Encoding of hunger by the neuronal epigenome slows aging in *Drosophila*

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Abstract: Hunger is, by necessity, an ancient motivational drive, yet the molecular nature of homeostatic pressures of this sort and how they modulate health and physiology are largely unknown. Here we show that the molecular encoding of hunger slows aging in *Drosophila*. We identify the branched-chain amino acids (BCAAs) as dietary hunger signals that extend lifespan despite increasing food intake when reduced, and in parallel show that optogenetic activation of a subset of hunger-promoting neurons is sufficient to recapitulate these effects. We find that remodeling of the neuronal histone acetylome is associated with dietary BCAA reduction, and that this requires BCAA metabolism in specific subsets of neurons. Preventing the histone acetylome from being molded by dietary BCAAs abrogates both increased feeding and extended lifespan. However, the mechanisms that promote feeding and modulate aging downstream of alterations in histone acetylation occur through spatially and temporally distinct responses; differential usage of the histone variant H3.3A in the brain is an acute response to hunger that promotes increased feeding without modulating lifespan, while a prolonged experience of hunger may slow aging by promoting a beneficial decrease of a set-point around which hunger levels are regulated. Identification of a molecular basis for the encoding of hunger and demonstration of its sufficiency in extending lifespan reveals that motivational states alone are deterministic drivers of aging and behavior.

38 **Introduction**

39 The relationship between an animal and its diet influences behavior and aging in remarkable
40 ways. The physiological need for nutrients motivates animals to forage and feed, and forced
41 limitation in food availability slows aging across taxa(*1*). Both effects derive not only from the
42 energetic content of the diet but also its composition(*2-4*). Many animals eat until they have
43 consumed a specific amount of protein, for example, and the protein:carbohydrate ratio that
44 results in part from seeking this target is a major factor in modulating lifespan(*5*). Remarkably,
45 an animal need not consume its diet to be affected by it. In mice, appetite is promoted by
46 environmental cues that predict future food consumption, presumably by influencing broader
47 neural states that specify nutrient-specific drive, or hunger(*6, 7*). Similarly, the taste and smell of
48 specific nutrients modulate lifespan in, among other species, the fruit fly, *Drosophila*
49 *melanogaster*, and the nematode, *Caenorhabditis elegans*(*8-11*).

50

51 It seems likely that the effects of diet on aging and behavior share mechanistic foundations in the
52 motivational states they promote, yet little is known about the molecular nature of these states.
53 Many animals, including humans, develop a motivational drive for protein, which has also been
54 described in *Drosophila* in response to starvation and mating(*12-14*). The existence of this drive
55 and its ability to influence physiology is demonstrated by the observation that perception of
56 protein-containing food without its consumption reverses the beneficial effects of protein-
57 restricted diets in *Drosophila* and *C. elegans.* (*11, 15, 16*). Serotonin, together with neurons that
58 express a specific serotonin receptor (5-HT2A), modulate this hunger state, and loss of 5-HT2A
59 increases lifespan up to 50% in nutrient-rich conditions(*17*). Observations like these led us to
60 consider the hypothesis that the neural states that encode the motivation to seek food and that

61 define hunger *per se* may be sufficient to slow aging, independent of any changes in nutrient
62 intake that may result.
63
64 Here we establish that hunger modulates aging in *Drosophila* and demonstrate that epigenomic
65 encoding of this motivational state promotes feeding and modulates lifespan through partially
66 distinct downstream mechanisms. We show that two models of hungry flies – by reducing
67 dietary branched-chain amino acids or by activation of neurons that evoke hunger - are long-
68 lived, despite consuming more calories and total protein. We establish that specific subsets of
69 *Drosophila* neurons use BCAA metabolism to promote the decoration of histone tails with
70 acetylation marks in the brain; a plasticity that is required to encode hunger in these models.
71 Finally, we present evidence to suggest that prolonged hunger alters a set-point around which
72 appetite is regulated and that this adaptation is an important component of a slowed aging
73 process.
74
75 **Results**
76 To investigate how neural states motivate feeding and modulate aging, we focused our
77 investigations on the branched-chain amino acids (BCAAs) primarily because reducing them in
78 the diets of mammals and flies increases protein appetite and extends lifespan(*18, 19*). We used a
79 chemically defined diet to titrate and manipulate the BCAAs without altering other dietary
80 components(*20*). We first designed a reference holidic diet (RD), around which we were able to
81 manipulate BCAAs within a range of concentrations consistent with standard diets used for
82 *Drosophila* aging studies that ensure against mal- and over-nutrition. We enforced equal
83 concentrations of all other non-essential amino acids and non-BCAA essential amino acids

84 across diets, which allowed us to study the effects of BCAAs without the confounding effects of
85 general amino acid deficiency that are mediated by well-described mechanisms (Fig. 1A)(*13, 21-*
86 *23*). This led to diets of modestly different caloricity. However, caloric content has been shown
87 to be less impactful than dietary composition in modulating lifespan and subsequent experiments
88 ruled out differences in calories as a cause of the dietary effects we observed(*2*).
89
90 Consistent with previous reports, dietary BCAA concentration modulated fly lifespan(*19, 24*)
91 (Fig. 1B). Lifespan extension was larger in female flies and was maximized on a diet containing
92 roughly 1/3rd of the BCAA content of the reference diet, which is consistent with lifespan
93 extension observed with dilution of the standard laboratory diet (Fig. 1B, Fig. 1C, Fig. S1A)(*2,*
94 *3*). We subsequently focused our investigation using female flies because of their known,
95 heightened lifespan and neuronal responses to protein availability, which we also observed(*25,*
96 *26*). We chose two diets from the range of those initially tested, hereafter termed "low -BCAA"
97 (8mM BCAAs/5.6% w/v total amino acid) and "high-BCAA" (44mM BCAAs/7.2% w/v total
98 amino acid), to investigate in detail.
99
100 Dietary BCAAs also modulated food intake. Using a method that determines how much volume
101 a group of flies consume in 24 hours by feeding them on blue-dyed food and then collecting their
102 excretions (termed "Con-Ex" for Consumption-Excretion)(*27, 28*), we found that flies on low-
103 BCAA diets consumed more food volume compared to those on high-BCAA diets (Fig. 1D, left
104 panel). This was due to an increase in food intake by flies on low-BCAA diets rather than a
105 decrease by flies on high-BCAA diets because food intake was also increased compared to flies
106 on our RD (Fig. S1B). Differences were unlikely a result of food taste; *Pox-Neuro* flies who have

107 extreme deficits in chemosensation also consumed more food on low-BCAA diets (Fig. 1D, right
108 panel). Increased volume intake by flies on low-BCAA diets resulted in significantly higher
109 caloric intake (Fig. 1E) but in similar amounts of total amino acids (ug/fly) eaten between flies
110 fed low- or high-BCAA diets (Fig. 1F, left panel), while BCAA intake was significantly reduced
111 (ug/fly) for flies fed the low-BCAA diet (Fig. 1F, right panel). Thus, flies fed our low-BCAA
112 diet consumed more calories and similar amounts of total amino acids yet lived significantly
113 longer.
114
115 **Dietary BCAAs influence hunger states**
116 The relative increase in food intake we observed when flies live on low-BCAA diets led us to
117 speculate that reducing dietary BCAAs created a food environment that promoted a heightened
118 and persistent state of hunger. Measuring hunger can be challenging in a simple model system;
119 animals eat for many reasons, and total food intake on homogenous mixtures of nutrients may
120 not be as indicative of hunger levels as feeding paradigms that quantify precisely when and how
121 often individual animals interact with specific food sources(*6, 13, 17, 29, 30*). We therefore
122 devised a refeeding assay to determine how BCAAs influence hunger. In this paradigm, starved
123 flies are refed a measured 3-hour bolus of test food, such as low- or high-BCAA, and are then
124 placed into a common food environment (termed "common garden") where we measure food
125 interactions using the Fly Liquid Food Interaction Counter (FLIC)(*31*) (Fig. 1G, Experiment 1,
126 top panel). We reasoned that if BCAAs did influence a state of hunger, then manipulating their
127 concentration in the test food during refeeding would alter future food interactions during
128 assessment in the common garden.
129

130 We observed that flies refed a low-BCAA diet subsequently interacted more often with the
131 sucrose/yeast food in the common garden than did flies refed a high-BCAA diet and, in fact,
132 interacted as frequently as did flies that were not refed at all (Fig. 1H). This was not due to a
133 reduction in total calories or amino acids consumed during the refeeding period: flies ingested
134 significantly more food volume and also more calories, as measured by Con-Ex, when refeeding
135 on a diet of low-BCAAs than they did when refeeding on a high-BCAA diet (Fig. 1I), although
136 total amino acid intake (ug/fly) was indistinguishable from flies refed high-BCAAs and BCAA
137 consumption (ug/fly) was significantly less (Fig. S1C).
138
139 While the three BCAAs are commonly investigated together because they share similarities in
140 their biochemical structures and functions, we next tested them individually for their role in
141 promoting feeding. We measured food intake after reduction of each BCAA from the high-
142 BCAA diet and found that decreasing isoleucine, but not valine or leucine or other pairwise
143 combinations of amino acids, was required to increase feeding in the 24hr ConEx assay (Fig. 1J,
144 Fig. S1D, S1E). Reducing dietary isoleucine increased the total calories consumed (Fig. S1E)
145 and was the only dietary manipulation that we observed as sufficient to promote increased total
146 amino acid intake (Fig. S1D). Furthermore, increasing isoleucine alone from our low-BCAA diet
147 to match the concentration in our high-BCAA diet was sufficient to decrease food intake (Fig.
148 1J), demonstrating that isoleucine acts as a dietary signal to modulate feeding.
149
150 Having established that isoleucine was sufficient to modulate volumetric food intake, we next
151 returned to our refeeding paradigm to determine how nutrient-specific drives may be affected by
152 this amino acid using the FLIC to measure behavior in a food-choice environment. Choices

153 between carbohydrate- or protein-rich food are thought to be tuned by increased protein demand
154 in starved animals, who exhibit a robust increase in interactions with protein-rich food compared
155 to carbohydrate-rich food, and to serve as a useful proxy for measuring protein-specific
156 hunger(*31-33*). We reasoned that if isoleucine has a specific influence on protein hunger, then
157 manipulating its concentration in the test food during refeeding would be expected to alter future
158 choices between protein or carbohydrate food during the subsequent assessment period. For
159 these experiments, we allowed flies a bolus of refeeding for three hours on either conventional
160 10% sugar-yeast food or the same food to which we added 1% isoleucine. We then measured
161 over the next 24hrs the frequency of individual fly interactions with either carbohydrate- or
162 protein-rich food in the choice environment (Fig. 1G, Experiment 2, bottom panel). Control flies
163 that were refed conventional sugar-yeast food exhibited more interactions with protein-rich food
164 compared to the carbohydrate-rich food, establishing that the three hours of refeeding before the
165 test period were insufficient to fully satiate the animals and that they maintained a heightened
166 level of protein drive (Fig. 1K, top). On the other hand, flies that were refed food with added
167 isoleucine had statistically indistinguishable interactions with carbohydrate- compared to
168 protein-rich food (Fig. 1K, bottom). Thus, levels of dietary isoleucine modulate protein-specific
169 appetite, suggesting that our low-BCAA diet could promote a heightened state of hunger,
170 perhaps by influencing protein drive.
171
172 **Hunger states influence lifespan**
173 We next asked whether restriction of isoleucine alone was capable of increasing lifespan. We
174 reduced all BCAAs or just isoleucine from our reference diet and found that isoleucine reduction
175 (but not valine or threonine) was sufficient to extend lifespan to a similar degree as reducing all

176 BCAAs, despite increased intake of calories, total amino acids, and carbohydrates (Fig. 1L, Fig.
177 S1F). This observation is consistent with our conjecture that the motivational state of hunger
178 itself, rather than the availability or energetic characteristics of the diet, might slow aging.
179 Further support for this idea was provided by the observations that our diets did not have
180 significant effects on egg laying, activity levels, triglyceride and protein levels, and activation of
181 mTOR/AKT pathways (Figs. S2, A-E), which are commonly associated with manipulations that
182 modulate lifespan through changes in nutrient availability or toxicity.
183
184 To determine whether hunger itself might slow aging, we sought a way to induce it independent
185 of dietary manipulations and examine its effect on lifespan. Neurons whose activity evokes
186 hunger in the fly have been described, but their role in modulating lifespan has not been
187 examined(*34*). These neurons can be manipulated by using flies that express the GAL4
188 transgenic activator under the R50H05 driver (R50H05-GAL4). By targeting expression of the
189 light-sensitive cation channel CsChrimson to these neurons, we created flies in which R50
190 hunger neurons were optogenetically activated at will to artificially generate hunger and to
191 increase feeding when adult flies were exposed to red light. We observed that these flies
192 consumed twice as much food as did flies of the same genotype that were kept in the dark or that
193 expressed a green-light sensitive opsin channel (GTACR), consistent with prior reports (Fig.
194 1M). Remarkably, we also found that inducing a heightened state of hunger by activating these
195 neurons for 12 hours each day during the light period significantly extended lifespan relative to a
196 genetic control strain, which was not observed in the dark where flies are modestly long-lived in
197 general – a known phenomenon that has been reported by our lab and others (Fig. 1N, Fig. S3).
198 We interpreted this as a stark confirmation of our conjecture that hunger itself slows aging.

