Internal needs drive the cephalic phase response in *Drosophila melanogaster*

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Manuscript information: Summary, 150 words; 7 figures; 4 supplemental figures; and 1 supplemental table

Keywords: Gustatory receptors, temperature-sensing neurons, circadian clock, clock genes, starvation, cephalic phase response, body temperature, temperature preference rhythms, hunger signals, and *Drosophila melanogaster*.

26 **HIGHLIGHTS:**

27 1. ***Drosophila* exhibit a cephalic phase response (CPR).**

28 2. **Sensory detection triggers CPR.**

29 3. **Hunger signals and clock genes selectively drive CPR.**

30 4. **Hunger signals and clock genes do not regulate the response after nutrient intake.**

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SUMMARY<150

Animals are motivated to eat based on their internal need. When animals are hungry, the sensory detection of food elicits bursts of physiological changes in their bodies. This is the cephalic phase response (CPR), which prepares animals to properly digest food before nutrients enter their bodies. Despite animals uniformly exhibiting a strong CPR, to what extent and how the internal state influences CPR is largely unclear. Here, we demonstrate in *Drosophila* that tasting food triggers CPR, which is strongly influenced by the internal state. We found that feeding an artificial sweetener or gustatory excitation by optogenetics trigger CPR, which is a rapid and partial recovery from the starved state. While clock genes and hunger signals profoundly drive CPR, they are not required for the process of response after nutrient intake. Therefore, we propose that CPR is the critical layer of regulatory mechanisms representing internal energy homeostasis and metabolism.

INTRODUCTION

Immediately after hungry animals see, smell, or chew food, even without absorbing nutrients, bursts of physiological changes are suddenly initiated within the body. This is the cephalic phase response (CPR), a flood of saliva and gastrointestinal secretions, which prepares animals for food digestion (Chen and Knight, 2016; Garrison and Knight, 2017; Pavlov, 1902; Power and Schulkin, 2008; Zafra et al., 2006). The introduction of food to the body firmly and suddenly disturbs the internal milieu; thus, the anticipation of food is an important process that helps animals prepare for digestion. If CPR is bypassed, food is digested slowly, which lengthens digestion and delays nutrient absorption (Pavlov, 1902). Importantly, CPR is observed not only in saliva and gastrointestinal secretions but also in thermogenesis, heart rate, and respiratory rate in mammals (LeBlanc, 2000; LeBlanc and Cabanac, 1989; Nederkoorn et al., 2000). For example, starvation or malnutrition in mammals causes lower body temperature (Cintron-Colon et al., 2017; Piccione et al., 2002), and biting food triggers heat generation, which is another example of CPR (LeBlanc et al., 1984; Power and Schulkin, 2008).

Animals are motivated to eat food depending on their internal state, such as time of the day or hunger. The circadian clock drives the daily feeding rhythms (Cedernaes et al., 2019; Hirayama et al., 2018; Longo and Panda, 2016) and anticipates meal timing. Daily feeding timing influences energy homeostasis and metabolism (Chaix et al., 2019; Greenhill, 2018). Hunger and satiety dramatically affect animal behavior and physiology (Hanci and Altun, 2016; Kain and Dahanukar, 2015; Keys et al., 1950; Piccione *et al*., 2002; Root et al., 2011; Sakurada et al., 2000; Soria-Gomez et al., 2014) and control feeding. Despite hungry animals uniformly exhibiting a strong CPR, how the internal state communicates with the brain, which enables animals to exhibit CPR, is largely unclear. Therefore, we postulate the internal state, modulated by circadian rhythms and hunger

signals, is involved in CPR. If this is the case, CPR is not only an anticipatory response but also an indispensable physiological response that requires further study. To address this question, we focused on the relatively simple, versatile model organism, *Drosophila melanogaster* and ask whether *Drosophila* exhibit CPR.

Here, we discovered that *Drosophila* exhibit CPR and that sensory detections trigger CPR, which is strongly influenced by the internal state. Hunger stress compels flies to seek a lower-temperature environment, causing lower body temperatures and metabolic rates (Berrigan and Partridge, 1997; Schilman et al., 2011; Umezaki et al., 2018b). Due to the low mass of small ectotherms, their body temperatures are nearly equivalent to their surrounding temperature (Stevenson, 1985a; Stevenson, 1985b). We showed here that tasting food initiates compelling the hungry flies to seek a warmer environment as CPR, that is, immediately increasing body temperatures and metabolic rates. In mammals, starvation results in lower body temperatures, and biting food triggers a rapid increase in heat generation, showing CPR in thermogenesis (LeBlanc, 2000; LeBlanc and Cabanac, 1989). Thus, the *Drosophila* CPR may be a functional, physiological response equivalent to the CPR observed in mammals. Furthermore, we found that clock genes, hunger signals, and light are required for CPR, but not the nutrient response. The data suggest that CPR is an indispensable physiological response that represents the internal state. Thus, our data shed new light on the role of CPR, which would highlight a crucial aspect of our understanding of feeding state and energy homeostasis.

115 **RESULTS**

116 **Starved flies quickly move to a warmer place right after a brief refeeding.**

117 *Drosophila* exhibit robust thermoregulatory and temperature preference behavior (Hamada et al.,
118 2008; Sayeed and Benzer, 1996; Umezaki *et al.*, 2018b). Small ectotherms such as *Drosophila*
119 avoid harmful hot or cold temperatures and choose appropriate temperatures to set their body
120 temperature (Dillon et al., 2009; Hamada *et al*., 2008; Sayeed and Benzer, 1996). To examine how
121 the internal state regulates temperature preference behavior, *white*^{1118} (*w*^{1118}) flies were fed fly
122 food containing carbohydrate, protein and fat sources all the time. They were separated into two
123 groups and one of them was starved overnight only with water (starved flies) (Fig. 1A). We then
124 tested temperature preference behavior of the fed and starved flies using a gradient of temperatures
125 ranging from 16-34 °C for 30 min (Hamada *et al.*, 2008). Fed flies preferred 25.2±0.2 ^{O}C (Fig. 1B:
126 Fed, white bar), whereas starved flies preferred 21.7±0.3 ^{O}C **(**Fig. 1B: Starvation overnight (STV),
127 gray bar). Thus, starvation causes a lower temperature preference, as we previously reported that
128 temperature preference behavior is greatly influenced by feeding state (Umezaki *et al.*, 2018b).

129 To determine how long of a refeeding is required to recover from the starved state to the
130 fed state, starved flies were offered fly food for 5, 10, 30 min, and 1 hr (Fig. 1A). We found that
131 the preferred temperature (Tp) of refed flies increased (Fig. 1B; orange bars) from that of the
132 starved flies (Fig. 1B; gray bar, statistics shown in green stars). Notably, Tps of the 10 min, 30
133 min, and 1 hr refed flies were similar to that of fed flies (Fig. 1B**:** white bar), suggesting a full
134 recovery. Importantly, refeeding with fly food for only 10 min is sufficient to recover to a similar
135 Tp as fed flies. On the other hand, Tp of the 5 min refed flies was intermediate between fed and
136 starved flies, suggesting a partial recovery (Fig. 1E, statistics shown in red stars, Table S1). Thus,
137 our data suggest that flies can quickly move to a warmer place after at least 5 min refeeding of fly

138 food and that 10 min refeeding of fly food is sufficient to recover from the starved state to the fed
139 state.
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142 **Sucralose intake results in intermediate recovery.**
143 We showed that only 5 min fly food refeeding let the starved flies increase Tp and that 10 min fly
144 food refeeding restored Tp to that of fed flies (Fig. 1B). We wondered whether this rapid Tp
145 recovery might be driven by food sensory cues and therefore hypothesized that gustatory cues
146 might play an essential role in this recovery. Sucralose is an artificial sweetener and non-
147 metabolizable sugar. Like glucose, sucralose activates sweet taste neurons in *Drosophila*
148 (Biolchini et al., 2017) and modulates taste behaviors like the proboscis extension reflex
149 *(Dahanukar et al., 2007; Dus et al., 2011; Park et al., 2017; Wang et al., 2016)*. We asked whether
150 sucralose could lead to the Tp recovery. The starved flies were refed sucralose for 10 min or 1 hr
151 (Fig. 1A). We found that starved flies partially increased Tp upon 10 min or 1 hr sucralose
152 refeeding, but Tp remained between those of fed and starved flies, indicating intermediate recovery
153 (Fig. 1C: blue, Table S1). Thus, our data suggest that sucralose intake results in an intermediate
154 recovery.
155 Because sucralose intake led to an intermediate recovery, starved flies might need both
156 gustatory cues and nutrients to recover Tp fully. To test this possibility, we used glucose, which
157 includes both sweetness (gustatory cues) and nutrients (i.e., metabolizable) (Fig. 1A). We
158 previously showed that overnight glucose refeeding restored the Tp of starved flies to that of fed
159 flies. Therefore, we shortened the glucose refeeding time to 5 min, 10 min and 1 hr (Umezaki *et*

160 *al*., 2018b). We found that 10 min or 1 hr glucose refeeding resulted in full recovery (Fig. 1D and
161 E: statistics shown in red NS, Table S1) and significantly differed from starved flies (Fig. 1D and
162 E: statistics shown in green stars, Table S1). Thus, our data showed gustatory cues trigger an
163 intermediate recovery and both gustatory and nutrient cues lead to a full recovery.

164 That said, it is still possible the starved flies might eat glucose more or faster than sucralose
165 during the first 10 min, which could cause a different Tp recovery. To exclude this possibility, we
166 examined how often starved flies touch glucose, sucralose, or water during the 30 min via Fly
167 Liquid-food Interaction Counter (FLIC) system (Ro et al., 2014). The FLIC system assays
168 interaction between the fly and liquid food, which reflects feeding/drinking episodes. We found
169 that starved flies touched glucose and sucralose food at a similar frequency and more often than
170 water during the 30 min test period (Fig. 1F, Table S1). The data suggest that flies most likely eat
171 glucose and sucralose at a similar speed and amount. Therefore, we concluded that the different
172 Tp recovery between sucralose and glucose is not due to differences in feeding rate or amount.
173 Instead, the gustatory cues trigger the intermediate Tp recovery, and gustatory cue plus nutrients
174 trigger a full Tp recovery.

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177 **Activation of sweet gustatory neurons trigger intermediate recovery.**

178 Sweet taste is detected by sweet gustatory receptors (Grs). We asked whether sweet Grs are
179 involved with recovery. Two different sweet Gr mutants, *Gr5a*$^{-/-}$; *Gr64a*$^{-/-}$ and *Gr5a*$^{-/-}$;;*Gr61a*$^{-/-}$,
180 *Gr64a-f*$^{-/-}$ cannot recognize sweetness in sugar choice assays (Dahanukar *et al*., 2007; Dus *et al*.,
181 2011; Fujii et al., 2015; Yavuz et al., 2014) and have reduced sugar sensitivity compared to the

182 control (Fujii *et al*., 2015; Yavuz *et al*., 2014). We found that sweet Gr mutants showed normal
183 starved response, in which Tp of starved flies was lower than that of fed flies (Fig. 2A and B, white
184 and gray bars, Table S1). However, starved flies did not increase Tp upon 10 min sucralose
185 refeeding (Fig. 2A and B, blue bars, Table S1). The data suggest that sweet Grs are required for Tp
186 recovery.

187 Sweet Grs are expressed in the sweet Gr-expressing neurons (GRNs) in the proboscis and
188 forelegs (Yarmolinsky et al., 2009). To further examine the involvement of sweet GRNs for Tp
189 recovery, we silenced sweet GRNs using *uas-Kir* (Baines et al., 2001) with *Gr64f-Gal4,* which is
190 expressed in all-sweet-GRNs ($Gr64f^+$ neurons) in both proboscis and forelegs (Dahanukar *et al*.,
191 2007; Thoma et al., 2016). Silencing of $Gr64f^+$ neurons did not impair the normal starved response
192 because Tp of starved flies was lower than that of fed flies (Fig. 2C, white and gray bars). However,
193 no Tp recovery was observed after 10 min of sucralose refeeding (Fig. 2C, blue bar, Table S1),
194 similar to the sweet Gr mutants (Fig. 2A and B). On the other hand, control flies (*Gr64f-Gal4*/+
195 and *uas-Kir*/+) showed a normal starved response and Tp recovery upon sucralose refeeding (Fig.
196 2D and E, gray and blue bars, respectively, Table S1). Therefore, our data indicate that sweet Grs
197 are required for an intermediate Tp recovery.

198 Because sucralose feeding causes intermediate recovery, we asked whether the artificial
199 activation of sweet GRNs trigger intermediate recovery. We used the optogenetic approach, a red-
200 light sensitive channelrhodopsin, CsChrimson (Klapoetke et al., 2014; Simpson and Looger,
201 2018). Starved flies were given water with 0.8 mM all-trans-retinal (ATR), which is the
202 chromophore required for the CsChrimson activation. These flies were not fed sucralose; instead,
203 gustatory neurons in starved flies were excited by red-light pulses (flashing on and off at 10 Hz)
204 for 10 min (Fig. 2L). In this case, even though flies were not refed, the gustatory neurons were

205 artificially excited so we could evaluate the effect of the excitation of gustatory neurons for
206 intermediate recovery.

