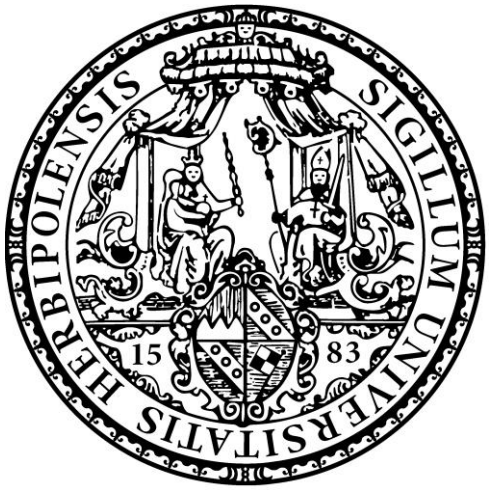
Functions of allatostatin A (AstA) and myoinhibitory peptides (MIPs) in the regulation of food intake and sleep in *Drosophila*

Funktion der Allatostatin A (AstA) und myoinhibitorische Peptide (MIP) in Bezug zu Nahrungsaufnahme und Schlaf bei *Drosophila*



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Contents

Summary

Neuropeptides and peptide hormones carrying neural or physiological information are intercellular signalling substances. They control most if not all biological processes in vertebrates and invertebrates by acting on specific receptors on the target cell. In mammals, many different neuropeptides and peptide hormones are involved in the regulation of feeding and sleep. In *Drosophila*, allatostatin A (AstA) and myoinhibitory peptides (MIPs) are brain-gut peptides. The AstA receptors are homologues of the mammalian galanin receptors and the amino acid sequences of MIPs are similar to a part of galanin, which has an orexigenic effect and is implicated in the control of sleep behaviour in mammals. I am interested in dissecting pleiotropic functions of AstA and MIPs in the regulation of food intake and sleep in *Drosophila*.

In the first part of the dissertation the roles of brain-gut peptide allatostatin A are analysed. Due to the genetic and molecular tools available, the fruit fly *Drosophila melanogaster* is chosen to investigate functions of AstA. The aims in this part are to identify pleiotropic functions of AstA and assign specific effects to the activity of certain subsets of AstA expressing cells in *Drosophila* adults. A new and restricted *AstA^{34}-Gal4* line was generated. The confocal imaging result showed that AstA neurons are located in the posterior lateral protocerebrum (PLP), the gnathal ganglia (GNG), the medullae, and thoracic-abdominal ganglion (TAG). AstA producing DLAa neurons in the TAG innervate hindgut and the poterior part of midgut. In addition, AstA are detected in the enteroendocrine cells (EECs).

Thermogenetic activation and neurogenetic silencing tools with the aid of the *UAS/Gal4* system were employed to manipulate the activity of all or individual subsets of AstA cells and investigate the effects on food intake, locomotor activity and sleep. Our experimental results showed that thermogenetic activation of two pairs of PLP neurons and/or AstA expressing

EECs reduced food intake, which can be traced to AstA signalling by using *AstA* mutants. In the locomotor activity, thermogenetic activation of two pairs of PLP neurons and/or AstA expressing EECs resulted in strongly inhibited locomotor activity and promoted sleep without sexual difference, which was most apparent during the morning and evening activity peaks. The experimental and control flies were not impaired in climbing ability. In contrast, conditional silencing of the PLP neurons and/or AstA expressing EECs reduced sleep specifically in the siesta. The arousal experiment was employed to test for the sleep intensity. Thermogenetically activated flies walked significantly slower and a shorter distance than controls for all arousal stimulus intensities. Furthermore, PDF receptor was detected in the PLP neurons and the PLP neurons reacted with an intracellular increase of cAMP upon PDF, only when PDF receptor was present. Constitutive activation of AstA cells by tethered PDF increased sleep and thermogenetic activation of the PDF producing sLNvs promoted sleep specifically in the morning and evening.

The study shows that the PLP neurons and/or EECs vis AstA signalling subserve an anorexigenic and sleep-regulating function in *Drosophila*. The PLP neurons arborise in the posterior superior protocerebrum, where the sleep relevant dopaminergic neurons are located, and EECs extend themselves to reach the gut lumen. Thus, the PLP neurons are well positioned to regulate sleep and EECs potentially modulate feeding and possibly locomotor activity and sleep during sending the nutritional information from the gut to the brain. The results of imaging, activation of the PDF signalling pathway by tethered PDF and thermoactivation of PDF expressing sLNvs suggest that the PLP neurons are modulated by PDF from sLNv clock neurons and AstA in PLP neurons is the downstream target of the central clock to modulate locomotor activity and sleep. AstA receptors are homologues of galanin receptors and both of them are involved in the regulation of feeding and sleep, which appears to be conserved in evolutionary aspect.

In the second part of the dissertation, I analysed the role of myoinhibitory peptides. MIPs are brain-gut peptides in insects and polychaeta. Also in *Drosophila*, MIPs are expressed in the CNS and EECs in the gut. Previous studies have demonstrated the functions of MIPs in the regulation of food intake, gut motility and ecdysis in moths and crickets. Yet, the functions of MIPs in the fruit fly are little known. To dissect effects of MIPs regarding feeding, locomotor activity and sleep in *Drosophila melanogater*, I manipulated the activity of $MIP^{W\ddot{U}}$ cells by

using newly generated *Mip$^{W\ddot{U}}$-Gal4* lines. Thermogenetical activation or genetical silencing of MIP$^{W\ddot{U}}$ celles did not affect feeding behaviour and resulted in changes in the sleep status.

My results are in contradiction to a recent research of Min Soohong and colleagues who demonstrated a role of MIPs in the regulation of food intake and body weight in *Drosophila*. They showed that constitutive silencing of MIP^{KR} cells increased food intake and body weight, whereas thermogenetic activation of MIP^{KR} cells decreased food intake and body weight by using *Mip^{KR}-Gal4* driver. Then I repeated the experiments with the *Mip^{KR}-Gal4* driver, but could not reproduce the results. Interestingly, I just observed the opposite phenotype. When MIP^{KR} cells were silenced by expressing UAS-tetanus toxin (*UAS-TNT*), the *Mip^{KR}>TNT* flies showed reduced food intake. The thermogenetic activation of MIP^{KR} cells did not affect food intake. Furthermore, I observed that the thermogenetic activation of MIP^{KR} cells strongly reduced the sleep duration.

In the third part of the dissertation, I adapted and improved a method for metabolic labelling for *Drosophila* peptides to quantify the relative amount of peptides and the released peptides by mass spectrometry under different physiological and behavioural conditions. qRT-PCR is a practical technique to measure the transcription and the corresponding mRNA level of a given peptide. However, this is not the only way to measure the translation and production of peptides. Although the amount of peptides can be quantified by mass spectrometry, it is not possible to distinguish between peptides stored in vesicles and released peptides in CNS extracts. I construct an approach to assess the released peptides, which can be calculated by comparing the relative amount of peptides between two timepoints in combination with the mRNA levels which can be used as semiquantitative proxy reflecting the production of peptides during this period.

After optimizing the protocol for metabolic labelling, I carried out a quantitative analysis of peptides before and after eclosion as a test. I was able to show that the EH- and SIFa-related peptides were strongly reduced after eclosion. This is in line with the known function and release of EH during eclosion. Since this test was positive, I next used the metabolic labelling in *Drosophila* adult, which were either fed *ad libitum* or starved for 24 hrs, and analysed the effects on the amount of AstA and MIPs. In the mRNA level, my results showed that in the brain *AstA* mRNA level in the 24 hrs starved flies was increased compared to in the *ad libitum*

fed flies, whereas in the gut the *AstA* mRNA level was decreased. Starvation induced the reduction of *Mip* mRNA level in the brain and gut. Unfortunately, due to technical problems I was unable to analyse the metabolic labelled peptides during the course of this thesis.

Zusamenfassung

Neuropeptide und Peptidhormone sind interzelluläre Botenstoffe, die neuronale und physiologische Informationen tragen. Sie kontrollieren die meisten - wenn nicht alle - biologische Prozesse in Wirbeltieren und Wirbellosen durch ihre Wirkung auf spezifische Rezeptoren an den Zielzellen. So sind bei Säugetieren z.B. viele unterschiedliche Neuropeptide an der Regulierung des Freßverhaltens und des Schlafs beteiligt. In *Drosophila* sind Allatostatin A (AstA) und myoinhibitorische Peptide (MIP) typische Gehirn-Darm- Peptide. Die AstA-Rezeptoren sind Homologe des Galanin-Rezeptors der Wirbeltiere, und die Aminosäurensequenz von MIP sind ähnlich zu einer Teilsequenz von Galanin, welches einen orexigenischen Effekt hat und mit der Kontrolle des Schlafverhaltens in Säugetieren verbunden ist. Ich bin interessiert an der Identifierung möglicher pleiotroper Funktionen von AstA und MIP in der Regulation von Nahrungsaufnahme und Schlaf in *Drosophila*.

Im ersten Teil der Dissertation wird die Rolle der Hirn-Darm- Peptide der AstA-Familie analysiert. Aufgrund der verfügbaren genetischen und molekularen Werkzeuge wurde die Taufliege *Drosophila melanogaster* als Modell ausgewählt, um die Funktionen von AstA zu erforschen. Der Fokus lag dabei darauf, die pleiotropen Funktionen von AstA zu identifizieren, und herauszufinden, ob den verschiedenen AstA-exprimierenden Zelltypen jeweils unterschiedliche Funktionen zukommen. Eine neue, eingeschränkte AstA-Gal4-Linie wurde generiert. AstA-exprimierende Neuronen lassen sich im posterio-lateralen Protocerebrum (PLP), dem Gnathalganglion (GNG), der Medulla und dem thorakal-abdominalen Ganglion(TAG) finden. DLAa-Neuronen im TAG innervieren den Enddarm und den vorderen Teil des Mitteldarms. Ausserdem wird AstA auch in enteroendokrinen Zellen (EEC) im Mitteldarm exprimiert.

Thermogenetische Aktivierung und neurogenetische Stillegung wurden zusammen mithilfe des

UAS/Gal4-Systems eingesetzt, um die Aktivität vieler oder einzelner Untergruppen von AstA-Zellen zu manipulieren und die Effekte auf Nahrungsaufnahme, Laufaktivität und Schlaf zu untersuchen. Unsere Ergebnisse zeigen, dass die thermogenetische Aktivierung der zwei Paare von PLP-Neuronen und/oder AstA-exprimierenden EEC Schlaf und Nahrungsaufnahme reduziert, was auf die signalisierende Funktion von AstA zurückzuführen ist. In der Laufaktivität führte die thermogenetische Aktivierung der zwei Paare von PLP-Neuronen und/oder AstA-exprimierende EEC zu starker Hemmung, und förderte Schlaf ohne geschlechtsspezifischen Unterschied, was während der Aktivitätsgipfel am Morgen und Abend am besten zu beobachten war. Die Experimental- sowie die Kontrollfliegen waren im generellen Klettervermögen nicht beeinträchtigt. In Kontrast dazu reduzierte eine konditionale Stillegung von PLP-Neuronen und allen *AstA-Gal4* exprimierenden Neuronen besonders den Siesta-Schlaf. Fliegen mit thermogenetisch aktivierten AstA-Zellen liefen wesentlich langsamer und weniger als die Kontrollgruppe bei allen Erregungsintensitäten. Außerdem wurde der PDF-Rezeptor in den PLP-Neuronen ermittelt. Die PLP-Neuronen reagierten auf PDF-Gabe mit einem intrazellulären Anstieg von cAMP nur dann, wenn der PDF-Rezeptor anwesend war. Konstitutive Aktivierung von AstA-Zellen durch "tethered" PDF steigerte den Schlaf, und thermogenetische Aktivierung von PDF-produzierenden sLNvs förderte Schlaf besonders am Morgen und Abend.

Die Studie zeigt, dass die PLP-Neuronen und/oder EECs via AstA eine anorexigenische und schlafregulierende Funktion in Drosophila ausübt. PLP-Neuronen verzweigen im posterio-superioren Protocerebrum, wo die für Schlaf relevanten dopaminergen Neurone lokalisiert sind. Die EECs erstrecken sich bis zum Darmlumen. Daher sind die PLP-Neuronen gut positioniert, um Schlaf zu regulieren, und EECs modulieren potenziell die Verdauung und möglicherweise auch Laufaktivität und Schlaf durch Vermittlung der Nahrungsinformationen vom Darm zum Gehirn. Die Ergebnisse von Imaging, Aktivierung des PDF-wegs durch "tethered" PDF und Thermoaktivierung von PDF-exprimierenden s-LNvs weisen darauf hin, dass die PLP-Neuronen durch PDF aus sLNv-Uhr-Neuronen moduliert werden. AstA in den PLP-Neuronen scheint ein indirektes Ausgangssignal der inneren Uhr das die Laufaktivität und Schlaf modelliert. Die AstA-Rezeptoren sind Homologe der Galanin-Rezeptoren; beide sind an der Regulierung von Ernährung und Schlaf beteiligt, was auf eine evolutionär bewahrte Funktion hindeutet.

Im zweiten Teil der Dissertation habe ich die Rolle der MIP analysiert. MIP sind Hirn-

Darm- Peptide der Insekten und Polychaeta. Auch in *Drosophila* wird MIP durch Neurone im ZNS und durch EEC im Darm exprimiert. Bisherige Studien haben Funktionen von MIP bei der Nahrungsaufnahme, Regulation der Darmbewegung und Häutung in Motten und Grillen demonstriert. Für *Drosophila* waren Funktionen von MIP nicht bekannt. Um mögliche Effekte von MIP bezüglich des Freßverhaltens, Laufaktivität und und Schlaf in *Drosophila melanogaster* zu finden, habe ich die Aktivität von $MIP^{WÜ}$-Zellen mit Hilfe der neu in unserem Labor hergestellten $Mip^{WÜ}$ -*Gal4*-Linien manipuliert. Dabei konnte ich keinen Effekt auf das Freßverhalten finden, nachdem ich die $MIP^{WÜ}$–Zellen thermogenetisch aktiviert oder genetisch stillgelegt habe. Allerdings führte dies zu Änderungen des Schlafstatuses.

Meine Ergebnisse stehen im Widerspruch zu einer neueren Veröffentlichung von Min Soohong und Kollegen, die eine Rolle der MIP in der Regulation von Nahrungsaufnahme und Körpergewicht von *Drosophila* nachweisen konnten. Sie zeigten dass konstitutive Stillegung der MIP^{KR}-Zellen Nahrungsaufnahme und Körpergewicht steigerte, während thermogenetische Aktivierung der MIP^{KR}-Zellen Nahrungsaufnahme und Körpergewicht durch MIP^{KR}-*Gal4*-Treiber verringerte. Ich habe daraufhin die Versuche mit der von Soohong eingesetzen Mip^{KR}-*Gal4*-Treiber wiederholt, konnte aber damit die Ergebnisse nicht bestätigen. Interessanterweise habe ich genau das Gegenteil beobachtet. Wenn ich MIP^{KR}-Zellen durch Expresseion von UAS-Tetanustoxin (UAS-TNT) ausgeschaltet habe, zeigten die $Mip^{KR} > TNT$-Fliegen eine reduzierte Nahrungsaufnahme. Eine thermogenetische Aktivierung der MIP^{KR}-Zellen hat die Nahrungsaufnahme nicht beeinflusst. Weiterhin habe ich beobachtet, dass die thermogenetische Aktivierung der MIP^{KR}-Zellen die Schlafdauer stark reduziert.

Im dritten Teil der Dissertation haben ich eine Methode zur metabolischen Markierung für *Drosophila*-Peptide adaptiert und verbessert, um die relative Menge von Peptiden und die Peptidausschüttung mittels Massenspektrometrie unter verschiedenen physiologischen Bedingungen und Verhaltenskontexten zu quantifizieren. qRT-PCR ist eine praktische Technik um die Transkription und die entsprechende mRNA-Menge für ein gegebenes Peptid zu messen. Dies ist allerdings kein zwingendes Maß für die Translation und Menge eines Peptids. Massenspektrometisch kann die Peptidmenge zwar quantifiziert werden, es kann aber nicht zwischen in Vesikel gespeicherten Peptiden und ausgeschütteten Peptiden in ZNS-Extrakten unterschieden werden. Ich habe nach einem Zugang zu den ausgeschütteten Peptiden gesucht, die durch Vergleich der relativen Menge der Peptide zwischen zwei Zeitpunkten kalkuliert werden können,

wenn die mRNA-Menge, welche ein semiquantitatives Proxy der Produktion der Peptide in dieser Periode darstellt, bekannt ist.

Nachdem ich das Protokoll für die metabolische Markierung optimiert hatte, habe ich als Test eine quantitative Peptidomanalyse vor und nach dem Adultschlupf durchgeführt. Dabei konnte ich zeigen, dass die EH- und SIFa-relatierte Peptide nach dem Schlupf stark reduziert sind. Dies passt gut überein mit der bekannten Funktion und Freisetzung von EH während des Schlupfs. Da dieser Test positiv war, habe ich dann als nächsten Schritt die metabolische Markierung in adulten *Drosophila* eingesetzt, die für 24h entweder *ad libitum* gefüttert oder gehungert wurden, und geschaut, wie sich dies auf die Menge der AstA und MIP auswirkt. Meine Ergebnisse zeigten, dass das *AstA* mRNA-Niveau im Gehirn der Fliegen, die 24 Stunden gehungert haben im Vergleich zu *ad libitum* gefütterten Fliegen steigt, während das *AstA* mRNA-Niveau im Darm sank. Hunger führte zur Reduzierung des *Mip* mRNA-Spiegels in Gehirn und Darm. Wegen technischer Probleme konnte ich die metabolisch markierten Peptide während meiner Forschungsphase leider nicht mehr analysieren.

Introduction

"As our feelings change, this mixture of peptides travels throughout your body and your brain. And they're literally changing the chemistry of every cell in your body."

Candace Pert, 1997

If language is a tool used in people to people communication, neuropeptides or peptide hormones may be one of the languages used for cell to cell communication by the nervous and endocrine system. Neurons are carrying information that is encoded in electrical signals. The signals travel along axons and arrive at axon terminals, where they often are transformed into chemical messages. These chemical messages are secreted in response to electrical and/or biochemical events at the terminus and then activate specific receptors on the membrane of neurons inside the central nervous system or on the peripheral targets. Activation of the receptors generate electrical or metabolic signals to initiate functional changes in the target neurons. Neuropeptides are one of the chemical messages. They are a large class of proteinaceous substances, which are produced from neurons or glia cells. They are released not only at the axon terminals, but also from the dendrites, cell somata as well as axon, and then act as neuromodulators within the nervous system, or as hormones on targets in peripheral tissues. Peptide hormones are another regulatory chemical messages. They are secreted into the blood or haemolymph by endocrine gland and circulated to the target cells which can respond to them. (Strand 1999, van den Pol 2012, Fricker 2012).

1 Discovery of the allatostatin A and myoinhibitory peptides in insects.

Substance P was the first neuropeptide to be discovered by v. Euler & Gaddum (1931). It was found not only in the brain, but also in the intestine. Substance P has an effect on the contraction of the intestinal smooth muscles, and on blood pressure. In 1971, Chang et al. (1971) identified the sequence of substance P, which is an 11 amino acid residues peptide with C-terminal amidation (Arg-Pro-Lys-Pro-Glu-Glu-Phe-Phe-Gly-Leu-Met-NH_2). At the beginning, as e.g. for substance P, the approach used to discover neuropeptides or peptide hormones was peptide extraction from brain, intestine or other tissues, and then subsequently biochemical purification with a bioassayto test for activity. Using this method Woodhead et al. (1989) isolated four amidated allatostatic neuropeptides from brains of cockroach *Diploptera punctata* virgin females and tested the synthesized allatostatin A (AstA) for allatostatic activity in vitro. All of them showed more than 40% inhibitory effect on the synthesis of juvenile hormone (JH) by the corpora allata (CA) of virgin females. The primary structures of these four AstA are similar and share the common C-terminal amino acid sequence Tyr-Xaa-Phe-Gly-Leu-NH_2 (Table 1). In 1989, Pratt et al. (1989) also identified a 13-residue allatostatic neuropeptide Ala-Pro-Ser-Gly-Ala-Gln-Arg-Leu-Tyr-Gly-Phe-Gly-Leu-NH_2 from *Diploptera punctata* females. In 1993, Donly et al. (1993) detected *AstA* mRNA in four cells in the pars intercerebralis (PI) of the *Diploptera punctata* brain, and isolated a cDNA from *Diploptera punctata* which encodes a prepropeptide consisting of 13 potential AstA-like peptides. Nine of them share the C-terminal Tyr-Xaa-Phe-Gly-Leu-NH_2 and twelve of them have the Phe-Gly-Leu-NH_2 structure. AstA not only located in the brain, Reichwald et al. (1994) but also detected AstA immunoreactivity pattern in intrinsic endocrine cells of the cockroach midgut.

The allatostatic peptide (allatostatin A (AstA)) in *Drosophila* was first identified using a reverse-physiology approach (Birgül et al. 1999). A G-protein coupled receptor (GPCR) was isolated by using degenerated oligonucleotide primers, which were deduced from the conserved regions of the mammalian galanin receptor. The structure of the *Drosohila* GPCR resembled mammalian galanin receptors. To identify the ligand of the *Drosohila* GPCR, the *Drosophila* GPCR and the mouse G-protein-gated inwardly rectifying potassium channels (GIRK) 1 were

co-expressed in frog oocytes. Application of crude *Drosophila* head extracts induced the activation of GIRK. After purification, an 8-residue peptide Ser-Arg-Pro-Tyr-Ser-Phe-Gly-Leu-amide was isolated, which showed high affinity in the frog oocyte expression system (Birgül et al. 1999). Later, after mining the "*Drosophila* Genome Project" database with various insect allatostatin related sequences, a cDNA was isolated, which encodes a *Drosophila* AstA prepropeptide containing four bioactive peptide copies (Table 1) (Lenz, Williamson & Grimmelikhuijzen 2000).

Table 1. Comparison of the allatostatin A peptides from *Drosophila* and *Diploptera*

Peptide	**Sequence**
Diploptera punctata	
AstA 1	Ala-Pro-Ser-Gly-Ala-Gln-Arg-Leu-**Tyr**-Gly-**Phe**-**Gly**-**Leu**-NH_2
AstA 2	Gly-Asp-Gly-Arg-Leu-**Tyr**-Ala-**Phe**-**Gly**-**Leu**-NH_2
AstA 3	Gly-Gly-Ser-Leu-**Tyr**-Ser-**Phe**-**Gly**-**Leu**-NH_2
AstA 4	Asp-Arg-Leu-**Tyr**-Ser-**Phe**-**Gly**-**Leu**-NH_2
Drosophila melanogaster	
AstA 1	Glu-Arg-**Tyr**-Ala-**Phe**-**Gly**-**Leu**-NH_2
AstA 2	Leu- Pro-Val-**Tyr**-Asn-**Phe**-**Gly**-**Leu**-NH_2
AstA 3	Ser- Arg-Pro-**Tyr**-Ser-**Phe**-**Gly**-**Leu**-NH_2
AstA 4	Thr-Thr-Arg-Pro-Gln-Pro-Phe-Asn-**Phe**-**Gly**-**Leu**-NH_2

Myoinhibitory peptides (MIPs) were first isolated from brain-corpora cardiaca (CC)-corpora allata (CA)-suboesophageal ganglion (SOG) system of the locust *Locusta migratoria* (Schoofs et al. 1991). Also here the extraction-purification-bioassay method was employed. Using high performance liquid chromatography (HPLC) the 28-32 min eluted fraction showed hindgut inhibitory activity, and was further purified. Using Edman sequencing (Edman 1949), the sequence of the first myoinhibitory peptide (MIP) was determinated: Ala-Trp-Gln-Asp-Leu-Asn-Ala-Gly-Trp-NH_2. The synthetic peptide showed the same inhibitory activity as the native MIP (Schoofs et al. 1991). In 1995, using the extraction-purification-biassay method Lorenz et al. (1995) identified four MIP-like peptides from the cricket *Gryllus bimaculatus*, which showed allatostatic activity (Table 2). Accordingly, Lorenz and colleagues named the cricket peptides allatostatin B (AstB).

