

1 **Neurotensin neurons in the central extended amygdala control energy balance**

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20 **SUMMARY**

21 Overeating and a sedentary life style are major causes of obesity and related metabolic disorders.
22 Identification of the neurobiological processes that regulate energy balance will facilitate
23 development of interventions for these disorders. Here we show that the Neurotensin-expressing
24 neurons in the mouse IPAC (IPAC^{Nts}), a nucleus of the central extended amygdala,
25 bidirectionally coordinate hedonic feeding and physical activity, thereby regulating energy
26 balance, metabolic processes and bodyweight. IPAC^{Nts} are preferentially activated by
27 consumption of highly palatable food or exposure to its taste and smell. Activating IPAC^{Nts}
28 promotes food intake in a palatability-dependent manner and decreases locomotion. Conversely,
29 inhibiting IPAC^{Nts} selectively reduces palatable food intake and dramatically enhances physical
30 activity and energy expenditure, and in parallel stimulates physiological responses that oppose
31 diet-induced obesity and metabolic dysfunctions. Thus, a single neuronal population,
32 Neurotensin-expressing neurons in the IPAC, acts to control obesogenic and leptogenic
33 processes by synergistically coordinating energy intake and expenditure with metabolism.

34

35 **INTRODUCTION**

36 In past decades obesity has become an epidemic and is currently one of the main causes of
37 premature death worldwide (Global et al., 2016; Mitchell et al., 2011). Genetic, environmental,
38 and behavioral factors all contribute to the onset and progression of weight gain and obesity.
39 Overeating and sedentary behavior, which are common in modern societies, support a positive
40 energy balance and in the long-term lead to weight gain and metabolic disorders. Treatments
41 involving lifestyle changes with the goal of losing weight and ameliorating metabolic diseases

42 often fail, in part because metabolic adaptations following weight loss act to restore homeostasis
43 and the original body weight (Fothergill et al., 2016; Trexler et al., 2014).

44

45 Homeostasis is regulated by specialized homeostatic neurons in the brain located in the
46 hypothalamus, parabrachial nucleus (PBN), and the nucleus tractus solitarii (NTS), which
47 receive orexigenic and anorexigenic inputs from the periphery. These neurons regulate energy
48 intake via homeostatic feeding (i.e., feeding on the basis of metabolic need), and regulate energy
49 expenditure to meet metabolic demands and maintain a stable body weight (Roh et al., 2016;
50 Rossi and Stuber, 2018; Saper et al., 2002; Sternson and Eiselt, 2017).

51

52 However, palatable foods can elicit feeding in the absence of a metabolic need, a phenomenon
53 known as hedonic eating (Morales and Berridge, 2020; Rossi and Stuber, 2018). In humans, the
54 degree of food intake positively correlates with food palatability – the hedonic evaluation of food
55 sensory cues such as smell, taste, and texture (Yeomans, 1998; Yeomans and Wright, 1991).

56 Foods rich in carbohydrates and fat are usually highly palatable (DiFeliceantonio et al., 2018).

57 As industrialization leads to an abundance of such highly palatable and calorie-dense foods,
58 hedonic eating, which results in excessive energy intake, is considered a major contributor to the
59 obesity epidemic.

60

61 In addition to excessive energy intake, insufficient energy expenditure is another major
62 contributor to the development of obesity (Hill et al., 2012; Tremblay and Willms, 2003), as
63 unused energy is stored in the body in the form of white adipose tissue (WAT) (Aldiss et al.,
64 2018). The main channels of energy expenditure are adaptive thermogenesis, which is regulated

65 by brown adipose tissue (BAT) activation in response to a thermal stimulus (e.g., cold
66 temperature), and activity-dependent thermogenesis (Srivastava and Veech, 2019). Strenuous
67 physical activity can induce the appearance of interspersed brown-like (beige) adipocytes in
68 WAT. These cells, unlike white adipocytes, are metabolically active and dissipate energy (Aldiss
69 et al., 2018; Srivastava and Veech, 2019).

70

71 While the neural circuits underlying homeostatic energy intake have been extensively
72 characterized (Sternson and Eiselt, 2017), those regulating hedonic feeding and energy
73 expenditure are less well understood (Gong et al., 2020; Hardaway et al., 2019; Riera et al.,
74 2017; Schneeberger et al., 2019; Zhang and van den Pol, 2017). Substantial evidence indicates
75 that structures belonging to the central extended amygdala (EAc), in particular the central
76 amygdala (CeA) and the bed nucleus of the stria terminalis (BNST), play important roles in the
77 maintenance of homeostasis (Cai et al., 2014; Douglass et al., 2017; Hardaway et al., 2019;
78 Jennings et al., 2013; Wang et al., 2019). The interstitial nucleus of the posterior limb of the
79 anterior commissure (IPAC) is another major structure of the EAc (Alheid, 2003). However,
80 unlike the BNST and CeA, which have been intensively studied in the context of motivated
81 behaviors including feeding, the function of the IPAC in behavior is largely unknown.

82

83 The IPAC encompasses a corridor of neurons extending from the caudal portion of the nucleus
84 accumbens (NAc) shell, merging into the lateral nuclei of the BNST (rostral IPAC) and further
85 reaching towards the CeA (caudal IPAC) (Alheid, 2003). Its afferent and efferent connections
86 are grossly similar to those of the lateral BNST and the CeA (Alheid et al., 1999; Gehrlach et al.,
87 2020; Shammah-Lagnado et al., 2001). For example, neurons in the IPAC receive dense

88 projections from the gustatory insular cortex and in turn project to the lateral hypothalamus
89 (LH), paraventricular nucleus of the hypothalamus (PVN), ventral tegmental area (VTA), NTS,
90 and other brainstem areas, which are structures implicated in regulating energy balance,
91 autonomic responses and reward processing (Sternson and Eisel, 2017). Thus, the IPAC is
92 anatomically poised to participate in regulating energy intake and/or expenditure.

93
94 It was recently shown that IPAC neurons are activated by innate or learned gustatory stimuli
95 (Tanaka et al., 2019, 2021). Nevertheless, the IPAC has a unique narrow elongated shape and
96 unclear anatomical borders with the rostral NAc and caudal BNST, making it difficult for
97 targeted *in vivo* manipulation based only on its anatomy. One approach to address this issue is to
98 use genetic markers to selectively label and study specific populations of IPAC neurons.

99 Previous studies indicate that the neuropeptide Neurotensin (Nts) is expressed and enriched in
100 the rostral IPAC region (Schroeder et al., 2019; Woodworth et al., 2018). Interestingly, Nts
101 infusion into specific brain areas, including the PVN, VTA, and NTS, modulates feeding
102 behavior, body temperature, and physical activities (for a recent review, see (Ramirez-Virella
103 and Leininger, 2021)). These findings indicate that central Nts has a role in regulating both
104 energy intake and energy expenditure, and further suggest that Nts-expressing IPAC neurons
105 may contribute to similar or related processes, as many of the Nts infusion areas are also the
106 projection targets of IPAC neurons.

107
108 In this study, we characterized the function of Nts-expressing neurons in the rostral IPAC, and
109 uncovered a critical role of these neurons in bidirectional control of energy intake and
110 expenditure, thereby regulating metabolism and body weight.

111

112 **RESULTS**

113 **IPAC^{Nts} neurons are specifically activated by palatable food**

114 To verify Nts expression in the IPAC, we bred *Nts^{Cre};Ai14* mice, in which Nts-expressing (Nts⁺)
115 neurons express the red fluorescence protein tdTomato (Leininger et al., 2011; Madisen et al.,
116 2010). We found that dense Nts⁺ cells form a narrow stripe in the most medial portion of the
117 rostral IPAC (Figure 1A, Figure S1A), which merge with the sparser Nts⁺ cells in the lateral
118 ventral (STLV) and juxtacapsular (STLJ) nuclei of the lateral BNST, forming a continuum. No
119 Nts⁺ neurons were observed in the ventral pallidum (VP), though it is rich in axon fibers
120 originating from Nts⁺ neurons (Figure 1A, Figure S1A).

121

122 Single molecule fluorescent *in situ* hybridization (smFISH) confirmed *Nts* expression in the
123 IPAC and lateral nuclei of the BNST, and its near absence in nearby striatal and VP territories.
124 Virtually all *Nts⁺* neurons in the IPAC (hereafter referred to as IPAC^{Nts} neurons) were
125 GABAergic (Figure 1B, C; Figure S1B). In addition, the expression pattern of *Cre* recapitulated
126 that of endogenous *Nts* in the IPAC of *Nts^{Cre}* mice (Figure S1C), thus validating the fidelity of
127 this line.

128

129 Food-restriction creates a negative energy balance and leads to the activation of homeostatic
130 circuits regulating energy intake to restore the balance (Atasoy et al., 2012). To test whether
131 IPAC^{Nts} neurons are involved in this process, we analyzed the expression of *c-Fos*, a marker for
132 neuronal activation, in these neurons in food-restricted (FR) or sated mice. We found that food-
133 restriction did not alter the *c-Fos* expression (Figure 1D, E, I).

134

135 Next, we exposed food-restricted mice to either regular chow or a high-fat diet (HFD)
136 (Methods). Interestingly, feeding on the HFD, but not regular chow, induced a marked increase
137 in *c-Fos* expression in IPAC^{Nts} neurons (Figure 1F-I; Figure S1D, E), although the mice
138 consumed comparable amounts of chow and HFD (Figure S1F, left) and had similar energy
139 intake (Figure S1F, right). In contrast, feeding on either the regular chow or HFD induced robust
140 *c-Fos* expression in the adjacent striatum (Figure S1D, E). These results suggest that IPAC^{Nts}
141 neurons are activated preferentially by the consumption of palatable food, but not by an energy
142 deficit or food consumption *per se*.

143

144 **IPAC^{Nts} neurons encode tastant palatability**

145 We reasoned that IPAC^{Nts} neurons encode food palatability. To test this hypothesis, we set out to
146 monitor the *in vivo* activities of these neurons in behaving mice consuming substances with
147 differing palatability. We first labelled these neurons with the genetically encoded calcium
148 indicator GCaMP6 (Chen et al., 2013), by injecting the IPAC of *Nts^{Cre}* mice with an adeno-
149 associated virus (AAV) expressing GCaMP6f in a *Cre*-dependent manner, then implanting an
150 optical fiber into the same location (Figure 2A, B, Figure S2A). This strategy allows recording
151 bulk GCaMP6 signals, which are readouts of average neuronal activities, *in vivo* from the
152 infected neurons with fiber photometry (Xiao et al., 2020; Yu et al., 2016).

153

154 In human subjects, a common experimental approach to infer palatability is to present
155 individuals with two foods with similar nutritional value (i.e., isocaloric) but different flavors.
156 The difference in food intake is used as a measure of palatability (Yeomans, 1998; Yeomans and

157 Wright, 1991). Using a similar approach, we tested the mice (which were under water restriction;
158 Methods) for their preference for non-caloric solutions – sucralose, water and quinine – using the
159 amount of intake as a proxy of tastant preference and thus palatability. These mice displayed a
160 preference for sucralose over water (Figure 2C, D) and for water over quinine (Figure 2I, J),
161 suggesting that sucralose and quinine are the most and least palatable tastants, respectively. Of
162 note, as all these liquids are non-caloric, the preference should not be influenced by nutritional
163 content.

164
165 *In vivo* fiber photometry revealed that IPAC^{Nts} neurons were robustly activated following liquid
166 consumption (Figure 2E-H, K-N; Figure S2B-D). Notably, the activation by sucralose was
167 greater than that by water (Figure 2F, G), and the activation by water was greater than that by
168 quinine (Figure 2L, M). This ranking of neuronal activation mirrored the tastant preference
169 measured behaviorally (Figure 2D, J). Thus, IPAC^{Nts} neuron activity scales with stimulus
170 palatability. Analysis of the licking behavior showed that mice licked more vigorously at the
171 spout delivering the preferred tastants (Figure 2 H, N), raising the possibility that IPAC^{Nts}
172 activity could represent the motion associated with licking. However, we found no correlation
173 between the amplitude of the neural response and lick rate in any of the mice (Figure S2E, F).
174 Together, these results suggest that IPAC^{Nts} neuron activities represent tastant palatability, rather
175 than the motor functions underlying licking.

176

177 **IPAC^{Nts} neurons integrate food stimulus information across sensory modalities**

178 Besides the taste, the smell of a food has a major impact on its palatability (Yeomans, 1998). In
179 mice, olfaction regulates feeding and metabolism (Patel et al., 2019; Riera et al., 2017).

180 Appetitive food-related smells prepare inner organs for a meal (Brandt et al., 2018) and modulate
181 the activity of homeostatic hypothalamic neurons (Chen et al., 2015).

182

183 To examine how IPAC^{Nts} neuron activities might be influenced by food-related smells, we used
184 fiber photometry, as described above (Figure 2A, B; Figure S3A), to measure the *in vivo*
185 responses of these neurons in food-restricted mice to the presentation of odors derived from
186 different substances: a high-fat diet (HFD), butyric acid (BA), and mineral oil (MO, used to
187 dissolve odorants and serving as a vehicle control; Methods; Figure 3A). While HFD is
188 appetitive, BA is typically found in spoiled food and responsible for its rotten smell, and is thus
189 aversive (Patel et al., 2019). Notably, we found that IPAC^{Nts} neurons were strongly activated by
190 the smell of HFD, but were only minimally activated by the smell of BA or MO alone (Figure
191 3B-D). These results suggest that IPAC^{Nts} neurons are tuned to multiple features of palatable
192 food, including both the taste and the smell.

193

194 **IPAC^{Nts} neurons represent the palatability of naturalistic food stimuli**

195 In humans, the preference for a food, or food palatability, tends to be idiosyncratic. For instance,
196 while some people prefer sweet food over spicy, others may do just the opposite. To assess the
197 palatability of naturalistic foods in mice, we presented them with two kinds of HFDs that had
198 identical nutritional value and macronutrient composition, but differed in lipid content, with one
199 derived from coconut oil (HFD^{CO}) and the other from olive oil (HFD^{OO}). Mice avidly consumed
200 either the HFD^{CO} or the HFD^{OO} pellets, even when sated (data not shown), indicating that both
201 are highly rewarding and more palatable than chow, which was available *ad libitum* in the home
202 cage. Interestingly, when both HFDs were offered, mice showed a clear preference for one of

203 them, with the majority favoring HFD^{CO} over HFD^{OO} (Figure 3E, F). Since these diets were
204 isocaloric, this preference should be dependent only on their palatability, but not the nutritional
205 value.

206
207 To determine whether IPAC^{Nts} neuron activities encode food palatability, we presented food-
208 restricted mice with odors derived from HFD^{CO}, HFD^{OO}, regular chow and MO. We found that
209 IPAC^{Nts} neurons showed higher responses to the odors from the HFDs (HFD^{CO} and HFD^{OO}) than
210 the odor from regular chow or MO (Figure 3G, H; Figure S3B), paralleling the observation that
211 all mice preferred HFDs over regular chow. Notably, when the idiosyncratic preferences of
212 individual mice for one of the two HFDs (Figure 3E, F) were considered, IPAC^{Nts} neuron
213 responses were larger for the odor of the favorite diet, irrespective of it being CO- or OO-
214 flavored (Figure 3I, left).

215
216 In a separate experiment, we examined the responses of IPAC^{Nts} neurons in food-restricted mice
217 to the smells of white chocolate (WCh) and dark chocolate (DCh) – which are isocaloric and
218 among the popular energy-dense “cafeteria foods” causing obesity in humans – as well as regular
219 chow and MO. We found that IPAC^{Nts} neurons preferentially responded to the smells of
220 chocolates, especially WCh (Figure S3C-E). These results, together with those from the cFos
221 experiments (Figure 1), strongly support the notion that IPAC^{Nts} neurons encode food
222 palatability or preference.

223
224 Palatable food’s sensory attributes are sufficient to override homeostatic regulation, thereby
225 driving excessive caloric intake (Morales and Berridge, 2020). To test whether IPAC^{Nts} neurons

226 would respond to palatable food cues even in the absence of homeostatic drive, we repeated the
227 above experiments in sated mice. We found that IPAC^{Nts} neurons still responded to food smells,
228 and the magnitude of the response was larger for energy-dense foods (HFD^{CO}, HFD^{OO}, WCh,
229 and DCh) than for chow or MO (Figure 3J, K; Figure S3B, C, F, G). Thus, IPAC^{Nts} neurons have
230 preferential responses to the odors of palatable foods, even in sated mice.

231
232 Hunger is known to increase the palatability of a food, a phenomenon called alliesthesia
233 (Cabanac, 1971). If IPAC^{Nts} neuron activity represents palatability, then such activity should be
234 modulated by animal's homeostatic state. Indeed, although IPAC^{Nts} neurons in hungry mice
235 showed increased response to the favored HFD, this increase disappeared in sated mice (Figure
236 3I, right). Moreover, the response of IPAC^{Nts} neurons – especially that to the HFD – was larger
237 when mice were in a hungry state than in satiety (Figure 3L). These results suggest that IPAC^{Nts}
238 neuron activity is modulated by both food palatability and the homeostatic state of the animal.

239
240 Together, our observations thus far unravel that IPAC^{Nts} neurons encode the palatability of both
241 simple tastants and complex, real-world food stimuli, and point to the possibility that these
242 neurons might have a role in regulating hedonic feeding, i.e., feeding in the absence of an energy
243 deficit.