199

200 **Molecular encoding of hunger by the neuronal epigenome**

201 We next turned to defining the molecular interactions among BCAAs, hunger neurons, and the
202 brain to determine how hunger might be molecularly encoded. Our attention was drawn to the
203 epigenome because alterations in histone proteins are involved in the generation of motivated
204 behaviors, are a hallmark of aging, and are regulated by nutrients(*35-38*). Furthermore, histone
205 acetylation is especially sensitive to the availability of dietary nutrients that generate acetyl-CoA,
206 and BCAAs and isoleucine specifically are metabolically fated to produce this substrate(*39, 40*).

207

208 We first examined whether dietary BCAAs influenced relevant histone post-translational
209 modifications. We found that histone H3K9 acetylation was significantly decreased in fly heads
210 within one week of feeding a low-BCAA diet (Fig. 2A). Subsequent experiments revealed that
211 H3K27 acetylation, but not H3K9 methylation, was also reduced when flies were fed low-BCAA
212 diets. All significant effects of dietary BCAAs on histone PTMs were abrogated by feeding flies
213 the histone deacetylase inhibitor, sodium butyrate (Fig. 2C). We also observed an unexpected
214 decrease in total histone H3 abundance in heads and brains, although H3 mRNA was increased
215 (Fig. 2A-C, Fig. S4). The significant decrease in total histone H3 was independent of changes in
216 histone H4, which were statistically non-significant between diets (Fig. 2D). Inducing hunger
217 independent of diet by activation of R50 hunger neurons also reduced histone H3 abundance in
218 fly heads (Fig. 2E), and reducing isoleucine alone, which was important for modulating feeding
219 and lifespan (e.g., Fig. 1), partially recapitulated the effects of BCAAs on histone H3 abundance,
220 suggesting that epigenetic changes may be causally linked to one or both hunger phenotypes
221 (Fig. 2D).

222

223 We next focused on the unexpected finding that histone H3 abundance was reduced by low-
224 BCAA diets. Increased H3 mRNA levels coupled with decreased protein abundance led us to
225 hypothesize that BCAAs may influence turnover of histone H3, such that it may happen more
226 quickly on low-BCAA diets. In the brain, histone H3 is marked for eviction from chromatin by
227 acetylation marks and can be replaced by the histone variant H3.3(*41, 42*). This variant
228 accumulates with age and is thought to decorate regions of actively transcribed chromatin(*43*).
229 We found that it is also transcriptionally increased in the heads of our flies fed low-BCAA diets
230 (Fig. 3A). To visualize the incorporation and removal of histone H3.3 into neuronal chromatin,
231 we used an inducible pan-neuronal driver (Nsyb-GeneSwitch-GAL4) to pulse and then chase
232 fluorescently tagged H3.3 into the brain(*41*). We observed higher H3.3-GFP signal in fly brains
233 after one week of feeding on low- vs. high-BCAA diets, indicating an increase in its stability or
234 persistence in chromatin (Fig. 3B, lower panel; quantified in Fig. 3D), which was not due to
235 changes in initial H3.3-GFP incorporation (Fig. 3B, top panel; quantified in Fig. 3C) or to
236 differences in protein degradation more generally (Fig. 3D). These data indicate differential
237 usage of histone H3 and the variant H3.3A in the fly brain following feeding on a low-BCAA
238 diet.

239

240 To determine whether the observed differences in histone acetylation or variant usage were
241 required for feeding or lifespan differences between the diets, we began with a pharmacological
242 approach to broadly inhibit histone deacetylases (HDACs), which prevented diet-dependent
243 differences in histone acetylation (Fig. 2C). Feeding flies the HDAC inhibitor sodium butyrate
244 for one week abrogated increased feeding on low-BCAA diets, indicating that the histone

245 acetylome could either encode hunger itself or be a permissive response to hunger that modulates
246 feeding (Fig. 3E). To distinguish between these possibilities, we fed flies HDAC inhibitors for
247 life and found that this extended the lifespan of flies on high-BCAA food. Dietary modulation of
248 histone acetylation therefore regulates both feeding and lifespan, supporting the idea that the
249 histone acetylome encodes hunger rather than feeding *per se* (Fig. 3H). We also tested whether
250 incorporation of histone variant H3.3 was required for the effects of hunger on feeding and
251 lifespan by using pan-neuronally expressed RNAi to knock-down the protein chaperone HIRA,
252 which is required for the exchange of H3 for H3.3(*44*). We observed that this also abolished
253 increased feeding on a low-BCAA diet (Fig. 3F), but surprisingly, it had no effect on lifespan
254 (Fig. 3G). Histone H3/H3.3 swapping is therefore required for modulating feeding in response to
255 hunger but is dispensable for hunger's effects on lifespan.
256
257 **Metabolic intermediates link BCAAs to feeding and lifespan**
258 How might BCAAs from the diet promote modifications to the neuronal epigenome? The
259 BCAAs are unusual amino acids because, in mammalian systems, they bypass metabolism in the
260 liver and are instead metabolized in target tissues by the enzymes branched-chain
261 aminotransferase (BCAT) and branched-chain alpha-ketoacid dehydrogenase (BCKDH)(*40, 45,*
262 *46*). We found that *BCAT* mRNA abundance was reduced in the heads of flies fed low-BCAA
263 diets (Fig. 4A). Knocking-down *BCAT*, a homolog of mammalian *BCAT2*, with the pan-neuronal
264 Nsyb-GAL4 driver reduced histone H3 abundance to statistically indistinguishable levels on
265 low- compared to high-BCAA diets (Fig. 4B). We consulted the Fly Cell Atlas and found that
266 *BCAT* is expressed in approximately 80 neurons that co-express the promoter used to label R50
267 hunger neurons, *SerT* (*47*) (Fig. S5). Strikingly, BCAT knock-down only in the R50 hunger

268 neurons also resulted in statistically indistinguishable levels of histone H3 abundance and
269 reduced H3K9ac on low- compared to high-BCAA diets (Fig. 4C). It also strongly reduced diet-
270 dependent differences in lifespan by significantly extending the lifespan of flies on high-BCAA
271 diet (Fig. 4D). This is unlikely to be a result of deficiencies in neuronal activity upon knockdown
272 of *BCAT* because optogenetic inhibition of the R50 hunger neurons had statistically non-
273 significant effects on the lifespan of flies fed high-BCAA diets (Fig. S6). These findings indicate
274 that R50 hunger neurons use BCAA metabolites to modulate aging via a mechanism that does
275 not require neuronal depolarization. We were surprised, however, to observe that *BCAT*
276 knockdown in hunger neurons did not abrogate feeding differences and may have in fact
277 exacerbated them (Fig. 4E). These results suggest complexity in the effector pathways that
278 increase feeding and modulate aging in response to hunger and reinforce the notion that they are,
279 at least in part, anatomically distinct.
280
281 **Considerations on the divergence of mechanisms linking hunger with feeding and aging**
282 A subset of the R50 hunger neurons produce the neuromodulator serotonin, and we observed that
283 expression of the genes *Trh*, which encodes tryptophan hydroxylase that functions as the first
284 and rate-limiting step in serotonin synthesis, and *Tph/Henna*, which encodes tryptophan
285 phenylalanine hydroxylase, were significantly increased in the heads of flies that were BCAA-
286 restricted (Fig. 5A). We also observed increased antibody staining of serotonin itself in the cell
287 bodies of the serotonergic PLP cluster, a subset of the R50 hunger neurons chosen for
288 quantification due to their easily accessible and recognizable anatomical location in the fly brain
289 (Fig. 5B-C, quantified in 5D). Given that inhibition of BCAT in the R50 neurons seemed to
290 exacerbate the feeding differences on our BCAA diets and that serotonin is known to both

291 promote and suppress feeding depending on which serotonergic neurons are manipulated(*34*), we
292 asked whether *BCAT* knockdown in the serotonergic network more broadly may produce
293 opposite effects on feeding. We used the Trh-GAL4 driver, which is putatively expressed in all
294 serotonergic neurons of the CNS, to target knock-down of *BCAT* and therefore BCAA
295 metabolism in these cells. This manipulation prevented increased feeding on low-BCAA diets,
296 suggesting that BCAA metabolites are used within distinct populations of serotonergic neurons
297 to regulate feeding and aging (Fig. 5E).
298
299 Intrigued by the possibility that hunger effects on feeding and aging may be regulated by
300 partially distinct pathways, we questioned if some of this complexity might also result from the
301 time scale on which they occur. While we consider lifespan to be an outcome of chronic dietary
302 effects, measurements of feeding quantify more acute responses that may or may not be stable
303 over longer time periods. We observed that the increased feeding on low-BCAA food that was
304 present after five days on the diet had receded after two weeks (Fig. 5F), resulting in reduced
305 amino acid intake overall (Fig. 5G). This result was mirrored by temporal changes in the
306 epigenome: patterns of changes in brain histone acetylation between diets after two weeks were
307 reversed or eliminated when compared with similar measures after only five days (Fig. 5H).
308 Thus, flies on low-BCAA diets presumably adapt to their food environment in a way that
309 decreases the amount of protein they require, which may have beneficial consequences for aging
310 (see model, Fig. S7).
311 **Discussion**
312 Here we present behavioral and molecular evidence in favor of the idea that the neural state of
313 hunger modulates aging. We show that this motivational state is molecularly encoded by the

314 neuronal epigenome, and that increased feeding and extended lifespan are consequences that
315 seem to be orchestrated by unique downstream responses to hunger. We found that the
316 mechanisms that increase feeding and extend lifespan in response to low-BCAAs share the use
317 of a BCAA-metabolism to histone acetylation pathway but diverge downstream of this
318 programming. Our findings that dietary BCAAs modulated use of the histone variant H3.3; that
319 this was required for increasing feeding, but dispensable for increasing lifespan; and that unique
320 populations of serotonergic neurons require BCAA metabolism to modulate either feeding or
321 aging, suggest that different neurons use different strategies to generate responses to hunger.
322 Such complexity is consistent with emerging reports of interactions between metabolism and the
323 epigenome that are driven in part by specialized enzymes that reside in distinct cellular
324 compartments and in some cases distinct nuclear sub-compartments(*48-50*). In *C. elegans,* H3.3
325 is important for engaging transcriptional networks that underlie neuronal plasticity in response to
326 environmental manipulations(*51, 52*). Perhaps neuronal plasticity is one strategy used by circuits
327 that promote feeding to generate behavioral responses to hunger. Notably, protein appetite was
328 previously shown to require plastic changes in a dopaminergic circuit, although a role for histone
329 variant swapping in this process has not been determined(*33*). Thus, the cell-type specificity of
330 the usage of histone variant H3.3 and the genomic loci to which it is targeted in response to
331 dietary BCAAs remain unknown.
332
333 The observation that BCAA reduction acutely increased appetite but that this eventually subsided
334 indicates that hunger may act as an allostatic stressor that, like other model homeostatic systems,
335 acutely increases feeding while chronic hunger may promote physiological changes that lower a
336 set-point around which appetite is regulated(*53, 54*). Perhaps it is this adaptative response,

337 mediated by modifications to the epigenome in discrete neural circuits, that slows aging? This
338 idea is consistent with several dietary manipulations that are known to extend lifespan: low
339 protein diets (and methionine restriction in particular) generate protein-specific appetites and
340 intermittent fasting paradigms putatively increase the frequency with which an animal
341 experiences hunger without affecting total caloric intake(*5, 55-59*).
342
343 Others have reported that the effects of BCAAs on feeding and lifespan are non-specific in
344 *Drosophila* and that such effects can be observed by restriction of other amino acids, which we
345 did not observe(*19*). These differences are likely due to experimental design; Juricic et al. used
346 holidic diets that were comparatively low in their overall amino acid content, which resulted in
347 reduced activation of TOR signaling. We chose diets that were amino acid replete and had no
348 effect on TOR activity, which revealed BCAA-specific effects, suggesting that interactions
349 between total amino acid abundance and reductions of individual amino acids warrant further
350 investigation.
351
352 Finally, our data support a role for chromatin reorganization as a link among hunger, feeding,
353 and aging, but its influence on transcriptional states in individual neurons remains an exciting
354 area to be explored. In the brain, transcriptional changes occur in response to dietary
355 manipulations, and yet, analysis of single "longevity genes", of which the list is continually
356 growing, is typically insufficient to generalize about how diet modulates aging. This prompts us
357 to speculate that the effects could require an overhaul of transcriptional programming more
358 broadly(*60-62*). Chromatin accessibility patterns, gene expression profiles, and intracellular
359 signaling pathways converge to determine transcriptional states which, although they are most

360 often studied in the context of neuronal-*fate* determination, may also tune neuronal *states* in
361 response to dietary or other environmental manipulations(*63, 64*). Future work that explores how
362 specific nutrients, and their interactions, interact with each of these processes to shape the
363 transcriptional environment of distinct neurons presents an important next step towards uniting
364 seemingly disparate effects of diet on physiology and will likely provide new insight into how
365 motivational states influence aging.