The discovery of *Drosophila* MIPs was based on the advent of "*Drosophila* Genome Project" database. After identifying a MIP sequence in the genome, Williamson et al. (2001) cloned a MIP/AstB prepropeptide containing five bioactive peptide copies MIP 1-5 (Table 2).

Table 2. Comparison of the vertebrate galanin with MIPs from insects.

Peptide	**Sequence**
Locusta migratoria	
MIP	Ala-**Trp**-Gln-Asp-**Leu**-**Asn**-Ala-Gly-**Trp**-NH_2
Gryllus bimaculatus	
MIP 1	**Gly**-**Trp**-Gln-Asp-**Leu**-**Asn**-Gly-Gly-**Trp**-NH_2
MIP 2	**Gly**-**Trp**-Arg-Asp-**Leu**-**Asn**-Gly-Gly-**Trp**-NH_2
MIP 3	Ala-**Trp**-Arg-Asp-**Leu**-Ser-Gly-Gly-**Trp**-NH_2
MIP 4	Ala-**Trp**-Glu-Arg-Phe-His-Gly-Ser-**Trp**-NH_2
Drosophila melanogaster	
MIP 1	Ala-**Trp**-Gln-Ser-**Leu**-Gln-**Ser**-Ser-**Trp**-NH_2
MIP 2	Ala-**Trp**-Lys-Ser-Met-**Asn**-Val-**Ala**-**Trp**-NH_2
MIP 3	Glu-Ala-Gln-**Gly**-**Trp**-Asn-Lys-Phe-Arg-Gly-**Ala**-**Trp**-NH_2
MIP 4	Glu-Pro-Thr-**Trp**-Asn-Asn-**Leu**-Lys-Gly-Met-**Trp**-NH_2
MIP 5	Asp-Gln-**Trp**-Gln-Lys-**Leu**-His-Gly-Gly-**Trp**-NH_2
Manduca sexta	
MIP 1	Ala-**Trp**-Gln-Asp-**Leu**-**Asn**-**Ser**-**Ala**-**Trp**-NH_2
MIP 2	**Gly**-**Trp**-Gln-Asp-**Leu**-**Asn**-**Ser**-**Ala**-**Trp**-NH_2
Galanin	**Gly**-**Trp**-Thr- **Leu**-**Asn**-**Ser**-**Ala**-Gly-Tyr...

2 Evolutionary aspects in the peptidergic regulation of feeding behaviour

Neuropeptides occur throughout the animal kingdom, ranging from hydra, one of the most basic animal, to mammals. Various neuropeptides have been already characterized in the Cnidarian *Hydra*, which has a very simple nervous system. Most of these isolated hydra-

neuropeptides induce the contraction and relaxation of muscle cells directly (Takahashi 2013).

Peptides can be grouped into sequence-related families, such as the FGL-AstA family and $W(X)_6W$-MIP/AstB family and these families can be highly conserved throughout evolution (Jékely 2013, Mirabeau & Joly 2013). One example for this is cholecystokinin (CCK). CCK is a brain-gut peptide in mammals. It is secreted by neurons in the brain and myenteric plexus as well as enteroendocrine cells (Liddle 1994). In rats, CCK induces satiety upon entry of the food into the intestine (van de Wall et al. 2005) and acts together with serotonin on CCK receptor to enhance the suppression of food intake induced by serotonin via serotonin receptor (Hayes & Covasa 2005). Injection of CCK into the cerebrospinal fluid of sheep decreased food intake (Della-Fera & Baile 1980). The anorexigenic effect of CCK was also reported in chicken. Intracerebroventricular (ICV) injection of CCK strongly inhibited food intake in neonatal chicks, which was dependent on the length of amino acid sequence (Furuse et al. 2000). In humans, CCK is characterized as a satiation signal in response to nutrients and inhibits gastric emptying (Steinert et al. 2017). In insects, sulfakinin (SK) is homologous to CCK (Nachman et al. 1986, Mirabeau & Joly 2013) and the introns are conserved in the CCK and SK receptors (Mirabeau & Joly 2013). SK acts as inhibitory regulator of food intake and is important for satiety signalling in insects (Maestro et al. 2001, Downer et al. 2007, Meyering-Vos & Müller 2007, Söderberg et al. 2012, Yu et al. 2013). Thus, not only the sequence but also anorexigenic function of CCK is conserved between vertebrates and insects.

Galanin is a further vertebrate brain-gut peptide and was first identified in 1983 (Tatemoto et al. 1983). It was reported to regulate feeding behaviour and to be a potential sleep promoter in mammals (Murck et al. 2004, Lang et al. 2015). Hypothalamic injection of galanin in rats and ICV injection of mammalian galanin in neonatal chicks increased feeding behaviour (Kyrkouli et al. 1986, Tachibana et al. 2008). Orexigenic effects of galanin are conserved in vertebrates, but there seem to be no galanin homologs in invertebrates. MIPs which were identified from insects such as *Locusta migratoria*, *Gryllus bimaculatus*,*Manduca sexta* and *Drosophila melanogaster* show a similar sequence to the N-terminal sequence of the vertebrate galanin (Table 2) (Blackburn et al. 1995). However, the G-protein coupled receptors (GPCRs) of AstA are considered as homolog of the vertebrate galanin receptors (Hewes & Taghert 2001, Jékely 2013, Mirabeau & Joly 2013, Felix et al. 2015). In *Drosophila melanogaster* larvae, ablation of AstA and its GPCRs reduced foraging behaviour (Wang et al. 2012). Anorexigenic effects

of AstA were reported in *Drosophila melanogaster* adult flies (Hergarden et al. 2012) and the cockroach *Blattella germanica* (Aguilar et al. 2003).

3 Production and release of neuropeptides and peptide hormones

For *Drosophila*, peptide mRNA is translated into an inactive preproneuropeptide precursor carrying an N-terminal signal peptide. Directed by the signal peptide, the preproneuropeptide precursor is translocated through the ribosome and into the lumen of the rough endoplasmic reticulum (rER). The signal peptide is cleaved-off by a signal peptidase during import into the rER lumen. The resulting proneuropeptide without signal peptide, can contain one or multiple copies of bioactive neuropeptides. Often neuropeptide copies are separated from each other by spacer regions without signalling function. The proneuropeptide is exported to the Golgi apparatus, where the early peptide processing may already start, for example if specific sites within the proneuropeptide are recognized by furin, which cleaves at Arg-Xaa-Lys/Arg-Arg site. Further peptide processing occurs within the dense-core vesicles (DCVs), which bud from the trans-Golgi. In *Drosophila*, first the prohormone convertase 2 encoded by *amontillado* cleaves on the C-terminal side of mono- or dibasic cleavage sites, usually Arg-Arg or Lys-Arg. The C-terminal cleavage sequences are subsequently removed by carboxypeptidase D encoded by *silver*. If a Gly residue is present on the C-terminus of the peptide after the cleavage action of the carboxypeptidase D, the amidating enzymes peptidyl α-hydroxyglycine-α-amidating lyase (PAL) 1 or PAL 2 & peptidylglycine-α-hydroxylating mono-oxygenase (PHM) cleaves between the C_α and the amine group of Gly, resulting in a C-terminal amidation (Fig. 1) (see Strand 1999, Pauls et al. 2014).

DCV containing neuropeptides are transported from the cell body to the axon and dendrites. Depolarization or other stimuli can trigger the fusion of large DCVs and subsequent release of neuropeptides. This calcium-dependent exocytosis requires higher frequencies of discharge or bursting activity than the fusion of synaptic vesicles. Unlike the release of neurotransmitters, the release of neuropeptides can occur not only from nerve endings, but also from dendrites and cell somata. After secretion, the released neuropeptides cannot be recycled into the

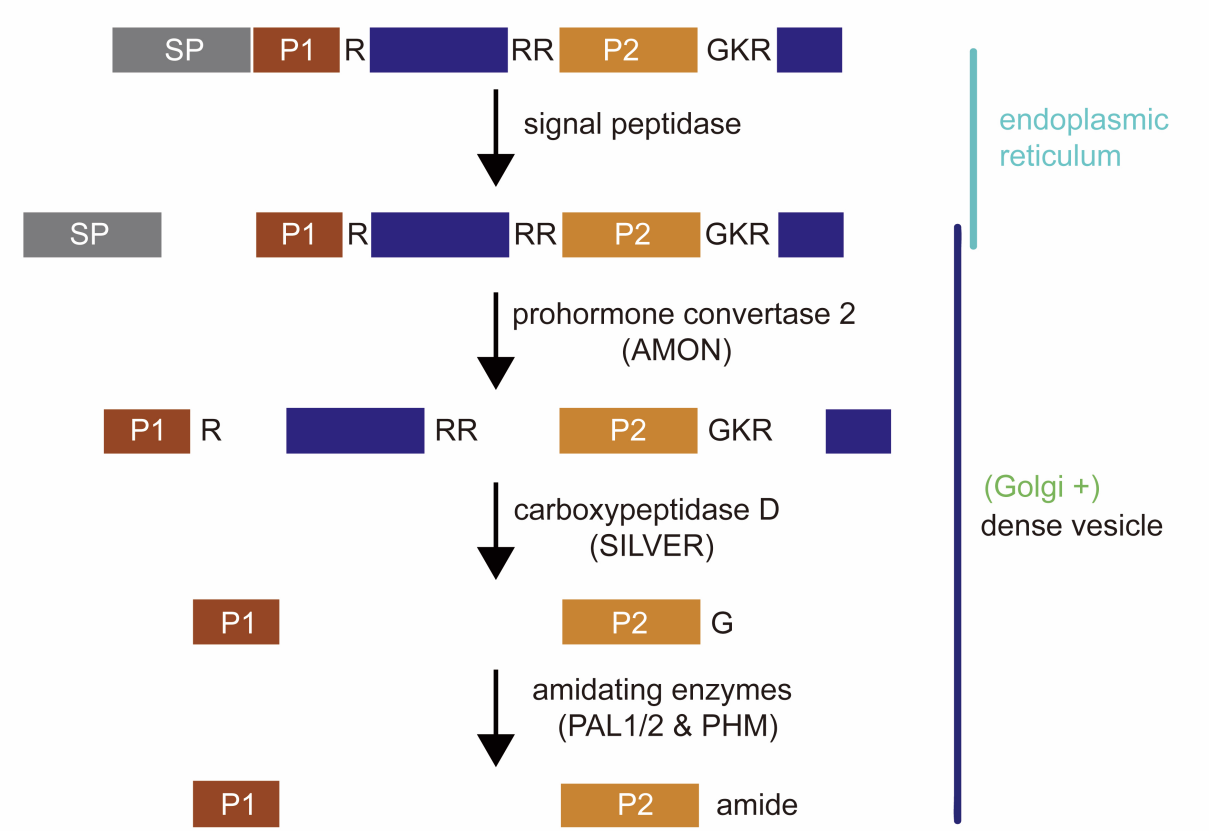

Fig 1. Model of *Drosophila* peptide processing. The prepropeptide carries an N-terminal signal peptide (SP), two bioactive peptide copies (P1 and P2) and spacer regions (blue). The SP is removed by signal peptidase during translocation into the endoplasmic reticulum. Then the resulting propeptide is transported to the Golgi apparatus. Further peptide processing occurs within the dense-core vesicles, which contain proneuropeptide and enzyms bud from the trans-Golgi. First, the prohormone convertase cleaves the C-terminal mono- or dibasic cleavage sites of propeptide. Then, the C-terminal cleavage sequences are subsequently removed by the carboxypeptidase D. In case of the presence of a Gly residue on the C-terminus of P2, the Gly is cleaved by amidating enzymes resulting in a C-terminal amidation. (modified from Pauls et al. 2014)

neurons and large DCVs cannot be regenerated from the sites of release. Large DCVs with newly synthesized neuropeptides must be delieved anew from the cell body (Burke et al. 1997, Strand 1999, Ludwig & Leng 2006, Fricker 2012, De-Miguel & Nicholls 2015).

Within the nervous system, neuropeptides can diffuse by volume transmission over a long distance far away from the site of release to act on the target. Neuropeptides have to exert their functions by activation of their receptors on cell surface. Typically, neuropeptide receptors are G-protein coupled receptors (GPCRs). The receptor of FMRFamide in *Helix aspersa* is an ionotropic receptor (Cottrell 1997) and some such as insulin act on tyrosine kinases-

coupled receptors (Ullrich et al. 1985, Ward & Lawrence 2009). Neuropeptide-receptor systems are not simply "one ligand binds to one receptor". One neuropeptide can have different receptors, different neuropeptides share the same receptor and different neuropeptides can bind to multiple receptors (Fricker 2012).

4 Function of neuropeptides and peptide hormones: energetics player

Neuropeptides and peptide hormones are signal molecules in cell-to-cell communication. Typically, each neuropeptide or peptide hormone is involved in many physiological processes and one biological process can be regulated by many different neuropeptides and peptide hormones.

4.1 Neuropeptides and peptide hormones are involved in the regulation of sleep and wakefulness.

Sleep and wakefulness are fundamental states and essential behaviours, which are conserved across animals from mammals to insects (Kryger et al. 2011). During sleep, the organism is inactive and has reduced sensory responsiveness to stimuli (Campbell & Tobler 1984). Many neuropeptides and peptide hormones are found to play important roles in maintaining sleep and wakefulness. Table 3 lists neuropeptides and peptide hormones which are involved in the regulation of sleep and wakefulness in mammals (Richter et al. 2014) and insects (Dubowy & Sehgal 2017, Schoofs et al. 2017). For example, in mammals orexin activates wakefulness-promoting regions in the brain and is essential for rapid eye movement sleep and wakefulness regulation. Deficiency of orexin results in narcolepsy (Richter et al. 2014, Chow & Cao 2016). In insects, for example, silencing of orcokinin-A and/or -B increased the frequency and duration of death feigning in response to mechanical stimuli in *Tribolium*, suggesting orcokinin-A and B are required for awakening ability (Jiang et al. 2015). In *Drosophila*, neuropeptide F (NPF) and NPF receptor (NPFR) regulate sleep and wakefulness. Overexpression of either NPF or NPFR increased sleep during the dark phase (He et al. 2013). However, thermogenetic activation of NPF expressing cells promoted wakefulness, which can be traced to NPF signalling via NPFR

(Chung et al. 2017). The regulation of sleep and wakefulness is usually associated with the circadian clock, for instance NPF is co-expressed with Period (PER) in clock neurons. Peptides such as pigment dispersing factor (PDF), Ion transport peptide (ITP), SIFamide (SIFa) and MIPs are involved in the regulation of sleep in combination with circadian rhythm (Details in section 5).

Table 3. Neuropeptides and peptide hormones involved in the regulation of sleep and wakefulness in mammals (Richter et al. 2014) and insects(Dubowy & Sehgal 2017, Schoofs et al. 2017).

Mammal		**Insects**
Adrenocorticotropic hormone	Melanin-concentrating hormone	Amnesiac
Brain-derived neurotrophic factor	Melanocyte-stimulating hormones	Calcitonin-gene related peptide
Cholecystokinin	Neuromedin S	Diuretic hormone 31
Cocaine- and amphetamine-regulated transcript	Neuropeptide B	Diuretic hormone 44
Corticotropin-releasing hormone	Neuropeptide S	Ion transport peptide
Cortistatin	Neuropeptide Y	Insulin-like peptide
Dynorphin	Neurotensin	Myoinhibitory peptide
Endomorphin 1	Nociceptin	Neuropeptide F
Epidermal growth factor	Obestatin	Orcokinin A and B
Galanin	Pituitary adenylyl cyclase-activating polypeptide	Pigment-dispersing factor
Ghrelin	Prolactin	SIFamide
Growth hormone	Somatostatin	short Neuropeptide F
Growth-hormone-releasing hormone	Substance P/Tachykinin 1	
Hypocretin/orexin	Transforming growth factor alpha	
Interleukin 1 beta	Tumor necrosis factor	
Leptin	Vasoactive intestinal peptide	

4.2 Neuropeptides and peptide hormones regulate energy homoeostasis

During wakefulness, daily activity is supported by stored energy and results in energy expenditure. Consumption of nutrients maintains energy homoeostasis of the organism. Expenditure of energy induces hunger, which can induce hyperactivity and suppresses sleep. During periods of reduced food availability, animals change their normal activity pattern and adapt with a longer period of wakefulness. Nutrient consumption induces satiety, then promotes sleep (MacFadyen et al. 1973, Antin et al. 1975, Borbely 1977, Viggiano et al. 2009, Keene et al. 2010, Murphy et al. 2016, Yang et al. 2015). Sleep/wakefulness and energy homoeostasis are two separated and yet interdependent processes. This notion suggests that animals need to coordinate their foraging/food intake and sleep/wake behaviour. Neuropeptides and peptide hormones may be shared in common neural regions and pathways between both processes (Sternson 2013).

For example, orexin, a wakefulness promoter, also regulates food intake (Sakurai et al. 1998). ICV injection of orexin increased food intake, and inhibition of orexin receptor reduced food intake in rats (Haynes et al. 2000). Deficiency of orexin containing neurons in the brain results in obesity despite lower food consumption compared to controls. In food deprived condition, the activity of orexin neurons is modulated to maintain wakefulness according to energy expenditure and stores. Mice with ablated orexin neurons did not increase wakefulness and activity in response to starvation (Yamanaka et al. 2003, Villano et al. 2017). Other neuropeptides and peptide hormones such as CCK, galanin, ghrelin, leptin and neuropeptide Y are involved in the regulation of not only sleep-wakefulness but also feeding in mammals (Richter et al. 2014).

In addition, digestion is also an important part energy homoeostasis. Digestive enzymes are required for the utilization of nutrients. In vertebrates, the brain controls digestion and gets the feedback signal from the released digestive enzyme (Penzlin 2005). In insects, base on nutrients in the gut, the digestive enzymes are released continuously and more digestive enzymes are released in fed insects than in starved insects (Lehane & Billingsley 1996, Woodring et al. 2007). Not only the amount and the ratio of nutrients (Lehane & Billingsley 1996), but also

neuropeptides control the release of digestive enzymes. For example, in the cricket *Gryllus bimaculatus*, application of AstA in vitro increased the release of amylase and trypsin enzyme from flat-sheet caecal preparation of 2 days old fed females, indicating that AstA is involved in regulating the release of digestive enzymes in the response to food intake (Woodring et al. 2009). In the cockroach *Blattella germanica*, synthetic AstA inhibited hindgut motility and activated the secretion of α-amylase in the midgut (Aguilar et al. 2003). In contrast, AstA inhibited the release of amylase and trypsin in the larvae of the fall armyworm *Spodoptera frugiperda* (Lwalaba et al. 2010).

5 Neuropeptides and peptide hormones in *Drosophila melanogaster*

5.1 Neuropeptides and peptide hormones in the regulation of feeding behaviour

In *Drosophila*, feeding behaviour can be subdivided into several steps: hunger-induced foraging, evaluation of detected food, initiation of ingestion and food consumption until satiety. This complex process is dependent on multiple factors in order to meet daily needs of energy, depending on locomotor activity, reproduction, metabolism and growth. In this section I will illustrate the role of some neuropeptides and peptide hormones in the regulation of feeding behaviour in *Drosophila* (Fig. 2).

5.1.1 Foraging and evaluation of food sources

For flies, feeding behaviour starts to search about for the food source and olfactory sensitivity plays an important role in this search of food. Odorant receptor neurons (ORNs) expressing odorant receptor genes project axons to glomeruli in the antennal lobe (AL), which is innervated by dendrites of the projection neurons. The olfactory information is then transmitted by the projection neurons from glomeruli to the other parts of the brain (Dethier 1976, Vosshall et al.

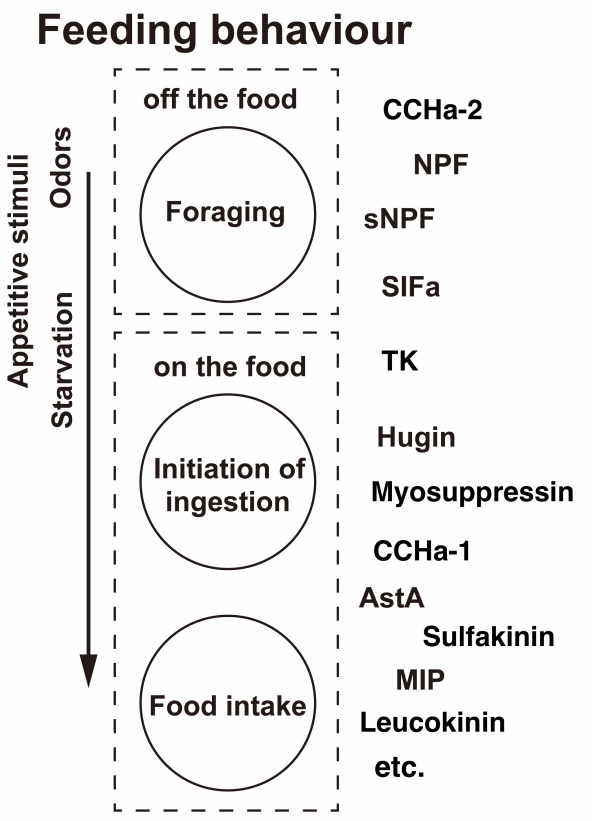

Fig 2. Schematic overview of feeding behaviour. Feeding behaviour can be subdivided into several steps, hunger-induced foraging, evaluation of detected food, initiation of food ingestion and food consumption until satiety. Many neuropeptides and peptide hormones are involved.

2000, Vosshall & Stocker 2007).

Starvation affects food-search behaviour because within the same time period more starved flies can find the food source than fed flies (Root et al. 2011, Zaninovich et al. 2013). Small neuropeptide F (sNPF) and its receptors (sNPFR) are expressed in ORNs, but not in projection neurons. sNPF and sNPFR1 are required for mediating the starvation-dependent modulation of food-search behaviour. Starved flies lacking sNPF signalling or sNPF receptor 1 (sNPFR1) in ORNs showed longer food-search times than starved control flies (Root et al. 2011). Furthermore, starvation increased calcium activity in three glomeruli (DM1, DM4 and DM2) of flies bearing *Or83b>GCaMP*, which was eliminated in *Or83b>GCaMP, sNPFRNAi* and *Or83b>GCaMP, sNPFRRNAi* flies. Presynaptically abolishing sNPF signalling in ORNs decreased postsynaptic projection neuron calcium activity in the DM1 glormerulus of starved flies containing *GH146-LexA*, *LexAop-GCaMP*, *Or83b-Gal4*, and *UAS-sNPF-RNAi* transgenes.

sNPFR1 but not *sNPF* mRNA levels in starved flies is four fold higher than in fed flies. High *Drosophila* insulin-like peptide levels suppress the expression of sNPFR1, resulting in an inhibition of starvation-dependent presynaptic facilitation and decreased food-search ability (Fig. 3). When flies are starved, the expression of sNPFR1 is increased due to low insulin levels. sNPF signalling induces the starvation-dependent presynaptic facilitation and then enhances the response of projection neurons in order to optimize the food search behaviour (Root et al. 2011).