244 245 **Activation of IPAC^{Nts} neurons preferentially increases the intake of palatable foods**

246 To assess whether activation of IPAC^{Nts} neurons could result in overfeeding, we selectively
247 activated these neurons in sated mice with optogenetics. For this purpose, we bilaterally injected
248 the IPAC of *Nts*^{Cre} mice with an AAV expressing the light-gated cation channel

249 channelrhodopsin (ChR2), or GFP (as a control), in a *Cre*-dependent manner. Optical fibres were
250 implanted over the infected areas for light delivery (Figure 4A, Figure S4A). Following recovery
251 from surgery and viral expression, sated mice received pellets of differing palatability (Figure
252 4B; Methods). During each food presentation, pulses of blue light were delivered into the IPAC.
253 We found that photostimulation in the ChR2 mice increased their intake of both regular chow
254 and the more palatable HFD, and, interestingly, the effect was larger for HFD than regular chow
255 (Figure 4C). Consistently, the number and length of feeding bouts were also increased by the
256 photostimulation (Figure S4B-D). Photostimulation in these mice occasionally resulted in
257 stereotyped appetitive behaviors such as licking the floor, foraging, and gnawing of inedible
258 bedding pellets (Supplementary Video 1 & 2). In contrast, photostimulation in the GFP mice had
259 no behavioral effect (Figure 4C; Figure S4C-E). These results indicate that activation of IPAC^{Nts}
260 neurons drives food intake in the absence of metabolic need and even more so for palatable food.
261
262 These data, together with the photometry results, led us to hypothesize that IPAC^{Nts} neuron
263 activity promotes feeding as a function of food palatability. To test this hypothesis, we activated
264 IPAC^{Nts} neurons with optogenetics in sated mice, as described above (Figure 4B, C), and
265 assessed the effects of this manipulation on the consumption of chow, HFD, WCh, DCh, and
266 sucrose. These foods have different palatability, as indicated by mice's differential preference for
267 them before the activation (baseline intake, Figure 4D). We found that activation of IPAC^{Nts}
268 neurons promoted the intake of all these foods (Figure 4E; S4E), and, remarkably, there was a
269 strong correlation between the activation-induced intake and baseline intake (Figure 4E). These
270 results suggest that the feeding-promoting effect of IPAC^{Nts} neuron activation is indeed
271 dependent on palatability.

272

273 As these foods have different nutritional values, which may influence consumption independent
274 of palatability, we repeated the above experiments with three pairs of foods that are isocaloric
275 but differ in palatability: (1) WCh and DCh (Figure 4F), (2) HFD^{CO} and HFD^{OO} (Figure 4G-I),
276 and (3) plain chow and chow flavored with quinine (Figure 4J, K; Methods). WCh was much
277 more preferred than DCh by all mice, likely due to the bitter taste of the latter (Figure 4D). The
278 preference for HFD^{CO} or HFD^{OO} was more idiosyncratic (see Figure 3E, F), but all mice in this
279 group preferred HFD^{CO} (Figure 4G). For the plain and quinine-flavored chow, the two kinds of
280 food pellets had identical texture and nutritional value, but the quinine-flavored was expected to
281 be less palatable (Nisbett, 1968).

282

283 We found that in all three cases, activation of IPAC^{Nts} neurons dramatically increased the intake
284 of the more palatable food and increased the intake of the less palatable counterpart in the pair to
285 a much lesser degree (Figure 4F-K). Of note, IPAC^{Nts} activation did not induce any feeding on a
286 random inedible object, such as an eraser (Figure 4L), suggesting the effect is food-specific.
287 Together, these results strongly indicate that IPAC^{Nts} neuron activity promotes food intake in a
288 palatability-dependent manner, and thus may play an important role in controlling hedonic
289 feeding.

290

291 **Activation of IPAC^{Nts} neurons is positively reinforcing and decreases locomotor activity**

292 Previous studies indicate that activating a BNST-to-LHA circuit, which regulates metabolic
293 feeding, is rewarding and can support intracranial self-stimulation (Jennings et al., 2013).

294 Interestingly, this phenomenon was dependent on the homeostatic state of the animal, as satiety

295 and food-restriction significantly decreased and increased, respectively, the degree of self-
296 stimulation (Jennings et al., 2013). To determine whether IPAC^{Nts} neurons act in a similar
297 manner, we presented the ChR2 and GFP mice – in which the IPAC^{Nts} neurons expressed ChR2
298 and GFP, respectively – with two ports (Figure S4F). Poking into one of the ports (the active
299 port) would lead to the delivery of light pulses into the IPAC, whereas poking into the other port
300 (the inactive port) would have no consequence. The ChR2 but not GFP mice consistently poked
301 into the active port to receive the photostimulation while completely ignoring the inactive port,
302 demonstrating that activation of IPAC^{Nts} neurons effectively supports self-stimulation (Figure
303 S4G, H). Interestingly, there was no difference in self-stimulation rates when the mice were
304 tested sated on regular chow or a high fat diet, or following food-restriction (FR) (Figure S4I, J;
305 Methods). These results indicate that activation of IPAC^{Nts} neurons is intrinsically rewarding.
306 Moreover, the rewarding effect is independent of animal's homeostatic state, and thus different
307 from the function of the circuits regulating metabolic feeding (Jennings et al., 2013).

308

309 In line with the self-stimulation results, photo-activation of IPAC^{Nts} neurons induced place
310 preference in a real-time place preference or aversion (RTPP/A) assay (Figure 4M, N; Methods).
311 Notably, we found that activation of IPAC^{Nts} neurons caused a significant reduction in mice's
312 movements in both the RTPP/A assay (Figure 4O) and an open field test (Figure 4P, Q;
313 Methods). These results show that IPAC^{Nts} neuron activation is positively reinforcing, and
314 moreover suggest that these neurons may regulate animal's physical activity, a major channel for
315 energy expenditure, in addition to influencing energy intake.

316

317 **Inhibition of IPAC^{Nts} neurons promotes energy expenditure**

318 To determine whether the activity of IPAC^{Nts} neurons is required for energy homeostasis, we
319 selectively blocked neurotransmitter release from these neurons with the tetanus toxin light chain
320 (TeLC) (Murray et al., 2011). For this purpose, we bilaterally injected the IPAC of *Nts^{Cre}* mice
321 with an AAV expressing TeLC, or GFP (as a control), in a Cre-dependent manner (Figure 5A,
322 B). Mice were returned to their home cage after the surgery. Although the TeLC mice and GFP
323 mice had similar body weight prior to the surgery (Figure S5A; “day 0” (d0) timepoint), the
324 TeLC group dramatically lost weight within 10 days post-surgery and did not regain it by d30
325 (Figure S5A; Figure 5C). On average, at d30, the GFP mice gained $4.0 \pm 2.0\%$ of their initial
326 body weight, whereas the TeLC mice lost $17.2 \pm 4.3\%$ (Figure S5A). Notably, the sudden loss of
327 mass in the TeLC mice did not lead to starvation, as their body weight stabilized between d10
328 and d30 (Figure S5A, right).

329
330 As a reduction in body weight is normally caused by an imbalance between energy intake and
331 expenditure, we next examined the source of the imbalance in these mice by monitoring their
332 food (and water) intake and energy expenditure for 72 h in metabolic cages (Methods). Prior to
333 the examination, the TeLC and GFP mice lost $15.1 \pm 2.1\%$ and gained $1.7 \pm 1.6\%$, respectively, of
334 their initial body weight (Figure S5B). No further change in body weight was observed in either
335 cohort within this 72-h period (Figure S5C), suggesting that the TeLC mice reached a new
336 energy balance after the initial weight loss. In addition, we found no difference in the gross food
337 or water intake in the TeLC mice compared to controls (Figure 5D, E), suggesting that the neural
338 circuits underlying hunger and thirst homeostasis were minimally affected by inhibition of
339 IPAC^{Nts} neurons. Interestingly, energy expenditure (EE) was significantly higher in the TeLC

340 mice than controls, in particular during the active phase (i.e., dark cycle) of the day for mice
341 (Figure 5F, G).

342
343 Energy is expended to sustain basal metabolic rate, the thermic effect of food and physical
344 activities. We reasoned that an increase in physical activity accounts for the increase in energy
345 expenditure in the TeLC mice, because mice with enhanced IPAC^{Nts} neuron activity – opposite
346 to the effect of TeLC – showed decreased locomotor activity (Figure 4P, Q). Indeed, we found
347 that the TeLC mice were 3.7-fold more active than controls (Figure 5H, I; Figure S5D). In
348 humans, the maximum volume of oxygen inhaled (VO_2^{Max}) and the respiratory exchange ratio
349 (RER, the ratio between VCO_2 produced and VO_2 inhaled) during exercise are gold standards for
350 endurance and cardiovascular fitness (Gollnick, 1985; Ramos-Jimenez et al., 2008). Athletes, for
351 example, typically have higher VO_2^{Max} and lower RER than untrained subjects during
352 incremental exercise (which increases in intensity over time until VO_2^{Max} is reached), with the
353 lower RER indicative of a higher lipid oxidation (i.e., fat burning) rate. Notably, we found that
354 the TeLC group had markedly higher VO_2 and lower RER than the control group during the
355 period in a day when mice had “incremental activities” (from the beginning of the dark phase to
356 when the highest VO_2 levels were reached) (Figure 5J-M; Figure S5E-G). These results suggest
357 that the TeLC mice were physically fitter than the controls.

358
359 To determine whether our manipulation affected anxiety-related behaviors, we subjected the
360 TeLC mice and GFP control mice to the elevated-plus maze (EPM) and open-field (OF) tests
361 (Figure S6). We found no difference between the two groups in measures of anxiety behaviors in
362 rodents, including the time spent on the open arms of the EPM (Figure S6A, B) or in the center

363 of the OF (Figure S6E, F), or the frequency of entering those spaces (Figure S6C, G).
364 Interestingly, the two groups also had similar locomotor activities during these anxiogenic tests
365 (Figure S6D, H), suggesting that the increase in physical activities in the TeLC mice is context-
366 or state-dependent, and may reflect volitional activities in a familiar or less stressful
367 environment.

368
369 Together, these results show that inhibition of IPAC^{Nts} neurons dramatically increases energy
370 expenditure, aerobic capacity, and physical fitness without affecting regular food intake,
371 suggesting that the activity of these neurons normally constrains energy expenditure by limiting
372 physical activity. However, the activity of these neurons is not critical for homeostatic energy
373 intake.

374

375 **Inhibition of IPAC^{Nts} neurons decreases food palatability**

376 Our photometry and optogenetic activation results indicate that IPAC^{Nts} neuron activity encodes
377 food palatability and is sufficient to promote hedonic feeding. We thus tested whether the
378 activity of these neurons is also required for this type of feeding. To this end, we switched the
379 diet from regular chow to HFD for the mice and subsequently assessed their food and water
380 intake (Figure 6A). We found that, in the four days following the switch, the GFP mice ate
381 significantly more than the TeLC mice (Figure 6B), however this was not observed when the
382 mice were fed with chow (Figure 5D). Accordingly, water intake was lower for TeLC mice on
383 HFD (Figure 6C). To assess the change in energy intake (EI) after the diet switch, we calculated
384 the daily calorie consumption when fed with chow (Figure 5D) or HFD (Figure 6B) and found
385 that the GFP, but not TeLC mice, dramatically increased EI when fed with HFD (Figure 6D). EI

386 increased by 40% in the GFP mice but remained unchanged in the TeLC mice (Figure 6E),
387 suggesting that the GFP, but not TeLC mice, ate above their metabolic needs and likely entered a
388 positive energy balance state. Indeed, after only 4 days of HFD, the body weight of GFP mice
389 dramatically increased while that of TeLC mice remained stable (Figure 6F). These data strongly
390 suggest that inhibition of IPAC^{Nts} neurons prevents overfeeding on palatable foods and acute
391 weight gain.

392

393 To determine whether an impairment in detecting nutritional value contributed to the preventive
394 effect on overfeeding, we tested these mice's preference for a sucralose solution versus water, or
395 a sucrose solution versus water (Figure 6G; Methods). Sucralose and sucrose were used because
396 they are both sweet tastants, but are noncaloric and caloric, respectively. Notably, the TeLC mice
397 showed decreased preference for sucralose, but normal preference for sucrose compared with the
398 GFP control mice (Figure 6H). This result suggests that IPAC^{Nts} neuron activity is required for
399 the orosensory perception of a palatable stimulus (i.e., palatability), but is dispensable for
400 detecting the nutritional value of the stimulus.

401

402 **Inhibition of IPAC^{Nts} neurons prevents obesity and facilitates glucose metabolism**

403 Following one week of feeding on HFD, TeLC mice still displayed markedly elevated energy
404 expenditure (Figure 6I, J) and locomotor activity (Figure S7A, B), which were accompanied by
405 increased VO₂ and VCO₂ (Figure S6C-F) and decreased RER (Figure 6K, L) compared with the
406 GFP mice. As a result, the TeLC mice had lower body weight-gain than the GFP mice during
407 this period (Figure 6M, first week).

408

409 Continued feeding on HFD (Figure 6A) effectively led to diet-induced obesity (DIO) in the GFP
410 mice, as these mice steadily gained weight over the course of several weeks (Figure 6M). In stark
411 contrast, the TeLC mice remained lean despite the HFD (Figure 6M), a phenotype that prompted
412 us to investigate possible beneficial effects of IPAC^{Nts} neuron inhibition on glucose metabolism.
413 We found that blood glucose levels were significantly lower in the TeLC mice than GFP mice in
414 a glucose tolerance test (Figure 6N; Methods). Moreover, the TeLC mice showed lower glucose
415 levels when measured in an insulin sensitivity test (Figure 6O; Methods). These results suggest
416 that inhibition of IPAC^{Nts} neurons confers resistance to DIO and ameliorates its detrimental
417 effects on glucose tolerance and insulin sensitivity.

418

419 The weight of the TeLC mice was 27.2% lower than the GFP mice at experiment endpoint (8
420 weeks of HFD; Figure 7A, B). Organ tissue analysis revealed no overt changes in the weight of
421 different organs in the TeLC mice compared with the GFP mice (Figure 7C). However, the
422 TeLC mice had 75.3% lower inguinal white adipose tissue (iWAT), 67.1% lower epididymal
423 WAT (eWAT), 66.1% lower mesenteric WAT (mWAT) and 46.0% lower brown adipose tissue
424 (BAT) (Figure 7D). Consistently, a lower amount of lipid droplets was found in the BAT (Figure
425 7E) and liver (Figure 7F) of the TeLC mice compared to controls. The adipocyte size in iWAT
426 and eWAT was correspondingly decreased in these mice (Figure 7G, H). Interestingly,
427 histological analysis revealed that the iWAT – but not the eWAT – of some TeLC mice
428 contained multilocular lipid droplets (Figure 7G), suggesting lipid browning. In line with this
429 observation, we found that the expression of *Ucp1* (*Uncoupling protein 1*) – which is usually
430 highly expressed in BAT, but not WAT – was significantly higher in the iWAT of TeLC mice
431 compared to controls (Figure 7I). Of note, *Ucp1* is critically involved in adaptive thermogenesis

432 by uncoupling mitochondrial oxidative metabolism from ATP production (Cannon and
433 Nedergaard, 2004).

434

435 Together, these data show that inhibition of IPAC^{Nts} neurons protects from obesity by reducing
436 caloric intake and increasing energy expenditure via elevated physical activity, thereby
437 improving fitness and metabolic health indicators, such as VO₂, RER, glucose metabolism and
438 lipid browning.

439

440 **DISCUSSION**

441 The easy availability of energy-dense foods is a main contributor to the current obesity
442 pandemic. Foods rich in sugars and lipids are highly palatable, increasing hedonic intake
443 (Morales and Berridge, 2020; Yeomans, 1998; Yeomans and Wright, 1991) that overrides
444 homeostasis and leads to weight gain. Recent studies have identified neurons in different brain
445 areas – such as the VP, NAc and peri-locus coeruleus – that encode or regulate hedonic feeding
446 (Gong et al., 2020; Ottenheimer et al., 2018; Taha and Fields, 2005), but our understanding of
447 the neural mechanisms underlying this behavior remains incomplete.

448

449 In this study, we show that IPAC^{Nts} neurons bidirectionally regulate palatable food intake and
450 energy expenditure, thereby regulating peripheral organ function and energy homeostasis. In
451 particular, these neurons respond to gustatory and olfactory food-related stimuli and encode their
452 palatability. The magnitude of the response was correlated with food preference, modulated by
453 the internal state of the animal, and independent of nutritional value. Furthermore, activation of
454 IPAC^{Nts} neurons increased food consumption even in sated mice in a palatability-dependent

455 manner, such that the effect was larger for more palatable foods and minimal for bitter foods or
456 tastants. However, the effect did not depend on the caloric content of the foods. Thus, the activity
457 of IPAC^{Nts} neurons likely contributes to dietary choice based on the hedonic, orosensory
458 properties of foods.

459

460 Consistent with this notion, blockage of IPAC^{Nts} neurons reduced palatable food intake and food
461 preference, but had no obvious effect on homeostatic feeding. Mice with IPAC^{Nts} neuron
462 inhibition ate only the calories needed for sustaining their metabolic needs, while control mice
463 overate on the HFD. Importantly, such effects of IPAC^{Nts} neuron inhibition seemed to be caused
464 by a decreased ability to perceive the HFD orosensory properties, not by an impairment in
465 detecting caloric compounds. These results are consistent with previous findings that separate
466 neural substrates mediate the detection of hedonic and nutritional reward properties. For
467 example, *Trpm5*-deficient mice are unable to sense sweet taste but are able to detect sucrose on
468 the basis of its caloric content (Beeler et al., 2012; de Araujo et al., 2008); and specialized
469 neurons in the NTS are known to encode the caloric value of glucose (Tan et al., 2020).

470

471 Lack of physical exercise, and thus energy expenditure, is another factor strongly contributing to
472 obesity onset and progression (Hill et al., 2012; Tremblay and Willms, 2003). We found that
473 activation of IPAC^{Nts} neurons caused a decrease in animals' locomotor activity. Conversely,
474 sustained inhibition of these neurons drastically increased locomotion, increased volume of
475 oxygen uptake (VO₂) and lowered Respiratory Exchange Ratio (RER). Both VO₂ and RER are
476 used to determine physical fitness in human subjects (Gollnick, 1985; Ramos-Jimenez et al.,

477 2008). A lower RER indicates a higher rate of lipid oxidation (i.e., fat burning), thus providing
478 an explanation for the decreased body weight following inhibition of IPAC^{Nts} neurons.

