# *Endocrine fine-tuning of daily locomotor activity patterns under non-starving conditions in Drosophila*

**Dennis Pauls[1,2 *], Mareike Selcho[1,2], Johanna Räderscheidt[1], Kelechi M. Amatobi[3], Agnes Fekete[3], Markus Krischke[3], Christiane Hermann-Luibl[1], Ayten Gizem Ünal[1], Nadine Ehmann[2], Pavel M. Itskov[4,5], Charlotte Helfrich-Förster[1], Ronald P. Kühnlein[6,7], Martin J. Mueller[3] and Christian Wegener[1 *]**

[1]Neurobiology and Genetics, Theodor-Boveri-Institute, Biocenter, University of Würzburg, D-97074 Würzburg, Germany

[2]Department of Animal Physiology, Institute of Biology, Leipzig University, Leipzig, Germany

[3]Pharmaceutical Biology, Julius-von-Sachs-Institute, Biocenter, University of Würzburg, D-97082 Würzburg, Germany

[4]Easy Behavior, Lisboa Portugal

[5]Department of Neuroscience, University of Uppsala, Sweden

[6]Institute of Molecular Biosciences, University of Graz, A-8010 Graz, Austria

[7]BioTechMed-Graz, A-8010 Graz, Austria, Field of Excellence BioHealth - University of Graz, Graz, Austria

***Correspondence**:

Dennis Pauls, Department of Animal Physiology, Institute of Biology, Faculty of Life Sciences, Leipzig University, Talstraße 33, 04103 Leipzig, Germany

E-mail: dennis.pauls@uni-leipzig.de

Christian Wegener, Neurobiology and Genetics, Theodor-Boveri-Institute, Biocenter, University of Würzburg, Am Hubland, 97074 Würzburg, Germany

E-mail: christian.wegener@biozentrum.uni-wuerzburg.de

Keywords: Octopamine, AKH, *Drosophila*, locomotor activity, starvation, fat body

## 1. Abstract

Animals need to balance competitive behaviours to maintain internal homeostasis. The underlying mechanisms are complex, but typically involve neuroendocrine signalling. Using *Drosophila*, we systematically manipulated signalling between energy-mobilising endocrine cells producing adipokinetic hormone (AKH), octopaminergic neurons and the energy-storing fat body to assess whether this neuroendocrine axis involved in starvation-induced hyperactivity also balances activity levels under *ad libitum* access to food.

Our results suggest that AKH signals via two divergent pathways that are mutually competitive in terms of activity and rest. AKH increases activity via the octopaminergic system during the day, while it prevents high activity levels during the night by signalling to the fat body. This regulation involves feedback signalling from octopaminergic neurons to AKH-producing cells (APCs). APCs are known to integrate a multitude of metabolic and endocrine signals. Our results add a new facet to the versatile regulatory functions of APCs by showing that their output contributes to shape the daily activity pattern under *ad libitum* access to food.

## 2. Introduction:

Foraging behaviour is fundamental to fitness and survival of all animals. When food is scarce, starving animals explore their environment in order to locate food sources. Depending on the amount of available energy and time of the day, foraging can suppress other essential behaviours like sleep. Upon food restriction, rodents and other vertebrate species increase their activity which can be interpreted as foraging [1,2]. In humans, anorexia nervosa is associated with sometimes even excessive hyperactivity, likely triggered by a decreased titre of the peptide hormone leptin [1,3]. Also in the fruit fly, *Drosophila melanogaster*, food deprivation induces locomotor hyperactivity (food search) [4,5], which is triggered by a signalling pathway comprising the peptide hormone adipokinetic hormone (AKH, an analogue to vertebrate glucagon) [4,6–8], and the biogenic amine octopamine (OA) [5,9]. AKH is produced and released by endocrine cells in the glandular part of the corpora cardiaca (CCA; a homologue of the vertebrate anterior pituitary gland and an analogue of mammalian pancreatic alpha cells) [10–12] and is required to maintain haemolymph sugar and lipid levels [4,7,8,11]. Upon starvation, carbohydrate levels in the haemolymph decrease, which is indirectly

sensed by the AKH-producing cells (APCs) through ATP-sensitive potassium channels and AMP-activated protein kinase (AMPK) [6,13]. Similar to glucagon, AKH acts on energy stores in the fat body by mobilising carbohydrates in form of the haemolymph sugar trehalose (hypertrehalosaemic effect), and by mobilising lipid depots (adipokinetic effect) [8,14–16].

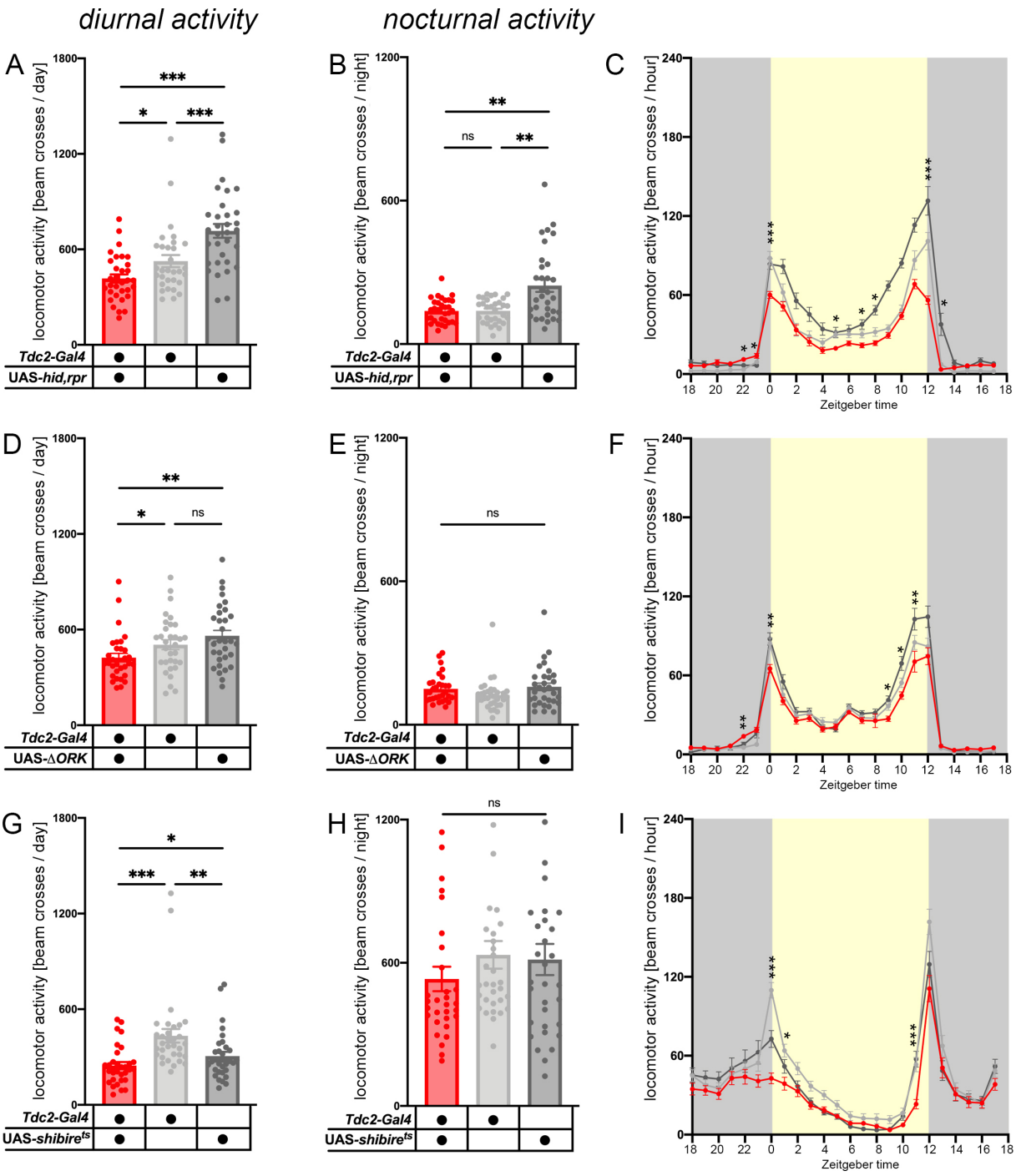
OA, an analogue to vertebrate noradrenaline, is considered to act as an insect stress or arousal hormone [17]. OA is produced by octopaminergic neurons (OANs) whose cells bodies are located mostly along the midline in the brain and ventral nerve cord [18]. Among other functions, OA exerts myotropic effects on skeletal muscles [19] and mediates the adaptation of muscle metabolism to the physiological demands of locomotion [20–23].

Both AKH and OA are crucial for starvation-induced hyperactivity, as this behaviour is absent in flies with impaired AKH or OA signalling [4,5,7–9]. AKH activates a subset of OANs which are required for starvation-induced hyperactivity [5,9]. These neurons express receptors for AKH and *Drosophila* insulin-like peptides (DILPs). DILPs act antagonistically to AKH, and suppress starvation-induced hyperactivity via the OANs [9]. Yu and colleagues (2016) showed that the AKH-dependent increase in locomotor activity upon starvation represents a direct neuromodulatory and behavioural effect of AKH and is not an indirect consequence of altered energy metabolism [9].

Remarkably, studies in various insect species suggest that AKH and also OA play a role in the regulation of locomotor activity when the insects have free access to food (reviewed in [24]). For example, the circulating haemolymph titres of AKH correlate positively with daily rhythms in walking activity in the linden bug *Pyrrhocoris apterus* [25,26]. In *Pyrrhocoris* [27,28], crickets [29] and the cockroach *Periplaneta americana* [30,31], injection into the haemolymph or topical application of AKH induces a strong increase in locomotor activity in *ad libitum* fed animals, independent of the time of day [28,30]. Also in *Drosophila*, genetic overexpression of AKH enhances locomotor activity when food is freely available [32]. Unlike for starvation-induced hyperactivity, however, it is unclear whether and how AKH:OAN signalling contributes to shape daily activity under *ad libitum* feeding conditions, or whether increased AKH signalling is a consequence of a locomotor-induced increase in energy demands. Cockroach AKHs are known to increase the spontaneous neuronal activity of octopaminergic dorsal unpaired median neurons in the central nervous system, which express a specific G-

102 protein coupled receptor (AKHR) [30,31]. Yet, the functional significance of AKHRs on
103 OANs under *ad libitum* feeding conditions has not been rigorously tested.
104
105 We here used the genetically tractable fruit fly to experimentally address the role of
106 AKH:OAN signalling for locomotor activity under non-starved conditions. We find that
107 even under these conditions AKH signalling to OANs participates in shaping diel
108 activity levels. Surprisingly, AKH-induced energy mobilisation appears to antagonize
109 the AKH:OAN-dependent promotion of activity by preventing abnormally high nocturnal
110 activity levels during an initial phase of starvation. In turn, OA modulates the activity of
111 APCs via a feedback signalling comprising OAMBII receptors. Taken together, our
112 results indicate that AKH:OAN and AKH:fat body signalling in *Drosophila* helps to
113 balance and adjust locomotor activity in the ambivalence between foraging and rest.
114
115 **3. Results**:
116 To validate our experimental conditions, we first confirmed that AKH is required for
117 starvation-induced hyperactivity and affects starvation resistance (Fig.S1-S2). As
118 previously shown [4,7,8], starved control flies showed a significant starvation-induced
119 hyperactivity compared to fed controls (Fig.S1-S2). This starvation-induced
120 hyperactivity was impaired in flies lacking APCs due to the targeted expression of pre-
121 apoptotic genes *head involution defective (hid*) and *reaper (rpr)* [33,34] using *Akh-Gal4*
122 [4]. Increased lipid stores in fat body cells contribute to the higher starvation resistance
123 observed in flies lacking AKH signalling [8,15,16]. In line with this, *Akh>hid,rpr* flies
124 showed higher starvation resistance during activity recording, as on day 3 more than
125 80% of flies were still alive, compared to less than 15% of starved control flies (Fig.S3).
126
127 **<u>Octopaminergic neurons modulate diurnal activity levels in fed flies</u>**
128 In addition to AKH, OA signalling is essential for starvation-induced hyperactivity [5].
129 We first tested whether the OA neuronal system is also involved in modulating
130 locomotor activity in flies that have permanent access to food. To manipulate OANs,
131 we used the *Tdc2-Gal4* line. Tdc2 (Tyrosine decarboxylase 2) is the enzyme necessary
132 for the rate-limiting decarboxylation of tyrosine to tyramine (TA) which then is further
133 hydroxylated by tyramine beta-hydroxylase (TßH) to OA [35]. *Tdc2-Gal4* specifically
134 marks OANs and tyraminergic neurons [36–39]. We first tested whether *Tdc2-Gal4*-
135 positive neurons (Tdc2Ns) are involved in the modulation of locomotor activity in fed

flies and ablated Tdc2Ns by the expression of UAS-*hid,rpr*. As expected, *Tdc2>hid,rpr* flies showed significantly reduced locomotor activity during the day, while activity levels during the night were unaffected (Fig.1A-C). We next blocked neuronal transmission of Tdc2Ns using UAS-*ΔOrk* [40] and UAS-*shibire*$^{ts}$ [41] to exclude any side effects on activity due to cell ablation. In line with the previous results, both electrical and chemical silencing of synaptic transmission reduced locomotor activity levels specifically during the day (Fig.1D-I).