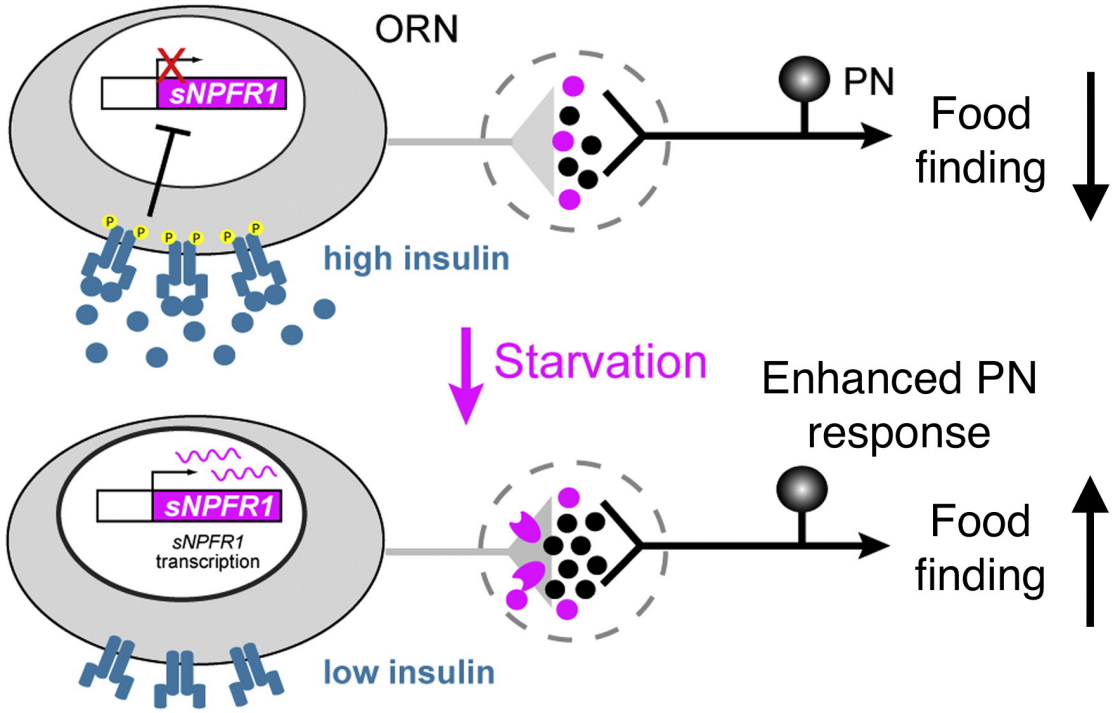

Fig 3. Model for starvation-dependent modulation of food finding. High level of insulin in satiated flies suppresses the expression of sNPFR1 in odorant receptor neurons, resulting in reduced starvation-dependent presynaptic facilitation and food finding. When flies are starved, increased expression of sNPFR1 due to low level of insulin induces the starvation-dependent presynaptic facilitation and then enhances the projection neuron response resulting in increased food finding. (modified from Root et al. 2011)

Files are attracted by food odours which show different attractiveness. For example, when yeast and banana are present, more flies are attracted by the yeast and banana is ignored. Starvation can increase the attractiveness of a food odour. Imaging results showed that food odours induce the activity of neuropeptide F (NPF) neurons in fed and starved flies. However silencing of NPF by *UAS-Kir 2.1* or NPF receptor (NPFR) by *UAS-npfr1*^{daRNA} abolished the food odour induced attraction. NPF is essentional for the attraction of food odours (Beshel

& Zhong 2013).

Ethyl acetate is a food related odorant, which is more attractive for starved flies than satiated flies (Martelli et al. 2017). Application of ethyl acetate increases the starvation-dependent postsynaptic calcium activity of DM3 glomerulus (Martelli et al. 2017). The neuropeptide SIFamide (SIFa) is expressed in the pars intercerebralis (PI) (Terhzaz et al. 2007) and innervates parts of the central complex, the area surrounding the esophagus (OE) and parts of the gnathal ganglia (GNG). The AL and GNG receives olfactory and gustatory information, respectively. SIFa is probably involved in the chemosensory process. When SIFa neurons were activated, satiated *SIFa2>TrpA1* flies were attracted by ethyl acetate at low concentration similar to starved flies. The increased starvation-dependent activity of DM3 in the response to ethyl acetate was abolished when SIFa was downregulated by *UAS-SIFa-dsRNA* in *SIFa2-Gal4* neurons (Martelli et al. 2017).

While flies are attracted by different food odours, hostile conditions prevent flies from foraging. However coerced by the starvation flies search food even under a deleteriously cold condition (Lingo et al. 2007). Overexpression of NPFR signalling in *npfr1-Gal4* forced fed *Drosophila* larvae to forage and downregulation of NPFR expression by *UAS-npfr1*^{dsRNA} in *npfr1-Gal4* or *elav-Gal4* reduced starvation-driven foraging under deleteriously cold condition (at 11°C). *Drosophila* insulin-like peptides (DILPs) as negative modulators regulate starvation-driven foraging under deleteriously cold condition (Fig 4) (Lingo et al. 2007).

When flies are searching food source, they are also evaluating the food quality in order to avoid noxious food. However when flies are in food-deprived condition for long time, the high-risk food source is more acceptable (Bateson 2002). Overexpression of NPF receptor (NPFR) promoted larvae to approach high-risk food source, even noxious food, resulting in increased tolerance to food quality. NPF receptor can be regulated by DILPs signalling pathway. Upregulation of insulin-like receptor (dInR) signalling in NPFR neurons by *UAS-dInR*^{ACT} decreased the starvation-driven attraction of the noxious food. In contrast, downregulation of dInR signalling in NPFR neurons by *UAS-dInR*^{DN} increased the starvation-driven feeding response to the noxious food (Fig 4) (Wu et al. 2005).

Other peptides such as adipokinetic hormone (AKH) (Lee & Park 2004, Yu et al. 2016),

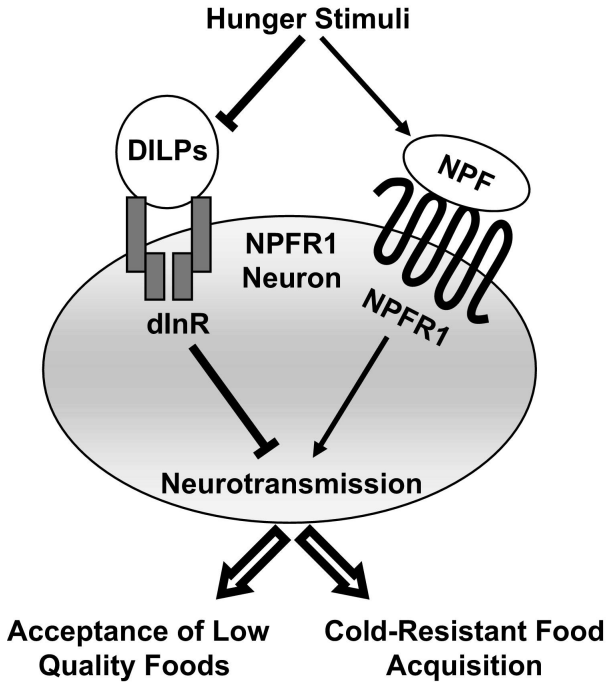

Fig 4. Model for the regulation of noxious food acquisition and food-searching under the deleteriously cold condition. Insulin suppressing the expression of NPF receptor decreases in starved flies and starvation increases NPF and NPF receptor signalling, which induces starvation-drivn behaviour such as acceptance of noxious food and food-search under the deleteriously cold condition (from Lingo et al. 2007).

CCHamide 1 (Farhan et al. 2013), MIPs (Min et al. 2016) and tachykinins (TK) (Ignell et al. 2009) are also important in odours-based and/or starvation-dependent food searching behaviour of *Drosophila*. For example, ablation of adipokinetic hormone (AKH) cells suppressed the starvation-induced hyperactivity (Lee & Park 2004). Flies lacking MIP signalling were more easily attracted by food odours such as yeast paste, grape juice and apple vinegar, whereas thermogenetic activation of MIP cells decreased the attractiveness (Min et al. 2016).

5.1.2 Initiation of food ingestion

After foraging and evaluation of the food source, flies extend the proboscis to initiate the food ingestion, stop ingesting after the first food-ingestion bout, and then start a new round. The appetitive information is received by gustatory receptors to drive the proboscis extension reflex (PER) (Dethier 1976, Wang et al. 2004, Shiraiwa & Carlson 2007, Masek & Scott 2010, Marella et al. 2012). Neuropeptides and peptide hormones such as hugin, AstA, MIPs and SIFa are involved in the regulation of the gustatory response to appetitive stimuli. Hugin expressing

neurons locate in the GNG of *Drosophila*, projecting to the protocerebrum, ventral nerve cord, ring gland and pharynx (Bader et al. 2007, Schlegel et al. 2016). The dendrites of hugin neurons receive input from gustatory organs. Gustatory signals are delivered to protocerebrum via GNG by hugin neurons. Blocking transmission of hugin neurons speed up food ingestion into the crop (Melcher & Pankratz 2005). Furthermore, wildtype flies avoid bitter taste food such as caffeine. The caffeine receptor GR66a neuron terminals locate close to hugin neurons. Flies with thermogenetically activated hugin neurons or lacking hugin neruons did not avoid caffeine compared to the control flies, indicating hugin neurons are required in bitter taste avoidance (Hückesfeld et al. 2016). These data suggest that hugin plays a role in the control of feeding initiation. Starvation enhances the PER of wildtype flies in response to sucrose (Inagaki et al. 2012). Activation of AstA cells by *UAS-NaChBac* (Hergarden et al. 2012) or thermogenetic activation of MIP cells (Min et al. 2016) suppressed the starvation-enhanced PER. Satiated flies with thermogenetically activited SIFa neurons (Martelli et al. 2017) or lacking MIP signalling (Min et al. 2016) showed increased PER as well as starved flies did.

5.1.3 Food intake

After initiation of food intake, flies ingest food until feeding termination and then leave food source. Many neuropeptides and peptide hormones are associated with promotion or suppression of food intake.

sNPF plays different roles in the regulation of food intake in insects. In *Drosophila*, sNPF has orexigenic effect. Overexpression of sNPF increased food intake, which resulted in larger body size and heavier flies whereas downregulation of sNPF signalling by *UAS-sNPF-RNAi* reduced food intake and body weight (Lee et al. 2004). Hong et al. (2012) demonstrated the molecular mechanism of sNPF signalling in regulating food intake (Fig. 5). sNPF modulates a *minibrain* (*mnb*) target gene encoding the Mnb enzyme through the PKA-CREB pathway. The increased Mnb activates Sir2 enzyme, which regulates the deacetylation of FOXO transcriptional factor. The deacetylated FOXO potentiates the expression of the *snpf* target gene, resulting in increased food intake. Increased food intake activates the insulin signalling pathway and increases the level of insulin signalling. The AKT-mediated insulin signalling increases the phosphorylation of the FOXO factor and inhibits the expression of sNPF, resulting in reduced

food intake.

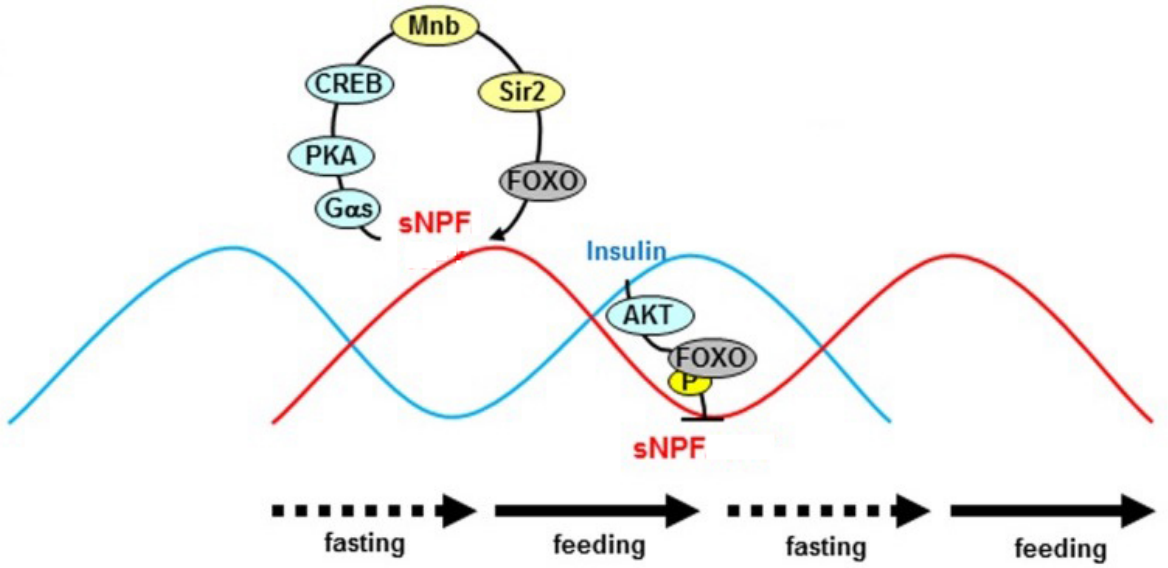

Fig 5. Model for the molecular mechanism of sNPF in the regulation of food intake in *Drosophila*. sNPF modulates *mnb* target gene through the PKA-CREB pathway. The increased Mnb enzyme activates Sir2 enzyme, which regulates FOXO transcriptional factor. The activated FOXO turns on the expression of sNPF, resulting in increased food intake. Increased food intake elevates insulin signalling, which suppresses the FOXO-mediated expression of sNPF resulting in decreased food intake. (modified from Hong et al. 2012)

Allatostatin A (AstA) is also involved in regulating food intake in insects. In *Drosophila* larvae, AstA promotes foraging behaviour.Ubiqutious downregulation of AstA or AstA receptor 1 (DAR-1) by RNAi reduced foraging distances but only when food was present (Wang et al. 2012). In adults, AstA influences food choice and intake. Activation of AstA cells by *UAS-NaChBac* in male and female flies induced the preference for protein (Hentze et al. 2015). Constitutive activation of AstA expressing cells by *UAS-NaChBac* inhibited food intake in starved *Drosophila* adult flies without impact on metabolism, energy storage or food perception. In contrast silencing of AstA expressing neurons increased food intake in starved flies. This anorexigenic effect of AstA can be rescued by activation of NPF expressing neurons (Hergarden et al. 2012).

Other peptides such as myoinhibitory peptides (details in the third chapter), myosuppressin, sulfakinin, leucokinin, tachykinin, CCHamides and others are also involved in the regulation of foraging and feeding. For example, *Drosophila* larval and adult CCHamide-2 mutant flies showed reduced food intake (Ren et al. 2015).

5.2 Neuropeptides and peptide hormones in regulating sleep

In *Drosophila melanogaster*, sleep research started with the studies of Hendricks et al. (2000) and Shaw et al. (2000). Flies were observed to have a state of quiescence with increased arousal threshold, lower sensory responsiveness to external stimuli and lower body position. During sleep, flies seem to be quarantined from the external world. In *Drosophila*, the neural mechanisms of sleep homoeostasis is little known. Based on genetic analysis, genes such as *sleepless* (Koh et al. 2008), *Fmr1* (Bushey et al. 2009) and the steroid hormone ecdyson (Ishimoto & Kitamoto 2010) were identified to be involved in the control of sleep homoeostasis. Recent studies showed that neuropil areas such as the mushroom body (MB), the pars intercerebralis (PI), the central complex as well as the clock neurons are important brain areas that regulate sleep (Potdar & Sheeba 2013).

5.2.1 The mushroom and pars intercerebralis in regulating sleep

In *Drosophila*, one important brain area implicated in sleep control is the mushroom body (MB) including calyx, peduncle and lobes (α/β, α'/β' and γ) (Joiner et al. 2006). Ablation of MB reduced sleep (Pitman et al. 2006) and sleep deprivation increased the sleep promoting circuits within MB (Sitaraman et al. 2015).

The pars intercerebralis (PI) in *Drosophila* is analogous to the mammalian hypothalamus. The epidermal growth factor receptor (EGFR) signaling in the mammalian hypothalamus is considered to be important in the circadian regulation of sleep (Kramer et al. 2001). In *Drosophila*, activation of the EGFR pathway by rhomboid increased sleep in a dose-dependent manner and blockade of *rhomboid* expression in the PI decreased sleep (Foltenyi et al. 2007). Crocker et al. (2010) identified a pathway through which *Drosophila* insulin like peptide 2 (Dilp2) neurons in the PI mediate the effect of octopamine on sleep, suggesting the PI is a structure regulating sleep and wakefulness in *Drosophila* similar to mammalian hypothalamus.

5.2.2 The central complex

The central complex is currently coming into focus as a major neuropil regulating sleep homoeostasis. In *Drosophila*, the central complex consists of the protocerebral bridge, the ellipsoid body, the fan shaped body (FB) and the noduli (Hanesch et al. 1989). Thermogenetic activation of the dorsal fan shaped body (dFB) promoted sleep, including the ExFl2 cells which project to the dFB. dFB neurons showed increased electrical activity after overnight sleep deprivation, while dFB neurons in rested flies were electrically silent (Donlea et al. 2011). *crossveinless-c* (*cv-c*) gene encodes for a Rho-GTPase-activating protein, which is required for the control of sleep homoeostasis because *cv-c* mutants exhibit increased wakefulness (Denholm et al. 2005, Donlea et al. 2014). Overexpression of Cv-c in dFB neurons can rescue the *cv-c* mutants, and silencing of dFB neurons by *UAS-cv-c*^{RNAi} reduced sleep time. The electrical properties of dFB neurons are regulated by Cv-c (Donlea et al. 2014). The majority of dFB neurons in cv-c mutants tends to be electrically silent. The input resistance and membrane time constant of dFB neurons was reduced in *cv-c* mutants, meaning that dFB neurons have a lower sensitivity to synaptic inputs and that the opportunities of dFB neurons for input intergration is limited overtime (Donlea et al. 2014). One dopamine neuron in the so-called PPL1 cluster projects to the dFB. Thermogenetic activation of dopamine neurons promoted wakefulness, which could be blocked by loss of dopamine receptor (Liu et al. 2012). Furthermore, dopamine neurons were more active during wakefulness (Liu et al. 2012). These data suggest that the dFB regulates sleep homoeostasis by switching dFB neurons between active and quiescent states. Pimentel et al. (2016) demonstrated that dopamine as a neuromodulator operates the dFB neuron switch. dFB neurons in wildtype flies belong to the electrically excitable category. Optogenetic stimulation of dopaminergic neurons or pressure ejections of dopamine onto dFB neuron dendrites induces transient hyperpolarization of dFB neurons and promotes wakefulness, and then dFB neurons return to the electrically excitable state. The dopamine 1-like receptor 2 (Dop1R2) and potassium conductances are required.

5.2.3 The circadian clock regulates sleep

5.2.3.1 The molecular clock mechanism

A functional circadian clock is considered to regulate the rhythmic sleep behaviour (Shaw et al. 2000). In 1971 the first gene affecting circadian rhythm was discovered in a single-gene *period* mutant (Konopka & Benzer 1971), other integral genes influencing the circadian clock were found successively: *timeless* (Sehgal et al. 1994), *clock* (Allada et al. 1998), *cycle* (Rutila et al. 1998) and *vrille* (Blau & Young 1999). All these genes and their proteins participate in several transcriptional feedback loops which comprise the foundation of the molecular clockwork which regulates the circadian oscillations at the molecular level (Hardin 2011).

5.2.3.2 The clock neuron network

The circadian clock neuron network consists of about 150 clock neurons expressing the clock genes described above. According to the anatomical position in the brain, clock neurons can be subdivided into the lateral neurons (LNs) and dorsal neurons (DNs). The LNs are a major group of clock neurons, which can be further subdivided into three lateral posterior neurons (LPNs), six dorsolateral neurons (LN_ds), four large ventrolateral neurons (l-LN_vs) and five small ventrolateral neurons (s-LN_vs) per brain hemisphere. The neuropeptide pigment-dispersing factor (PDF) is expressed in four of the s-LN_vs; the 5^{th} s-LN_v is PDF negative. The DNs can be classified into three clusters: DN1, DN2 and DN3. DN1 can be further subdivided into two anterior DN1 neurons ($DN1_a$) and about fifteen posterior DN1 neurons ($DN1_p$). There two DN2 and about 40 DN3 in each hemisphere (Helfrich-Förster et al. 2007, Shafer et al. 2006). (Fig. 6)

Clock neurons generate the normal rhythmic behaviour. The PDF positive s-LN_vs are defined as the morning cells (Grima et al. 2004, Stoleru et al. 2004), which promote morning locomotor activity with DN1 (Zhang et al. 2010), whereas the PDF negative 5^{th} s-LN_v, LN_ds and a subset of the DN1 are evening cells, which are connected to the evening activity (Grima et al. 2004, Stoleru et al. 2004, Murad et al. 2007). s- and l-LN_vs send fibers into the accessory medulla (aMe) and project to the superior protocerebrum. Light information is delivered from the compound eyes and the H-B eyelet via the aMe to the clock network. LN_ds and DNs innervate

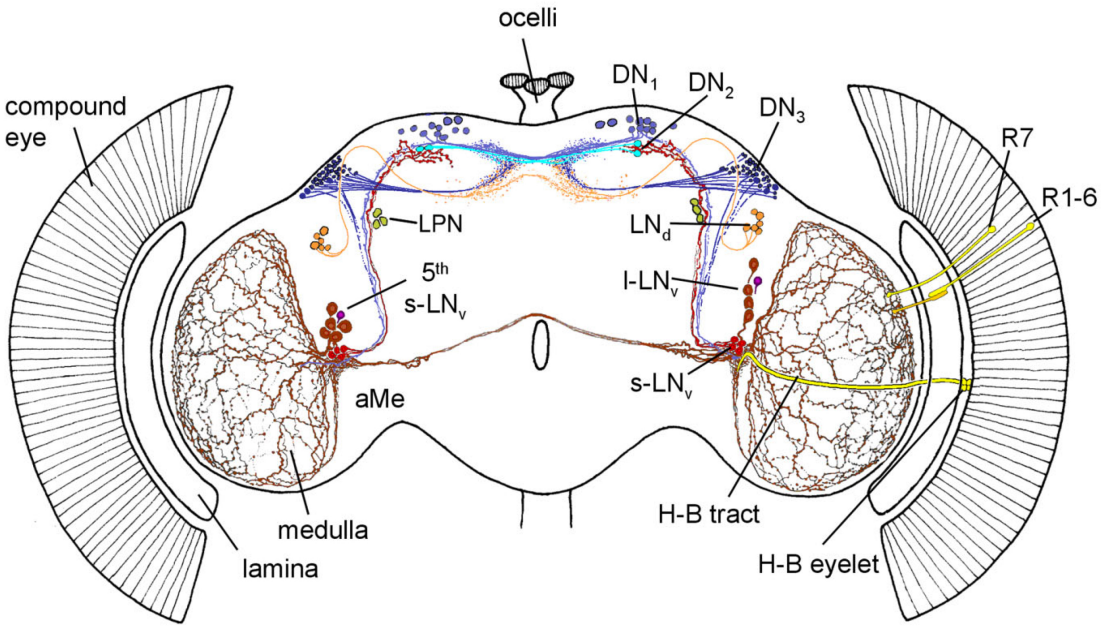

Fig 6. Schematic overview of clock gene expessing neurons and circadian clock network of *Drosophila melanogaster*. The lateral (LNs) and dorsal neuons (DNs) are two main groups of circadian clock neurons. The LNs consist of the LPNs, LN_ds (orange), l-LN_vs, s-LN_vs (red) and 5^{th} s-LN_vs (violet). The DNs consist of DN1, DN2 and DN3 (blue). l- and s-LN_vs send fibers into the accessory medulla and project to the superior protocerebrum. All DNs arborize in the superior protocerebrum (from Helfrich-Förster et al. 2007)

the superior protocerebrum where the PI and FB are located (Helfrich-Förster et al. 2007).

