Allatostatin A Signalling in *Drosophila* Regulates Feeding and Sleep and Is Modulated by PDF

Jiangtian Chen¹, Wencke Reiher¹, Christiane Hermann-Luibl¹, Azza Sellami², Paola Cognigni³, Shu Kondo⁴, Charlotte Helfrich-Förster¹, Jan A. Veenstra², Christian Wegener¹*

¹ Neurobiology and Genetics, Theodor-Boveri-Institute, Biocenter, University of Würzburg, Würzburg, Germany, ² INCIA, UMR 5287 CNRS, University of Bordeaux, Talence, France, ³ Department of Zoology, University of Cambridge, Cambridge, United Kingdom, ⁴ Genetic Strains Research Center, National Institute of Genetics, Shizuoka, Japan

* Current address: Centre for Neural Circuits and Behaviour, The University of Oxford, Oxford, United Kingdom

* christian.wegener@biozentrum.uni-wuerzburg.de

**Abstract**

Feeding and sleep are fundamental behaviours with significant interconnections and cross-modulations. The circadian system and peptidergic signals are important components of this modulation, but still little is known about the mechanisms and networks by which they interact to regulate feeding and sleep. We show that specific thermogenetic activation of peptidergic Allatostatin A (AstA)-expressing PLP neurons and enteroendocrine cells reduces feeding and promotes sleep in the fruit fly *Drosophila*. The effects of AstA cell activation are mediated by AstA peptides with receptors homolog to galanin receptors subserving similar and apparently conserved functions in vertebrates. We further identify the PLP neurons as a downstream target of the neuropeptide pigment-dispersing factor (PDF), an output factor of the circadian clock. PLP neurons are contacted by PDF-expressing clock neurons, and express a functional PDF receptor demonstrated by cAMP imaging. Silencing of AstA signalling and continuous input to AstA cells by tethered PDF changes the sleep/activity ratio in opposite directions but does not affect rhythmicity. Taken together, our results suggest that pleiotropic AstA signalling by a distinct neuronal and enteroendocrine AstA cell subset adapts the fly to a digestive energy-saving state which can be modulated by PDF.

**Author Summary**

Feeding and sleep are fundamental behaviours that are controlled by diverse neuropeptides. While feeding is associated with wake periods, sleep prevents feeding. Both feeding and sleep are timed to specific parts of the day by internal clocks, presumably to optimise behaviour and metabolic processes. We investigated the functions of Allatostatin A (AstA)
peptides in the fruit fly. AstA is produced by neurons as well as by endocrine cells in the midgut epithelium. Thermogenetic activation of subsets of AstA-producing cells in the brain and midgut revealed that AstA affects both feeding and sleep in opposite directions: feeding is reduced by AstA signalling, while sleep is promoted. Others could previously show that insect AstA also inhibits gut motility and release of digestive enzymes from the gut. An attractive conclusion is that AstA signalling helps to put flies in a digestive energy-saving state. Interestingly, a set of six AstA-expressing PLP neurons are in close contact to neurons central to the circadian clock network, and express functional receptors for the clock output signal PDF. This opens the possibility that PLP neurons are at the interface between clock, feeding and sleep—a hypothesis that needs to be tested in the future.

**Introduction**

Neuropeptides and peptide hormones transfer a wide variety of neuronal or physiological information from one cell to the other by activating specific receptors on their target cells [1]. Most if not all peptides are pleiotropic and can orchestrate diverse physiological, neuronal or behavioural processes [2,3]. In vertebrates, such a pleiotropic effect is especially prominent in the regulation of feeding and sleep. Many different peptides (e.g. orexin/hypocretin, ghrelin, obestatin) modulate different aspects of both behaviours [4,5], which reciprocally influence each other [6,7]. The temporal pattern of neuroendocrine activity and neuropeptide release is shaped by sleep homeostasis and the circadian clock which, in turn, reciprocally affects feeding and sleep-wake cycles [7–9]. Significant progress has been made in this field during recent years. Still little characterised, however, is the neuronal architecture that enables the relevant peptidergic neurons to integrate energy status, circadian time and sleep-wake status in order to coordinate the timing of sleep, locomotor activity and feeding. Information about the output signals by which endogenous clocks provide time- and non-circadian information to relevant peptidergic cells is still limited.

During the last years, the fruit fly *Drosophila* has become an important model for research into the regulation of feeding and sleep [10–13]. *Drosophila* offers advanced genetic tools, a small brain with only about 100,000 neurons and a quantifiable sleep- and feeding behaviour that shows characteristics very similar to that of mammals [11,14,15]. These features greatly facilitate the analysis of the neuronal and endocrine underpinnings of feeding and sleep. Like in most animals, feeding and sleep follow a circadian pattern in the fruit fly [16–18] with little characterised neuronal and hormonal pathways downstream of the central clock. Like in mammals, a number of neuropeptides have been shown to be involved in the regulation of feeding [11,12] or sleep [19,20] in *Drosophila*. Yet, so far, only sNPF [21–24] and likely also NPF [25,26] are implicated in the regulation of both feeding and sleep. Also Insulin-like peptide (DILP)-expressing neurons (IPCs) in the pars intercerebralis affect feeding and sleep, yet only feeding seems to be directly dependent on DILP signalling [27].

Recent work by Hergarden and colleagues demonstrated that neurons expressing neuropeptides of the allatostatin A (AstA) family regulate feeding behaviour of the fruit fly [28]. Constitutive activation of AstA cells contained in the AstA<sup>1-Gal4</sup> expression pattern by ectopic expression of the bacterial low threshold voltage-gated NaChBac channel [29] potently inhibited starvation-induced feeding. In contrast, constitutive inactivation of AstA<sup>1</sup> cells by expression of the inwardly rectifying Kir2.1 potassium channel [30] increased feeding under restricted food availability. NaChBac activation of AstA<sup>1</sup> cells also inhibited the starvation-induced increase of the proboscis extension reflex (PER), a behavioural indicator for glucose
responsiveness [28]. The AstA\textsuperscript{1} expression pattern includes a large number of brain neurons plus gut-innervating thoraco-abdominal ganglion (TAG) neurons and enteroendocrine cells (EECs) in the posterior midgut [28]. This broad expression pattern is consistent with earlier described patterns of AstA-like immunoreactivity [31–34] and suggests multiple functions for AstA. Earlier work had demonstrated an effect of AstA on gut motility [35]. Two AstA receptors, DAR-1 (= AlstR) and DAR-2 are characterised for Drosophila [36–39]. Different genome-based phylogenetic GPCR analyses independently demonstrated their homology with the galanin receptor family of vertebrates [40–43].

Using anatomical subdivision and genetic manipulation of neuronal activity, we aimed to identify AstA functions and-if possible-assign them to subsets of AstA expressing cells. Our results revealed new interconnected AstA functions that link feeding and sleep and identify AstA-expressing PLP neurons and EECs as a target of the central clock output factor PDF. Pleiotropic AstA signalling seems capable of coordinating multiple aspects of physiology and behaviour in a coherent manner to adapt the fly to a digestive energy-saving state. The functional range of AstA signalling in the fly is thus reminiscent of the pleiotropy found in mammalian galanin signalling [44–46].

**Results**

To be able to restrict genetic manipulations to subgroups of AstA-expressing cells in *Drosophila*, we first generated an *AstA\textsuperscript{34}*-Gal4 line that specifically drive ectopic expression of effector genes in restricted subsets of AstA-expressing cells.

**Expression pattern of the AstA\textsuperscript{34}-Gal4 line**

To test the specificity of *AstA\textsuperscript{34}-Gal4* expression in adult flies, we co-immunolabelled *AstA\textsuperscript{34}>*GFP flies against GFP and AstA. The observed AstA immunoreactivity (IR) pattern was consistent with earlier descriptions [31–33] (Fig 1), and we adopted the nomenclature of Yoon and Stay (1995). S1 Table provides a summary of the localisation of *AstA\textsuperscript{34}-Gal4*-driven GFP expression in relation to the AstA IR.

In each brain hemisphere of *AstA\textsuperscript{34}>*GFP flies, GFP was consistently detected in two to three of the three AstA-IR PLP interneurons with somata in the posterior lateral protocerebrum (Fig 1A and 1B). These cells sent a primary neurite dorsally just anterior of the calyx which typically trifurcated and then extensively arborised throughout the whole superior lateral (SLP), superior intermediate (SIP) and superior medial (SMP) protocerebrum (Fig 1A and 1B, S1 and S2 Movies). In the anterior-posterior axis, this large arborisation field extended from the height of the fan-shaped body to just anterior of the calyx. Furthermore, GFP was found in two to four cells per hemisphere with somata in the lateral cell body rind close to the lateral horn. These LCBR neurons were AstA immunonegative and are not contained in the *AstA\textsuperscript{1}-Gal4* line (Fig 1A and 1B). In addition, a varying small number of AstA-IR neurons in the medulla showed generally weak GFP expression (Fig 1A and 1C). In some preparations, single medulla neurons were found that exhibited a stronger GFP signal (Fig 1C).

In the thoraco-abdominal ganglion (TAG), three pairs of AstA-IR DLAA cells within the posterior abdominal region ([27], Fig 1A and 1D) sent neurites via the median abdominal nerve to innervate the hindgut and the posterior-most midgut (Fig 1F, 1G and 1I). Regions with innervations include the pyloric valve and the rectal valve, which control transit of gut contents and urine from the midgut to the ileum and from the ileum to the rectum. Processes of the DLAA neurons innervating the rectum in part extend through the muscle layer (Fig 1G), thus their peptide signals might target the rectal epithelium. The DLAA neurons consistently exhibited strong *AstA\textsuperscript{34}*-driven GFP expression, while the brain neurons showed a more...
Fig 1. AstA (magenta) and GFP (green) immunolabeling of nervous systems and guts of adult AstA$^{34}$>GFP (A–D, F–H) and tsh-Gal80; AstA$^{34}$>GFP (E, I) flies. (A) GFP expression is detectable in two paired groups of brain neurons. In each hemisphere, one group with two somata in the posterior lateral protocerebrum (PLP cells, solid arrowheads in A''') and a second group with two to four somata in the lateral cell body rind (LCBR cells, open arrowheads in A''') are labelled (see also C). The LCBR neurons are anti-AstA-negative. Some of the AstA-IR medulla neurons also express GFP. In the abdominal TAG, the six AstA-IR DLAa cells show strong anti-GFP staining and project through the median abdominal nerve towards the
variable GFP labelling intensity between preparations (see Fig 1C). In many preparations, one or a few variably positioned non-AstA-IR interneurons within the TAG additionally showed a weak GFP signal.

Outside of the CNS, two pairs of peripheral AstA-IR neurons with somata located on the segmental nerves leading to the wings and the halteres [33] expressed GFP (Fig 1A). Furthermore, GFP was detectable in most if not all AstA-IR EECs in the posterior part of the midgut (Fig 1F–1H). The staining results are summarized in S1 Table.

In comparison to the AstA<sup>34</sup>-Gal4 pattern, the expression pattern of AstA<sup>1</sup>-Gal4 included the following AstA-IR neurons per brain hemisphere: all three PLP neurons, 2 neurons in the superior protocerebrum, ~ 30 medulla neurons, and three neurons with cell bodies in the GNG (gnathal (= subesophageal) ganglion) thought to be important for sucrose responsiveness [28]. Thus, AstA<sup>1</sup>-Gal4 drives expression in a larger number of AstA brain neurons though it is lacking the AstA-negative LCBR brain neurons (S1 Table). The expression in the TAG is identical in both AstA-Gal4 lines, while AstA<sup>34</sup>-Gal4 includes a larger fraction of AstA EECs in the midgut. A schematic summary of the expression patterns is given in S2 Fig.

**Activation of the AstA PLP neurons and EECs is sufficient to reduce food intake**

To test for a possible role of AstA<sup>34</sup> cells in the control of food intake, we employed the CAFE assay [47] and measured food intake while AstA<sup>34</sup> cells were conditionally activated by the thermogenetic effector TrpA1. TrpA1 is a temperature sensor widely used to conditionally activate neurons by temperatures above 28°C [48,49]. Male AstA<sup>34</sup>→TrpA1 flies were raised on food at 20 or 22°C, and then assayed over a period of two days. At 29°C, but not at 20°/22°C, food consumption was significantly lowered in AstA<sup>34</sup>→TrpA1 flies (Fig 2A). A similar reduction of food intake at 29°C was detected for AstA<sup>1</sup>→TrpA1 flies (Fig 2B). This effect is not sex-specific, as a similar significant reduction in food intake was also observed in females (S3 Fig).