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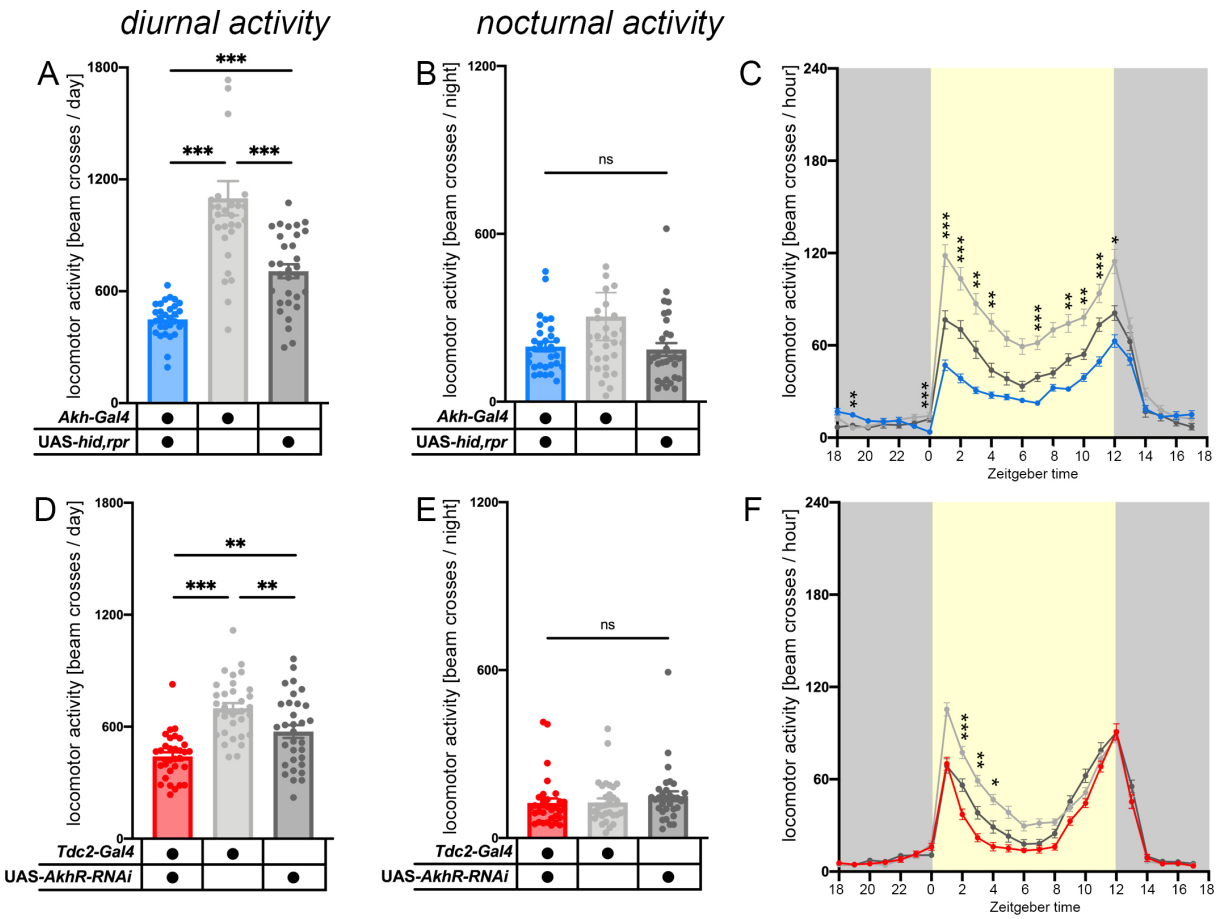
146 ***Figure 1: Tdc2-Gal4-positive neurons modulate activity during the day under non-starvation***
147 ***conditions**. **(A-C)** Activity levels are reduced during the day in Tdc2>hid,rpr flies compared to genetic*
148 *controls (p<0.05 to Gal4/+ and p<0.001 to UAS/+). **(D-F)** Neuronal silencing of Tdc2Ns (Tdc2-Gal4-*
149 *positive neurons) revealed a similar effect with reduced activity of experimental flies during the day. **(G-***
150 ***I)** Conditional thermogenetic silencing of Tdc2Ns using UAS-shi$^{ts1}$ at restrictive temperature also*
151 *reduced activity levels during the day (p<0.001 to Gal4/+ and p<0.01 to UAS/+). n=26-32, significance*
152 *levels: p>0.05 ns, p<0.05 *, p<0.01 **, p<0.001 ***. Error bars indicate SEM.*

153 Our results are consistent with an earlier study showing that *Tdc2*$^{R054}$ and *TβH*$^{nm18}$
154 mutants increase sleep mainly during the day [42,43], but partially differ from results
155 published by Crocker and Sehgal: here Kir2.1-mediated electrical silencing of *Tdc2Ns*
156 consistently decreased locomotor activity, but also increased night sleep [42].
157 Nonetheless, the results show that OA/TA signalling promotes locomotor activity and
158 hence wakefulness during the night [42,44] or under starvation [5,9], and in addition
159 shapes the general locomotor activity level during the day when flies have permanent
160 access to food.

161 **<u>AKH:OAN signalling is important for the modulation of day-time activity</u>**

162 Our results and data from *Tdc2* mutants [42] showed that impaired OA signalling
163 reduced locomotor activity specifically during the day. We thus hypothesized that OANs
164 signal primarily during the day but not during the night to modulate locomotor activity
165 under *ad libitum* feeding conditions. This is consistent with the view of OA (and TA)
166 being a stress hormone that is released during physical activity [17,23]. AKH positively
167 modulates OANs in cockroaches and fruit flies [9,30], which predicts that ablation of
168 APCs and down-regulation of the AKH receptor (AKHR) specifically in Tdc2Ns will
169 result in reduced locomotor activity during the day (when OA signalling is effective),
170 but unaltered locomotor activity during the night (when OA signalling is not present
171 under *ad libitum* feeding conditions). Indeed, similar to the ablation of Tdc2Ns (Fig.1A-
172 C), *Akh>hid,rpr* and *Tdc2>AkhR-RNAi* flies showed significant reduced locomotor
173 activity during the day, while locomotor activity was unaffected during the night (Fig.2A-
174 F). In order to demonstrate that the effect of the AKHR knockdown in Tdc2Ns was
175 specific to day-time locomotor activity and not due to a general impairment of motor
176 capability, we performed a startle-induced negative geotaxis (SING) climbing assay
177 [45]. The performance of *Tdc2>AkhR-RNAi* flies was indistinguishable from genetic
178 controls in this climbing assay, suggesting that motor capability was not generally

179 affected by our genetic manipulation (Fig.S4). Interestingly, while the ablation of APCs
180 led to a reduction in activity throughout day-time (Fig.2A-C), the average activity profile
181 in *Tdc2>AkhR-RNAi* flies suggests that the reduction in activity is mainly based on a
182 reduced activity immediately after the morning activity bout, while locomotor activity
183 around the evening peak was indistinguishable from genetic controls (Fig.2F). This
184 day-time specific reduction of locomotor activity was partially rescued when we
185 restricted the expression of *AkhR-RNAi* to Tdc2Ns in the brain, using *tshGal80* (Fig.S5)
186 [46]. Expression of GFP in *Tdc2,tshGal80*>10x*myr::GFP* flies revealed a strong
187 reduction of labelled Tdc2Ns in the ventral nerve cord (VNC). From ~39 *Tdc2-Gal4*-
188 positive VNC neurons [47] ~7 cells (7.4 ± 2.07, n=5) escaped *tshGal80*-dependent
189 suppression (Fig.S6), while the number of Tdc2Ns in the brain was not affected. These
190 results suggest that Tdc2Ns in the VNC are involved in the AKH:OAN signalling. To
191 confirm the expression of AKHR in cells in the VNC we performed RT-PCR. We found
192 AKHR to be expressed in isolated adult VNCs, as well as in adult brains and the larval
193 fat body (Fig.S7).
194




195
196 ***Figure 2: AKH:OAN signalling is important for the modulation of day-time activity. (A-C)***
197 *Akh>hid,rpr flies showed significantly reduced activity (all p<0.001) specifically during the day.* ***(D-F)***

198 *Knockdown of AKHR in Tdc2Ns (Tdc2-Gal4-positive neurons) specifically affected locomotor activity*
199 *during the day (p<0.01 to Gal4/+ and p<0.001 to UAS/+). n=26-32, significance levels: p>0.05 ns, p<0.05*
200 **, p<0.01 **, p<0.001 ***. Error bars indicate SEM.*
201
202 The results so far are consistent with data from the cockroach *Periplaneta americana*,
203 where AKHR is expressed in thoracic and abdominal octopaminergic dorsal unpaired
204 median neurons and signals both via $G_q$ and $G_s$ to activate cAMP and $Ca^{2+}$-dependent
205 second messenger pathways [30,31]. To confirm AKH signalling to OANs located in
206 the VNC, we bath-applied AKH to isolated VNCs (detached from the brain) and
207 recorded changes in intracellular cAMP or free $Ca^{2+}$ levels, respectively, in Tdc2Ns
208 expressing the genetically encoded Epac1-camps50A [48] or GCaMP6m [49] sensors,
209 focusing on the mesothoracic cluster.
210 All mesothoracic Tdc2Ns responded to the synthetic adenylate cyclase agonist
211 NKH477 or the nicotinic receptor agonist carbamylcholine with robust increase in
212 cAMP/free $Ca^{2+}$ levels showing that neurons were functional in our preparations. Upon
213 bath-application of $10^{-5}$M AKH, Tdc2Ns in the mesothoracic cluster responded with a
214 significant cAMP increase, while application of haemolymph-like HL3.1 saline solution
215 as vehicle control had no effect (Fig.S8) [50]. In contrast, free intracellular $Ca^{2+}$ levels
216 did not significantly change in Tdc2Ns in the VNC upon AKH bath application
217 compared to HL3.1 vehicle control (Fig.S10). These results suggest that activated
218 AKHR modulate OAN activity via cAMP-dependent pathways. Of note, the observed
219 AKH-induced increase in cAMP levels occurred with a time lag of at least 15 minutes
220 after bath application of the peptide (Fig.S8). We next overexpressed AKHR in Tdc2Ns
221 and repeated cAMP imaging in the mesothoracic cluster. Overexpression of AKHR did
222 not significantly increase cAMP beyond the level observed in controls (Fig.S9).
223 Although the response to exogenous AKH occurred significantly faster, responses
224 were still delayed with a mean lag time of around 10 minutes (Fig.S9). This suggests
225 that AKH induces slow cAMP elevations in OANs even if AKHR is overexpressed in
226 these neurons, potentially in a dose-dependent manner.
227