207 To this end, we expressed CsChrimson in either all-sweet-GRNs (Gr64f$^+$ neuron),
208 proboscis (Gr5a$^+$ neurons), or legs (Gr64a$^+$ neurons). All flies showed normal starved response in
209 that Tps of fed and starved flies were significantly different (Fig. 2F-I, white and gray bars, Table
210 S1). With excitation of all-sweet-GRNs (Gr64f$^+$ neuron) by red-light pulses, starved flies showed
211 a Tp between fed and starved flies, suggesting an intermediate recovery (Fig. 2F, yellow bar, Table
212 S1). However, neither excitation of gustatory neurons in the proboscis nor legs induced Tp
213 recovery (Fig. 2G and H, yellow bars, Table S1). Notably, control flies (*UAS-CsChrimson*/+) did
214 not show Tp recovery upon red light with ATR application (Fig. 2I). Taken together, our data
215 suggest that the excitation of all-sweet-GRNs leads to intermediate recovery.

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218 **Sensory cues trigger intermediate recovery.**

219 We next asked whether sweet taste is required for the intermediate recovery, as compared to other
220 taste cues like bitter taste; therefore, we wondered whether these cues induce intermediate recovery.
221 We expressed CsChrimson in the bitter sensing Gr (Gr66a)-expressing (Gr66a$^+$) neurons
222 (Dahanukar *et al.*, 2007). The flies show normal starved response: Tps of fed and starved flies
223 were significantly different (Fig. 2J, white and gray bars, Table S1). The excitation of Gr66a$^+$
224 neurons caused a significant increase in Tp of starved flies (Fig. 2J, yellow bar, Table S1),
225 suggesting that the excitation of bitter taste neurons triggers Tp recovery. Furthermore, we also
226 investigated the potential role of olfaction. We used mutants of the odorant receptor co-receptor,

227 *Orco* (*Orco*^{1}), which shows olfactory defectiveness (Larsson et al., 2004). Tps of fed and starved
228 *Orco*^{1} flies were significantly different (Fig. 2K, white and gray bars, Table S1), showing normal
229 starved response. However, they failed to show a Tp recovery upon sucralose refeeding (Fig. 2K,
230 blue bar, Table S1). Thus, our data suggest that bitter taste and olfactory response are possible
231 other cues for Tp recovery.

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234 **Intermediate Tp recovery is CPR.**

235 Starved flies show a lower Tp, indicating lower body temperature (Umezaki *et al*., 2018b). Sweet
236 taste and activation of sweet- or bitter-sensing neurons initiate starved flies to increase Tp,
237 indicating a warmer body temperature. Similarly, in mammals, starvation or malnutrition causes
238 lower body temperatures (Cintron-Colon *et al*., 2017; Piccione *et al*., 2002), and biting food, even
239 before food enter the stomach, quickly initiate heat generation, namely CPR (LeBlanc *et al*., 1984;
240 Power and Schulkin, 2008). Thus, in both flies and mammals, starvation causes lower body
241 temperatures, and feeding cues quickly induce heat generation before the nutrition enter the bodies.
242 Based on the reasons above, we propose that intermediate Tp recovery upon the sensory signals in
243 flies is likely CPR. In this article, we further elucidate how sensory information elicits CPR and
244 how the internal state regulates CPR using flies.

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247 **The activation of temperature-sensing neurons triggers CPR.**

248 To understand how gustatory information elicits CPR, we focused on the temperature-sensing
249 neurons; the warm-sensing neurons, anterior cells (ACs) (Hamada *et al*., 2008), and the cold-
250 sensing *R11F02-Gal4*-expressing neurons, which control temperature preference behavior (Ni et
251 al., 2016; Umezaki et al., 2018a). We previously showed that thermal activation of threshold of
252 ACs, which is ~25°C, is lowered in the starved condition, which results in a lower Tp (Umezaki
253 *et al*., 2018b). Because hungry flies increase their Tp after detecting food quickly (Fig. 1 and 2),
254 we asked whether ACs or *R11F02-Gal4*-expressing neurons are involved in CPR.

255 To this end, we silenced ACs or *R11F02-Gal4*-expressing neurons by *uas-Kir*. We found
256 that Tps of fed flies and starved flies were significantly different, showing the normal starved
257 response (Fig. 3A and B, white and gray bars, Table S1). However, the 10 min sucralose refeeding
258 did not cause CPR (Fig. 3A and B, blue bars, Table S1). Notably, the control flies, *TrpA1^{SH}-Gal4*/+,
259 *R11F02-Gal4*/+, and *uas-Kir*/+, showed normal starved effect and CPR (Fig. 3C-E). Therefore,
260 these data suggest that ACs and *R11F02-Gal4*-expressing neurons are required for CPR upon
261 sucralose refeeding.

262 To further ensure the involvement of ACs and *R11F02-Gal4*-expressing neurons in CPR,
263 we used optogenetics to excite them artificially by red-light pulse exposure for 10 min. We
264 compared Tp of starved flies with and without ATR under the red light. Tp of starved flies with
265 ATR was significantly increased (Fig. 3F and G, yellow bars, Table S1) compared to those of
266 starved flies without ATR (Fig. 3F and G, gray bars, Table S1). Thus, our data suggest that the
267 excitation of ACs or *R11F02-Gal4*-expressing neurons causes CPR upon sucralose refeeding.

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270 **Gustatory and temperature-sensing neurons regulate response after 10 min glucose intake.**

271 We showed that activations of gustatory or temperature-sensing neurons are required for CPR (Fig.
272 2 and 3). We wondered if they are also crucial for the response after glucose refeeding, which
273 enables the starved flies to fully recover. To this end, we used all sweet GRNs silenced flies (*Gr64f-*
274 *Gal4>uas-Kir*), two Grs mutants (*Gr5a*$^{-/-}$; *Gr64a*$^{-/-}$ and *Gr5a*$^{-/-}$;;*Gr61a*$^{-/-}$, *Gr64a-f*$^{-/-}$), and the warm
275 or cold neuron silenced flies (*TrpA1*^{SH}*Gal4* or *R11F02-Gal4>uas-Kir*). We found that all of these
276 starved flies did not increase Tp upon 10 min glucose intake (Fig. S1A-E, green bars, Table S1),
277 but increased Tp upon 10 min refeeding with fly food, which includes carbohydrate, fat, and
278 protein (Fig. S2A-E, orange bars, Table S1). The data suggest that gustatory and temperature-
279 sensing neurons are required for recovery upon carbohydrate but dispensable for other nutrition
280 such as fat or protein (see discussion). Since flies have sensory neurons that detect fatty acid (Ahn
281 et al., 2017; Brown et al., 2021; Masek and Keene, 2013) and also amino acids-responsive neurons
282 (Chen and Dahanukar, 2017; Croset et al., 2016; Ganguly et al., 2017; Steck et al., 2018), these
283 neurons may control the response after fly food intake. This is likely why the sweet- or
284 temperature-sensing-blind flies still exhibit the normal recovery after fly food intake (Fig. S2A-
285 E). On the other hand, the control flies and olfactory mutant (*Orco*^{1}) showed normal responses for
286 both 10 min glucose and fly food intake (Fig. S1F-J, green bars, S2F-J, orange bars, Table S1).
287 The data suggest that in contrast to the gustatory neurons, olfactory neurons are not involved in
288 the Tp recovery upon glucose intake.

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291 **Neuropeptide F is a crucial factor for CPR.**

292 Animals uniformly exhibit a strong CPR triggered by sensory signals. We showed in flies that
293 sensory cues trigger CPR. Though it is known that internal state strongly influences eating
294 motivation, the relationship between the internal state and CPR is largely unclear. Hunger
295 represents the energy state in the body, which induces a release of hunger signals such as a
296 neuropeptide Y (NPY). NPY promotes food-seeking and feeding behavior in mammals and flies
297 (Nassel and Wegener, 2011). While intracerebroventricular NPY injection causes CPR
298 (Geoghegan et al., 1993), injecting NPY antagonists suppress CPR in dogs (Lee et al., 1994),
299 suggesting that NPY is a regulator for CPR (Lee *et al*., 1994). Therefore, we first focused on
300 neuropeptide F (NPF) and small neuropeptide F (sNPF), which are the *Drosophila* homologue and
301 orthologue of mammalian NPY, respectively (Nassel and Wegener, 2011) and asked if they were
302 involved in CPR. We found in NPF or sNPF mutants that Tp of fed and starved flies were
303 significantly different, showing a normal starved response (Fig. 4A-D: white vs. gray bars, Table
304 S1), but they failed to show CPR upon 10 min sucralose refeeding (Fig. 4A and C, blue bars, Table
305 S1). Thus, NPF and sNPF are required for CPR upon sucralose refeeding.

306 What about the glucose refeeding? The control flies can fully recover Tp from the starved
307 state after refeeding with glucose (Fig. 1D). We asked whether NPF and sNPF are involved in
308 recovery upon glucose refeeding. Surprisingly, we found starved NPF or sNPF mutants
309 significantly increased Tp after 1 hr or 10 min of glucose refeeding, respectively (Fig. 4B and D,
310 green and dark green bars, Table S1). Furthermore, NPF and sNPF mutants also increased Tp after
311 10 min fly food refeeding (Fig. S3A and B, orange bars, Table S1). The data suggest that NPF and
312 sNPF are not critical for response after nutrient intake. It is noteworthy that NPF and sNPF mutants
313 could not completely recover from starved state after 10 min normal food intake or 1 hour glucose
314 intake, suggesting that these mutants' recovery rate may be slower than that of w^{1118} flies.

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317 **Factors involving hunger regulate CPR but not recovery upon nutrient intake.**

318 Based on these results above, we hypothesized that hunger signals might influence CPR and
319 nutrient responses differently. To test this hypothesis, we focused on several factors involved in
320 hunger state. The important hunger signals (Lin et al., 2019), Diuretic hormone 44 (DH44) and
321 Adipokinetic hormone (AKH), are homologs of the mammalian corticotropin-releasing hormone
322 (Dus et al., 2015) and glucagon (Kim and Rulifson, 2004; Lee and Park, 2004), respectively. We
323 found that control flies (*Dh44-Gal4*/+, *Akh-Gal4*/+, and *UAS-Kir*/+) showed normal CPR upon
324 sucralose refeeding (Fig. 4E, I and S; blue bars, Table S1), response after glucose refeeding (Fig.
325 4F, J, and T, green bars, Table S1), and response after fly food refeeding (Fig. S3 C, E, and J,
326 orange bars, Table S1). Although DH44 or AKH neurons-silenced flies failed to show CPR upon
327 sucralose refeeding, they could increase Tp after 10 min glucose or fly food refeeding (Fig. 4H
328 and L, green and S3D and F, orange, Table S1). The data suggest that DH44 or AKH neurons are
329 required for CPR but not the response after nutrient refeeding.

330 Insulin-like peptide 6 (Ilp6) is a homologue of mammalian insulin-like growth factor 1
331 (IGF1), and *ilp6* mRNA expression increases in starved flies (Bai et al., 2012; Okamoto et al.,
332 2009; Slaidina et al., 2009). Unpaired3 (Upd3) is a cytokine that increases dietary stress
333 (Woodcock et al., 2015). We found that *Ilp6* and *Upd3* mutants failed to show CPR upon sucralose
334 refeeding (Fig. 4M and O, blue bars, Table S1) but showed a normal response after nutrient intake
335 (Fig. 4N and P, green bars and S3G and H, orange bars, Table S1). The data therefore suggest that
336 *Ilp6* and *Upd3* are required for CPR but not for nutrient response.

337 We also investigated the role of the satiety factor, *Unpaired2* (*Upd2*), a functional leptin
338 homologue in mammals in CPR (Rajan and Perrimon, 2012). *Upd2* mutants failed to show recovery
339 after sucralose, glucose, or fly food refeeding (Fig. 4R, green bars and S3I, orange bars, Table S1).
340 Thus, our data suggest that some factors involved in hunger state processing are required for CPR
341 upon sucralose refeeding but not nutrient response. The data suggest that that CPR and nutrient
342 response are differently regulated.
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345 **Flies exhibit CPR at all times of the day.**
346 Animals anticipate eating food at a specific time of the day, which is tightly controlled by the
347 circadian clock (Chaix *et al*., 2019). Flies show rhythmic feeding pattern; a peak in the morning
348 (Ro *et al*., 2014) or two peaks in the morning and evening (Xu et al., 2008). Because food cues
349 trigger CPR, we wondered if the induction of CPR depends on the timing of food consumption
350 and if the timing of CPR and feeding is coordinated. To determine this possibility, we examined
351 CPR at several time points during the daytime, Zeitgeber time (ZT) 1-3, 4-6, 7-9, and 10-12 in
352 light and dark (LD) conditions. Importantly, flies exhibit clock-dependent temperature preference
353 rhythms (TPR) (Kaneko et al., 2012), in which Tp gradually increases during daytime and peaks
354 in the evening. We found that both w^{1118} and *yellow white* (y^1w^1) flies showed significant starved
355 responses at all of the tested time points, with a higher Tp in the fed state and lower Tp in the
356 starved state (Fig. 5A and B: black vs. gray line Table S1).
357 We found that starved y^1w^1 flies showed CPR any time of the day (Fig. 5B1, gray vs. blue
358 line, Table S1). On the other hand, starved w^{1118} flies showed a significant CPR only at ZT 4-6 and
359 10-12 (Fig. 5A1, gray vs. blue line, Table S1). However, it is likely because starved w^{1118} flies

360 showed an advanced phase shift of TPR with a peak at ZT 7-9 (Fig. 5A, gray), the highest Tp
361 might simply mask the CPR at ZT 7-9. Thus, the results suggest that w^{1118} and y^1w^1 flies can exhibit
362 CPR upon sucralose refeeding anytime of the day.