366 **Materials and Methods**

367 **Fly stocks and husbandry**

368 Fly stocks were maintained on a standard cornmeal-based larval growth medium and in a
369 controlled environment (21C, 60% humidity) with a 12:12hr light:dark cycle. We controlled the
370 developmental larval density by manually aliquoting 32 ul of collected eggs into individual
371 bottles containing 25-50mL of food at 25C. Following eclosion, mixed sex flies were kept on
372 SY10% medium (10% w/v sucrose and 10% w/v yeast) for 2-4 days until they were sorted by
373 sex and transferred onto holidic food or kept on SY10% food, as needed, for experiments.
374 Experimental flies were flipped to fresh food e/o day until completion of the experiment. Unless
375 otherwise noted, we used mated female flies that were between 7-12 days old for all
376 experiments. The following stocks were used for experiments: *Canton-S and* w^{1118} were obtained
377 from the Bloomington *Drosophila* Stock Center. *UAS-CsChrimson* (BDSC #55135 and #55136),
378 *UAS-H3.3A-GFP* (BDSC #68241), *UAS-HIRA RNAi* (BDSC #35346), *GMR50H05-GAL4*
379 (BDSC #38764), *Trh-GAL4* (BDSC #38388), *UAS-BCAT RNAi* (VDRC KK110229) were
380 purchased from BDSC or the Vienna *Drosophila* Resource Center, as indicated. *PoxN* mutants
381 were provided by J. Alcedo (Wayne State University). *Nsyb-GAL4* was provided by L. Buttitta
382 (University of Michigan, MI) and Nsyb-GeneSwitch-GAL4 was obtained from A. Sehgal

383 (Perelman School of Medicine, PA). *UAS-GTACR* was provided by M. Dus (University of
384 Michigan, MI). All transgenic lines used in this study, with the exception of *UAS-H3.3A-GFP,*
385 *UAS-HIRA RNAi, Nsyb-GAL4, and Nsyb-GeneSwitch-GAL4* were back-crossed 10 generations to
386 *w-;Canton-S* prior to experiments.
387
388 **Holidic food**
389 Holidic media were prepared according to previous protocols with some modifications[20]. For all
390 experiments, we used the Yaa ratio of amino acids, but increased each amino acid, sucrose, and
391 agar (see Extended Data Table 1). Briefly, agar, sucrose, branched-chain amino acids and amino
392 acids with low solubility (L-Leucine, L-Isolecuine, L-Valine, L-Tyrosine) were added to
393 solutions containing metal ions and cholesterol. The mixtures were autoclaved for 20 minutes.
394 Filter-sterilized acetate buffer and solutions of the remaining amino acids, vitamins, nucelotides,
395 inositol, choline, and preservatives were added while mixtures were stirred on a hot plate not to
396 exceed 65C. After mixing, 2mL of food was dispensed into vials and stored at 4C until use, but
397 no longer than 3 weeks.
398
399 **Drug administration**
400 Sodium Butyrate was purchased from abcam (ab120948) and Trichostatin A was purchased from
401 Cayman (Item No. 89730). Prior to the lifespan experiment, TSA was dissolved in 100% ethanol
402 to a concentration of 1 mM, aliquoted, and frozen at -20°C. Food was prepared fresh e/o day as
403 follows: a TSA aliquot was thawed and sodium butyrate was added as powder to the TSA stock.
404 Appropriate holidic diet food was melted and allowed to cool slightly, then the inhibitor cocktail
405 was added (final concentration of TSA was 10 μm and sodium butyrate was 10 mm), gently

406 mixed, and dispensed to new vials. For Con-Ex or western blot experiments using only sodium
407 butyrate, holidic food was melted and sodium butyrate was added as powder to a final
408 concentration of 100 mm. Roughly 250 ul of sodium butyrate-containing food was layered on
409 top of appropriate holidic food vials and cooled before use.
410
411 For the pulse-chase GeneSwitch experiment, RU486 (mifepristone) was dissolved in 80% (v/v)
412 ethanol at 10 mM concentration and marked by blue dye (5% [w/v] FD&C Blue No. 1; Spectrum
413 Chemical) and stored at -20°C. Holidic food + RU486 was prepared by melting appropriate
414 holidic foods and adding RU486 to a final concentration of 200 μM.
415
416 **Lifespan measurements**
417 Lifespans were measured using established protocols[65]. We established 6-10 replicate vials for
418 each treatment, with 20 flies per vial. Flies were transferred to fresh media every 2-3 days, at
419 which time dead flies were removed and recorded using the DLife system [65]. Flies were kept in
420 constant temperature (25C) and humidity (60%) conditions with a 12:12hr light:dark cycle.
421 Normally, we conducted at least two experimental replicates of each lifespan experiment.
422
423 **Optogenetic Assays**
424 Flies expressing UAS-CsChrimson in desired neuronal populations were reared as described
425 above. Upon eclosion, females were sorted to SY10% medium containing 800 μm all-*trans*-
426 retinal (from a stock solution of 100mM ATR in 100% ethanol) and kept in the dark for 2 days.
427 Flies were then flipped to appropriate holidic diet food containing 400 μm ATR (and flipped to
428 fresh ATR-containing food e/o day for the duration of the experiment) and moved to a custom

429 rig containing 627nm (red) LEDs or 530nm (green) LEDs (Luxeon) or kept in the dark in the
430 same incubator (Fig. S3). The custom rig is fully enclosed to prevent leakage of light and houses
431 48 individual vials surrounded by mirrors. Custom hardware and firmware were designed to
432 allow the experimenter to control the LED intensity and a range of other light stimulus
433 parameters. Lifespan and Con-Ex experiments used a stimulus frequency of 40 Hz and a pulse
434 width of 800 ms. Flies were exposed to this protocol every day for 12 hours per day during the
435 light period, followed by 12 hours of darkness.
436
437 **Consumption-excretion feeding assays**
438 Con-Ex experiments were carried out as previously described[27,28]. Experimental female flies
439 were sorted to appropriate BCAA diets (10 flies per vial, 8-10 replicates for each treatment)
440 when they were >4 days old. Food was changed every 2-3 days until the appropriate
441 experimental time-point was reached (5 days for standard experiments, unless otherwise
442 indicated). After the dietary pre-treatment period, blue test food was prepared in removable caps
443 by adding 1% (w/v) FD&C Blue No. 1 to the appropriate diets. Flies were moved to fresh vials
444 with the removable blue-food caps on the top of the vials and were allowed to feed and excrete
445 for 24 hours. Caps and flies were removed after the 24-hour test period and flies were counted.
446 Excreted dye was collected by vortexing each vial with 3mL of water. Concentration of the dye
447 was determined by absorbance at 630nm and compared to a standard curve of known
448 concentrations. For short-term Con-Ex experiments lasting <3 hours, both excretions and flies
449 were collected and measured. To measure internal blue concentration, groups of flies were
450 homogenized in 1mL of phosphate-buffered saline containing 0.1% Trixon X-100 (IBI
451 Scientific) for 30s at 30Hz using a QIAGEN TissueLyser. Concentration of internal extracts

452 were determined by absorbance at 630nm and both internal and excreted concentrations were
453 summed to determine total consumption.
454
455 **Fly Liquid-Food Interaction Counter (FLIC) assays**
456 Flies were tested on the Fly Liquid-Food Interaction Counter (FLIC) system as previously
457 described[31](Sable Systems International). Female flies were starved for 20-24 hours in vials
458 containing a kimwipe with 2mL of Milli-Q water. For single-choice FLIC experiments, flies
459 were flipped to low-BCAA, high-BCAA, or fresh starvation vials at 10AM the morning of
460 testing and allowed to feed for 3 hours. Re-feeding food was spiked with blue dye to ensure
461 feeding, and flies with visually blue bellies were selected for the experiment. FLIC *Drosophila*
462 Feeding Monitors (DFMs, Sable Systems International, model DFMV3) were loaded with a food
463 solution containing 5% sucrose and 5% yeast extract (w/v) in 4 mg/l $MgCl_2$. After re-feeding,
464 flies were briefly anesthetized on ice and aspirated into the DFM chambers. We began recording
465 immediately after loading flies (generally, loading all DFMs requires <10 minutes) and measured
466 FLIC interactions for 3 hours. For FLIC experiments that contained a choice environment in the
467 common garden, re-feeding food was prepared fresh for each experiment by melting SY10%
468 medium with or without 1% L-Isoleucine (w/v). FLIC DFMs were loaded with food solutions
469 containing either 2% sucrose OR [2% yeast extract + 1% sucrose] in 4 mg/l $MgCl_2$ and FLIC
470 interactions were recorded for 24 hours. Each DFM was loaded with flies from at least two
471 treatment groups to reduce technical bias from individual DFM signals. FLIC data were analyzed
472 using custom R code, which is available at https://github.com/PletcherLab/FLIC_R_Code.
473 Default thresholds were used for analysis except for the following: minimum feeding threshold =
474 10, tasting threshold = (0,10). Animals that did not participate (i.e. returned zero values) were

475 excluded from analysis. FLIC data are generally non-normal, and thus are expressed as a box-
476 cox transformation to the 0.25 power of the total interactions, which yielded normal
477 distributions.
478
479 **Western Blots**
480 Experimental flies were flash frozen in liquid nitrogen after appropriate diet or light exposures.
481 Heads and bodies were separated using a metal sieve on dry ice, and 10 heads were pooled for
482 each biological replicate. Heads were first pulverized to a fine powder using a plastic pestle on
483 dry ice. Protein extraction was carried out on ice using RIPA buffer (Sigma Aldrich)
484 supplemented with protease inhibitor cocktail (Sigma), phosphatase inhibitor cocktail (Sigma),
485 sodium orthovanadate (NEB, 1mM), sodium fluoride (NEB, 1 mM), and sodium butyrate
486 (100uM). 150uL of ice cold buffer was added to heads followed by immediate homogenization
487 with a motorized pestle for 10 seconds on ice. Lysates were incubated on ice for 10 minutes
488 followed by 20 seconds of sonication and centrifugation at 16000xg at 4C for 10 minutes.
489 Protein lysates were added 1:1 to 2X protein sample buffer (1mM Tris-HCL pH 6.8, 10% SDS,
490 1% Bromophenol blue, and 1M DTT) and denatured at 95°C for 10 minutes. Protein was
491 separated by SDS-PAGE on a 4-12% gel (Biorad) at 200V for 30 minutes, followed by
492 electrophoretic transfer to PVDF or nitrocellulose membrane at 70V for 1 hour. Blots were
493 incubated in 5% milk in .1% TBS-T at room temperature for one hour, followed by overnight
494 incubation with primary antibodies overnight at 4°C. Membranes were washed with .1% TBS-T
495 and incubated with HRP-conjugated secondary antibodies (abcam) at room temperature for 1-4
496 hours. Membranes were washed again with .1% TBS-T and then incubated briefly in ECL
497 substrate (SuperSignal West Femto, ThermoFisher) before imaging. Band detection and

498 quantification was performed using Image Lab software (Bio-Rad). Rabbit anti-histone H3
499 (abcam, ab1791, 1:20000), Rat anti-histone H3K9ac (active motif, #61663, 1:500), Rabbit anti-
500 LaminB1(CST, #13435S, 1:2000) Rabbit anti-Beta-Tubulin (abcam, ab179513, 1:500), rabbit
501 anti-pS6K(T398) (#9209S 082813, 1:1000), mouse anti-H4K9me3 (Millipore #05-1242,
502 1:1000), rabbit anti-H3K27ac (D. Lombard, 1:2000), rabbit anti-ubiquitin (Santa Cruz 1:5000),
503 Rabbit anti-histone H4 (abcam, ab10158 1:5000), mouse anti-GAPDH (proteintech #60004
504 1:1000), rabbit anti-dS6K (T. Neufeld 1:3500), rabbit anti-pAKT (CST 1:1000), rabbit anti-AKT
505 (CST 1:1000), and rabbit anti-GFP (abcam, 1:1000) were used for primary antibody staining.
506

507 **RNA extraction and RT-qPCR**

508 Experimental flies were flash frozen in liquid nitrogen after appropriate diet exposures. Heads
509 and bodies were separated using a metal sieve on dry ice, and 10 heads were pooled for each
510 biological replicate. Total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific,
511 #15596026) following the manufacturer's instructions. Flies were collected into nuclease-free
512 lysing tubes with matrix D beads (MPbio, #6913-500-129984) and 300 ul of TRIzol, then
513 homogenized for two 15-s pulses at 6.5 M/s. Lysates were incubated at room temperature for 10
514 min, and then 100 ul of chloroform (Sigma-Aldrich) was added for phase separation. 175 ul of
515 RNA containing supernatant was transferred to nuclease-free tubes and mixed with an equal
516 volume of isopropanol (Sigma-Aldrich) to precipitate the RNA, and centrifuged at 12000xg for
517 15 min at 4°C. The pellet was washed twice with 70% cold EtOH (Sigma-Aldrich), air dried, and
518 redissolved in 25 ul of nuclease-free water. RNA concentration and quality was determined using
519 a NanoDrop One (Thermo Fisher Scientific). Complementary DNA (cDNA) was prepared from
520 1 ug of total RNA using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher