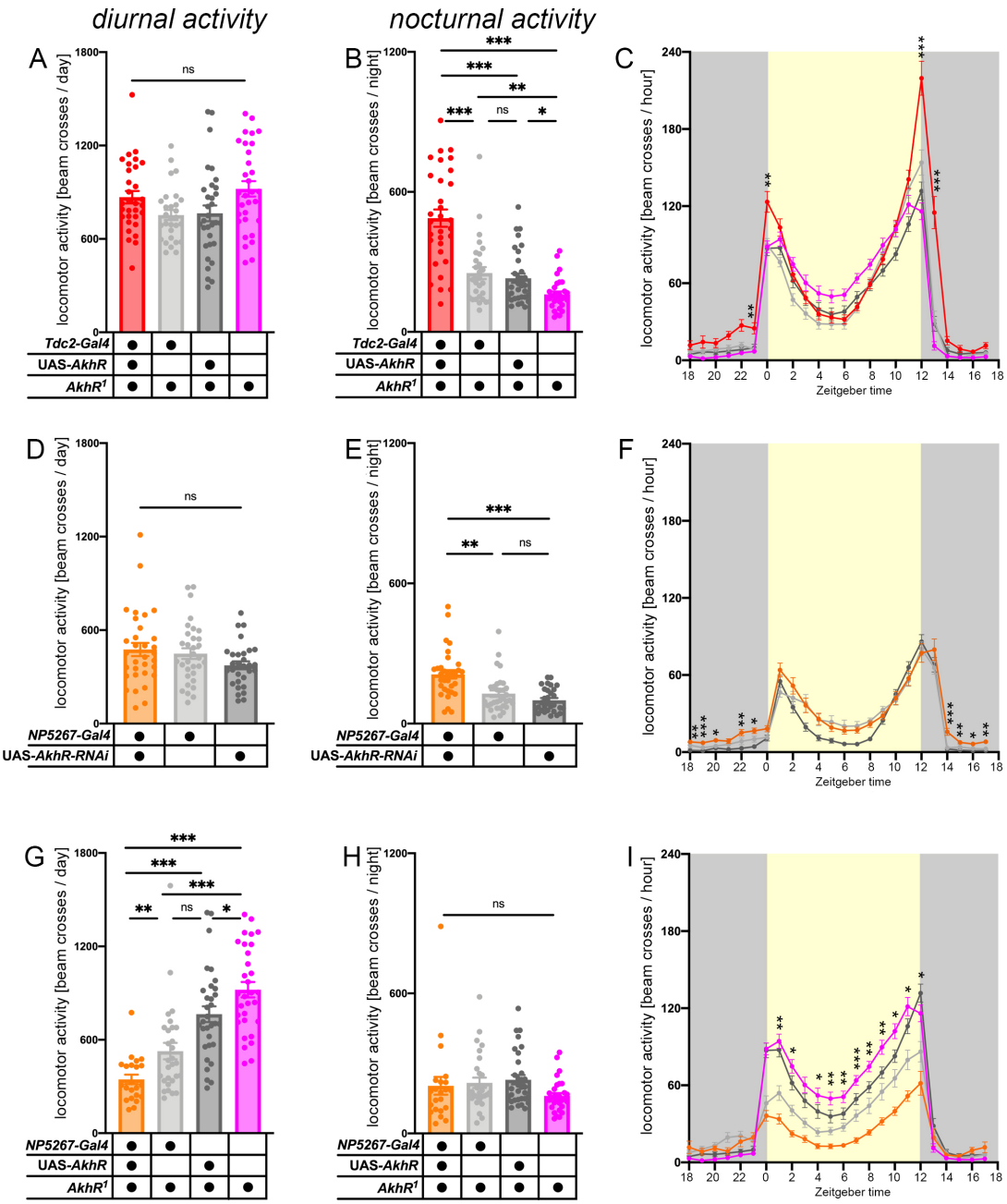



***Figure 3: AKH balances day and night locomotor activity via different signalling routes. (A-C)*** *Genetic rescue of AKHR in Tdc2Ns (Tdc2-Gal4-positive neurons) rescued locomotor activity during the day (all p>0.05); however, AkhR¹,Tdc2>AkhR,AkhR¹ flies showed significantly higher activity during morning and evening activity bouts (all p<0.001 at ZT0 and ZT12). In addition, experimental flies showed significantly increased activity levels during the night (all p<0.001).* ***(D-F)*** *Flies with a fat body-specific knockdown of AKHR showed increased levels of activity during the night (p<0.01 to Gal4/+ and p<0.001 to UAS/+.* ***(G-I)*** *Specific rescue of AKHR in the fat body elicited the opposite effect seen to the rescue in OANs (see A-C). Expression of AKHR specifically in the fat body rescued activity levels during the night; in contrast, locomotor activity was significantly reduced during the day (all p<0.01). Note that the experiments in A-C and G-I were conducted at the same time. The controls shown are identical in the*

240 *two experiments. n=13-32 for behavioural data, significance levels: p>0.05 ns, p<0.05 *, p<0.01 **,*
241 *p<0.001 ***. Error bars indicate SEM.*
242
243 To provide further genetic evidence for the modulatory role of AKHR in OANs, we
244 rescued AKHR expression specifically in Tdc2Ns in an *AKHR*$^{1}$ mutant background and
245 monitored locomotor activity (Fig.3A-C) [15]. Rescue flies showed significantly
246 increased locomotor activity during the usual activity bouts in the morning and evening
247 and during the early and late night, compared to AKHR-deficient controls (Fig. 3A-C).
248 No difference in locomotor activity was apparent during the midday siesta, which is
249 reflected by the lack of significant day-time activity differences compared to genetic
250 controls.
251
252 Taken together, the results so far suggest that AKH modulates OANs in the VNC (and
253 potentially in the brain) [5,9] to increase locomotor activity during the day when flies
254 have permanent access to food. Yet, the AKHR is most strongly expressed by the fat
255 body [15,24] where it mediates the hypertrehalosaemic effect of AKH and mobilizes
256 stored energy in form of the haemolymph sugar trehalose (Fig.7). To test whether
257 AKH:fat body signalling affects locomotor activity under *ad libitum* feeding conditions,
258 we first knocked down AKHR expression specifically in the fat body using *NP5267-*
259 *Gal4*. *NP5267>AkhR-RNAi flies* showed significantly increased locomotor activity
260 during the night (Fig.3D-F). Further, specific rescue of AKHR in the fat body in a *AkhR*$^{1}$-
261 mutant background significantly reduced locomotor activity during morning and
262 evening activity bouts as well as during midday siesta (phenocopying the impairment
263 of AKH:OAN signalling; see Fig.1 and Fig.2), while locomotor activity appeared to be
264 normal during the night (Fig.3G-I).
265
266 **<u>OAN signalling modulates AKH cells in the corpora cardiaca</u>**
267 Our results so far support a role for AKH:OAN signalling under *ad libitum* feeding
268 conditions. We next wondered whether OANs in turn provide feedback signals to the
269 APCs in the corpora cardiaca (CCA). Thus, we traced the projections of Tdc2-positive
270 cells in whole body sections of flies using a Tdc2 antibody (Fig.4A-B). Besides
271 arborizations in the brain and VNC, we found Tdc2-positive fibres projecting to the
272 CCA-hypocerebral ganglion complex (CCA-HG complex, also known as stomodaeal
273 ganglion; Fig.4B, inlet) presumably via the stomodaeal nerve (Fig.4B, asterisk). Thus,
274 we assumed that OA may be released in vicinity of the AKH cells. Among the different

275 OA receptors, OAMBII has been responsible for most OA actions [17], and increases
276 intracellular $Ca^{2+}$ levels in heterologous expression systems as well as in *Drosophila*
277 neurons [51–53]. Using primers targeting different exons of the OAMBII gene [54], we
278 confirmed the expression of the OAMB-K3 isoform (Fig.4D, OAMB-K3 contains exon
279 7 but not 8) in the CCA-HG complex, while signals for the OAMB-AS isoform (OAMB-
280 AS contains exon 8 instead of 7) were not detected (Fig.4C). To test whether APCs
281 express functional OA receptors, we expressed the genetically encoded $Ca^{2+}$ sensor
282 GCaMP6m in APCs and imaged the effect of bath-applied OA. For these experiments,
283 we used the whole larval central nervous system with attached ring glands as it was
284 not feasible to dissect intact adult CCA-HG-brain preparations. Since at low trehalose
285 levels the $Ca^{2+}$ concentration is generally high in APCs, we adopted a procedure
286 developed by Kim and Rulifson (2004) to image $Ca^{2+}$ changes in these cells:
287 preparations were first pre-incubated in 80mM trehalose solved in HL3.1 which
288 decreased free intracellular $Ca^{2+}$ concentration to basal level (Fig.4E: arrow No.1 at
289 100s). Subsequent application of 1mM OA + 80mM trehalose (Fig.4E: arrow No.2 at
290 300s) significantly increased intracellular $Ca^{2+}$ levels indicated by an increase of
291 fluorescence intensity (Fig.4E, blue line). In contrast, no changes in $Ca^{2+}$ levels were
292 visible after bath application of the control solution (Fig.4E, grey line), suggesting that
293 APCs respond with increased intracellular $Ca^{2+}$ levels to OA (Fig.4F).
294 As our results suggest that OANs signal to APCs via the OAMBII receptor, we next
295 performed a knockdown of OAMBII in these cells (*Akh>OAMBII-RNAi*). The
296 knockdown resulted in significantly increased nocturnal activity (Fig.4G-I), suggesting
297 that OANs modulate AKH release. Modulation of APC activity by OA is likely a common
298 feature among insects, as OA was found to modulate AKH secretion in the locust
299 [55,56]. In locusts, the CCA is not innervated by OANs, and OA likely acts in a hormonal
300 fashion [57]. In *Drosophila*, however, *Tdc2*-positive fibres innervate the CCA-HG
301 complex (Fig. 4A,B) and OA may act in a paracrine fashion.
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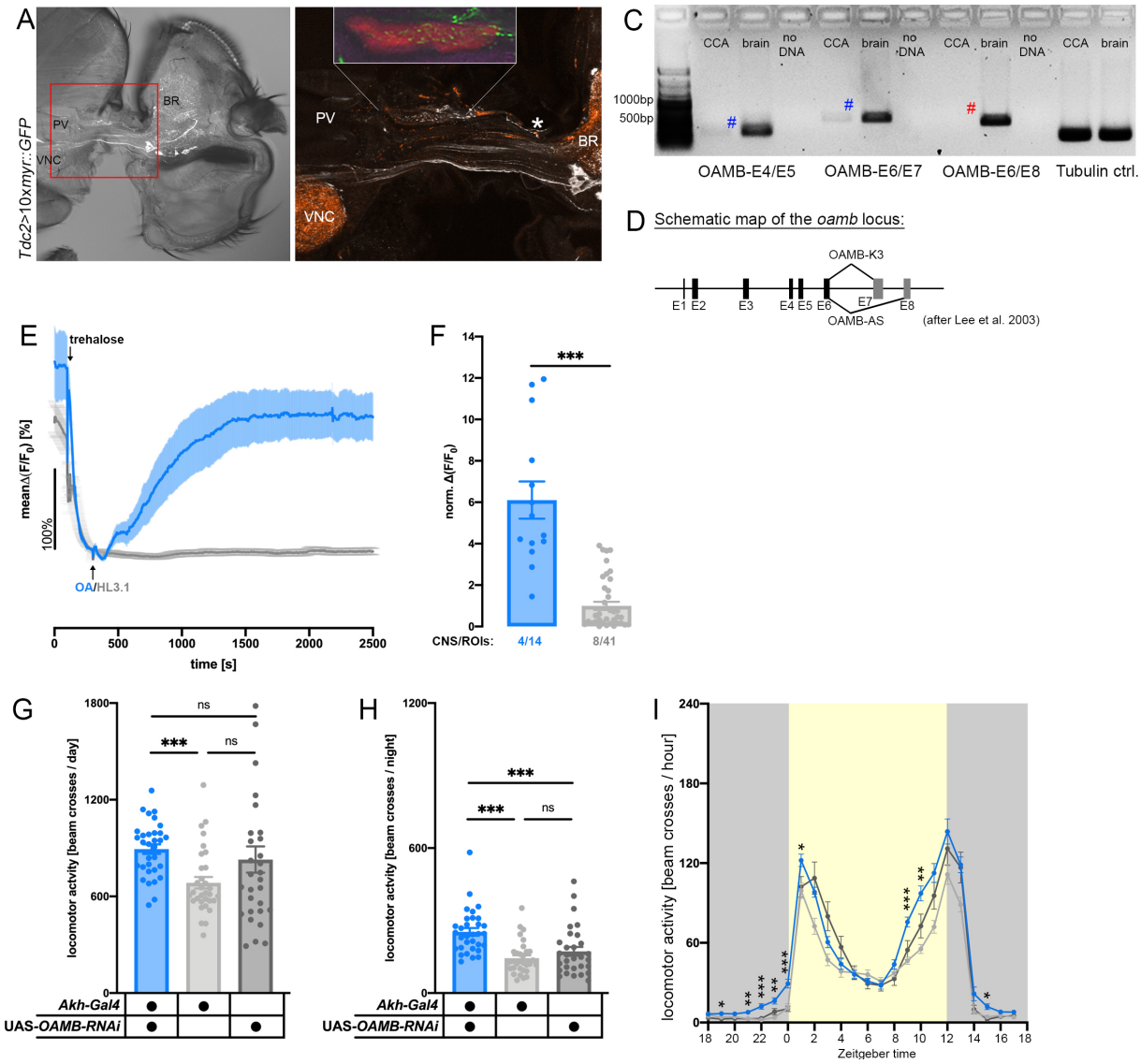
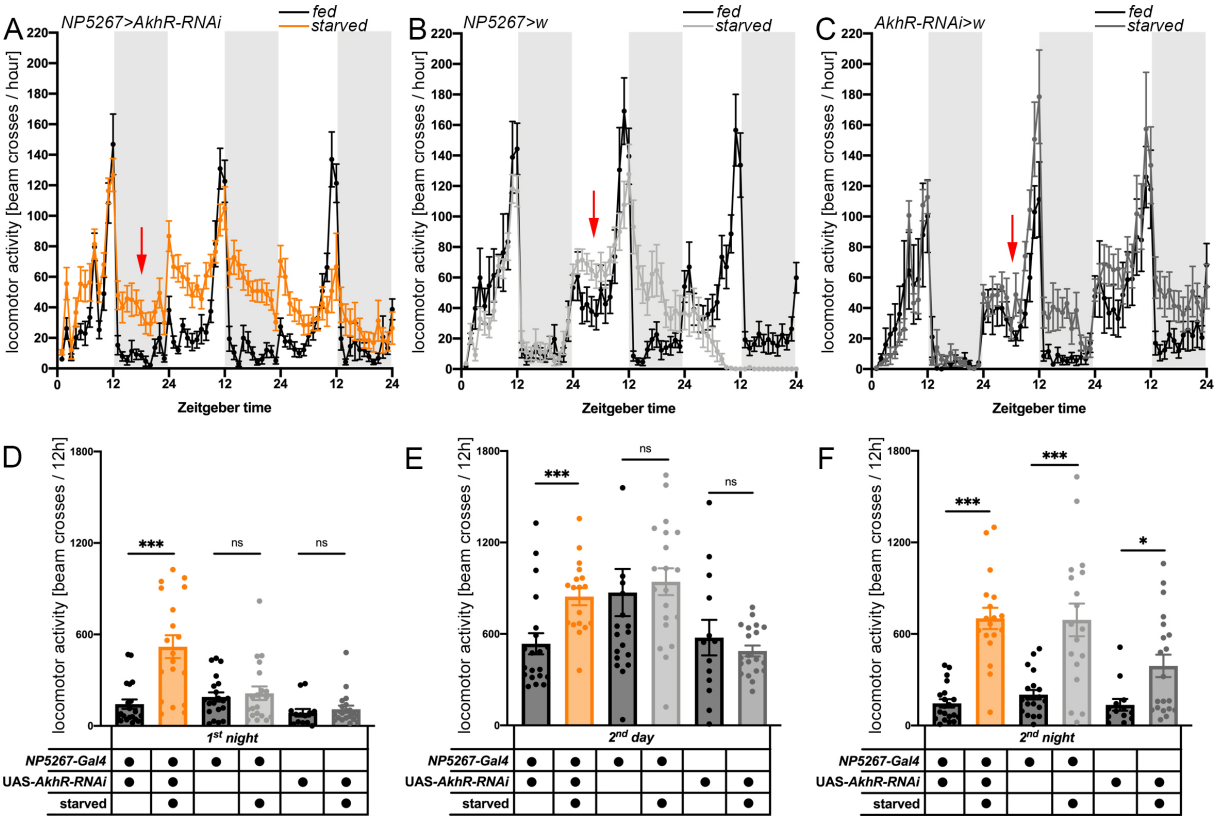



