived group. The
836 y-axis shows the number of minutes that flies slept in intervals of 30min. Stars indicate significance
837 level (* denotes p<0.05; ** p< 0.01; *** p< 0.001; **** p< 0.0001.

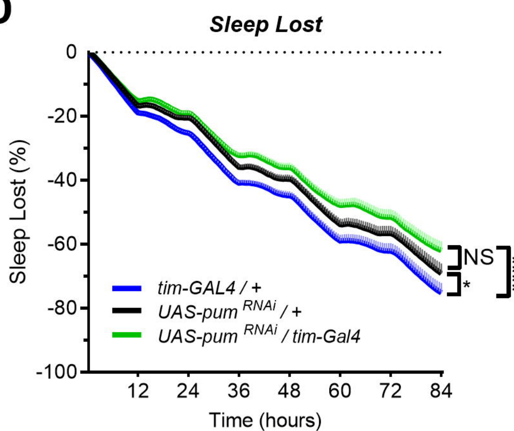
838

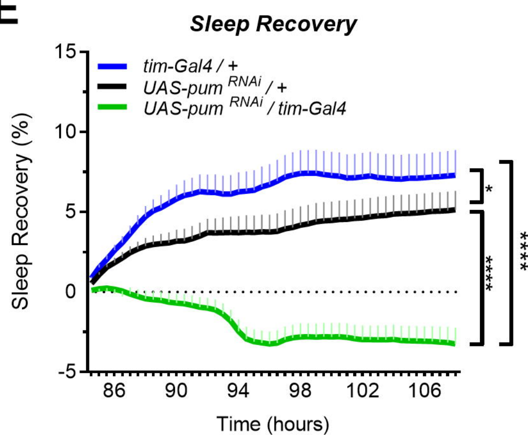
839 **Figure S3: *Pum*^{RNAi} acute SD time course from qRT-PCR flies confirmed acute SD differences**
840 **in sleep rebound** Sleep comparison of *UAS-pum*^{RNAi}/*tim*-Gal4 (experimental) vs UAS-*pum*^{RNAi}/+
841 ("sibling" controls) during acute and chronic SD. Flies were removed from the monitors after two
842 hours of sleep recovery and immediately freeze for qRT-PCR analysis. **(A,C)** Depiction of the acute
843 sleep deprivation and sleep rebound period for both genotypes. The y-axis shows the number of
844 minutes that flies slept in intervals of 30 min. The data shown represents one experiment with the
845 following sample sizes (N): UAS-*pum*^{RNAi}/+ Non-Deprived (N=31) and Deprived (N=27); UAS-
846 *pum*^{RNAi}/*tim*-Gal4 Non-Deprived (N=31) and Deprived (N=32). **(B,D)** Depiction of the sleep
847 deprivation period and sleep rebound pattern for *tim*-Gal4/+ (parental) flies, UAS-*pum*^{RNAi}/+ (sibling)
848 and UAS-*pum*^{RNAi}/*tim*-Gal4 exposed to chronic (84hrs) mechanical SD. The data shown represents two
849 experiments with the following sample sizes (N): UAS-*pum*^{RNAi}/+ Non-Deprived (N=62) and
850 Deprived (N=34); UAS-*pum*^{RNAi}/*tim*-Gal4 Non-Deprived (N=61) and Deprived (N=54). Error bars
851 represent means ± SEM.

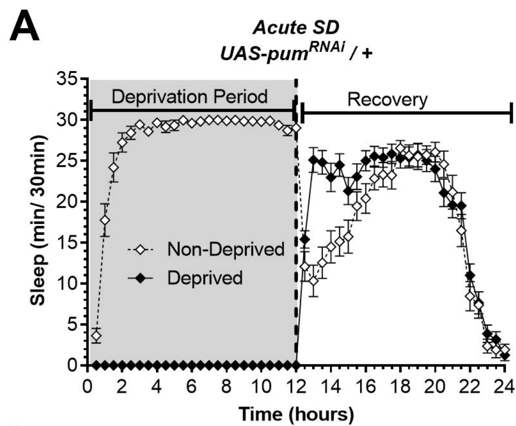

A
tim-Gal4/ +
Non-Deprived
Deprived
Chronic Sleep Deprivation Period
Sleep Recovery
Sleep (min/30min)
Time (hours)
Rebound