These results are consistent with a previous report showing reduced starvation-induced feeding upon constitutive activation of AstA cells by AstA<sup>1</sup>→NaChBac in a different feeding assay [28]. These findings indicate that the LCBR neurons (lacking in AstA<sup>1</sup>) and AstA cells in the GNG (lacking in AstA<sup>34</sup>) are dispensable to reduce food intake. Thus, activation of only the AstA<sup>34</sup> subset appears sufficient to reduce food intake.
To restrict the activation pattern further, we created *tsh-Gal80; AstA<sup>34>TrpA1</sup> (UAS-TrpA1/tsh-Gal80; AstA<sup>34>Gal4/+</sup>) flies. *tsh-Gal80* suppresses *Gal4* expression in the thoracic and abdominal part of the CNS [50–52], and limited *TrpA1* expression to *AstA<sup>34</sup>* central brain neurons and EECs (Fig 1E and 1I). Thermogenetic activation of this *AstA<sup>34</sup>* cell subset by a shift to 29°C was sufficient to reproduce the feeding phenotype found in *AstA<sup>34>TrpA1</sup>* flies (Fig 2C), indicating that the *AstA* neurons in the TAG and periphery are dispensable for feeding inhibition. A role for the *AstA* neurons in the optic lobe seems very unlikely due to their anatomy and since *AstA<sup>34>Gal4</sup>* driven expression in these neurons was inconsistent and weak and comprised only few of the many *AstA* optic lobe neurons. Thus, we conclude that the *AstA*-producing PLP cells and/or EECs are sufficient to control food intake.

So far, we had observed feeding inhibition upon activation of *AstA* cells. Inhibition of *AstA<sup>1</sup>* cells by constitutive expression of *UAS-Kir2.1* [30] has previously been reported to increase feeding under restricted food availability [28]. To exclude developmental effects due to constitutive silencing, we next conditionally manipulated *AstA* cells using the TARGET system [53]. At both 18°C and 30°C, *tubGal80<sup>ts</sup>;AstA<sup>34>Kir2.1</sup> flies showed a similar food intake as controls.

![Graphs and images showing food intake measurements and immunostaining](https://example.com/graphs.png)
under non-restricted food availability in the CAFE assay (S4 Fig). This suggests to us that signalling from PLP neurons or EECs is not essential for normal feeding behaviour and that PLP neurons and EECs are not core components of a feeding circuit. Rather, AstA cells modulate feeding circuits, and likely become functionally active only under specific circumstances, e.g. when flies are satiated or feeding will interfere with other behaviours. A similar situation has been found for *hugin*-expressing neurons in the *Drosophila* larva. When activated via TRPA1, they inhibit fictive pharyngeal pumping. When silenced or ablated, fictive pharyngeal pumping is unchanged compared to controls, suggesting a modulatory role of the anorexigenic *hugin* pyrokinin peptide [54].

**Reduced food intake upon AstA cell activation can be traced to Allatostatin A signalling**

Peptides are typically co-localised with other peptides or classic transmitters [55,56]. To identify whether the observed feeding phenotype upon activation of AstA cells is due to AstA or a co-localised peptide/transmitter, we used AstA<sup>SK4</sup> null mutant flies generated by germline-specific CRISPR/Cas9 [57]. In contrast to controls, AstA<sup>SK4</sup> mutants are devoid of any AstA-IR in the nervous system and gut (Fig 2E and 2F). We then thermogenetically activated the AstA<sup>1</sup> neurons in an AstA null mutant background and found no difference in food uptake compared to controls (Fig 2D). Similar observations were made when reducing AstA expression by RNAi in AstA<sup>34&gt;TrpA1/ AstA-RNAi</sup> flies (S5A Fig). Together, these experiments show that PLP neurons or EECs signal via AstA peptides to reduce food intake. The general lack of AstA without activation of AstA cells did, however, not reduce feeding under the experimental conditions; controls in wildtype and AstA<sup(SK4)</sup> mutant background showed similar amounts of ingested food (Fig 2D).

**Activation of AstA PLP neurons and EEC decreases locomotor activity and promotes sleep**

Locomotor activity affects energy expenditure and consequently also appetite, and is in turn altered by hunger and feeding. We therefore asked whether activation of AstA cells affects locomotor activity. Flies were kept in small glass tubes on agar-sucrose food and their locomotor activity was monitored using the DAM system. Compared to controls, the average locomotor activity of AstA<sup>1&gt;TrpA1</sup> and AstA<sup>34&gt;TrpA1</sup> flies was strongly and significantly reduced at 29°C, but not at 22°C in both sexes (Fig 3A and 3B, S6 Fig). In contrast, AstA<sup>1&gt;TrpA1</sup> flies were not impaired in climbing ability in a startle-induced negative geotaxis assay, independent of being fed or starved for 24h at 29°C (Fig 3F), showing that the flies were not suffering from impaired locomotor ability or energy deficiency due to decreased feeding.

To analyse locomotor activity in the CAFE assay, we video-monitored activity of AstA<sup>1&gt;TrpA1</sup> males in a slightly modified setup using Petri dishes instead of a 24 well plate. Prior to testing, flies were starved for 24h at 29°C but had free access to water. After placement into the Petri dish, we filmed pairs of flies at 29°C for 4 hours and visually categorised their behaviour (not moving, moving, feeding). Fig 3C shows that AstA<sup>1&gt;TrpA1</sup> spent much less time moving as well as feeding compared to AstA<sup>x w<sup>1118</sup></sup> controls, with individual variations within both strains (S7 Fig). Nevertheless, AstA<sup>1&gt;TrpA1</sup> flies were fully capable of locating the capillary and did not stay there longer than controls, which would have allowed them to feed without moving (S7 Fig).

We next monitored the locomotor activity of *ish-Gal80; AstA<sup>34&gt;TrpA1</sup>* flies (Fig 3D) and found a reduction of locomotor activity similar to AstA<sup>1&gt;TrpA1</sup> flies upon thermogenetic activation. (Fig 3A). Activation of PLP neurons and/or the AstA EECs seems thus sufficient to
reduce locomotor activity. The inhibitory effect is again mediated by AstA peptide signalling, since thermogenetic activation of AstA cells in AstA
1
TrpA1 flies in the AstA
SK4
null mutant background did not significantly alter locomotor activity (Fig 3E). The rhythmicity and period of locomotor activity [58] was not affected by activation of AstA cells in AstA
1
TrpA1 and AstA
34
TrpA1 flies at 29°C and constant darkness (Fig 4). Strikingly, however, subjective even-ing activity was lost. (Fig 4A and 4B). A general lack of AstA without activation of AstA cells did not influence locomotor activity, as controls in wildtype and AstA
SK4
mutant background showed similar activity levels (Fig 3E).

A strongly reduced locomotor activity is suggestive of abnormal sleep. Applying the widely used 5 min inactivity criterion [59], we found that in fact thermogenetic activation of the AstA
1
and AstA
34
cells strongly promotes sleep, which is most apparent during the morning and evening activity peaks in both males (Fig 5) and females (S8 Fig). At 29°C, but not at 22°C, AstA
1
TrpA1 and AstA
34
TrpA1 flies showed a significant increase in both total amount of sleep and sleep bout duration (Fig 5). Thermogenetic activation of AstA
1
and AstA
34
cells
Fig 4. Locomotor activity in the DAM system under constant conditions. (A) Typical double-plotted actograms of AstA\textsuperscript{34} > TrpA1, AstA\textsuperscript{1} > TrpA1 and control flies kept for three days at 22˚C and L:D 12:12, then switched to 29˚C and constant darkness (DD, red arrows). (B) Average actograms for all rhythmic flies tested (non-rhythmic flies were excluded). Flies with activated AstA cells showed not only a decreased activity, but also a lack of evening activity. (C) The rhythmicity and period is unchanged compared to controls. (D-E) Total sleep amount and sleep bound duration is significantly increased upon AstA\textsuperscript{1} (D) and Asta\textsuperscript{34} (E) cell activation in DD.

doi:10.1371/journal.pgen.1006346.g004
Fig 5. Thermogenic activation of AstA cells strongly promoted sleep. At 22˚C, AstA1>TrpA1 (A) and AstA34>TrpA1 male flies (C) did not sleep more than controls. Activation of the TrpA1 channel at 29˚C resulted in increased sleep time of AstA1>TrpA1 (B) and AstA34>TrpA1 (D) flies especially during the time of the morning and evening activity. For both AstA1>TrpA1 (E) and AstA34>TrpA1 (F), mean sleep bout duration and the total amount of sleep per day was significantly increased when AstA cells were thermogenetically activated.

doi:10.1371/journal.pgen.1006346.g005
significantly increased total sleep and sleep bout duration also under constant darkness (Fig 4D and 4E), and constant light conditions known to disrupt the clock (S9 Fig). Next we silenced AstA cells by constitutive expression of UAS-Kir2.1 [30], yet without effect on activity or sleep (S10 Fig). However, when we conditionally silenced AstA cells using the TARGET system [53] and UAS-Kir2.1, sleep was significantly affected especially during the midday siesta time (Fig 6). This is in line with a significant increase in total activity (S11A Fig). A similar increase in locomotor activity and decrease in sleep upon UAS-Kir2.1 silencing was also observable in constant darkness, while rhythmicity and period of the locomotor rhythm was not affected (S12 Fig). An alternative neuronal silencer, UAS-ΔORK [60], did also not reduce sleep when constitutively expressed (S13 Fig). Under conditional expression, however, UAS-ΔORK lead to a significant increase in sleep only during the evening activity, and unexpectedly to decreased sleep during the early siesta time (S13 Fig).

To test for sleep intensity, we determined the arousal threshold during the day in two different assays (Fig 7). For the first assay, AstA<sup>34</sup>TrpAI flies were put into glass tubes as used in the DAM monitor, and kept for three days at 29°C to thermogenetically activate AstA cells. On day four, the tubes were placed onto a loudspeaker at 29°C. Five separated 5Hz sine wave stimuli were given with increasing intensity every hour during the light phase from Zeitgeber Time 1 (ZT1) to ZT12, and velocity and distance walked for 2 min after each stimulus was measured. As expected [61], the arousal-related parameters were dynamic during the day and varied somewhat between genotypes in the controls (Fig 7A and 7B). Notwithstanding, AstA<sup>34</sup>TrpAI flies walked on average significantly slower and covered less distance for all stimulus intensities and at all times during the light phase than controls (Fig 7A and 7B). Again, this phenotype is unlikely to be caused by impaired locomotor ability since the maximum speed reached by individual flies was similar between AstA<sup>34</sup>TrpAI flies and controls (S14 Fig).

For the second assay, flies were put in small groups into Petri dishes and kept again for three days at 29°C. On day four, we monitored their activity in the Petri dishes placed on a shaker at 29°C during the light phase to better mimic the situation during the CAFE assay. The Petri dish was hourly agitated in a series of five 2s shakes with increasing speed separated by a 5 min break during which fly behaviour was manually analysed for the fraction of aroused flies after each stimulus. Again, control flies showed a dynamic arousal threshold that was higher during the afternoon "siesta" as expected (Fig 7C, S3 Movie), and a distinctly smaller percentage of aroused flies was observed for AstA<sup>34</sup>TrpAI flies at all time points and intensities. Strikingly, the percentage of aroused flies was steadily decreasing during the course of the day and was lowest at the time of the evening peak activity (Fig 7C).