5.2.3.3 Neuropeptides involved in the circadian regulation of sleep

The neuropeptide pigment-dispersing factor (PDF) is a key signal molecule of the clock neuron network (Renn et al. 1999). PDF is essential for circadian rhymicity (Renn et al. 1999, Mertens et al. 2005, Hyun et al. 2005). Immunostainings against the neuropeptides expressed in different clock neurons as well ectopical expression of fluorescent markers enabled the visualization of the arborization pattern of the various clock neurons and different clock neuron clusters. s-LN_vs send projections to the superior protocerebrum (Helfrich-Förster et al. 2007). Hyperpolarization of the LN_vs by expressing the EKO potassium channel (White et al. 2001) under the *pdf-Gal4* driver promoted sleep during day- and night-time, whereas depolarization of the LN_vs by down-regulating the ubiquitous leak channel *Shaw* (Hodge et al. 2005) or a dominant-negative Na^+/K^+-ATPase α subunit (Sun et al. 2001) decreased sleep significantly during the daytime. PDF is required in the LN_vs to promote wakefulness, because *pdf* mu-

tants showed increased sleep time during the light phase in LD and DD, and down-regulation of PDF receptor in LN_vs by *UAS-pdfrRNAi* promoted sleep (Parisky et al. 2008). Furthermore, l-LN_vs are sufficient to promotes wakefulness, which is dependent on the input of the light (Shang et al. 2008). GABAergic neurons are another input pathway that modulate LN_vs and sleep, because overexpression of the *Rdl* encoding $GABA_A$ receptor in the LN_vs promoted sleep (Parisky et al. 2008).

Ion transport peptide (ITP) is another neuropeptide which has a function in the control of circadian clock regulated behaviour. ITP is expressed in the 5th PDF negative l-LN_vs, one of the l-LN_ds, and in some non-clock neurons in the brain. Like PDF, ITP is released in the dorsal protocerebrum. Double knock down of PDF and ITP reduced sleep in the siesta and during the dark phase. This phenotype is not observed, when PDF or ITP alone is knocked down alone (Hermann-Luibl et al. 2014).

Neuropeptide F (NPF) is homologous to neuropeptide Y (NPY) in mammals, which is required for the modulation of sleep-wakefulness homeostasis (Wiater et al. 2011). Overexpression of either NPF or NPFR increased sleep during the dark phase (He et al. 2013). However, thermogenetic activation of NPF producing cells or NPF receptor expressing cells by *UAS-TrpA1* promoted wakefulness, which can be traced to NPF signalling via NPFR (Chung et al. 2017). NPF is co-expressed with Period (PER) in the LN_ds. Furthermore NPF is required by starvation-induced hyperactivity (Chung et al. 2017).

sNPF is also important for sleep homoeostasis. Thermogenetic activation of sNPF neurons by *UAS-TrpA1* promoted daytime sleep, whereas silencing of sNPF neurons by *UAS-Kir 2.1* decreased sleep which can be traced to sNPF signalling (Shang et al. 2013). GABAergic neurons may be the upstream of sNPF neurons to regulate sleep, because the daytime sleep-promoting effect induced by sNPF can be suppressed by the *Rdl* encoding $GABA_A$ receptor in sNPF neurons - *sNPF>rdl-RNAi* flies showed increased daytime sleep (Shang et al. 2013).

Oh et al. (2014) reported a novel sleep homeostatic pathway modulated by sex peptide receptor (SPR) and its ligand MIPs, which is essential for sleep stabilization. Lack of SPR expression (*spr* mutants) or depletion of MIPs by RNAi under *elav* driver promoted wakefulness. SPR is expressed in the LN_vs and specific downregulation of MIP signalling by *UAS-SPR-RNAi* under

control of the *pdf-Gal4* driver was sufficient to promote wakefulness. After a phase of sleep deprivation, wildtype flies show sleep rebound. Interestingly, a sleep rebound was not detected in *spr* mutants, *elav>Mip-RNAi* or *pdf>Spr-RNAi* flies (Oh et al. 2014). MIPs are likely secreted prior to and during the darkness and MIPs can reduce the activity of PDF neurons by downregulation of the cAMP levels via SPR (Oh et al. 2014). Thus, the light input activates l-LN$_v$s to keep flies awake (Shang et al. 2008). As sleep pressure increases, MIPs are secreted to modulate the activity of PDF neurons in order to ensure the siesta sleep. In the dark phase, MIPs are secreted to maintain the stabilization of sleep state.

5.2.3.4 Regulation of circadian output pathways in sleep

Despite the knowledge described above, there is still little known about how the circadian clock output circuits controls sleep. s-LN$_v$s project to the superior protocerebrum where they contact DN1 neurons. DN1 might thus be a downstream target of PDF to control sleep. Diuretic hormone 31 (DH31), which is homologous to the vertebrate neuropeptide calcitonin gene-related peptide (Jékely 2013), is expressed in the DN1 together with PDFR. DH31 decreases sleep late in the dark phase. This suppressed sleep behaviour was observed when the DH31 receptor or PDFR was activated in DN1 neurons (Kunst et al. 2014).

The PI might be another downstream clock target involved in sleep regulation. PI cells are linked to s-LN$_v$s via DN1$_p$ neurons (Cavanaugh et al. 2014). The neuropeptide SIFamide (SIFa), SIFamide Receptor (SIFR) (Park et al. 2014) and diuretic hormone 44 (Cavanaugh et al. 2014) are expressed in PI cells. DH44 plays role in the control of circadian rhythmicity (Cavanaugh et al. 2014, Cavey et al. 2016). Ablation of SIFa neurons and SIFa and SIFR signalling promoted sleep behaviour in PI cells (Park et al. 2014).

6 Aim of the dissertation

The aim of this dissertation is to characterise functions of two peptide families, AstA and MIPs using the genetic model system *Drosophila melanogaster*. I hypothesised that AstA and MIPs generally play important roles in the regulation of food intake, sleep, locomotor activity,

digestion and metabolism.

The first two chapters of the dissertation focus on the regulatory effect of AstA and MIP cells on feeding-related behaviour and sleep/wakefulness activity. The questions I asked are: is AstA or MIPs influencing feeding or sleep behaviour. If so, I wanted to assign the effects to subsets of the expressing cells and characterize the peptidergic pathway in the regulation of feeding and sleep behaviour - how AstA or MIP expressing cells modulate feeding and sleep, what are the up- and downstream target and which internal or external stimuli are involved in the regulatory pathway. The third part of the dissertation focus on the development of stable isotope labeling methods to quantify production and release of peptides.

Functional characterization of neuropeptides and their target receptors helps us better understand the neural circuits and cellular mechanisms behind the regulation of sleep/wakefulness and feeding behaviour, and furthermore provides many opportunities to discover new drug targets for the treatment of disorders in further. As well the expected results will provide an important component to understand the evolution of relevant regulatory circuits within the animal world.

Materials and Methods

1 Flies

The fly strains used in this dissertation are summarized in Table 4. Flies were kept on standard *Drosophila* medium at a 12:12 hours light-dark cycle (LD 12:12) and 25°C, except for the crossing used in TrpA1 and Kir 2.1 experiments, which were kept at 20°C and 18°C, respectively.

Table 4. Fly stains used in this dissertation

Fly Strains	Reference
Wildtype and mutants	
Canton-S	Bloomington Stock Center
han^{5304}	Bloomington Stock Center (Hyun et al. 2005)
w;;pdfr-myc	Kindly provided by Paul Taghert (Im & Taghert 2010)
w^{1118}	Bloomington Stock Center
Gal4-lines	
386y-Gal4	Kindly provided by Paul Taghert (Im & Taghert 2010)
$AstA^{1}$*-Gal4*	Kindly provided by D. Anderson, Caltech, CA, USA (Hergarden et al. 2012)
elav-Gal4	Bloomington Stock Center
Mip^{KR}*-Gal4*	Kindly provided by Jongkyeong Chung (Min et al. 2016)
w;nsyb-Gal4	Kindly provided by T. Langenhan
prospero-Gal4	Kindly provided by J. F. Ferveur
UAS-lines	
UAS-AstA-RNAi	VDRC Stock #103215 KK

UAS-Dcr-2	VDRC Stock #60007
UAS-Epac1camps	(Shafer et al. 2008)
w;UAS-DenMark	Kindly provided by Bassem Hassan (Nicolaï et al. 2010)
10× UAS-IVS-myr::GFP	Bloomington Stock Center (Pfeiffer et al. 2010)
w;UAS-Kir2.1	(Baines et al. 2001)
w;;UAS-ΔOrk-ΔC1	Bloomington Stock center (Nitabach et al. 2002)
w;;UAS-ΔOrk-ΔNC1	Bloomington Stock center (Nitabach et al. 2002)
UAS-tethered-PDF-M6a	Kindly provided by Joel Levine (Choi et al. 2009)
UAS-tethered-PDF-SCR	Kindly provided by Joel Levine (Choi et al. 2009)
UAS-TNT-E	Kindly provided by Sean Sweeney
w;UAS-TrpA1	Bloomington Stock Center
Gal80-lines	
elav-Gal80	Kindly provided by LY and YN Jan (Yang et al. 2009)
w;nsyb-Gal80	Kindly provided by Stephen F. Goodwin (Rezával et al. 2012)
w;tsh-Gal80/CyO	Kindly provided by J. Simpson
UAS-tubGal80^{ts}	(McGuire et al. 2004)

2 Creation of AstA promoter-Gal4 transgenic flies

The creation of AstA promoter-Gal4 transgenic flies were performed by Jan Veenstra and Azza Sellami (Chen et al. 2016). The putative *Drosophila melanogaster* allatostatin A (AstA) 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 (Details in 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* promotoer in the *pAkh-Gal4* vector (Isabel et al. 2005). 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 central nervous system (CNS) and midgut (S1 Fig). We chose the 2.74 kb promoter line *AstA*^{34}*-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).

3 Creation of AstA mutant flies

The generation of AstA mutants by germline-specific expression of CRISPR associated protein 9 (Cas9) and guide RNA (gRNA) transgenes was done by Shu Kondo (Kondo & Ueda 2013) and Hentze et al. (2015) has already described the detail. Mutant stocks were established from two alleles, *AstA*^{SK1} (used by (Hentze et al. 2015)) and *AstA*^{SK4} (*w*^{1118}*;;AstA*^{SK4} used throughout this study), in which the start codon of the *AstA* gene is removed.

4 Creation of *Mip-Gal4* transgenic flies

The *Mip-Gal4* vector was generated by Jan Veenstra. The putative *Drosophila melanogaster* myoinhibitory peptides (MIPs) promoter region was amplified from genomic DNA by PCR using one pair of primer GGAGGAATTCagcagcaaaaagtcggaaaa, GGAGAATTCGGATCCgtgaatttacgggcacgagt, that amplified 2.4 kb upstream of the transcription initiation site. The resulting PCR products were cloned into pCR-TOPO. The inserts were digested with *MunI* and *BamHI*, gel purified and exchanged with the *Akh* promotoer in the *pAkh-Gal4* vector (Isabel et al. 2005). We amplified the *P*{*pMip-Gal4*} plasmids which were injected into *Drosophila w*^{1118} embryos by BestGene Inc. (Chino Hills, CA, USA) and we got 6 independent P-element transformant *Mip*$^{W\ddot{U}}$*-Gal4* lines, namely *Mip*$^{W\ddot{U}}$*-2-* to *Mip*$^{W\ddot{U}}$*-7-Gal4*.

5 Immunostaining

Tissue of feeding 3rd instar larvae or one week old adult flies was dissected in HL3.1 solution (Feng et al. 2004) and fixed in 4% PFA/PBS (pH 7.2) at room temperature for 45 min (guts

and larval CNS) or 90 min (adult CNS). The tissue was blocked with PBT containing 10% normal goat serum overnight at 4°C. After six times washes with PBT (PBS with 0.3% Triton X), the tissue was incubated in primary antibody solution on a shaker for 2 days at 4°C, then several hours at room temperature. Primary antibodies were diluted in PBT containing 3% normal goat serum. Table 5 shows the antibodies used in this dissertation. Samples were then washed six times with PBT, after which they were incubated with secondary antibodies diluted 1:200 in PBT containing 3% normal goat serum for one day at 4°C. Samples were again washed six 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 (Schindelin et al. 2012) was applied for maximum intensity projection and contrast enhancement. Figures were generated with Adobe Photoshop CS2.

Table 5. Antibodies used in this thesis

Primary Antibody	**Donor**	**Dilution**	**Reference**
anti-AstA-7	rabbit	1:2000	Jena Bioscience GmbH, Germany
anti-ELAV mAb	rat	1:100	7E8A10, developed by GM Rubin, obtained from the Developmental Studies Hybridoma Bank, University of Iowa, IA, USA
anti-GFP	mouse	1:1000	A11120, Invitrogen GmbH, Karslruhe, Germany
anti-mCherry	rat	1:1000	Frederick MD, USA
anti-MIPs	rabbit	1:1000	generated by Manfred Eckert, Jena (Predel et al. 2001)
anti-Myc-tag mAb	mouse	1:1000	New England Biolabs, Frankfurt, Germany
anti-PDFc7	mouse	1:2000	Developmental Studies Hybridoma Bank, IA, USA, donated by Justin Blau
Secondary Antibody	**Immunogen**	**Dilution**	**Source**
Alexa Fluor 488	anti-mouse	1:200	Invitrogen, MA, USA

Alexa Fluor 488	anti-rat	1:200	Invitrogen, MA, USA
Alexa Fluor 635	anti-rabbit	1:200	Invitrogen, MA, USA
DyLight 647	anti-rat	1:200	Dianova GmbH, Hamburg, Germany

6 Feeding Assay

6.1 Capillary Feeder (CAFE) assay

The CAFE protocol followed (Ja et al. 2007). 4-5 old male or female flies were anesthetized on ice and transferred into 24-well plates (one fly per well) containing several small holes on the bottom of each well for air exchange. A piece of moist filter paper was added to each well providing flies with water separately from the food. Capillaries (5 µl glass capillary pipettes, Megro GmbH & Co. KG, Wesel, Germany) were filled with liquid food. One capillary per well was inserted through a hole in the lid of the well plate so that the bottom of the capillary was easily accessible to the fly. Food capillaries in wells without flies were used to control for evaporation. The average amount of evaporated liquid in these control capillaries was substracted from the other capillaries in the CAFE assays. Two plates were put into one airtight, humid container, which was placed into an incubator with an LD 12:12 at 22°C or 29°C. Fresh liquid food was prepared 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. Flies acclimated themselves to the new environment and food for one day (day 1). Food consumption was not measured for the first day (day 1). Values measured for day 2 and 3 (descent of the meniscus) were summed up for each fly.

6.2 Fly Liquid-food Interaction Counter (FLIC) assay

FLIC assay was performed as described in (Ro et al. 2014). Three *Drosophila* Feeding Monitors (DFM) were connected to a computer via a Master Control Unit. Both channels were filled with

liquid-food containing 5% sucrose in ultrapure water. 4-5 days old male flies were anesthetized on ice and transferred into feeding arenas for single choice feeding assay and their feeding activity was recorded for 3 days in LD 12:12 at 20°C. Liquid-food was prepared fresh and refilled each day at the same time. According to the original report, we checked the electrical signal of individual fly and set threshold as 40. Electrical signals higher than 40 were considered as feeding. Like in the CAFE assay, total feeding bouts were calculated for day 2 and day 3 accordingly.

7 Startle-induced negative geotaxis assay

Four to five days old flies of each genotype were kept for 24 hrs 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 50 ml 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 recorded. Each trial was repeated 10 times.

8 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 into 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. An infrared beam crossed the tube at the midpoint. As a fly walked back and forth within its tube, the infrared beam was interrupted. Light beam interruptions were counted for individual flies at 1 min intervals representing fly activity. Flies were monitored under LD 12:12 with 365 lux light intensity at 22°C and subsequently at 29°C; average minute-by-minute activities during the day were recorded for both conditions. Activity and sleep data was analysed using ActogramJ (Schmid et al. 2011) and a custom-made Excel macro by Taishi Yoshii (Gmeiner et al. 2013).

9 Arousal assay

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

Tube assay: During this assay, each fly was individually housed in a 65 mm long glass tube (Trikinetics). For each experiment, 5 tubes laying on a loudspeaker (VISATON WS 25E, 8Ω) were used for each genotype. 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 fly behaviour was recorded by a camera (The IMAGINGSOURCE® Bremen, Germany. Lense: PENTAX TV LENS 25 mm 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 the loudspeaker to generate a 5 Hz sine wave to agitate the flies. The interval between the individual stimulus 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 male 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 stimulus was between 5-8 min. Fly behaviour was recorded at 1 frame/s for 12 hrs and the locomotor activity was measured by eye, then arousal thresholds (percentage of flies moving) were calculated for each stimulus.

10 cAMP live imaging

The live-cell cAMP imaging was performed by Christiane Hermann-Luibl (Details in Chen et al. 2016).

11 Quantitative Real-Time PCR

Canton-S flies were raised on nornal food in LD12:12. 4-5 days old flies were transfered into vials with normal food (fed) or 2% agarose (food deprived). After 24 hour, brain, TAG and guts were dissected from each group.

The Quick-RNA™ MicroPrep KIT from Zymo research (Irvine, USD) was used to extract mRNA. All steps were performed following the protocol of manufacturer.

The QuantiTect® Reverse Transcription Kit from Qiagen (Venlo, Netherlands) was used for cDNA synthesis. All step were performed following the protocol of manufacturer. cDNA samples were stored at -20°C.

For quantification of mRNA level, specific primers were designed using FlyBase (www.flybase.org) and primer BLAST function of NCBI (www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 6). All primers were ordered from Sigma-Aldrich® (Munich, Germany). α-*tubulin* was chosen as house keeping gene for normalization. qPCR was performed in Rotor-Gene Q (Qiagen Hilden, Germany) by using SensiMix™ SYBR® No-ROX Kit from Bioline (London, UK). The qPCR reaction recipe is shown in Table 7 and the cycle program is shown in Table 8.

Table 6. Primers used in this dissertation.

Target	**Orientation**	**Sequence (5'→3')**
AstA	forward	TGGAATTCGCTCAGCAGTAG
	reverse	AGTAGGAGGTGGGCGTGAAG
Dar-1	forward	CAAACCTTCCGCAGAGTC
	reverse	GAGGATGACATGAATGGGC
Dar-2	forward	GGATGATGAGGACGGAGAAC
	reverse	GTAATCCACCACCACGTCG
Mip	forward	GCGAGGAGATATATAGTCAGCTATGG
	reverse	GCCACCAAATTACCGCAAG
Spr	forward	CGCCGTTCAAAGATACATCTAC
	reverse	GAAACGCCAGCAATGCAATATAC
α-tubulin	forward	TCTGCGATTCGATGGTGCCCTTAAC
	reverse	GGATCGCACTTGACCATCTGGTTGGC

Table 7. Recipe for qPCR

SensiMix™ SYBR® No-ROX (Bioline)	10 μl
forward Primer 400 nM	0.8 μl
reverse Primer 400 nM	0.8 μl
cDNA Template	1.0 μl
H_2O	7.4 μl
Total Reaction Volume	20 μl

Table 8. Program for qPCR

Temperature (°C)	**Time (s)**	**Cycles**
95	120	1
95	5	40
63	10	
72	15	
4	Hold	

12 Metabolic labelling

12.1 Yeast labelling

Minimal medium consisted of 1.7 g yeast nitrogen base without amino acids and ammonium sulfate (e.g.BD Difco 233520), 20 g sucrose (analysis grade, nitrogen-free), 1 g ^{15}N-labelled ammoniumsulfate (for ^{15}N-labelling) or 1 g normal ammoniumsulfate (for ^{14}N-labelling) and 1 l Aqua bidest. 5 ml minimal medium was inoculated with yeast (*Saccharomyces cerevisiae*, Sigma, type II YSC2) on a shaker with 230-270 rpm at 30°C overnight. 1 l minimal medium was inoculated by 500 μl of the overnight culture on a shaker with 230-270 rpm at 30°C overnight. After centrifugation at 2400 g for 20 min at 4°C, the supernatant was removed which was used for a second incubation as above to collect further yeast and the yeast pellet was resuspended in 20 ml PBS. After centrifugation at 2400 g for 20 min at 4°C, the supernatant was removed and the yeast was stored at -80°C for next step.

12.2 Fly labelling

60 female and 30 male flies were placed in a vial filled with 2-3 cm 2% agarose overnight for egg laying, and then 40 eggs were transported into a new vial filled with 2-3 cm 2% agarose. A piece of nitrogen free filter paper (Macherey-Nagel, Germany) was placed into the vial. 1ml yeast-sugar solution containing 3.4 g (^{15}N- or ^{14}N-) labeled yeast, 0.9 g sucrose and 7ml ultrapure water was added into one vial everyday at the same time point (ZT 2). For each example, two vials obtained ^{15}N-labelled solution and the other two vials obtained ^{14}N-labelled solution. Flies were incubated at 20°C in LD 12:12 until dissection.

12.3 Tissue dissection and extraction

Brain, TAG and gut from pharate adults which were either very close to eclosion or freshly eclosed with unexpanded wings were quickly dissected in HL3.1 solution (Feng et al. 2004) on ice. The same amount of ^{15}N-labelled and ^{14}N-labelled tissue was pooled into one pre-cooled

low-binding tube kept in a labtop cooler.

Sample 1: Brain/TAG of ^{14}N-labeled pharate adult+Brain/TAG of ^{15}N-labeled eclosed flies
Sample 2: Brain/TAG of ^{15}N-labeled pharate adult+Brain/TAG of ^{14}N-labeled eclosed flies
Sample 3: Gut of ^{14}N-labeled pharate adult+Gut of ^{15}N-labeled eclosed flies
Sample 4: Gut of ^{15}N-labeled pharate adult+Gut of ^{14}N-labeled eclosed flies

After dissection 30 μl methanol/water/Trifluoroacetic acid (TFA) (90/9/1 v/v/v) was added into the tube kept on ice. For nervous system samples, the tissue was sonicated in a cold ultrasonic water bath for 1 minute until the tissue was largely disintegrated. For gut samples, the tissue was incubated on ice for 30 min. Samples (nervous system or gut) were centrifuged for 15 min at 15,000 g. The supernatant was transferred into a fresh tube, then centrifuged for 15 min at 15,000 g. The supernatant was transferred into a fresh tube and dried down in a vacuum concentrator to get rid of the methanol.

Jens Vanselow and Andreas Schlosser analysed the extracted peptides by nanoLC–MS/MS on an Orbitrap mass spectrometer (EASY-nLC 1000 coupled to LTQ Orbitrap Velos, Thermo). The ratio (^{14}N/^{15}N) of each identified peptide was calculated using Mascot Distiller (Matrix Science) software.

13 Statistics

OriginPro 9.1G and the R environment (http://www.r-project.org/) were used for plotting and statistical analysis. One-way ANOVA with post-hoc Tukey's HSD tests was 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.

I. Allatostatin A signalling in *Drosophila* regulates feeding and sleep and is modulated by PDF

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Figures	
1	Wencke Reiher
2-7	Jiangtian Chen
8A, B and E	Jiangtian Chen
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9 and 10	Jiangtian Chen
Supplementary Figures	
S1	Christian Wegener
S2	Wencke Reiher
S3-S16	Jiangtian Chen
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Supplementary Movie 1-3	Jiangtian Chen
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Writing of Materials & Methods	Wencke Reiher, Jiangtian Chen, Christian Wegener and Christiane Hermann-Luibl
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Writing of Discussion	Christian Wegener, Jiangtian Chen and Wencke Reiher
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Primary Supervisor's Name Date Place Signature

1 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 (Boonen et al. 2009). Most if not all peptides are pleiotropic and can orchestrate diverse physiological, neuronal or behavioural processes (Nässel & Winther 2010, Kastin 2013). 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 (Brown et al. 2015, Richter et al. 2014), which reciprocally influence each other (Penev 2012, Saper 2006). 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 (Penev 2012, Saper 2006, Bonnefont 2010). 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 (Cirelli 2009, Itskov & Ribeiro 2013, Pool & Scott 2014, Sehgal & Mignot 2011). *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 (Itskov & Ribeiro 2013, Huber et al. 2004, Shaw et al. 2000). 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 (Ro et al. 2014, Seay & Thummel 2011, Xu et al. 2008) 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 (Itskov & Ribeiro 2013, Pool & Scott 2014) or sleep (Griffith 2013, Kunst et al. 2015) in *Drosophila*. Yet, so far, only sNPF (Chen et al. 2013, Hong et al. 2012, Lee et al. 2004, Shang et al. 2013) and likely also NPF (He et al. 2013, Wu et al. 2003) 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 (Erion et al. 2012).