363 Next, we focused Tp recovery on the nutrient response. Starved w^{1118} and y^1w^1 flies
364 successfully increased Tp after glucose refeeding during the 10 min (Fig. 5A2 and B2, green line,
365 Table S1). The 1 hr glucose refeeding led to similar Tp as fed w^{1118} flies (Fig. 5A3, green line). The
366 data show flies can exhibit CPR and nutrient response at any time of the day, and therefore, CPR
367 and feeding schedule do not occur in parallel.

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370 **Circadian clock genes are required for CPR but not nutrient response.**

371 To determine whether the circadian clock is involved in CPR, we used null mutants of clock genes,
372 *period*^{01} (*per*^{01}) and *timeless*^{01} (*tim*^{01}). Although they showed significant starved responses (Fig. 5C
373 and D, black and gray lines, Table S1), neither starved *per*^{01}nor *tim*^{01} mutants could exhibit CPR
374 upon sucralose refeeding (Fig. 5C1 and D1, blue lines, Table S1). Nonetheless, they showed a full
375 recovery upon glucose refeeding in LD anytime of the day (Fig. 5C2 and D2, green lines, Table
376 S1). Therefore, our data suggest that clock genes are required for CPR but not nutrient response
377 under the LD condition.

378 That said, starved *per*^{01} and *tim*^{01} mutants might eat sucralose less frequently than glucose,
379 which could cause a failure to show CPR. Therefore, we examined how often starved *per*^{01} and
380 *tim*^{01} mutants touched glucose, sucralose, or water during the 30 min using FLIC assays (Ro *et al*.,
381 2014) (Fig. S4A-C). Interestingly, starved *per*^{01} or *tim*^{01} mutants touched water significantly more

382 often than glucose or sucralose (Fig. S5B and C, Table S1). Although starved *tim*^{01} flies touched
383 glucose slightly more than sucralose only during 10 min, this phenotype is not consistent with *per*^{01}
384 and *w*^{1118} flies. Thus, we concluded in *per*^{01} and *w*^{1118} flies that the different response between CPR
385 and response after glucose intake is not due to feeding speed or amount.

386 Because clock genes are critical for CPR upon sucralose refeeding, we wondered if internal
387 clock oscillation is required for CPR. To this end, we leveraged constant light (LL) conditions to
388 disrupt the circadian molecular clock oscillation (Allada and Chung, 2010; Krzeptowski et al.,
389 2018). However, even though we kept flies in LL for 2 days after eclosion, *w*^{1118} flies still sustained
390 CPR at CT 7-9, and *y*^{1}*w*^{1} flies showed sustained CPR any time of the day in LL (Fig. 6A1 and B1,
391 gray vs. blue lines, Table S1). The data suggest that internal clock oscillations might not be
392 required for CPR upon sucralose refeeding.

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395 **Light is required for CPR upon sucralose refeeding but not nutrient response.**

396 Furthermore, we tested flies in constant darkness (DD) and found that *w*^{1118} lost the ability for CPR
397 upon sucralose refeeding (Fig. 6C1, gray vs. blue line, Table S1), suggesting that light is required
398 for CPR. Thus, flies can exhibit CPR anytime during the day in the presence of light but not in the
399 dark. Notably, in both LL and DD conditions, starved *w*^{1118} and *y*^{1}*w*^{1} flies showed recovery upon
400 nutrient intake (Fig. 6A2, B2 and, C2 gray vs. green lines, Table S1). Thus, light is required for
401 CPR upon sucralose refeeding but not nutrient response.

402

403

404 **DISCUSSION**

405 When we are hungry, sensory detection of food (sight, smell, or chew food) initiates digestion even
406 before the food goes to the stomach. This is CPR, which contributes to the physiological regulation
407 of digestion, nutrient homeostasis, and daily energy homeostasis (Zafra *et al*., 2006). The failure
408 to induce CPR causes slower digestion, a delay in nutrient absorption, and lower metabolism.
409 However, the mechanisms by which animals exhibit CPR are largely unclear. To examine these
410 questions, we established the fly model for CPR and found that sensory cues trigger CPR, which
411 is profoundly controlled by the internal state. We showed that the excitation of the sweet, bitter, or
412 temperature-sensing neurons triggers CPR. We found that hunger signals and circadian clock genes
413 are required for CPR but do not regulate nutrient response. The data suggest that CPR and nutrient
414 response are separately controlled and that CPR is driven by the internal need. Thus, we propose
415 that CPR represents another layer of regulation of energy homeostasis.

416

417

418 **How do flies set Tp for CPR?**

419 The temperature-sensing neurons, ACs and the cold-sensing *R11F02-Gal4*-expressing neurons,
420 control temperature preference behavior (Hamada *et al*., 2008; Ni *et al*., 2016; Umezaki *et al*.,
421 2018a). We previously showed that the starved flies chose a lower Tp, controlled by ACs but not
422 cold-sensing *R11F02-Gal4*-expressing neurons (Umezaki *et al*., 2018b). ACs express Transient
423 receptor potential A1 (TrpA1), which responds to a warm temperature >25 degrees (Hamada *et al*.,
424 2008; Tang et al., 2013). The set point of ACs in fed flies, which is ~25 ^{o}C, is lowered in the starved
425 flies. Therefore, the lower ACs' setpoint is likely corresponding to the lower Tp in starved flies.
426 On the other hand, we showed here that starved flies partially increase Tp upon a food cue without

427 nutrient (Fig. 1C and E), resulting in CPR. We wondered which ACs or cold-sensing neurons are
428 responsible for CPR. However, since the Tp of CPR is between those of fed and starved flies (Figs.
429 2, 3 and S2), the temperature differences between CPR flies and starved flies are very small. It is
430 challenging to detect the smaller temperature differences using the calcium imaging experiments.
431 Instead, our behavioral data show that both ACs and cold-sensing neurons are responsible for CPR,
432 suggesting that the temperature setpoints of these neurons are likely changed by food cue, which
433 could result in CPR.

434

435

436 **Internal state drives CPR**.

437 We show that sensory signals trigger CPR only when factors involving hunger (or clock genes) are
438 present. At a specific time of the day, animals are eager to eat food. Therefore, the "hungry"
439 internal state functions as a gatekeeper and opens the gate of circuits when the hungry flies detect
440 the food information, which leads to CPR (Fig. 7, blue arrow). On the other hand, neither hunger
441 signals nor time of day information are required for the nutrient response (Fig. 7, green arrow).
442 The data suggest that the internal state is more linked to CPR than nutrient response. It is
443 noteworthy that the sensory signals contribute to both CPR and response after 10 min glucose
444 intake (Figs. 7 blue and green arrows, S1 and S2). Therefore, the differences between CPR and
445 nutrient response are differentiated at the level of the internal state but not the sensory level. Thus,
446 our data suggest that CPR and nutrient response are separately controlled and that CPR is closely
447 linked to the internal state.

448

449

450 **How do hunger signals or clock genes contribute to CPR?**

451 Mammalian NPY and *Drosophila* homologue NPF modulate the outputs of gustatory signals
452 (Herness and Zhao, 2009; Inagaki et al., 2014; Wang *et al*., 2016). Hunger signal
453 hormones/peptides investigated here are important for taste modulation. For example, Akh
454 receptor is expressed in a subset of the gustatory neurons, which may modulate taste information
455 for carbohydrate metabolism (Bharucha et al., 2008). Therefore, the hunger signals likely modulate
456 the downstream of the sensory neurons, which contribute to CPR.

457 Circadian clock genes control and coordinate the expression of many genes in the body.
458 Therefore, we expect that the lack of clock genes disturbs the molecular and neural networks of
459 cellular homeostasis, including metabolism, which are essential for animal life. For example,
460 gustatory neurons express clock genes, and impaired clock function in gustatory neurons disrupts
461 daily rhythms in feeding behavior (Chatterjee et al., 2010). Temperature-sensing neurons transmit
462 warm or cold temperature information to central clock neurons (Alpert et al., 2020; Jin et al., 2021;
463 Marin et al., 2020; Tang et al., 2017; Yadlapalli et al., 2018). Therefore, the disrupted central clock
464 in the clock mutants may respond to the temperature signals imprecisely. There are many possible
465 reasons why the lack of clock gene expression to the central clock in the brain likely cause
466 abnormal CPR.

467 Furthermore, hunger signals may contribute to the output of the circadian regulations.
468 DH44 is located in the dorsomedial region in the fly brains, pars intercerebralis (PI), and the DH44-
469 expressing neurons play a role in the output pathway of the central clock (Barber et al., 2021; King
470 et al., 2017). Insulin-producing cells (IPCs) are also located beside DH44-expressing neurons
471 (Brogiolo et al., 2001; Cao and Brown, 2001; Ikeya et al., 2002; Rulifson et al., 2002). IPCs receive

472 various information, including circadian (Barber *et al*., 2021; Cavanaugh et al., 2014) and
473 metabolic signals (Bai *et al*., 2012; Okamoto *et al*., 2009; Rajan and Perrimon, 2012; Slaidina *et*
474 *al*., 2009) and subsequently transduce the signals downstream and release Ilps. Both Upd2 and
475 Ilp6 expressed in the fat body respond to metabolic states and remotely regulate Ilps expression
476 (Bai *et al*., 2012; Okamoto *et al*., 2009; Rajan and Perrimon, 2012; Slaidina *et al*., 2009).
477 Therefore, each hunger signal might have its specific function for CPR. Further studies will be
478 needed to describe the entire process.

479

480

481 **The role of light for CPR in both flies and mammals.**

482 Flies exhibit CPR in the presence of light but not in darkness (Figs. 5 and 6), suggesting that visual
483 inputs are required for CPR or that light may modulate sensory processing. This makes sense in
484 terms of body temperature regulation for diurnal ectotherms since they often move under the sun
485 and increase their body temperature by sun basking (Heinrich, 1993). Notably, in starved
486 mammals, the sight of food under the light is crucial in eliciting CPR (Fukuda and Morita, 2017).
487 In fact, visual cues are essential to evaluate how much food is consumed (Scheibehenne et al.,
488 2010). Thus, food signals could trigger CPR under light in both flies and mammals.

489

490

491 **Both mammals and flies show CPR in body temperature regulation.**

492 Starved flies choose a lower Tp. Because flies are small ectotherms, lower Tp means a lower
493 body temperature (Stevenson, 1985a; Stevenson, 1985b), which leads to a reduced metabolic rate

494 (Klepsatel et al., 2019). Therefore, both body temperature and metabolic states are lower in
495 starved flies than those of fed flies, resulting in energy savings for survival. Since flies move
496 slowly in the lower temperature than in the warm temperature and their metabolic state is low
497 (Giraldo et al., 2019), starved flies may need to exit from the starved state as soon as possible.

498 As soon as hungry animals taste food, the sensory signals trigger CPR. They can move to
499 a warmer place to prepare to warm up their body temperature (Fig. 7, blue arrows). This CPR is
500 an intermediate response in which flies generally prefer medium warmer Tp yet do not reach the
501 high Tp of fed flies. CPR may allow flies to choose a warmer place to enable higher metabolism,
502 which allows them to move to the next step, such as foraging and actively moving to find a mate
503 before competitors arrive. As such, CPR may be a strategy for the fly's survival. As soon as
504 nutrients enter the body, flies can choose a similar Tp as fed flies. (Fig. 7, green arrows).

505 Similarly, starvation or malnutrition in mammals causes lower body temperature (Cintron-
506 Colon *et al*., 2017; Piccione *et al*., 2002), and biting food triggers heat generation, which is CPR
507 (LeBlanc *et al*., 1984; Power and Schulkin, 2008). Thus, while starvation causes lower body
508 temperatures in both flies and mammals, feeding cues initiate CPR in heat generation and nutrient
509 intake, resulting in full recovery from the hungry state. Our data suggest that the *Drosophila* CPR
510 may be a functional and physiological response equivalent to the CPR observed in other animals.
511 Thus, *Drosophila* may shed new light on the regulation of CPR and provide a deeper understanding
512 of the relationship between CPR and metabolism.

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516

517 **Acknowledgements**

518 We are grateful to Drs. Anupama Dahanukar, Hubert Amrein, Greg Suh, and Paul A. Garrity as
519 well as Bloomington *Drosophila* fly stock center for the fly lines. We thank the members of the
520 Hamada laboratory for their critical comments and advice on the manuscript, Dr. Richard A. Lang
521 and his laboratory members for their comments and kindly sharing reagents, Dr. Satoshi
522 Namekawa for kindly sharing reagents, and Mrs. Matthew Batie and Nathan T. Petts for designing
523 and constructing the behavioral testing apparatuses and the red light illumination device. This
524 research was supported by a Trustee Grant and RIP funding from Cincinnati Children's Hospital,
525 JST (Japan Science and Technology)/Precursory Research for Embryonic Science and Technology
526 (PRESTO), the March of Dimes, and NIH R01 grant GM107582 to F. N. H. S.H.S. is a Latin
527 American Fellow in the Biomedical Sciences supported by the Pew Charitable Trusts. Research in
528 the laboratory of J.C.C. is supported by NIH R01 grant DK124068.

529

530

531 **Contributions**

532 F.N.H and Y.U designed the experiments. Y.U, E.N., T.N. and J.S. performed the temperature
533 preference behavioral assays and data analysis. Y.U., S.H.S., S.S.U. and J.C. performed the
534 feeding experiments and the data analysis. F.N.H and Y.U wrote the manuscript.