479

480 The combination of reduced HFD intake and increased energy expenditure in mice with IPAC^{Nts}
481 neuron inhibition likely confers protection from the detrimental effects of chronic HFD feeding
482 in the DIO model. Indeed, mice with impaired IPAC^{Nts} transmission remained lean and had
483 improved glucose homeostasis compared with controls when challenged with DIO, suggesting
484 that suppressing IPAC^{Nts} neuron activity effectively protects from metabolic diseases.

485

486 The reduced body weight of mice with IPAC^{Nts} neuron inhibition was mostly represented as a
487 dramatic reduction in white adipose storage. Indeed, histological analysis in these mice revealed
488 that iWAT and eWAT adipocytes were visibly smaller than those in control mice, and
489 furthermore, exhibited features of BAT (e.g., multilocular appearance). Interestingly, IPAC^{Nts}
490 neuron inhibition increased the expression of *Ucp1* in the iWAT, which is usually highly
491 expressed in BAT (but not WAT) and regulates thermogenesis (Aldiss et al., 2018). Thus,
492 inhibition of IPAC^{Nts} neurons induces changes in not only energy intake and expenditure, but
493 also energy storage. Importantly, inhibition of IPAC^{Nts} neurons reduces lipid droplet
494 accumulation in the BAT and liver in the DIO model, which is characteristic of BAT dysfunction
495 (i.e., BAT “whitening”) (Shimizu et al., 2014) and nonalcoholic fatty liver disease (Recena
496 Aydos et al., 2019) associated with obesity in humans.

497

498 Collectively, our results identify IPAC^{Nts} neurons as a crucial substrate for regulating energy
499 balance-related behaviors, including hedonic food intake and energy expenditure/deposit

500 homeostasis. In particular, given previous findings in humans that BAT is linked to metabolic
501 health (Matsushita et al., 2014), and exercise is known to improve metabolic conditions and
502 reduce the risk for or improve the prognosis of metabolic diseases (Cormie et al., 2017; Moore et
503 al., 2016; Moore et al., 2012; Naylor et al., 2020), our results suggest that manipulation of
504 IPAC^{Nts} neurons might have important implications in the prevention or treatment of these
505 disorders, including bodyweight-related disorders and cancer.

506

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515

516 **AUTHOR CONTRIBUTIONS**

517 A.F. and B.L. conceived and designed the study. A.F. conducted the experiments and analyzed
518 data. A.C. assisted with the photometry experiments with food odors and the data analysis. S.
519 Boyle set up behavioral rigs and generated Matlab code for controlling behavioral devices and
520 analyzing photometry data. R.S. assisted with the smFISH experiments. R.R. and J.H. assisted
521 with operating the metabolic cages. E.C.G. assisted with the GTT and ITT experiments. R.S. and
522 E.C.G. collected tissue samples and performed qPCR experiments. J.G. assisted with the EPM

523 and OF experiments. S. Beyaz provided critical reagents. T.J. supervised the experiments by
524 E.C.G. and assisted with interpreting metabolic data. S.D.S. supervised the experiments by A.C.
525 and assisted with analyzing and interpreting the data. A.F. and B.L. wrote the paper with inputs
526 from all authors.

527

528 **DECLARATION OF INTERESTS**

529 The authors declare no competing interests.

530

531 **RESOURCE AVAILABILITY**

532 **Lead Contact**

533 Further information and requests for resources and reagents should be directed to and will be
534 fulfilled by the Lead Contact, Bo Li (bli@cshl.edu).

535

536 **Data and code availability**

537 The custom code that support the findings from this study are available from the Lead Contact
538 upon request.

539

540 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

541 Adult male and female mice of at least 2 months old were used for all the experiments. Mice
542 were housed under a 12-h light/dark cycle (7 a.m. to 7 p.m. light) in groups of 2-5 animals, with
543 food and water available *ad libitum* before being used for experiments, unless otherwise
544 specified. All behavioral experiments were performed during the light cycle. Littermates were
545 randomly assigned to different groups prior to experiments. All experimental procedures were

546 approved by the Institutional Animal Care and Use Committee of Cold Spring Harbor
547 Laboratory (CSHL) and performed in accordance with the US National Institutes of Health
548 guidelines in an AAALACi accredited facility. The *Nts^{Cre}* mouse line (Stock No: 017525) and
549 *Ai14* (Stock No: 007908) were purchased from Jackson Laboratory. All mice were bred onto a
550 C57BL/6J background.

551

552 **METHOD DETAILS**

553 **Immunohistochemistry**

554 Immunohistochemistry experiments were conducted following standard procedures (Stephenson-
555 Jones et al., 2016). Briefly, mice were anesthetized with Euthazol (0.4 ml; Virbac, Fort Worth,
556 Texas, USA) and transcardially perfused with 30 ml of PBS, followed by 30 ml of 4%
557 paraformaldehyde (PFA) in PBS. Brains were extracted and further fixed in 4% PFA overnight
558 followed by cryoprotection in a 30% PBS-buffered sucrose solution for 36-48 h at 4 °C. Coronal
559 sections (50- μ m) were cut using a freezing microtome (Leica SM 2010R, Leica). Sections were
560 first washed in PBS (5 minutes), incubated in PBST (0.3% Triton X-100 in PBS) for 30 minutes
561 at room temperature (RT) and then washed with PBS (3 x 5 minutes). Next, sections were
562 blocked in 5% normal donkey serum in PBST for 30 minutes at RT and then incubated with
563 primary antibodies overnight at 4 °C. Sections were washed with PBS (3 x 5 minutes) and
564 incubated with fluorescent secondary antibodies at RT for 2 h. Next, sections were washed twice
565 in PBS, incubated with DAPI (4',6-diamidino-2-phenylindole, Invitrogen, catalogue number
566 D1306) (0.5 μ g/ml in PBS) for 5 minutes. After washing with PBS (3 x 5 minutes), sections
567 were mounted onto slides with Fluoromount-G (eBioscience, San Diego, California, USA).

568 Images were taken using an LSM 710 or LSM780 confocal microscope (Carl Zeiss, Oberkochen,
569 Germany) and visualized and processed using ImageJ and Adobe Illustrator.

570

571 The primary antibody used was chicken anti-GFP (Aves Labs, GFP1020, dilution 1:1000).

572 Appropriate fluorophore-conjugated secondary antibodies (Life Technologies) were used to
573 detect the primary antibodies used.

574

575 **Fluorescent *in situ* hybridization**

576 Single molecule fluorescent *in situ* hybridization (ACDBio, RNAscope) was used to detect the
577 expression of *Nts*, *Gad2*, *Slc17a6* (*Vglut2*), *cFos*, and *Cre* in the IPAC and surrounding tissues of
578 adult mice. For tissue preparation, mice were first anesthetized under isoflurane and then
579 decapitated. Their brain tissue was first embedded in cryomolds (Sakura Finetek, Ref 4566)
580 filled with M-1 Embedding Matrix (Thermo Scientific, Cat. No. 1310) then quickly fresh-frozen
581 on dry ice. The tissue was stored at -80 °C until it was sectioned with a cryostat. Cryostat-cut
582 sections (16- μ m) containing the IPAC were collected and quickly stored at -80 °C until
583 processed. Hybridization was carried out using the RNAscope kit (ACDBio).

584

585 The day of the experiment, frozen sections were post-fixed in 4% PFA in RNase-free PBS
586 (hereafter referred to as PBS) at RT for 15 minutes, then washed in PBS, dehydrated using
587 increasing concentrations of ethanol in water (50%, once; 70%, once; 100%, twice; 5 minutes
588 each). Sections were then dried at RT and incubated with Protease IV for 30 minutes at RT.
589 Sections were washed in PBS three times (5 minutes each) at RT, then hybridized. Probes
590 against *Nts* (Cat. No. #420441), *Gad2* (Cat. No. #439371), *Slc17a6* (*Vglut2*) (Cat. No. #319171),

591 *c-Fos* (Cat. No. #316921), and *Cre* (Cat. No. #312281) were applied with a 1:50 dilution to
592 IPAC sections. Hybridization was carried out for 2 h at 40°C. After that, sections were washed
593 twice in 1x Wash Buffer (Cat. No. 310091) (2 minutes each) at RT, then incubated with three
594 consecutive rounds of amplification reagents (30 minutes, 15 minutes and 30 minutes, at 40°C).
595 After each amplification step, sections were washed twice in 1x Wash Buffer (2 minutes each) at
596 RT. Finally, fluorescence detection was carried out for 15 minutes at 40°C. Sections were then
597 washed twice in 1x Wash Buffer, incubated with DAPI for 2 minutes, washed twice in 1x Wash
598 Buffer (2 minutes each), then mounted onto slides with Fluoromount-G (eBioscience, San Diego,
599 California, USA). Images were taken using an LSM 710 or LSM780 confocal microscope (Carl
600 Zeiss, Oberkochen, Germany) and visualized and processed using ImageJ and Adobe Illustrator.
601

602 *Detection of c-Fos with fluorescent in situ hybridization.* For the mice in the food-restriction
603 (FR) groups, food was removed at 5 p.m. the day before the testing day. Food was reintroduced
604 to the mice 18-20 h after food-restriction (between 11 a.m. and 2 p.m.). The foods used were
605 regular chow (PicoLab Rodent Diet 20, Cat. No. #5053*) and HFD (Bioserv HFD, Bioserv, Cat.
606 No. # S3282). At 30 minutes after the food reintroduction, food consumption was recorded, and
607 the mice were sacrificed. The brain tissue was processed for RNAscope. Water (Hydrogel
608 (ClearH20)) was available ad libitum until 3 h before the mice were sacrificed. Mice in the sated
609 group had food (regular chow) and Hydrogel (ClearH20) freely available until 3 h before the
610 mice were sacrificed. Mice and their brain tissues in different groups underwent the experimental
611 procedure in parallel to minimize variability.

612

613 **Viral vectors**

614 The AAV5-Ef1a-DIO-hChR2(H134R)-eYFP and AAV9-CAG-Flex-GFP were produced by the
615 University of North Carolina vector core facility (Chapel Hill, North Carolina, USA). The
616 AAV9-EF1a-DIO-hChR2(H134R)-eYFP-WPRE-hGH were made by the Penn Vector Core
617 (Philadelphia, PA, USA). The AAV2/9-CAG-DIO-TeLC-eGFP was previously described
618 (Murray et al., 2011) and custom-packed by the Penn Vector Core (Philadelphia, PA, USA). The
619 AAV1.Syn.Flex.GCaMP6f.WPRE.SV40, were produced by Addgene (Watertown, MA, USA).
620 All viral vectors were aliquoted and stored at -80°C until use.

621

622 **Stereotaxic surgery**

623 All surgery was performed under aseptic conditions and body temperature was maintained with a
624 heating pad. Standard surgical procedures were used for stereotaxic injection and implantation,
625 as previously described (Stephenson-Jones et al., 2016; Zhang and Li, 2018). Briefly, mice were
626 anesthetized with isoflurane (1–2% in a mixture with oxygen, applied at 1.0 L/minute), and
627 head-fixed in a stereotaxic injection frame, which was linked to a digital mouse brain atlas to
628 guide the targeting of different brain structures (Angle Two Stereotaxic System,
629 myNeuroLab.com). Lidocaine (20 μl) was injected subcutaneously into the head and neck area
630 as a local anesthetic.

631

632 To prepare mice for the photometry, optogenetics and inhibition experiments, we first made a
633 small cranial window (1–2 mm^2) in each mouse, bilaterally. We then lowered a glass
634 micropipette (tip diameter, $\sim 5 \mu\text{m}$) containing viral solution to reach the IPAC (coordinates: 0.35
635 mm anterior to Bregma, 1.40 mm lateral from midline, and 4.50 mm vertical from brain surface).
636 0.1–0.15 μL of viral solution was delivered with pressure applications (5–20 psi, 5–20 ms at 1

637 Hz) controlled by a Picospritzer III (General Valve) and a pulse generator (Agilent). The rate of
638 injection was ~20 nl/minute. The pipette was left in place for 10–15 minute following the
639 injection, and then slowly withdrawn. Infection of the IPAC was performed in both hemispheres
640 in mice dedicated to optogenetic and inhibition experiments, and unilaterally in mice used for
641 photometry.

642

643 We subsequently implanted optic fibers above injection locations (coordinates: 0.35 mm anterior
644 to Bregma, 1.40 mm lateral from midline, and 4.30 mm vertical from brain surface). A head-bar
645 was also mounted for head-restraint. We waited for a minimum of 4 weeks following the viral
646 injection and before starting experiments on these mice.

647

648 ***In vivo* fiber photometry and data analysis**

649 To record the activity of IPAC^{Nts} neurons *in vivo* in behaving animals, we used a commercial
650 fiber photometry system (Neurophotometrics Ltd., San Diego, CA, USA) to measure GCaMP6f
651 signals in these neurons through an optical fiber (Fiber core diameter, 200 μm ; Fiber length, 5.0
652 mm; NA, 0.37; Inper, Hangzhou, China) unilaterally implanted above the IPAC. A patch cord
653 (fiber core diameter, 200 μm ; Doric Lenses) was used to connect the photometry system with the
654 implanted optical fiber. The intensity of the blue light ($\lambda = 470 \text{ nm}$) for excitation was adjusted to
655 ~20 μW at the tip of the patch cord. We simultaneously recorded the isosbestic signal (using a
656 415 nm LED) in order to monitor potential motion artifacts as previously described (Kim et al.,
657 2016). Emitted GCaMP6f fluorescence was bandpass filtered and focused on the sensor of a
658 CCD camera. Mean values of fluorescent signal from each fiber were calculated and saved using
659 Bonsai software (Bonsai), and were exported to MATLAB for further analysis. Photometry

660 signals and relevant behavioral events were aligned based on an analogue TTL signal and timing
661 data generated by the Bpod.

662

663 To correct photobleaching of fluorescence signals, we used a sliding window correction method
664 to subtract the gradual reduction in baseline signal. We used Mathworks' `tsmovavg` function to
665 find the average fluorescence values calculated over a 10-second sliding time window. This gave
666 us an average smoothed measurement of fluorescence at each timepoint throughout the bleaching
667 process (`average_window(t)`) that we used for $\Delta F/F_0$ normalization, where ΔF is the change in
668 fluorescence and F_0 is baseline fluorescence. From there, we found the lowest average value in
669 the 30 seconds before time t to get baseline fluorescence values, $F_0(t) = \min(\text{average_window}((t$
670 $- 30):t))$. We treated these values as baseline fluorescence in our $\Delta F/F_0$ calculation: $\Delta F/F_0 =$
671 $(F(t) - F_0(t)) / F_0(t)$, where F is the raw fluorescence data and F_0 is our normalized baseline
672 fluorescence. This gave us a corrected $\Delta F/F_0$ that takes into account the slowly decreasing
673 baseline and is not affected by large peaks of signal. This method of bleaching correction works
674 very well for correcting slow bleaching and is resilient to brief disruptions in signal due to
675 artifacts. It is not ideal for analyzing signal with large slow fluctuations, as the sliding window
676 calculation can mask this type of signal. However, this correction method is ideal for our free-
677 moving photometry data, since the responses to stimuli in the IPAC are transient and do not last
678 more than the length of the 30 second sliding window. The Z-score of $\Delta F/F_0$ was then calculated
679 using the mean and standard deviation of the signal during the baseline periods (the pooled 10
680 second time windows before each stimulus), $Z\text{-score}(\Delta F/F_0) = (\Delta F/F_0 - \text{mean}(\text{baseline}$
681 $\Delta F/F_0)) / \text{standard deviation}(\text{baseline } \Delta F/F_0)$.

682

683 A small number of trials had artifacts due to coiling of the photometry fibers or movement of the
684 animals. To find these trials, we searched the isosbestic control channel for large changes in
685 fluorescence and automatically flagged for review any trial with a fluctuation (increase or
686 decrease) of greater than 3 times the standard deviation of signals in the control channel. We
687 discarded trials with significant artifacts during the stimulus period. This method left most trials
688 intact, but minimized the effect of movement artifacts on the signal.

689

690 *Photometry experiments in free moving mice (Figure 2)*. Mice were water-restricted starting at 5
691 p.m. the day before the training day. On the training day, the mice learned to acquire water by
692 licking at two adjacent spouts, with each spout delivering equal volume of water upon each lick.
693 Water will be delivered only if mice licked a spout. The spout also served as part of a custom
694 “lickometer” circuit, which registered a lick event each time a mouse completed the circuit by
695 licking the spout. A custom software written in MATLAB (The MathWorks, Inc., Natick,
696 Massachusetts, USA) was used to control the delivery of liquids and record licking
697 events through a Bpod State Machine (Sanworks, Stony Brook, NY, USA) (Xiao et al., 2020).
698 The training consisted one session of 100 trials.

699

700 The next day, which was the testing day, the mice were tested with two pairs of liquids: a
701 sucralose solution vs. water, and a quinine solution vs. water. Each pair of liquids was available
702 in interleaved trials (25 trials each pair, 50 trials in total; inter-trial-intervals, random between 8
703 and 14 s), and each liquid was delivered from one of the two spouts in equal volume (6 μ l) upon
704 each lick. The sucralose and quinine solutions were delivered from the same spout in consecutive

705 days. The tubing and spouts were carefully washed between delivering of different liquids.

706 Volume calibration was carried out prior to every testing.

707

708 *Photometry experiments with olfactometer.* Mice were under head-restraint in front of the output

709 of a custom-built olfactometer. Before the testing, mice were habituated to the setup for 1 hour.

710 The odors were presented using the olfactometer, which contains an eight-way solenoid that

711 controls oxygen flow through eight vials. The vials contained odorants dissolved in mineral oil.

712 The odors presented were: butyric acid (Sigma, Cat. No. #103500; 100 μ l dissolved in 5 ml

713 mineral oil), olive oil-based HFD (Envigo; 1 g in 5 ml mineral oil), coconut oil-based HFD

714 (Envigo; 1 g in 5 ml mineral oil), white chocolate (Lindt; 1 g in 5 ml mineral oil), dark chocolate

715 (Ghirardelli; 1 g in 5 ml mineral oil), regular chow (PicoLab Rodent Diet 20, Cat. No. #5053*; 1

716 g in 5 ml mineral oil), and mineral oil as a control (Sigma, Cat. No. #M3516). Food pellets were

717 crumbled and homogenized to mineral oil for 10 minutes using a vortex mixer. Odorized oxygen

718 was diluted 10:1 into a continuous carrier stream for a total flow of 4 l/minute. To prevent odor

719 accumulation, air was collected behind the animal with a vacuum pump. Odor presentations were

720 3 s every 30 s while constantly measuring calcium signals in IPAC^{Nts} neurons. Every testing

721 session consisted of 10 trials per odor.