Recent work by Hergarden and colleagues demonstrated that neurons expressing neuropeptides of the allatostatin A (AstA) family regulate feeding behaviour of the fruit fly (Hergarden et al. 2012). Constitutive activation of AstA cells contained in the *AstA*^{1}*-Gal4* expression pattern by ectopic expression of the bacterial low threshold voltage-gated NaChBac channel (Nitabach et al. 2006) potently inhibited starvation-induced feeding. In contrast, constitutive inactivation of AstA^{1} cells by expression of the inwardly rectifying Kir2.1 potassium channel (Baines et al. 2001) increased feeding under restricted food availability. NaChBac activation of AstA^{1} cells also inhibited the starvation-induced increase of the proboscis extension reflex (PER), a behavioural indicator for glucose responsiveness (Hergarden et al. 2012). The AstA^{1} expression pattern includes a large number of brain neurons plus gut-innervating thoracico-abdominal ganglion (TAG) neurons and enteroendocrine cells (EECs) in the posterior midgut (Hergarden et al. 2012). This broad expression pattern is consistent with earlier described patterns of AstA-like immunoreactivity (Veenstra 2009, Veenstra et al. 2008, Yoon & Stay 1995, Reiher et al. 2011) and suggests multiple functions for AstA. Earlier work had demonstrated an effect of AstA on gut motility (Vanderveken & O'Donnell 2014). Two AstA receptors, DAR-1 (=AlstR) and DAR-2 are characterised for *Drosophila* (Birgül et al. 1999, Lenz, Søndergaard & Grimmelikhuijzen 2000, Lenz, Williamson & Grimmelikhuijzen 2000, Larsen et al. 2001). Different genome-based phylogenetic GPCR analyses independently demonstrated their homology with the galanin receptor family of vertebrates (Mirabeau & Joly 2013, Felix et al. 2015, Jékely 2013, Hewes & Taghert 2001)

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 pigment dispersing 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 (Lang et al. 2007, 2015, Steiger 2007).

2 Results

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

2.1 Expression pattern of the *AstA^{34}-Gal4* line

To test the specificity of *AstA^{34}-Gal4* expression in adult flies, we co-immunolabelled *AstA^{34}>GFP* flies against GFP and AstA. The observed AstA immunoreactivity (IR) pattern was consistent with earlier descriptions (Veenstra 2009, Veenstra et al. 2008, Yoon & Stay 1995) (Fig 7), and we adopted the nomenclature of Yoon & Stay (1995). S1 Table provides a summary of the localization of *AstA^{34}-Gal4*-driven GFP expression in relation to the AstA IR.

In each brain hemisphere of *AstA^{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 7A and 7B). 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 7A and 7B, 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^{1}-Gal4* line (Fig 7A and 7B). In addition, a varying small number of AstA-IR neurons in the medulla showed generally weak GFP expression (Fig 7A and 7C). In some preparations, single medulla neurons were found that exhibited a stronger GFP signal (Fig 7C).

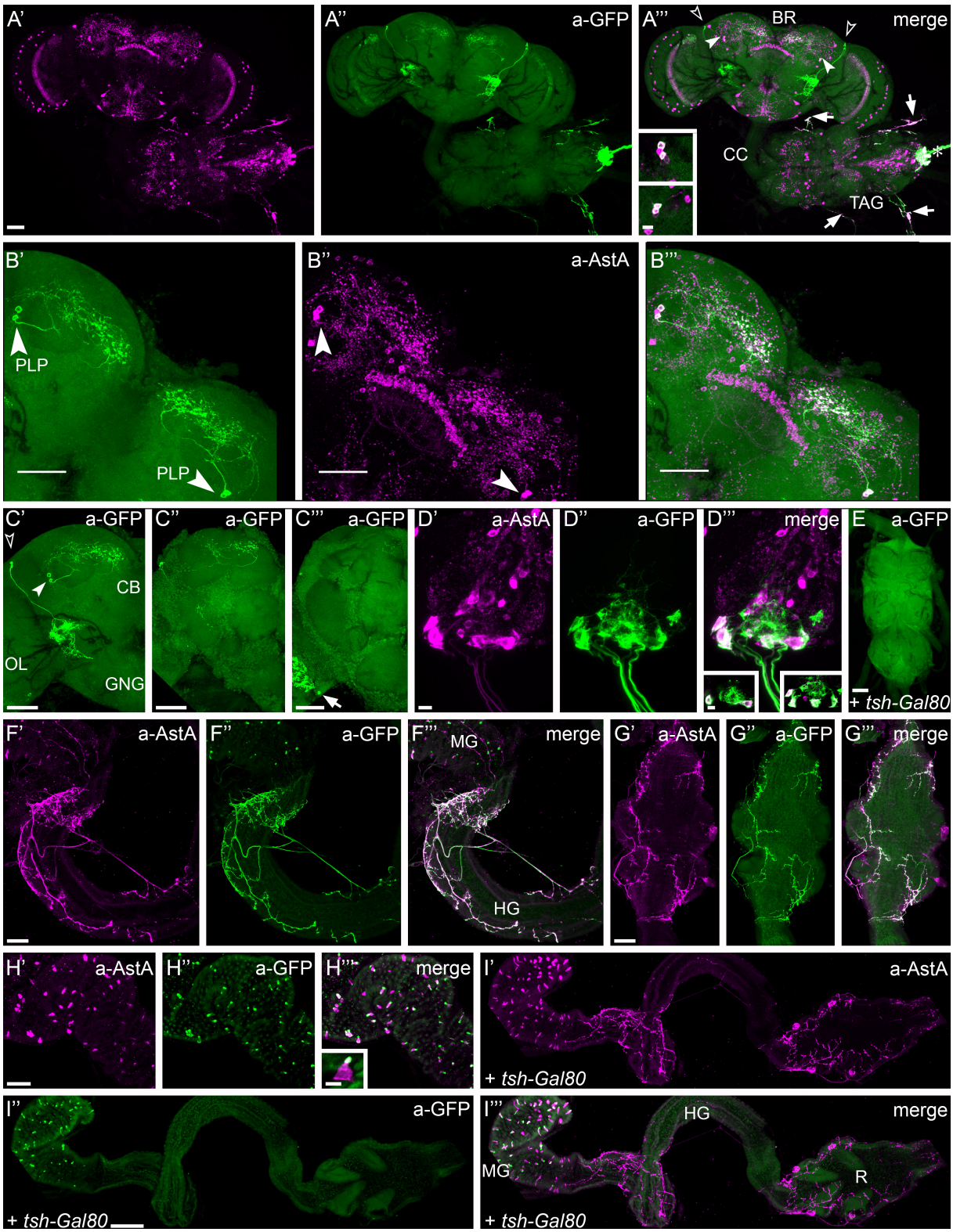
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Fig 7. AstA (magenta) and GFP (green) immunolabeling of nervous systems and guts of adult *AstA^{34}>GFP* (A–D, F–H) and *tsh-Gal80; AstA^{34}>GFP* (E, I) flies. (A) GFP expression is detectable in two paired groups of brain neurons. In each hemisphere, 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 gut (asterisk in A"'). Four peripheral cells located on nerves that exit the TAG dorso-laterally also exhibit co-labeling (arrows in A"'). Inset in A"': Single optical sections showing double-labelled PLP cells of both hemispheres. (B) Close-up of the PLP neurons (solid arrowheads in B',B") from A, maximum projection of the horizontal sections between the level of central body and calyx. The extensive arborisations in the superior protocerebrum are visible. (C) The GFP signal in brains of different individuals illustrates the variability of expression intensity among PLP, LCBR and medulla neurons. The PLP cells (solid arrowhead in C') innervate the superior protocerebrum, while the ramifications of the LCBR neurons (open arrowhead in C') lie mainly within the posterior lateral protocerebrum and the posterior slope. C' and C" show a strong and moderate GFPexpression intensity, respectively. The arrow in C"' marks a strongly stained neuron in the medulla, which occurred only in a few preparations (D) Detail of the abdominal TAG. Three pairs of AstA-IR DLAa neurons co-express GFP and run through the median abdominal nerve to innervate the hindgut and posterior midgut (see F). The membrane-targeted GFP distinctly marks the projections of these neurons. Two single optical sections (insets in D"') reveal six co-labelled cell bodies. (E) *AstA^{34}-Gal4* expression in the TAG is absent with *tsh-Gal80*. (F) AstA and GFP labeling are present in neuronal processes at the hindgut, which extend onto the posterior midgut (Malpighian tubules have been removed during dissection). (G) The rectal part of the hindgut is likewise innervated by double-labelled neurons. (H) GFP is expressed in most of the AstA-producing EECs that are scattered within the epithelium of the posterior midgut. The maximum intensity projection of a single EEC in the inset of H"' illustrates that the main GFP signal is restricted to the narrow apical portion of the cells. (I) GFP expression is absent from gut neurons in individuals carrying *tsh-Gal80*, but remains in the AstA EECs. (Malpighian tubules have been removed during dissection.) Scale bars: in A-C and E-H 50 μm; in D 10 μm; in I 100 μm. BR brain, CB central brain, CC cervical connective, IL ileum, MG midgut, OL optic lobe, PV pyloric valve, R rectum, RV rectal valve, GNG gnathal ganglia, TAG thoracico-abdominal ganglion.

In the thoracico-abdominal ganglion (TAG), three pairs of AstA-IR DLAa cells within the posterior abdominal region ((Erion et al. 2012), Fig 7A and 7D) sent neurites via the median abdominal nerve to innervate the hindgut and the posterior-most midgut (Fig 7F, 7G and 7I). 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 7G), thus their peptide signals might target the rectal epithelium. The DLAa neurons consistently exhibited strong AstA^{34}-driven GFP expression, while the brain neurons showed a more variable GFP labelling intensity between preparations (see Fig 7C). 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 central nervous system (CNS), two pairs of peripheral AstA-IR neurons with somata located on the segmental nerves leading to the wings and the halteres(Yoon & Stay 1995) expressed GFP (Fig 7A). Furthermore, GFP was detectable in most if not all AstA-IR EECs in the posterior part of the midgut (Fig 7F–7H). The staining results are summarized in S1 Table.

In comparison to the *AstA^{34}-Gal4* pattern, the expression pattern of *AstA^{1}-Gal4* included the following AstA-IR neurons per brain hemisphere: all three PLP neurons, 2 neurons in the superior protocerebrum, $\sim$ 30 medulla neurons, and three neurons with cell bodies in the GNG (gnathal (= subesophageal) ganglion) thought to be important for sucrose responsiveness (Hergarden et al. 2012). Thus, *AstA^{1}-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^{34}-Gal4* includes a larger fraction of AstA EECs in the midgut. A schematic summary of the expression patterns is given in S2 Fig.

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

To test for a possible role of AstA^{34} cells in the control of food intake, we employed the CAFE assay (Ja et al. 2007) and measured food intake while AstA^{34} 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 (Hamada et al. 2008, Pulver et al. 2009). Male *AstA^{34}>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^{34}>TrpA1* flies (Fig 8A). A similar

reduction of food intake at 29°C was detected for *AstA^{1}>TrpA1* flies (Fig 8B). 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^{1}>NaChBac* in a different feeding assay (Hergarden et al. 2012). These findings indicate that the LCBR neurons (lacking in *AstA^{1}*) and AstA cells in the GNG (lacking in *AstA^{34}*) are dispensable to reduce food intake. Thus, activation of only the AstA^{34} subset appears sufficient to reduce food intake.

To restrict the activation pattern further, we created *tsh-Gal80; AstA^{34}>TrpA1* (*UAS-TrpA1/tsh-Gal80; AstA^{34}-Gal4/+*) flies. *tsh-Gal80* suppresses *Gal4* expression in the thoracic and abdominal part of the CNS (Clyne & Miesenböck 2008, Yu et al. 2010, Tsubouchi et al. 2012), and limited *TrpA1* expression to AstA^{34} central brain neurons and EECs (Fig 7E and 7I). Thermogenetic activation of this AstA^{34} cell subset by a shift to 29°C was sufficient to reproduce the feeding phenotype found in *AstA^{34}>TrpA1* flies (Fig 8C), 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^{34}-Gal4* 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^{1} cells by constitutive expression of *UAS-Kir2.1* (Baines et al. 2001) has previously been reported to increase feeding under restricted food availability (Hergarden et al. 2012). To exclude developmental effects due to constitutive silencing, we next conditionally manipulated AstA cells using the TARGET system (McGuire et al. 2004). At both 18°C and 30°C, *tubGal80ts;AstA^{34}>Kir2.1* flies showed a similar food intake as controls 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 (Schoofs et al. 2014).

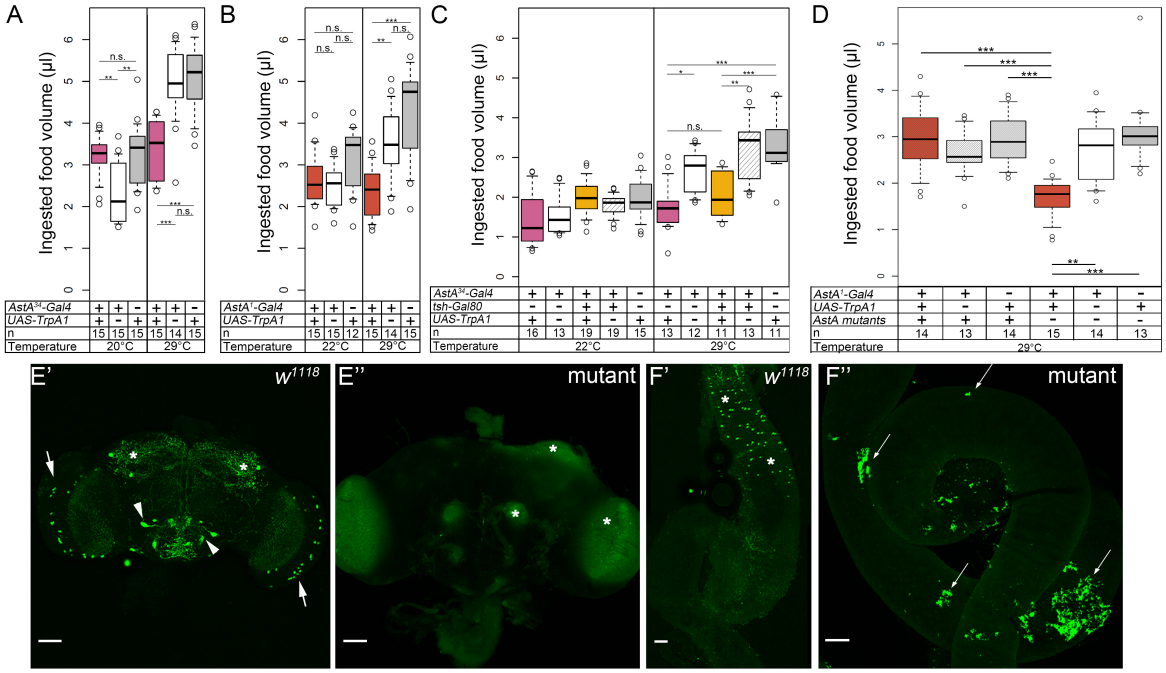


Fig 8. Thermogenetic activation of the AstA cells resulted in reduced food intake. The total food volume consumed within two days was measured via the CAFE assay. At 20°C, *AstA*^{34}>*TrpA1* (A), *AstA*^{1}>*TrpA1* (B) and *tsh-Gal80; AstA*^{34}>*TrpA1*(C) flies did not consume less food than the controls. Activation of the TrpA1 channel at 29°C resulted in significantly reduced food intake in *AstA*^{34}>*TrpA1* (A), *AstA*^{1}>*TrpA1* (B) flies compared to the controls. Food intake of *tsh-Gal80; AstA*^{34}>*TrpA1* (C) flies was significantly lower than in control flies, but not different from *AstA*^{34}>*TrpA1* flies. (D) Thermogenetical activation of AstA^{1} cells did not reduce food intake in flies with a *AstA*^{SK4} null mutant background, in contrast to flies with an *AstA* wildtype background tested in parallel. (E) Maximum projections of confocal stacks of an adult brain of a *w*^{1118} control (E') and an *AstA*^{SK4} mutant (E") immunostained against AstA. (F) AstA immunostaining in the midgut of a *w*^{1118} control (F') and *AstA*^{SK4} mutant (F"). AstA-IR neurons are visible in the optic lobes (arrow), gnathal ganglion (arrow head) and superior protocerebrum (asterisks) in the brain (E'), and in EECs in the posterior midgut (asterisks in F'). In contrast, AstA-IR cells are absent in the mutant, indicating a global lack of AstA peptides. The mutant tissues were scanned at very high gain to detect even weak potential AstA immunostaining. This lead to detection of autofluorescence signals in the brain (asterisks in E") and gut associated fat body (arrows in F"). Scale bars: 50 μm.

2.3 Reduced food intake upon AstA cell activation can be traced to Allatostain A signalling

Peptides are typically co-localised with other peptides or classic transmitters (Hökfelt et al. 1987, Nässel & Homberg 2006). To identify whether the observed feeding phenotype upon activation

of AstA cells is due to AstA or a co-localised peptide/transmitter, we used $AstA^{SK4}$ null mutant flies generated by germline-specific CRISPR/Cas9 (Kondo & Ueda 2013). In contrast to controls, $AstA^{SK4}$ mutants are devoid of any AstA-IR in the nervous system and gut (Fig 8E and 8F). We then thermogenetically activated the $AstA^1$ neurons in an *AstA* null mutant background and found no difference in food uptake compared to controls (Fig 8D). Similar observations were made when reducing *AstA* expression by RNAi in $AstA^{34}>TrpA1/AstA\text{-}RNAi$ 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 expermental conditions; controls in wildtype and $AstA^{SK4}$ mutant background showed similar amounts of ingested food (Fig 8D).

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

Locomoteor 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 $AstA^1>TrpA1$ and $AstA^{34}>TrpA1$ flies was strongly and significantly reduced at 29°C, but not at 22°C in both sexes (Fig 9A and 9B, S6 Fig). In contrast, $AstA^1>TrpA1$ 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 9F), showing that the flies were not suffering from impaired locomotor ability or energy deficiency due to decreased feeding.

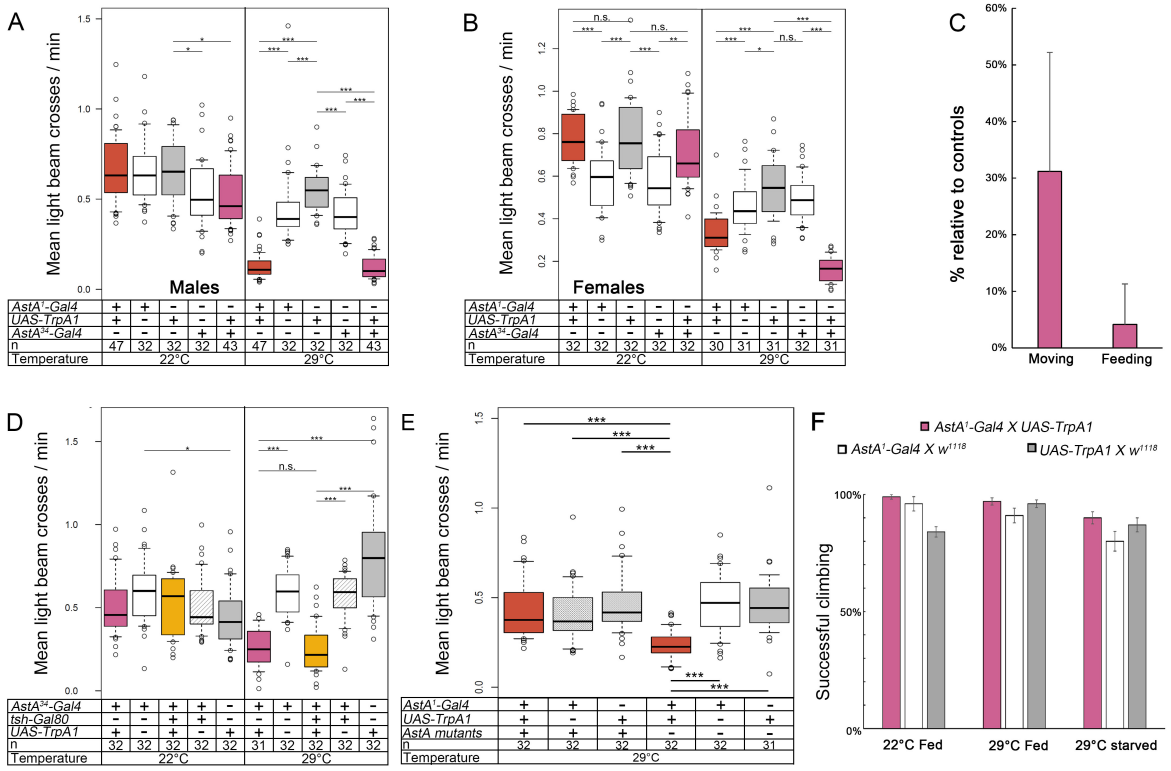


Fig 9. Thermogenetic activation of AstA cells resulted in strongly inhibited locomotor activity. At 29°C, the activity levels of $AstA^1>TrpA1$ and $AstA^{34}>TrpA1$ males (A) and females (B) were significantly lower than in controls. At 22°C, $AstA^1>TrpA1$ and $AstA^{34}>TrpA1$ flies showed the same activity level than the controls. (C) Manual behavioural categorisation of individual flies monitored for 4h by a camera in the CAFE assay (n=3, see S7 Fig). Both movement and food consumption of $AstA^1>TrpA1$ flies were strongly reduced relative to $AstA^1\times w^{1118}$ controls at 29°C. n= 3 (D) The activity level of *tsh-Gal80;* $AstA^{34}>TrpA1$ flies was similar to $AstA^{34}>TrpA1$ males and lower compared to controls. (E) Activation of $AstA^1$ cells did not reduce locomotory activity in flies with an $AstA^{SK4}$ null background, in contrast to flies with an *AstA* wildtype background tested in parallel. (F) In a negative geotaxis assay, starved or satiated $AstA^1>TrpA1$ flies show a similar locomotor performance compared to control flies at 22°C and 29°C. $*$ $p \leq 0.05$. $**$ $p \leq 0.01$, $***$ $p \leq 0.001$.

To analyse locomotor activity in the CAFE assay, we video-monitored activity of $AstA^1>TrpA1$ 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 9C shows that $AstA^1>TrpA1$ spent much less time moving as well as feeding compared to $AstA^1\times w^{1118}$ controls, with individual variations within both strains (S7 Fig). Nevertheless, $AstA^1>TrpA1$ 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).