### Starvation decreases sleep in flies with activated AstA cells

Sleep and feeding are interconnected behaviours, and it is interesting to ask whether flies with activated AstA cells are prevented from eating more because their locomotor activity is reduced, leading to insufficient foraging activity although flies are "hungry". Alternatively, flies with activated AstA cells may eat less because they need less energy intake since they move less, and thus are "satiated". To find out which scenario applies, we monitored food intake in AstA<sup>1</sup>TrpAI and AstA<sup>34</sup>TrpAI flies that prior to the CAFE assay at 22°C had been kept under assay conditions for one day at 22°C and then for two days at 29°C to activate AstA signalling. Under these conditions, both AstA<sup>1</sup>TrpAI and AstA<sup>34</sup>TrpAI flies showed no feeding rebound after release from thermogenetical activation of AstA signalling (S15 Fig). This suggests that flies with activated AstA signalling are not in a hunger state, and further indicates that the observed feeding phenotype is not due to impaired locomotor ability. To test this
Fig 6. Conditional silencing of AstA^{34} cells by ectopic expression of the inward rectifying K^+ channel Kir2.1 decreases sleep. (A+B) Averaged sleep over 24h of tubGal80^{ts}; AstA^{34}-Gal4 x UAS-Kir 2.1 experimental flies and controls. (C+D) Average sleep bout duration and total amount of sleep calculated from A+B. At 18°C, tubGal80^{ts} inhibits ectopic expression of Kir2.1 and experimental flies show a similar sleep behaviour as controls (A, C-D). At 30°C, Kir2.1 is expressed in AstA^{34} cells and causes a significant reduction of total sleep and average sleep bout duration (C+D), both during the light and dark phase (B). p ≤ 0.05. ** p ≤ 0.01, *** p ≤ 0.001.

doi:10.1371/journal.pgen.1006346.g006
further, we monitored the locomotor activity of fed (agarose with sugar) and starved (agarose without sugar) flies with thermogenetically activated AstA neurons. Wildtype flies respond to prolonged starvation with a phase of hyperactivity, interpreted as a hunger-driven food search [62,63]. Likewise, AstA^{34}\triangleright TrpA1 flies on starvation medium increased locomotor activity/reduced sleep compared to flies on food (Fig 7D). This provides further evidence that flies with activated AstA cells kept on food do not feel hungry. Off food, these flies become hungry as judged by their observed hyperactivity which argues against a general locomotor impairment.

Fig 7. Mechanically- and starvation-induced activity. (A-B) An increasing level of mechanical stimuli by a loudspeaker (shown as dashed lines) were used to arouse flies in a glass tube (for details see material and methods). Thermogenetic activation of AstA cells in AstA^{34}\triangleright TrpA1 flies resulted in decreased average velocity (A) and a shorter distance walked (B) in a 2 min window after each stimulus compared to controls (n = 5). (C) An increasing level of mechanical stimuli were used to arouse flies in a Petri dish on a shaker (for details see material and methods). In general, the percentage of aroused flies increased with increasing shaking speed. While the arousal threshold for control flies seems to increase during the siesta phase during the middle of the day to decrease again towards the evening activity peak, there is a steady decline of the percentage of aroused flies during the day (n = 15). (D) Starvation-induced locomotor hyperactivity in flies with thermogenetically activated AstA cells reduces sleep during the morning and evening activity. Flies were kept at 20°C in LD12:12 on normal food, and then transferred to DAM glass tubes and switched to 29°C and feeding/starvation-conditions at ZT8 at the start of locomotor activity monitoring (n = 32).

doi:10.1371/journal.pgen.1006346.g007
in flies with activated AstA cells. The same phenotype was also seen with AstA^TrpA1 flies (S16 Fig). Obviously, the sleep-promoting effect of AstA neurons can at least partially be overcome by starvation, arguing against a direct dependence between the sleep-promoting and anorexic effect of AstA cells.

**Genetic distinction between AstA-expressing PLP neurons and EECs**

So far, we could show that thermogenetic activation of AstA-signalling from PLP neurons and/or EECs inhibits feeding and promotes sleep. To distinguish between these AstA cell subsets, we next aimed to further restrict the thermogenetic activation to AstA EECs only, using pan-neuronal elav-Gal80 [64]. To our surprise, elav-Gal80 not only efficiently suppressed GFP expression in AstA neurons, but also in EECs (see S16C and S16D Fig). Since elav was reported to be specifically expressed in neurons and glia [65,66], we tested for elav expression in the midgut by immunostaining with an anti-ELAV monoclonal antibody which strongly and specifically stained EECs in the midgut (S17A and S17B Fig). A similar pattern was found when expressing GFP with an elav-Gal4 driver line (S17E Fig). This indicates that the widely used panneuronal elav-Gal4 drivers cannot be regarded as neuron/nervous system-specific, and suggests a role for elav in EEC differentiation. A second Gal80 line used to restrict Gal4 expression to the nervous system is nsyb-Gal80 [67]. We found no nsyb>GFP expression in EECs, and tried to restrict AstA^GFP expression to the EECs by co-expression of nsyb-Gal80. Co-expression of Gal80 inhibited the expression of GFP in AstA neurons, but to our surprise also in the midgut EECs. In line with that, nsyb-Gal80 completely suppressed the behavioural effects observed upon thermoactivation of the AstA^GFP cells (S18 Fig). These results caution against the assumption that Gal80 patterns always fully replicate the respective Gal4 pattern. Prospero is a EEC-specific marker for the gut [68,69], but expresses also broadly in the adult CNS [70] which prevented the use of prospero-Gal4 for thermogenetic activation. Thus, we were unable to further genetically differentiate between PLP neurons and AstA EECs.

**The AstA-expressing PLP neurons are a direct target of the clock output factor PDF**

During our morphological analysis (Fig 1) we noticed that the PLP neurites in the superior protocerebrum make branches in the same area as the PDF-expressing small ventral lateral neurons (sLNvs), a main component of the central circadian clock. The neuropeptide PDF is a major synchronisation and output factor of the circadian clock [71] which affects the timing of sleep and feeding [18,72]. We therefore wanted to know whether the PLP neurons represent downstream targets of circadian PDF-signalling. Confocal microscopy first showed that indeed the projections of sLNvs and PLP neurons are overlapping in the superior protocerebrum (Fig 8A and 8B). While the sLNv projections represent mainly output sites [73], the PLP neurites seem to be postsynaptic as indicated by the expression of the postsynaptic marker DenMark:mcherry (Fig 8A and 8B). Using live cAMP imaging, we next asked whether PLP neurons express functional PDF receptors. Synthetic PDF was bath-applied to acutely isolated brains that expressed the cAMP sensor Epac-camps in the AstA^GFP neurons. A similar approach had previously been very successful to demonstrate functional PDF receptors on clock neurons [74]. The PLP neurons reacted with a fast increase in intracellular cAMP upon 10 μM PDF (Fig 8C and 8D), while control applications of saline had no effect. This PDF-mediated cAMP increase appeared to be by direct activation of PDF receptors on the PLP neurons since a similar cAMP increase was also seen after blocking neuronal conduction by tetrodotoxin (TTX, Fig 8C and 8D). PDF application had no effect on the PLP neurons in a PDF receptor mutant background (han^5304 [75], Fig 8C and 8D). We also found that the PDFR expression reporter
Fig 8. The AstA-expressing PLP neurons are a downstream target of the clock output factor PDF. (A') A 2 μm confocal section through the superior protocerebrum containing the arborisations of the PLP neurons immunolabelled against AstA. (A'') The same section contains the terminals of the PDF-expressing sLNv clock neurons visualised by immunostaining against PDF. (A''') Both peptidergic arborisations are in close apposition to each other. (B) The PLP arborisations in the superior protocerebrum are more extensively labelled by the postsynaptic marker UAS-DenMark (B') than by AstA staining (B''). Fine branches that are only DenMark-labelled but AstA-negative are evident in the merged confocal section (B'''), suggesting that these branches represent input sites of the PLP neurons. (B''') The DenMark labeling of PLP arborisations in an AstA1>DenMark brain extends over a large area in the superior protocerebrum and is not restricted to the site of contact with the sLNv. The asterisk marks DenMark-labeled dendrites of the AstA cells in the ventral brain/gnathal ganglia. Maximum projection of the PLP neurons. Scale bars: 10 μm, B'''' 50 μm. (C-D) Ex vivo live-cAMP imaging of central brain Allatostatin-A neurons. (C) Average inverse FRET traces (CFP/YFP) of Allatostatin-A neurons reflecting intracellular changes in cAMP levels. Substances were bath applied drop-wise between recording seconds 100 and 110 (black bar). Application of 10μM of the adenylate cyclase
pdfr-myc \[76\] is weakly but consistently expressed in the PLP neurons (Fig 8E). Only very few further neurons in that area of the superior protocerebrum were weakly myc-positive; strongly myc-positive neurons comparable in staining intensity to the sLNvs were absent in that part of the brain. These results suggest that the PLP neurons represent downstream targets of circa-dian PDF signalling.

**Activation of AstA cells by tethered PDF increases sleep**

To investigate the functional significance of PDF-PLP neuron signalling, we first aimed to down-regulate the expression of the PDF receptor by RNAi in AstA neurons. In preliminary test, however, none of the tested VDRC or Janelia PDFR RNAi-lines had an effect on circadian locomotor activity when expressed in PDF and other clock neurons (Pamela Menegazzi, pers. commun.), indicating a general lack-of-effect in these lines. We therefore switched to constitutive activation of PDF signalling by expressing membrane-tethered PDF (t-PDF) in AstA cells. A similar approach has been successfully used to study the sleep effects of calcitonin gene-related peptide/DH31 [77]. t-PDF activated co-expressed PDFR in heterologous cell culture and rescued rhythmicity when specifically expressed in clock neurons in a pdf
\[525\]01
mutant background [78] When expressed in AstA cells, t-PDF induced a significant increase in total sleep compared to Gal4/UAS controls and flies expressing a scrambled non-functional version of t-PDF (Fig 9A, 9B, 9D and 9E). In accordance, total activity in AstA>t-PDF flies was significantly reduced to about half of that of controls (Fig 9E). The effect of t-PDF expression, compared to Gal4/UAS controls, was most pronounced during the evening activity when no or little native PDF is released, and small during the peak time of native PDF release in the morning hours ([79,80], Fig 9A). Compared to flies expressing a scrambled version of PDFR, the effect of t-PDF expression was most pronounced during the light phase, and less pronounced during the dark phase when also the PDFR-SCR control flies slept most of the time (Fig 9D and 9E).

This suggests that t-PDF-induced PDFR signalling activates AstA cells, in line with the reported activating effect of t-PDF on sLNvs [81] and that this ectopic activation is most effective when native PDF release is absent. The timing of the activity peaks was unaltered. In addition, AstA>t-PDF flies fed significantly less than the PDFR-SCR and UAS-TRPA1 control (Fig 9C), again in line with the notion that t-PDF increases the activation of AstA cells. No significant difference, however, was detectable for the AstA-Gal4 control. We note that for the t-PDF-SCR expressing flies the total amount of sleep and the sleep bout duration was considerably lower than for other controls (Fig 9B), mostly due to a low amount of sleep during the day (Fig 9A).
Fig 9. Effect of ectopic expression of tethered PDF (t-PDF) in AstA<sup>34</sup> cells on sleep (A-B, D-E) and food intake (C,F). (A-C) Experiments using heterozygous controls. (A) t-PDF expression induced a small increase in sleep especially during the time of the evening activity. (B) Total sleep amount but not sleep bout duration was significantly increased by t-PDF expression. (C) t-PDF expression did not significantly reduce food intake over genetic controls. (D-F) Experiments using t-PDF-SCR as a control. (D) t-PDF expression induced increased sleep mostly during the light phase and lights-on anticipation compared to the AstA<sup>34</sup>t-PDF-SCR control. (E) Quantification shows that mean sleep bout duration and the total amount of sleep was significantly increased by t-PDF expression. (F) t-PDF expression also significantly reduced food intake. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.

doi:10.1371/journal.pgen.1006346.g009
The observed changes in sleep after expression of t-PDFR are considerably smaller but go in the same direction than the changes observed upon activation of AstA\textsuperscript{34} cells (Fig 5), suggesting that PDF positively modulates rather than strongly activates AstA\textsuperscript{34} cell activity. To test this assumption, we thermogenetically activated the PDF-expressing sLNvs using the R6-Gal4 driver line [82]. As expected if PDF activates AstA cells, activating sLNvs increased sleep and not activity. Yet, the effect was limited to the time of morning and evening peak activity and was -again- much smaller compared to thermogenetic activation of the AstA cells (Fig 10A). Total sleep and sleep bout duration over the day was not significantly altered (Fig 10A’’). We cannot exclude that this mild effect is at least in part caused by co-activation of one or two large LNvs which weakly express R6-Gal4 [83] and have been shown to promote arousal [72].

Based on these results and the anatomical and imaging data, we conclude that PDF from the sLNvs positively modulates PLP neurons without affecting the phase and general timing of AstA-regulated behaviours.