535

536

537 **Declaration of Interest**

538 The authors declare no competing interests

539

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810 **FIGURES**

811 **Figure 1. Sucralose refeeding triggers an intermediate recovery, which differs from the**
812 **response after glucose intake.**

813 **(A)** A schematic diagram of the experimental conditions. Flies were reared under fly food.
814 Emerged flies were kept with fly food for one or two days (orange) and then starved with water-
815 absorbed paper for one or two overnights (gray). Starved flies were refed with either fly food
816 (orange), 2 mL of sucralose (blue), or glucose solution (green) absorbed paper, and then the
817 temperature preference (Tp) behavior assays were examined. Details are provided in the
818 experimental procedures.

819 **(B-D)** Comparison of Tp of *white*^{1118} (w^{1118}) control flies among fed (white bar), starved (STV;
820 gray bar), and refed state (orange, blue, or green bar). Starved flies were refed with fly food
821 (orange bar) for 5, 10, 30, or 60 min (1 hr) (B), 2.8 mM sucralose solution (blue bar) for 10 min
822 or 1 hr (C), or 2.8 mM (equivalent to 5%) glucose solution (green bar) for 5 min, 10 min, or 1 hr
823 (D). Behavioral experiments were performed at zeitgeber time (ZT) 4-7. ZT0 and ZT12 are light-
824 on and light-off, respectively. Dots on each bar show individual Tp in assays. Italicized numbers
825 indicate the number of trials. Per normality test, the Shapiro-Wilk test was performed. One-way
826 ANOVA was performed to compare Tp among each refeeding condition. Tukey's post hoc test
827 was used to compare between experiments and starved condition (green stars). Data is show as
828 average Tp with SEM. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$. ****$p < 0.0001$. NS indicates no
829 significance.

830 **(E)** Comparison of Tp among the following condition in w^{1118} (control) flies; fed (white bar),
831 starved overnight (O/N) (Water or STV; gray bar), refed with fly food (orange bar), sucralose
832 (blue bars), or glucose (green bars). The same data are used in Fig. 1 B-D. Per normality test, the

833 Shapiro-Wilk test was performed. One-way ANOVA was performed to compare Tp among each
834 refeeding condition. Red (or green) stars show Tukey's post hoc test, which was compared
835 between experiments and fed (or starved) conditions, respectively. *p < 0.05. **p < 0.01. ***p <
836 0.001. ****p < 0.0001. NS indicates no significance.
837 **(F)** Feeding assay: number of touches to the water (gray), 2.8 mM (equivalent to 5%) glucose
838 (green), or 2.8 mM sucralose in each solution (blue) was examined using w^{1118} flies which
839 starved for 1 day. Cumulative number of touches for 0-30 min were plotted. Two-way ANOVA
840 was performed for the multiple comparison test. Blue and green stars show Fisher's LSD post
841 hoc test compared sucralose (blue stars) or glucose (green stars) solution feeding to water
842 drinking. All data shown are averages with SEM. *p < 0.05. **p < 0.01. ***p < 0.001. ****p <
843 0.0001.
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846 **Figure 2. Gustatory neurons are important for intermediate recovery.**
847 **(A-E and K)** Comparison of Tp of flies among fed (white bar), starved (STV; gray bar), and
848 refed state (blue bar). The starved flies were refed with sucralose (blue bars) for 10 min.
849 **(F-J)** Comparison of Tp of flies among fed (white bar), starved (STV; gray bar), and starved
850 state with all-trans-retinal (ATR; yellow bars), which is the chromophore and required for the
851 CsChrimson activation. Gustatory neurons in starved flies were excited by red-light pulses
852 (flashing on and off at 10 Hz) for 10 min. Behavioral experiments were performed at ZT4-7.
853 Dots on each bar show individual Tp in assays. Italicized numbers indicate the number of trials.
854 Per normality test, the Shapiro-Wilk test was performed. One-way ANOVA was performed for
855 statistical analysis. Green stars show Tukey's post hoc test that was compared between each

856 experiment and starved condition. Compiled data were shown in Fig. S2. All data shown are
857 averages with SEM. *p < 0.05. **p < 0.01. ***p < 0.001. ****p < 0.0001. NS indicates no
858 significance.
859 **(L)** Schematic diagram of the optogenetic activation assay.
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862 **Figure 3. Activation of temperature-sensing neurons triggers CPR.**
863 **(A-E)** Comparison of Tp of flies among fed (white bar), starved (STV; gray bar), and refed state
864 (blue bar). Starved flies were refed with sucralose (blue bars) for 10 min. Behavioral experiments
865 were performed at ZT4-7. Per normality test, the Shapiro-Wilk test was performed. One-way
866 ANOVA or Kruskal-Wallis test was performed for statistical analysis. Green stars show Tukey's
867 post hoc test or Dunn's test that was compared between each experiment and starved condition.
868 Compiled data were shown in Fig. S2. All data shown are averages with SEM. *p < 0.05. **p <
869 0.01. ***p < 0.001. ****p < 0.0001. NS indicates no significance. The same data of *UAS-Kir/+*
870 in Fig. 2E are used in Fig. 3E.
871 **(F and G)** Comparison of Tp between starved (STV; gray bar) and starved flies with all-trans-
872 retinal (ATR; yellow bars). Warm neurons (F) or cold neurons (G) in starved flies were
873 expressed CsChrimson that was excited by red-light pulses for 10 min. Behavioral experiments
874 were performed at ZT4-7. Per normality test, the Shapiro-Wilk test was performed. Student t-test
875 or Kolmogorov-Smirnov test was used for statistical analysis. (G) *tubGal80^{ts}; R11F02-*
876 *Gal4>uas-CsChrimson* flies were reared at 18°C, and emerged adult flies were collected and
877 kept at 29°C. Details are in the materials and methods.
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880 **Figure 4. Hunger signals are necessary for CPR.**

881 Comparison of Tp of flies among fed (white bar), starved (STV; gray bar), and refed state (blue,
882 green or dark green). Starved flies were refed with sucralose (blue bars) or glucose (green) for 10
883 min. These behavioral experiments were performed at ZT4-7. Dots on each bar showed
884 individual Tp in assays. Italicized numbers indicate the number of trials. Per normality test, the
885 Shapiro-Wilk test was performed. One-way ANOVA was performed for statistical analysis.
886 Green stars show Tukey's post hoc test that was compared between each experiment and starved
887 condition. Compiled data were shown in Fig. S3. All data shown are averages with SEM. *p <
888 0.05. **p < 0.01. ***p < 0.001. ****p < 0.0001. NS indicates no significance.

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891 **Figure 5. Clock genes are necessary for CPR in LD.**

892 Comparison of Tp of flies among fed (white circles), starved (STV; gray triangles), and refed
893 state (blue, green or dark green squares). Starved flies were refed with sucralose (blue squares)
894 or glucose for 10 min (green squares) or 1 hr (dark green squares). After refeeding for 10 min or
895 1 hr, the temperature preference behavior assays were immediately performed at ZT1-3, 4-6, 7-9,
896 and 10-12 in LD. Per normality test, the Shapiro-Wilk test was performed. One-way ANOVA or
897 Kruskal-Wallis test was performed for statistical analysis. Green stars show Tukey's post hoc
898 test or Dunn's test that was compared between each experiment and starved condition at the
899 same time point. All data shown are averages with SEM. *p<0.05. **p<0.01. ***p<0.001. ****p
900 < 0.0001.

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903 **Figure 6. Light is crucial for CPR.**

904 Comparison of Tp of flies among fed (white circles), starved (STV; gray triangles), and refed
905 state (blue or green squares). After flies were raised in LD condition, adult flies were under the
906 LL (A1, A2, B1, and B2) or DD (C1 and C2) conditions for two days. Starved flies were refed
907 with sucralose (blue squares) or glucose for 10 min (green squares). Right after, the temperature
908 preference behavioral assay was performed under the light (A1, A2, B1, and B2) or in the dark
909 (C1 and C2) at circadian time (CT)1-3, 4-6, 7-9, and 10-12. Per normality test, the Shapiro-Wilk
910 test was performed. One-way ANOVA or Kruskal-Wallis test was performed for statistical
911 analysis. Green stars show Tukey's post hoc or Dunn's test that was compared between each
912 experiment and starved condition at the same time point. As for Fig. 6C, fed flies kept in DD
913 showed rhythmic TPR in daytime ($p<0.05$). All data shown are averages with SEM. *$p<0.05$.
914 **$p<0.01$. ***$p<0.001$. ****$p < 0.0001$.

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917 **Figure 7. MODEL**

918 A schematic diagram for the Tp recovery by CPR (blue arrows) and nutrient-dependent
919 regulation (green arrows).

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922 **Figure S1. Gustatory or temperature-sensing neurons are indispensable for the recovery**
923 **after glucose intake (Related to Figs. 2 and 3).**

924 Comparison of Tp of flies among fed (white bar), starved (STV; gray bar), and refed state (green
925 or dark green bar). The starved flies were refed with glucose for 10 min (green) and 1 hr (dark
926 green). These behavioral experiments were performed at ZT4-7. Dots on each bar showed
927 individual Tp in assays. Italicized numbers indicate the number of trials. Per normality test,
928 Shapiro-Wilk test was performed. One-way ANOVA or Kruskal-Wallis test was performed for
929 statistical analysis. Green stars show Tukey's post hoc test or Dunn's test that was compared
930 between each experiment and starved condition. All data shown are averages with sem. *p <
931 0.05. **p < 0.01. ***p < 0.001. ****p < 0.0001. NS indicates no significance.
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934 **Figure S2: Tp recovery upon sucralose, glucose, fly food refeeding using gustatory or**
935 **temperature-sensing neurons silenced flies. (Related to Figs. 2, 3 and S1)**
936 Comparison of Tp of flies among fed (white bar), starved (STV; gray bar), and refed state
937 (orange, blue, green or dark green bar). The starved flies were refed with normal fly food for 10
938 min (orange), sucralose for 10 min (blue), glucose for 10 min (green) or 1 hr (dark green). The
939 same data were used with Figs. 2, 3 and S1. These behavioral experiments were performed at
940 ZT4-7. Dots on each bar showed individual Tp in assays. Italicized numbers indicate the number
941 of trials. Per normality test, Shapiro-Wilk test was performed. One-way ANOVA or Kruskal-
942 Wallis test was performed for statistical analysis. Green stars show Tukey's post hoc test or
943 Dunn's test that was compared between each experiment and starved condition. All data shown
944 are averages with SEM. *p < 0.05. **p < 0.01. ***p < 0.001. ****p < 0.0001. NS indicates no
945 significance.
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948 **Figure S3: Tp recovery upon sucralose, glucose, fly food refeeding using flies, which deleted**

949 **the hunger signals or suppressed the hunger-stress-responsive neurons. (Related to Fig. 4)**

950 Comparison of Tp of flies among fed (white bar), starved (STV; gray bar), and refed state

951 (orange, blue, green or dark green bar). Starved flies were refed with normal fly food for 10 min

952 (orange), sucralose for 10 min (blue), glucose for 10 min (green) or 1 hr (dark green). The same

953 data were used in Fig. 4. These behavioral experiments were performed at ZT4-7. Dots on each

954 bar showed individual Tp in assays. Italicized numbers indicate the number of trials. Per

955 normality test, the Shapiro-Wilk test was performed. One-way ANOVA or Kruskal-Wallis test

956 was performed for statistical analysis. Green stars show the Tukey post hoc test or Dunn's test

957 that was compared between each experiment and starved condition. All data shown are averages

958 with SEM. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$. ****$p < 0.0001$. NS indicates no significance.

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961 **Figure S4. The number of touches on water, sucralose, and glucose using *yw*, *per*^{01}, and**

962 ***tim*^{01} flies. (Related to Figs. 1, 5 and 6)**

963 Feeding assay: the number of touches to water (gray), 2.8 mM (equivalent to 5 %) glucose

964 (green), or 2.8 mM sucralose solution (blue) were examined using *yw*, *per*^{01}, or *tim*^{01} flies that

965 were starved for 1 day. The cumulative number of touches for 0-30 min was plotted. Two-way

966 ANOVA was performed for the multiple comparison test. Blue and green stars show Fisher's

967 LSD post hoc test that was compared between sucralose (blue stars) or glucose (green stars)

968 solution feeding to water drinking. Black stars show the statistical difference between sucralose

969 and glucose feeding. All data shown are averages with SEM. *$p < 0.05$. **$p < 0.01$. ***$p <$

970 0.001. ****$p < 0.0001$.