***Figure 4: AKH release is modulated by OAN feedback signalling. (A-B)*** *Whole body sections of flies displayed arborizations of Tdc2-positive neurons (white) in the CCA-HG complex (inlet in (B)). (B) shows a magnification of the red squared region in (A). The CCA-HG complex is highlighted in red in the inlet. White: anti-Tdc2; orange: anti-Synapsin.* ***(C-D)*** *RT-PCR analysis confirmed that the OA receptor OAMBII-K3 isoform is expressed in the CCA-HG complex (blue #), but not the OAMBII-AS isoform (red #).* ***(E-F)*** *Average changes in $Ca^{2+}$ levels of larval AKH cells to bath applied OA and HL3.1, respectively (Akh>GCaMP6m). AKH cells were pre-incubated with 80mM trehalose to reduce intracellular $Ca^{2+}$ levels to baseline (arrow at 100s). AKH cells responded to bath applied OA (arrow at 300s) with a strong increase in intracellular $Ca^{2+}$ levels (blue line; (F)); in contrast, no significant responses were obvious in response to the vehicle control (grey line).* ***(G-I)*** *Akh>OAMBII-RNAi flies showed significantly increased activity during the night (all $p<0.001$). n=29-32 for behavioural data, significance levels: $p>0.05$ ns, $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***. [CCA= corpora cardiaca, Ctrl= control, BR=brain, E=exon, HG=hypocerebral ganglion, OA=octopamine, PV=proventriculus, ROI=region of interest, VNC=ventral nerve cord]. Error bars indicate SEM.*

321 **AKH:fat body signalling prevents an increase of activity during the night**
322 Our results suggest that - opposite to the stimulatory locomotor effect of AKH:OAN
323 signalling - AKH:fat body signalling prevents an increase of activity with the most
324 pronounced effect during the night. Previous studies suggested that haemolymph
325 trehalose levels are at a trough at the end of the night and immediately after lights on
326 [58]. We therefore hypothesized that AKH modulates locomotor activity in a balanced
327 state-dependent manner. AKH:OAN signalling may promote activity during the day,
328 while AKH:fat body signalling stabilises rest during the night.
329
330 To test this hypothesis, we monitored locomotor activity of *NP5267>AkhR-RNAi* for
331 three days, but this time under starved conditions. We assumed that flies lacking
332 AKH:fat body signalling cannot compensate for the lack of food with energy
333 mobilization from the fat body and thus will show an earlier onset of nocturnal
334 starvation-induced hyperactivity than control flies. Flies were recorded from ZT0
335 (lights-on; ZT=Zeitgeber time) in tubes that contained agarose providing water to
336 prevent dehydration, but lacked sugar or other nutrients. All flies showed starvation-
337 induced hyperactivity during the experiment (Fig.5A-C). For *NP5267>AkhR-RNAi* flies,
338 however, this hyperactivity started earlier and became already obvious during the first
339 night around 12h after starvation onset (Fig.5A; red arrow). In contrast, control flies
340 became hyperactive not until the second day (Fig.5B-C; red arrows), with starvation-
341 induced hyperactivity fully developed first during the second night in both genetic
342 controls (Fig.5D-F). Thus, while *NP5267>AkhR-RNAi* flies were significantly
343 hyperactive from the first night on in response to starvation, endogenous AKH:fat body
344 signalling prevented undue high nocturnal activity levels in consequence of food
345 search in control flies during the first night, presumably by mobilizing energy from the
346 fat body into the haemolymph (Fig.5D-F).




347
348
349 ***Figure 5: Onset of starvation-induced hyperactivity. (A-F)*** *NP5267>AkhR-RNAi flies showed an*
350 *early onset of starvation-induced hyperactivity during the first night (after ~12h of starvation; red arrow).*
351 *In contrast, AKH:fat body signalling in control flies allowed a short-term adaptation under starvation and*
352 *prevented abnormally high night activity. Red arrows indicate first obvious appearance of starvation-*
353 *induced hyperactivity. n=13-32; significance levels: p>0.05 ns, p<0.05 *, p<0.01 **, p<0.001 ***. Error*
354 *bars indicate SEM.*
355

356 **<u>Impaired AKH-dependent carbohydrate mobilisation is compensated by dietary</u>**
357 **<u>uptake</u>**

358 So far, our results suggest that AKH signalling modulates locomotor activity not only
359 under starved conditions, but also when animals have free and permanent access to
360 food. The effect was most obvious during the night when feeding-related behaviours
361 are normally absent or strongly reduced [58]. We assumed that during these nocturnal
362 non-feeding phases, flies usually compensate the reduced dietary carbohydrate intake
363 via the hypertrehalosemic effect of AKH. However, flies deficient of AKH:fat body
364 signalling are unable to maintain high trehalose levels [4,7,11]. This may lead to
365 increased AKH release, which in turn will promote foraging activity via the integrated
366 increase of AKH:OAN-signalling (Fig.5A) especially towards the end of the night,
367 analogous to but less pronounced as starvation-induced hyperactivity. To find out

368 whether APCs of flies with impaired AKH signalling are more active during the night,
369 we first used calcium-dependent nuclear import of LexA (CaLexA) [59], an integrator
370 of long-term $Ca^{2+}$ activity. As expected, CaLexA-driven GFP expression increased
371 significantly upon 24 hours of starvation in *AKH>CaLexA* flies (Fig.S11B-C, F-G),
372 showing that the method is apt to monitor APC activity.
373 On food, we found a weak and statistically insignificant increase in CaLexA-dependent
374 GFP expression in *Akh>CaLexA* controls between end of the day (ZT10) and middle
375 (ZT18) or end (ZT22) of the night (Fig.S11E, F-G). In *Akh;amon-RNAi>CaLexA* flies
376 with reduced AKH signalling [11], we found a slight but insignificant increase in
377 CaLexA-driven GFP expression compared to controls (Fig. S11D, F-G). These results
378 suggest that APCs generally have a low activity on food, and speak against a
379 significantly increased need for energy mobilization in flies with impaired AKH
380 signalling under our experimental conditions. We therefore tested whether the loss of
381 AKH-dependent carbohydrate mobilization is compensated by dietary carbohydrate
382 uptake. First, we quantified haemolymph sugar levels during the light phase under
383 starved and *ad libitum* feeding conditions using Ultra performance liquid
384 chromatography - tandem mass spectrometry. Indeed, APC-ablated flies under fed
385 conditions showed similar haemolymph levels of sucrose, fructose, trehalose and
386 glucose, although trehalose and fructose levels were somewhat reduced (Fig.6A-D).
387 Under starved conditions, however, trehalose levels became depleted in APC-ablated
388 flies (Fig.6C), as previously shown [7]. Also, glucose levels were strongly though not
389 significantly reduced (Fig.6D). In contrast, fructose and sucrose levels did not
390 significantly differ compared to controls and were generally very low (Fig.6A-B), likely
391 because the haemolymph titres of theses sugars originate mostly from food uptake.
392 These results fully support our assumption that flies deficient in AKH signalling
393 compensate impaired carbohydrate mobilisation by food uptake, and explain the
394 similar activity levels of APCs between controls and AKH down-regulated flies as
395 determined with CaLexA.
396
397 Next, we quantified trehalose, glucose, and diacylglycerol levels every four hours
398 during the day under *ad libitum* feeding conditions. In line with the results in Fig.6C,
399 trehalose levels of *Akh>hid,rpr* flies were lower but not significantly reduced compared
400 to both controls (Fig.6E). Again, as expected from previous measurements (Fig.6D),
401 the glucose concentration were similar in both *Akh>hid,rpr* and control flies (Fig.6F).

402 Yet, the temporal pattern differed. *Akh>hid,rpr* flies showed constant low levels of
403 trehalose during the night, while control flies displayed increased levels of trehalose
404 during rest phases (the siesta and night) suggesting trehalose mobilization from the fat
405 body (Fig.6E). The glucose level stayed constant within a certain range in controls,
406 with a drop at the end of the night in *Akh>w* control flies (Fig.6F). In *Akh>hid,rpr* flies,
407 however, the glucose titre was low at ZT2 and steadily increased until ZT14 to then
408 drop at ZT18 and increase again to a high level at ZT22. This suggests feeding activity
409 in *Akh>hid,rpr* flies especially around ZT22, given that *Akh>hid,rpr* flies are impaired
410 in mobilizing carbohydrates. Interestingly, no obvious differences in haemolymph
411 diacylglycerol levels were detectable between the genotypes (Fig.6G). Strong
412 increases of diacylglycerol occurred at times when the glucose titre dropped even in
413 *Akh>hid,rpr* flies (Fig.6F), providing further evidence for an AKH-independent lipid
414 mobilisation pathway [15].
415
416 To directly address food uptake, we used an automated two-choice flyPAD assay [60]
417 and monitored feeding in individual flies on 5mM sucrose vs. 10% yeast (Fig.6H-J,
418 S12). Generally, flies consumed significantly more sucrose and yeast during the day
419 than during the night (Fig.S12). Though not significantly different, sucrose consumption
420 was by trend elevated in *Akh>hid,rpr* flies both during day and night. Analysis at a
421 higher temporal resolution revealed significantly increased sucrose feeding
422 in *Akh>hid,rpr* flies at the end of the night and after lights-on during morning activity
423 compared to controls (Fig.6H). A less pronounced increase in feeding was also visible
424 around the end of the day (coinciding with the start of evening activity), as well as
425 during the night though levels did not reach significance except for ZT 10-11 (Fig.6H).
426 Thus, genetic ablation of APCs led to an earlier onset of feeding activity with a slight
427 general increase of feeding throughout the night. In contrast, total yeast consumption
428 was not significantly affected in *Akh>hid,rpr* flies during the day and night (Fig.S12).
429 Unlike for sucrose feeding, a consistently and significantly elevated level of yeast
430 feeding in *Akh>hid,rpr* flies was also not visible at higher temporal resolution across
431 the day (Fig.6I). Likewise, total feeding was not affected by APC ablation (Fig.6J).
432 These results show that ablation of APCs exerts a specific effect on sucrose but not
433 yeast feeding under our conditions. The observed time course of sucrose feeding
434 in *Akh>hid,rpr* flies (Fig.6H) is compatible with their glucose haemolymph profile
435 (Fig.6F) which shows increasing concentrations during the day, and a drop in the

middle of the night when feeding activity was basically absent in *Akh>hid,rpr* flies (Fig.6H).

Taken together, our results propose that *Akh>hid,rpr* flies are not able to mobilize trehalose from the fat body and rescue haemolymph carbohydrate titres by increased food uptake at certain times of the day.