**Discussion**

Our study shows that AstA cells via AstA signalling subserve an anorexigenic and sleep-promoting function in *Drosophila*. In mammals, a variety of neuropeptides and peptide hormones affect both sleep and feeding [4,5], and our results provide evidence that also further such peptides exist in the fly besides sNPF and possibly NPF [21,24,25]. More specifically, our results with a new AstA\textsuperscript{34}-Gal4 driver line show that activation of AstA-expressing PLP brain neurons or numerous EECs in the midgut strongly reduces food intake and promotes sleep. These behavioural effects are congruent with the anatomy of these cells. PLP interneurons are well positioned to modulate sleep as they widely arborise in the posterior superior protocerebrum, a projection area of sleep-relevant dopaminergic neurons [84,85], superior (dorsal) fan-shaped body neurons [86–88] and neurons of the pars intercerebralis [89]. AstA EECs in *Drosophila* are “open type” EECs [31,32], possessing apical extensions that reach the gut lumen and likely express gustatory receptors [90]. AstA-expressing EECs are thus potentially able to humorally signal nutritional information from the gut to brain centres regulating feeding and possibly also sleep and locomotor activity. If AstA is involved in inhibiting feeding and promoting sleep, one could expect AstA mutants to display decreased sleep and increased feeding in the absence of any other manipulation of AstA cells. We observed, however, that a functional loss of the AstA gene did neither affect feeding nor locomotor activity under the experimental conditions with unrestricted access to a food source. This may suggest that AstA signalling is not part of a core feeding network, but represents an extrinsic modulator which becomes activated under specific yet so far uncharacterised conditions. Alternatively, as suggested by the observed difference in effect of constitutive vs. conditional electrical silencing of AstA cells, flies may be able to genetically or neuronally compensate for a constitutive loss of AstA signalling during development.

In larval *Drosophila*, AstA inhibits midgut peristalsis and affects K\textsuperscript{+} transport [35] in order to concentrate ingested food. Together with our finding of a sleep-promoting and feeding-inhibiting effect of AstA, we propose that pleiotropic AstA signalling serves to coordinate behaviour and gut physiology to allow for efficient digestion. After food intake, AstA from the PLP neurons or EECs cause inhibition of further feeding, and -as the need for food search behaviour is relieved and nutrients need to be taken up- promotes sleep and inhibits gut peristalsis. Based on the gut content, enteroendocrine AstA is released and hormonally activates DAR-2 on key metabolic centers to tune adipokinetic hormone and insulin signalling [91], and -at least in other insects- stimulates digestive enzyme activity in the midgut [92,93].
Fig 10. Activation of the PDF-expressing sLNvs promotes sleep specifically during the time of morning and evening peak activity. (A) At 22˚C, R6>TrpA1 flies showed the same sleep pattern than controls. Activation of the TrpA1 channel at 29˚C resulted in increased sleep time specifically during the time of the morning and, to a lesser amount, the evening activity (B). (C) Both mean sleep bout duration and the total amount of sleep per day was not affected by activation of the sLNvs.

doi:10.1371/journal.pgen.1006346.g010
The AstA receptors are homologues of the vertebrate galanin receptors [40–43] that have pleiotropic functions [44]. When activated in specific brain areas, galanin signalling has a strong orexigenic effect [45] and has also been implicated in the control of arousal and sleep in mammals [45]. In zebrafish, transgenic heat-shock induced expression of galanin decreased swimming activity, the latency to rest at night and decreased the responsiveness to various stimuli [94]. Furthermore, the allatostatin/galanin-like receptor NPR-9 inhibits local search behaviour on food in the nematode C. elegans [95]. Similar to AstA in Drosophila [35], galanin modulates intestinal motility and ion transport [44]. Thus, in broad terms, the involvement of DARs/galanin receptors in modulating feeding, gut physiology and arousal/sleep appears to be evolutionarily conserved.

The neuronal clock network in Drosophila is intrinsically and extrinsically modulated by a variety of peptides (sNPF, NPF, calcitonin-gene related peptide/DH31, ion transport peptide, myoinhibiting peptides and PDF), which all affect sleep and locomotor activity and in part also act as clock output factors [24,77,96–100]. Our imaging results and constitutive activation of the PDF signalling pathway by t-PDF now suggest that the PLP neurons are modulated by PDF originating from the sLNv clock neurons. Unlike the peptides above, AstA from PLP neurons is outside and downstream of the central clock and seems not to modulate the clock network. Due to their anatomy and position, PLP neurons thus appear well-suited candidate cells by which clock neurons could modulate the complex cross-regulatory network regulating sleep, locomotor activity and perhaps also feeding. The rather mild effects on sleep and feeding of either t-PDF expression in AstA cells or thermogenetic activation of the sLNvs implies that this pathway is not the major output target of the central clock (if there is any) to modulate feeding and locomotor activity/sleep. We found no shift in the circadian period or phase of feeding and locomotory activity/sleep upon AstA cell activation, suggesting that the main function of PDF-to-AstA cell signalling is not to time the respective behaviours but to modulate their amplitude. Similar non-timing functions of PDF have been demonstrated for other behaviours, including geotaxis and rival-induced mating duration [101,102].

At first sight, our data suggesting that PDF activates PLP neurons to promote sleep seem to contradict earlier findings [72]. Since pdf01 mutants show increased sleep during the photo-phase, the arousing effect appears to be the dominant effect of PDF which is due to signalling between ventral lateral clock neurons (LNvs) [72], with a major contribution of the PDF-expressing large LNvs [103]. The PLP neurons are only contacted by the sLNvs, which upon activation induced a time-specific increase in sleep, but did not increase arousal. Thus, the sLNv-PLP pathway likely represents a sleep-promoting clock output branch. Besides PDF, the sLNvs but not the lLNvs also co-localise the sleep-promoting peptide sNPF [24]. A recent report shows that hormonal PDF released from abdominal PDF neurons serves to couple the central clock with a peripheral clock in the oenocytes [104]. Furthermore, the posterior midgut is innervated by the abdominal PDF neurons [32], and PDFR is expressed in the midgut [105]. It is thus possible that the AstA-expressing EECs represent additional PDF targets and may contribute to the PDF-related effects of AstA cells.

In conclusion, the lack of effect on feeding upon AstA cell silencing under non-restricted food availability and an unaltered circadian locomotor rhythmicity after AstA cell silencing suggests that AstA signalling is neither a primary signal in feeding regulation nor in the clock output pathway timing rhythmic behaviour. Rather—like mammalian galanin signalling [45]—it seems to be one out of several modulatory pathways that allow to adapt the intensity of feeding and locomotor activity/sleep to specific physiological or environmental conditions. For example, decreased locomotor activity to save energy and increased digestion efficiency to maximise energy uptake may be most important during restricted food conditions, at which AstA cell silencing leads to increased feeding [28]. While our results allow now to raise such
speculations, it is clear that more research is needed to reveal the conditions at which AstA signalling is functional and the modulatory PDF input is strongest.

Materials and Methods

Flies

Following strains were used: w;AstA1-Gal4 [28], kindly provided by D. Anderson, Caltech, CA, USA, w;tsh-Gal80/CyO (kindly provided by J. Simpson), elav-Gal4 (Bloomington Stock Center), elav-Gal80 [64], kindly provided by LY and YN Jan), w;nsyb-Gal80 [67], kindly provided by Stephen F. Goodwin—originally from J. Simpson), prospero-Gal4 (kind gift of J. F. Ferveur), nsyb-Gal4 (kindly provided by T. Langenhan), 386y-Gal4 and w;Pdfr-myc ([76,106], kindly provided by Paul Taghert), UAS-Dcr-2 (VDRC Stock #60007) UAS-AstA-RNAi (VDRC Stock #103215 KK), w;UAS-DenMark [107], kind gift of Bassem Hassan), UAS-Epac1camps [74], 10xUAS-IVS-myr::GFP ([108], Bloomington Stock Center), UAS-tethered-PDF (UAS-t-PDF-M6a containing one transgene copy, and control UAS-t-PDF-SCR A2 [78], kindly provided by Joel Levine), w;UAS-TrpA1 (Bloomington Stock Center), w;UAS-Kir2.1 ([30], w; UAS-ΔOrk-ΔC1 and w;UAS-ΔOrk-ΔNC1 ([60], Bloomington Stock center), UAS-tubGal80 [53] han [53], and Canton-S wildtype and w [118] for control crossings (all from Bloomington Stock Center). Flies were kept on standard Drosophila medium (Supplementary file 1) at a 12:12 h light-dark cycle (LD, in which lights-on is defined as ZT0 and lights-off as ZT12) and 25°C, except for the crossings used in TrpA1 experiments, which were kept at 20 or 22°C.

Creation of AstA promoter-Gal4 transgenic flies

The putative D. melanogaster allatostatin A promoter region was amplified from genomic DNA by PCR using three different primer sets that amplified 1.03 kb, 2.05 kb and 2.74 kb upstream of the transcription initiation site (see S1 Text). The resulting PCR products were cloned into pCR-TOPO. The inserts were digested with MunI and BamHI, gel purified and exchanged with the Akh promoter in the pAkh-Gal4 vector [109]. The resulting P{pAstA--Gal4} plasmids were injected into Drosophila embryos by BestGene Inc. (Chino Hills, CA, USA) and at least 5 independent P-element transformant lines per construct were obtained. While the short 1.03 kb promoter fragment failed to direct GAL4 expression to AstA-immunoreactive (IR) cells, longer 2.05 and 2.74 kb promoter fragments lead to GAL4 expression in varying subsets of AstA-IR cells in the larval CNS and midgut (S1 Fig). We chose the 2.74 kb promoter line AstA34-Gal4 for our experiments, since it showed the most restricted and AstA-specific cellular distribution among the different 2.74 kb promoter lines generated by P element transposition (S1 Fig).

Creation of AstA mutant flies

The generation of AstA mutants by germline-specific expression of Cas9 and guide RNA (gRNA) transgenes [57] was already described [91]. Mutant stocks were established from two alleles, AstA SK1 (used by [91]) and AstA SK4 (w [118];AstA SK4 used throughout this study), in which the start codon of the AstA gene is removed.

Immunostaining

Tissue of feeding 3rd instar larvae or adult flies (approx. 1 week after eclosion at 25°C) was dissected in HL3.1 solution [110] and fixed in 4% PFA/PBS (pH 7.2) at room temperature for 45 min (guts and larval CNS) or 90 min (adult CNS). After several washes with PBT (= PBS with 0.3% Triton X) followed by an overnight blocking step with PBT containing 10% normal goat
serum at room temperature, the tissue was incubated in primary antibody solution on a shaker for 1d at 4°C, then several hours at room temperature. Primary antibodies were diluted in PBT containing 3% normal goat serum. 1d of washing steps with PBT followed, after which the samples were incubated with secondary antibodies diluted 1:300 in PBT containing 3% normal goat serum. Samples were again washed several times with PBT, then twice with PBS and finally mounted onto microscope slides using 80% glycerol/20% PBS. Images were acquired with a Leica TCS SPE or SP8 confocal microscope (Leica, Wetzlar, Germany). Fiji [111] was applied for maximum intensity projection and contrast enhancement. Figures were generated with Adobe Photoshop CS2.

Primary antibodies used were a mouse anti-GFP IgG mAb (1:100, A11120, Invitrogen GmbH, Karlsruhe, Germany), a rat anti-ELAV mAb (1:100, 7E8A10, developed by GM Rubin, obtained from the Developmental Studies Hybridoma Bank, University of Iowa, IA, USA), monoclonal rat anti-mCherry (1:1000, Molecular Probes, Frederick MD, USA), mouse anti-Myc-tag mAb (1:1000, 9B11 mouse, mAb New England Biolabs, Frankfurt, Germany) and a polyclonal rabbit antiserum directed against Dippu-AstA-7 (1:2000, [112], Jena Bioscience GmbH, Germany) which recognizes the C-terminal YXFGL-amide of AstA peptides including that of Drome-AstA-1–3 [113]. Alexa Fluor 532 and 647- or DyLight 488-conjugated IgG (H +L) secondary antibodies were purchased from Dianova GmbH, Hamburg, Germany.