521 Scientific) and the resulting cDNA was diluted 1:50 before use. No-RT reactions were prepared
522 and run as controls to ensure no genomic DNA contamination. Quantitative polymerase chain
523 reactions (qPCRs) contained 1X PowerUp SYBR Green PCR Master Mix (Applied Biosystems),
524 500 nM appropriate primers, and 5 ul 1:50 cDNA for a total reaction volume of 10 ul. C_T values
525 were calculated using an absolute threshold of ΔRn=0.1 and relative expression was determined
526 using the comparative C_T approach [66]. Primers used in this study are:
527 *RpL-32-RA*_Forward (cgg atc gat atg cta agc tgt) and
528 *RpL-32-RA*_Reverse (gcc ctt gtt cga tcc gta)
529 *H3.3A*_Forward (GAAGAAGCCACATCGCTACC) and
530 *H3.3A*_Reverse (CACAGATTGGTGTCCTCGAA)
531 *H3*_Forward (ACCGAGCTTCTAATCCGCAAG) and
532 *H3*_Reverse (ACCAACCAGGTAGGCTTCGC)
533 *CG1673*_Forward (TGCGCTTTTACTTCCAAGCAGCA) and
534 *CG1673*_Reverse(GGGCCTAGGTTCTACTGACGGGT)
535 *Trh*_Forward(GTGCTCCAGTTTTGACTTCGG) and
536 *Trh*_Reverse(TTTACGGTACACGGGGTCCT)
537 *Tph*_Forward(CCTCTGTACTATGTGGCCGA) and
538 *Tph*_Reverse(TCGAGTCGAGAACCTCAACA)
539
540 **Brain Immunohistochemistry**
541 Adult brain immunostaining was performed as previously described[67]. Adult brains were
542 dissected and fixed in PBS, pH 7.4 containing 3.7% formaldehyde for approximately 1 hr. Fixed
543 brains were washed quickly 3 times followed by 3 – 20 min washes in 0.1% PBS-T, or 0.5%

544 PBS-T for histone immunostaining, with gentle shaking at room temperature. Brains were
545 blocked using 5% normal goat serum (NGS) in 0.1% PBS-T for at least 30 min at room
546 temperature with gentle rocking, then incubated in primary antibody diluted in 5% NGS for two
547 nights at 4°C. After primary antibody incubation, brains were washed 3 times in PBS-T and
548 incubated in secondary antibody diluted 1:500 in 5% NGS for one night at 4°C. Brains were
549 washed 3 times in PBS-T and mounted between a glass microscope slide and a #1.5 cover glass
550 separated by a custom bridge in VECTASHIELD Antifade Mounting Medium (Vector
551 Laboratories). Samples were imaged on a Nikon A1 Confocal Microscope using either a 20X air
552 or 40X oil lens objective. All treatments were mounted under the same cover slip and at least two
553 slides per experiment were imaged. Image processing was performed using ImageJ (NIH). ROIs
554 were drawn by hand around appropriate cell bodies and values are background-subtracted.
555 Images are representative maximum intensity projections compiled from 1-2 μm thick sections
556 of the indicated number of z-stacks and are contrast matched. Rabbit anti-serotonin (Sigma,
557 S5545 1:6000), mouse anti-nc82 (DSHB 1:20) and rabbit anti-histone H3 (abcam, ab1791
558 1:20000) were used for primary antibody staining, and Alexa Fluor 488 and 594 were used for
559 secondary antibody staining (Life Technologies 1:1000).

560

561 **Egg laying assay**

562 Following eclosion, male and female flies were allowed to mate on SY10% food for 48 hours.
563 Groups of 5 males and 5 females were sorted to appropriate holidic diets. Food was changed
564 every day for the first 2 days and e/o day thereafter and eggs were counted from the old media.
565 Egg counts were obtained from 8-10 vials per treatment.

566

567 **Triacylglyceride and Total Protein Quantification**

568 Experimental flies were flash frozen in liquid nitrogen after exposure to appropriate diets for one
569 week. Two experimental flies per biological replicate were homogenized in 200 µl PBS/0.05%
570 Triton-X with protease inhibitor cocktail (Sigma). The homogenate was added into Infinity
571 Triglyceride Reagent (Thermo Electron Corp.) according to manufacturer's instructions. In
572 parallel, the homogenate was added to BCA working reagent (Pierce BCA Protein Assay Kit)
573 according to manufacturer's instructions. TAG concentrations were determined by the
574 absorbance at 520nm and estimated by a known triglyceride standard. Total protein
575 concentrations were determined by absorbance at 562 nm and estimated by a known protein
576 albumin standard.

577

578 **Activity Assay**

579 Activity recordings and data processing were performed using the *Drosophila* Activity Monitor
580 System (TriKinetics). After diet exposure for 5 days, adult flies were individually tested in 5 mm
581 x 65 mm polycarbonate tubes with the appropriate diet food at one end of the testing tube. The
582 first day of data was removed from the final analysis in order to allow for acclimation to
583 experimental housing conditions. Total activity counts were calculated for each fly by summing
584 all activity counts recorded during the light and dark cycle, respectively. Experiments were
585 performed at 25°C and 60% humidity under a 12-hour light:12-hour dark cycle.

586

587

588

589 **Statistics**

590 Pairwise comparisons between treatment survivorship curves were carried out using the
591 statistical package R with DLife, as previously described[65]. P-values were obtained using the
592 log-rank test. For all other comparisons involving only one level, we used Student's t-test to
593 detect significant differences between two treatments or one-way ANOVA followed by Tukey's
594 post-hoc test after verifying normality and equality of variances. T-tests were two-tailed during
595 initial characterization experiments, or one-tailed in future experiments where the predicted
596 direction of change was known. For comparisons involving more than one level, we used two-
597 way ANOVA to detect significant interactions between the levels and followed up with Tukey's
598 post-hoc when significance was detected ($p<0.05$). In cases where data were non-normally
599 distributed (FLIC data), we performed a box-cox transformation to the 0.25 power before
600 computing P-values. In cases where experimental replicates were pooled, a two-way ANOVA
601 with blocking for experiment was performed to ensure non-significant experimental effects. P-
602 values for experiments with less than 3 biological replicates per treatment are not reported. For
603 all dot and bar plots, error bars represent the SEM. All statistical tests and graphing were
604 performed using R. Specific details of statistical analyses are presented in the figure legends.

605

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765 **Author contributions:**

766 Conceptualization: KJW, SDP

767 Methodology: KJW, SDP

768 Investigation: KJW, RAH, EH, SDP

769 Visualization: KJW, EH

770 Funding acquisition: KJW, SDP

771 Project administration: KJW, SDP

772 Supervision: KJW, SDP

773 Writing – original draft: KJW, SDP

774 Writing – review & editing: KJW, SDP

775

776 **Competing interests:** The senior author (S.D.P) is a share holder in the company, Flidea, which
777 has developed technology related to the FLIC feeding system. **Data and materials availability:**
778 The datasets generated during the current study are available from the corresponding author on
779 reasonable request.nFLIC data were analyzed using custom R code, which is available at
780 https://github.com/PletcherLab/FLIC_R_Code.

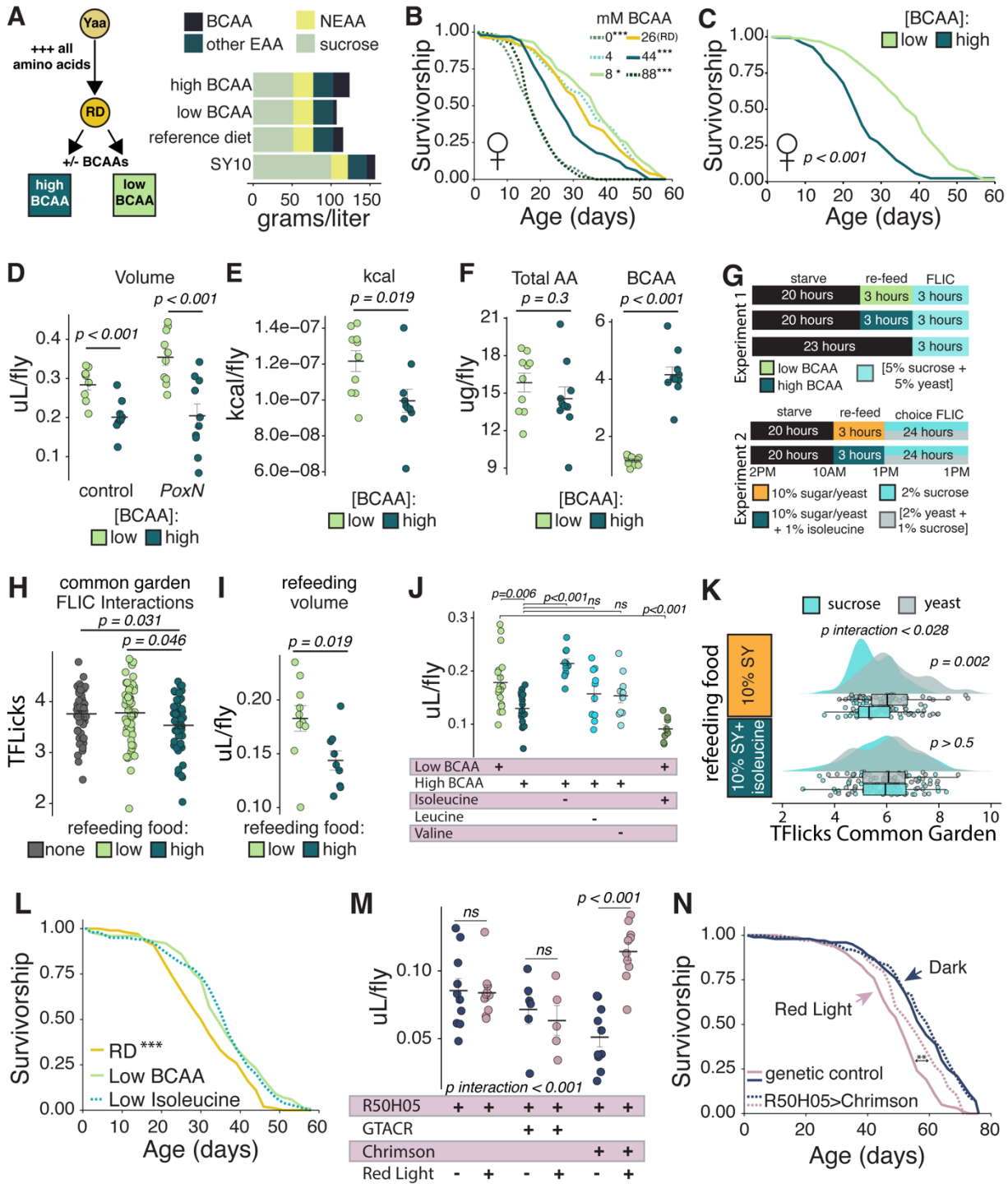

Figure 1

781

782

783 **Figure 1. Hunger extends lifespan independent of appetite in dietary and genetic models of**
784 **hungry flies. (A)** Schematic (left) and relative composition (right) of experimental diets. Details
785 in Supplementary Tables S1-2 and Methods. Yaa=baseline diet as in (*20*), RD=reference diet,
786 containing 51.36g/L carbohydrate, 34.9g/L non-essential amino acids, 22.56g/L non-BCAA
787 essential amino acids and, 12.39g/L BCAAs. **(B)** Lifespan of *Canton-S* flies on diets of indicated
788 BCAA concentration (log-rank test, p-values derived by comparison to RD, N=159-172). **(C)**
789 Lifespan of *Canton-S* flies on low- or high-BCAA diets (log-rank test, N=173-175). **(D-F)** 24-hr
790 Con-Ex measurement of w^{1118} or *PoxN* flies on indicated diets. Volume (D), kcal (E), and total or
791 BCAA-only amino acid intake (F) (two-tailed Student's t-test). **(G)** Schematic of FLIC
792 experimental designs used in H-I (top panel) and K (bottom panel). **(H)** FLIC interactions in
793 common garden (one-way ANOVA with Tukey's post-hoc. Experimental replicates are pooled,
794 N=52-55). **(I)** Con-Ex measurement of volumetric intake during re-feeding period (two-tailed
795 Student's t-test). **(J)** Con-Ex measurement on diets with individual BCAA reductions (one-way
796 ANOVA with Tukey's post-hoc). **(K)** FLIC interactions in food choice environment (2% sucrose
797 or [2% yeast + 1% sucrose]) after re-feeding SY food +/- 1% isoleucine (two-way ANOVA with
798 Tukey's post-hoc. Experimental replicates are pooled, N=59-60). **(L)** Lifespan of *Canton-S* flies
799 on RD, low-BCAA, or low-isoleucine diets (log-rank test, p-values derived by comparison to
800 low-BCAA diet, N=171-177). **(M-N)** Con-Ex and lifespan measurements of flies carrying
801 R50H05-GAL>UAS-CsChrimson or R50H05-GAL4/w-;CS controls exposed to red light for 12
802 hours per day or kept in constant darkness. (M) 24-hr Con-Ex measurement (two-way ANOVA
803 with Tukey's post-poc). (N) Lifespan measurement (log-rank test, N=101-123). All FLIC data
804 are expressed as box-cox transformation to the 0.25 power (termed TFLicks) to achieve
805 normality. *p<0.05, **p<0.01,***p<0.001.