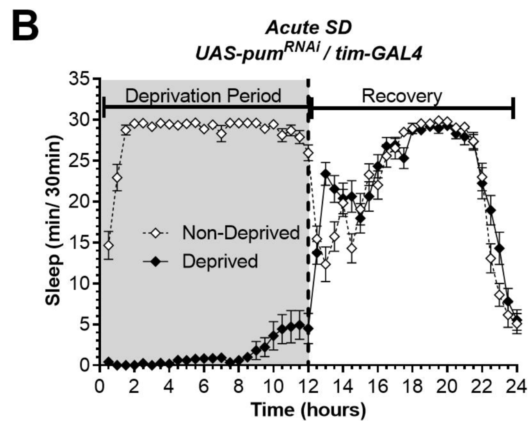

B
UAS-PUM RNAi / +
Non-Deprived
Deprived
Chronic Sleep Deprivation Period
Sleep Recovery
Sleep (min/30min)
Time (hours)
Rebound

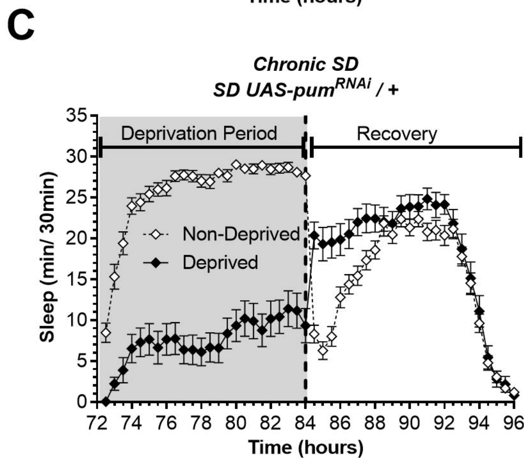

C
UAS-PUM RNAi / tim-Gal4
Non-Deprived
Deprived
Chronic Sleep Deprivation Period
Sleep Recovery
Sleep (min/30min)
Time (hours)
Rebound

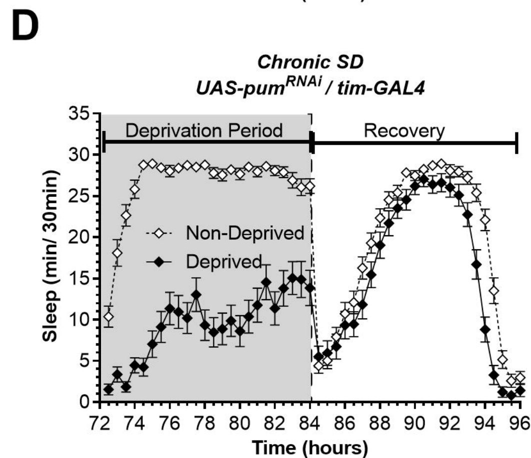

D
Sleep Lost
Sleep Lost (%)
Time (hours)
NS
*
****
tim-GAL4 / +
UAS-pum RNAi / +
UAS-pum RNAi / tim-Gal4


E
Sleep Recovery
tim-Gal4 / +
UAS-pum RNAi / +
UAS-pum RNAi / tim-Gal4
Sleep Recovery (%)
Time (hours)
*
****
****


A
Acute SD
UAS-pum^RNAi / +
Deprivation Period
Recovery
Non-Deprived
Deprived
Sleep (min/ 30min)
Time (hours)


B
Acute SD
UAS-pum^RNAi / tim-GAL4
Deprivation Period
Recovery
Non-Deprived
Deprived
Sleep (min/ 30min)
Time (hours)


C
Chronic SD
SD UAS-pum^RNAi / +
Deprivation Period
Recovery
Non-Deprived
Deprived
Sleep (min/ 30min)
Time (hours)