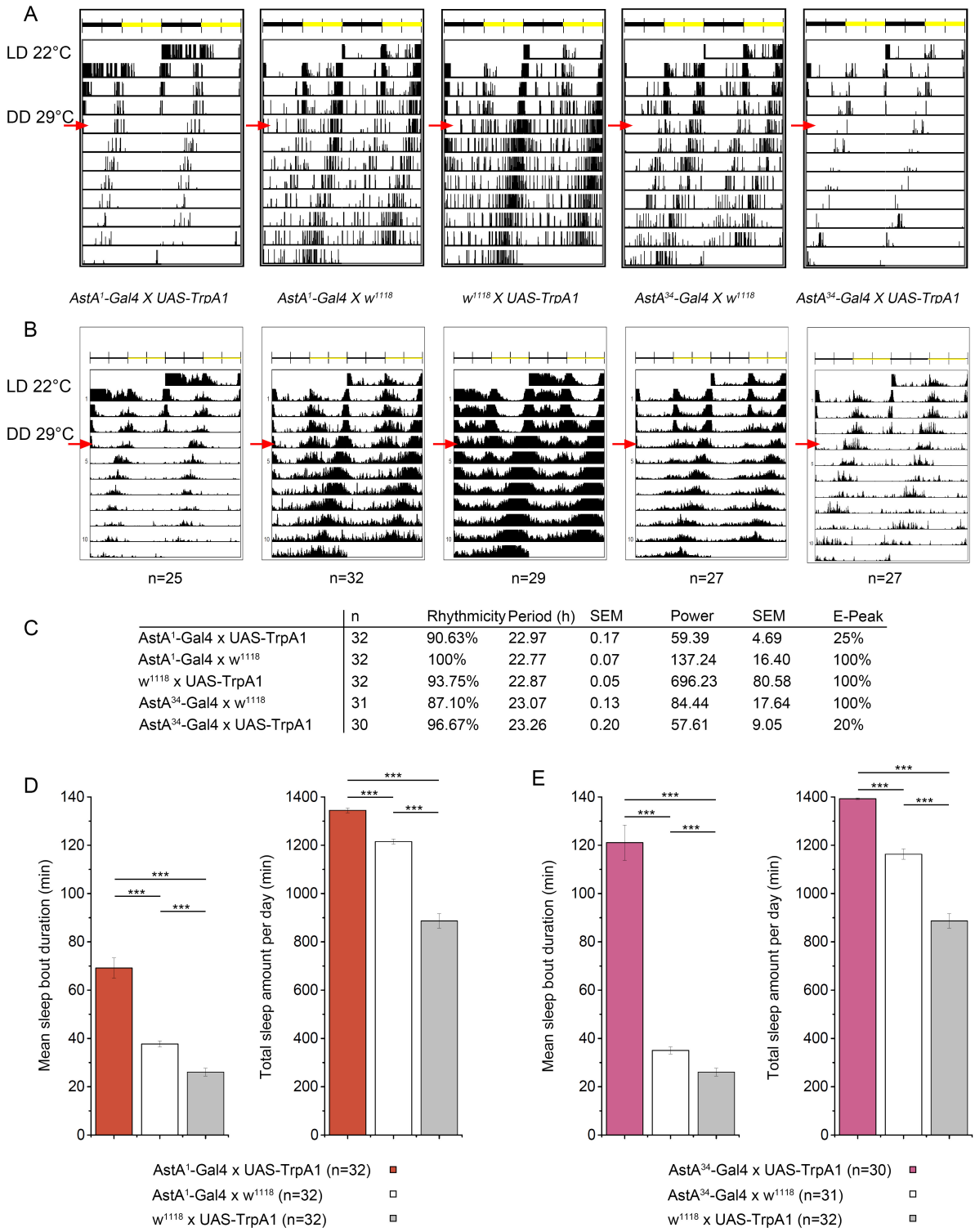
We next monitored the locomotor activity of *tsh-Gal80; AstA^{34}>TrpA1* flies (Fig 9D) and found a reduction of locomotor activity similar to *AstA^{1}>TrpA1* flies upon thermogenetic activation. (Fig 9A). Activation of PLP neurons and/or the AstA EECs seems thus sufficient to reduce locomotor activity. 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 significant alter locomotor activity (Fig 9E). The rhythmicity and period of locomotor activity (Helfrich-Förster 2005) was not affected by activation of AstA cells in *AstA^{1}>TrpA1* and *AstA^{34}>TrpA1* flies at 29°C and constant darkness (Fig 10). Strikingly, however, subjective evening activity was lost. (Fig 10A and 10B). 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 9E).

A strongly reduced locomotor activity is suggestive of abnormal sleep. Applying the widely used 5 min inactivity criterion (Ho & Sehgal 2005), 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 11) 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 11). Thermogenetic activation of AstA^{1} and AstA^{34} cells significantly increased total sleep and sleep bout duration also under constant darkness (Fig 10D and 10E), and constant light conditions known to disrupt the clock (S9 Fig). Next we silenced AstA cells by constitutive expression of *UAS-Kir2.1* (Baines et al. 2001), yet without effect on activity or sleep (S10 Fig). However, when we conditionally silenced AstA cells using the TARGET system (McGuire et al. 2004) and UAS-Kir2.1, sleep was significantly affected especially during the midday siesta time (Fig 12). 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 (Nitabach et al. 2002), 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 13). For the first assay,*AstA^{34}>TrpA1* 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 (van Alphen et al. 2013), the arousal-related parameters were dynamic during the day and varied somewhat between genotypes in the controls (Fig 13A and 13B). Notwithstanding, $AstA^{34}>TrpA1$ 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 13A and 13B). Again, this phenotype is unlikely to be caused by impaired locomotor ability since the maximum speed reached by individual flies was similar between $AstA^{34}>TrpA1$ 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 13C, S3 Movie), and a distinctly smaller percentage of aroused flies was observed for $AstA^{34}>TrpA1$ 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 13C).



C

	n	Rhythmicity	Period (h)	SEM	Power	SEM	E-Peak
AstA¹-Gal4 x UAS-TrpA1	32	90.63%	22.97	0.17	59.39	4.69	25%
AstA¹-Gal4 x w¹¹¹⁸	32	100%	22.77	0.07	137.24	16.40	100%
w¹¹¹⁸ x UAS-TrpA1	32	93.75%	22.87	0.05	696.23	80.58	100%
AstA³⁴-Gal4 x w¹¹¹⁸	31	87.10%	23.07	0.13	84.44	17.64	100%
AstA³⁴-Gal4 x UAS-TrpA1	30	96.67%	23.26	0.20	57.61	9.05	20%

Fig 10. Locomotor activity in the DAM system under constant conditions. (A) Typical double-plotted actograms of *AstA*^{34}>*TrpA1*, *AstA*^{1}>*TrpA1* and control flies kept for three days at 22°C and LD 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^{1} (D) and AstA^{34} (E) cell activation in DD.

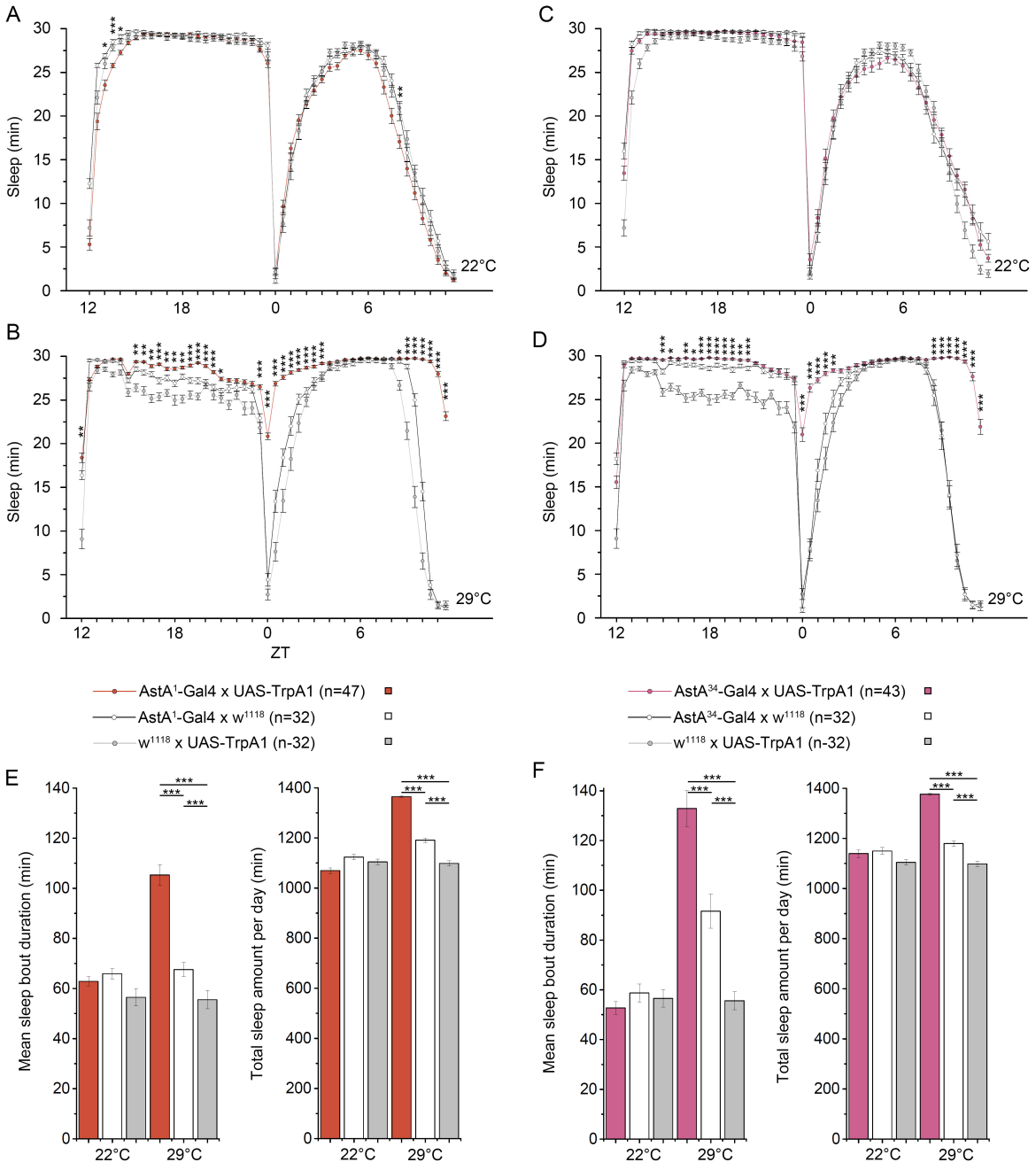


Fig 11. Thermogenetic activation of AstA cells strongly promoted sleep. At 22°C, *AstA*^{1}>*TrpA1* (A) and *AstA*^{34}>*TrpA1* male flies (C) did not sleep more than controls. Activation of the TrpA1 channel at 29°C resulted in increased sleep time of *AstA*^{1}>*TrpA1* (B) and *AstA*^{34}>*TrpA1* (D) flies especially during the time of the morning and evening activity. For both *AstA*^{1}>*TrpA1* (E) and *AstA*^{34}>*TrpA1* (F), mean sleep bout duration and the total amount of sleep per day was significantly increased when AstA cells were thermogenetically activated.

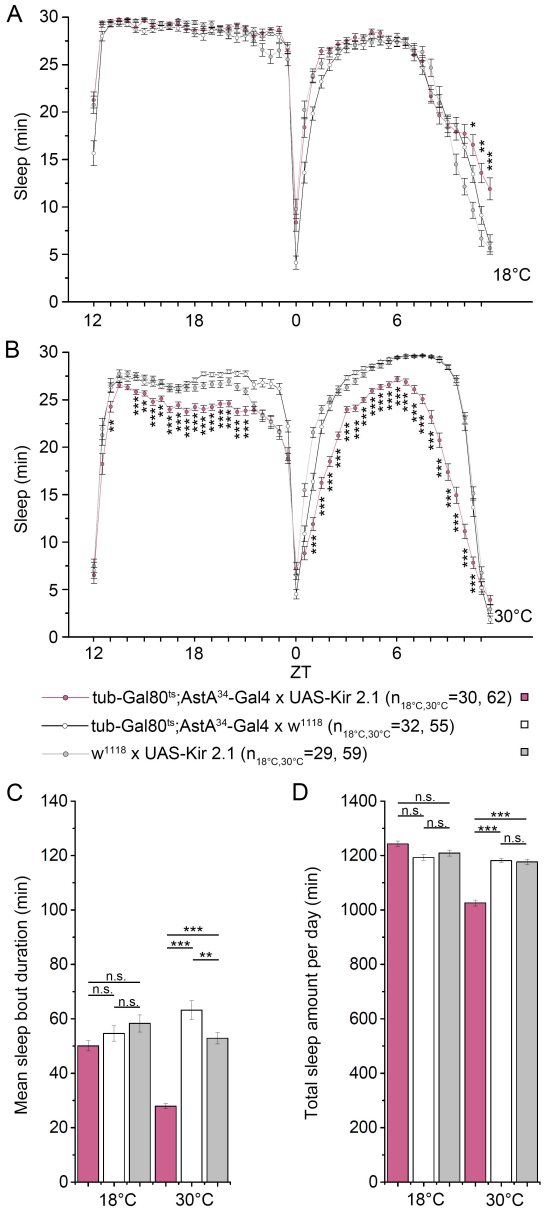


Fig 12. Conditional silencing of AstA^{34} cells by ectopic expression of the inward rectifying K+ channel Kir2.1 decreases sleep. (A+B) Averaged sleep over 24h of *tubGal80^{ts}; AstA^{34}>Kir2.1* experimental flies and controls. (C+D) Average sleep bout duration and total amount of sleep calculated 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). $*$ $p \leq 0.05$. $**$ $p \leq 0.01$, $***$ $p \leq 0.001$.

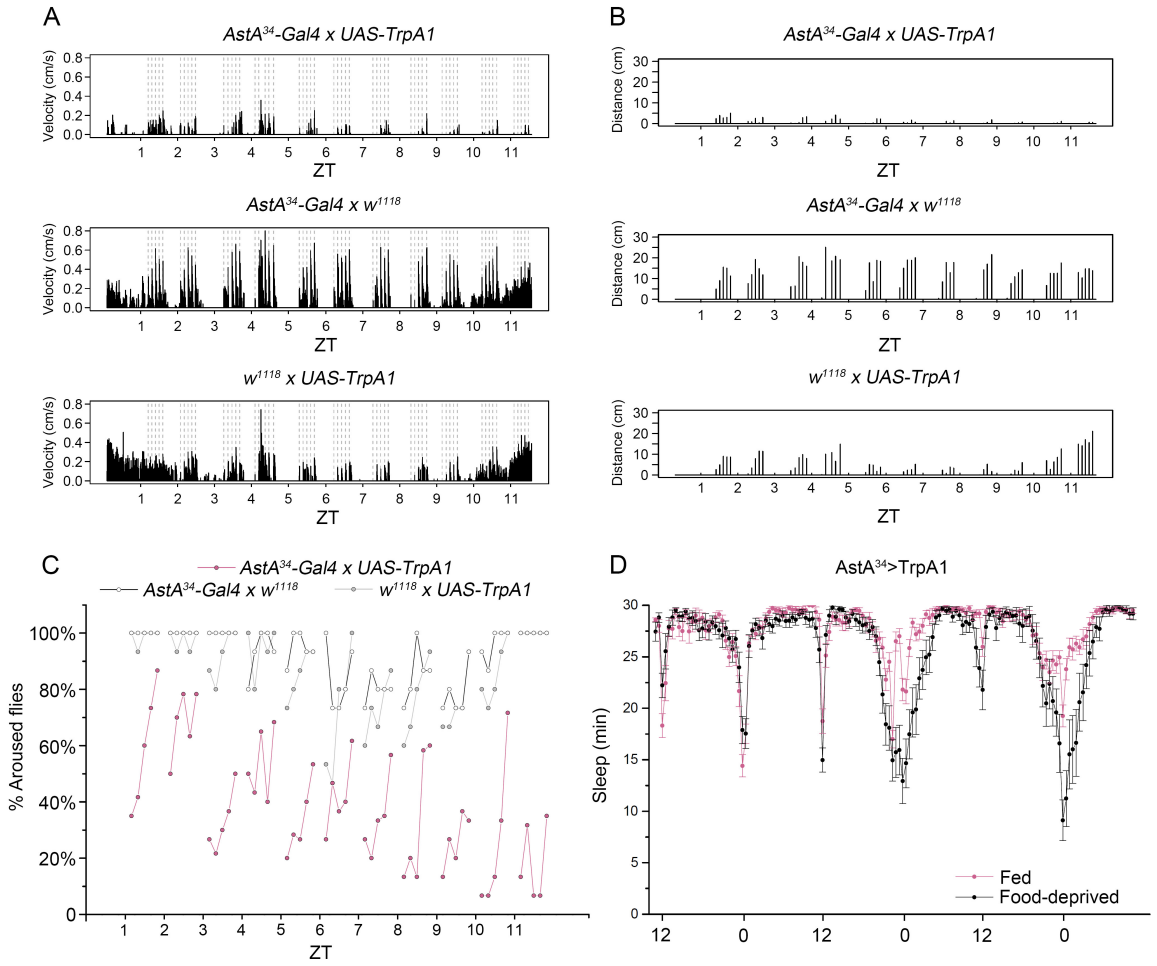


Fig 13. Mechanically- and starvation-induced activity. (A-B) An increasing level of mechanical stimuli by a loudspeaker (shown as dashed lines) were used to arouse flies in a glass tube (for details see material and methods). Thermogenetic activation of AstA cells in *AstA^{34}>TrpA1* flies resulted 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).

2.5 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 $AstA^{1}>TrpA1$ and $AstA^{34}>TrpA1$ 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 $AstA^{1}>TrpA1$ and $AstA^{34}>TrpA1$ 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 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 (Lee & Park 2004, Keene et al. 2010). Likewise, $AstA^{34}>TrpA1$ flies on starvation medium increased locomotor activity/reduced sleep compared to flies on food (Fig 13D). 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 in flies with activated AstA cells. The same phenotype was also seen with $AstA^{1}>TrpA1$ 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.

2.6 Genetic distinction between AstA-expressing PLP neurons and EECs

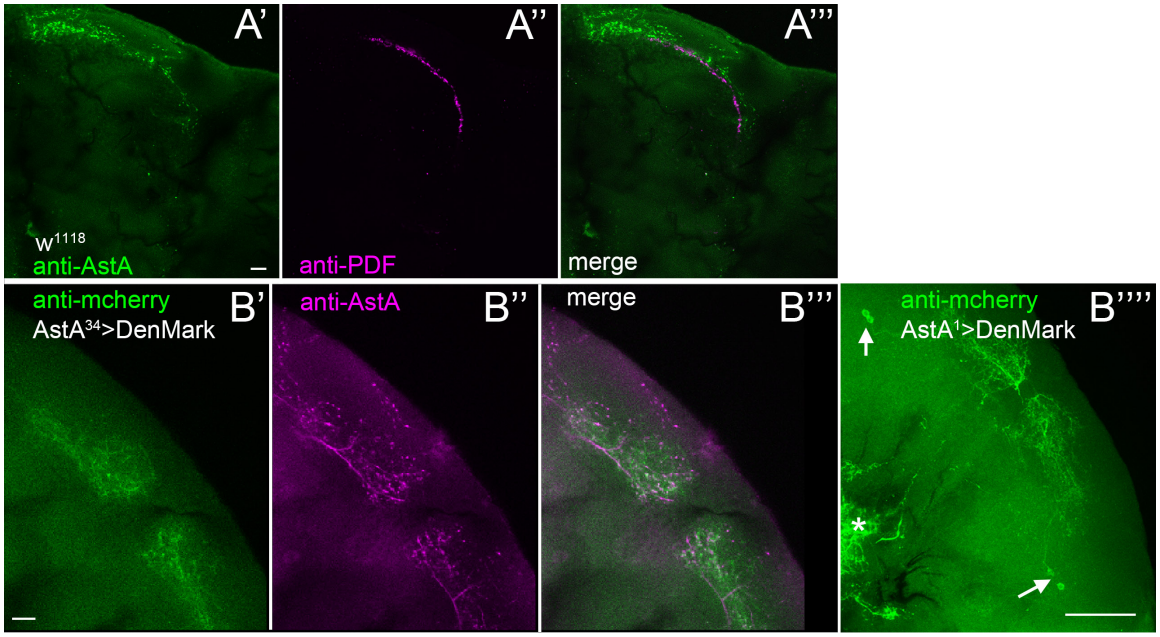
So far, we could show that thermogenetic activation of AstA-signalling from PLP neurons and/or EECs inhibits feeding and promotes sleep. To distinguish between these AstA cell subsets, we next aimed to further restrict the thermogenetic activation to AstA EECs only, using panneuronal *elav-Gal80* (Yang et al. 2009). 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 (Yao & White 1994, Berger et al. 2007), 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* (Rezával et al. 2012). We found no *nsyb>GFP* expression in EECs, and tried to restrict $AstA^{34}$*>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 $AstA^{34}$ 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 (Micchelli & Perrimon 2006, Beehler-Evans & Micchelli 2015), but expresses also broadly in the adult CNS (Chintapalli et al. 2007) which prevented the use of *prospero-Gal4* for thermogenetic activation. Thus, we were unable to further genetically differentiate between PLP neurons and AstA EECs.

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

During our morphological analysis (Fig 7) 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 (Hermann-Luibl & Helfrich-Förster 2015) which affects the timing of sleep and feeding (Xu et al. 2008, Parisky et al. 2008). 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 14A and 14B). While the sLNv projections represent mainly output sites (Yasuyama & Meinertzhagen 2010), the PLP neurites seem to be postsynaptic as indicated by the expression of the postsynaptic marker DenMark:mcherry (Fig 14A and 14B). 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 $AstA^{34}$ neurons. A similar approach had previously been very successful to demonstrate functional PDF

receptors on clock neurons (Shafer et al. 2008). The PLP neurons reacted with a fast increase in intracellular cAMP upon 10μM PDF (Fig 14C and 14D), 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 14C and 14D). PDF application had no effect on the PLP neurons in a PDF receptor mutant background (han^{5340} (Hyun et al. 2005), Fig 14C and 14D). We also found that the PDFR expression reporter pdfr-myc (Im & Taghert 2010) is weakly but consistently expressed in the PLP neurons (Fig 14E). 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 circadian PDF signalling.