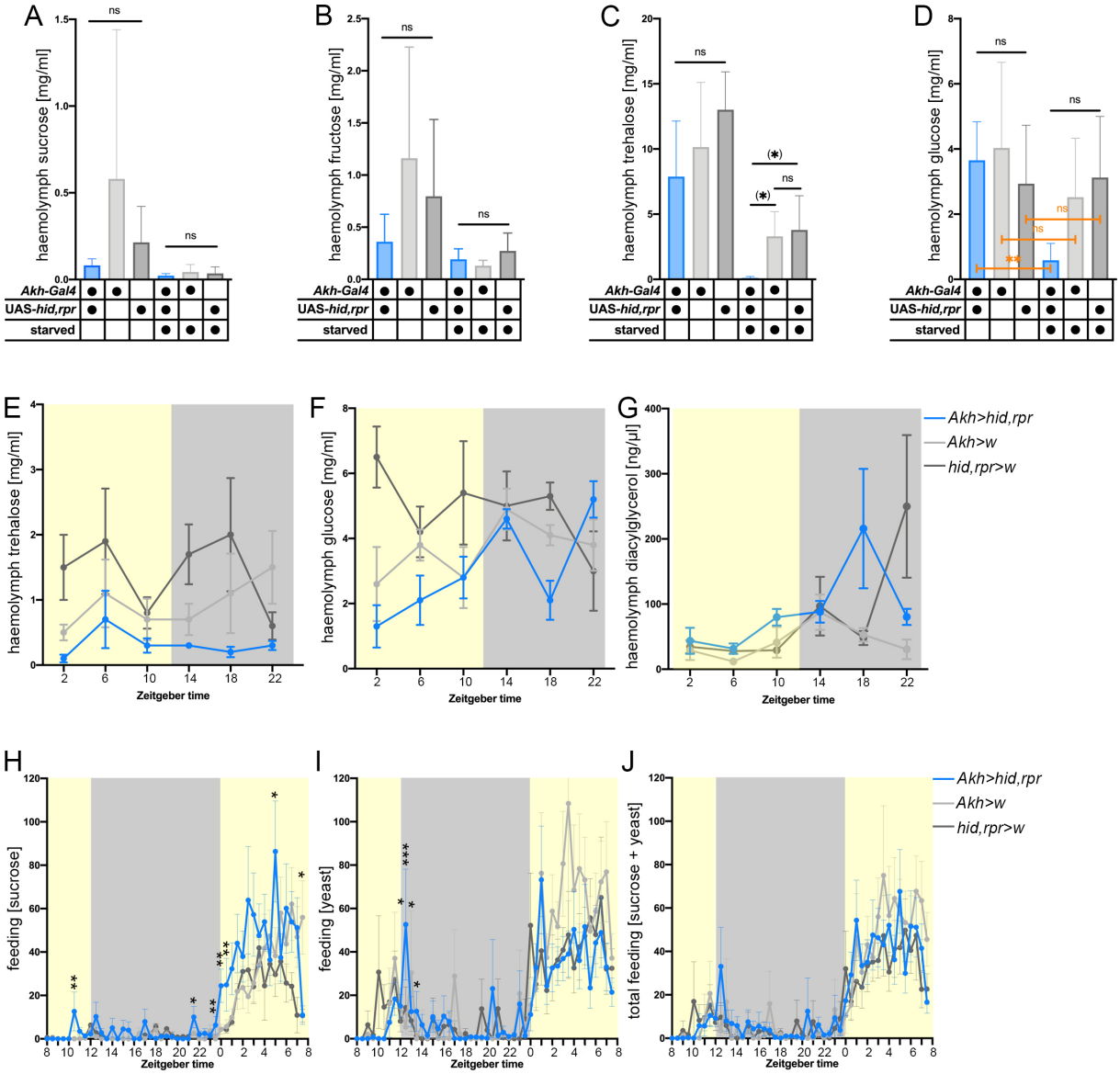



***Figure 6: Carbohydrate haemolymph titres and feeding activity. (A-D)*** *Haemolymph sugar levels analyzed by UPLC-MS/MS in Akh>hid,rpr flies and controls: while sucrose and fructose levels did not differ between genotypes and conditions (starved vs. fed), trehalose levels were depleted in experimental flies under starvation conditions (p=0.078 to Gal4/+ and p=0.063 to UAS/+). In addition, glucose levels were reduced in Akh>hid,rpr flies between fed and starved conditions (p<0.01), without any effect in control flies (p>0.05; all N=3-4).* ***(E)*** *In control flies, haemolymph trehalose levels rise during*

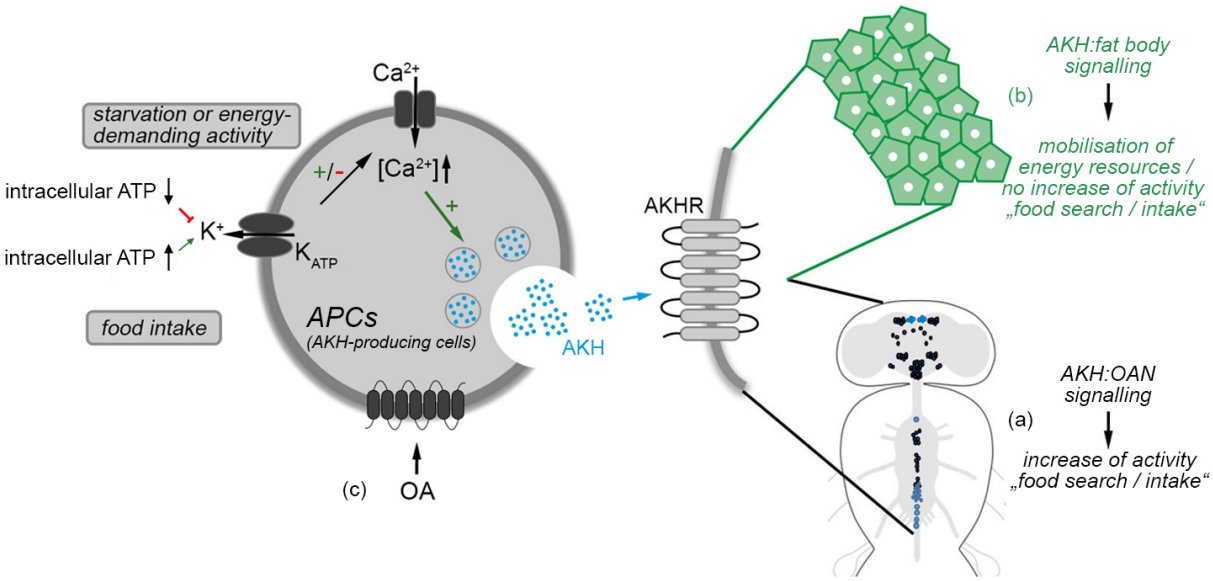
*the night. In contrast, trehalose levels stay low during the night in NP5267>AkhR-RNAi flies.* ***(F)*** *Haemolymph glucose levels increase throughout 24h in experimental flies indicating higher food intake compared to controls.* ***(G)*** *In contrast to haemolymph sugar levels, no obvious change was detectable on haemolymph lipid levels.* ***(H-J)*** *Time course of feeding (number of sips per 30 min) for sucrose* ***(H)****, yeast extract* ***(I)*** *or sucrose and yeast combined* ***(J)*** *in Akh>hid,rpr flies and controls. Significant differences between APC-ablated flies and controls are visible for sucrose feeding mostly at the end of the night and the first hours after lights on, while feeding on yeast or total food consumption was not significantly altered. n=35-36 for feeding data, significance levels: p>0.05 ns, p<0.05 *, p<0.01 **, p<0.001 ***, (p<0.06 (*)). [UPLC-MS/MS=Ultra performance liquid chromatography - tandem mass spectrometry]. Error bars indicate SEM.*

## 4. Discussion:

The circadian activity and rest pattern of animals is determined by a multitude of intertwined systems and mechanisms at different levels. The general organisation is in large set out by the circadian clock and the sleep homeostat [61]. Yet, internal and external signals can dramatically alter activity/rest cycles and induce locomotor activity to meet physiological demands or to escape from harmful situations. A particularly well studied example is the starvation-induced locomotor activity in *Drosophila,* which is triggered by the action of the peptide hormone AKH on neurons that release the arousal signal OA [4,5,9]. On food, AKH signalling was thought to not affect general activity levels, as AKH or AKHR mutants show similar activity levels as controls [8,62], and display normal circadian activity patterns [4,63]. Our study now suggests that the unaltered activity levels and activity/rest patterns in AKH and AKHR mutants is an outcome of the combined loss of both branches of AKH signalling to OANs (regulating locomotor activity via feedforward and feedback signalling) and to the fat body (regulating energy mobilisation). By individually manipulating either of the two AKH signalling branches, we were able to uncover that AKH signalling balances and fine-tunes day and night locomotor activity levels when flies have unrestricted access to food.

When AKH:OAN signalling was genetically impaired, flies were significantly less active during the day. During the night, activity levels remained unaffected. In contrast, when AKH:fat body signalling was impaired, the opposite was observed. Activity remained unaffected during the day, but flies were more active during the night. Concomitantly,

487 *AkhR*[1] mutant flies with rescued AKH:OAN signalling were significantly more active
488 during the night, while day activity was unaffected. Again, the opposite was observed
489 when selectively AKH:fat body signalling was restored in *AkhR*[1] mutants: flies were
490 less active during the day, while normal activity levels were present during the night.
491 Based on our results and available literature data, we propose the following model for
492 the modulatory action of AKH on balancing activity under given conditions (Fig.7).
493 When the fly needs to mobilize energy, for example due to increased physical activity
494 during the day, AKH is released into the circulation to mobilize carbohydrate and lipid
495 fat body stores, and simultaneously activates OANs in the CNS (see Fig.2) [9,64]. The
496 resulting increase in haemolymph trehalose and glucose titres negatively feeds-back
497 to APCs, reducing their secretory activity via opening of ATP-sensitive potassium
498 channels [6,65]. This reduces AKH:fat body signalling, as long as fat body energy
499 stores are not depleted (under fed conditions), and simultaneously prevents a strong
500 activity-promoting effect of time-delayed AKH:OAN signalling. Under starved
501 conditions, however, energy stores become depleted, which leads to a strong and long-
502 lasting increase in the haemolymph AKH titre once carbohydrate levels fall, which in
503 turn stimulates hyperactivity via the activation of OANs [4,7–9].
504




505
506
507 ***Figure 7: AKH signalling to OANs and the fat body balance competing rest and feeding drives.***
508 *In response to a negative balance in haemolymph carbohydrate homeostasis (e.g. under starved*
509 *conditions), AKH is released from neuroendocrine cells in the corpora cardiaca and functions via two*
510 *mutually competitive signalling pathways: (a) AKH signalling to OANs induces food-search behaviour in*
511 *response to starvation (during the day); (b) at the same time AKH mobilizes energy from the fat body to*

*restore haemolymph sugar levels; (c) APC activity is regulated by OA-feedback signaling. This will inhibit the activation of locomotor activity by OANs and thereby prevents abnormally high activity levels during the night. As a stress hormone, the release of AKH presumably balances not only locomotor activity to feeding; AKH release will rather induce a general hypermetabolic response, an increase in respiration and immune response [57]. [AKH: adipokinetic hormone; AKHR: AKH receptor; ATP: adenosine triphosphate; OA: octopamine; OAN: octopaminergic neuron]*

When AKH:fat body signalling is genetically impaired, flies cannot efficiently mobilise fat body energy stores when haemolymph carbohydrate levels are low. In that case, during the day, haemolymph sugar levels are restored by food uptake, as suggested by the observed increase in trehalose and glucose titres during the light phase and the increased sucrose feeding during morning activity, the main time of food intake [58]. As a consequence, impairing AKH:fat body signalling in flies with free access to food does not induce hyperactivity as flies can perform compensatory feeding. However, flies are non-continuous feeders that normally feed little during the night [58,66,67]. During prolonged nocturnal rest phases, the loss of AKH-dependent carbohydrate mobilization leads to increasing AKH release, which in turn positively modulates OANs and subsequently leads to the observed increase in late nocturnal activity, and earlier and more pronounced onset of morning feeding activity in APC-ablated flies kept on food. This scenario is in line with the previous finding that loss of AKHR induces upregulation of *Akh* expression [8]. It also fits with our observation of a drop in glucose levels in the middle of the night, followed by an increase at the end of the night coinciding with the earlier onset of feeding activity in flies with ablated APCs. These flies obviously compensate the loss of carbohydrate mobilisation by feeding during the nocturnal phase, but without triggering increased nocturnal locomotor activity as also AKH:OAN signalling is impaired. A time-restriced increased feeding in AKH-deficient flies is also suggested by the transcriptional up-regulation of the orexigenic peptides NPF and CCHa2 in AKH mutant flies, which paradoxically was not found to be coupled to increased total feeding [71]. Wildtype flies increase *Akh* mRNA levels 3h after the dark-light or light-dark transition, the time points at which starved flies showed pronounced starvation-induced hyperactivity [68]. Interestingly, times of increased sucrose feeding in flies with ablated APCs occurred around ZT3 (significant increase, coupled with low glucose titres) and ZT15 (small and insignificant increase, coupled with high glucose titres) in our study.

546 When AKH:OAN signalling is specifically impaired under *ad libitum* feeding conditions,
547 flies are still sufficiently able to mobilise their energy stores (via AKH:fat body
548 signalling) to meet the metabolic requirements during rest at night, as evidenced by
549 the observed normal nocturnal activity patterns. However, when AKH:OAN signalling
550 is impaired during the light phase, the increased energy demand during diurnal
551 physical activity triggers AKH release which is then no longer able to stimulate activity-
552 promoting OA signalling, explaining the observed reduced locomotor activity in
553 *Tdc2>AkhR-RNAi* flies.
554
555 AKH has been shown to directly trigger strong calcium responses in AKHR-expressing
556 interoceptive SEZ (subesophageal zone) neurons (ISNs) involved in regulating the
557 homeostatic drive to eat and drink [69]. Thus, the observed effects on locomotor activity
558 through the manipulation of AKH signaling in our study might not only be related to
559 foraging activity, but also to the search of water, supporting the idea of a more general
560 effect of AKH on locomotor activity.
561
562 The observed effects of manipulating the two AKH signalling branches are not very
563 strong, and our study does not suggest that AKH is a major factor in shaping circadian
564 locomotor activity patterns. Yet, AKH appears to be one of presumably several factors
565 that fine-tune locomotor activity patterns to meet physiological requirements under
566 dietary stress-free conditions. OANs and APCs receive a multitude of converging and
567 interconnected inputs, especially from insulin-producing cells (IPCs), with extensive
568 feedbacks (see [70–72]). For example, OANs in the SEZ express receptors for AKH
569 and DILPs, and integrate the nutritional status by responding to AKH and DILPs in an
570 antagonistic fashion [9]. In turn, APCs express a range of OA receptors [72]. In
571 addition, APCs are negatively modulated by insulin-producing cells (IPCs), suggesting
572 feedforward signalling from IPCs to OANs via AKH signalling in parallel to direct
573 IPC:OAN signalling [72,73]. AKH signalling is also required for the carbohydrate-
574 dependent release of DILP3 [74], and represses expression of DILP2,3 and 5 [71].
575 Moreover, feeding and diet as well as gustatory perception also influence activity and
576 sleep [73–75], and AKHR is expressed by gustatory neurons including those that
577 express the sugar-sensing receptor Gr5a [16]. AKH production or release is also
578 modulated by diurnal myokine (unpaired 2)-secretion from muscles [76]. Thus, any
579 manipulation of AKH or OA signalling likely results in complex neuroendocrine