D
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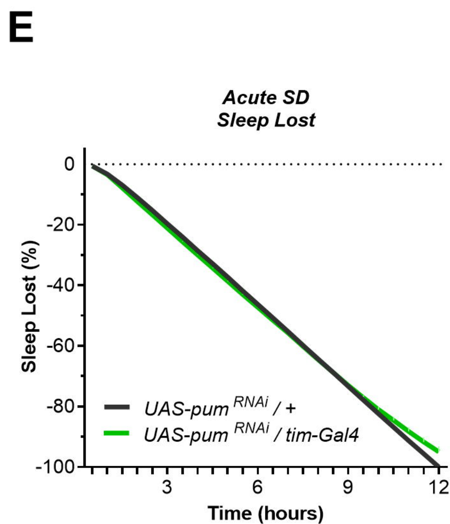
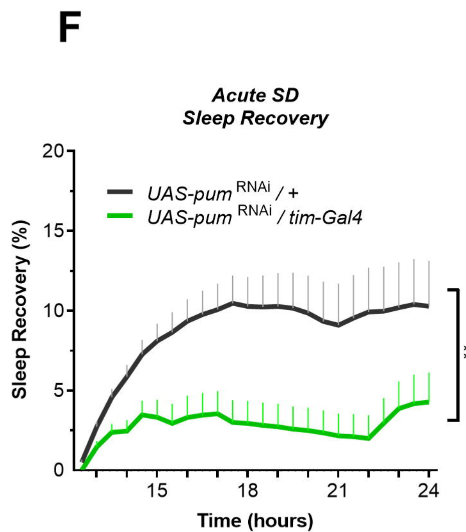
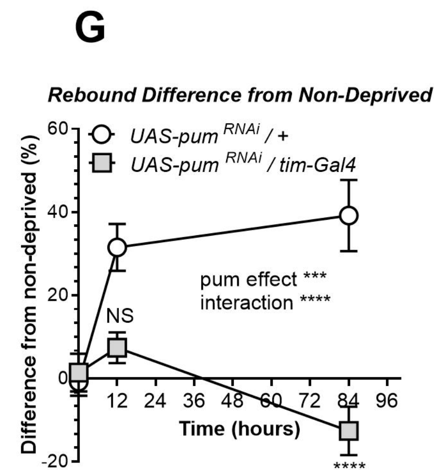
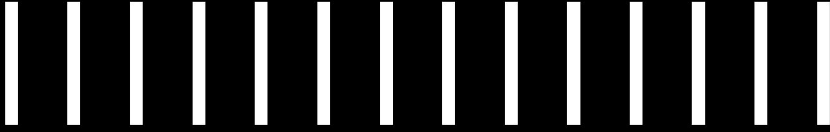
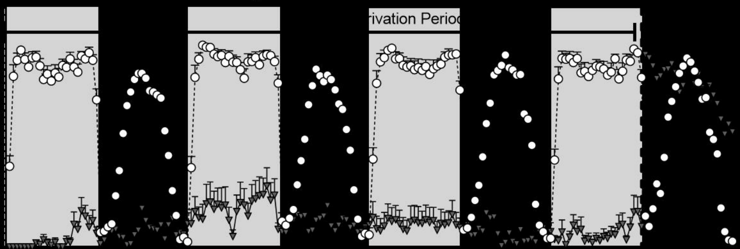
UAS-pum^RNAi / tim-GAL4
Deprivation Period
Recovery
Non-Deprived
Deprived
Sleep (min/ 30min)
Time (hours)

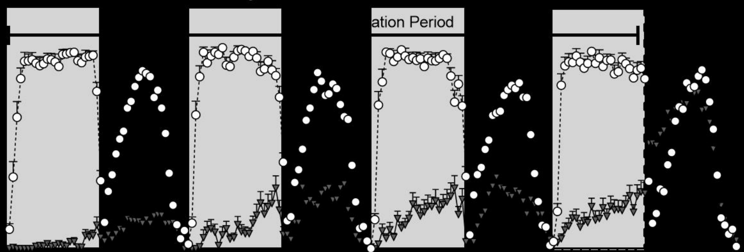

E
Acute SD
Sleep Lost
Sleep Lost (%)
UAS-pum RNAi / +
UAS-pum RNAi / tim-Gal4
Time (hours)

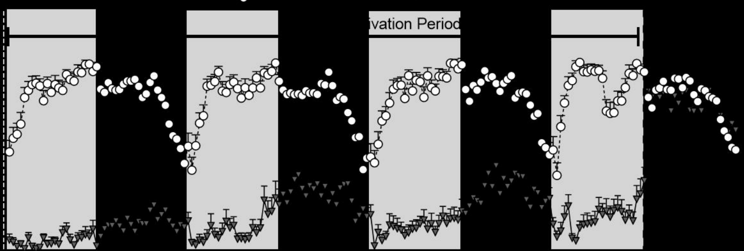

F
Acute SD
Sleep Recovery
UAS-pum RNAi / +
UAS-pum RNAi / tim-Gal4
Sleep Recovery (%)
**
Time (hours)

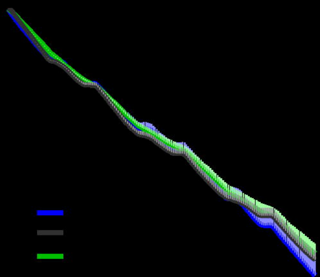

G
Rebound Difference from Non-Deprived
UAS-pum RNAi / +
UAS-pum RNAi / tim-Gal4
Difference from non-deprived (%)
pum effect ***
interaction ****
NS
****
Time (hours)