722

723 For the photometry experiments with the olfactometer, we used a custom-made fiber photometry

724 system to measure GCaMP6f signals *in vivo*. Green and red emitted fluorescence signals were

725 filtered and split to separate photodetectors and digitally sampled at 6100 Hz via a data

726 acquisition board (National Instruments, Model # NI USB-6211). Peaks were extracted by

727 custom Matlab software with an effective sampling rate of 211 Hz. Signals from each signal

728 were corrected for photobleaching by fitting the decay with a double exponential, and then
729 normalized to a Z score. The red signals represent autofluorescence and was used to monitor and
730 correct for potential movement artifacts (which were essentially absent as the signals were
731 collected in head-fixed mice). The signals in the green channel were transformed back to
732 absolute fluorescence and DF/F was computed. The resulting traces from each recording session
733 were converted to a Z score to compare between subjects. All data analysis was performed using
734 custom written code in Matlab.

735

736 **Liquid preference tests**

737 To test water-restricted mice's preference between a sucralose solution (0.13%) and water, or
738 between a quinine solution (0.5 mM) and water (Figure 2), mice were water restricted overnight,
739 and then were presented with two bottles, each containing one of the liquids in a pair. The mice
740 were tested in two consecutive days. The test in each day lasted for 20 minutes, with the bottles
741 switched their positions at 10 minutes to minimize a potential positional effect.

742

743 To test sated mice's preference between a sucralose solution (0.004%; 0 cal/ml) and water, or
744 between a sucrose solution (1%; 0.04 cal/ml) and water (Figure 6), sated mice were singly
745 housed with food and water available *ad libitum* for a week before the start of the experiment.
746 Water was dispensed through a bottle in the cage. After this, a second bottle containing either the
747 sucralose or sucrose solution was added to the cage. Mice were allowed to first habituate to the
748 newly added solution for 24 h, after which their consumption of the solution and water over a 48-
749 h period was measured. The testing of sucralose and sucrose was separated by a 48-h period,

750 during which the mice had access only to water. The positions of the bottles were switched every
751 24 h to minimize a potential positional effect.

752

753 **Food preference test**

754 Mice were familiarized with HFD^{CO} and HFD^{OO} for 4 days, during which HFD^{CO} or
755 HFD^{OO}(1g/mouse) was available for 3 h in home cage on alternating days, with chow and water
756 available *ad libitum*. The day prior to the testing, mice were singly housed. On the test day, both
757 HFD^{CO} and HFD^{OO} were delivered to the cage and intake was measured at 3 h after the delivery.

758

759 **Optogenetics and feeding behavior**

760 Mice sated on regular chow were habituated to the box for testing the effects of optogenetics on
761 feeding behavior for 10 minutes on the day prior to the testing. Food was provided to the floor of
762 the box. On the testing day, feeding behavior was assessed for 5 minutes with laser off, then 5
763 minutes with light on (20Hz, 7-10 mW measured at the tip of the fiber), and then another 5
764 minutes with laser off. Food was provided to the floor and weighed before and after each of the
765 5-minute sessions. The foods used were grain-based pellets (similar to the regular chow; 45
766 mg/pellet, Bioserv, F0165, 3.43 cal/g), sucrose (45 mg/pellet, Bioserv, F0021, 3.83 cal/g), high
767 fat diet (HFD) (soft pellet, Bioserv, S3282, 5.49 cal/g), white chocolate (Lindt, 5.5 cal/g), dark
768 chocolate (Ghirardelli, 5.5 cal/g), HFD^{CO} (Envigo custom diet, 4.5 cal/g), HFD^{OO} (Envigo
769 custom diet, 4.5 cal/g). Plain and quinine-flavored grain-based pellets were prepared by
770 immersing the pellets in either water or a 10 mM quinine solution for 10 minutes. Pellets were
771 dried overnight and used for testing the following day. Diets were presented on consecutive days
772 to sated mice.

773

774 To score feeding bouts, videos generated from the feeding behavioral assays were analyzed
775 frame by frame using Behavioral Observation Research Interactive Software (BORIS) (Gamba,
776 2016). A feeding bout was defined as an event lasting for at least 3 seconds from pellet pickup to
777 either pellet drop or pellet consumed.

778

779 **Real-time place aversion or preference test**

780 Freely moving mice were initially habituated to a two-sided chamber ($23 \times 33 \times 25$ cm; made
781 from Plexiglas) for 10 minutes, during which their baseline preference for the left or right side of
782 the chamber was assessed. During the first test session (10 minutes), we assigned one side of the
783 chamber (counterbalanced across mice) as the photostimulation side, and placed the mice in the
784 non-stimulation side to start the experiment. Once the mouse entered the stimulation side, photo-
785 stimulation (5-ms pulses, 20 Hz, 7-10 mW (measured at the tip of optic fibers)), generated by a
786 473-nm laser (OEM Laser Systems Inc., Bluffdale, Utah, USA), was immediately turned on.
787 Photostimulation was turned off as soon as the mouse exited the stimulation side. In the second
788 test session (10 minutes) we repeated this procedure but assigned the other side of the chamber
789 as the stimulation side. The behavior of the mice was videotaped with a CCD camera interfaced
790 with Ethovision software (Noldus Information Technologies), which was also used to control the
791 laser stimulation and extract behavioral parameters (position, time, distance and velocity).

792

793 **Self-stimulation tests**

794 Freely moving mice were placed in a chamber equipped with two ports. Poking into one of the
795 ports (the active port) triggered photo-stimulation for 2 s in the IPAC (5-ms pulses, 20 Hz, 10

796 mW; $\lambda = 473$ nm), whereas poking into the other port (the inactive port) did not trigger photo-
797 stimulation. Mice were allowed to freely poke the two ports and were tested in 1-h sessions.

798

799 For testing the impact of nutritional state on self-stimulation behavior, Chr2 mice were trained
800 for two consecutive days, 1 hour per day, to nose poke on a fixed ratio (FR1) to self-stimulate
801 IPAC^{Nts} neurons while sated on regular chow (PicoLab Rodent Diet 20, Cat. No. #5053*). Each
802 nose poke produced a 2-second train of stimulation (5-ms pulses, 20 Hz, 10 mW; $\lambda = 473$ nm).
803 Mice were then tested on consecutive days when fed a high fat diet (“HFD”, Bioserv, Cat. No. #
804 S3282; Physiological value: 5.49 Kcal/g) or after being food-restricted overnight. High-fat diet
805 was provided to mice already sated on chow for 2h prior testing. Self-stimulation experiments
806 were carried out during the light cycle, between 9 a.m. and 5 p.m.

807

808 **Metabolic testing**

809 Mice were singly housed and habituated to the metabolic cages (CLAMS, Columbus) for at least
810 a week before testing, under a 12-h light/dark cycle (6 a.m. to 6 p.m. light). Mouse locomotor
811 activity, energy expenditure (EE), oxygen consumption (VO_2), carbon dioxide production
812 (VCO_2), Respiratory exchange ratio (RER), food and water intake were recorded. The mice were
813 first fed with regular chow (PicoLab Rodent Diet 20, Cat. No. #5053*; physiological value, 3.43
814 kcal/g) and then with HFD (Bioserv, Cat. No. # S3282; physiological value, 5.49 kcal/g). Diets
815 and water were available *ad libitum*. Gas sensor calibration (CO_2 , O_2) of the apparatus was
816 performed before each test. Mouse bodyweight was recorded prior to and after every testing
817 session.

818

819 **Insulin tolerance test (ITT) & glucose tolerance test (GTT)**

820 Singly housed mice were transferred to a clean cage, with food removed for 6 hours (9 a.m. – 3
821 p.m.) before each test. All tests started at 3 pm. For ITT, mice were injected intraperitoneally
822 (i.p.) with 0.5 U/kg body weight insulin (Humulin, Eli Lilly; NDC Code: 0002-8215) in 0.9%
823 sterile saline. For GTT, mice were injected i.p. with 1 g/kg bodyweight glucose (Sigma G5767-
824 25G) in 0.9% sterile saline. There was a 48-h gap between tests, during which food and water
825 were available *ad libitum*. Blood glucose levels were measured in duplicates at 0, 15, 30, 45, 60,
826 90, and 120 minutes after injection using OneTouch Ultra 2 Glucometer (OneTouch).

827

828 **RNA extraction and qPCR**

829 Approximately 50 mg fat tissue was harvested using sterile instruments, and was frozen in 500
830 µl Trizol (Thermo Fisher, Cat. No. #15596026) on dry ice and stored at -80 °C until further
831 processing. The tissue was homogenized by adding a stainless-steel bead (Qiagen, Cat. No.
832 #69989) into each tube and shaking the tubes in the TissueLyser (TissueLyser II, Qiagen, Cat.
833 No. #85300) 2 times for 2 minutes each at 30 Hz. After incubating the homogenate for 5 minutes
834 on ice, 100 µl Chloroform (Sigma-Aldrich, Cat. No. #C2432-1L) was added and the tubes were
835 shaken briefly. After incubating for 3 minutes on ice, the tubes were spun at 12000 g at 4 °C for
836 15 minutes. Subsequently, the clear top layer was transferred into a fresh tube and 1/10 volume
837 of 3 M sodium acetate (Bioworld, Cat. No. #41920024-4) and Glycogen (Thermo Scientific, Cat.
838 No. #R0551) at a final concentration of 1 µg/ul and 250 µl isopropanol (Fisher Scientific, Cat.
839 No. #S25372) were added. The tubes were inverted to mix the contents and after 10 minutes
840 incubation on ice, the tubes were spun at 12000 g at 4 °C for 10 minutes. The supernatant was
841 discarded, and the RNA pellet resuspended in 500 µl 75% ethanol. After centrifuging at 7500 g

842 at 4 °C for 5 minutes, the supernatant was discarded, and the RNA pellet left to air dry for 5
843 minutes and then resuspended in 25 µl RNase-free water. cDNA was synthesized from 500 ng
844 total RNA using Taqman Reverse Transcription reagents (Thermo Fisher, Cat. No. #N8080234).
845 Quantitative RT-PCR was performed using QuantStudio™ 6 Flex Real-Time PCR System, using
846 Taqman Fast Advanced Master Mix (Thermo Fisher, Cat. No. #4444556) and Taqman Primers.
847 The $2\Delta\Delta C_t$ method was used to quantify relative amounts of product with a housekeeping gene
848 (Gapdh) as endogenous control. Primers used were Gapdh (Thermo Fisher, Assay ID:
849 Mm99999915_g1, Cat. No. #4331182) and Ucp1 (Thermo Fisher, Assay ID: Mm01244861_m1,
850 Cat. No. #4331182).

851

852 **H&E Staining**

853 Tissues were fixed in 4% PFA for 24 h at 4°C, washed in PBS three times at room temperature
854 and dehydrated in 70% ethanol. Subsequently, tissues were embedded in paraffin, cut using a
855 microtome serially to produce 5-µm sections and stained with Hematoxylin and Eosin (H&E).
856 Pictures were taken using a Zeiss Observer microscope equipped with 10x, 20x and 40x lenses.

857

858 **Oil Red O staining**

859 Livers were fixed in 4% PFA for 24 h at 4°C, washed in PBS three times at room temperature
860 and cryopreserved in 30% sucrose. Tissues were embedded in OCT tissue tek (Sakura, Cat. No.
861 #4583) and 10-µm sections were cut using a Leica Cryostat. Oil Red O staining was performed
862 as previously described (Mehlem et al., 2013) including the counterstaining with Hematoxylin
863 (Abcam, Cat. No. # ab220365). Pictures were taken using a Zeiss Observer microscope
864 equipped with 10x, 20x and 40x lenses.

865

866 QUANTIFICATION AND STATISTICAL ANALYSIS

867 All statistics are described where used. Statistical analyses were conducted using GraphPad Prism
868 7 Software (GraphPad Software, Inc., La Jolla, CA). Parametric tests were used whenever possible
869 to test differences between two or more means. Non-parametric tests were used when data
870 distributions were non-normal. The Shapiro-Wilk Test was used to test for normality. For two-
871 way ANOVA tests, normality of data was assumed. All t-tests were two-tailed. Statistical
872 hypothesis testing was conducted at a significance level of 0.05.

873

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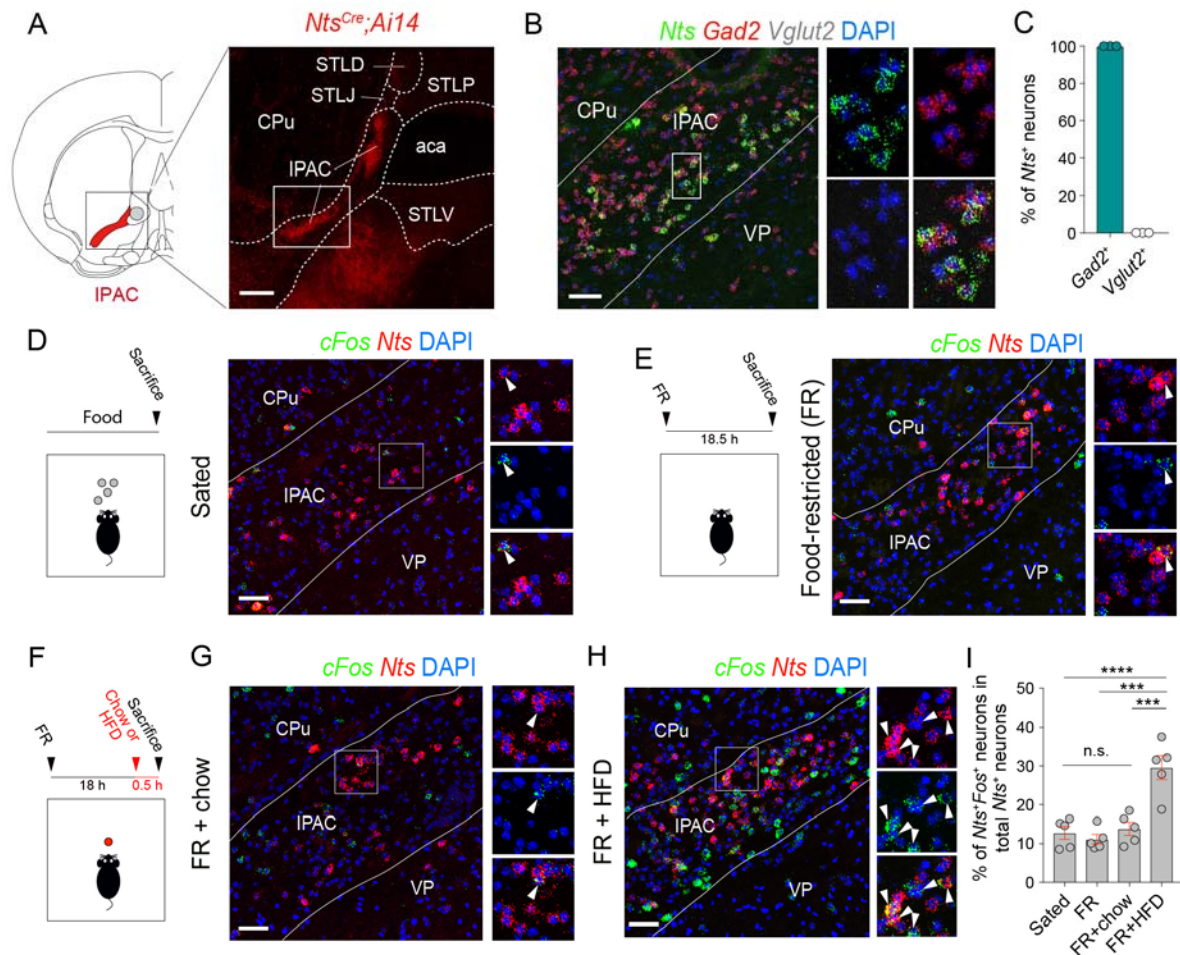
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- 1047



10
 11 **Figure 1. IPAC^{Nts} neurons are selectively activated by palatable food**
 12 (A) Left: a schematic of a coronal brain section containing the IPAC (red). Right: a confocal
 13 image of a coronal brain section from a representative *Nts^{Cre};Ai14* mouse, showing the
 14 distribution of Nts neurons in the IPAC (red). Scale bar: 200 μ m. aca, anterior commissure;
 15 STLP/STLV/STLD/STLJ, lateral posterior/ventral/dorsal/juxtacapsular division of the bed
 16 nucleus of the stria terminalis; CPu, caudoputamen; VP, ventral pallidum.
 17 (B) Left: a representative confocal image of *in situ* hybridization for *Nts*, *Gad2* and *Vglut2*, and
 18 DAPI staining for nuclei. Right, high-magnification images of the boxed area on the left,
 19 showing *Nts*⁺ cells in the IPAC expressed *Gad2* but not *Vglut2*. Scale bar: 50 μ m.
 20 (C) Quantification of the result in (B) (n = 3 mice).
 21 (D) Left: a schematic of the approach. Right: representative confocal images of *in situ*
 22 hybridization for *cFos* and *Nts* in the brain sections from sated mice. On the rightmost are high-
 23 magnification images of the boxed area, showing only few *Nts*⁺ cells in the IPAC expressed *cFos*
 24 (arrow heads). Scale bar: 50 μ m.
 25 (E) Same as (D), except that the result was from food-restricted (FR) mice.
 26 (F) A schematic of the approach.
 27 (G) Representative confocal images of *in situ* hybridization for *cFos* and *Nts* in the brain sections
 28 from FR mice just fed with chow. On the right are high-magnification images of the boxed area,
 29 showing only few *Nts*⁺ cells in the IPAC expressed *cFos* (arrow heads). Scale bar: 50 μ m.