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992 **Materials and Methods**

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994 All flies were reared under 12 hr-light/12 hr-dark (LD) cycles at 25°C and 60-70% humidity in
995 an incubator (DRoS33SD, Powers Scientific Inc.) with an electric timer (light on: 8am; light off:
996 8pm). The light intensity is 1000-1400 lux. All flies were reared on custom fly food recipe, with
997 the following composition per 1 L of food: 6.0 g sucrose, 7.3 g agar, 44.6 g cornmeal, 22.3 g
998 yeast and 16.3 mL molasses as described previously (Umezaki *et al.*, 2018a). *white*^{1118} (w^{1118})
999 and *yellow*^{1} *white*^{1} ($y^{1}w^{1}$) flies were used as control flies. *EP5Δ; Gr64a*^{1} (*Gr5a*$^{-/-}$*; Gr64a*$^{-/-}$) was
1000 kindly provided by Dr. Anupama Dahanukar (Dahanukar *et al.*, 2007). *R1; Gr5a-LexA; +;*
1001 *ΔGr61a, ΔGr64a-f* (*Gr5a*$^{-/-}$*; Gr61a*$^{-/-}$*, Gr64a-f*$^{-/-}$) were kindly provided by Dr. Hubert Amrein
1002 (Fujii *et al.*, 2015; Yavuz *et al.*, 2014). *Dh44-Gal4* was kindly provided by Dr. Greg Suh (Dus *et*
1003 *al.*, 2011). *TrpA1*^{SH}*-gal4* was kindly provided by Dr. Paul A. Garrity (Hamada *et al.*, 2008).
1004 Other fly lines were provided by Bloomington Drosophila stock center and Vienna Drosophila
1005 Stock Center.

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1008 **Temperature preference behavioral assay**

1009 The temperature preference behavior assays were examined using a gradient of temperatures,
1010 ranging from 16-34°C and were performed for 30 min in an environmental room maintained at
1011 25°C /60%–70% humidity as described previously (Umezaki *et al.*, 2018b). We prepared a total
1012 of 40-50 flies (male and female flies were mixed) for fed condition experiments and 90-100 flies
1013 for overnight(s) starved and refed condition experiments for one trial. Flies were never reused in
1014 subsequent trials. In starved and refed experiments, we prepared double the amount of flies

1015 required for one trial, as almost half of them died by starvation stress. Others climbed on the wall
1016 and ceiling (starved flies are usually hyperactive (Yang et al. 2015 PNAS)), even though we
1017 applied slippery coating chemicals (PTFE; Cat# 665800, Sigma or byFormica PTFE Plus,
1018 https://byformica.com/products/fluon-plus-ptfe-escape-prevention-coating) to the plexiglass
1019 covers.

1020 The behavioral assays were performed for 30 min at Zeitgeber time (ZT) 4-7 (Light on
1021 and light off are defined as ZT0 and ZT12, respectively), and starvation was initiated at ZT9-10
1022 (starved for 1, 2 or 3 O/N are 18-21 hrs, 42-45 hrs or 66-69 hrs, respectively). For the starvation
1023 assays, the collected flies were maintained with our fly food for at least one day for their
1024 maturation and subsequently moved into plastic vials with 3 mL of distilled water which is
1025 absorbed by a Kimwipe paper. For the refeeding experiments, starved flies were transferred into
1026 plastic vials with 2 mL of 2.8 mM sugar solution (Sucralose water/ Glucose water) for a certain
1027 time period. Sugar solution is absorbed by the half size of a Kimwipe.

1028 After the 30-min behavioral assay, the number of flies whose bodies were completely
1029 located on the apparatus were counted. Flies whose bodies were partially or completely located
1030 on the walls of the apparatus cover were not included in the data analysis. The percentage of flies
1031 within each one-degree temperature interval on the apparatus was calculated by dividing the
1032 number of flies within each one-degree interval by the total number of flies on the apparatus. The
1033 location of each one-degree interval was determined by measuring the temperature at 6 different
1034 points on the bottom of the apparatus. Data points were plotted as the percentage of flies within a
1035 one-degree temperature interval. The weighted mean of Tp was calculated by totaling the
1036 product of the percentage of flies within a one-degree temperature interval and the corresponding
1037 temperature (e.g., fractional number of flies x 15.5°C + fractional number of flies x 16.5°C

1038 +…… fractional number of flies x 34.5°C). If the SEM of the averaged Tp was not < 0.3 after
1039 the five trials, we performed additional trials around 10 times until the SEM reached < 0.3.
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1042 **Temperature preference rhythm (TPR) assay**
1043 For the TPR assays, we performed temperature preference behavior assays in different time
1044 windows during the day (zeitgeber time (ZT) or circadian time (CT) 1-3, 4-6, 7-9, and 10-12) as
1045 described previously (Goda et al., 2014; Kaneko *et al.*, 2012). Because starvation duration
1046 directly affects flies' Tp (Umezaki *et al.*, 2018b), starvation was initiated at each time window to
1047 adjust the starvation duration at each time point, which means flies were starved for 24 hrs or 48
1048 hrs but not 1 or 2 O/N. Each behavioral assay was not examined during these time periods (ZT or
1049 CT0-1 and 11.5-13) because of large phenotype variation around light on and light off.
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1052 **Optogenetic activation**
1053 For the optogenetic activation of the target neurons for behavioral assays, red-light-sensitive
1054 channelrodopsin, *UAS-CsChrimson*, was crossed with each Gal4 drivers. Flies were reared on fly
1055 food at 25°C and 60-70% humidity under LD cycles in an incubator (DRoS33SD, Powers
1056 Scientific Inc.) with an electric timer. After flies emerged, we collected adult flies and kept them
1057 with fly food for 1-2 days. Next day, flies were transferred into water with or without 0.8 mM
1058 all-trans retinal (ATR; #R2500, Sigma) diluted in dimethyl sulfoxide (DMSO; #472301, Sigma)
1059 for 2 O/N. To activate flies expressing UAS-CsChrimson crossed with Gal4 drivers, we
1060 employed 627 nm red-colored light-emitted diode (LED) equipped with pulse photoillumination

1061 system (10 Hz, 0.08 $mWmm^{-2}$). Flies were irradiated with pulsed red light for 10 min, which is
1062 equivalent to refeeding time period. This photoillumination system was used in an incubator
1063 (Sanyo Scientific, MIR-154), and followed by temperature preference behavioral assays.
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1066 **Optogenetic assay for *tubGal80^{ts}; R11F02-Gal4>uas-CsChrimson***
1067 Because *R11F02-Gal4>uas-CsChrimson* flies died during the pupal stage, Gal4/Gal80^{ts} system
1068 was used to restrict *uas-CsChrimson* expression to the adult stage for optogenetc assays against
1069 the cold-sensing neurons (*R11F02-Gal4*-expressing neurons). Gal80^{ts} is a temperature sensitive
1070 allele of Gal80, which binds Gal4 and inhibits *CsChrimson* gene expression at 18°C. However,
1071 Gal80 is degraded at 29°C; in turn, the Gal4 activates *CsChrimson* gene expression (Southall et
1072 al. 2008 CSH protocol). *tubGal80^{ts}; R11F02-Gal4>uas-CsChrimson* flies were reared with fly
1073 food at 18°C. Emerged adult flies were collected and kept with fly food at 29°C. Next day, flies
1074 were transferred to water condition (starved condition) with or without 0.8 mM ATR for 2 O/N.
1075 Starved flies with or without ATR application were exposed to pulsed red light for 10 min
1076 (equivalent to refeeding period) and then immediately loaded in the behavior apparatus for
1077 behavioral assays to measure their Tp.
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1079
1080 **Feeding assay**
1081 To measure single fly feeding, we used *Drosophila* Fly Liquid Interaction Counter (FLIC)
1082 system (Ro et al. 2013 Plos one; Sable systems International https://www.sablesys.com). Groups
1083 of 1-2-day-old male and female flies were starved for 24 hrs, between ZT4-5 (12-1pm).

1084 Individual flies were then loaded into FLIC monitors. Flies were acclimatized to the monitors for
1085 30 min, with access to water provided in the food wells. To start the feeding assay, water was
1086 replaced with 2.8 mM Sucralose or 2.8 mM Glucose solution (equivalent to 5% Glucose conc.)
1087 and the number of licks (touches) were recorded for 30 min. Assays were performed at ZT4-7.
1088 To account for potential confounding effect of startle response when changing the food, wells
1089 where water was replaced with new water were used as a control. FLIC raw data was analyzed
1090 using the FLIC R code master (Pletcher Lab, available
1091 at https://github.com/PletcherLab/FLIC_R_Code). The numbers of licks were obtained and
1092 summed into 5-min windows bin, while the cumulative licks were obtained by the consecutive
1093 sum of the licks in these bins.
1094

Fig. 1

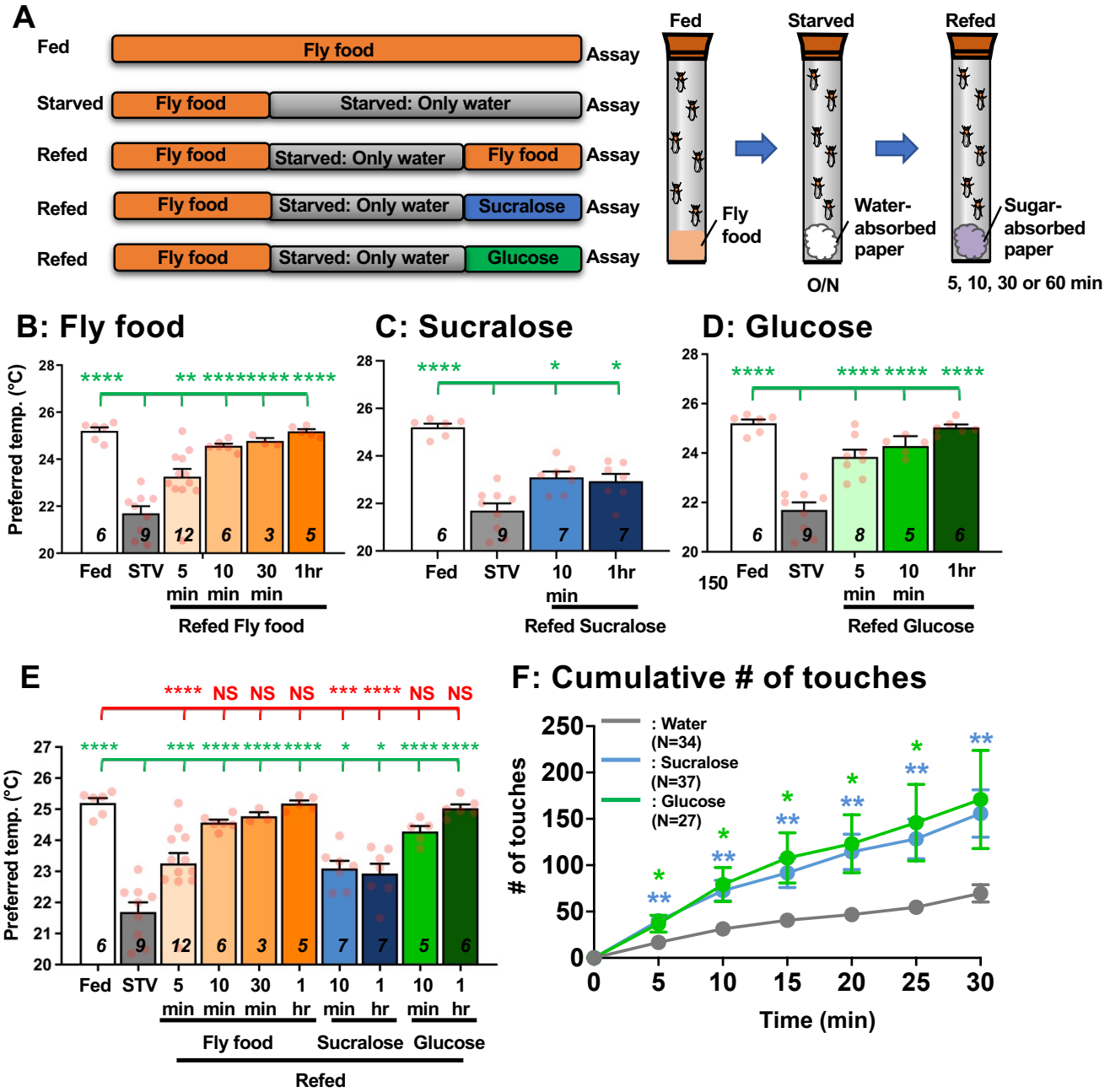

A
Fed
Fly food
Assay
Starved
Fly food
Starved: Only water
Assay
Refed
Fly food
Starved: Only water
Fly food
Assay
Refed
Fly food
Starved: Only water
Sucralose
Assay
Refed
Fly food
Starved: Only water
Glucose
Assay
Fed
Starved
Refed
Fly food
Water-absorbed paper
O/N
Sugar-absorbed paper
5, 10, 30 or 60 min
B: Fly food
Preferred temp. (°C)
Fed
STV
5 min
10 min
30 min
1hr
Refed Fly food
C: Sucralose
10 min
1hr
Refed Sucralose
D: Glucose
150
Refed Glucose
E
NS
Fly food
Sucralose
Glucose
Refed
F: Cumulative # of touches
: Water (N=34)
: Sucralose (N=37)
: Glucose (N=27)
# of touches
Time (min)