A'
A''
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anti-AstA
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merge
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AstA34>DenMark
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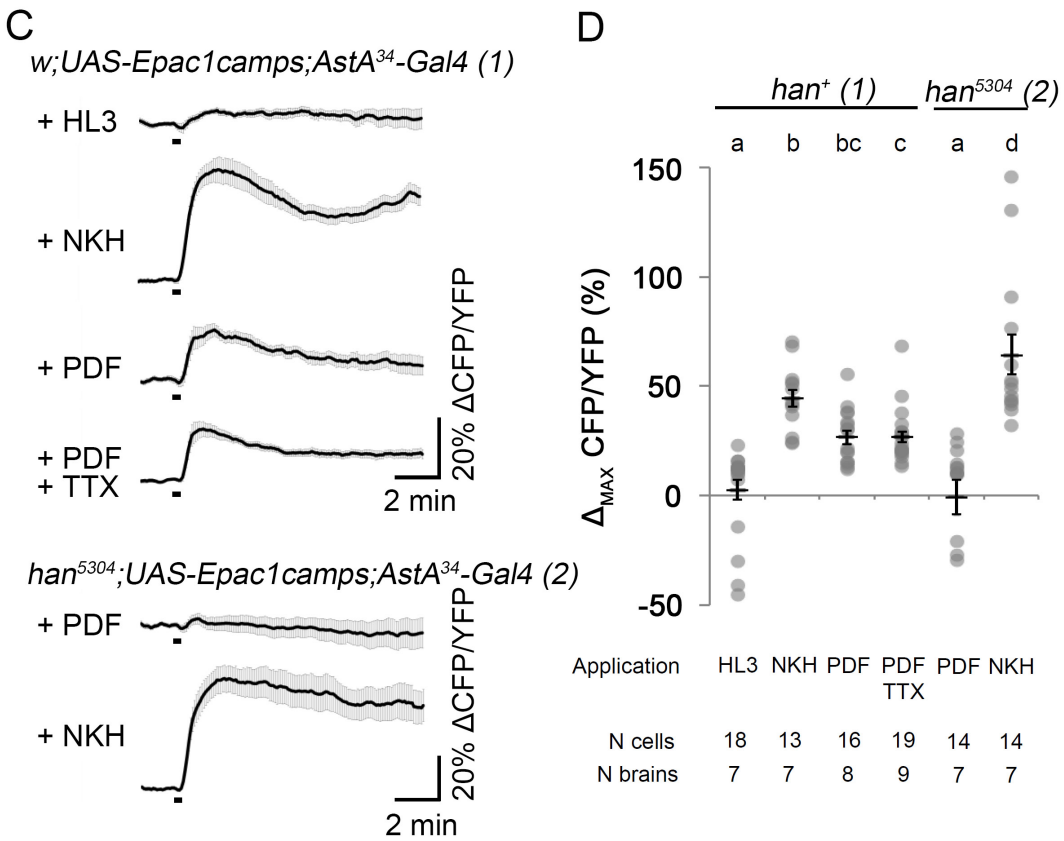
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w;UAS-Epac1camps;AstA34-Gal4 (1)
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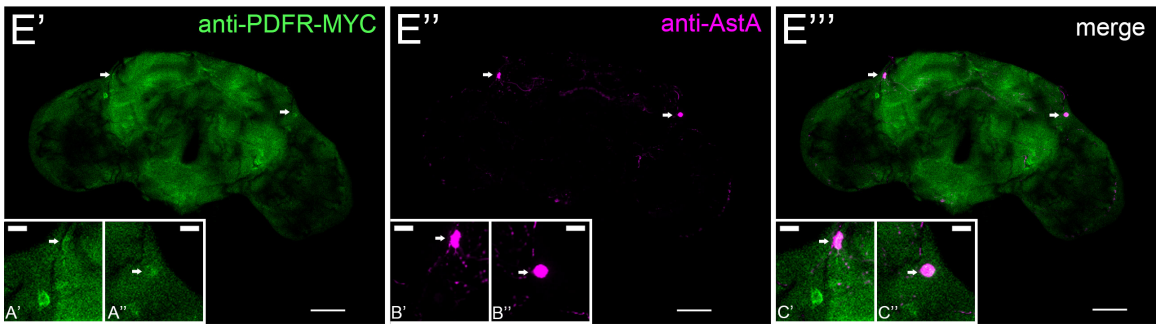
E'
anti-PDFR-MYC
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Fig 14. The AstA-expressing PLP neurons are a downstream target of the clock output factor PDF. (A') A 2 μm confocal section through the superior protocerebrum containing the arborisations of the PLP neurons immunolabelled against 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 a *AstA^{1}>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 10 μM of the adenylate cyclase activator NKH_{477} led to a robust increase in cAMP, indicating that the general procedure was working. 10 μM PDF peptide also evoked an increase in cAMP, indicating a functional connection between PDF expressing cells and PLP neurons. To test whether this functional connection was direct, brains were incubated in 2 μM TTX for 15min prior to imaging and 10 μM PDF were then coapplied together with 2 μM TTX. The neurons responded with similarly increasing levels in cAMP, indicating that the signalling from the PDF neurons to the PLP neurons is not mediated by interneurons. (D) Maximum inverse FRET changes were quantified for each individual neuron (gray dots) and averaged for each pharmacological treatment (mean $\pm$ SEM: black horizontal lines). Statistical comparison revealed significant increases in cAMP levels compared to the negative control (HL3) for NKH477, PDF as well as PDF+TTX. Error bars represent SEM and letters indicate statistical significances. Statistics in D): Kruskal-Wallis $H_{(3)}$=39.507; Bonferroni-corrected Wilcoxon pairwise-comparison with negative control (HL3): NKH p=0.006, PDF p=0.018, PDF+TTX p<0.001; Bonferroni-corrected Wilcoxon pairwise-comparison with positive control (NKH^{477}): PDF p=0.066, PDF+TTX p=0.012; Bonferroni-corrected Wilcoxon pairwise-comparison with PDF: PDF+TTX p=1.0. (E) The PDFR expression marker PDFR-myc (E') is weakly expressed in the AstA-immunopositive ((E") PLP neurons (E"'). Single confocal section. The inserts show the PLP somata in larger magnification. Scale bars: 50 μm, inserts 10 μm.

2.8 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^{34} cells. A similar approach has been successfully used to study the sleep effects of calcitonin gene-related peptide/DH31 (Kunst et al. 2014). t-PDF activated co-expressed PDFR in heterologous cell culture and rescued rhythmicity when specifically expressed in clock neurons in a *pdf^{01}* mutant background (Choi et al. 2009).

When expressed in AstA^{34} 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 15A, 15B, 15D and 15E). In accordance, total activity in *AstA^{34}>t-PDF* flies was significantly reduced to about half of that of controls (S10B Fig). 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 ((Fernández et al. 2008, Park et al. 2000), Fig 15A). 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 15D and 15E).

This suggests that t-PDF-induced PDFR signalling activates AstA^{34} cells, in line with the reported activating effect of t-PDF on sLNvs (Choi et al. 2012) 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^{34}>t-PDF* flies fed significantly less than the PDFR-SCR and UAS-TRPA1 control (Fig 15C), again in line with the notion that t-PDF increases the activation of AstA^{34} cells. No significant difference, however, was detectable for the *AstA^{34}-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 15B), mostly due to a low amount of sleep during the day (Fig 15A).

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^{34} cells (Fig 11), suggesting that PDF positively modulates rather than strongly activates AstA^{34} cell activity. To test this assumption,

we thermogenetically activated the PDF-expressing sLNvs using the *R6-Gal4* driver line (Helfrich-Förster et al. 2007). 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 16A and 16B). Total sleep over the day was not significantly altered (Fig 16C). 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* (Shafer & Taghert 2009) and have been shown to promote arousal (Parisky et al. 2008).

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.

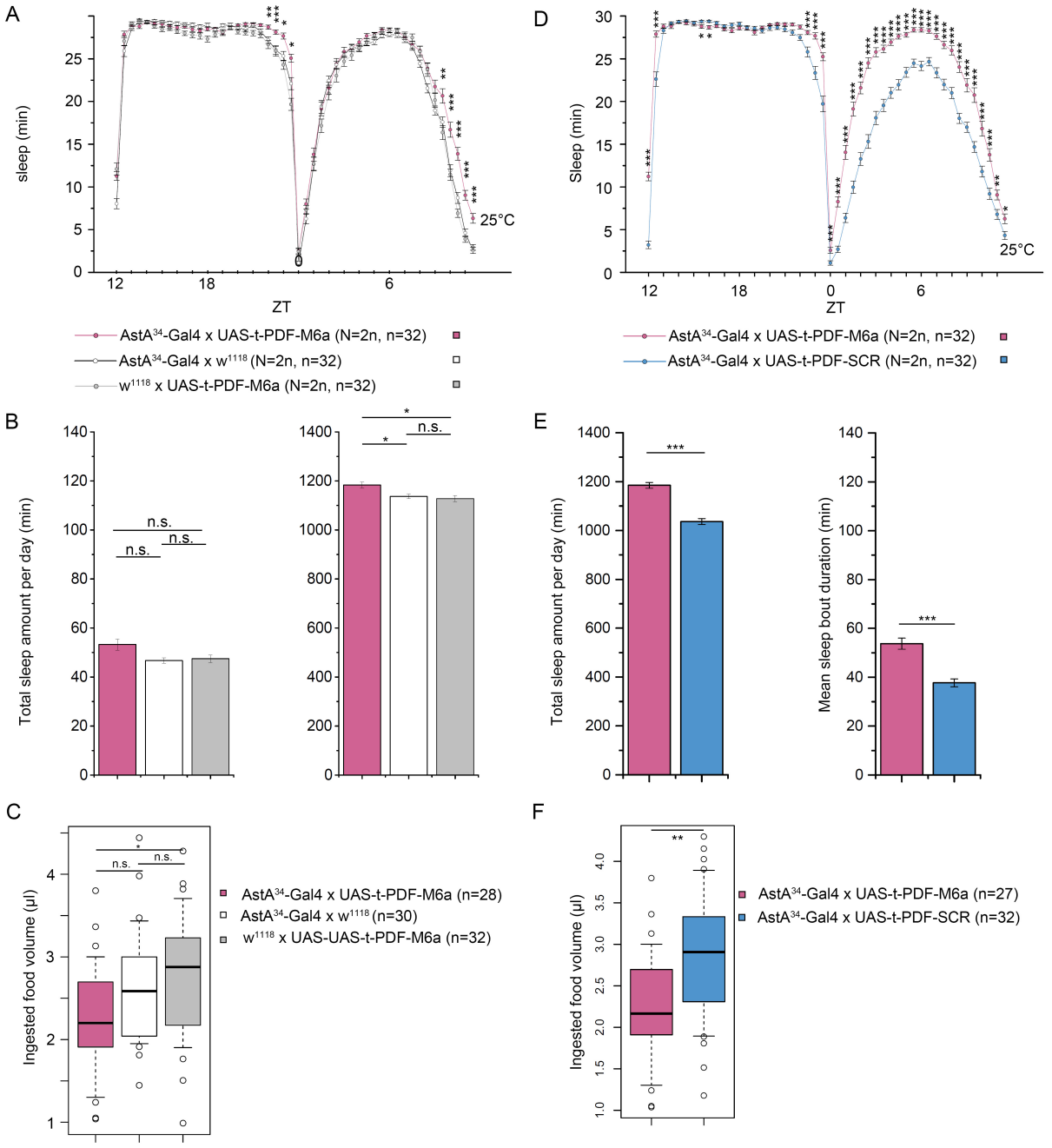


Fig 15. Effect of ectopic expression of tethered PDF (t-PDF) in AstA^{34} cells on sleep (A-B, D-E) and food intake (C,F). (A-C) Experiments using heterozygous controls. (A) t-PDF expression induced a small increase in sleep especially during the time of the evening activity. (B) Total sleep amount but 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^{34}>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. $*$ $p \leq 0.05$. $**$ $p \leq 0.01$, $***$ $p \leq 0.001$.

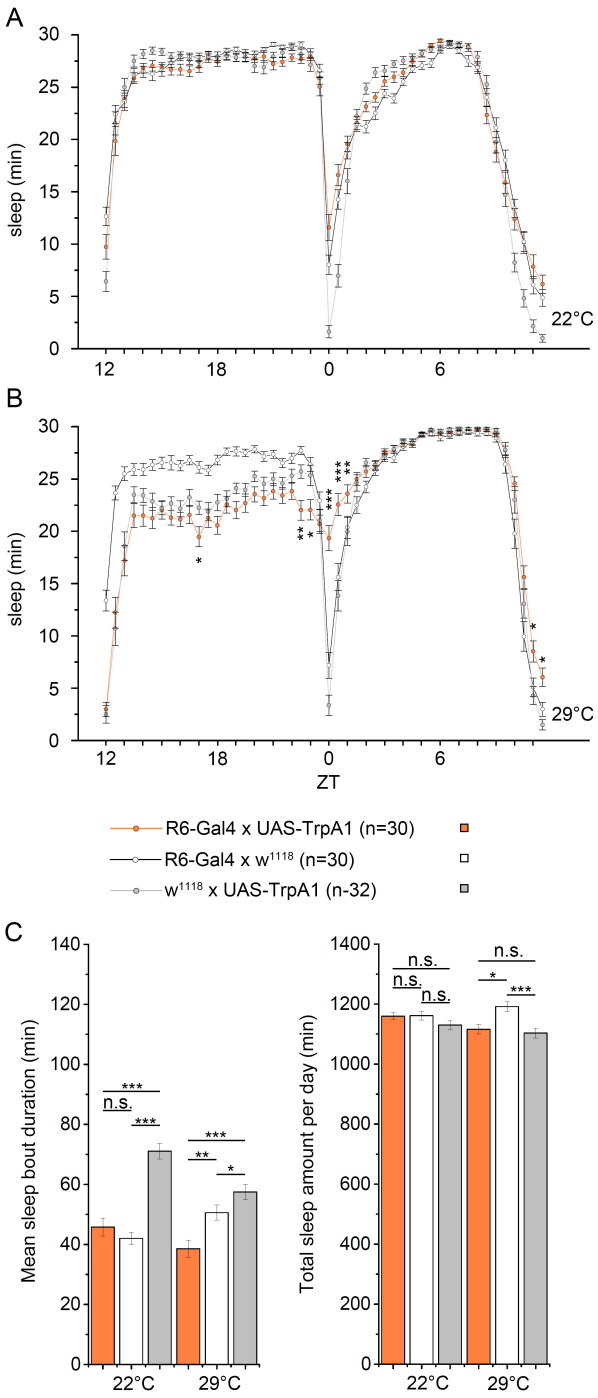


Fig 16. Activation of the PDF-expressing sLNvs promotes sleep specifically during the time of morning and evening peak activity. A) At 22°C, *R6>TrpA1* flies showed the same sleep pattern than controls. (B) Activation of the TrpA1 channel at 29°C resulted in increased sleep time specifically during the time of the morning and, to a lesser amount, the evening activity. (C) Mean sleep bout duration but not the total amount of sleep per day was affected by activation of the sLNvs.

3 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 (Brown et al. 2015, Richter et al. 2014), and our results provide evidence that also further such peptides exist in the fly besides sNPF and possibly NPF (Chen et al. 2013, Shang et al. 2013, He et al. 2013). More specifically, our results with a new *AstA^{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 (Liu et al. 2012, Ueno et al. 2012), superior (dorsal) fan-shaped body neurons (Donlea et al. 2014, Wolff et al. 2015, Young & Armstrong 2010) and neurons of the pars intercerebralis (Foltenyi et al. 2007). AstA EECs in *Drosophila* are "open type" EECs (Veenstra 2009, Veenstra et al. 2008), possessing apical extensions that reach the gut lumen and likely express gustatory receptors (Park & Kwon 2011). 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^+ transport (Vanderveken & O'Donnell 2014) 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 (Hentze et al. 2015), and -at least in other insects- stimulates digestive enzyme activity in the midgut (Aguilar et al. 2003, Fusé et al. 1999).

The AstA receptors are homologues of the vertebrate galanin receptors (Mirabeau & Joly 2013, Felix et al. 2015, Jékely 2013, Hewes & Taghert 2001) that have pleiotropic functions (Lang et al. 2007). When activated in specific brain areas, galanin signalling has a strong orexigenic effect (Lang et al. 2015) and has also been implicated in the control of arousal and sleep in mammals (Lang et al. 2015). 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 (Woods et al. 2014). Furthermore, the allatostatin/galanin-like receptor NPR-9 inhibits local search behaviour on food in the nematode *C. elegans* (Bendena et al. 2008). Similar to AstA in *Drosophila* (Vanderveken & O'Donnell 2014), galanin modulates intestinal motility and ion transport (Lang et al. 2007). 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 (Shang et al. 2013, Kunst et al. 2014, Hermann et al. 2012, Hermann-Luibl et al. 2014, Lee et al. 2006, Oh et al. 2014, Yao & Shafer 2014). 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 (Mertens et al. 2005, Kim et al. 2013).

At first sight, our data suggesting that PDF activates PLP neurons to promote sleep seem to contradict earlier findings (Parisky et al. 2008). Since pdf^{01} mutants show increased sleep during the photophase, the arousal effect appears to be the dominant effect of PDF which is due to signalling between ventral lateral clock neurons (LNvs) (Parisky et al. 2008), with a major contribution of the PDF-expressing large LNvs (Shang et al. 2008). 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 (Shang et al. 2013). 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 (Krupp et al. 2013). Furthermore, the posterior midgut is innervated by the abdominal PDF neurons (Veenstra et al. 2008), and PDFR is expressed in the midgut (Talsma et al. 2012). 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 (Lang et al. 2015) - 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 (Hergarden et al. 2012). 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.

II. Functions of myoinhibitory peptides in feeding and sleep

1 Introduction

Myoinhibitory peptides (MIPs) were first isolated in *Locusta migratoria* (Schoofs et al. 1991). The primary structure was characterized as Ala-Trp-Gln-Asp-Leu-Asn-Ala-Gly-Trp-NH_2 (A**W**QDLNAG**Wa**) and MIPs are recognized by the sequence W(X6)Wamide which is similar to a part of the mammalian galanin (Table 2). They are sometimes also referred to as B-type allatostatin (AstB) according to their allatostatic ability in crickets (Lorenz et al. 1995). However, the encoding genes and amino acid sequences of AstB are not related to allatostatin A (AstA). In *Manduca sexta*, *Locusta migratoria* and *Periplaneta americana*, MIPs were shown to have a myoinhibitory effect, inhibition of spontaneous muscle contractions of the gut and oviduct preparations (Schoofs et al. 1991, Blackburn et al. 1995, 2001, Predel et al. 2001).The inhibitory effect on muscle constractions is supposed to influence food intake in insects (Aguilar et al. 2006). Thus, the myoinhibitory action of MIPs/AstB appears to be a common function, while the allatostatic action seems to be restricted to only few hemimetabolous taxa. Therefore, the designation MIPs will be used in this dissertation. Activation of MIP signaling in *Platynereis* postlarvae increased gut peristalsis and frequency of pharynx contractions resulting in increased food intake (Williams et al. 2015). In *Manduca sexta*, *Bombyx mori* and *Drosophila melanogaster*, according to the localization in the brain, prothoracic gland and epiproctodeal glands, and the level of release, MIPs are suggested to be co-released with crustacean cardioactive peptide (CCAP) and to initiate ecdysis (Gammie & Truman 1997, Davis et al. 2003, Kim et al. 2006).

In *Drosophila*, MIPs are brain-gut peptides expressed in the central nervous system (CNS) and intestine (Williamson et al. 2001, Veenstra et al. 2008, Reiher et al. 2011). Gene CG6456 encodes for five MIPs (MIP 1-5) shown in Table 2 (Vanden Broeck 2001). One MIP receptor, sex peptide receptor (SPR) is characterised for *Drosophila* (Johnson et al. 2003). Recently, Soohong Min and colleagues demonstrated a peptidergic pathway where neurons expressing MIPs regulate body size and food intake in *Drosophila* (Min et al. 2016). Constitutive silencing by *UAS-tetanus toxin (UAS-TNT)* (Sweeney et al. 1995) of MIP cells contained in the *Mip^{KR}-Gal4* expression pattern increased body size and food intake. In contrast, activation of MIP^{KR} cells by the thermogenetic effector TrpA1 (Hamada et al. 2008) decreased body size and food intake, which could be traced to MIP signalling. A subset of MIP expressing neurons (IAM neurons) were suggested to play a critical role in the regulation of body size (Min et al. 2016). Furthermore, Yangkyun Oh and colleagues demonstrated a peptidergic modulatory pathway where MIPs maintain sleep homeostasis by regulating PDF release

via SPR (Oh et al. 2014). Earlier work has shown that MIPs in *Drosophila* larva and adult is expressed broadly in the midgut, suggesting that it may have an effect on gut motility (Williamson et al. 2001, Veenstra 2009, Reiher et al. 2011).

We generated six new *Mip^{WÜ}-Gal4* lines (*Mip^{WÜ}-2-Gal4*, *Mip^{WÜ}-3-Gal4*, *Mip^{WÜ}-4-Gal4*, *Mip^{WÜ}-5-Gal4*, *Mip^{WÜ}-6-Gal4* and *Mip^{WÜ}-7-Gal4*) based on P-element transformation of *w^{1118}* flies with a promoter construct produced by Jan Veenstra (U Bordeaux) to dissect the roles of MIPs regarding food intake, locomotor activity and sleep. Confocal imaging showed that the new *Mip^{WÜ}-Gal4* lines have a more specific expression pattern of MIPs than the *Mip^{KR}-Gal4* line. Neurogenetic experiments showed that neither conditional activation nor silencing of MIP^{WÜ} cells in the different *Mip^{WÜ}-Gal4* lines affect food intake, while manipulation of MIP^{WÜ} cells induced in changes in the sleep status. However, constitutive silencing of MIP^{KR} cells by using Korean *Mip^{KR}-Gal4* line decreased food intake and conditional activation of MIP^{KR} cells did not affect food intake, which are not consistent with Min et al. (2016)'s results. In addition, thermoactivation of MIP^{KR} cells decreased sleep. The flies showed extreme hyperactivity during the dark phase.

2 Results

To be able to manipulate MIP-expressing cells in *Drosophila*, we generated six *Mip$^{W\ddot{U}}$-Gal4* lines, *Mip$^{W\ddot{U}}$-2-Gal4*, *Mip$^{W\ddot{U}}$-3-Gal4*, *Mip$^{W\ddot{U}}$-4-Gal4*, *Mip$^{W\ddot{U}}$-5-Gal4*, *Mip$^{W\ddot{U}}$-6-Gal4*, *Mip$^{W\ddot{U}}$-7-Gal4*, by using the same promoter construct which was injected into *Drosophila* wildtype embryos based on the P-element transformation.

2.1 Expression pattern of the newly generated *Mip$^{W\ddot{U}}$-Gal4* lines.

In order to test the expression specificity of our different *Mip$^{W\ddot{U}}$-Gal4*, I analysed *Mip$^{W\ddot{U}}$-2-Gal4>GFP* adult flies for co-expression of GFP and MIPs using immunostaining. The results showed that the observed MIP immunoreactivity (IR) expression pattern was consistent with previous studies (Veenstra et al. 2008, Kolodziejczyk & Nässel 2011, Reiher et al. 2011, Min et al. 2016). In each brain hemisphere of *Mip$^{W\ddot{U}}$-2-Gal4* flies, GFP was consistently detected in three to four MIP-IR superior posterior lateral (SPL) interneurons (Fig 17C) in the superior lateral protocerebrum (SLP), about four local interneurons (LNs) in the antennal lobe (AL) (Fig 17B, asterisks), one large lateral MIP-immunoreactive optic lobe (LMIo) neuron (Fig 17E) in the optic lobe close to the medulla and accessory medulla, and one large inferior contralateral interneuron (ICLI) (Fig 17D) in the gnathal ganglia (GNG). The LMIo neuron branched very widely in the medulla and projected to the SLP. A pair of ICLI innervated the GNG and sent axonal projections to the superior protocerebrum (Fig 17D, arrows) and SLP. Furthermore, GFP was detected in four MIP-IR superior anterior median (SAM) interneurons (Fig 17B, white arrows) and four MIP-IR interior anterior median (IAM) interneurons (Fig 17B, red arrows) in the central brain (CB).

In the thoracico-abdominal ganglion (TAG), three MIP-IR cells in the prothoracic neuromeres (PN) (Fig 17F), six MIP-IR cells in the mesothoracic neuromeres (MN) (Fig 17G) and four MIP-IR cells in the abdominal neuromeres (Abd) (Fig 17H) were detected. Processes of these neuron widely innervated the TAG. A GFP signal was also detected in some cells of the midgut (Fig 17I), however few of them were MIP-IR positive. Same expression patterns were also detected in other *Mip$^{W\ddot{U}}$-Gal4* lines (*Mip$^{W\ddot{U}}$-3-Gal4>GFP, MIP$^{W\ddot{U}}$-4-Gal4>GFP, MIP$^{W\ddot{U}}$-5-Gal4>GFP and MIP$^{W\ddot{U}}$-6-Gal4>GFP*) (data not shown).