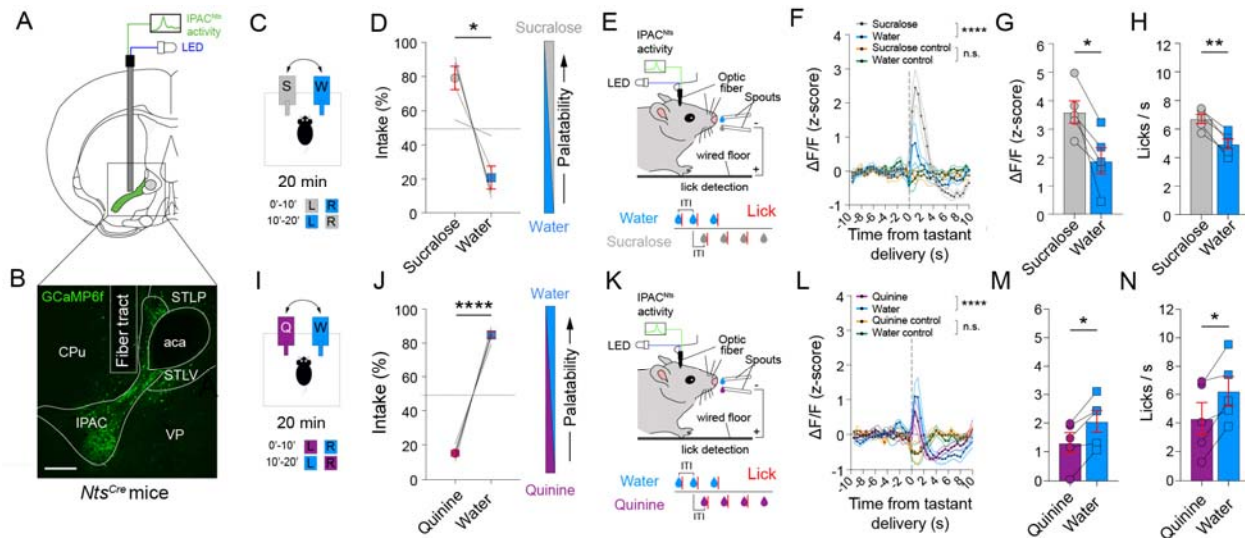
30 (H) Same as (G), except that the result was from FR mice just fed with HFD, and that many *Nts*⁺
31 cells in the IPAC expressed *cFos*.

32 (I) Quantification of the results in (D-H). N = 5 mice in each group; $F_{(3,16)} = 17.51$, $p < 0.0001$;
33 *** $p < 0.001$, **** $p < 0.0001$; one-way ANOVA followed by Sidak's multiple comparisons
34 test.

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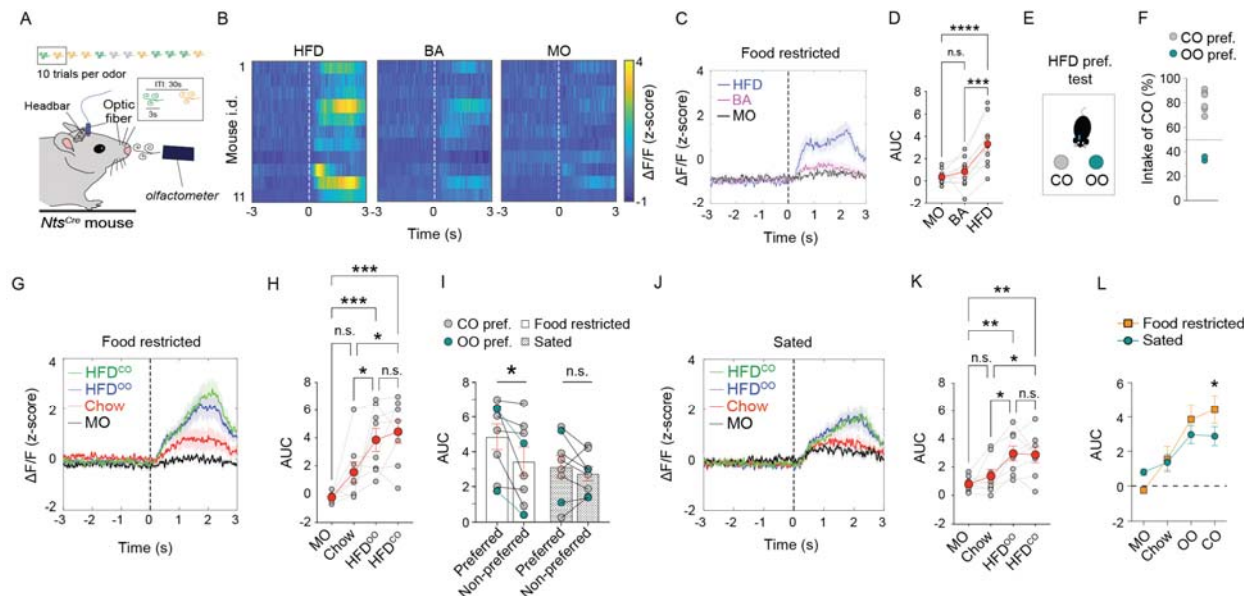
36 Data are presented as mean \pm s.e.m.

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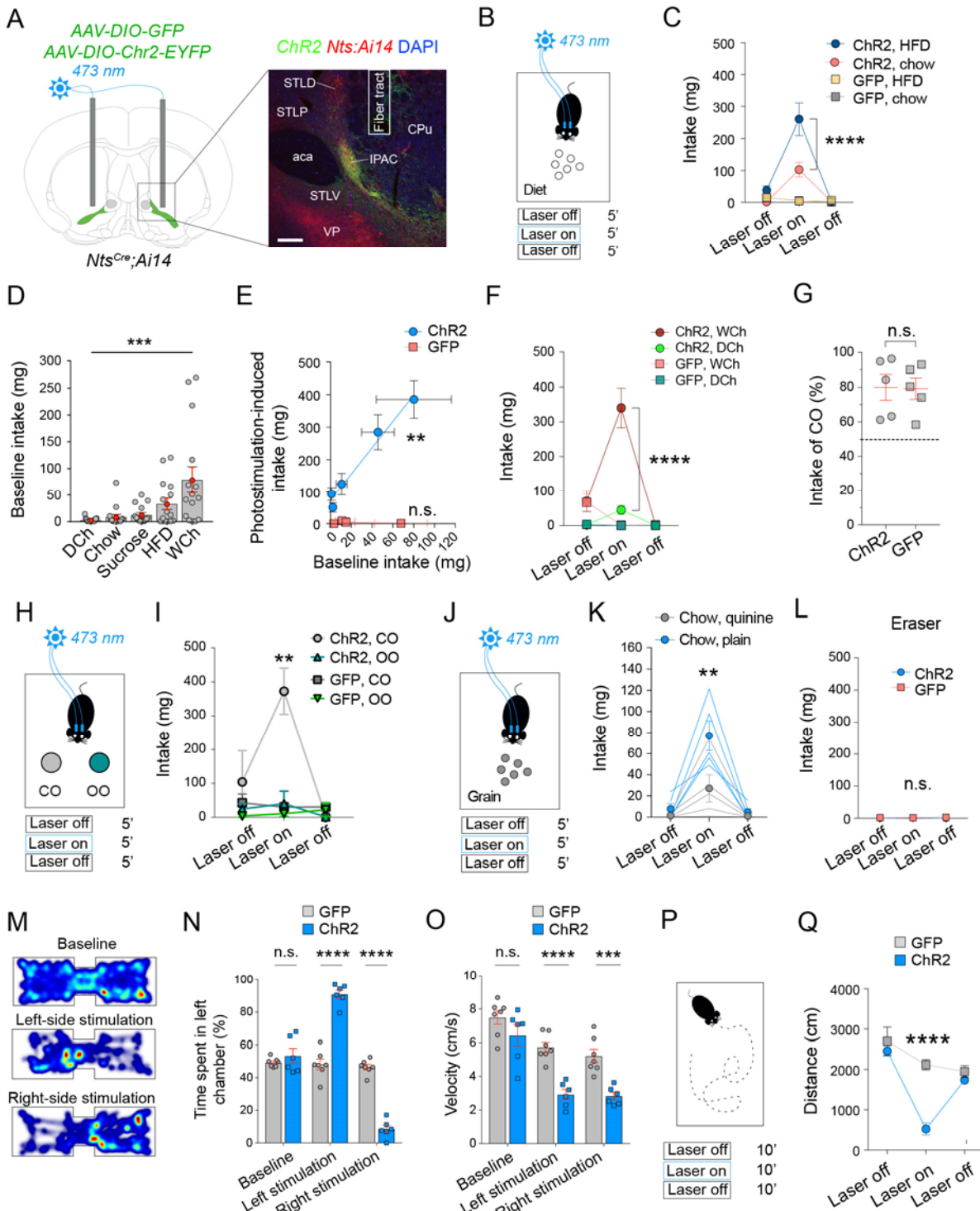


38
 39 **Figure 2. IPAC^{Nts} neurons encode the palatability of tastants**
 40 (A) A schematic of the approach.
 41 (B) A confocal image showing GCaMP6 expression in IPAC^{Nts} neurons and an optical-fiber tract
 42 in a representative mouse. Scale bar 200 μ m.
 43 (C) A schematic of the design of the 2-bottle preference test. L, left bottle, R, right bottle.
 44 (D) Quantification of the intake of sucralose or water relative to total fluid intake (n = 5 mice, *p
 45 = 0.0128, paired t-test).
 46 (E) A schematic of the experimental design.
 47 (F) GCaMP6 and control (isosbestic) signals from IPAC^{Nts} neurons in mice (n = 5) consuming
 48 sucralose solution or water. GCaMP6 signals, $F(39, 156) = 8.45$, ****p < 0.0001; control
 49 signals, $F(39, 156) = 1.19$; p = 0.2278 (n.s.); two-way repeated-measures (RM) ANOVA, liquid
 50 x epoch interaction.
 51 (G) Peak GCaMP6 signals from IPAC^{Nts} neurons after the delivery of sucralose solution or water
 52 (n = 5 mice, *p = 0.0171, paired t-test).
 53 (H) Licking behavior after the delivery of sucralose solution or water, measured in a 3-s window
 54 following the first lick (n = 5 mice, **p = 0.0019, paired t-test).
 55 (I) A schematic of the design of the 2-bottle preference test. L, left bottle, R, right bottle.
 56 (J) Quantification of the intake of quinine or water relative to total fluid intake (n = 5 mice,
 57 ****p < 0.0001, paired t-test).
 58 (K) A schematic of the experimental design.
 59 (L) GCaMP6 and control (isosbestic) signals from IPAC^{Nts} neurons in mice (n = 5) consuming
 60 quinine solution or water. GCaMP6 signals, $F(39, 156) = 4.054$; ****p < 0.0001; control signals,
 61 $F(39, 156) = 0.7166$; p = 0.8883 (n.s.); two-way RM ANOVA, liquid x epoch interaction.
 62 (M) Peak GCaMP6 signals from IPAC^{Nts} neurons after the delivery of quinine solution or water
 63 (n = 5 mice, *p = 0.0145, paired t-test).
 64 (N) Licking behavior after the delivery of quinine solution or water (n = 5 mice, *p = 0.0218,
 65 paired t-test).

66
 67 Data are presented as mean \pm s.e.m.
 68



69
 70 **Figure 3. IPAC^{Nts} neurons encode the palatability of food odors**
 71 (A) A schematic of the experimental setup.
 72 (B) Heatmaps of average GCaMP6 responses of IPAC^{Nts} neurons in individual mice aligned to
 73 odor presentation (dashed line). HFD, high fat diet; BA, butyric acid; MO, mineral oil.
 74 (C) Average GCaMP6 signals from IPAC^{Nts} neurons in food-restricted mice aligned to HFD, BA
 75 and MO odor presentation (dashed line).
 76 (D) Quantification of the area under the curve (AUC) of the responses in individual mice
 77 between 0 and 3 s. N = 11 mice, $F_{(2,18)} = 21.06$, $p < 0.0001$; *** $p < 0.001$, **** $p < 0.0001$; One-
 78 way RM ANOVA followed by Holm-Sidak's multiple comparisons test.
 79 (E) A schematic of the preference test.
 80 (F) Mice's intake of HFD^{CO} relative to the total intake of HFD^{CO} and HFD^{OO}.
 81 (G) Average GCaMP6 signals from IPAC^{Nts} neurons in food-restricted mice aligned to the
 82 presentation of different odors (dashed line).
 83 (H) Quantification of the AUC of the responses in individual mice between 0 and 3 s. N=8 mice,
 84 $F_{(3,21)} = 11.96$, $p < 0.0001$; n.s., $p > 0.05$, * $p < 0.05$, *** $p < 0.001$; one-way RM ANOVA
 85 followed by Holm-Sidak's multiple comparisons test.
 86 (I) IPAC^{Nts} neurons responded more to the preferred HFD than the non-preferred in food-
 87 restricted mice (left), but not sated mice (right). N = 8 mice, $F_{(1,7)} = 8.769$, $p = 0.0211$; n.s., $p >$
 88 0.05 , * $p < 0.05$; two-way RM ANOVA followed by Holm-Sidak's test.
 89 (J) Average GCaMP6 signals from IPAC^{Nts} neurons in sated mice aligned to the presentation of
 90 different odors (dashed line).
 91 (K) Quantification of the AUC of the responses in individual mice between 0 and 3 s. N=8 mice,
 92 $F_{(3,21)} = 8.546$, $p = 0.0007$; n.s., $p > 0.05$, * $p < 0.05$, ** $p < 0.01$; one-way RM ANOVA followed
 93 by Holm-Sidak's multiple comparisons test.
 94 (L) Average responses of IPAC^{Nts} neurons in (H) and (K) are replotted for visual inspection.
 95 $F_{(3,21)} = 5.394$, $p = 0.0065$; * $p < 0.05$, two-way RM ANOVA followed by Holm-Sidak's multiple
 96 comparisons test.
 97
 98 Data are presented as mean \pm s.e.m.



99
100 **Figure 4. Activation of IPAC^{Nts} neurons selectively increases feeding on palatable foods**
101 (A) Left: a schematic of the approach. Right: a confocal image showing ChR2 expression in
102 IPAC^{Nts} neurons and an optical-fiber tract in a representative mouse. Scale bar 200 μ m.
103 (B) A schematic of the paradigm for testing the effects of optogenetics on feeding behavior.
104 (C) Light delivery into the IPAC preferentially increased the intake of HFD over chow in the
105 ChR2, but not GFP mice. ChR2 mice (n = 9): $F_{(2,16)} = 12.64$, $p = 0.0005$, **** $p < 0.0001$; GFP

106 mice ($n = 8$): $F_{(2,14)} = 0.6838$, $p = 0.5208$; two-way RM ANOVA followed by Sidak's multiple
107 comparison test.

108 (D) Food intake of sated mice ($n = 15$) at baseline (laser-off period) ($***p = 0.0003$, Friedman
109 RM test).

110 (E) Analysis of the relationship between food intake at baseline and during photostimulation.
111 ChR2 mice: $n = 9$, $**p = 0.0018$, Pearson's test; GFP mice: $n = 6-8$, $p > 0.05$ (n.s.), Spearman's
112 test.

113 (F) Effect of light delivery into the IPAC on chocolate consumption. ChR2 mice ($n = 9$): $F_{(2,16)} =$
114 19.12 , $p < 0.0001$, $****p < 0.0001$; GFP mice ($n = 6$): $F_{(2,10)} = 5.6$, $p = 0.0234$; between WCh
115 and DCh during laser on, $p > 0.05$; two-way RM ANOVA followed by Sidak's multiple
116 comparisons test.

117 (G) Mice's intake of HFD^{CO} relative to the total intake of HFD^{CO} and HFD^{OO} ($n = 5$ mice per
118 group, $p = 0.9385$ (n.s.), unpaired t-test).

119 (H) A schematic of the paradigm for testing the effect of optogenetics on food preference in
120 sated mice.

121 (I) Effect of light delivery into the IPAC on the consumption of HFD^{CO} or HFD^{OO}. ChR2 mice (n
122 $= 5$): $F_{(2,8)} = 9.443$, $p = 0.0078$, $**p < 0.01$; GFP mice ($n = 5$): $F_{(2,8)} = 0.9049$, $p = 0.4423$; two-
123 way RM ANOVA followed by Sidak's multiple comparisons test.

124 (J) A schematic of the paradigm for testing the effect of optogenetics on food consumption in
125 sated mice.

126 (K) Effect of light delivery into the IPAC of the ChR2 mice on the consumption of quinine-
127 flavored chow or plain chow ($n = 5$): $F_{(2,8)} = 9.476$, $p = 0.0078$, $**p < 0.01$, two-way RM
128 ANOVA followed by Sidak's multiple comparisons test.

129 (L) Effect of light delivery into the IPAC of the ChR2 ($n = 9$) or GFP ($n = 6$) mice on the
130 consumption of inedible items (i.e., pencil eraser) ($F_{(2,26)} = 1.066$, $p = 0.3591$ (n.s.), two-way RM
131 ANOVA).

132 (M) Heatmaps for the activity of a representative mouse at baseline (top), or in a situation
133 whereby entering the left (middle) or right (bottom) side of the chamber triggered
134 photostimulation in the IPAC.

135 (N) Preference of ChR2 ($n = 6$) and GFP mice ($n = 7$) for the left chamber side ($F_{(2,22)} = 137.9$; p
136 < 0.0001 ; $****p < 0.0001$; two-way RM ANOVA followed by Sidak's multiple comparisons
137 test).