Capillary Feeder (CAFE) assay

The CAFE protocol followed [47]. 4–5 old male or female flies were anesthetized on ice and transferred into 24-well plates (1 fly per well) containing several small holes in each well to allow for air exchange. A piece of moist filter paper was added to each well to provide the flies with water separately from the food. Capillaries (5 μl glass capillary pipettes, Megro GmbH & Co. KG, Wesel, Germany) were filled with liquid food and one capillary per well was inserted through a hole in the lid of the well plate so that the bottom was easily accessible to the fly. Food capillaries in wells without flies were used to control for evaporation. The amount of evaporated liquid in these control capillaries was subtracted from the other capillaries in the capillary assays. The plates were put into an airtight, humid container and placed into an incubator with a 12:12 h LD at 22°C or 29°C. Liquid food was prepared fresh every day and contained: 5.4% sucrose, 3.6% yeast extract (BioChemica, AppliChem, Darmstadt, Germany) and 0.03% BPB (Bromophenol blue sodium salt, electrophoresis grade, AppliChem, Darmstadt, Germany) (all m/v) in ultrapure water. Capillaries were exchanged each day at the same time. Food consumption was not measured for the first day to give the flies some time to acclimatize to the change of environment and food. Values measured for day 2 and 3 (descent of the meniscus) were summed up for each fly.

Startle-induced negative geotaxis assay

Four to five days old flies of each genotype were kept for 24h at 22°C on normal food, 29°C with normal food or at 29°C with water only. For each trial, 10 male flies were transferred to a 50ml falcon tube. These tubes were tapped gently on the table, and the number of flies which climbed over an 8 cm marker within 10 seconds was calculated. For each experiment, this was repeated 10 times.

Locomotor activity and sleep measurement

Drosophila Activity Monitors (DAM, TriKinetics Inc., Waltham, MA, USA) were used to measure locomotor activity. 4–5 days old adult males or females were transferred to separate glass tubes containing an agar-sucrose food medium (prepared from 2% agar and 4% sucrose in...
ultrapure water by brief boiling), after which the tubes were closed with foam plugs. The tubes were inserted into holes in the monitor and centered. As a fly walked back and forth within its tube, it interrupted an infrared beam that crossed the tube at its midpoint. Light beam interruptions were counted for individual flies at 1-min intervals as a measure of fly activity. Flies were monitored under a 12:12 h LD with 365 lux light intensity at 22°C and subsequently at 29°C; average minute-by-minute activities during the day were calculated for both conditions. Activity and sleep data was analysed using ActogramJ [114] and a custom-made Excel macro by Taishi Yoshii [115].

**Arousal assay**

Flies were kept in a 29°C incubator, LD12:12, for 3d prior to the experiments. Two different arousal setups were used, with flies kept in tubes or Petri dishes, respectively.

**Tube assay:** During the assay, each fly was individually housed in a 65mm glass tube (Trikinetics). For each experiment, we used 5 tubes for each genotype, which were laid on a loudspeaker (VISATON WS 25E, 8Ω). On the 4th day from ZT1 to ZT12, stimuli of increasing intensity between 0.4 and 2.0 volt (steps of 0.4 Volt) were consecutively delivered and behaviour was recorded by a camera (PENTAX TV LENS 25mm 1:1.4) at 1 Hz using IC Capture 2.2 software. Stimuli were generated with a PHILIPS PM 5139 function generator coupled to a TAURUS A2100 stereo amplifier and a loudspeaker to generate a 5Hz sine wave to wake up the flies. The interval between the individual stimuli was between 5–8 min. Average walking velocity (cm/s) and stimulus-induced walking distance for a 2 min window after each stimulus was analysed with MetaMorph version 7.8.0 (Molecular Devices, Sunnyvale, CA, USA) from ZT1 to ZT 12.

**Petri dish assay:** 5 flies were housed in a Petri dish filled with 2% agarose containing 4% sucrose on a shaker (Edmund Bühler KL-2, Tübingen, Germany). On the 4th day from ZT1 to ZT 12, five mechanical shakes with increasing speed (50, 100, 200, 300 and 400 rpm) were delivered for 2 seconds. The interval between the individual stimuli was between 5–8 min. Fly behaviour was recorded at 1 frame/s for 12h and locomotor activity was measured by eye, then arousal thresholds (percentage of flies moving) were calculated for each stimulus.

**cAMP live imaging**

The ratiometric cAMP sensor UAS-Epac1camps [116] was expressed under the control of the AstA34-Gal4 driver line. Homozygous 5–7 days old male w;UAS-Epac1camps;AstA34-Gal4 flies were freshly dissected in cold Hemolymph-like saline (HL3 [117]) and isolated brains were mounted with the posterior surface up on the bottom of a Petri-dish containing HL3. Brains were allowed to recover from dissection for 15min prior to imaging. Live-imaging was conducted using an epifluorescent imaging setup (Zeiss Axios Examiner D1, Specta-X hybrid solid state LED source, or a VisiChrome High Speed Polychromator System with a ZEISS Axioskop2 FS plus, Visiion Systems GmbH, Puchheim, Germany) equipped with a 40x dipping objective (Zeiss 20x/1.0 DIC M27). Central brain AstA neurons were brought into focus and regions of interest (ROIs) were defined on single cell bodies using the Visiview Software (version 2.1.1, Visiion Systems, Puchheim, Germany). Time lapse frames were imaged with 0.2Hz by exciting CFP. CFP and YFP emissions were separately recorded with sCMOS cameras (pc.edge 4.2., PCO AG, Kelheim, Germany, connected via a Cairn TwinCam) or with a CCD-camera (Photometrics, CoolSNAP HQ, Visiion Systems GmbH using a beam splitter). After measuring baseline FRETs for 100s, substances were bath applied drop-wise between recording seconds 100 and 110. PDF peptide was synthesized by Iris Biotech GmbH (Marktredwitz, Germany) and was applied in a concentration of 10μM in 0.1% DMSO in HL3. The water-soluble forskolin derivate
NKH477 served as positive control in a concentration of 10μM, while HL3 alone was applied as negative control. Both negative and positive controls also contained 0.1% DMSO. For tetrodotoxin (TTX) treatments, brains were incubated for 15min in 2μM TTX in HL3 prior to imaging and substances were coapplied together with 2μM TTX (as described in [118]). Intensity data for CFP and YFP emissions of each ROI were exported into Excel and inverse FRET (iFRET) was calculated over time according to the following equation: \[ \text{iFRET} = \frac{\text{CFP}}{\text{YFP} - \text{CFP} 	imes 0.357} \] [74]. Thereby, raw CFP and YFP emission data were first background corrected and YFP data were further corrected by subtracting the CFP spillover into the YFP signal, which was determined as 35.7% of the CFP signal. Individual neuronal traces were finally normalized to baseline and were averaged for each treatment. Maximum iFRET changes were quantified for each individual neuron, then averaged for each pharmacological treatment and statistically compared.

**Statistics**

Plotting and statistical analysis were performed using OriginPro 9.1G and the R environment (http://www.r-project.org/). One-way ANOVA with post-hoc Tukey's HSD tests were applied if criteria for normal distribution (Shapiro-Wilk normality test, \( p > 0.05 \)) and homogeneity of variances (Levene's test, \( p > 0.05 \)) were met, otherwise Kruskal-Wallis and post-hoc Mann-Whitney U tests (with Holm correction) were applied. Exceptions are stated in the figure legends.

**Supporting Information**

S1 Text. Recipe for standard Drosophila medium and primers. (DOC)

S1 Table. Expression patterns of AstA\(^{34}\)-Gal4 and tsh-Gal80; AstA\(^{34}\)-Gal4. (DOC)

S1 Fig. UAS-LacZ expression pattern in the larval CNS in relation to AstA immunostaining for the different generated AstA-Gal4 lines. AstA-LacZ overlap: Gal4-UAS-LacZ expressing cells are AstA-immunopositive, AstA-LacZ false positives: Gal4-UAS-LacZ expressing cells are not AstA-immunopositive, AstA-LacZ false negatives: AstA-immunopositive cells not contained in the Gal4-UAS-LacZ expression pattern. The following primer sets were used to amplify the respective promoter regions:

- pAstA1:
  5'-GCGCAATTGATGGCT ATTTCCCAGCTCCT-3'
  5'-GCCGGATCCAGAGGT TCCGCGGACTAAAT-3'

- pAstA2:
  5'-GCGCAATTGAGTAGAAGCT GCGCCAGAAG-3'
  5'-GCCGGATCCAGAGGT TCCGCGGACTAAAT-3'

- pAstA3:
  5'-GCGCAATTGGGGAATAACCTCCGAAAACC-3'
  5'-GCCGGATCCAGAGGT TCCGCGGACTAAAT-3'

(TIF)

S2 Fig. Schematic summary of the expression pattern of the two AstA-Gal4 drivers used, in conjunction with tsh-Gal80. AstA neurons in the posterior lateral protocerebrum (PLP cells) are in red, the dorsolateral abdominal AstA neurons (DLAa) in the thoracico-abdominal ganglion and the peripheral neurons (PN) are in blue, other AstA neurons in the ventral brain (VBN) and lateral optic lobes (LOL) are in green. AstA-expressing enteroendocrine cells (EECs) in the posterior midgut are represented by red triangles. (TIF)
S3 Fig. Thermogenetic activation of the AstA cells of female adults resulted in reduced food intake. (A) AstA\textsuperscript{1}\textgreater TrpA1. (B) AstA\textsuperscript{34}\textgreater TrpA1 and respective controls. * p \leq 0.05, ** p \leq 0.01, *** p \leq 0.001.

(TIF)

S4 Fig. Conditional silencing of AstA\textsuperscript{34} cells by temperature-dependent expression of Kir2.1 did not alter food consumption: tubGal80\textsuperscript{ts};AstA\textsuperscript{34}\textgreater Kir2.1 flies with silenced AstA cells at 29°C and with normally active AstA cells at 18°C consumed the same amount of food then the controls.

(TIF)

S5 Fig. AstA RNA-interference rescued reduced food intake but not locomotor activity in flies with thermogenetically activated AstA cells. At 22°C, food intake (A) and locomotor activity (B) of AstA\textsuperscript{34}\textgreater TrpA1 flies did not show significant differences to controls. At 29°C, thermogenetic activation of AstA\textsuperscript{34} cells resulted in lower food intake (A) and locomotor activity (B). AstA\textsuperscript{34}\textgreater TrpA1/UAS-dcr-2; AstA-RNAi flies were not significantly different in food consumption to AstA\textsuperscript{34}\textgreater TrpA1, but showed a significantly reduced locomotor activity. * p \leq 0.05. ** p \leq 0.01, *** p \leq 0.001.

(TIF)

S6 Fig. Thermogenetic activation of AstA cells resulted in a strongly inhibited locomotion. Examples of double-plotted single fly actogramms underlying the results shown in Fig 4. Flies were initially kept at 22°C, then temperature was raised to 29°C at the time point indicated by a red arrow.

(TIF)

S7 Fig. Thermogenetic activation of AstA\textsuperscript{1} cells reduced food intake and locomotion in the CAFE assay. Individual flies were filmed for 4 hours in a modified CAFE assay (three flies per genotype). Behaviour was categorized as "not moving", "moving" and "feeding". AstA\textsuperscript{1}\textgreater TrpA1 flies with activated AstA\textsuperscript{1} cells moved and consumed less than controls.

(TIF)

S8 Fig. Thermogenetic activation of AstA cells strongly promoted sleep also in female flies. At 20°C, AstA\textsuperscript{34}\textgreater TrpA1 (top left) and AstA\textsuperscript{1}\textgreater TrpA1 females (bottom left) did not sleep more than controls. Activation of the TrpA1 channel by 29°C resulted in increased sleep time of AstA\textsuperscript{34}\textgreater TrpA1 (top right) and AstA\textsuperscript{1}\textgreater TrpA1 (bottom right) females during the light phase from ZT0 to ZT12.

(TIF)

S9 Fig. Thermogenetic activation of AstA\textsuperscript{1} cells (A-B) or AstA\textsuperscript{34} cells (C-D) increased sleep under LL conditions known to impair the clock and to induce arrhythmicity.

(TIF)

S10 Fig. Constitutive silencing of AstA cells by ectopic expression of Kir2.1 did not alter sleep behaviour in AstA\textsuperscript{34}\textgreater Kir2.1 (A) and AstA\textsuperscript{1}\textgreater Kir2.1 (B) flies. The mean sleep bout duration (C) and the total amount of sleep per day (D) of AstA\textsuperscript{34}\textgreater Kir2.1 and AstA\textsuperscript{1}\textgreater Kir2.1 is not significantly different to all controls.

(TIF)

S11 Fig. A) Conditional silencing of AstA\textsuperscript{34} cells increases the mean locomotor activity. B) Ectopic expression of t-PDF in AstA\textsuperscript{34} cells decreases the mean locomotor activity.