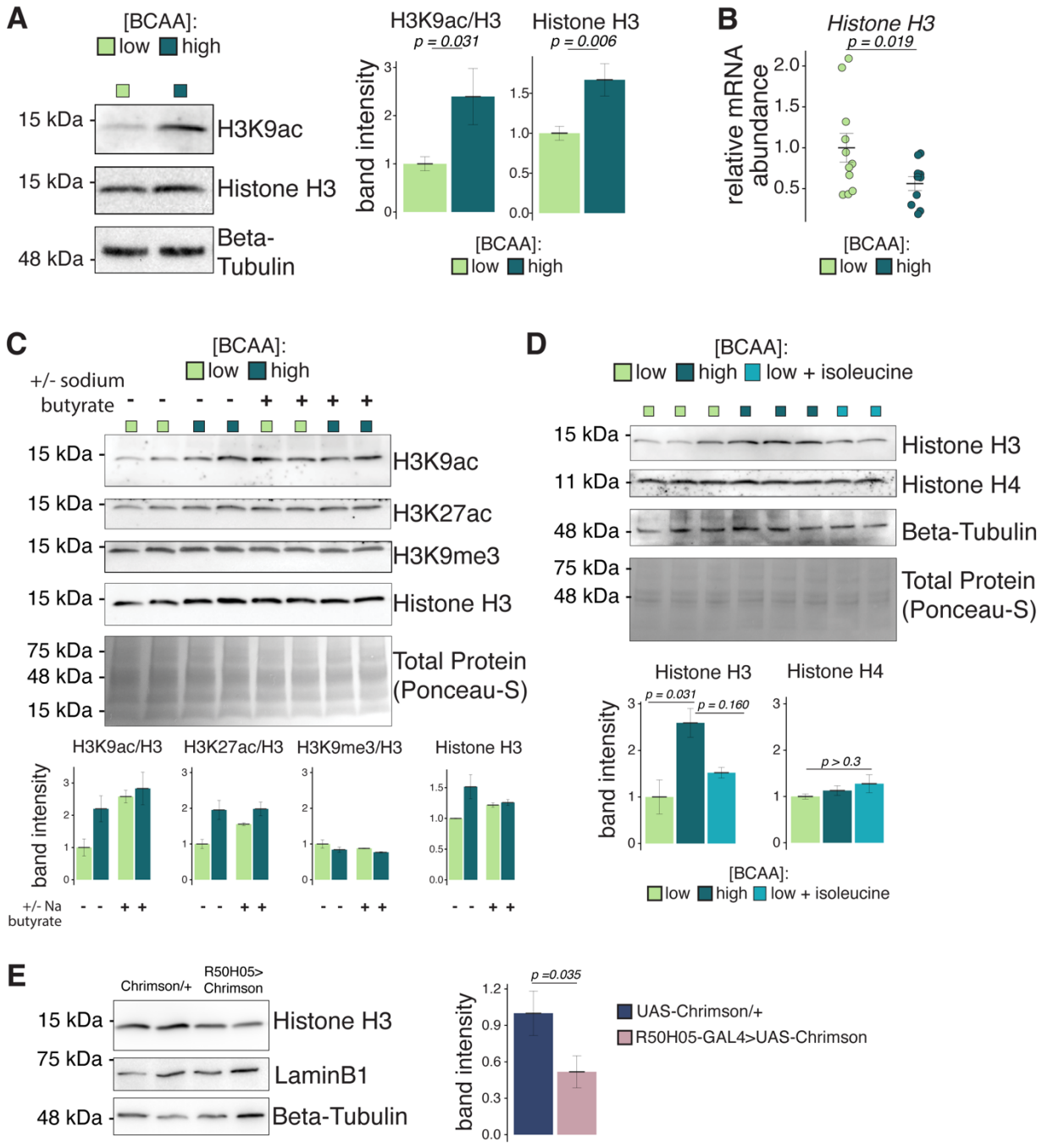
A
[BCAA]:
low high
15 kDa
H3K9ac
15 kDa
Histone H3
48 kDa
Beta-Tubulin
H3K9ac/H3
p = 0.031
Histone H3
p = 0.006
band intensity
[BCAA]:
low high
B
Histone H3
p = 0.019
relative mRNA abundance
[BCAA]:
low high
C
[BCAA]:
low high
+/- sodium butyrate
15 kDa
H3K9ac
15 kDa
H3K27ac
15 kDa
H3K9me3
15 kDa
Histone H3
75 kDa
48 kDa
15 kDa
Total Protein (Ponceau-S)
H3K9ac/H3
H3K27ac/H3
H3K9me3/H3
Histone H3
band intensity
+/- Na butyrate
D
[BCAA]:
low high low + isoleucine
15 kDa
Histone H3
11 kDa
Histone H4
48 kDa
Beta-Tubulin
75 kDa
48 kDa
Total Protein (Ponceau-S)
Histone H3
Histone H4
p = 0.031
p = 0.160
p > 0.3
band intensity
[BCAA]:
low high low + isoleucine
E
Chrimson/+
R50H05> Chrimson
15 kDa
Histone H3
75 kDa
LaminB1
48 kDa
Beta-Tubulin
p =0.035
band intensity
UAS-Chrimson/+
R50H05-GAL4>UAS-Chrimson

Figure 2

806

807 **Figure 2. Dietary BCAAs reshape the neuronal epigenome by altering histone acetylation**
808 **and histone H3 abundance. (A)** Representative western blot for H3K9ac and total histone H3 in
809 *Canton-S* fly heads after exposure to indicated diets for 5-7 days, quantified in right panel (one-
810 way ANOVA. 10 heads per biological replicate, experimental replicates are pooled, N=11). **(B)**
811 RT-qPCR measurement of relative mRNA abundance in *Canton-S* heads after 5-7 days on low-
812 or high-BCAA, values are normalized to low-BCAA treatment (one-way ANOVA. 10 heads per
813 biological replicate, experimental replicates are pooled, N=10-11). **(C)** Western blot for histone
814 PTMs in *Canton-S* fly heads after exposure to indicated diets +/- 100 mM sodium butyrate for 5-
815 7 days, bands are quantified in bottom panel and normalized to low-BCAA treatment (10 heads
816 per biological replicate, N=2). **(D)** Western blot for histone H3 and H4 in *Canton-S* heads on
817 low-BCAA, high-BCAA, or [low-BCAA + high isoleucine] diets, quantified in bottom panel and
818 normalized to low-BCAA treatment (one-way ANOVA with Tukey's post-hoc. 10 heads per
819 biological replicate, N=2-3). **(E)** Western blot for histone H3 in heads of flies carrying R50H05-
820 GAL>UAS-CsChrimson or UAS-CsChrimson/w-;CS controls exposed to red light for 12 hours,
821 quantified in right panel and normalized to UAS-CsChrimson/w-;CS control (one-way ANOVA.
822 10 heads per biological replicate, N=4-5).
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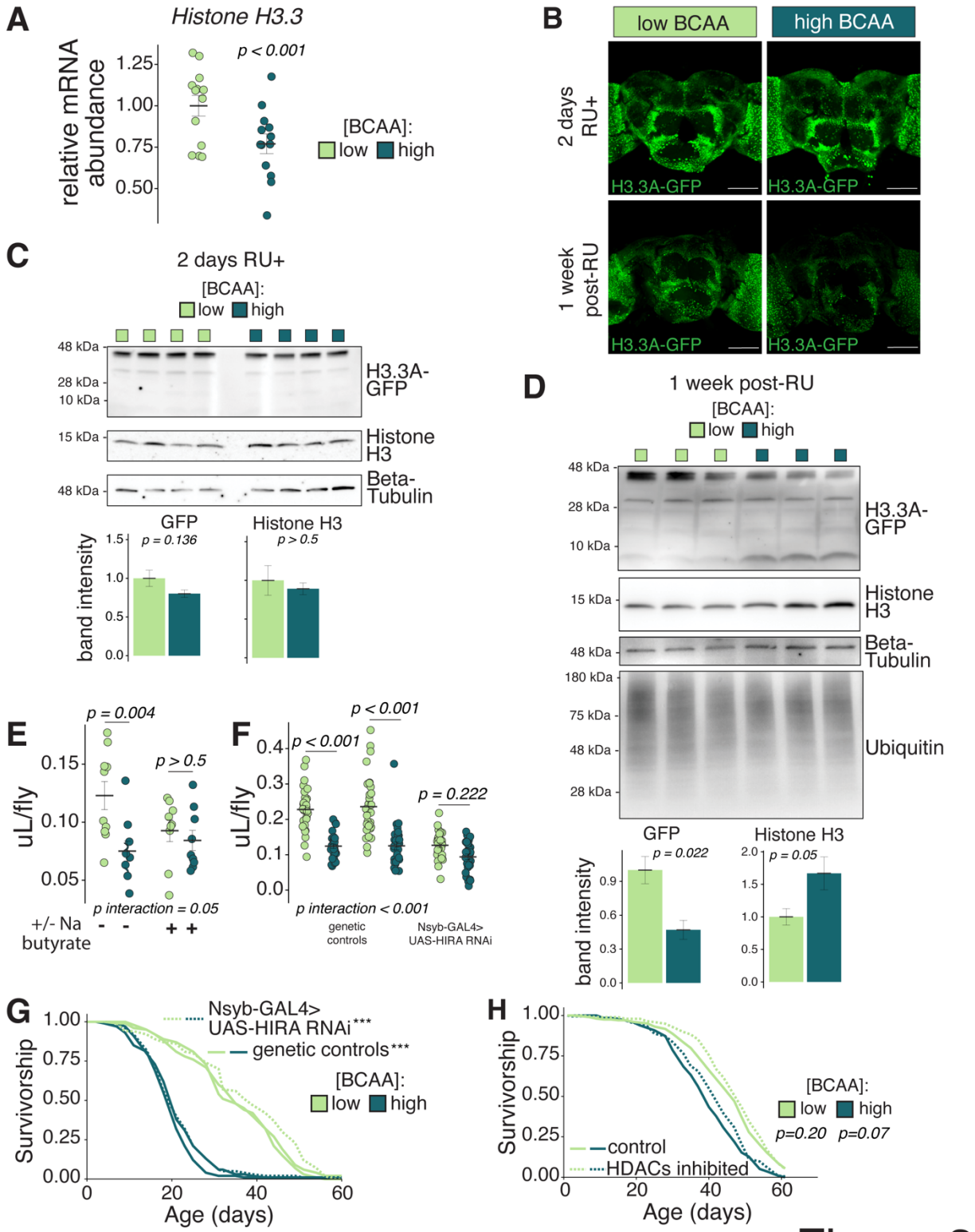
A
Histone H3.3
relative mRNA abundance
p < 0.001
[BCAA]:
low high
B
low BCAA
high BCAA
2 days RU+
1 week post-RU
H3.3A-GFP
C
2 days RU+
[BCAA]:
low high
48 kDa
28 kDa
10 kDa
H3.3A-GFP
15 kDa
Histone H3
48 kDa
Beta-Tubulin
GFP
Histone H3
p = 0.136
p > 0.5
band intensity
D
1 week post-RU
[BCAA]:
low high
48 kDa
28 kDa
10 kDa
H3.3A-GFP
15 kDa
Histone H3
48 kDa
Beta-Tubulin
180 kDa
75 kDa
48 kDa
28 kDa
Ubiquitin
GFP
Histone H3
p = 0.022
p = 0.05
band intensity
E
p = 0.004
p > 0.5
uL/fly
p interaction = 0.05
+/- Na butyrate
- - + +
F
p < 0.001
p < 0.001
p = 0.222
uL/fly
p interaction < 0.001
genetic controls
Nsyb-GAL4> UAS-HIRA RNAi
G
Nsyb-GAL4> UAS-HIRA RNAi***
genetic controls***
[BCAA]:
low high
Survivorship
Age (days)
H
[BCAA]:
low high
p=0.20 p=0.07
control
HDACs inhibited
Survivorship
Age (days)