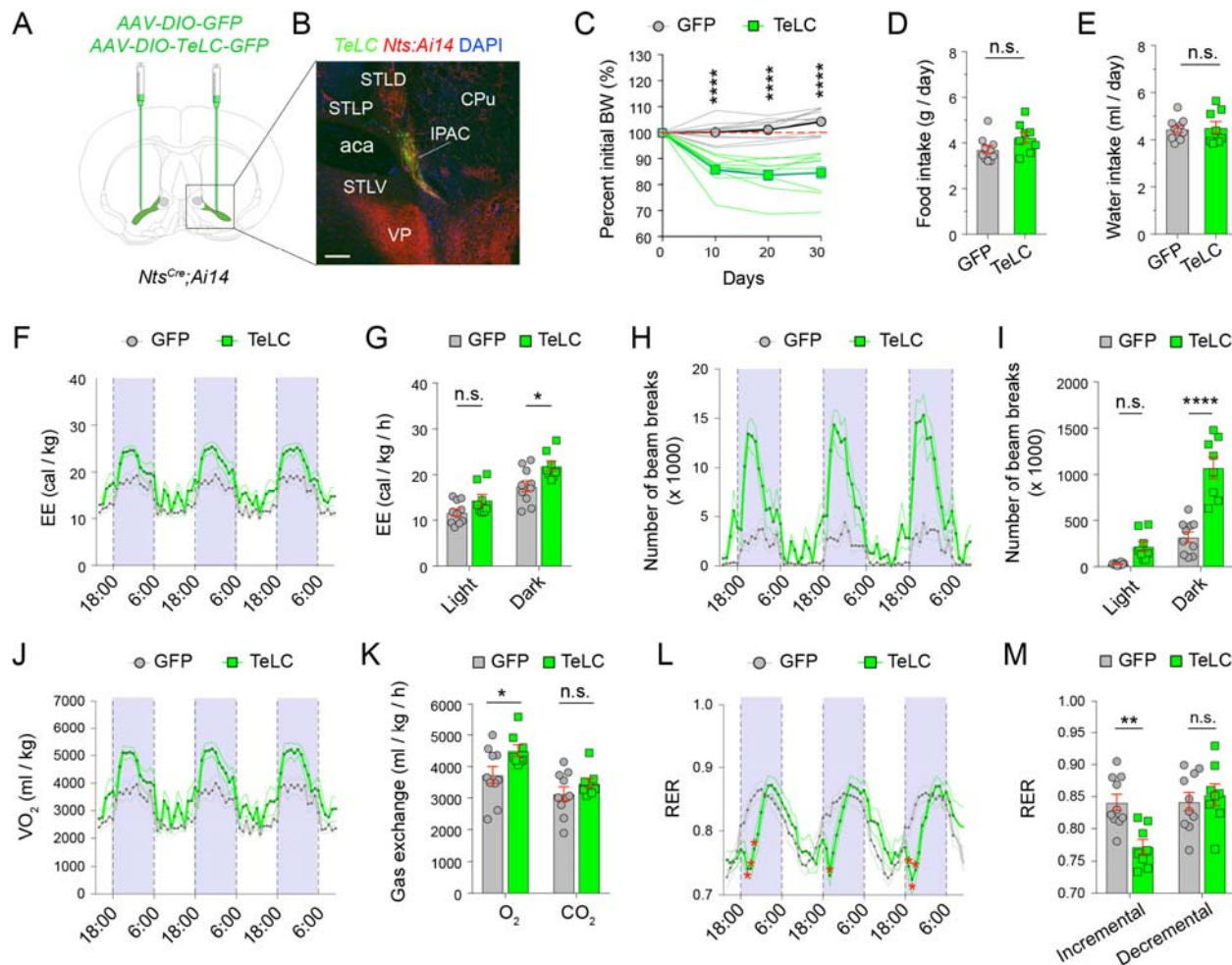
138 (O) Velocity of the ChR2 ($n = 6$) and GFP ($n = 7$) mice in the RTPP/A task ($F_{(2,24)} = 7.116$, $p =$
139 0.0041 ; $**p < 0.001$; $****p < 0.0001$; two-way RM ANOVA followed by Sidak's multiple
140 comparisons test).

141 (P) A schematic of the open field test.

142 (Q) Distance traveled during the open field test for the ChR2 ($n = 8$) and GFP ($n = 6$) mice
143 ($F_{(2,24)} = 13.37$, $p < 0.0001$; $****p < 0.0001$; two-way RM ANOVA followed by Sidak's
144 multiple comparisons test).

145

146 Data are presented as mean \pm s.e.m.



147

148 **Figure 5. Inhibition of IPAC^{Nts} neurons increases energy expenditure**

149 (A) A schematic of the approach.

150 (B) A confocal image showing TeLC expression in IPAC^{Nts} neurons in a representative mouse.

151 Scale bar 200 μ m.

152 (C) Changes in bodyweight (BW) in the GFP mice (n = 11) and TeLC mice (n = 10) following
153 viral injection (d0) ($F_{(3,37)} = 44.28$, $p < 0.0001$; **** $p < 0.0001$; two-way RM ANOVA followed
154 by Sidak's multiple comparisons test).

155 (D) Daily food (chow) intake of the GFP mice (n = 10) and TeLC mice (n = 8) ($p = 0.0785$ (n.s.),
156 unpaired t-test).

157 (E) Daily water intake of the GFP mice (n = 10) and TeLC mice (n = 8) ($p = 0.8023$ (n.s.),
158 unpaired t-test).

159 (F) Energy expenditure of the GFP mice (n = 10) and TeLC mice (n = 8) over 72 h. Data are
160 plotted in 1-h intervals. White and purple areas represent light (6:00-18:00) and dark cycles
161 (18:00-6:00), respectively ($F_{(70,1120)} = 2.029$, $p < 0.0001$, two-way RM ANOVA).

162 (G) Average energy expenditure of the mice in (F) during light and dark cycles ($F_{(1,16)} = 5.934$, p
163 $= 0.0269$; * $p < 0.05$, n.s., $p > 0.05$; two-way RM ANOVA followed by Sidak's multiple
164 comparisons test).

165 (H) Locomotor activity of the GFP (n = 10) and TeLC mice (n = 8) over 72 h ($F_{(70,1120)} = 7.699$, p
166 < 0.0001 , two-way RM ANOVA).

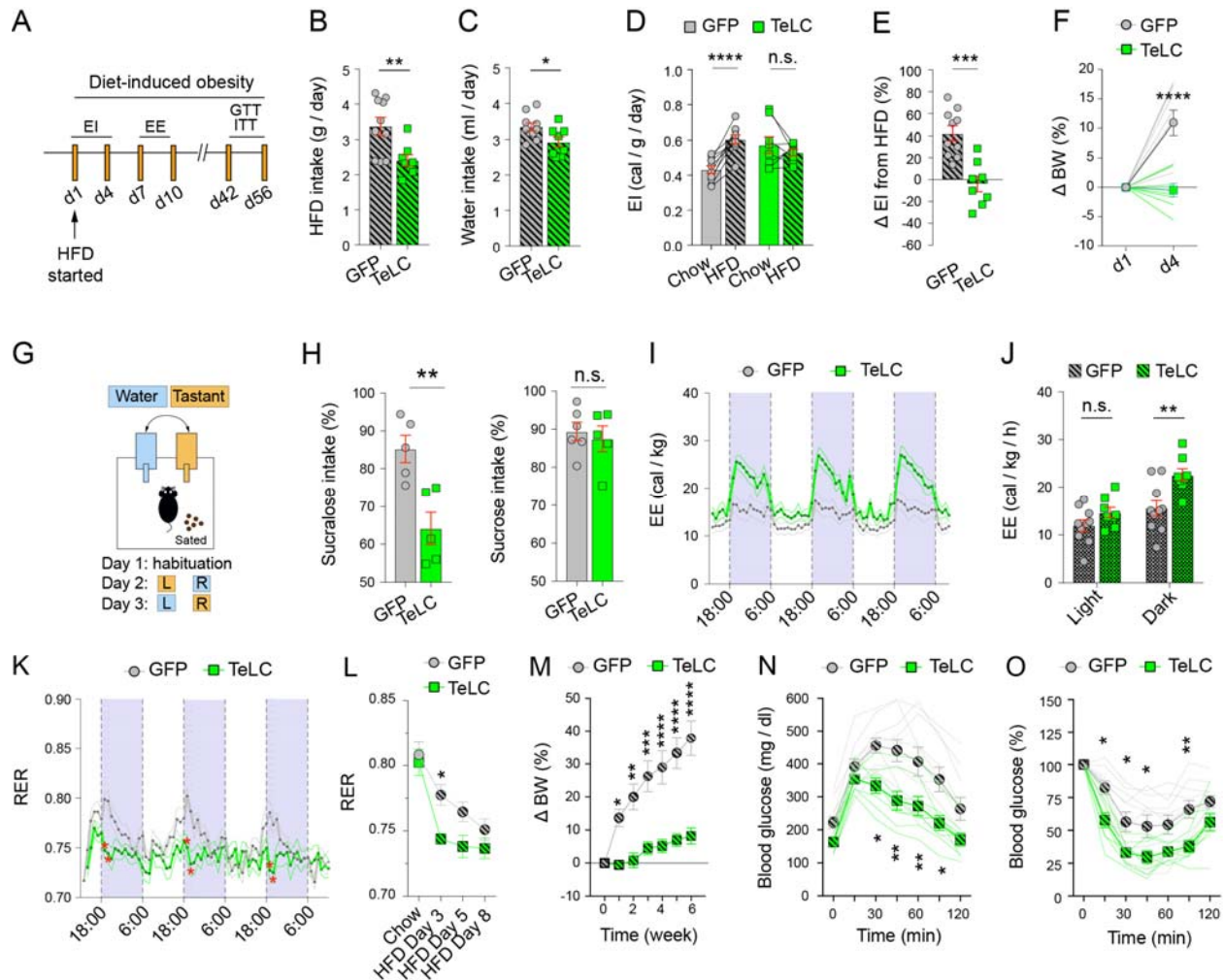
167 (I) Average locomotor activity of the mice in (H) during light and dark cycles ($F_{(1,16)} = 37.84$, $p <$
168 0.0001 ; **** $p < 0.0001$, n.s., $p > 0.05$, two-way RM ANOVA followed by Sidak's multiple
169 comparisons test).

170 (J) The volume of oxygen consumed (VO_2) by GFP ($n = 10$) and TeLC mice ($n = 8$) over 72 h
171 ($F_{(70,1120)} = 2.221$, $p < 0.0001$, two-way RM ANOVA).

172 (K) O_2 and carbon dioxide (CO_2) exchange during incremental activities in the dark cycle (18:00-
173 6:00) (GFP mice, $n = 10$, TeLC mice, $n = 8$; $F_{(1,16)} = 20.24$, $p = 0.0004$; * $p < 0.05$, n.s., $p > 0.05$;
174 two-way RM ANOVA followed by Sidak's multiple comparisons test).

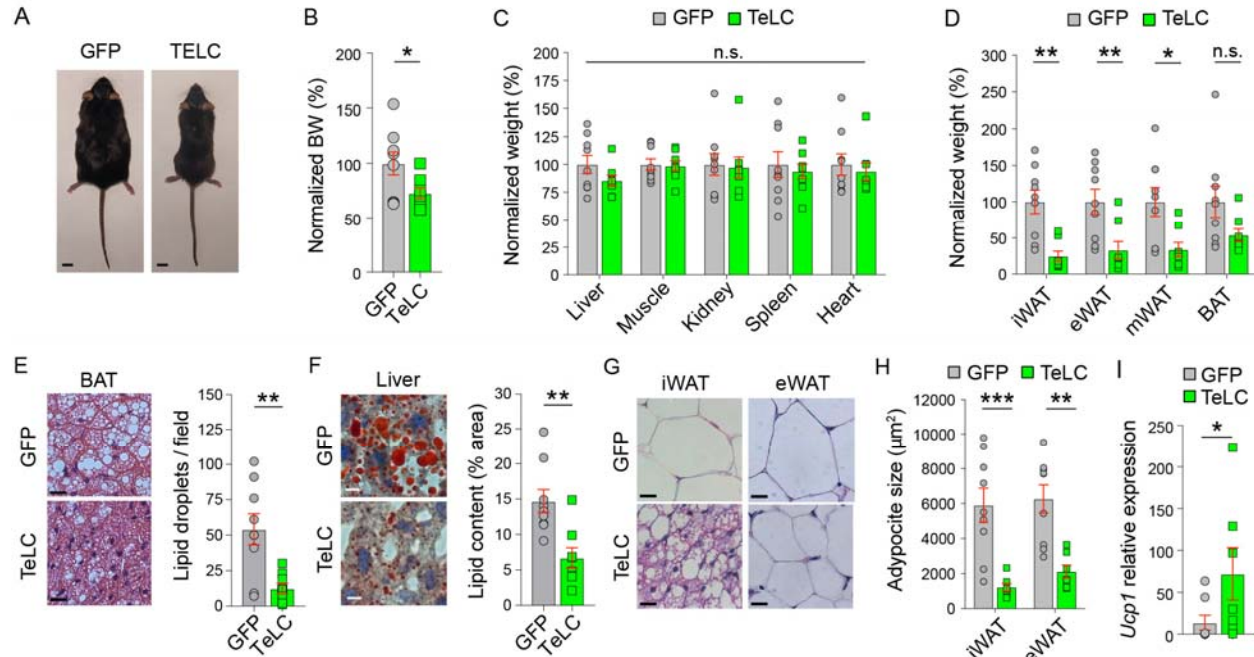
175 (L) Respiratory exchange ratio (RER) of GFP ($n = 10$) and TeLC mice ($n = 8$) over 72 h
176 ($F_{(70,1120)} = 5.042$, $p < 0.0001$, two-way RM ANOVA).

177 (M) Average RER during incremental and decremental activities in the dark cycle (18:00-6:00)
178 on the 3rd day for the mice in (L) ($F_{(1,16)} = 20.24$, $p = 0.0005$; ** $p < 0.01$, n.s., $p > 0.05$, two-way
179 RM ANOVA followed by Sidak's multiple comparisons test). The effect was similar in other
180 days.
181
182



183
 184 **Figure 6. Inhibition of IPAC^{Nts} neurons protects from HFD-induced weight gain**
 185 (A) A schematic of the experimental design. GTT and ITT, glucose and insulin tolerance tests,
 186 respectively.
 187 (B) Daily HFD intake (GFP mice, n = 10, TeLC mice, n = 8; **P = 0.0073, unpaired t-test).
 188 (C) Daily water intake of the same mice in (B) (*P = 0.0305, unpaired t-test).
 189 (D) Comparison of energy intake from chow and HFD diets (derived from Figure 5D; GFP, n =
 190 10, ****p < 0.0001; TeLC mice, n = 8, p = 0.3562 (n.s.); paired t-test).
 191 (E) Change in energy intake after the switch from chow to HFD, in the same mice as those in (D)
 192 (**P = 0.0002, unpaired t-test).
 193 (F) Acute changes in bodyweight (BW) following 4 days of HFD, in the same mice as those in
 194 (D) (F_(1,16) = 19.45, p = 0.0004, ****p < 0.0001, two-way RM ANOVA followed by Sidak's
 195 multiple comparisons test).
 196 (G) A schematic of the design of the 2-bottle preference test. L, left bottle, R, right bottle.
 197 (H) Left: quantification of the intake of sucralose relative to total fluid intake (GFP mice, n = 5,
 198 TeLC mice, n = 5; **p = 0.0055, unpaired t-test). Right: quantification of the intake of sucrose
 199 relative to total fluid intake (GFP mice, n = 6, TeLC mice, n = 5; p = 0.6488 (n.s.), unpaired t-
 200 test).

201 (I) Energy expenditure of the GFP mice (n = 10) and TeLC mice (n = 8) over 72 h, when fed
202 HFD. Data are plotted in 1-h intervals. White and purple represent light (6:00-18:00) and dark
203 cycles (18:00-6:00), respectively ($F_{(71, 1136)} = 7.087$, $p < 0.0001$, two-way RM ANOVA).
204 (J) Average energy expenditure of the HFD-fed mice in (I) during light and dark cycles ($F_{(1, 16)} =$
205 6.527 , $p = 0.0212$; $**p < 0.01$, n.s., $p > 0.05$; two-way RM ANOVA followed by Sidak's
206 multiple comparisons test).
207 (K) RER values of the HFD-fed mice over 72 h (GFP mice, n = 10, TeLC mice, n = 8; $F_{(71, 1136)} =$
208 2.337 , $p < 0.0001$; $*p < 0.05$; two-way RM ANOVA followed by Sidak's post hoc multiple
209 comparisons test).
210 (L) Quantification of the changes in the RER (GFP mice, n = 10, TeLC mice, n = 8; $F_{(1, 16)} =$
211 10.71 , $p = 0.0048$; $*p < 0.05$; two-way RM ANOVA followed by Sidak's multiple comparisons
212 test).
213 (M) Change in bodyweight (BW) after switching to HFD diet (GFP mice, n = 10, TeLC mice, n
214 = 8; $F_{(6, 120)} = 16.9$, $p < 0.0001$; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$; two-way
215 RM ANOVA followed by Sidak's multiple comparisons test).
216 (N) Blood glucose levels following glucose administration during GTT (see A) (GFP mice, n =
217 10, TeLC mice, n = 8; $F_{(6, 96)} = 4.37$, $p = 0.0006$; $*p < 0.05$, $**p < 0.01$; two-way RM ANOVA
218 followed by Sidak's multiple comparisons test).
219 (O) Blood glucose levels following insulin administration during ITT in the same mice as those
220 in (N) ($F_{(6, 96)} = 2.794$, $p = 0.0151$; $*p < 0.05$, $**p < 0.01$; two-way RM ANOVA followed by
221 Sidak's multiple comparisons test).
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Figure 7. Inhibition of IPAC^{Nts} neurons reduces adiposity

227 (A) Representative images of a GFP and a TeLC mouse at endpoint of the experiment (8 weeks
228 of DIO). Scale bar: 1 cm.

229 (B) Quantification of the bodyweight (BW) of the mice during DIO, which was normalized to
230 the BW of GFP mice (GFP mice, n = 9, TeLC mice, n = 8; *p = 0.0381, unpaired t-test).

231 (C) Quantification of the weight of different organs in the mice treated with DIO, with the
232 weight of each organ normalized to that of GFP mice (GFP mice, n = 9, TeLC mice, n = 8; $F_{(1,15)}$
233 = 0.4306, n.s., $p > 0.05$, two-way ANOVA).