(TIF)
S12 Fig. Conditional silencing of AstA<sup>34</sup> cells by UAS-Kir2.1 decreased sleep in constant darkness (DD). (A) Actograms of single flies show that locomotor activity increases during the subjective day and night upon silencing of AstA<sup>34</sup> cells. Rhythmicity and period is not affected. Both the duration of sleep bouts (B) and total sleep (C) is reduced.

(TIF)

S13 Fig. The effect on sleep of constitutive (A–B) and conditional (C–E) silencing of AstA<sup>34</sup> cells by UAS-ΔORK under LD12:12 (A–B) Constitutive silencing let to a slight increase in the total amount of sleep (B), mostly due to increased sleep during the day (A). This effect is opposite of the expected decrease upon AstA cell silencing. (C–E) Conditional silencing did not affect total sleep or sleep bout duration (E), yet sleep is increased during the end of the evening activity and decreased during the first half of the photophase.

(TIF)

S14 Fig. Velocity of flies after mechanical arousal by a loudspeaker at 29°C (compare to Fig 7 which shows the average velocity of several flies). While AstA<sup>34</sup> > TrpA1 flies walked less (leading to a reduced average velocity), the maximum speeds when moving where not different to control flies, suggesting that the reduced locomotor activity is not due to motor impairment.

(TIF)

S15 Fig. Lack of a feeding rebound after releasing the activation of AstA cells. AstA<sup>34</sup> > TrpA1 and AstA<sup>1</sup> > TrpA1 flies were kept for 1 day at 22°C, then 2 days at 29°C in the CAFE assay. Afterwards, flies were put back again to 22°C, and food consumption was summed up for the first 3, 6 and 24h.

(TIF)

S16 Fig. Starvation-induced locomotor hyperactivity in flies with thermogenetically activated AstA<sup>1</sup> cells reduces sleep especially during morning activity. Flies were kept at 20°C in LD12:12 on normal food, and then transferred to DAM glass tubes and switched to 29°C and feeding/starvation-conditions at ZT8 at the start of locomotor activity monitoring (n = 32).

(TIF)

S17 Fig. Expression pattern of ELAV, elav-Gal80 and elav-Gal4 in L3 midguts. A and B): GFP expression (anti-GFP staining, green in A'/B') driven by the peptidergic cell marker 386y-Gal4 (Taghert et al. 2001, Reiher et al. 2011) colocalises with a-ELAV immunoreactivity (magenta, A''/B'') as seen in the merged pictures A'''/B'''. Scale bar = 50 μm. C and D): 386y-Gal4 driven GFP expression (C) is suppressed by co-expression of elav-GAL80 (D). Widefield pictures taken with a CCD camera with an exposure time of 3.75 s (C'), 2.5 s (C''), 5 s (D') and 7.5 s (D'). All other camera settings were kept constant. E): elav-Gal4-driven native GFP expression in EECs. Arrows point to neurons in the proventricular ganglion.

(TIF)

S18 Fig. nsyb-Gal80 rescued reduced food intake and locomotor activity. Thermogenetic activation of AstA<sup>34</sup> cells resulted in significant lower food consumption (A) and locomotor activity (B) compared to controls. Food intake (A) and locomotor activity (B) of nsyb-Gal80; AstA<sup>34</sup> > TrpA1 were not significantly different to controls, but significantly higher than AstA<sup>34</sup> > TrpA1 at 29°C. * p ≤ 0.05. ** p ≤ 0.01, *** p ≤ 0.001.

(TIF)

S1 Movie. 3D rotation of the PLP neuron arborisations in the dorsal protocerebrum, anti-GFP staining in an adult AstA<sup>34</sup> > GFP fly.

(AVI)
S2 Movie. 3D rotation of AstA neuron immunoreactivity in the dorsal protocerebrum, same brain as in Suppl. video 1. (AVI)

S3 Movie. The behaviour of AstA^{34)>TrpA1} (top), AstA^{34} x w^{1118} (right) and w^{1118} x UAS-TrpA1 (left) flies upon increasing mechanical stimuli in the shaker assay at ZT1 to ZT12. (MP4)

Acknowledgments
We thank Susanne Klühspies, Gertrud Gramlich and Sylwia Febocolon for excellent technical assistance, Pamela Menegazzi and Dirk Rieger for help with locomotor activity analysis, Taishi Yoshii (Okayama, Japan) for the sleep-analyser software and Mareike Selcho and Basil el Jundi (Lund, Sweden) for helpful discussions. We also thank D. Anderson, J-F. Ferveur, S.F. Goodwin, L.Y. and Y.N. Jan, T. Langenhan, J. Levine, J. Simpson, P. Taghert and the VDRC and Bloomington stock center for the kind gift of flies.

Author Contributions
Conceptualization: CW JC WR JAV PC CHF CHL.
Formal analysis: JC WR CHL.
Funding acquisition: CW CHF WR.
Investigation: JC WR CHL.
Methodology: JC WR CHL PC AS SK JAV CHF CW.
Resources: AS JAV SK CW.
Supervision: CW JAV CHF PC.
Validation: JC WR CW.
Visualization: JC WR CW CHL AS.
Writing – original draft: CW WR JC.
Writing – review & editing: CW JC WR CHL AS PC CHF JAV.

References
1. Boonen K, Creemers JW, Schoofs L. Bioactive peptides, networks and systems biology. BioEssays. 2009; 31: 300–314. doi: 10.1002/bies.200800055 PMID: 19260025
2. Nässel DR, Winther ÅME. Drosophila neuropeptides in regulation of physiology and behavior. Prog Neurobiol. 2010; 92: 42–104. doi: 10.1016/j.pneurobio.2010.04.010 PMID: 20447440
3. Kastin AJ, editor. Handbook of Biologically active peptides [Internet]. 2nd edition. Boston: Academic Press; 2013. Available: http://www.sciencedirect.com/science/article/pii/B9780123850959010010
4. Brown JA, Woodworth HL, Leinninger GM. To ingest or rest? Specialized roles of lateral hypothalamic area neurons in coordinating energy balance. Front Syst Neurosci. 2015; 9: 9. doi: 10.3389/fnsys.2015.00009 PMID: 25741247
5. Richter C, Woods IG, Schier AF. Neuropeptidergic control of sleep and wakefulness. Annu Rev Neurosci. 2014; 37: 503–531. doi: 10.1146/annurev-neuro-062111-150447 PMID: 25032501
6. Penev PD. Update on energy homeostasis and insufficient sleep. J Clin Endocrinol Metab. 2012; 97: 1792–1801. doi: 10.1210/jc.2012-1067 PMID: 22442266
7. Saper CB. Staying awake for dinner: hypothalamic integration of sleep, feeding, and circadian rhythms. Prog Brain Res. 2006; 153: 243–252. doi: 10.1016/S0079-6123(06)53014-6 PMID: 16876579

8. Arble DM, Copinschi G, Vitaterna MH, Van Cauter E, Turek FW. Circadian Rhythms in Neuroendocrine Systems. In: Levine GF, WPE, editor. Handbook of Neuroendocrinology. San Diego: Academic Press; 2012. pp. 271–305. Available: http://www.sciencedirect.com/science/article/pii/B9780123750976100125

9. Bonnefont X. Circadian timekeeping and multiple timescale neuroendocrine rhythms. J Neuroendocrin. 2010; 22: 209–216. doi: 10.1111/j.1365-2826.2010.01955.x PMID: 20070481

10. Cirelli C. The genetic and molecular regulation of sleep: from fruit flies to humans. Nat Rev Neurosci. 2009; 10: 549–560. doi: 10.1038/nrn2683 PMID: 19617891

11. Itskov PM, Ribeiro C. The dilemmas of the gourmet fly: the molecular and neuronal mechanisms of feeding and nutrient decision making in Drosophila. Front Neurosci. 2013; 7: 12. doi: 10.3389/fnins.2013.00012 PMID: 23407678

12. Pool A-H, Scott K. Feeding regulation in Drosophila. Curr Opin Neurobiol. 2014; 29: 57–63. doi: 10.1016/j.conb.2014.05.008 PMID: 24937262

13. Sehgal A, Mignot E. Genetics of sleep and sleep disorders. Cell. 2011; 146: 194–207. doi: 10.1016/j.cell.2011.07.004 PMID: 21784243

14. Huber R, Hill SL, Holladay C, Biesiadetzki M, Tononi G, Cirelli C. Sleep homeostasis in Drosophila melanogaster. Sleep. 2004; 27: 628–639. PMID: 15282997

15. Shaw PJ, Cirelli C, Greenspan RJ, Tononi G. Correlates of sleep and waking in Drosophila melanogaster. Science. 2000; 287: 1834–1837. PMID: 10710313

16. Ro J, Harvanek ZM, Pletcher SD. FLIC: High-Throughput, Continuous Analysis of Feeding Behaviors in Drosophila. Roman G, editor. PLoS ONE. 2014; 9: e101107. doi: 10.1371/journal.pone.0101107 PMID: 24978054

17. Seay DJ, Thummel CS. The circadian clock, light, and cryptochrome regulate feeding and metabolism in Drosophila. J Biol Rhythms. 2011; 26: 497–506. doi: 10.1177/0748730411420080 PMID: 22215608

18. Xu K, Zheng X, Sehgal A. Regulation of feeding and metabolism by neuronal and peripheral clocks in Drosophila. Cell Metab. 2008; 8: 289–300. doi: 10.1016/j.cmet.2008.09.006 PMID: 18840359

19. Griffith LC. Neurmodulatory control of sleep in Drosophila melanogaster: integration of competing and complementary behaviors. Curr Opin Neurobiol. 2013; 23: 819–823. doi: 10.1016/j.conb.2013.05.003 PMID: 23743247

20. Kunst M, Tso MCF, Ghosh DD, Herzog ED, Nitabach MN. Rhythmic control of activity and sleep by class B1 GPCRs. Crit Rev Biochem Mol Biol. 2015; 50: 18–30. doi: 10.3109/10409238.2014.985185 PMID: 25410535

21. Chen W, Shi W, Li L, Zheng Z, Li T, Bai W, et al. Regulation of sleep by the short neuropeptide F (sNPF) in Drosophila melanogaster. Insect Biochem Mol Biol. 2013; 43: 809–819. doi: 10.1016/j.ibmb.2013.06.003 PMID: 23796436

22. Hong S-H, Lee K-S, Kwak S-J, Kim A-K, Bai H, Jung M-S, et al. Minibrain/Dyrk1a regulates food intake through the Sir2-FOXO-sNPF/NPY pathway in Drosophila and mammals. PLoS Genet. 2012; 8: e1002857. doi: 10.1371/journal.pgen.1002857 PMID: 22876196

23. Lee K-S, You K-H, Choo J-K, Han Y-M, Yu K. Drosophila short neuropeptide F regulates food intake and body size. J Biol Chem. 2004; 279: 50781–50789. doi: 10.1074/jbc.M407842200 PMID: 15385546

24. Shang Y, Donelson NC, Vecsey CG, Guo F, Rosbash M, Griffith LC. Short Neuropeptide F Is a Sleep-Promoting Inhibitory Modulator. Neuron. 2013; 80: 171–183. doi: 10.1016/j.neuron.2013.07.029 PMID: 24049110

25. He C, Yang Y, Zhang M, Price JL, Zhao Z. Regulation of sleep by neuropeptide Y-like system in Drosophila melanogaster. PLoS One. 2013; 8: e74237. doi: 10.1371/journal.pone.0074237 PMID: 24040211

26. Wu Q, Wen T, Lee G, Park JH, Cai HN, Shen P. Developmental control of foraging and social behavior by the Drosophila neuropeptide Y-like system. Neuron. 2003; 39: 147–161. PMID: 12848939

27. Erion R, DiAngelo JR, Crocker A, Sehgal A. Interaction between sleep and metabolism in Drosophila with altered octopamine signaling. J Biol Chem. 2012; 287: 32406–32414. doi: 10.1074/jbc.M112.360875 PMID: 22829591

28. Hergarden AC, Tayler TD, Anderson DJ. Allatostatin-A neurons inhibit feeding behavior in adult Drosophila. Proc Natl Acad Sci U S A. 2012; 109: 3967–3972. doi: 10.1073/pnas.120778108 PMID: 22345563
29. Nitabach MN, Wu Y, Sheeba V, Lemon WC, Strumbos J, Zelensky PK, et al. Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. J Neurosci Off J Soc Neurosci. 2006; 26: 479–489. doi: 10.1523/JNEUROSCI.3915-05.2006

30. Baines RA, Uhler JP, Thompson A, Sweeney ST, Bate M. Altered electrical properties in Drosophila neurons developing without synaptic transmission. J Neurosci Off J Soc Neurosci. 2001; 21: 1523–1531.