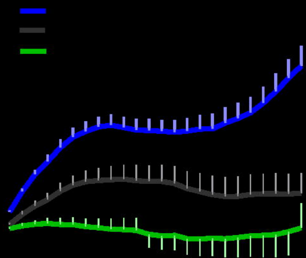


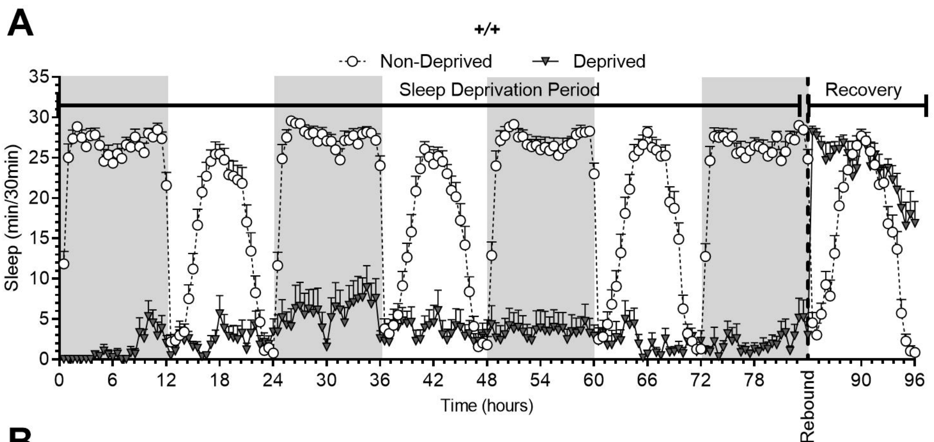


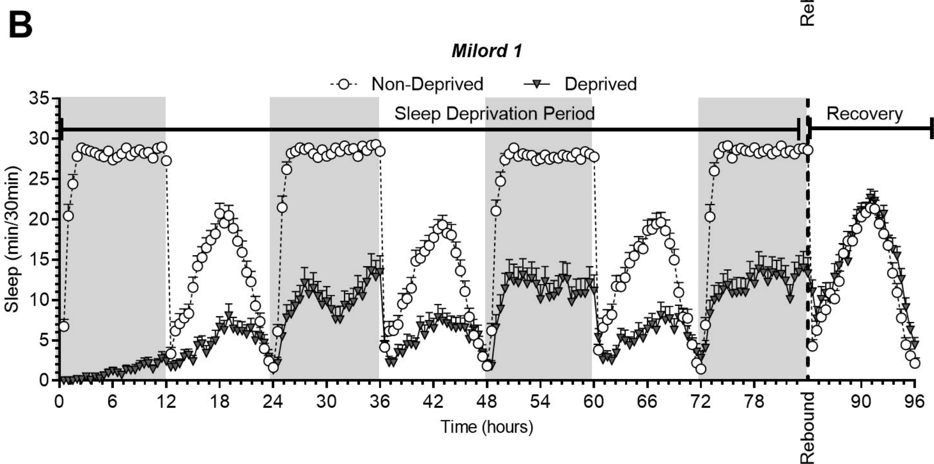


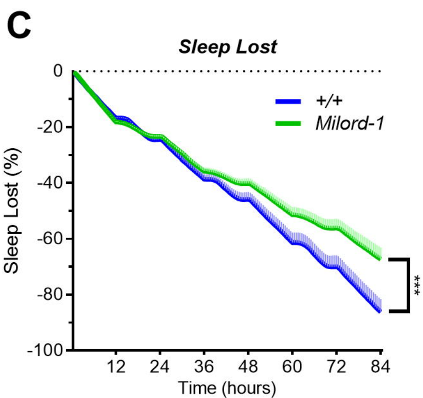


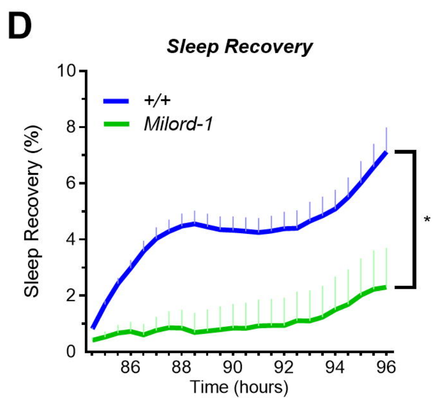





A
+/+
Non-Deprived
Deprived
Sleep Deprivation Period
Recovery
Sleep (min/30min)
Time (hours)
Rebound


B
Milord 1
Non-Deprived
Deprived
Sleep Deprivation Period
Recovery
Sleep (min/30min)
Time (hours)
Rebound


C
Sleep Lost
+/+
Milord-1
Sleep Lost (%)
Time (hours)
***


D
Sleep Recovery
+/+
Milord-1
Sleep Recovery (%)
Time (hours)
*