580 changes. This complexity may also underlie differences in the effect and effect size
581 observed between different studies. It has to be noted that 4% sucrose served as food
582 source during the locomotor activity recordings in our study. While this is standard for
583 circadian research and allows flies a long-life span, it represents a state of dietary
584 restriction. Future research has to show how the observed locomotor phenotypes
585 manifest on more complete food sources. At least, the locomotor activity-promoting
586 effect of OA seems not to be mediated by DILP-signalling [44]. DILP-signalling seems
587 to rather function upstream and inhibits AKH and OA signalling as DILP2-3,5 mutants
588 show increased day-time activity dependent on AKH and OA signalling [77]. These
589 findings strengthen the view that AKH and AKH:OAN signalling are directly important
590 to balance activity/rest modulation during day and night phases. It remains to be seen
591 to which extend the observed locomotor phenotype following genetic manipulations
592 can be reproduced by endogenous changes in AKH signaling on food.
593
594 **OA and AKH signalling is intertwined**
595 A subset of OANs in the SEZ balance sleep and courtship in male flies. Activation of
596 these octopaminergic MS1 neurons suppressed sleep, while silencing the same
597 neurons led to decreased female-induced sleep loss and impaired mating behaviour
598 in male flies [78]. Furthermore, other OANs in the SEZ are important to integrate AKH
599 and DILP signals to modulate activity levels in response to the nutritional situation [9].
600 The octopaminergic ASM cluster located in the supraesophageal zone has been
601 identified to further include wake-promoting OANs [79].
602 Consistent with findings from the cockroach [30,31], we now provide evidence in
603 support of OANs in the VNC to be part of an AKH signalling pathway that modulates
604 activity levels under *ad libitum* feeding conditions. *Tdc2>AkhR-RNAi* flies showed
605 reduced activity levels during day-time, while this phenotype was reduced in flies
606 expressing *tshGal80*, which blocks expression in OANs located in the VNC. However,
607 around 7 Tdc2Ns in the VNC were still labelled despite the expression of *tshGal80*.
608 Future studies will clarify whether the slightly lower day activity displayed by
609 *Tdc2,tshGal80>AkhR-RNAi* flies is based on those persisting cells in the VNC or due
610 to OANs in the central brain normally expressing AKHR. An involvement of OANs in
611 the VNC in AKH signalling is further suggested by the observed slow but quite strong
612 increase in intracellular cAMP in mesothoracic OANs upon bath-application of AKH
613 onto dissected VNCs. The slow cAMP increase might suggest indirect action, yet

614 transgenic (over)expression of AKHR in the OANs only mildly shortened the lag time
615 from 15 to around 10 min. Interestingly, the AKH-induced increase in intracellular $Ca^{2+}$
616 activity of larval prothoracic gland cells sets in only after 15 min of peptide bath
617 application [80]. Clearly, further studies addressing AKHR expression in OANs and the
618 time course of AKH signalling are required to clarify whether the observed cAMP
619 increase in OANs is due to direct or indirect action of AKH. An attempt to address
620 AKHR expression in OANs by available intragenic Gal4/LexA lines did not provide a
621 conclusive answer in our hands (data not shown). In support of an evolutionary
622 conserved activity-promoting action of AKH in the VNC, injection of AKH into the
623 mesothoracic neuropil of the moth *Manduca sexta* leads to increased activity of
624 motoneurons [81].
625 Our results provide several lines of evidence for a modulation of APC activity by OA
626 and the OAMBII-K3 receptor. A recent cell-specific transcriptomic study supports the
627 expression of OAMB in APCs, yet found expression of further OA receptors (Octbeta1-
628 3R, Oct-TyrR; [72]). At the moment, we can only speculate about the functional
629 significance of OA modulation of APCs and the role of the different OA receptors
630 therein.
631

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884

885 **6. Material and Methods**:

886 6.1 Fly strains:

887 Flies were cultured according to standard methods. Vials were kept under constant
888 conditions at 25°C and 60% humidity in a 12:12 light:dark cycle. Genetic controls were
889 obtained by crossing Gal4-driver and UAS-effector lines to $w^{1118}$. Transgenic and
890 mutant fly lines used in this study are listed in supplementary table S13.

891

892 6.2 Behavioural assays:

893 6.2.1 Locomotor activity recording

894

895 3-7d old flies were recorded individually in *Drosophila* activity monitors (DAM,
896 Trikinetics, Waltham, USA) under standard 12:12 light:dark conditions with light
897 intensities around 60-100lux at ~20°C. Flies were kept in small glass tubes with 2%
898 agarose and 4% sugar on one side. Under starved conditions, flies were kept on 2%

899 agarose to avoid dehydration. 32 flies of a genotype (half male and female) and
900 appropriate controls were measured simultaneously. Fly activity was defined by the
901 number of infrared light beam crosses within 12 hours. Activity patterns were separated
902 into day (ZT0-ZT12; ZT0=lights-on, ZT12=lights-off) and night (ZT12-ZT24), and data
903 was pooled from six days and nights, respectively. For average activity profiles, fly
904 activity was defined as beam crosses per hour. Within these average activity profiles,
905 asterisks label those time points at which experimental flies differ significantly from
906 both genetic controls. For thermogenetic inhibition or activation via UAS-*shibire*$^{ts}$ and
907 UAS-*dTrpA1,* experiments were performed at ~31°C under the same light regime.
908
909 6.2.2 Survival
910
911 Survival analysis was performed with the same DAM system raw data as above. The
912 number of flies alive at the end of day was counted based on present or absent beam
913 crossings.
914
915 6.2.3 Startle-induced negative geotaxis (SING)
916
917 Negative geotaxis was performed to screen for general motor impairments. The
918 experiments were performed as described in Ali et al. 2011, with minor modifications:
919 Groups of 10 male and female flies, respectively, were separated into Falcon tubes 5
920 minutes before the experiment. Flies were tapped to the conic bottom of the Falcon
921 tube and then recorded with a DMK22BUC09 video camera with a Pentax C2514-M
922 objective (The Imaging Source Europe GmbH, Germany). Data was averaged over 5
923 trials per group (with an interspace period of 1 minute) and represents the percentage
924 of flies reaching a label 8.5 cm above the bottom within 5 and 10 seconds.
925
926 6.2.4 Flypad feeding assay
927
928 Feeding was automatically monitored using a 2-channel flyPAD system
929 (www.flypad.rocks) adapted for long-term measurement of food intake. The flyPAD
930 relies on capacitative measurements [60]. For each experimental run, four males and
931 four females of each genotype were in parallel anesthesized on ice, and then
932 transferred to individual arenas in the late afternoon prior to the evening activity phase.

933 In the arenas, flies had the choice between 2% agarose (Carl Roth, Karlsruhe,
934 Germany) containing 5 mM sucrose (molecular biology grade, Carl Roth, Karlsruhe,
935 Germany, "sucrose") or 10% yeast extract (AppliChem, Darmstadt, Germany, "yeast").
936 Food was provided in glass tubes with an enlarged diameter of 7mm to allow for long-
937 term monitoring. The flyPADs were turned upside-down and placed on evenly-
938 illuminated white LED plates under control of a timer to generate a light regime of
939 LD12:12 in a dark box at 21°C. Feeding (number of sips) was analysed in 30 min bins
940 under exclusion of flies that did not feed during the entire experiment. The data was
941 analysed using custom made Matlab scripts [60].
942
943 6.3 Live cell imaging:
944 6.3.1 Imaging setup
945
946 For $Ca^{2+}$ imaging, specimens were imaged with an AXIO Examiner D1 upright
947 microscope (Carl Zeiss, Göttingen, Germany) with a Zeiss W Plan-Apochromat 40x
948 1.0 or 20x 0.8 water immersion objective and a SPECTRA 4 hybrid solid state LED
949 source (Lumencor, Beaverton, USA). Images were captured with a PCO.edge 4.2m
950 sCMOS camera (PCO AG, Kelheim, Germany). For cAMP imaging, we used an
951 Axioskop 2FS plus microscope (Carl Zeiss, Göttingen, Germany) with a Visichrom
952 high-speed polychromator system and a Xenon arc lamp (75W, Visitron Systems,
953 Puchheim, Germany). Images were recorded with a CCD camera (CoolSnap HQ,
954 Roper Scientific, Tuscon, USA) and a Photometrics DualView2 beam splitter allowing
955 to separately record CFP and YFP emissions. Images were analysed using
956 VisiView3.0 software (Visitron Systems, Puchheim, Germany).
957
958
959 6.3.2 Bath application experiments
960
961 Intracellular $Ca^{2+}$ and cAMP levels were monitored by UAS-*Epac1-camps50A* [48] and
962 UAS-*GCaMP6m* [49]. 1-2 brains were dissected, mounted in 405µl haemolymph-like
963 HL3.1 saline solution [50] in the imaging chamber and allowed to settle for 30-45
964 minutes. For $Ca^{2+}$ and cAMP imaging in OANs, AKH (+ 0.1% DMSO) or HL3.1 (+0.1%
965 DMSO; as vehicle control), were bath-applied after 100s. Application of

966 carbamylcholine (carbachol; for $Ca^{2+}$ imaging; Fig.S6) and the water-soluble forskolin
967 derivate NKH477 served as positive control (data not shown).
968 Prior to $Ca^{2+}$ imaging in AKH cells, $Ca^{2+}$ levels were reduced to background levels by
969 applying trehalose (80mM) after 100 seconds [6]. 80mM trehalose was added to all
970 subsequent solutions applied during the experiments. At 300 seconds, either HL3.1
971 (+0.1% DMSO) as vehicle control or 1mM OA dissolved in HL3.1 (+0.1% DMSO) was
972 bath-applied. Application of potassium chloride (end concentration: 80 mM) after 2980
973 seconds served as viability control.
974 Changes in the GCaMP fluorescence intensity within the region of interest were
975 calculated as: $\Delta F/F_0=(F_n-F_0)/F_0$, where $F_n$ is the fluorescence at time point n and $F_0$ the
976 baseline fluorescence intensity calculated from the first 30 seconds. For Epac1
977 imaging, the CFP/YFP ratio was calculated for each time point: Δ(CFP/YFP) with
978 $CFP=CFP_n/CFP_0$ and $YFP=(YFP_n-(CFP_n*\text{spillover into YFP})/YFP_0$, where $CFP_n$ or
979 $YFP_n$ is the fluorescence intensity at a given time point, and $CFP_0$ or $YFP_0$ is the
980 baseline fluorescence intensity calculated from the first 30 seconds. CFP spillover was
981 determined as 35.7% of the YFP signal.
982 Region of interests (ROI) were drawn over the cell bodies and the background was
983 subtracted for every ROI. All imaging experiments were performed during the light
984 phase.
985
986 <u>6.4 Immunostainings:</u>
987 6.4.1 Whole mounts
988
989 Immunofluorescence stainings of *Tdc2>10xmyr::GFP* and
990 *Tdc2,tshGal80>10xmyr::GFP* preparations were performed as described [82]. In short,
991 ventral nerve cords of 3-7 days old flies were dissected in HL3.1 (pH 7.2-7.4).
992 Afterwards, specimens were fixated in 4% paraformaldehyde in PBS (0.1M; phosphate
993 buffer saline) for two hours, washed four times in PBS with 0.3% Triton-X 100 (PBT),
994 and blocked with 5% normal goat serum in PBT. Then, specimens were incubated with
995 rabbit anti-GFP serum (A6455, Invitrogen, USA; 1:1000) and mouse monoclonal
996 antibodies against Synapsin (3C11; [83]; 1:50) in blocking solution for one night at 4°C.
997 On day two, preparations were washed six times with PBT and incubated for one night
998 at 4°C with secondary antibodies goat anti-rabbit Alexa 488 (Invitrogen, USA; 1:250)
999 and goat anti-mouse DyLight 649 (Jackson ImmunoResearch, USA; 1:250). Finally,

1000 specimens were rinsed six times in PBT and mounted in 80% glycerol in PBS. Until
1001 scanning with a Leica TCS SPE or SP8 confocal microscope (Leica microsystems,
1002 Wetzlar, Germany), specimens were stored in darkness at 4°C.
1003
1004 6.4.2 Tissue sections
1005
1006 To visualize the arborisations of OANs on the CCA-HG complex, whole body sections
1007 were performed (as described in [22]). In short, whole flies with opened cuticle were
1008 fixated with 4% paraformaldehyde in PBS for two hours and afterwards washed three
1009 times with PBS. Subsequently flies were embedded in hot 7% low melting agarose.
1010 After hardening, specimens were cut with a vibratome into 90-95 µm sections. Staining
1011 of the sections was continued as described in 6.4.1 in a 24 well plate. Rabbit anti-Tdc2
1012 (pab0822-p, Covalab, France; 1:200) and mouse anti-Synapsin (3C11; 1:50) were
1013 used as primary antibodies visualized by goat anti-rabbit Alexa488 (1:200) and goat
1014 anti-mouse DyLight649 (1:200) sera.
1015
1016 6.5 CaLexA labelling:
1017
1018 For CaLexA labelling, equal numbers of 4-11 day old males and female flies were used.
1019 The proventriculus plus attached retrocerebral complex (corpora allata and fused
1020 corpora cardiaca-hypocerebral ganglion CCA-HG complex) were dissected in HL3.1
1021 saline as previously described [84] and immediately imaged with an AXIO Examiner
1022 D1 upright microscope (Carl Zeiss, Göttingen, Germany) with a Zeiss W Plan-
1023 Apochromat 40x 1.0 water immersion objective, and a SPECTRA 4 hybrid solid state
1024 LED source with 475 nm excitation maximum (Lumencor, Beaverton, USA). Images
1025 were captured with a PCO.edge 4.2m sCMOS camera (PCO AG, Kelheim, Germany)
1026 at 16bit intensity resolution. All settings were kept constant throughout the
1027 experiments. Images were analysed using Fiji [85]. A region of interest was drawn
1028 around the CCA-HG and in the direct vicinity (background). The mean intensity of the
1029 CCA-HG was then calculated and the mean background was subtracted. In addition,
1030 the number of labeled cells was counted.
1031
1032
1033