234 (D) Quantification of the weight of different adipose tissues in the same mice as those in (C),
235 with the weight normalized to that of GFP mice ($F_{(1,15)} = 9.757$, $p = 0.0070$; *p < 0.05, **p <
236 0.01, n.s., $p > 0.05$; two-way RM ANOVA followed by Sidak's multiple comparisons test).

237 (E) Left: representative images of BAT tissue stained for H&E from a GFP (top) and a TeLC
238 (bottom) mouse treated with DIO. Scale bar: 20 μ m. Right: quantification of the number of large
239 lipid droplets in the two groups (GFP mice, n = 9; TeLC mice, n = 8; **p = 0.0037, unpaired t-
240 test).

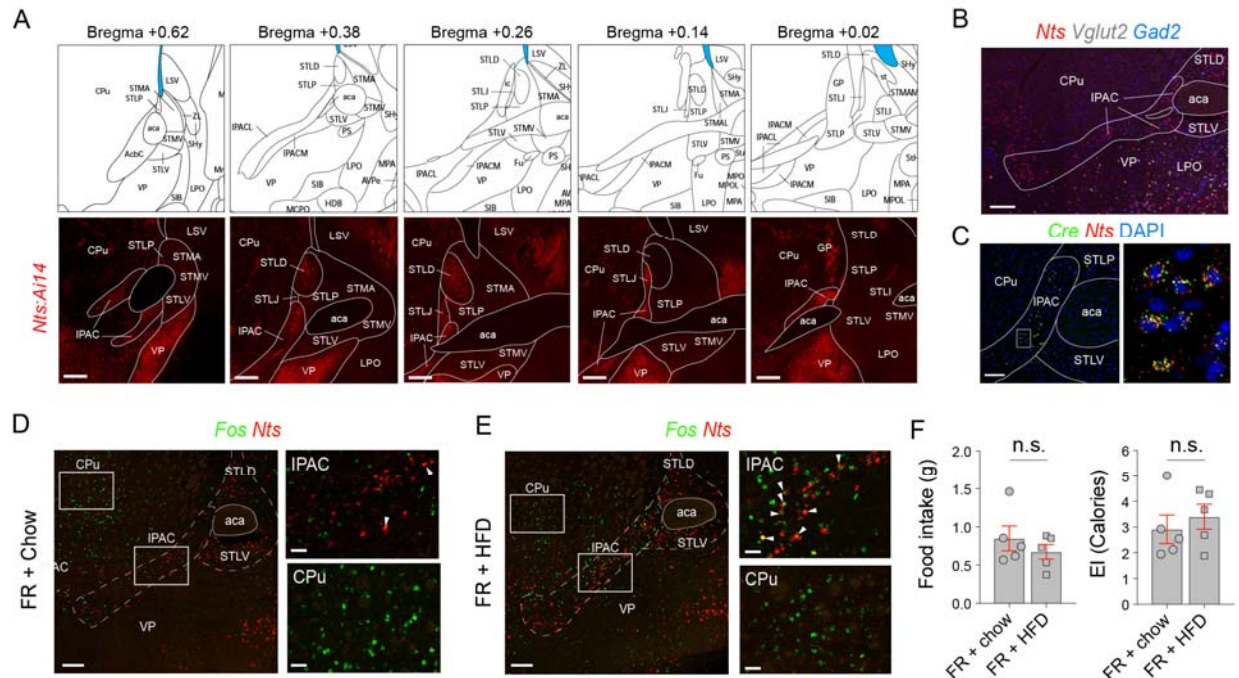
241 (F) Left: representative images of liver tissue stained with Red-Oil from a GFP (top) and a TeLC
242 (bottom) mouse treated with DIO. Scale bar: 10 μ m. Right: quantification of the area occupied
243 by lipid droplets in the two groups (GFP mice, n = 9, TeLC mice, n = 8; **p = 0.0025, unpaired
244 t-test).

245 (G) Representative images of WAT tissues stained for H&E from a GFP (top) and a TeLC
246 (bottom) mouse treated with DIO. Scale bar: 20 μ m.

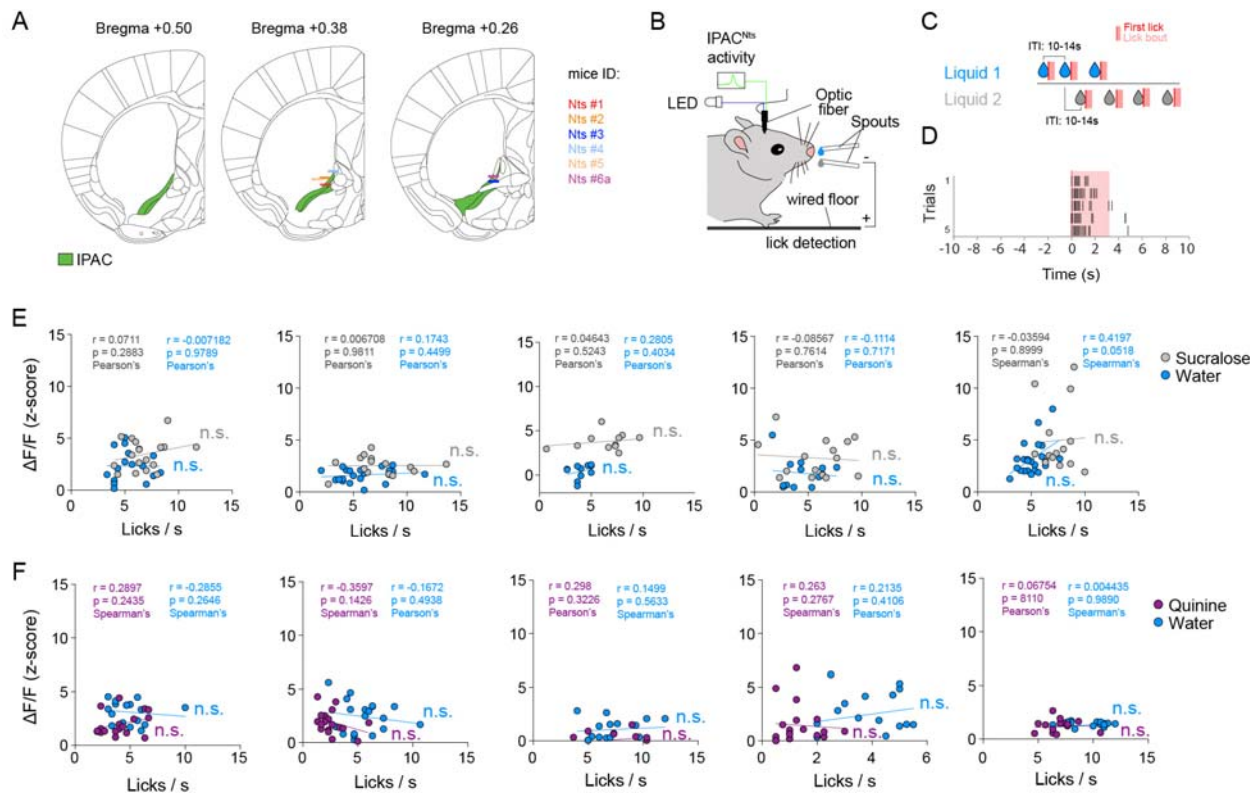
247 (H) Quantification of adipocyte size in iWAT and eWAT based on histological images as those
248 in (G) (GFP mice, n = 8; TeLC mice, n = 7; $F_{(1,14)} = 19.8$, $p = 0.0005$; **p < 0.01, ***p < 0.001;
249 two-way RM ANOVA followed by Sidak's multiple comparisons test).

250 (I) Expression of *Ucp1* in iWAT tissue from GFP and TeLC mice treated with DIO (GFP mice,
251 n = 8, TeLC mice, n = 7; *p = 0.0289, Mann Whitney U-test).

252



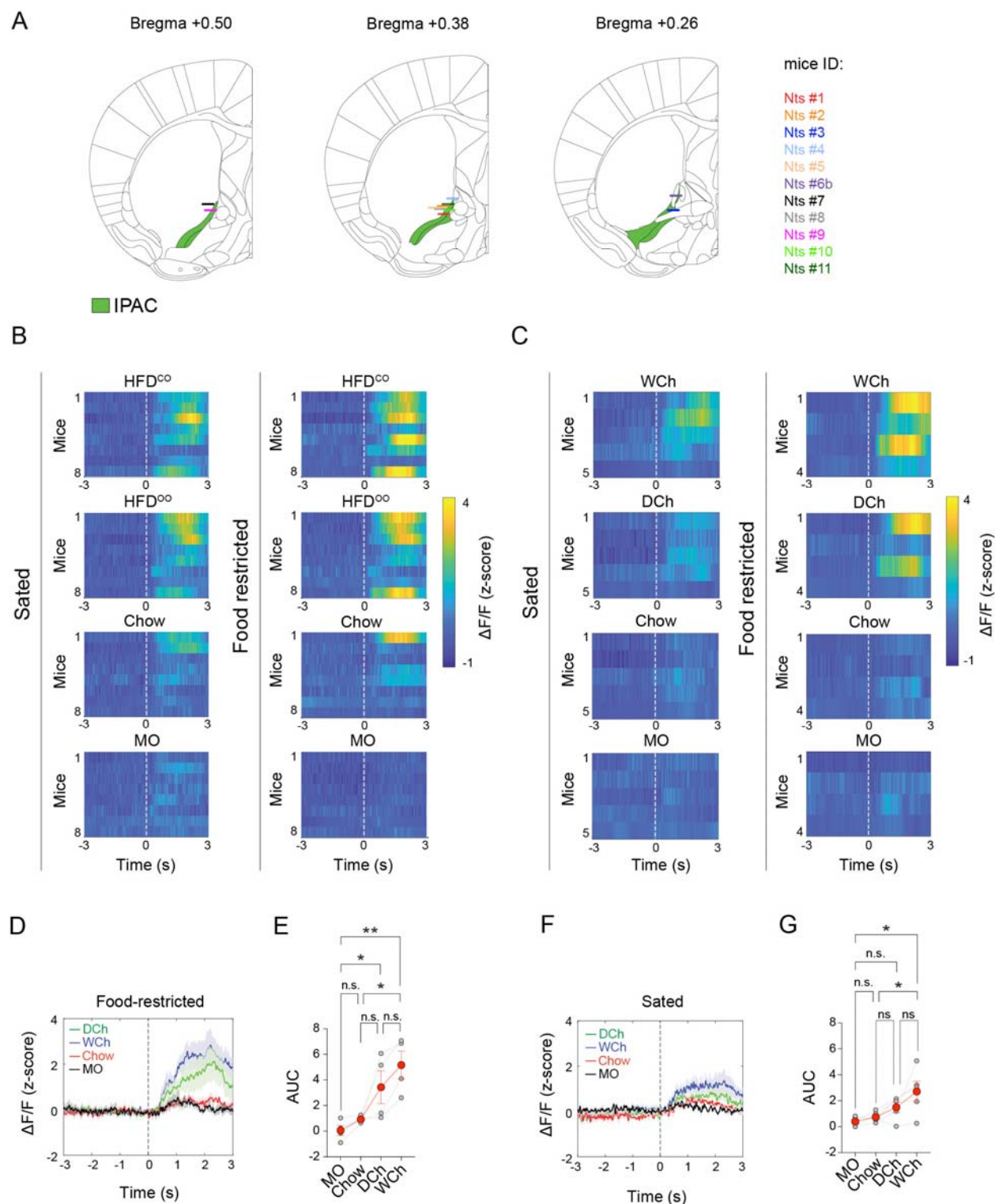
253
 254 **Figure S1. Characterization of IPAC^{Nts} Neurons, Related to Figure 1**
 255 (A) Top: coronal brain plates (from The Mouse Brain in Stereotaxic Coordinates, by Franklin
 256 and Paxinos) depicting the IPAC along the anteroposterior axis. Bottom: confocal images of
 257 coronal brain sections – which correspond to the plates on the top – from a representative
 258 *Nts*^{Cre};*Ai14* mouse, showing the distribution of *Nts* neurons in the IPAC (red). Scale bars: 200
 259 μ m. aca, anterior commissure; STLP/STLV/STLD/STLJ/STLI, lateral
 260 posterior/ventral/dorsal/juxtacapsular/intermedial nucleus of the bed nucleus of the stria
 261 terminalis; STMA/STMV, medial anterior/ventral nucleus of the bed nucleus of the stria
 262 terminalis; CPU, caudoputamen; VP, ventral pallidum; LPO, lateral preoptic area; LSV, lateral
 263 septal nucleus.
 264 (B) A representative confocal image of *in situ* hybridization for *Nts*, *Vglut2* and *Gad2* in a brain
 265 section containing the IPAC. Scale bar: 200 μ m.
 266 (C) Representative confocal images of *in situ* hybridization for *Nts* and *Cre* in the brain sections
 267 containing the IPAC from *Nts*^{Cre} mice. Scale bar: 100 μ m.
 268 (D) Representative confocal images showing *Nts* and *cFos* expression in the IPAC and
 269 surrounding tissues in brain sections prepared from food-restricted (FR) mice just fed chow. On
 270 the right are high-magnification images of the boxed areas on the left, showing only few *Nts*⁺
 271 cells in the IPAC expressed *cFos* (top panel, arrow heads), and many cells in the CPU expressed
 272 *cFos* (bottom panel). Scale bars: 200 μ m (left panel), 50 μ m (right panels).
 273 (E) Representative confocal images showing *Nts* and *cFos* expression in the IPAC and
 274 surrounding tissues in brain sections prepared from FR mice just fed HFD. On the right are high-
 275 magnification images of the boxed areas on the left, showing many *Nts*⁺ cells in the IPAC
 276 expressed *cFos* (top panel, arrow heads), and many cells in the CPU expressed *cFos* (bottom
 277 panel). Scale bars: 200 μ m (left panel), 50 μ m (right panels).
 278 (F) Quantification of food (left) and energy (right) intake in FR mice just fed chow or HFD (food
 279 intake, $p = 0.3799$ (n.s.); energy intake (EI), $p = 0.5295$ (n.s.); unpaired t-test).



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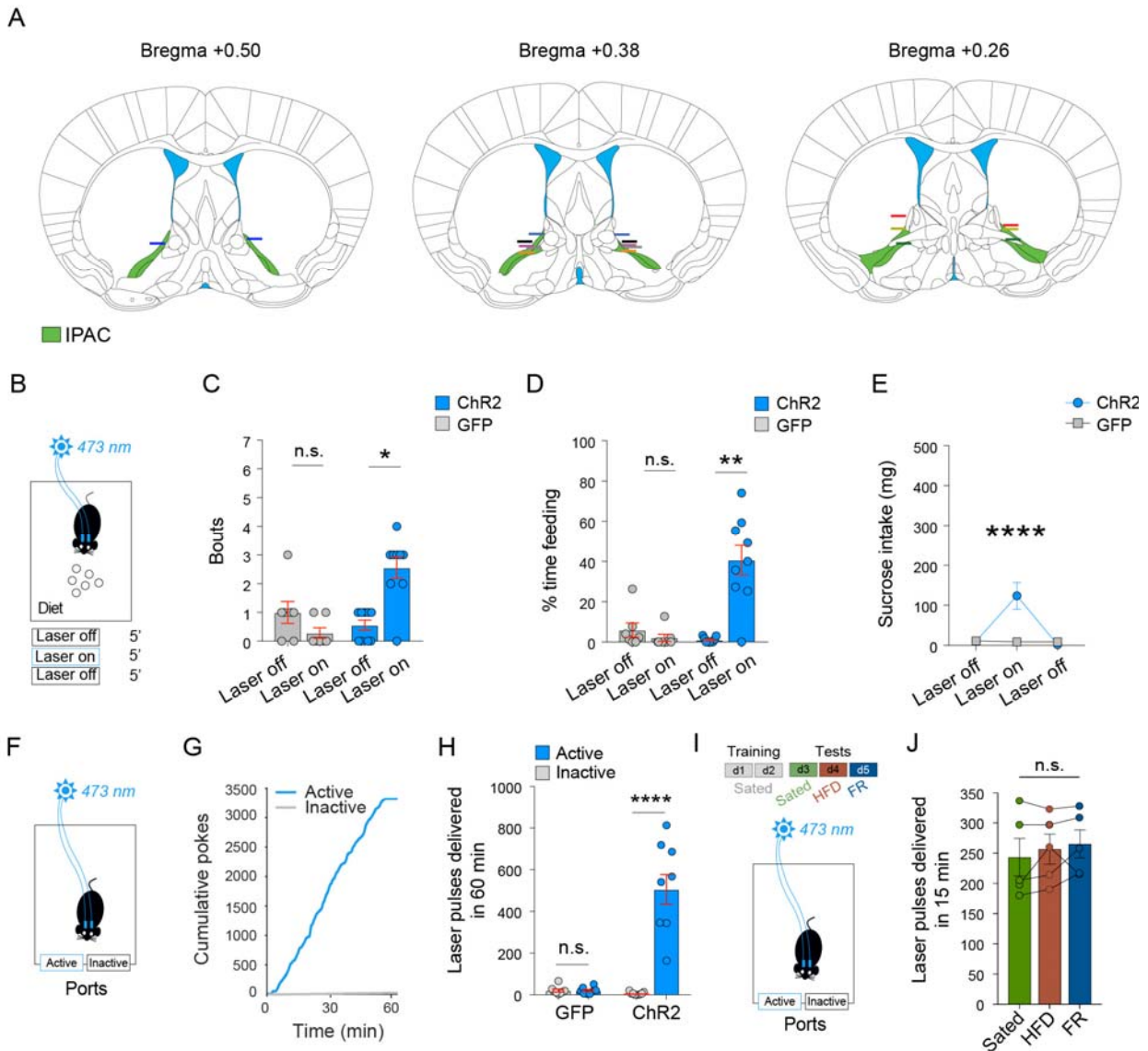
Figure S2. IPAC^{Nts} Neurons Do Not Represent Motion, Related to Figure 2

(A) Schematics showing the locations of optic fiber placement in the mice used in Figure 2.
 (B, C) Schematics of the experimental setup (B) and task structure (C).
 (D) Raster plot showing licking behavior following liquid delivery.
 (E, F) Analysis of the relationship between IPAC^{Nts} neuron responses and mouse licking behavior. Each plot represents the result from one mouse, and each dot represents data from one trial. The amplitude of peak GCaMP6 signals and average lick rate in a 3-s window following the first lick in each trial were used for the analysis. $P > 0.05$ (n.s.) for all mice, Pearson's or Spearman's correlation analysis.