Figure 3

832 **Figure 3. Utilization of the histone variant H3.3A in the brain is modulated by BCAAs to**
833 **stimulate feeding but is not required to extend lifespan. (A)** RT-qPCR quantification of
834 relative mRNA abundance of *H3.3A* in *Canton-S* heads after 5-7 days on low- vs. high-BCAA
835 diet, values are normalized to low-BCAA treatment (one-way ANOVA. 10 heads per biological
836 replicate, experimental replicates are pooled). **(B-D)** Pulse-chase of fluorescently labelled H3.3A
837 in brains (B) or heads (C-D) of flies carrying *Nsyb-GeneSwitch-GAL4>H3.3A-GFP* on low- or
838 high-BCAA diets. (B) Representative confocal images of brains immediately following the 2-day
839 pulse of H3.3A-GFP induced by BCAA food + RU486 (top panel) and 1 week after the pulse
840 (bottom panel). Scale bar 100μm. (C) Western blot of H3.3A-GFP (predicted molecular weight =
841 42 kDa) and histone H3 immediately following the 2-day pulse of H3.3A-GFP induced by
842 BCAA food + RU486, quantified in bottom panel (one-way ANOVA, 10 heads per biological
843 replicate). (D) Western blot of H3.3A-GFP (one-way ANOVA), histone H3 (one-tailed Student's
844 t-test), and ubiquitin (one-way ANOVA, p=0.571) 1 week after the H3.3A-GFP pulse, quantified
845 in bottom panel (10 heads per biological replicate). **(E)** Con-Ex measurement of 24-hr food
846 intake after 5 days on BCAA diets +/- 100 mM sodium butyrate (two-way ANOVA with
847 Tukey's post-hoc). **(F)** Con-Ex measurement of 24-hr food intake after 5-7 days on BCAA diets
848 from flies carrying *Nsyb-GAL4>UAS-HIRA RNAi* or controls (*Nsyb-GAL4/w-;CS* and *UAS-*
849 *HIRA RNAi/w-;*CS) (two-way ANOVA with Tukey's post-hoc, experiment replicates are
850 pooled). **(G)** Lifespans of flies carrying *Nsyb-GAL4>UAS-HIRA RNAi* or controls (*Nsyb-*
851 *GAL4/w-;CS* and *UAS-HIRA RNAi/w-;CS*) (log-rank test, N=184-202). **(H)** Lifespan of flies on
852 BCAA diets +/- 100 mm sodium butyrate (log-rank test, N=186-196).
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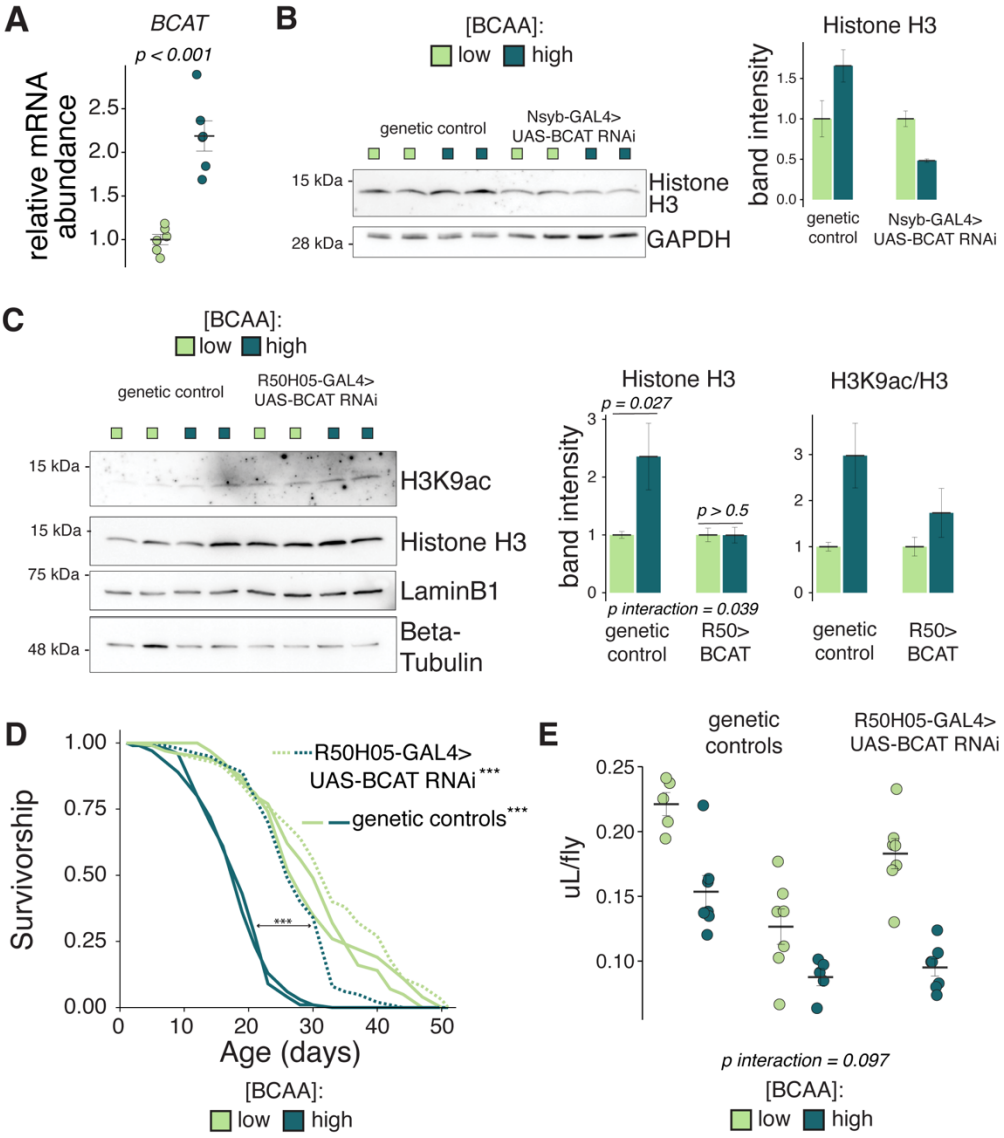

Figure 4

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856

857 **Figure 4. Hunger neurons use BCAA metabolism to regulate histone abundance and**
858 **modulate lifespan independent of feeding. (A)** RT-qPCR quantification of relative mRNA
859 abundance of *BCAT* (*CG1673)* in *Canton-S* heads after 5-7 days on BCAA diets, values are
860 normalized to low-BCAA treatment (one-way ANOVA, 10 heads per biological replicate). **(B)**
861 Western blot of histone H3 in heads of flies carrying *Nsyb-GAL>UAS-BCAT RNAi* or control
862 (*Nsyb-GAL4/w-;CS)*, quantified in right panel and normalized to low-BCAA treatment (two-way
863 ANOVA with Tukey's post-hoc, N=2). **(C)** Western blot of histone H3 and H3K9ac in heads of
864 flies carrying *R50H05-GAL4>UAS-BCAT RNAi* or control (*R50H05-GAL4/w-;CS)*, quantified in
865 right panel (two-way ANOVA with Tukey's post-hoc, experimental replicates pooled, N=5). **(D)**
866 Lifespans of flies carrying *R50H05-GAL4>UAS-BCAT RNAi* or controls (*R50H05-GAL4/w-;CS*
867 or *UAS-BCAT RNAi/w-;CS)* on low- or high-BCAA diets (log-rank test, N=91-100,
868 ***p<0.001,). **(E)** Con-Ex measurement of 24-hr food intake after 5 days on BCAA diets in flies
869 carrying *R50H05-GAL4>UAS-BCAT RNAi* or controls (*R50H05-GAL4/w-;CS* or *UAS-BCAT*
870 *RNAi/w-;CS)* (two-way ANOVA).
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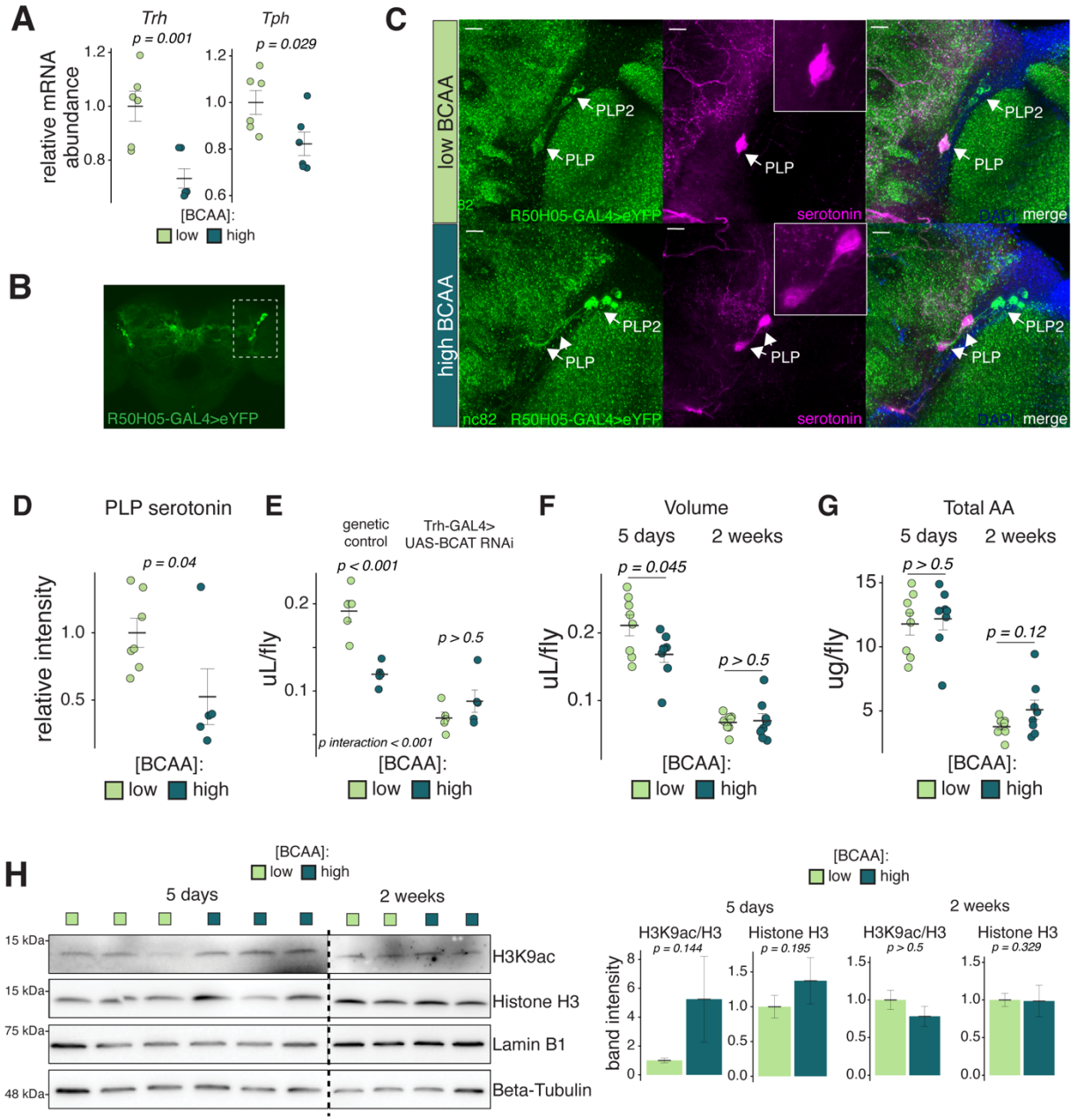
A
Trh
p = 0.001
Tph
p = 0.029
relative mRNA abundance
[BCAA]:
low
high
B
R50H05-GAL4>eYFP
C
low BCAA
high BCAA
PLP2
PLP
R50H05-GAL4>eYFP
serotonin
merge
nc82
D
PLP serotonin
p = 0.04
relative intensity
E
genetic control
Trh-GAL4> UAS-BCAT RNAi
p < 0.001
p > 0.5
p interaction < 0.001
uL/fly
F
Volume
5 days
2 weeks
p = 0.045
p > 0.5
G
Total AA
p > 0.5
p = 0.12
ug/fly
H
15 kDa
75 kDa
48 kDa
H3K9ac
Histone H3
Lamin B1
Beta-Tubulin
H3K9ac/H3
p = 0.144
p = 0.195
p > 0.5
p = 0.329
band intensity

Figure 5

881 **Figure 5. Spatially and temporally distinct processes link hunger to feeding and aging. (A)**
882 RT-qPCR quantification of relative mRNA abundance of *Trh* and *Tph* in *Canton-S* heads after 5-
883 7 days on BCAA diets, values are normalized to low-BCAA treatment (one-way ANOVA. 10
884 heads per biological replicate). **(B-D)** Immunostaining for serotonin in PLP neuron cell-bodies in
885 flies carrying *R50H05-GAL4>UAS-GTACR.eYFP* after 5-7 days on BCAA diets. (B) Maximum
886 intensity projection of ten 1.5 µm stacks highlighting the PLP neurons (white box) in the
887 posterior fly brain. Scale bar 100µm. (C) Representative confocal images of immunostaining for
888 serotonin in PLP neurons of flies carrying *R50H05-GAL4>UAS-GTACR.eYFP (*green=nc82,
889 magenta=serotonin, blue=DAPI, scale bar=10µm). Images are maximum intensity projections of
890 ten 1.5 µm stacks through the PLP neurons (D) Quantification of (C) as described in Methods
891 (one-way ANOVA, experimental replicates are pooled). **(E)** Con-Ex measurement of 24-hr food
892 intake after 5 days on BCAA diets in flies carrying *Trh-GAL4>UAS-BCAT RNAi* or control
893 (*Trh-GAL4/w-;CS)* (two-way ANOVA). **(F-G)** Con-Ex measurement of 24-hr food intake after 5
894 days or 2 weeks on BCAA diets in *Canton-S* flies; volume (F) and total amino acid intake (G)
895 (two-tailed Student's t-test). (H) Western blot of histone H3 and H3K9ac in heads of *Canton-S*
896 flies after 5 days vs. 2 weeks on BCAA diets, quantified in right panel (one-tailed Student's t-
897 tests, 10 heads per biological replicate, experimental replicates are pooled, N=3-5).
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Supplementary Materials for

Encoding of hunger by the neuronal epigenome slows aging in *Drosophila*

Weaver, KJ[1], Holt, RA[2], Henry, E[3], Pletcher, SD[1]