1034 6.6 PCR:

1035 Total RNA was extracted from adult Canton-S flies using the Quick-RNA MicroPrep Kit
1036 (Zymo Research, Irvine, USA) according to manufacturer's instructions. Central brains,
1037 VNCs, and HG-CCA (for OAMB 15 flies per group; for AKHR five flies per group) were
1038 dissected in HL3.1 medium, and transferred to 300 µl RNA lysis buffer on ice. Tissues
1039 were homogenized with a plastic pestle. Total RNA was eluted in 8 µl RNAse-free
1040 water. For cDNA synthesis, the QuantiTect Reverse Transcription Kit from Qiagen
1041 (Hilden, Germany) was used. All steps were performed following the manufacturer's
1042 protocol. Genomic DNA was removed by adding 1 µl of gDNA wipe-out buffer to 6 µl
1043 of the eluted RNA. Following incubation at 42°C for 2min, the samples were placed for
1044 2min at 4°C and 3 µl of a mastermix composed of 2 µl RT buffer, 0.5 µl RT primer Mix
1045 and 0.5 ml reverse transcriptase was added. Reverse transcription was performed for
1046 30 min at 42°C, followed by 3 min at 95°C and 2min at 4°C. Finally, 20 µl water were
1047 added; cDNA samples were stored at -20 °C. To analyse OAMB and AKHR expression
1048 in the isolated tissues, cDNAs were PCR-amplified using specific primers (Table 1) and
1049 a JumpStart REDTaq ReadyMix Reaction Mix (Sigma-Aldrich, MO, USA). α-tubulin and
1050 water ("no DNA") were used as internal control. The PCR programme consisted of
1051 5min at 95 °C, followed by 35 cycles of 30 s at 95°C, 30 s at 61°C (57,5°C for AKHR),
1052 60 s at 72°C and finally 5 min at 72°C.

1053

1054 ***Table 1: Primer pairs used for PCR in this study***

| **Primer** | **Sequence from 5´ to 3´** |
|---|---|
| OAMB E4E5 fw | CGTTCTGGTCATCGGTGG |
| OAMB E4E5 rv | CGTGGACATTATGCTTGG |
| OAMB E6E7 / E6E8 fw | CACCGCCGCAGAACAGAC |
| OAMB E6E7 rv | CGCTACTGATTACCGCAT |
| OAMB E6E8 rv | CGCTCGTGACCCACATCC |
| | |
| AKHR fw | GGACTCTACAACATTCGC |
| AKHR rv | CCTCCTCCATTCAGCAGC |
| α-tubulin fw | TCTGCGATTCGATGGTGCCCTTAAC |
| α-tubulin rv | GGATCGCACTTGACCATCTGGTTGGC |

1055

1056 6.7 Ultra performance liquid chromatography-tandem mass spectrometry (UPLC-
1057 MS/MS) measurements:
1058 6.7.1 Haemolymph sampling
1059
1060 For each sample, 20 flies were perforated by a thin needle at the thorax and put in a
1061 perforated 0.5ml Eppendorf cap, which was fitted into a 1.5ml Eppendorf cap.
1062 Haemolymph was collected within the 1.5ml Eppendorf cap after 1min of centrifugation
1063 in a benchtop centrifuge at 5000 rpm, and immediately deep-frozen until analysis.
1064
1065 6.7.2 UPLC-MS/MS analysis of sugars and diacylglycerols
1066
1067 Sugars were analysed using a Waters Acquity ultra-high-performance liquid
1068 chromatograph coupled to a Waters Micromass *Quattro Premier* triple quadrupole
1069 mass spectrometer (Milford, MA, USA) with an electrospray interface.
1070 Chromatographic separation was performed on a Waters Acquity BEH amide column
1071 (1.7 µm, 2.1×100mm; Waters, Milford, MA, USA) according to Waters application note
1072 WA60126 with a modified flow rate of 0.2 ml/min. The analytes were eluted in a linear
1073 solvent-strength gradient (75% to 45% acetonitrile containing 0.1% ammonium
1074 hydroxide in 10min) at a column temperature of 35°C. Sugars were detected in the
1075 negative electrospray mode at a source temperature of 120°C and a capillary voltage
1076 of 3.25 kV. Nitrogen was used as desolvation and cone gas with flow rates of 800 l $h^{-}$
1077 $^{1}$ at 350°C and 25 l $h^{-1}$. The mass spectrometer was operated in multiple reaction
1078 monitoring (MRM) mode using Argon as collision gas at a pressure of approximately 3
1079 x $10^{-3}$ bar. Cone voltage (CV) and collision energy (CE) were optimized for maximum
1080 signal intensity of each individual compound during ionization and collision induced
1081 dissociation (CID) for a dwell time of 50ms per transition (Table 2). Glucose-6,6-$d_2$ and
1082 Trehalose-1,1′-$d_2$ were used as internal standards for quantification of mono- and
1083 disaccharides.
1084
1085
1086
1087
1088
1089

1090 ***Table 2: Table with specific cone voltage and collision energy for each transition***

| Compound Name | Parent (m/z) | Daughter (m/z) | CV (V) | CE (eV) |
|---|---|---|---|---|
| Glucose/Fructose | 179 | 89 | 18 | 10 |
| Glucose-6,6-$d_2$ | 181 | 91 | 18 | 10 |
| Sucrose/Trehalose | 341 | 179 | 32 | 15 |
| Trehalose-1,1′-$d_2$ | 343 | 180 | 32 | 15 |

1091

1092 Determination of diacylglycerols were performed according to [86]. Prior to analysis,
1093 haemolymph samples were dried and recovered in 75 µl isopropanol containing 1
1094 µg/ml 1,2-didecanoyl-glycerol used as an internal standard.

1095

1096 6.8 Statistics:

1097

1098 Data was analysed for normal distribution using the Shapiro-Wilk test. For statistical
1099 comparison between genotypes, a pairwise t-test was used for normally distributed
1100 data, a pairwise Wilcoxon rank sum test was used for not normally distributed data
1101 (both including a Bonferroni-Holm correction for pairwise comparisons). All statistical
1102 analyses were done with R Studio version 1.0.136. (www.r-project.org). For the
1103 statistical comparison of sugar levels quantified by LC-MS, we used a simple t-test
1104 based on the relatively small sample size (N=3-5, with n=20 per N). Data were plotted
1105 using OriginPro 2016G, GraphPad Prism 8, R Studio and Photoshop CS6. Activity
1106 levels are presented as box plots, with 50% of values for a given genotype being
1107 located within the box. Box and whiskers represent the entire set of data. Outliers are
1108 indicated as open circles. The median performance index is indicated as a thick line
1109 within the box plot. Data presented as barplots show the mean values and standard
1110 error of the mean (SEM). Significance levels between genotypes shown in the figures
1111 refer to the raw p-values obtained in the statistical tests.

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1113 **Acknowledgements**:

1114

1115 The authors thank Bryon Hughson, Wolf Hütteroth, Robert Kittel and Pamela
1116 Menegazzi for fruitful discussions and comments on the manuscript, Gertrud Gramlich
1117 and Susanne Klühspies for excellent technical assistance, and Jay Hirsh and the
1118 Bloomington Stock centre for providing flies. This work was supported by intramural

support of the University of Würzburg (to C.W.) and the University of Graz (to R.P.K.), and the Deutsche Forschungsgemeinschaft (SFB1047 "Insect timing", project B2 to C.W., DP1979/3-1 to D.P., PA3241/2-1 to M.S., and FO207/14-1 to C.H.F.). The authors declare no competing interests. D.P., M.S., J.R., R.P.K. and C.W. conceived and designed the experiments. D.P., M.S., J.R., K.A., M.K., C.H.-L., A.G.Ü., N.E., P.M.I. and C.W. performed the experiments. D.P., M.S., J.R., A.F., M.K., C.H.-L., P.M.I., C.H.F., M.J.M., and C.W. analysed the results. D.P. and C.W. wrote the article. All authors provided comments and approved the manuscript.

1133 **Supplement Figures:**

1134 ***(S1-S2)*** *In response to starvation Akh>hid,rpr flies show only a minor increase in*
1135 *activity, while there was no starvation-induced increase in activity within the first 48h in*
1136 *contrast to control flies.*

1137 ***(S3)*** *The loss of AKH signalling increased the starvation susceptibility as control flies*
1138 *died earlier compared to Akh>hid,rpr flies.*

1139 ***(S4)*** *The knockdown of AKHR in OANs did not affect motor behaviours in general, as*
1140 *experimental flies performed indistinguishable from genetic controls in the startle-*
1141 *induced climbing assay (all p>0.05; all N=11).*

1142 *(**S5**) Specific knockdown of AKHR in Tdc2Ns (Tdc2-Gal4-positive neurons) of the VNC*
1143 *specifically affected locomotor activity.*

1144 *(**S6**) Expression of tshGal80 restricted the expression pattern of Tdc2-Gal4 within the*
1145 *VNC to ~7.4 ± 2.07 cells (n=5).*

1146 *(**S7**) RT-PCR analysis revealed that AKHR is expressed in larval fat bodies, the adult*
1147 *brain and VNC. Significance levels: p>0.05 ns, p<0.05 *, p<0.01 **, p<0.001 ***. [CS=*
1148 *CantonS, VNC=Ventral nerve cord].*

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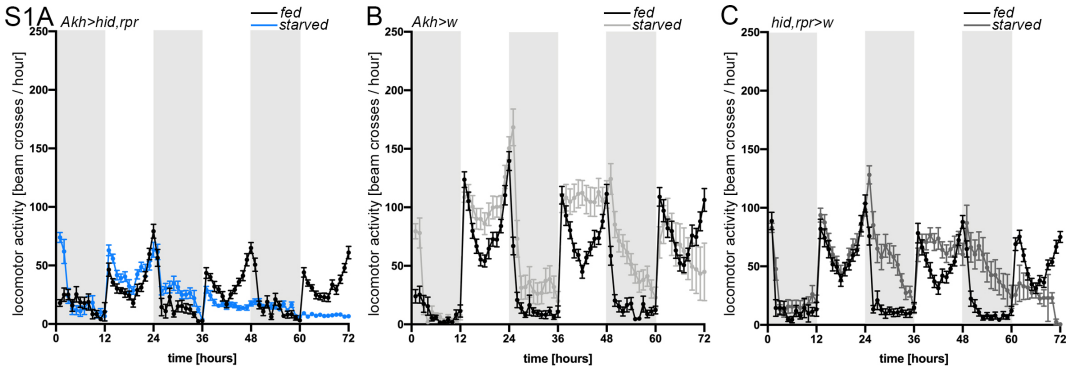
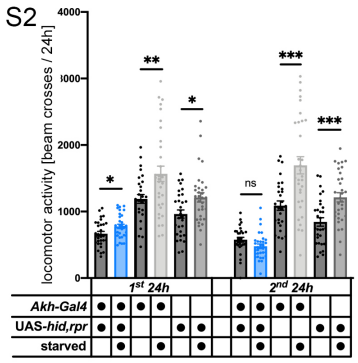
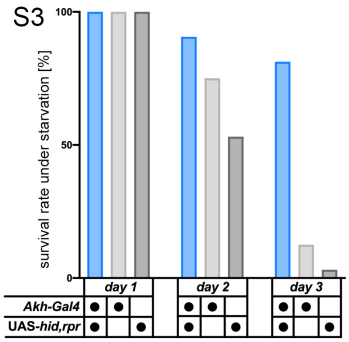
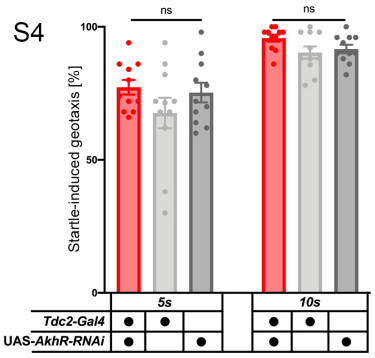
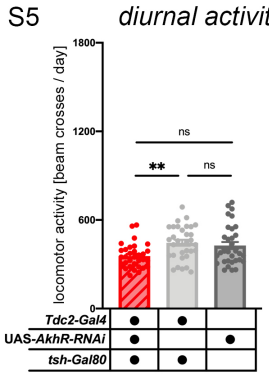
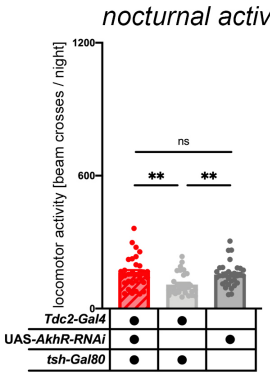