Fig. 2

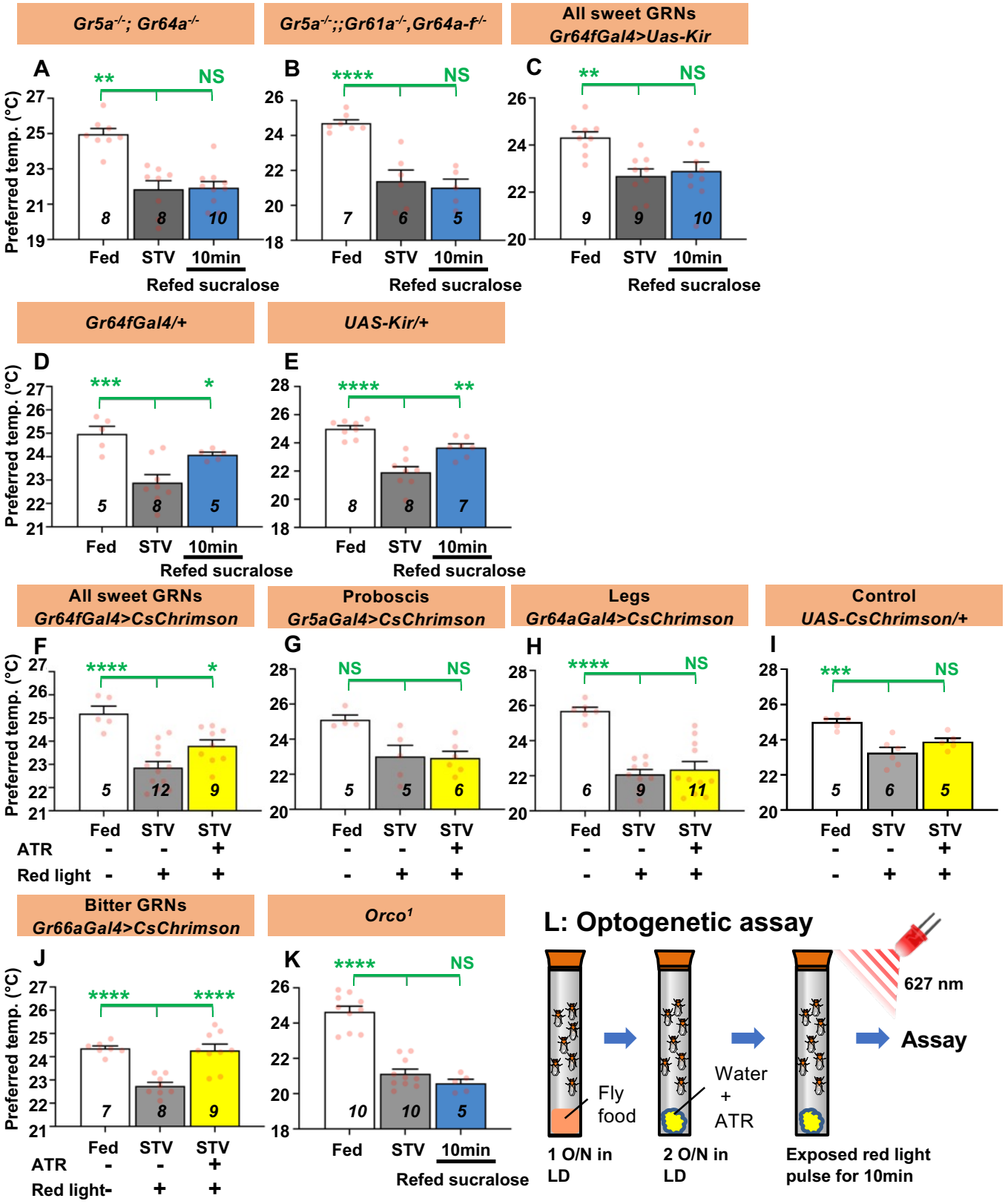

Gr5a-/-; Gr64a-/-
Gr5a-/-;;Gr61a-/-,Gr64a-f-/-
All sweet GRNs
Gr64fGal4>Uas-Kir
A
B
C
Preferred temp. (°C)
**
NS
****
Fed
STV
10min
Refed sucralose
Gr64fGal4/+
UAS-Kir/+
D
E
***
*
All sweet GRNs
Gr64fGal4>CsChrimson
Proboscis
Gr5aGal4>CsChrimson
Legs
Gr64aGal4>CsChrimson
Control
UAS-CsChrimson/+
F
G
H
I
ATR
Red light
Bitter GRNs
Gr66aGal4>CsChrimson
Orco1
J
K
L: Optogenetic assay
627 nm
Assay
Fly food
Water + ATR
1 O/N in LD
2 O/N in LD
Exposed red light pulse for 10min

Fig. 3

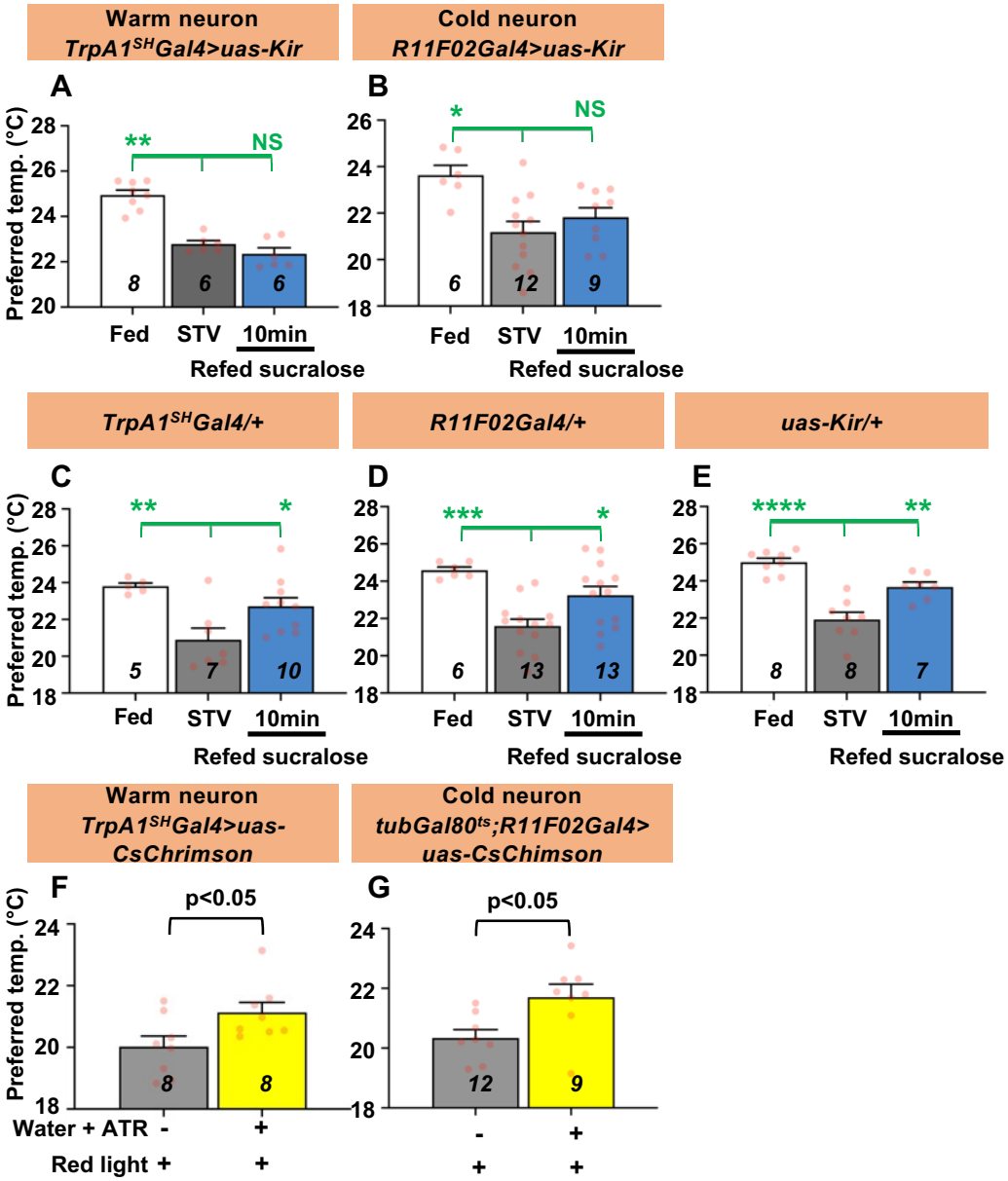

Warm neuron
TrpA1SHGal4>uas-Kir
Cold neuron
R11F02Gal4>uas-Kir
A
B
Preferred temp. (°C)
**
NS
*
NS
8
6
6
6
12
9
Fed
STV
10min
Refed sucralose
TrpA1SHGal4/+
R11F02Gal4/+
uas-Kir/+
C
D
E
**
*
***
*
****
**
5
7
10
6
13
13
8
8
7
Warm neuron
TrpA1SHGal4>uas-CsChrimson
Cold neuron
tubGal80ts;R11F02Gal4>uas-CsChimson
F
G
p<0.05
p<0.05
8
8
12
9
Water + ATR
Red light

Fig. 4

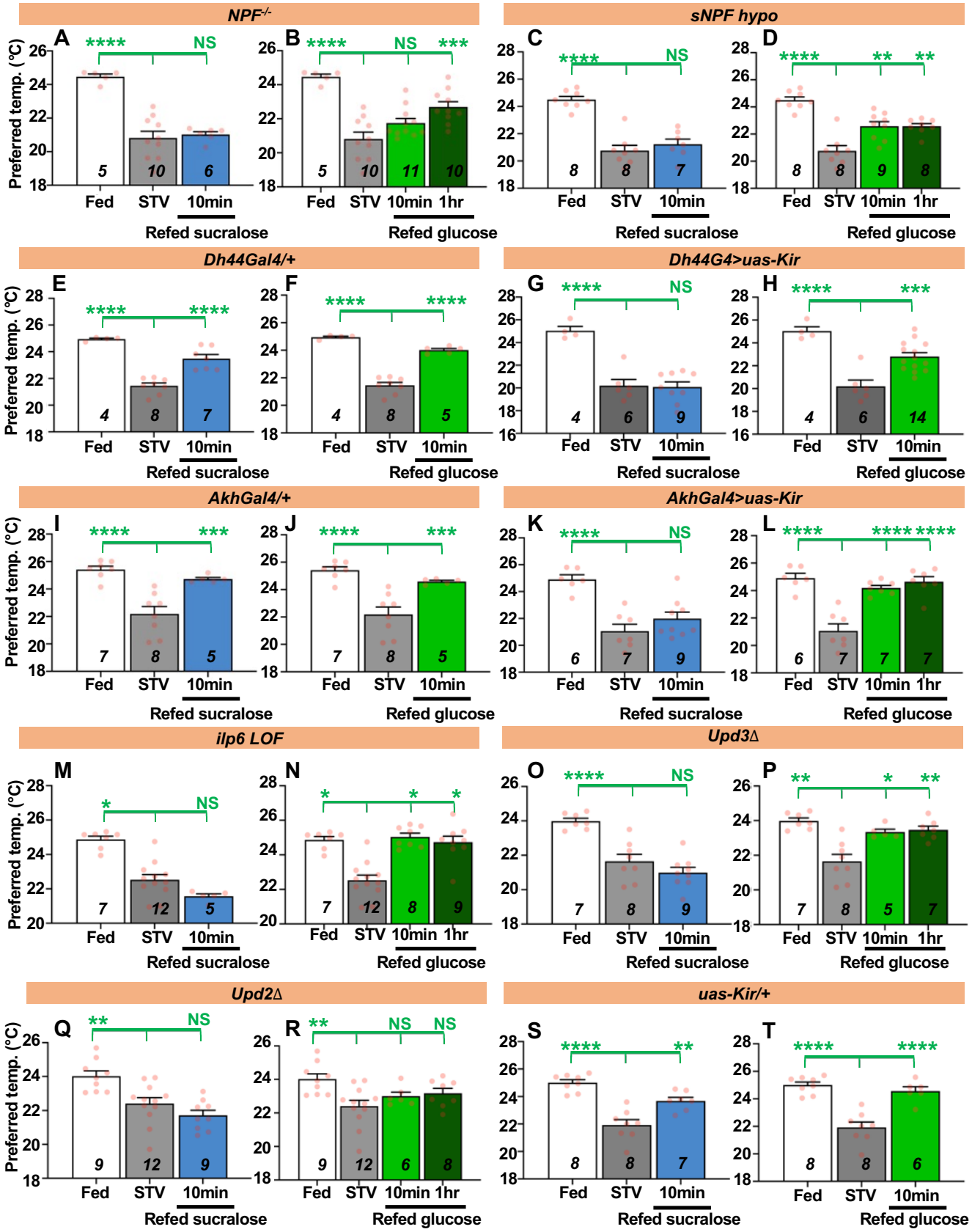

NPF-/-
sNPF hypo
Dh44Gal4/+
Dh44G4>uas-Kir
AkhGal4/+
AkhGal4>uas-Kir
ilp6 LOF
Upd3Δ
Upd2Δ
uas-Kir/+
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
Preferred temp. (°C)
Fed
STV
10min
1hr
Refed sucralose
Refed glucose
NS