31. Veenstra JA. Peptidergic paracrine and endocrine cells in the midgut of the fruit fly maggot. Cell Tissue Res. 2009; 336: 309–323. doi: 10.1007/s00441-009-0769-y PMID: 19319573

32. Veenstra JA, Agricola H-J, Sellami A. Regulatory peptides in fruit fly midgut. Cell Tissue Res. 2008; 334: 499–516. doi: 10.1007/s00441-008-0708-3 PMID: 18972134

33. Yoon JG, Stay B. Immunocytochemical localization of Diploptera punctata allatostatin-like peptide in Drosophila melanogaster. J Comp Neurol. 1995; 363: 475–488. PMID: 8847412

34. Reiher W, Shirras C, Kahnt J, Baumeister S, Isaac RE, Wegener C. Peptidomics and Peptide Hormone Processing in the Drosophila Midgut. J Proteome Res. 2011; 10: 1881–1892. doi: 10.1021/pr101116g PMID: 21214272

35. Vanderveken M, O’Donnell MJ. Effects of diuretic hormone 31, drosokinin, and allatostatin A on transepithelial K+ transport and contraction frequency in the midgut and hindgut of larval Drosophila melanogaster. Arch Insect Biochem Physiol. 2014; 85: 76–93. doi: 10.1002/arch.21144 PMID: 24408875

36. Birgül N W C. Reverse physiology in Drosophila: identification of a novel allatostatin-like neuropeptide and its cognate receptor structurally related to the mammalian somatostatin/galanin/opioid receptor family. EMBO J. 1999; 18: 5882–5900. PMID: 10545101

37. Lenz C, Williamson M, Grimmelikhuijzen CJ. Molecular cloning and genomic organization of a second probable allatostatin receptor from Drosophila melanogaster. Biochem Biophys Res Commun. 2000; 273: 571–577. doi: 10.1006/bbrc.2000.2964 PMID: 10873647

38. Lenz C, Søndergaard L, Grimmelikhuijzen CJ. Molecular cloning and genomic organization of a novel receptor from Drosophila melanogaster structurally related to mammalian galanin receptors. Biochem Biophys Res Commun. 2000; 269: 91–96. doi: 10.1006/bbrc.2000.2251 PMID: 10694483

39. Larsen MJ, Burton KJ, Zantello MR, Smith VG, Lowery DL, Kubiak TM. Type A allatostatins from Drosophila melanogaster and Diploptera punctata activate two Drosophila allatostatin receptors, DAR-1 and DAR-2, expressed in CHO cells. Biochem Biophys Res Commun. 2001; 286: 895–901. doi: 10.1006/bbrc.2001.5476 PMID: 11527383

40. Mirabeau O, Joly J-S. Molecular evolution of peptidergic signaling systems in bilaterians. Proc Natl Acad Sci. 2013; 110: E2028–E2037. doi: 10.1073/pnas.1219956110 PMID: 23671109

41. Felix RC, Trindade M, Pires IRP, Fonseca VG, Martins RS, Silveira H, et al. Unravelling the Evolution of the Allatostatin-Type A, KISS and Galanin Peptide-Receptor Gene Families in Bilaterians: Insights from Anopheles Mosquitoes. PLoS ONE. 2015; 10: e0130347. doi: 10.1371/journal.pone.0130347 PMID: 26135459

42. Jékely G. Global view of the evolution and diversity of metazoan neuropeptide signaling. Proc Natl Acad Sci. 2013; 110: 8702–8707. doi: 10.1073/pnas.1221933110 PMID: 23637342

43. Hewes RS, Taghert PH. Neuropeptides and neuropeptide receptors in the Drosophila melanogaster genome. Genome Res. 2001; 11: 1126–1142. doi: 10.1101/gr.169901 PMID: 11381038

44. Lang R, Gundlach AL, Kofer B. The galanin peptide family: receptor pharmacology, pleiotropic biological actions, and implications in health and disease. Pharmacol Ther. 2007; 115: 177–207. doi: 10.1016/j.pharmthera.2007.05.009 PMID: 17604107

45. Lang R, Gundlach AL, Holmes FE, Hobson SA, Wynick D, Hökfelt T, et al. Physiology, signaling, and pharmacology of galanin peptides and receptors: three decades of emerging diversity. Pharmacol Rev. 2015; 67: 118–175. doi: 10.1124/pr.112.006536 PMID: 25428932

46. Steiger A. Neurochemical regulation of sleep. J Psychiatr Res. 2007; 41: 537–552. PMID: 16777143

47. Ja WW, Carvalho GB, Mak EM, de la Rosa NN, Fang AY, Liong JC, et al. Prandiology of Drosophila and the CAFE assay. Proc Natl Acad Sci U S A. 2007; 104: 8253–8256. doi: 10.1073/pnas.0702726104 PMID: 17494737

48. Hamada FN, Rosenzweig M, Kang K, Pulver SR, Ghezzi A, Jegla T, et al. An internal thermal sensor controlling temperature preference in Drosophila. Nature. 2008; 454: 217–220. doi: 10.1038/nature07001 PMID: 18548007

49. Pulver SR, Pashkovskiy SL, Hornstein NJ, Garrity PA, Griffith LC. Temporal dynamics of neuronal activation by Channelrhodopsin-2 and TRPA1 determine behavioral output in Drosophila larvae. J Neurophysiol. 2009; 101: 3075–3088. doi: 10.1152/jn.00071.2009 PMID: 19339465
50. Clyne JD, Miesenböck G. Sex-specific control and tuning of the pattern generator for courtship song in Drosophila. Cell. 2008; 133: 354–363. doi: 10.1016/j.cell.2008.01.050 PMID: 18423205

51. Yu JY, Kanai MI, Demir E, Jefferis GSXE, Dickson BJ. Cellular organization of the neural circuit that drives Drosophila courtship behavior. Curr Biol. 2010; 20: 1602–1614. doi: 10.1016/j.cub.2010.08.025 PMID: 20832315

52. Tsoubouchi A, Caldwell JC, Tracey WD. Dendritic filopodia, Ripped Pocket, NOMPC, and NMDARs contribute to the sense of touch in Drosophila larvae. Curr Biol CB. 2012; 22: 2124–2134. doi: 10.1016/j.cub.2012.09.019 PMID: 23103192

53. McGuire SE, Mao Z, Davis RL. Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in Drosophil a. Sci STKE Signal Transd uct Knowl Environ. 2004; 2004: pl6. doi: 10.1126/st ke.2202004 pl6

54. Schoofs A, Hückesfeld S, Schlegel P, Miroschnikow A, Peters M, Zeymer M, et al. Selection of Motor Programs for Suppressing Food Intake and Inducing Locomotion in the Drosophila Brain. Sengupta P, editor. PLoS Biol. 2014; 12: e1001893 . doi: 10.137 1/journal.pbi o.1001893 PMID: 24960360

55. Hökfelt T, Millhorn D, Seroogy K, Tsuruo Y, Ceccatelli S, Lindh B, et al. Coexistence of peptides with classical neurotransmitters. Experientia. 1987; 43: 768–780 . doi: 10.100 7/BF0194 5354 PMID: 2885215

56. Nässel DR, Homberg U. Neuropeptides in interneurons of the insect brain. Cell Tissue Res. 2006; 361: 1–24. doi: 10.1007/s00441-006-0210-8 PMID: 16761145

57. Kondo S, Ueda R. Highly Improved Gene Targeting by Germline-Specific Cas9 Expression in Dro- sophila. Genetics. 2013; 195: 715–721. doi: 10.1534/genetics.113.156737 PMID: 22400268

58. Helfrich-Förster C. Neurobiology of the fruit fly’s circadian clock. Genes Brain Behav. 2005; 4: 65–76. PMID: 15720403

59. Ho KS, Sehgal A. Drosophil a melanogaster: an insect model for fundamental studies of sleep. Methods Enzymol. 2005; 393: 772–793. doi: 10.1016/S0076-6879(05 )93041-3 PMID: 15817324

60. Nitabach MN, Blau J, Holmes TC. Electrical silencing of Drosophila pacemaker neurons stops the free-running circadian clock. Cell. 2002; 109: 485–495. PMID: 12086605

61. van Alphen B, Yap MHW, Kirszenblat L, Kottler B, van Swindere n B. A Dynamic Deep Sleep Stage in Drosophila . J Neurosci. 2013; 33: 6917–6927. doi: 10.1523 /JNEUROSC I.0061-13.2 013 PMID: 23595750

62. Lee G, Park JH. Hemolymph sugar homeosta sis and starvation-induc ed hyperactivity affected by genetic manipu lations of the adipokinet ic-hormone-encoding gene in Drosophi la melanogaster. Genetics. 2004; 167: 311–323. PMID: 15166157

63. Keene AC, Duboue ER, McDonal d DM, Dus M, Suh GSB, Waddell S, et al. Clock and cycle Limit Starvation-Induced Sleep Loss in Drosophil a. Curr Biol. 2010; 20: 12091215 . doi: 10.101 6/j.cub.2010.05.029

64. Yang C, Rumpf S, Xiang Y, Gordon MD, Song W, Jan LY, et al. Control of the Postmati ng Behavioral Switch in Drosophila Females by Internal Sensory Neuron s. Neuron. 2009; 61: 519–526. doi: 10.1016/j.neuro n.2008.12.021 PMID: 19249273

65. Yao KM, White K. Neural specificity of elav expressio n: definin g a Drosophi la promoter for directing expression to the nervous system. J Neurochem . 1994; 63: 41–51. PMID: 8207445

66. Berger C, Renner S, Lüer K, Technau GM. The commonly used marker ELAV is transient ly expressed in neuroblast s and glial cells in the Drosophila embryonic CNS. Dev Dyn. 2006; 239: 2546–2552. doi: 10.1002/dv dy.21372 PMID: 17994541

67. Micchelli CA, Perrimon N. Evidence that stem cells reside in the adult Drosophila midgut epithelium. Nature. 2006; 439: 475–479. doi: 10.1038/nature04371 PMID: 16340959

68. Beehler-Evans R, Micchelli CA. Generation of enteroen docrine cell diversity in midgut stem cell line- ages. Development. 2015; 142: 654–664. doi: 10.1242/dev.114959 PMID: 25670792

70. Chintapalli VR, Wang J, Dow JAT. Using FlyAtlas to identify better Drosophila melanogaster models of human disease. Nat Genet. 2007; 39: 715–720. doi: 10.1038/ng2049 PMID: 17534367

71. Hermann-Luib C, Helfrich-Förster C. Clock network in Drosophila. Curr Opin Insect Sci. 2015; 7: 65– 70. doi: 10.1016/j.cois.2014.11.003

72. Parisky KM, Agosto J, Pulver SR, Shang Y, Kuklin E, Hodge JLL, et al. PDF cells are a GABA- responsive wake-promoting component of the Drosophila sleep circuit. Neuron. 2008; 60: 672–682. doi: 10.1016/j.neuron.2008.10.042 PMID: 19038223
73. Yasuyama K, Meinertzhagen IA. Synaptic connections of PDF-immunoreactive lateral neurons projecting to the dorsal protocerebrum of Drosophila melanogaster. J Comp Neurol. 2010; 518: 292–304. doi: 10.1002/cne.22210 PMID: 19941354

74. Shafer OT, Kim DJ, Dunbar-Yaffe R, Nikolaev VO, Lohse MJ, Taghert PH. Widespread receptivity to neuropeptide PDF throughout the neuronal circadian clock network of Drosophila revealed by real-time cyclic AMP imaging. Neuron. 2008; 58: 223–237. doi: 10.1016/j.neuron.2008.02.018 PMID: 18439407

75. Hyun S, Lee Y, Hong S-T, Bang S, Paik D, Kang J, et al. Drosophila GPCR Han is a receptor for the circadian clock neuropeptide PDF. Neuron. 2005; 48: 267–278. doi: 10.1016/j.neuron.2005.08.025 PMID: 16242407

76. Im SH, Taghert PH. PDF receptor expression reveals direct interactions between circadian oscillators in Drosophila. J Comp Neurol. 2010; 518: 1925–1945. doi: 10.1002/cne.22311 PMID: 20394051