Correspondence to: spletch@umich.edu, kjweaver@umich.edu

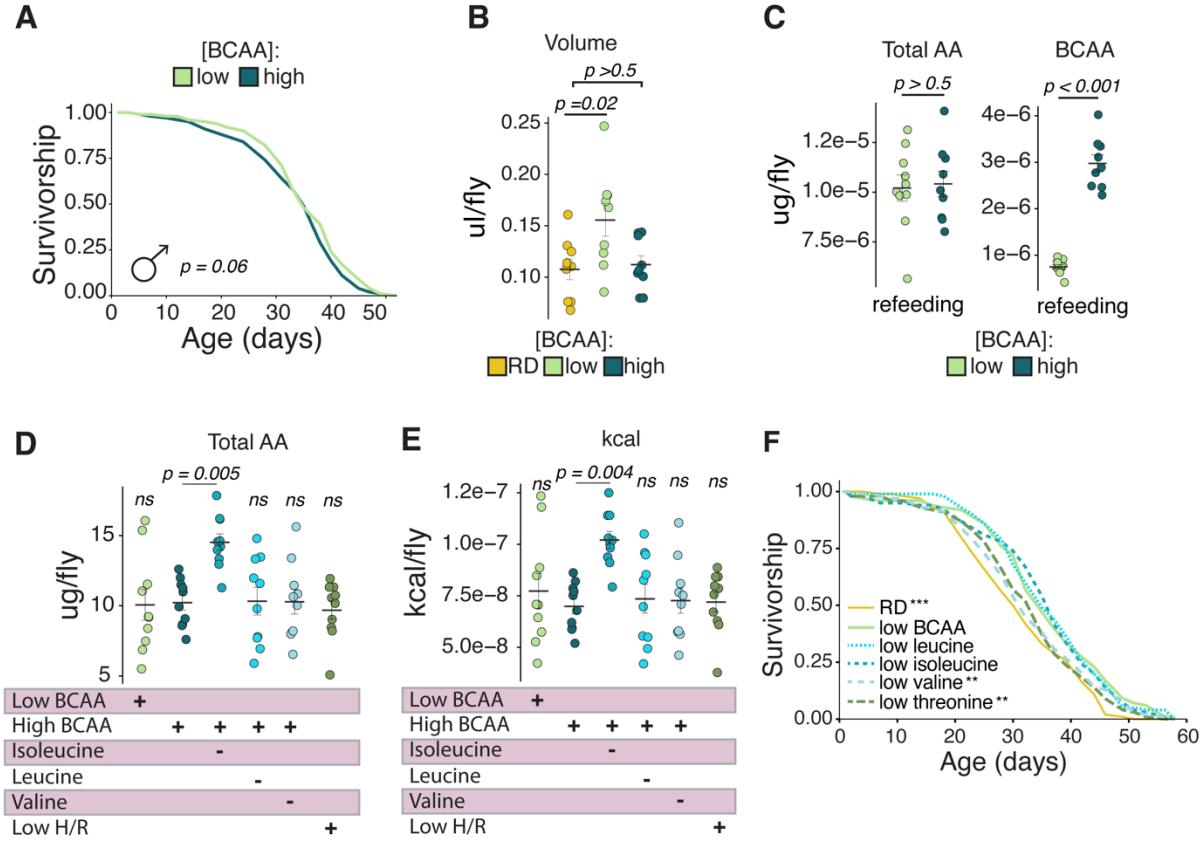
A
[BCAA]:
low high
Survivorship
1.00
0.75
0.50
0.25
0.00
p = 0.06
0 10 20 30 40 50
Age (days)
B
Volume
p >0.5
p =0.02
ul/fly
0.25
0.20
0.15
0.10
[BCAA]:
RD low high
C
Total AA
BCAA
p > 0.5
p < 0.001
ug/fly
1.2e−5
1.0e−5
7.5e−6
4e−6
3e−6
2e−6
1e−6
refeeding
refeeding
[BCAA]:
low high
D
Total AA
p = 0.005
ns ns ns ns
ug/fly
15
10
5
Low BCAA +
High BCAA + + + +
Isoleucine -
Leucine -
Valine -
Low H/R +
E
kcal
p = 0.004
ns ns ns ns
kcal/fly
1.2e−7
1.0e−7
7.5e−8
5.0e−8
Low BCAA +
High BCAA + + + +
Isoleucine -
Leucine -
Valine -
Low H/R +
F
Survivorship
1.00
0.75
0.50
0.25
0.00
RD ***
low BCAA
low leucine
low isoleucine
low valine **
low threonine **
0 10 20 30 40 50 60
Age (days)

Figure S1

19 **Figure S1. Isoleucine is necessary and sufficient to increase feeding and modulate aging.**

20 **(A)** Lifespan of male *Canton-S* flies on low- or high-BCAA diets, related to Figure 1B (log-rank

21 test, N= 217-231**). (B)** Total volume consumed by female *Canton-S* flies on RD, low-, or high-

22 BCAA diets for 1 week (one-way ANOVA with Tukey's post-hoc). **(C)** Total amino acid and

23 BCAA consumed during 3-hour refeeding low- or high-BCAA food, related to Figure 1I (one-

24 way ANOVA). **(D-E)** Total amino acid **(D)** and kcal **(E)** consumed by *Canton-S* flies on

25 indicated diets, related to Figure 1J. **(F)** Lifespans of *Canton-S* flies on indicated diets (log-rank

26 test, p-values derived by comparison to low-BCAA, N=170-177, **$p<0.01$, ***$p<0.001$).

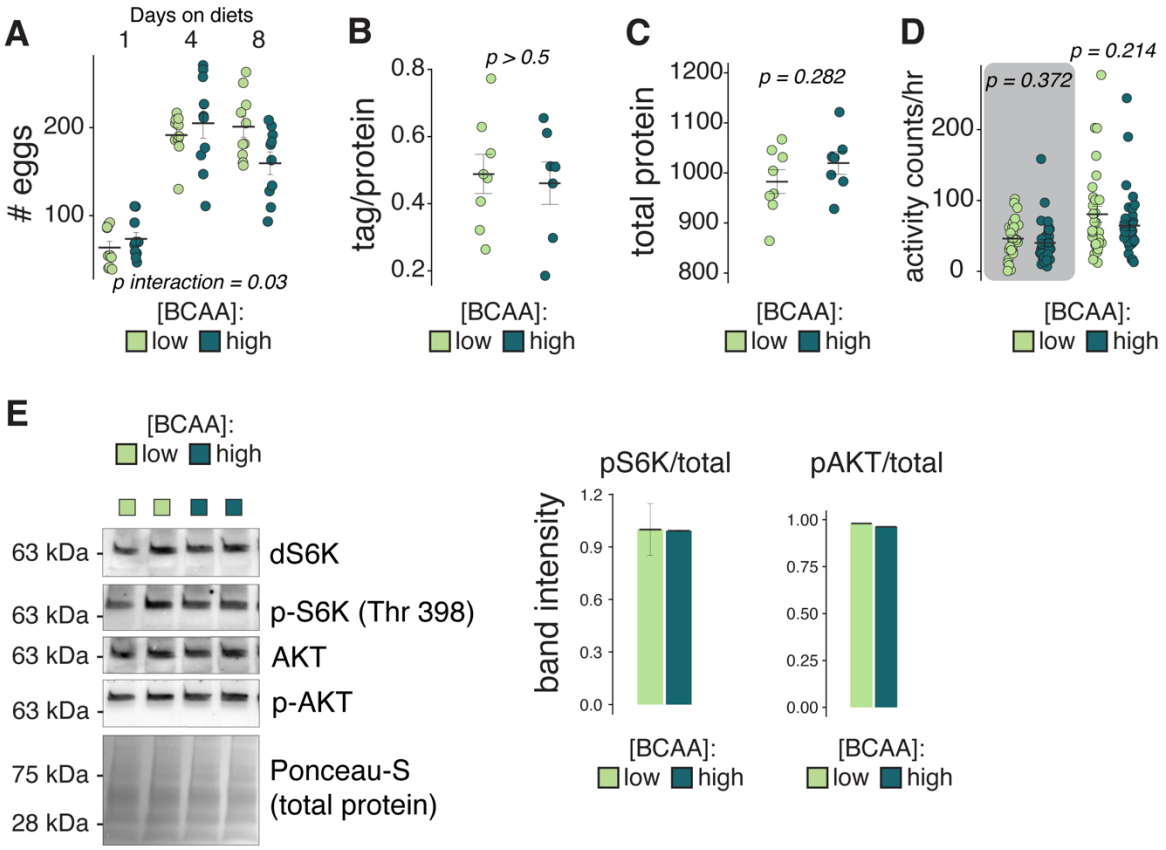

Figure S2

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29 **Figure S2. Physiological measures typically associated with aging are unchanged by dietary**
30 **BCAAs. (A)** Eggs laid by *Canton-S* flies on indicated diets after 1, 4, or 8 days on diet (two-way
31 ANOVA). **(B)** Total triacylglycerides in *Canton-S* flies after 1 week on BCAA diets (one-way
32 ANOVA, 2 flies per biological replicate). **(C)** Total protein in *Canton-S* flies after 1 week on
33 BCAA diets (one-way ANOVA, 2 flies per biological replicate). **(D)** Total activity counts of
34 *Canton-S* flies after 5 days on BCAA diets during dark (left) and light (right) period (one-way
35 ANOVAs). **(E)** Western blot and quantification of S6K and AKT activation in heads of *Canton-*
36 *S* flies after 1 week on BCAA diets (N=2).
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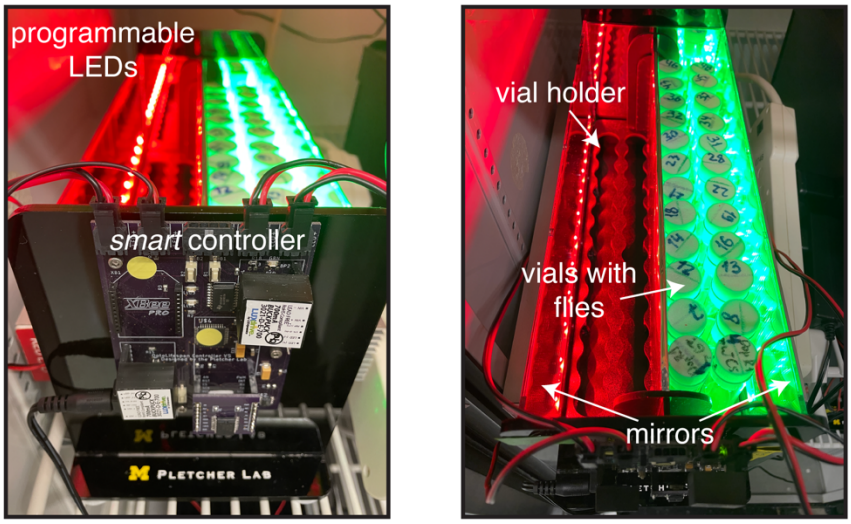

Figure S3

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53 **Figure S3. Custom rig for optogenetic experiments. (A)** Representative rig used for

54 optogenetic lifespan and Con-Ex experiments. Details can be found in methods.

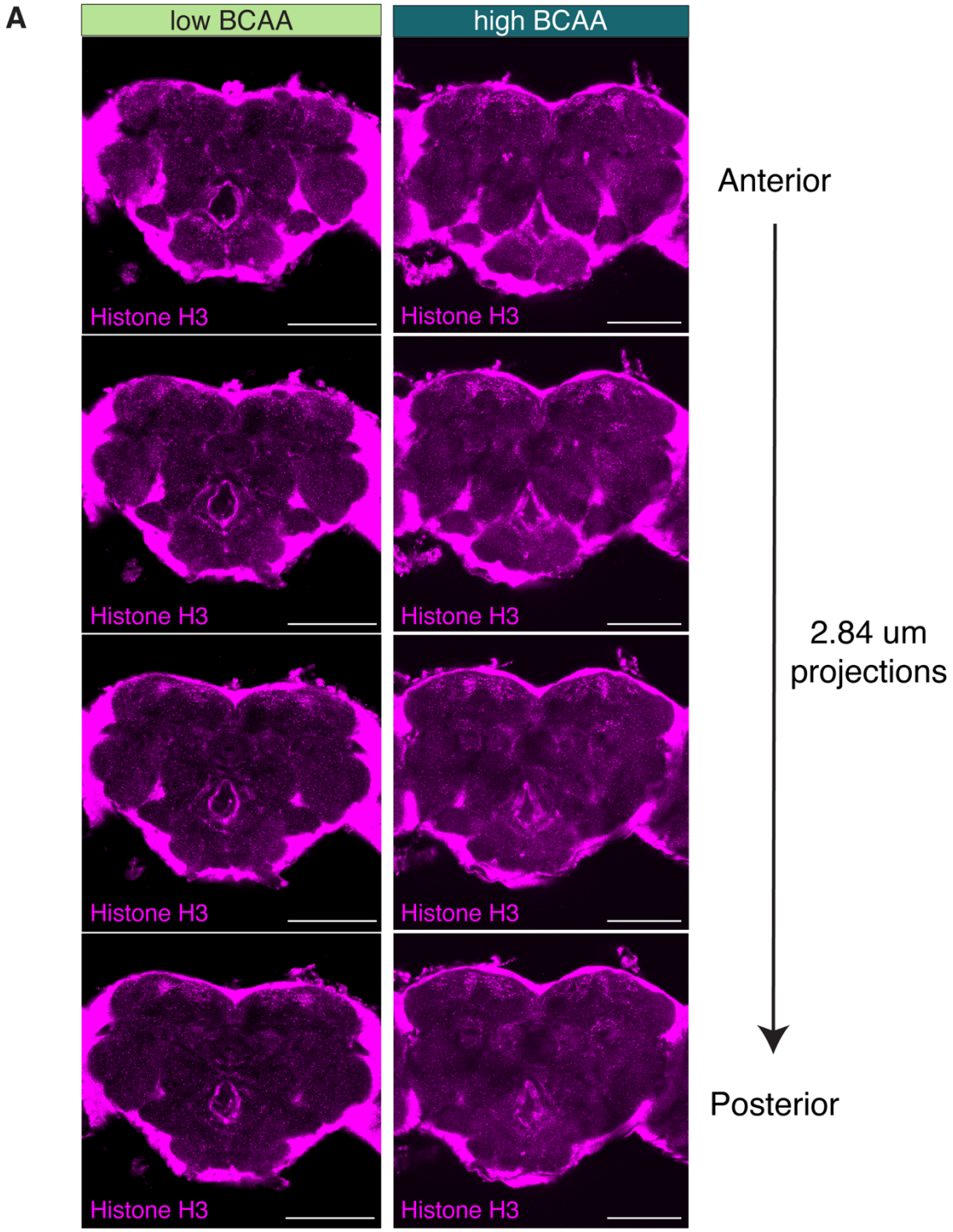

Figure S4

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57 **Figure S4. Histone H3 abundance is modulated by dietary BCAAs in discrete anatomical**

58 **locations. (A)** Immunostaining for histone H3 in *Canton-S* flies after 5-7 days on BCAA diets.