Fig. 5

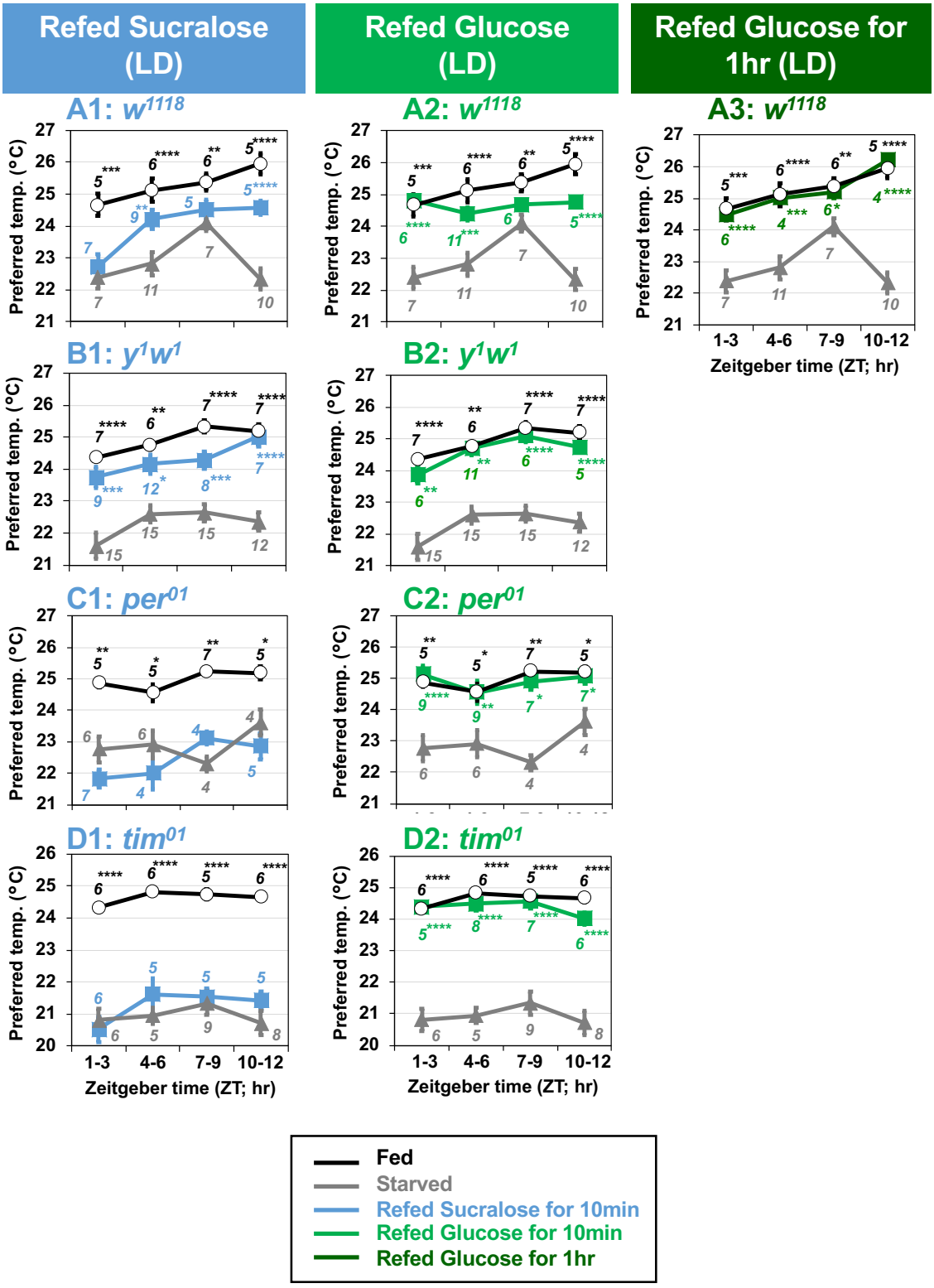

Refed Sucralose (LD)
Refed Glucose (LD)
Refed Glucose for 1hr (LD)
A1: w1118
A2: w1118
A3: w1118
B1: y1w1
B2: y1w1
C1: per01
C2: per01
D1: tim01
D2: tim01
Preferred temp. (°C)
Zeitgeber time (ZT; hr)
1-3
4-6
7-9
10-12
Fed
Starved
Refed Sucralose for 10min
Refed Glucose for 10min
Refed Glucose for 1hr

Fig. 6

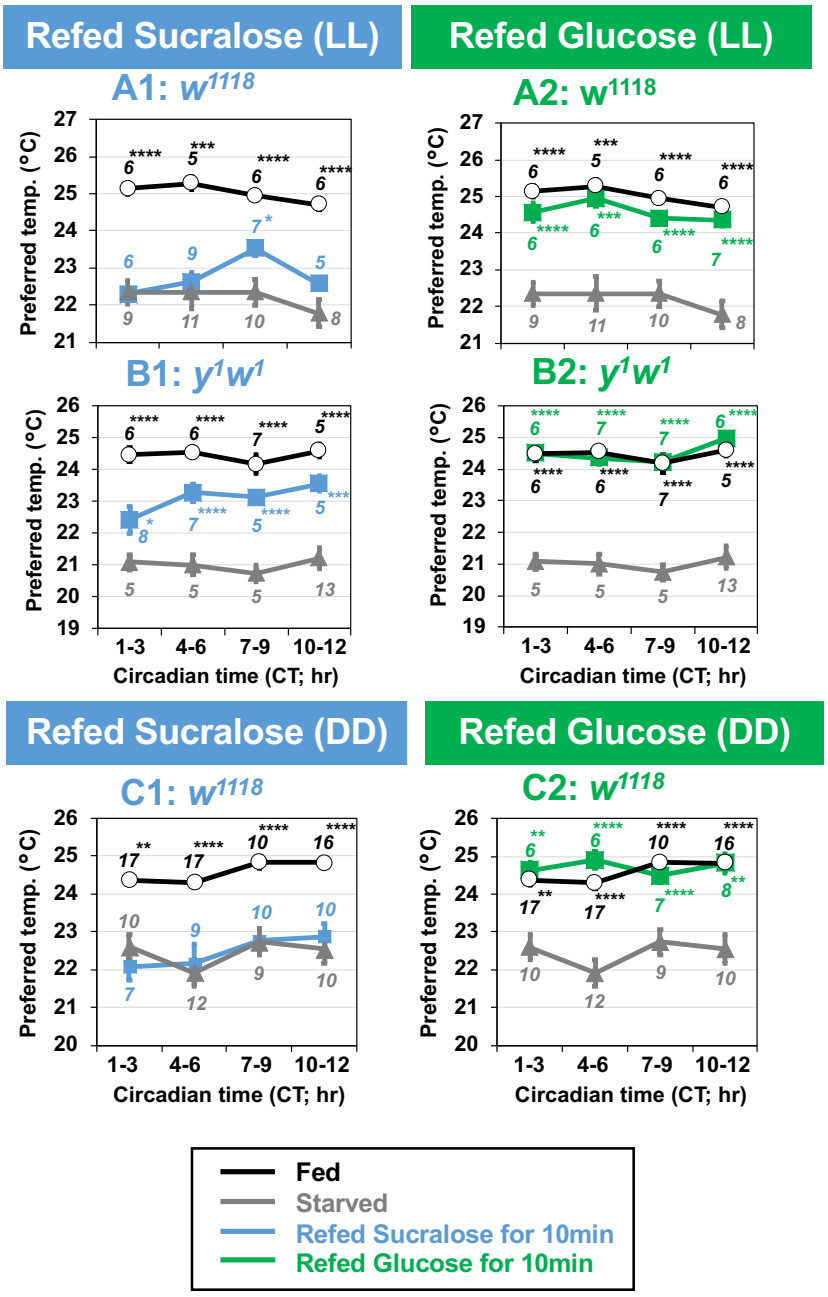

Refed Sucralose (LL)
Refed Glucose (LL)
A1: w1118
A2: w1118
B1: y1w1
B2: y1w1
Refed Sucralose (DD)
Refed Glucose (DD)
C1: w1118
C2: w1118
Preferred temp. (°C)
Circadian time (CT; hr)
1-3
4-6
7-9
10-12
Fed
Starved
Refed Sucralose for 10min
Refed Glucose for 10min

Fig. 7

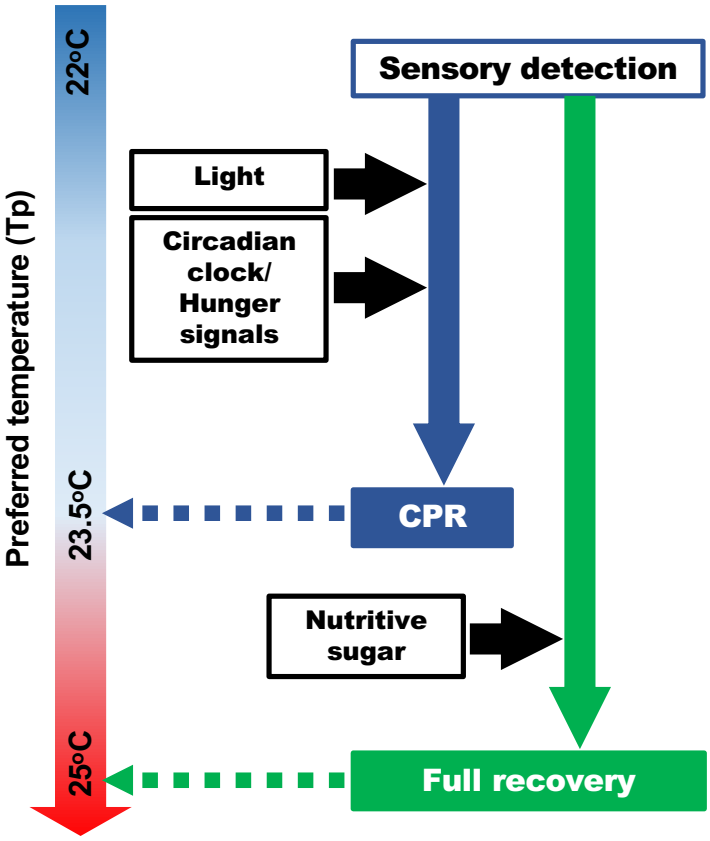

Preferred temperature (Tp)
22°C
23.5°C
25°C
Sensory detection
Light
Circadian clock/ Hunger signals
CPR
Nutritive sugar
Full recovery

Fig. S1

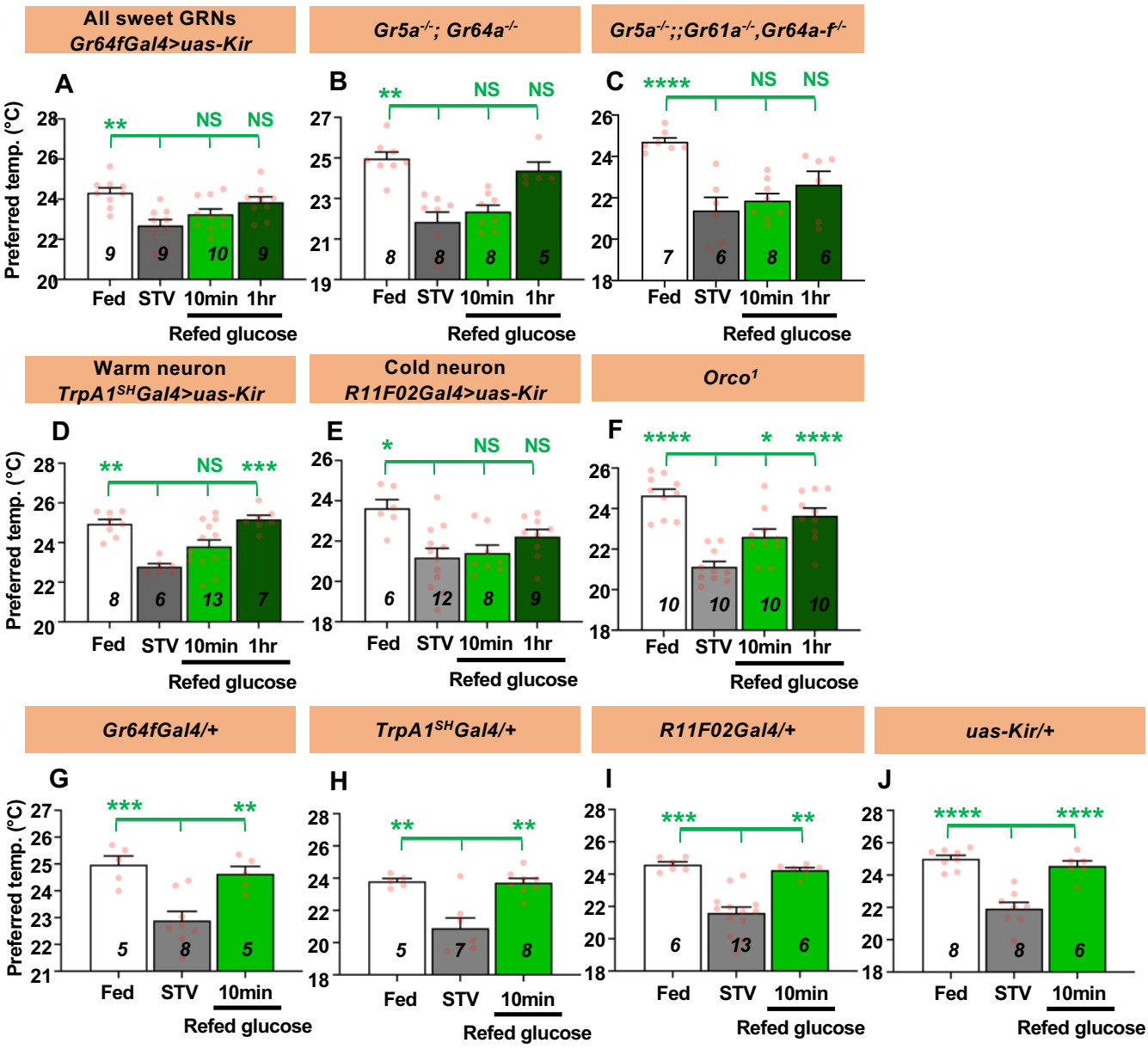

All sweet GRNs
Gr64fGal4>uas-Kir
Gr5a-/-; Gr64a-/-
Gr5a-/-;;Gr61a-/-,Gr64a-f-/-
A
B
C
Preferred temp. (°C)
Fed
STV
10min
1hr
Refed glucose
Warm neuron
TrpA1SHGal4>uas-Kir
Cold neuron
R11F02Gal4>uas-Kir
Orco1
D
E
F
Gr64fGal4/+
TrpA1SHGal4/+
R11F02Gal4/+
uas-Kir/+
G
H
I
J