77. Kunst M, Hughes ME, Raccuglia D, Felix M, Li M, Barnett G, et al. Calcitonin gene-related peptide neurons mediate sleep-specific circadian output in Drosophila. Curr Biol CB. 2014; 24: 2652–2664. doi: 10.1016/j.cub.2014.09.077 PMID: 25455031

78. Choi C, Fortin J-P, McCarthy EV, Oksman L, Kopin AS, Nitabach MN. Cellular dissection of circadian peptide signals with genetically encoded membrane-tethered ligands. Curr Biol CB. 2009; 19: 1167–1175. doi: 10.1016/j.cub.2009.06.029 PMID: 19592252

79. Fernández MP, Berni J, Ceriani MF. Circadian Remodeling of Neuronal Circuits Involved in Rhythmic Behavior. PLoS Biol. 2008; 6: e69. doi: 10.1371/journal.pbio.0060069 PMID: 18366255

80. Park JH, Helfrich-Förster C, Lee G, Liu L, Rosbash M, Hall JC. Differential regulation of circadian pacemaker output by separate clock genes in Drosophila. Proc Natl Acad Sci USA. 2000; 97: 3608–3613. PMID: 10725392

81. Choi C, Cao G, Tanenhaus AK, McCarthy EV, Jung M, Schleyer W, et al. Autoreceptor control of peptide/neurotransmitter corelease from PDF neurons determines allocation of circadian activity in Drosophila. Cell Rep. 2012; 2: 332–344. doi: 10.1016/j.celrep.2012.06.021 PMID: 22938867

82. Helfrich-Förster C, Shafer OT, Wülbeck C, Grieshaber E, Rieger D, Taghert P. Development and morphology of the clock-gene-expressing lateral neurons of Drosophila melanogaster. J Comp Neurol. 2007; 500: 47–70. doi: 10.1002/cne.21146 PMID: 17099895

83. Shafer OT, Taghert PH. RNA-interference knockdown of Drosophila pigment dispersing factor in neuronal subsets: the anatomical basis of a neuropeptide’s circadian functions. PloS One. 2009; 4: e8298. doi: 10.1371/journal.pone.0008298 PMID: 20011537

84. Liu Q, Liu S, Kodama L, Driscoll MR, Wu MN. Two dopaminergic neurons signal to the dorsal fan-shaped body to promote wakefulness in Drosophila. Curr Biol CB. 2012; 22: 2114–2123. doi: 10.1016/j.cub.2012.09.008 PMID: 23022067

85. Ueno T, Tomita J, Tanimoto H, Endo K, Ito K, Kume S, et al. Identification of a dopamine pathway that regulates sleep and arousal in Drosophila. Nat Neurosci. 2012; 15: 1516–1523. doi: 10.1038/nn.3238 PMID: 23064381

86. Donlea JM, Pimentel D, Miesenböck G. Neuronal machinery of sleep homeostasis in Drosophila. Neuron. 2014; 81: 860–872. doi: 10.1016/j.neuron.2013.12.013 PMID: 24559676

87. Wolff T, Iyer NA, Rubin GM. Neuroarchitecture and neuroanatomy of the Drosophila central complex: A GAL4-based dissection of protocerebral bridge neurons and circuits. J Comp Neurol. 2015; 523: 997–1037. doi: 10.1002/cne.23705 PMID: 25380328

88. Young JM, Armstrong JD. Structure of the adult central complex in Drosophila: organization of distinct neuronal subsets. J Comp Neurol. 2010; 518: 1500–1524. doi: 10.1002/cne.22284 PMID: 20187142

89. Foltenyi K, Greenspan RJ, Newport JW. Activation of EGFR and ERK by rhomboid signaling regulates the consolidation and maintenance of sleep in Drosophila. Nat Neurosci. 2007; 10: 1160–1167. doi: 10.1038/nn.1957 PMID: 17694052

90. Park J-H, Kwon JY. Heterogeneous Expression of Drosophila Gustatory Receptors in Enteroendocrine Cells. PLoS ONE. 2011; 6: e29022. doi: 10.1371/journal.pone.0029022 PMID: 22194978

91. Hentze JL, Carlsson MA, Kondo S, Nässel DR, Rewitz KF. The Neuropeptide Allatostatin A Regulates Metabolism and Feeding Decisions in Drosophila. Sci Rep. 2015; 5: 11680. doi: 10.1038/srep11680 PMID: 26123697

92. Aguilar R, Maestro JL, Vilaplana L, Pascual N, Piulachs M-D, Béliers X. Allatostatin gene expression in brain and midgut, and activity of synthetic allatostatins on feeding-related processes in the cockroach Blattella germanica. Regul Pept. 2003; 115: 171–177. PMID: 14556958

93. Fusé M, Zhang JR, Partridge E, Nachman RJ, Orchard I, Bendena WG, et al. Effects of an allatostatin and a myosuppressin on midgut carbohydrate enzyme activity in the cockroach Diploptera punctata. Peptides. 1999; 20: 1285–1293. PMID: 10612442
94. Woods IG, Schoppik D, Shi VJ, Zimmerman S, Coleman HA, Greenwood J, et al. Neuropeptidergic signaling partitions arousal behaviors in zebrafish. J Neurosci Off J Soc Neurosci. 2014; 34: 3142–3160. doi: 10.1523/JNEUROSCI.3529-13.2014

95. Bendena WG, Boudreau JR, Papanicolau T, Maltby M, Tobe SS, Chin-Sang ID. A Caenorhabditis elegans allatostatin/galanin-like receptor NPR-9 inhibits local search behavior in response to feeding cues. Proc Natl Acad Sci. 2008; 105: 1339–1342. doi: 10.1073/pnas.0709492105 PMID: 18216257

96. Hermann C, Yoshii T, Dusik V, Helfrich-Förster C. Neuropeptide F immunoreactive clock neurons modify evening locomotor activity and free-running period in Drosophila melanogaster. J Comp Neurol. 2012; 520: 970–987. doi: 10.1002/cne.22742 PMID: 21826659

97. Hermann-Luibl C, Yoshii T, Senthilan PR, Dircksen H, Helfrich-Förster C. The Ion Transport Peptide Is a New Functional Clock Neuropeptide in the Fruit Fly Drosophila melanogaster. J Neurosci. 2014; 34: 9522–9536. doi: 10.1523/JNEUROSCI.0111-14.2014 PMID: 25031396

98. Lee G, Bahn JH, Park JH. Sex- and clock-controlled expression of the neuropeptide F gene in Drosophila. Proc Natl Acad Sci U S A. 2006; 103: 12580–12585. doi: 10.1073/pnas.0601171103 PMID: 16894172

99. Oh Y, Yoon S-E, Zhang Q, Chae H-S, Daubnerová I, Shafer OT, et al. A Homeostatic Sleep-Stabilizing Pathway in Drosophila Composed of the Sex Peptide Receptor and Its Ligand, the Myoinhibitory Peptide. Shaw P, editor. PLoS Biol. 2014; 12: e1001974. doi: 10.1371/journal.pbio.1001974 PMID: 25333796

100. Yao Z, Shafer OT. The Drosophila Circadian Clock Is a Variably Coupled Network of Multiple Peptidergic Units. Science. 2014; 343: 1516–1520. doi: 10.1126/science.1251285 PMID: 24675961

101. Mertens I, Vandingene A, Johnson EC, Shafer OT, Li W, Trigg JS, et al. PDF receptor signaling in Drosophila contributes to both circadian and geotactic behaviors. Neuron. 2005; 48: 213–219. doi: 10.1016/j.neuron.2005.09.009 PMID: 16242402

102. Kim WJ, Jan LY, Jan YN. A PDF/NPF Neuropeptide Signaling Circuitry of Male Drosophila melanogaster Controls Rival-Induced Prolonged Mating. Neuron. 2013; 80: 1190–1205. doi: 10.1016/j.neuron.2013.09.034 PMID: 24314729

103. Shang Y, Griffith LC, Rosbash M. Light-arousal and circadian photoreception circuits intersect at the large PDF cells of the Drosophila brain. Proc Natl Acad Sci U S A. 2008; 105: 19587–19594. doi: 10.1073/pnas.0809577105 PMID: 19060186

104. Krupp JJ, Billette J-C, Wong A, Choi C, Nitabach MN, Levine JD. Pigment-Dispersing Factor Modulates Pheromone Production in Clock Cells that Influence Mating in Drosophila. Neuron. 2013; 79: 54–68. doi: 10.1016/j.neuron.2013.05.019 PMID: 23849197

105. Talsma AD, Christov CP, Terriente-Felix A, Linneweber GA, Perea D, Wayland M, et al. Remote control of renal physiology by the intestinal neuropeptide pigment-dispersing factor in Drosophila. Proc Natl Acad Sci. 2012; 109: 12177–12182. doi: 10.1073/pnas.1200247109 PMID: 22778427

106. Taghert PH, Hewes RS, Park JH, O’Brien MA, Han M, Peck ME. Multiple amidated neuropeptides are required for normal circadian locomotor rhythms in Drosophila. J Neurosci. 2001; 21: 6673–6686. PMID: 11517257

107. Nicolai LJ, Ramaekers A, Ramaekers T, Drozdzecki A, Mauss AS, Yan J, et al. Genetically encoded dendritic markers shed light on neuronal connectivity in Drosophila. Proc Natl Acad Sci Acad Sci. 2010; 107: 20553–20558. doi: 10.1073/pnas.100198107 PMID: 21059961

108. Pfeiffer BD, Ngo T-TB, Hibbard KL, Murphy C, Jenett A, Truman JW, et al. Refinement of tools for targeted gene expression in Drosophila. Genetics. 2010; 186: 735–755. doi: 10.1534/genetics.110.119917 PMID: 20697123

109. Isabel G, Martin JR, Chidami S, Veenstra JA, Rosay P. AKH-producing neuroendocrine cell ablation decreases trehalose and induces behavioral changes in Drosophila. J Physiol—Regul Integr Comp Physiol. 2005; 28B: R531–538. PMID: 15374818

110. Fung Y, Ueda A, Wu C-F. A modified minimal hemolymph-like solution, HL3.1, for physiological recordings at the neuromuscular junctions of normal and mutant Drosophila larvae. J Neurogenet. 2004; 18: 377–402. doi: 10.1080/01677060490894522 PMID: 15763995

111. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012; 9: 676–682. doi: 10.1038/nmeth.2019 PMID: 22743772

112. Wittzheim H, Homberg U, Agricola H. Distribution of Dip-allatostatin I-like immunoreactivity in the brain of the locust Schistocerca gregaria with detailed analysis of immunostaining in the central complex. J Comp Neurol. 1996; 369: 419–437. doi: 10.1002/(SICI)1096-8861(19960603)369:3<419::AID-CNEN7>3.0.CO;2-8 PMID: 8743422
113. Santos JG, Vömel M, Struck R, Homberg U, Nässel DR, Wegener C. Neuroarchitecture of peptidergic systems in the larval ventral ganglion of Drosophila melanogaster. PLoS One. 2007; 2: e695. PMID: 17668072

114. Schmid B, Helfrich-Förster C, Yoshii T. A new ImageJ plug-in “ActogramJ” for chronobiological analyses. J Biol Rhythms. 2011; 26: 464–467. doi: 10.1177/0748730411414264 PMID: 21921300

115. Gmeiner F, Kołodziejczyk A, Yoshii T, Rieger D, Nässel DR, Helfrich-Förster C. GABAB receptors play an essential role in maintaining sleep during the second half of the night in Drosophila melanogaster. J Exp Biol. 2013; 216: 3837–3843. doi: 10.1242/jeb.085563 PMID: 24068350

116. Nikolaev VO, Büinemann M, Hein L, Hannawacker A, Lohse MJ. Novel single chain cAMP sensors for receptor-induced signal propagation. J Biol Chem. 2004; 279: 37215–37218. doi: 10.1074/jbc.C400302200 PMID: 15231839

117. Stewart BA, Atwood HL, Renger JJ, Wang J, Wu CF. Improved stability of Drosophila larval neuromuscular preparations in haemolymph-like physiological solutions. J Comp Physiol [A]. 1994; 175: 179–191.

118. Lelito KR, Shafer OT. Imaging cAMP dynamics in the Drosophila brain with the genetically encoded sensor Epac1-Camps. Genetically encoded functional indicators. Springer; 2012. pp. 149–168.