292
 293 **Figure S3. Characterization of IPAC^{Nts} Responses to Food Odors, Related to Figure 3**
 294 (A) Schematics showing the locations of optic fiber placement in the mice used in Figure 3.
 295 (B, C) Heatmaps of the response of IPAC^{Nts} neurons in individual mice to odors derived from
 296 different food sources, under sated or food-restricted condition, as indicated. Dashed lines
 297 indicate the onset of odor presentation.

298 (D) Average GCaMP6 signals from IPAC^{Nts} neurons in food-restricted mice aligned to the
299 presentation of different odors (dashed line).
300 (E) Quantification of the area under the curve (AUC) of the responses in individual mice
301 between 0 and 3 s. N = 4 mice. $F_{(3,9)} = 10.36$, $p = 0.0028$; * $p < 0.05$, ** $p < 0.01$, n.s., $p > 0.05$;
302 one-way RM ANOVA followed by Holm-Sidak's multiple comparisons test.
303 (F) Average GCaMP6 signals from IPAC^{Nts} neurons in sated mice aligned to the presentation of
304 different odors (dashed line).
305 (G) Quantification of the AUC of the responses in individual mice between 0 and 3 s. N = 5
306 mice. $F_{(3,12)} = 5.169$, $p = 0.0160$; * $p < 0.05$, n.s., $p > 0.05$; one-way RM ANOVA followed by
307 Holm-Sidak's multiple comparisons test.
308
309



310

311 **Figure S4. Characterization of the Effects of Activating IPAC^{Nts} Neurons, Related to**

312 **Figure 4**

313 (A) Schematics showing the locations of optic fiber placement in the mice used in Figure 4.

314 (B) A schematic of the paradigm for testing the effects of optogenetics on feeding behavior.

315 (C) Light delivery into the IPAC increased the number of feeding bouts in the ChR2 but not GFP

316 mice. ChR2 mice, n = 9, *p = 0.0117; GFP mice, n = 7, p = 0.1250 (n.s.); Wilcoxon matched-

317 pairs signed rank test.

318 (D) Light delivery into the IPAC increased the duration of feeding bouts in the ChR2 but not

319 GFP mice. ChR2 mice, n = 9, **p = 0.0078; GFP mice, n = 7, p = 0.1250 (n.s.); Wilcoxon

320 matched-pairs signed rank test.

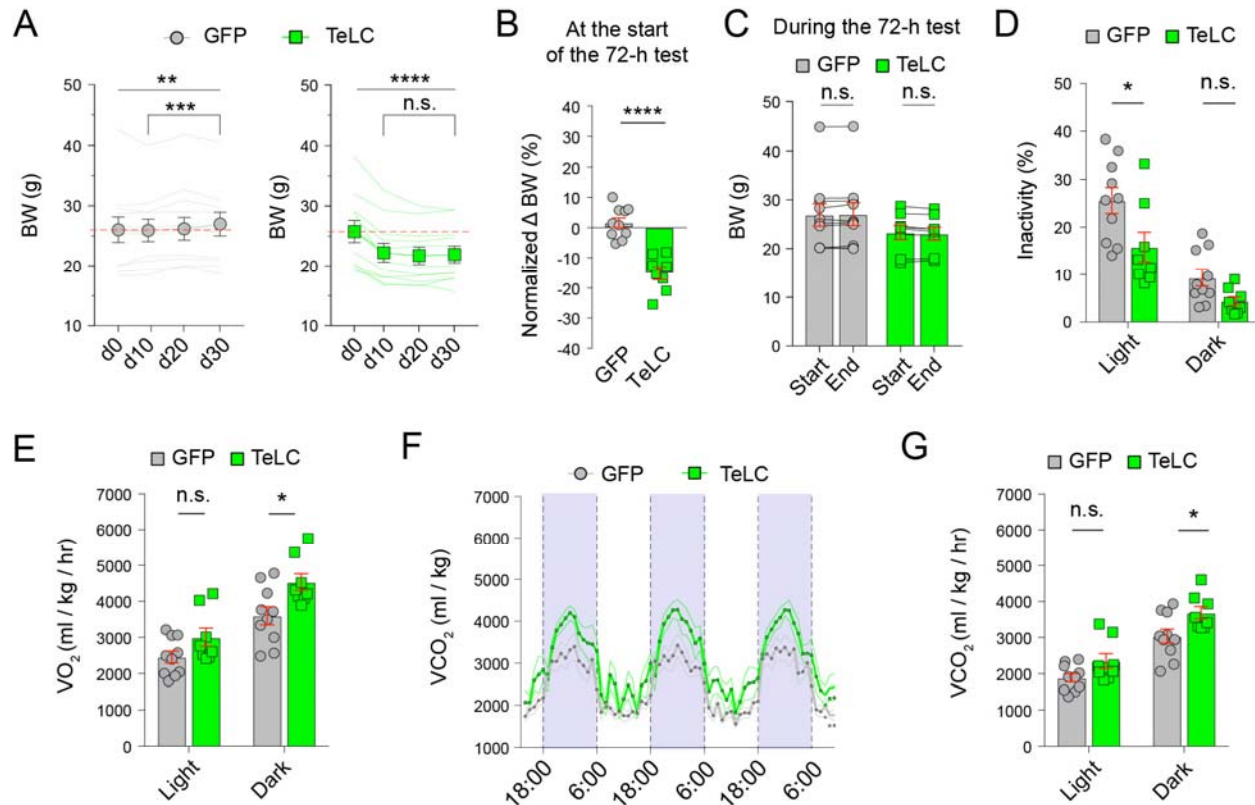
321 (E) Light delivery increased sucrose intake in the ChR2 (n = 9), but not GFP (n = 8) mice (F(2,

322 30) = 10.16, p = 0.0004; ****p < 0.0001; two-way RM ANOVA followed by Sidak's multiple

323 comparisons test).

324 (F) A schematic of the self-stimulation paradigm.

325 (G) Cumulative curves for the poking responses of a representative ChR2 mouse at a port where
326 poking triggered the photostimulation (active) and a port where poking did not trigger the
327 photostimulation (inactive).
328 (H) Quantification of the poking responses as shown in (G). The ChR2 mice, but not the GFP
329 mice, poked the port for photostimulation in the IPAC (ChR2 mice, $n = 9$, **** $p = 0.0001$,
330 paired t-test; GFP mice, $n = 8$, $p = 0.3750$ (n.s.), paired Wilcoxon test).
331 (I) A schematic of the design for testing self-stimulation under different states.
332 (J) Quantification of the self-stimulation in the ChR2 mice under different states ($n = 5$ mice,
333 $F_{(2,8)} = 1.463$, $p = 0.2875$ (n.s.), one-way ANOVA).
334
335



336
337 **Figure S5. Inhibition of IPAC^{Nts} Neurons Increases Energy Expenditure, Related to Figure**
338 **5**

339 (A) Changes in bodyweight (BW) following viral injection (d0) (GFP mice: n = 11, F(3, 30) =
340 6.588, p = 0.0015; **p < 0.01; ***p < 0.001; TeLC mice: n = 10, F(3, 27) = 28.11, p < 0.0001;
341 ****p < 0.0001, n.s., p > 0.05; one-way RM ANOVA followed by Sidak's multiple comparisons
342 test).

343 (B) Changes in BW from the initial BW at d0 (GFP mice, n = 11; TeLC mice, n = 10; ****P <
344 0.0001, unpaired t-test).

345 (C) BW was stable during the 72-h test (GFP mice: n = 10, p = 0.2969 (n.s.), Wilcoxon matched-
346 pairs signed rank test; TeLC mice: n = 8, p = 0.1319 (n.s.), paired t-test).

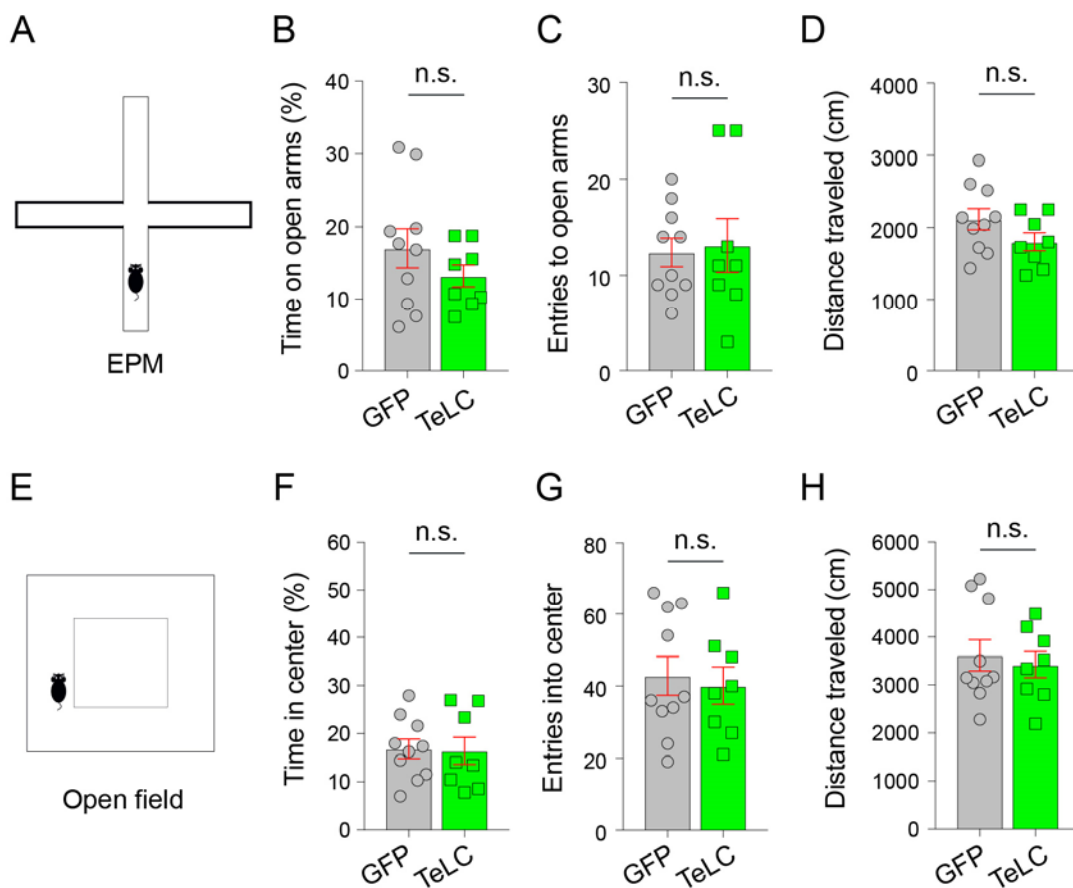
347 (D) Fraction of time spent without moving (inactivity) (GFP mice, n = 10; TeLC mice, n = 8;
348 F_(1,16) = 6.172, p = 0.0244; *p < 0.05, n.s., p > 0.05; two-way RM ANOVA followed by Sidak's
349 multiple comparisons test).

350 (E) Oxygen consumption (VO₂) during light and dark cycles (GFP mice, n = 10; TeLC mice, n =
351 8; F_(1,16) = 5.604, p = 0.0309; *p < 0.05, n.s., p > 0.05; two-way RM ANOVA followed by
352 Sidak's multiple comparisons test).

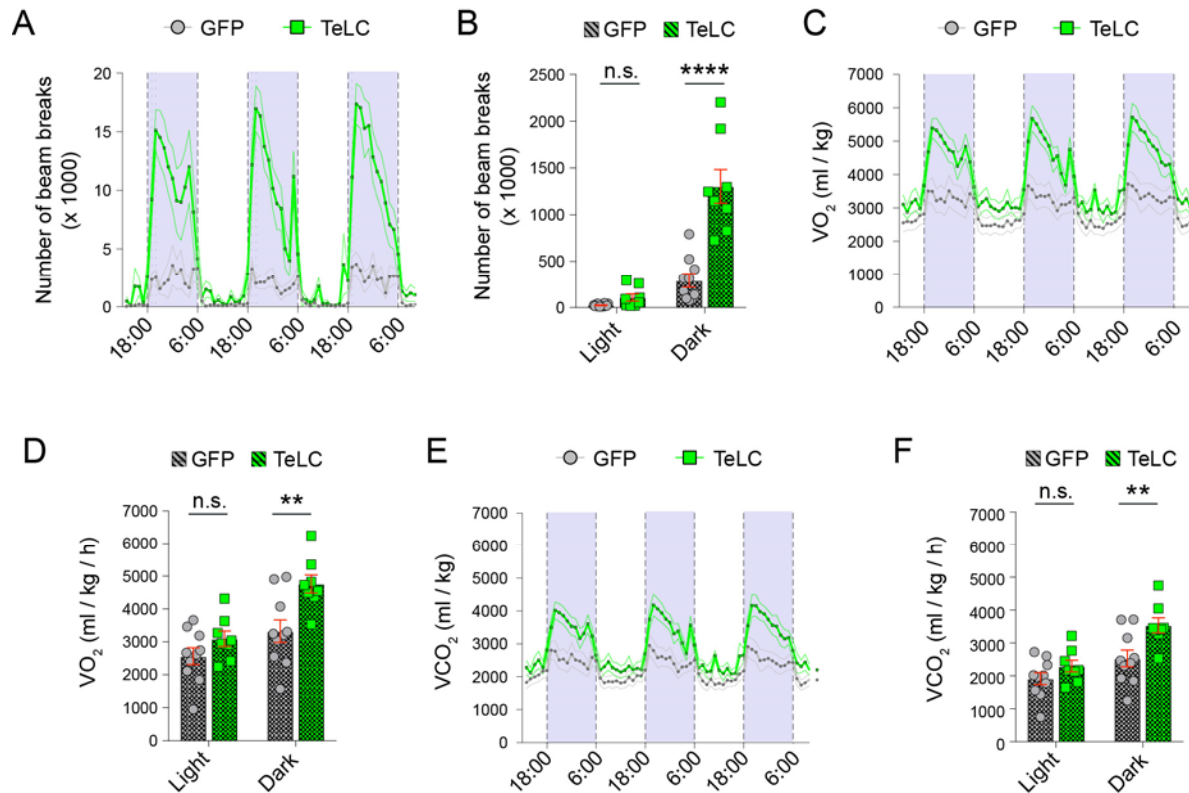
353 (F) The volume of carbon dioxide production (VCO₂) by GFP (n = 10) and TeLC mice (n = 8)
354 over the 72-h period. Data are plotted in 1-h intervals. White and purple represent light (6:00-
355 18:00) and dark cycles (18:00-6:00), respectively (F_(70,1120) = 1.508, p = 0.0053, two-way RM
356 ANOVA).

357 (G) Carbon dioxide production (VCO₂) during light and dark cycles (GFP mice, n = 10; TeLC
358 mice, n = 8; F_(1,16) = 5.603, p = 0.0309; *p < 0.05, n.s., p > 0.05; two-way RM ANOVA
359 followed by Sidak's multiple comparisons test).

360



361
362 **Figure S6. Characterization of Anxiety-Related Behaviors, Related to Figure 5**
363 (A) A schematic of the elevated plus maze (EPM) test.
364 (B) Time spent on the open arms (GFP mice, n = 10; TeLC mice, n = 8; n.s., p = 0.2664,
365 unpaired t-test).
366 (C) Entries to the open arms (n.s., p = 0.8111, unpaired t-test).
367 (D) Distance travelled (n.s., p = 0.1345, unpaired t-test).
368 (E) A schematic of the open field test.
369 (F) Time spent in the center (n.s., p = 0.9068, unpaired t-test).
370 (G) Entries into the center (n.s., p = 0.7295, unpaired t-test).
371 (H) Distance travelled (n.s., p = 0.6640, unpaired t-test).



372
 373 **Figure S7. Inhibition of IPAC^{Nts} neurons in mice fed with HFD, Related to Figure 6**
 374 (A) Locomotor activity of the GFP (n = 10) and TeLC mice (n = 8) over 72 h. Data are plotted in
 375 1-h intervals. White and purple represent light (6:00-18:00) and dark cycles (18:00-6:00),
 376 respectively ($F_{(71, 1136)} = 11.46$, $p < 0.0001$, two-way RM ANOVA).
 377 (B) Average locomotor activity of the mice in (A) during light and dark cycles ($F_{(1, 16)} = 27.12$, p
 378 < 0.0001 ; **** $p < 0.0001$, n.s., $p > 0.05$, two-way RM ANOVA followed by Sidak's multiple
 379 comparisons test).
 380 (C) The volume of oxygen consumed (VO₂) by GFP (n = 10) and TeLC mice (n = 8) over 72 h
 381 ($F_{(71, 1136)} = 7.204$, $p < 0.0001$, two-way RM ANOVA).
 382 (D) Oxygen consumption (VO₂) during light and dark cycles (GFP mice, n = 10; TeLC mice, n =
 383 8; $F_{(1, 16)} = 6.05$, $p = 0.0257$; ** $p < 0.01$, n.s., $p > 0.05$; two-way RM ANOVA followed by
 384 Sidak's multiple comparisons test).
 385 (E) The volume of carbon dioxide production (VCO₂) by GFP (n = 10) and TeLC mice (n = 8)
 386 over the 72-h period ($F_{(1, 16)} = 5.738$, $p < 0.0001$, two-way RM ANOVA).
 387 (F) Carbon dioxide production (VCO₂) during light and dark cycles (GFP mice, n = 10; TeLC
 388 mice, n = 8; $F_{(1, 16)} = 5.276$; $p = 0.0355$; ** $p < 0.01$, n.s., $p > 0.05$; two-way RM ANOVA
 389 followed by Sidak's multiple comparisons test).