S1A
Akh>hid,rpr
fed
starved
B
Akh>w
C
hid,rpr>w
locomotor activity [beam crosses / hour]
time [hours]

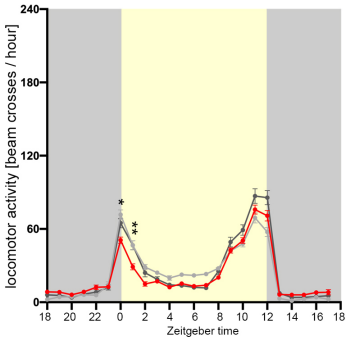

S2
locomotor activity [beam crosses / 24h]
1st 24h
2nd 24h
Akh-Gal4
UAS-hid,rpr
starved

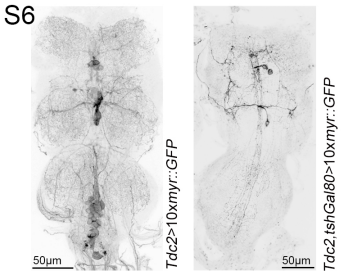

S3
survival rate under starvation [%]
day 1
day 2
day 3
Akh-Gal4
UAS-hid,rpr

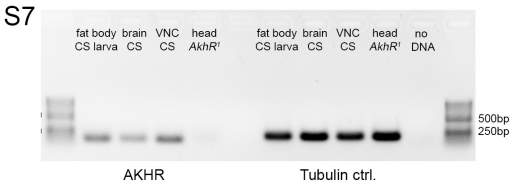

S4
Startle-induced geotaxis [%]
5s
10s
Tdc2-Gal4
UAS-AkhR-RNAi

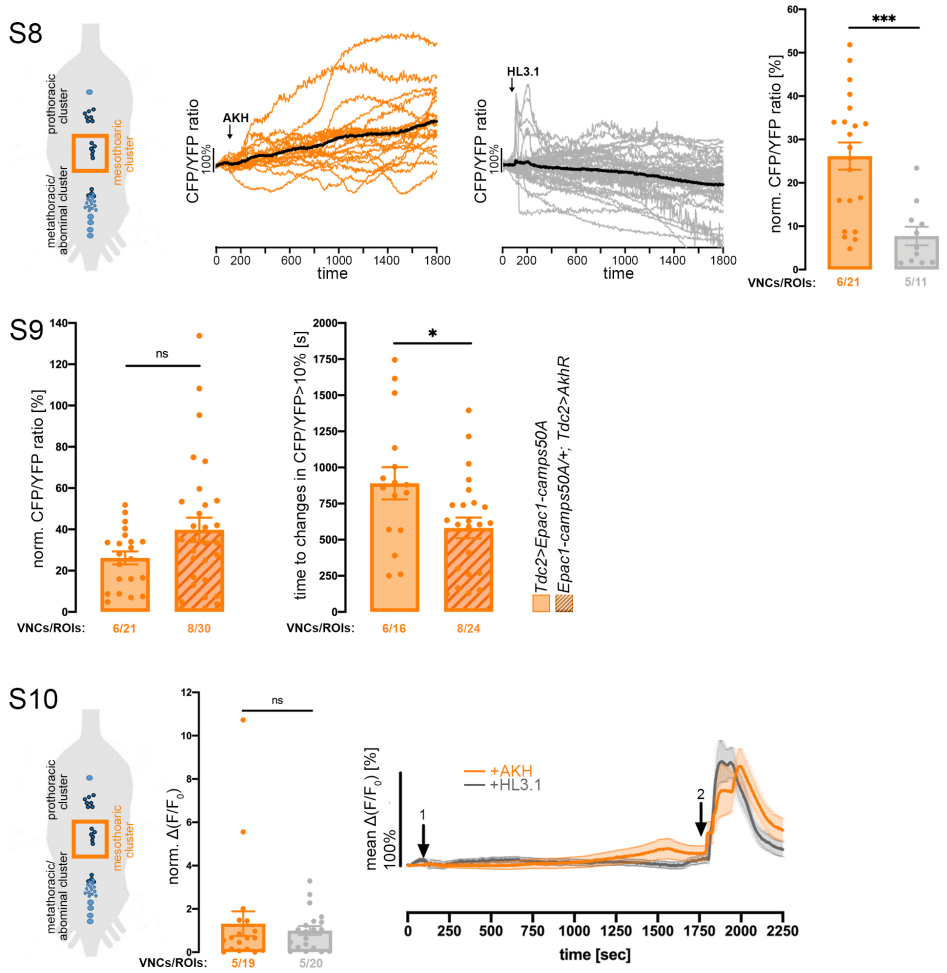

S5
diurnal activity
locomotor activity [beam crosses / day]
Tdc2-Gal4
UAS-AkhR-RNAi
tsh-Gal80


nocturnal activity
locomotor activity [beam crosses / night]
Tdc2-Gal4
UAS-AkhR-RNAi
tsh-Gal80


locomotor activity [beam crosses / hour]
Zeitgeber time


S6
Tdc2>10xmyr::GFP
Tdc2,tshGal80>10xmyr::GFP
50µm
50µm


S7
fat body CS larva
brain CS
VNC CS
head AkhR1
fat body CS larva
brain CS
VNC CS
head AkhR1
no DNA
500bp
250bp
AKHR
Tubulin ctrl.

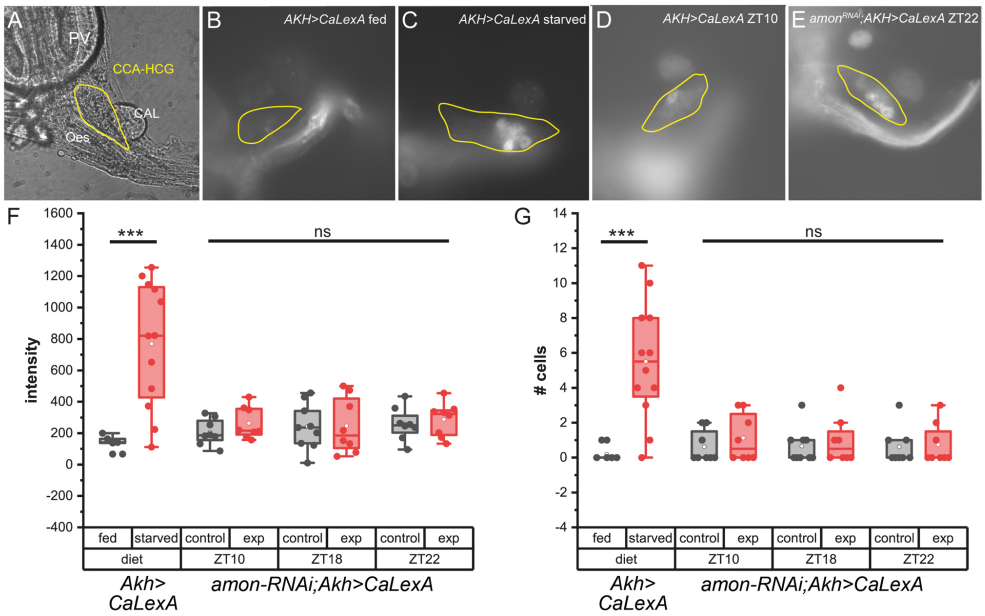



*(**S8**) cAMP imaging experiments in Tdc2Ns (Tdc2-Gal4-positive neurons) of the mesothoracic cluster (orange box) in the VNC revealed that cells significantly responded with a cAMP increase to AKH bath application.*

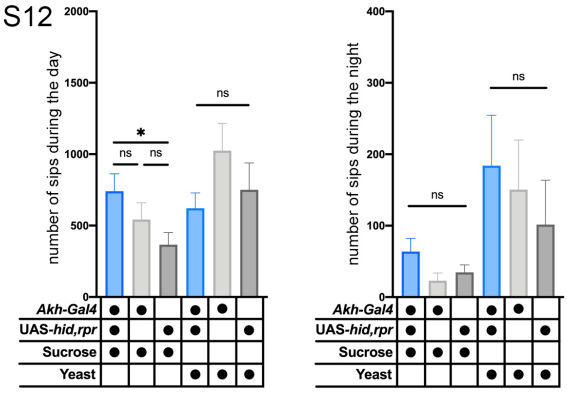
*(**S9**) Overexpression of AKHR did not affect response levels and did only slightly reduce the lag time in response to AKH in Tdc2Ns.*

***(S10)** $Ca^{2+}$-imaging in Tdc2Ns of the mesothoracic cluster in the VNC revealed that these cells did not respond with a significant $Ca^{2+}$ increase to AKH bath-application (arrow 1 at 100s). Application of carbamylcholine at 1800s (arrow 2) served as a positive control for viability of the cells. [ROI=Region of interest, VNC=Ventral nerve cord]. Error bars indicate SEM.*

S11




S12




***(S11)*** *CaLexA-induced GFP expression in AKH-producing cells (APCs).* ***(A)*** *shows a typical preparation of the CCA-HG complex including the corpora allata (CA), esophagus (ES) and proventriculus (PV).* ***(B-E)*** *Examples for CaLexA-induced GFP expression in Akh>CaLexA flies on food (B) and 24h off food (C), as well as Akh>CaLexA controls at ZT10 (D) and amonRNAi;Akh>CaLexA experimental flies at ZT22 (E).* ***(F-G)*** *CaLexA-induced GFP expression in APCs under fed (n=6) or starved (n=12) conditions (Akh>CaLexA, left two columns) and on food at different time points during the day (Akh>CaLexA = controls, amonRNAi; Akh>CaLexA = exp (experimental flies), n=8-9 per condition) quantified as GFP fluorescence intensity in (F), and number of GFP-expressing APCs in (G). Significance levels: p>0.05 ns, p<0.05 *, p<0.01 **, p<0.001 ***. [APCs= AKH-producing cells; ZT= Zeitgeber time].*

***(S12)*** *Total feeding indicated by the number of sips was not affected by APCs for both diurnal and nocturnal sucrose and yeast consumption. n=35-36; signifcance levels p<0.05 *, p<0.01 **, p>0.001 ***. Error bars indicate SEM.*

1182 S13 Fly lines

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| Name | Genotype | Inserted chromosome | Reference |
|---|---|---|---|
| UAS-*hid,rpr* | *w*, P[UAS-*hid*], P[UAS-*rpr*] | 1 | Zhou et al. 1997 |
| UAS-*shibire*$^{ts}$ (*shi*$^{ts}$) | w; P{UAS-shi$^{ts1}$.K}3 | 3 | Kitamoto 2001 |
| UAS-*OambII-RNAi* | y1 v1; P{TRiP.JF01673}attP2 | 3 | Shankar et al. 2015 |
| UAS-*AkhR-RNAi* | y$^1$v$^1$; P{TRiP.JF032056} attp2 | 3 | Braco et al. 2012 |
| UAS-*Eac1-camps50A* | w1118;P{UAS-Epac1-camps}50A | 2 | Nikolaev et al. 2004 |
| UAS-*GCaMP6m* | w1118;P{y[+t7.7] w[+mC]=20XUAS-IVS-GCaMP6medium}attP40 | 2 | Chen et al. 2013 |
| UAS-10x*myr::GFP* | w*;P{10XUAS-IVS-myr::GFP}attP2 | 2 | Pfeiffer et al. 2010 |
| UAS-*amonRNAi*$^{78b}$ | w[*]; P{w[+mC]=UAS-amon-RNAi}78b | 2 | Rhea et al. 2010 |
| UAS-*CaLexA* | LexAop-CD8-GFP-2A-CD8-GFP/LexAop-CD8-GFP-2A-CD8-GFP; UAS-mLexA-VP-NFATC del (4-2), LexAop-CD2-GFP/TM6B | 2;3 | Masuyama et al. 2012 |
| *AkhR*$^1$ | y* float w[*]; AKHR[1] / CyO | 2 | Grönke et al. 2007 |
| *AkhR*$^{revA}$ | y* float w[*]; rev[Pfw[þmC] ¼ EPgAKHR[G6244]]A / CyO | 2 | Grönke et al. 2007 |
| UAS-*AkhR* | w[*]; P{w[+mC] AKHR[Scer/UAS]}#32A /TM3 Sb[1] e[1] | 3 | Grönke et al. 2007 |
| *AkhR*$^1$; UAS-*AkhR* | y* w[*]; AKHR[1] UAS-dAKH | 2 | Grönke et al. 2007 |
| *Akh-Gal4* | y1 w*; P{Akh-gal4.L}2 | 2 | Lee and Park 2004 |
| *Tdc2-Gal4* | w*; P{Tdc2-GAL4.C}2 | 2 | Cole et al. 2005 |
| *Tdc2-Gal4* | w*;; P{Tdc2-GAL4.S}attP2 | 3 | Cole et al. 2005 |
| *Tdc2-Gal4; tshGal80* | w*; P{Tdc2-GAL4.C}2 P{GAL80}$^{tsh-GAL80}$ | 2 | Selcho et al. 2014 |
| *AkhR*$^1$*; Tdc2-Gal4* | y* float w[*]; AKHR[1]; *Tdc2-GAL4* | 2,3 | this study |
| *NP5267-Gal4* | y[*] w[*]; P{w[+mW.hs]=GawB} NP5267 / CyO, P{w[-]=UAS-lacZ.UW14}UW14 | 2 | Hayashi et al. 2002 |

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