59 Representative images are montages of maximum intensity projections through the central brain,

60 each consisting of two 1.42μm stacks (scale bar = 100μm).

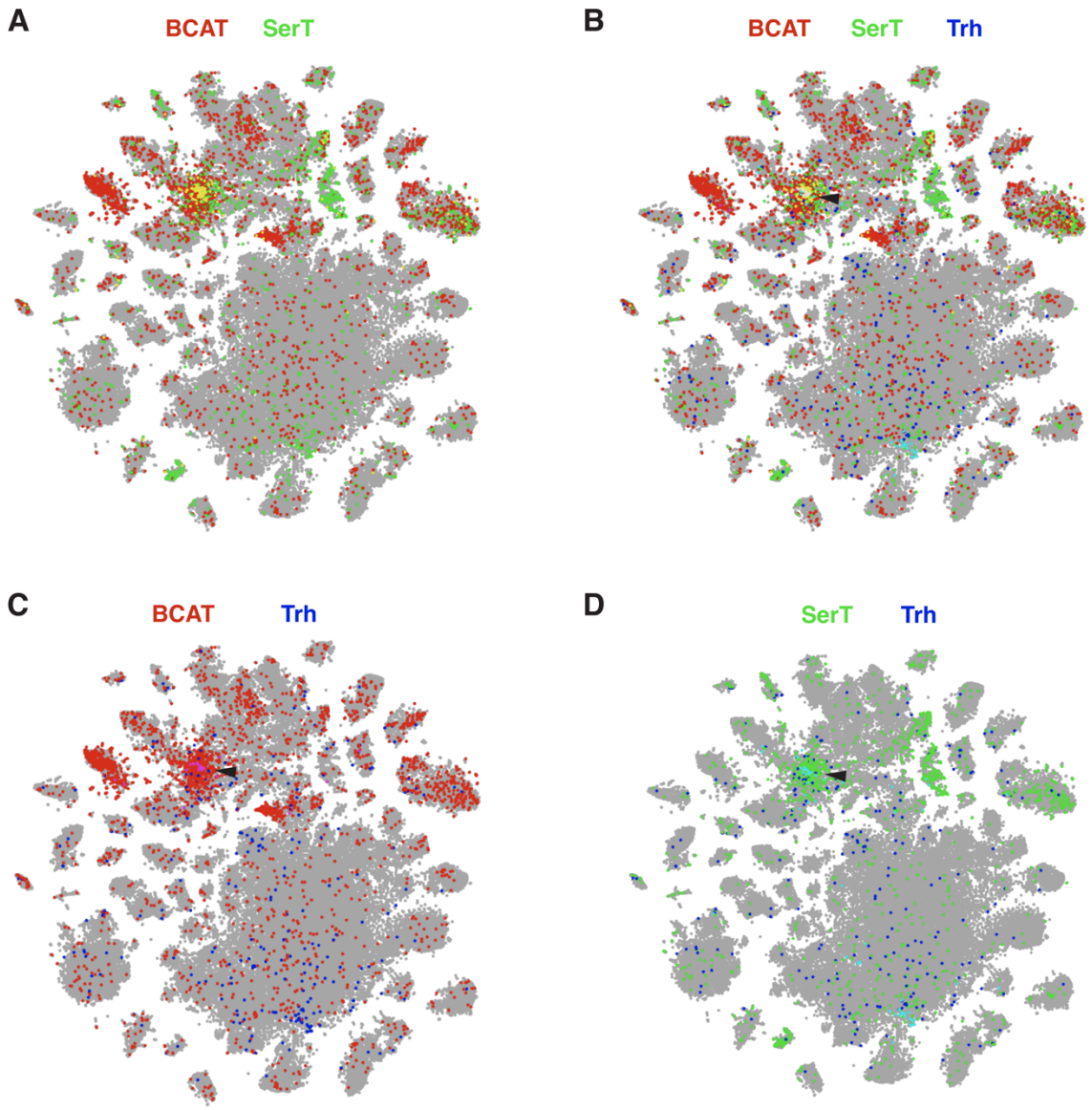

Figure S5

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63 **Figure S5. tSNE plots of single-cell gene expression in fly heads. (A-D)** tSNE plots from the
64 Fly Cell Atlas (www.flycellatlas.org) generated using the publicly available 10x droplet based
65 single-cell sequencing dataset from fly heads and visualized using SCope[47]. **(A)** Cells that
66 express *BCAT* (red) and *SerT* (green) with a cluster of co-expressing cells shown in yellow
67 (approximately 80 cells were detected to express both *BCAT* and *SerT*). **(B)** Cells that express
68 *BCAT* (red), *SerT* (green), *Trh* (blue), and co-expressing cells shown in white and highlighted
69 with arrowhead. **(C)** Cells that express *BCAT* (red) or *Trh* (blue) and co-expressing cells shown
70 in pink. **(D)** Cells that express *SerT* (green) or *Trh* (dark blue) and co-expressing cells shown in
71 light blue.
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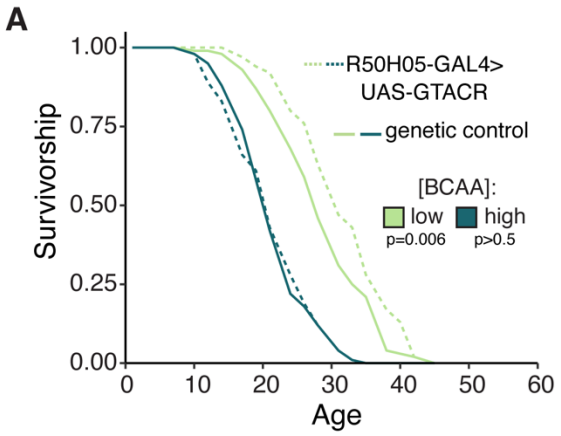

Figure S6

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88 **Figure S6. Lifespan of flies with optogenetic inhibition of R50 hunger neurons.** Flies
89 carrying *R50H05-GAL4>UAS-GTACR* or *UAS-GTACR/w-;CS* as control were aged on low- or
90 high-BCAA diets and exposed to 530nm light constantly for the duration of the experiment (log-
91 rank test, N=93-100).
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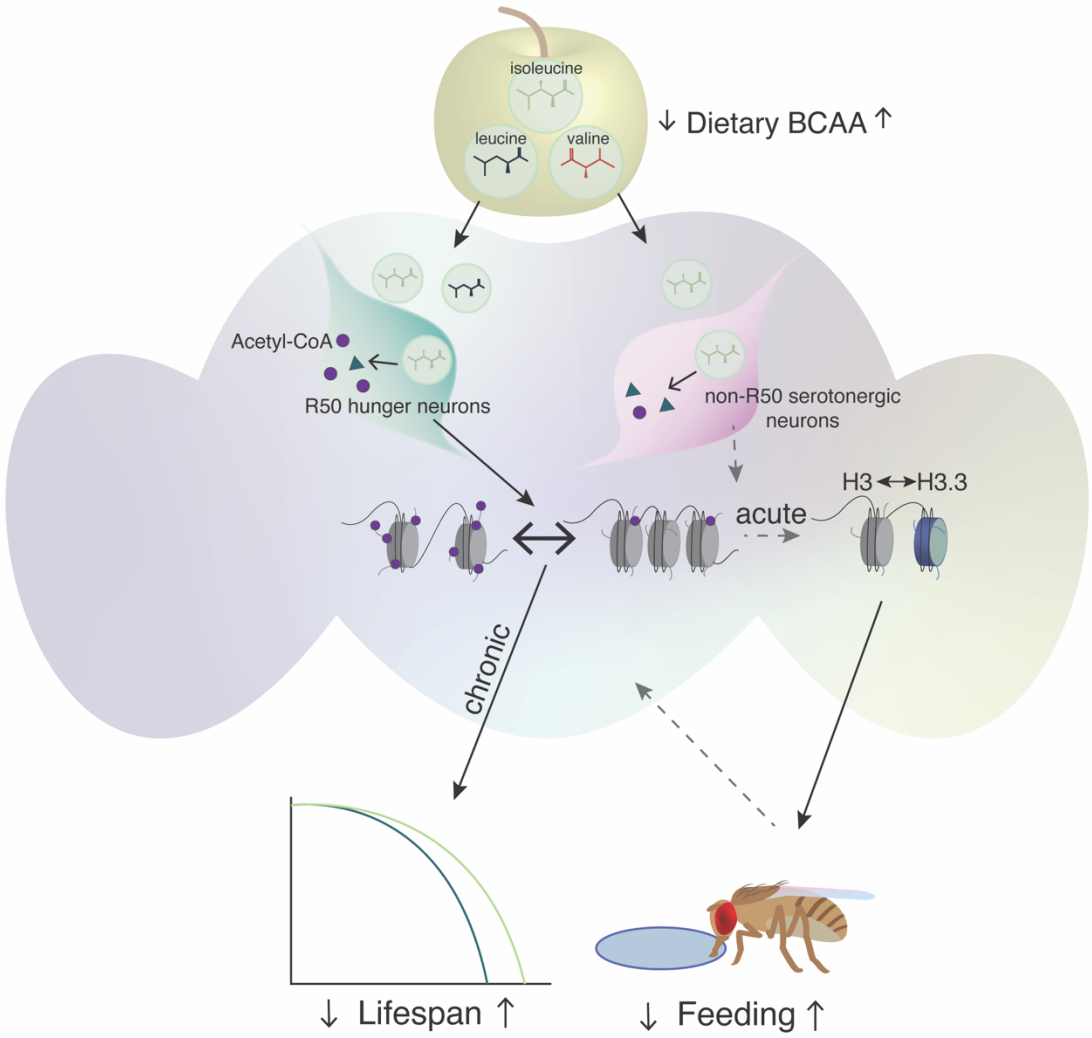

Figure S7

Figure S7. Proposed model. The motivational state of hunger modulates feeding and aging via distinct pathways.

97
98 **Table S1. Composition of diets used for experiments**

Diet/ Component	Yaa (Piper, et al.)	RD	Low BCAA	High BCAA	High BCAA –Isoleucine	High BCAA – Leucine	High BCAA – Valine	Low BCAA + Isoleucine	Low Histidine/ Arginine
Total volume (l)	1	1	1	1	1	1	1	1	1
Agar (g)	7	11.25	11.25	11.25	11.25	11.25	11.25	11.25	11.25
Isoleucine (g)	1.16	3.48	1.16	5.8	1.16	5.8	5.8	5.8	3.48
Leucine (g)	1.64	4.92	1.64	8.2	8.2	1.64	8.2	1.64	4.92
Valine (g)	1.33	3.99	1.33	6.65	6.65	6.65	1.33	1.33	3.99
Tyrosine (g)	0.84	2.52	2.52	2.52	2.52	2.52	2.52	2.52	2.52
Sucrose (g)	17.12	51.36	51.36	51.36	51.36	51.36	51.36	51.36	51.36
Cholesterol solution (ml)	15	15	15	15	15	15	15	15	15
CaCl2 (1000X, ml)	1	1	1	1	1	1	1	1	1
MgSO4 (1000X, ml)	1	1	1	1	1	1	1	1	1
CuSO4 (1000X, ml)	1	1	1	1	1	1	1	1	1
FeSO4 (1000X, ml)	1	1	1	1	1	1	1	1	1
ZnSO4 (1000X, ml)	1	1	1	1	1	1	1	1	1
Acetate buffer (10X, ml)	100	100	100	100	100	100	100	100	100
Nucleic acids/lipids (125X, ml)	8	8	8	8	8	8	8	8	8
EAA solution (ml)	60.51	181.5	181.5	181.5	181.5	181.5	181.5	181.5	181.5
NEAA solution (ml)	60.51	181.5	181.5	181.5	181.5	181.5	181.5	181.5	181.5
Glutamate solution (ml)	18.21	54.5	54.5	54.5	54.5	54.5	54.5	54.5	54.5
Cysteine solution (ml)	5.28	15.5	15.5	15.5	15.5	15.5	15.5	15.5	15.5
Vitamin solution (47.6X, ml)	21	21	21	21	21	21	21	21	21
Folic acid (1000X, ml)	1	1	1	1	1	1	1	1	1
Propionic acid (ml)	6	6	6	6	6	6	6	6	6
Tegosept (ml)	15	15	15	15	15	15	15	15	15

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125
126 **Table S2. Concentration of amino acid stock solutions used in diets**

	BCAA diets (g/200ml)	Low Histidine/Arginine (g/200ml)
Essential Amino Acids		
F (L-phenylalanine)	3.03	3.03
H (L-histidine)	2.24	0.746
K (L-lysine)	5.74	5.74
M (L-methionine)	1.12	1.12
R (L-arginine)	4.7	1.566
T (L-threonine)	4.28	4.28
W (L-tryptophan)	1.45	1.45
Non-essential Amino Acids		
A (L-alanine)	5.25	5.25
D (L-aspartate)	2.78	2.78
G (glycine)	3.58	3.58
N (L-asparagine)	2.78	2.78
P (L-proline)	1.86	1.86
Q (L-glutamine)	6.02	6.02
S (L-serine)	2.51	2.51
C (L-cysteine)	2.5	2.5
E (L-glutamate, Na salt)	5	5
Added as solid	**(g/l)**	**(g/l)**
I (L-isoleucine)	See Extended Data Table 1	See Extended Data Table 1
L (L-leucine)	See Extended Data Table 1	See Extended Data Table 1
V (L-Valine)	See Extended Data Table 1	See Extended Data Table 1
Y (L-tyrosine)	2.52	2.52

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