Fig. S2

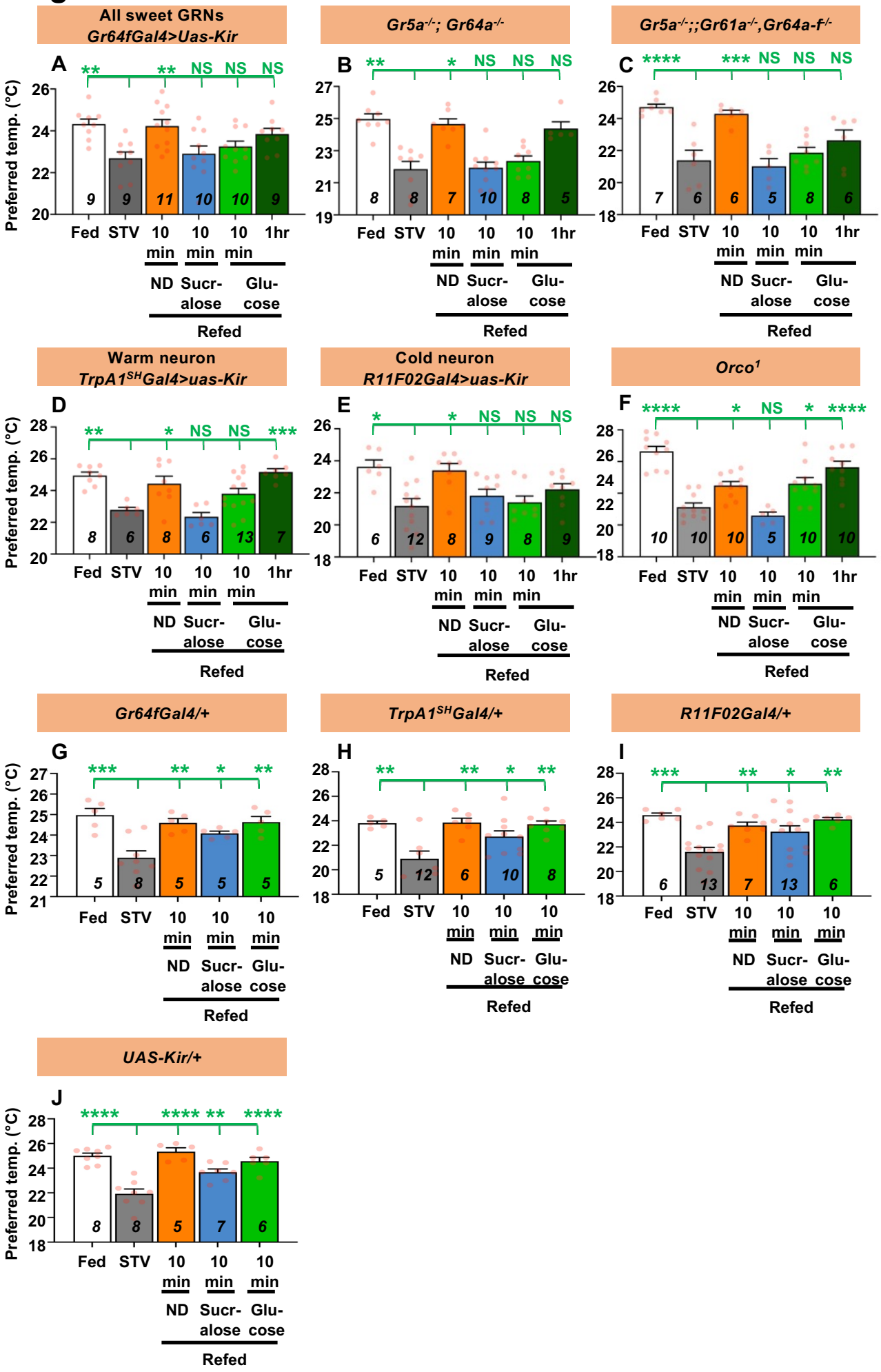

All sweet GRNs
Gr64fGal4>Uas-Kir
Gr5a-/-; Gr64a-/-
Gr5a-/-;;Gr61a-/-,Gr64a-f-/-
Warm neuron
TrpA1SHGal4>uas-Kir
Cold neuron
R11F02Gal4>uas-Kir
Orco1
Gr64fGal4/+
TrpA1SHGal4/+
R11F02Gal4/+
UAS-Kir/+
A
B
C
D
E
F
G
H
I
J
Preferred temp. (°C)
Fed
STV
10 min
1hr
ND
Sucr-alose
Glu-cose
Refed
NS

Fig. S3

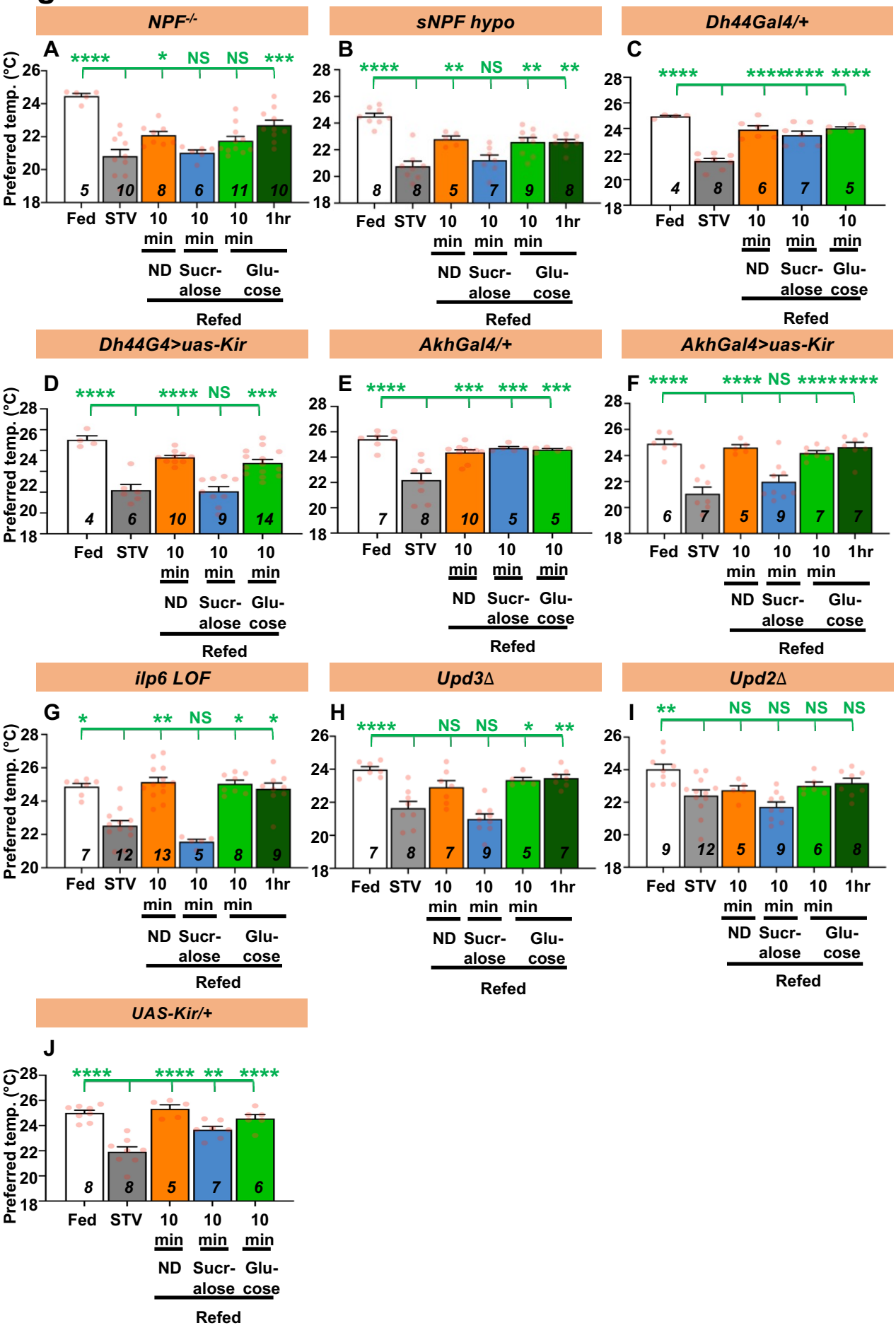
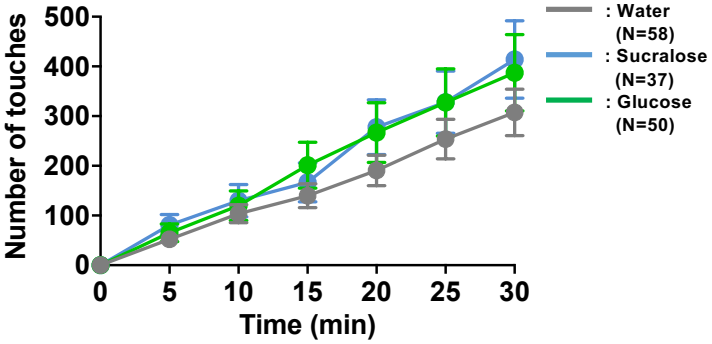
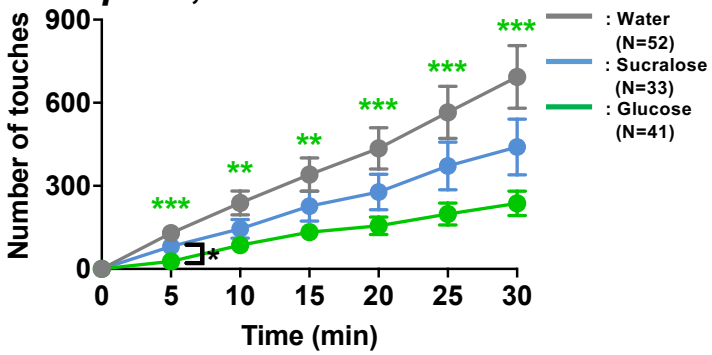
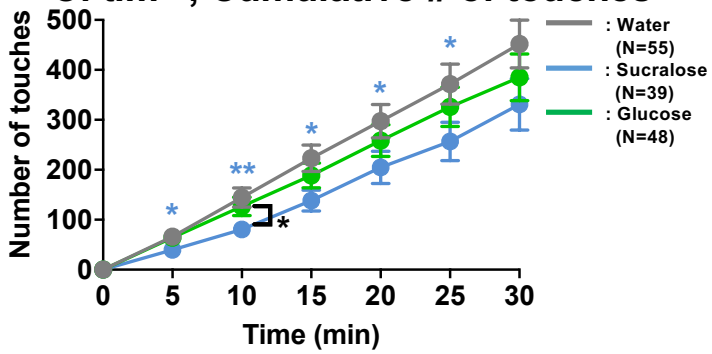

NPF-/-
A
**** * NS NS ***
Preferred temp. (°C)
26 24 22 20 18
5 10 8 6 11 10
Fed STV 10 min 10 min 10 min 1hr
ND Sucr-alose Glu-cose
Refed
sNPF hypo
B
**** ** NS ** **
28 26 24 22 20 18
8 8 5 7 9 8
Fed STV 10 min 10 min 10 min 1hr
ND Sucr-alose Glu-cose
Refed
Dh44Gal4/+
C
**** ******** ****
28 26 24 22 20 18
4 8 6 7 5
Fed STV 10 min 10 min 10 min
ND Sucr-alose Glu-cose
Refed
Dh44G4>uas-Kir
D
**** **** NS ***
28 26 24 22 20 18
4 6 10 9 14
Fed STV 10 min 10 min 10 min
ND Sucr-alose Glu-cose
Refed
AkhGal4/+
E
**** *** *** ***
28 26 24 22 20 18
7 8 10 5 5
Fed STV 10 min 10 min 10 min
ND Sucr-alose Glu-cose
Refed
AkhGal4>uas-Kir
F
**** **** NS ********
28 26 24 22 20 18
6 7 5 9 7 7
Fed STV 10 min 10 min 10 min 1hr
ND Sucr-alose Glu-cose
Refed
ilp6 LOF
G
* ** NS * *
28 26 24 22 20
7 12 13 5 8 9
Fed STV 10 min 10 min 10 min 1hr
ND Sucr-alose Glu-cose
Refed
Upd3Δ
H
**** NS NS * **
26 24 22 20 18
7 8 7 9 5 7
Fed STV 10 min 10 min 10 min 1hr
ND Sucr-alose Glu-cose
Refed
Upd2Δ
I
** NS NS NS NS
26 24 22 20 18
9 12 5 9 6 8
Fed STV 10 min 10 min 10 min 1hr
ND Sucr-alose Glu-cose
Refed
UAS-Kir/+
J
**** **** ** ****
28 26 24 22 20 18
8 8 5 7 6
Fed STV 10 min 10 min 10 min
ND Sucr-alose Glu-cose
Refed

Fig. S4

A: *yw*, Cumulative # of touches



B: *per*^{01}, Cumulative # of touches



C: *tim*^{01}, Cumulative # of touches