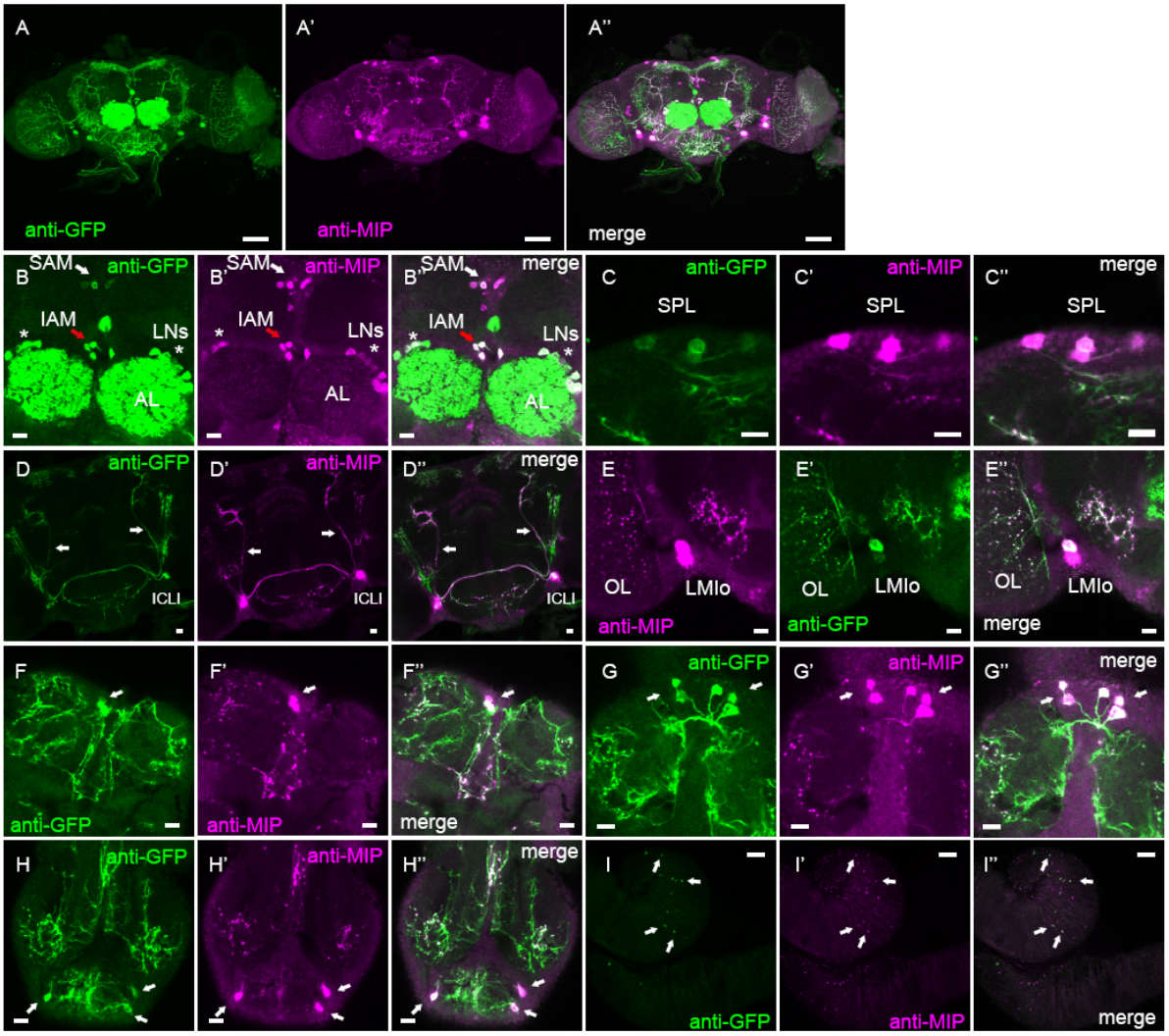


Fig 17. MIPs (magenta) and GFP (green) immunolabeling of nervous systems (A-H) and guts (I) of adult *Mip$^{W\ddot{U}}$-2-Gal4>GFP* flies. (A) Overview of expression pattern in the brain with adult *Mip$^{W\ddot{U}}$-2-Gal4>GFP* flies. (B-E) Details of the subset of MIP neurons in the brain. (B) GFP expression is detected in MIP-IR SAM (white arrows), IAM (red arrows), LNs (asterisks), (C) SPL, (E) LMlo and (D) ICLI cells. (D) The ICLI cells innervate the GNG and send projections (white arrows) to the SLP. The arborization of LMlo cells are visible in (E). (F-H) Detail of the TAG. Three MIP-IR neurons (white arrows) in the PN (F), six MIP-IR neurons (white arrows) in the MN (G) and three MIP-IR neurons (white arrows) in the Abd (H) co-labeled with GFP arborise widely in the neuromeres. (I) GFP expression is detected in few MIP-IR cells (white arrows) in the gut. Scale bars: in A and I 50μm; in B-H 10μm. (Abd abdominal neuromere, AL antennal lobe, IAM interior anterior median, ICLI inferior contralateral interneuron, GNG gnathal ganglia, LNs local interneuron, LMlo lateral MIP-immunoreactive optic lobe, MN mesothoracic neuromere, PN prothoracic neuromere, SAM superior anterior median, SLP superior lateral protocerebrum, SPL superior posterior lateral, TAG thoracico-abdominal ganglion)

Min et al. (2016) described the MIP-expression pattern in the CNS using a different *Mip-Gal4* driver, *Mip^{KR}-Gal4* (Fig 18B). The location of MIP neurons were mapped by anti-GFP stainings in

Mip^{KR}>*mCD8GFP* flies, anti-MIP antibody and *Mip* mRNA in situ hybridization. GFP was detected in six MIP-IR SAM, four IAM and SGS neurons. In each brain hemisphere, GFP was detected in three MIP-IR SPL, three LMIo, one ICLI and one ALS neurons. Compared to the expression patter of our *Mip*^{WÜ}-*Gal4* drivers (Fig 18A), there are two major differences. 1. GFP was not detected in SGS and ALS neurons of all our *Mip*^{WÜ}-*Gal4* lines. 2. A lot of unspecific MIP-immuno-negative neurons were contained in the *Mip*^{KR}-*Gal4* line.

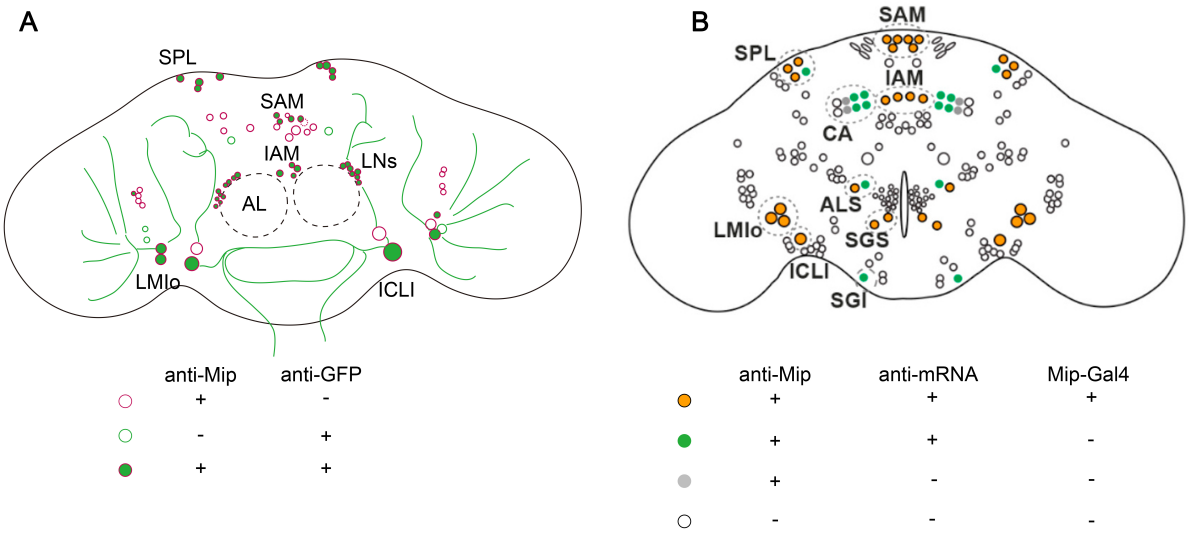


Fig 18. Schematic summary of the expression pattern under *Mip*^{WÜ}-*Gal4* (A) and *Mip*^{KR}-*Gal4* (B) driver. AL antennal lobe, IAM interior anterior median, ICLI inferior contralateral interneuron, LMIo lateral MIP-immunoreactive optic lobe, LNs local interneurons, SAM superior anterior median, SLP superior lateral protocerebrum, SPL superior posterior lateral (B modified from Min et al. 2016).

2.2 Effect of *Mip*^{WÜ}-*Gal4* cell manipulation on food intake.

To investigate the role of MIP^{WÜ} cells in food intake, I employed the CAFE assay (Ja et al. 2007) and measured the volume of liquid food intake while MIP^{WÜ} cells were conditionally activated by the thermogenetic effector TrpA1 at 29°C. *Mip*^{WÜ}-*2*>*TrpA1*, *Mip*^{WÜ}-*3*>*TrpA1*, *Mip*^{WÜ}-*4*>*TrpA1*, *Mip*^{WÜ}-*5*>*TrpA1*, *Mip*^{WÜ}-*6*>*TrpA1* and *Mip*^{WÜ}-*7*>*TrpA1* flies were raised on normal food at 20°C, and then, 4-5 days old male flies were assayed over a period of two days at 29°C after one day acclimation to the new environment and food conditions. At 29°C, *Mip*^{WÜ}-*2*>*TrpA1* (Fig 19A), *Mip*^{WÜ}-*3*>*TrpA1* (Fig 19B), *Mip*^{WÜ}-*4*>*TrpA1* (Fig 19C), *Mip*^{WÜ}-*5*>*TrpA1* (Fig 19D), *Mip*^{WÜ}-*6*>*TrpA1* (Fig 19E) and *Mip*^{WÜ}-*7*>*TrpA1* (Fig 19F) flies did not show significant differences in food intake compared to the control flies. These results are not consistent with the previous report (Min

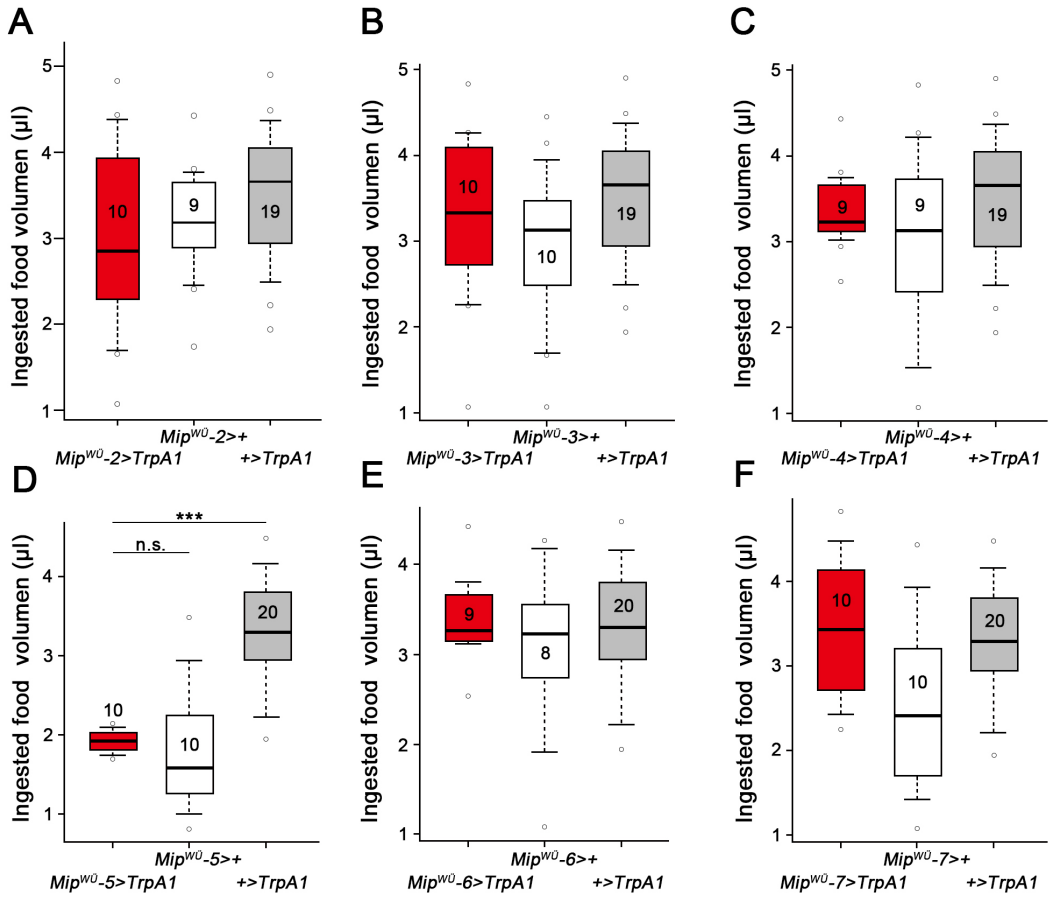


Fig 19. Thermogenetic activation of MIP^{WÜ} cells did not affect food intake. The total volume of liquid food consumed within two days was measured in the CAFE assay. At 29°C, *Mip^{WÜ}-2>TrpA1* (A), *Mip^{WÜ}-3>TrpA1* (B), *Mip^{WÜ}-4>TrpA1* (C), *Mip^{WÜ}-5>TrpA1* (D), *Mip^{WÜ}-6>TrpA1* (E) and *Mip^{WÜ}-7>TrpA1* (F) flies ingested as much food as the control flies. Numbers in each box indicate the amount of tested flies. $***$ p $\leq$ 0.001, n.s. not significant.

et al. 2016) about the activation of MIP^{KR} cells by expression of TrpA1 in the CAFE assay which found that the activation of MIP^{KR} cells resulted in about 70% less food ingestion compared to the control. Min et al. (2016) also showed that silencing of MIP^{KR} cells by constitutive expression of *UAS-tetanus toxin (UAS-TNT)* increased food intake compared to the control expressing an inactive form of TNT (*Mip^{KR}>impTNT*). I repeated these silencing experiments with our *Mip^{WÜ}-2-Gal4* line.To exclude developmental effects due to constitutive silencing, I conditionally silenced MIP^{WÜ}-2 cells using the TARGET system (McGuire et al. 2004). *tub-Gal80ts;Mip^{WÜ}-2>Kir2.1* and control flies were raised on normal food at 18°C, and then a CAFE assay was performed at 30°C. At 30°C, *tub-Gal80ts;Mip^{WÜ}-2>Kir2.1* flies showed a similar food intake as the control in the CAFE assay (Fig 20). Taken together, results suggest that MIP^{WÜ} cells do not play a prominent role in regulating

food intake.

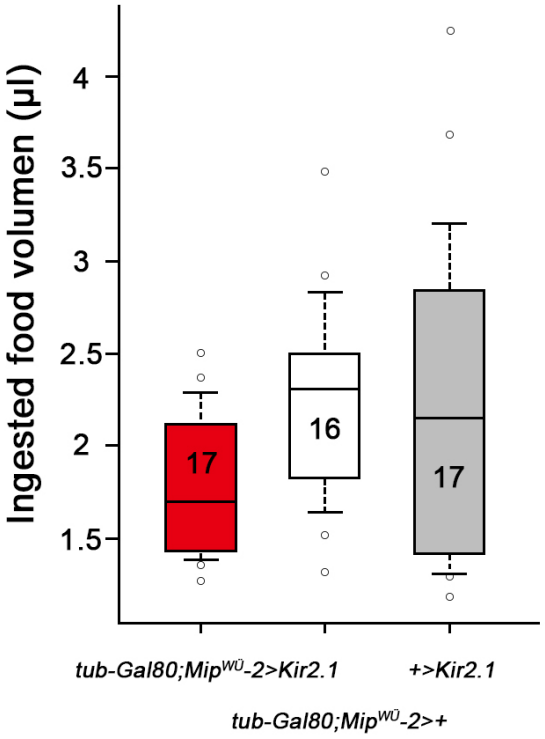


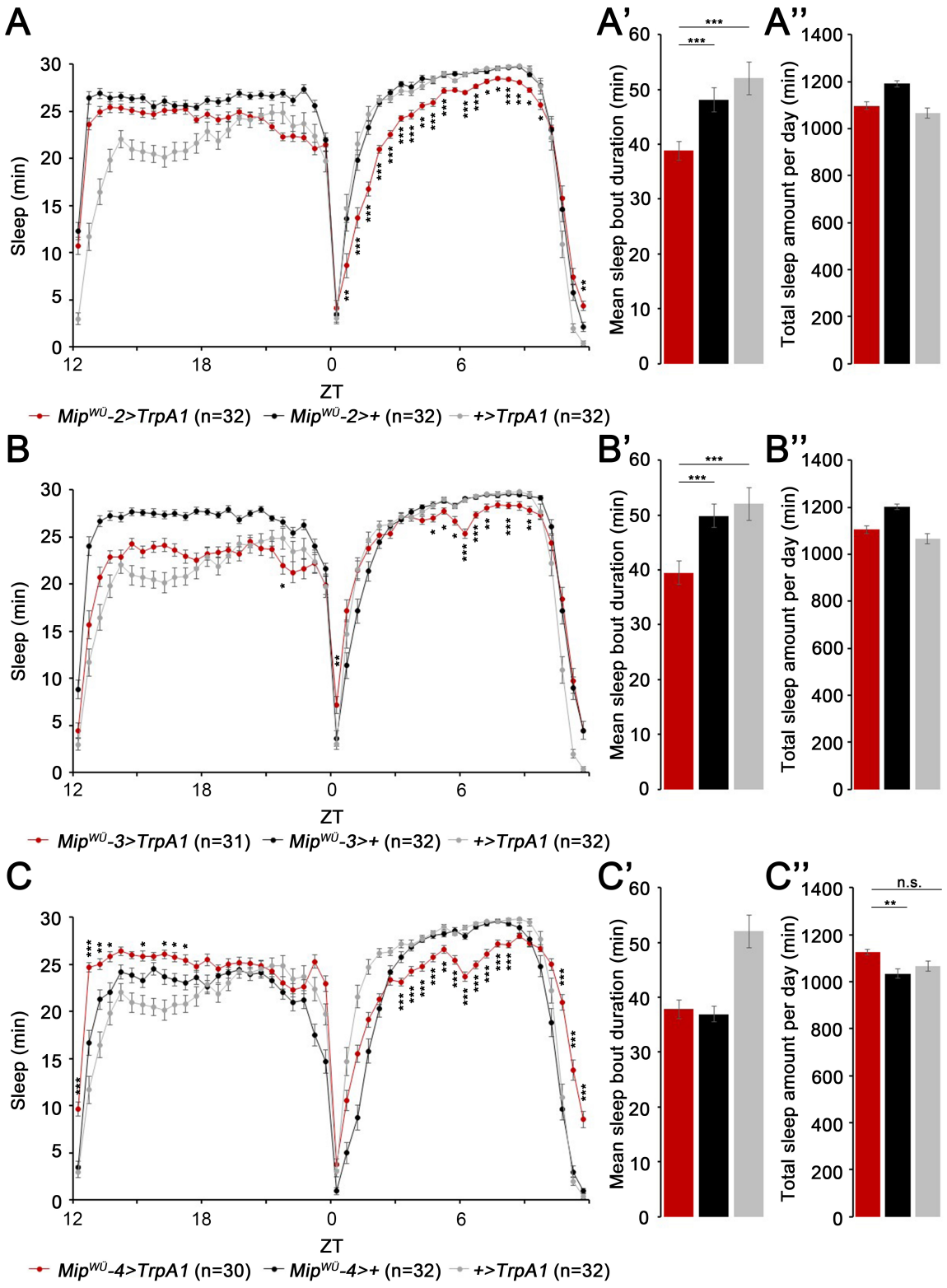
Fig 20. Conditional silencing of MIP^{WÜ}-2 cells by ectopic expression of the inward recifying K$^+$ channel Kir2.1 did not affect food intake. The total volume of liquid food consumed within two days was measured in the CAFE assay. At 30°C, *Mip^{WÜ}-2>TrpA1* flies ingested as much food as the control flies. Numbers in each box indicate the amount of tested flies.

2.3 Manipulation of MIP^{WÜ} cells resulted in changes in the sleep status.

MIPs and SPR play an important role in the homeostatic sleep stabilization. Flies lacking *Spr* (*Spr$^{-/-}$* mutants) or having reduced *Mip* (*elav>Mip-RNAi*) showed a reduction of the total sleep amount but not activity during wakefulness, and were not able to recover sleep in the morning following a mechanical sleep deprivation of 12 hours during the dark phase in LD 12:12 (Oh et al. 2014). I therefore asked whether the manipulation of MIP^{WÜ} cells influence locomotor activity and sleep, and recorded the locomotor activity of 4-5 days old males. *Mip^{WÜ}-2>TrpA1*, *Mip^{WÜ}-3>TrpA1*, *Mip^{WÜ}-4>TrpA1*, *Mip^{WÜ}-5>TrpA1*, *Mip^{WÜ}-6>TrpA1*, *Mip^{WÜ}-7>TrpA1* and the control flies were kept in small glass tubes on agar-sucrose food in the DAM system at 29°C. I analysed sleep using the 5 min inactivity criterion (Ho & Sehgal 2005) and found that thermogenetic activation of MIP^{WÜ}-2

or 3 cells reduced sleep time in the siesta (Fig 21A and 21B) and sleep bout duration (Fig 21A' and 21B'). In contrast, thermogenetic activation of MIP$^{W\ddot{U}}$-6 cells increased sleep time in the late dark phase, in the morning and in the evening (Fig 21E). Sleep bout duration and total sleep amount of *Mip$^{W\ddot{U}}$-6>TrpA1* flies were significant longer than in the controls (Fig 21E' and 21E''). Interestingly, thermogenetic activation of MIP$^{W\ddot{U}}$-4 cells increased sleep time from ZT10 to ZT17, whereas it decreased sleep time during the siesta (Fig 21C). The sleep bout duration and total sleep amount of *Mip$^{W\ddot{U}}$-4>TrpA1* flies were not significantly different compared to the controls (Fig 21C' and 21C''). *Mip$^{W\ddot{U}}$-5>TrpA1* flies just showed increased duration of sleep during the first hour after lights-on (Fig 21D). Sleep bout duration or total amount of sleep of *Mip$^{W\ddot{U}}$-5>TrpA1* flies were not significantly different to the controls (Fig 21D' and 21D''). Thermogenetic activation of MIP$^{W\ddot{U}}$-7 cells (Fig 21F) just increased sleep duration slightly at ZT11, but did not affect sleep bout duration or total amount of sleep compared to the controls (Fig 21F' and 21F''). It can be seen that there is no consistent trend for thermogenetically activated MIP$^{W\ddot{U}}$ cells with sleep behaviour, suggesting that thermogenetic activation of MIP$^{W\ddot{U}}$ cells do not affect sleep.

Next I conditionally silenced MIP$^{W\ddot{U}}$-2 cells using *tub-Gal80ts;Mip$^{W\ddot{U}}$-2>Kir2.1* flies and the locomotor activity was recorded in DAM system at 30°C. *tub-Gal80ts;Mip$^{W\ddot{U}}$-2>Kir2.1* flies showed increased duration of sleep during the dark phase compared to the control (Fig 22A). The total sleep amount (Fig 22C) but not the sleep bout duration (Fig 22B) of *tub-Gal80ts;Mip$^{W\ddot{U}}$-2>Kir2.1* flies was significantly higher than in the control.


A
A'
A''
Sleep (min)
ZT
12
18
0
6
Mean sleep bout duration (min)
Total sleep amount per day (min)
***
Mip^WÜ-2>TrpA1 (n=32)
Mip^WÜ-2>+ (n=32)
+>TrpA1 (n=32)
B
B'
B''
Mip^WÜ-3>TrpA1 (n=31)
Mip^WÜ-3>+ (n=32)
+>TrpA1 (n=32)
C
C'
C''
n.s.
**
Mip^WÜ-4>TrpA1 (n=30)
Mip^WÜ-4>+ (n=32)
+>TrpA1 (n=32)

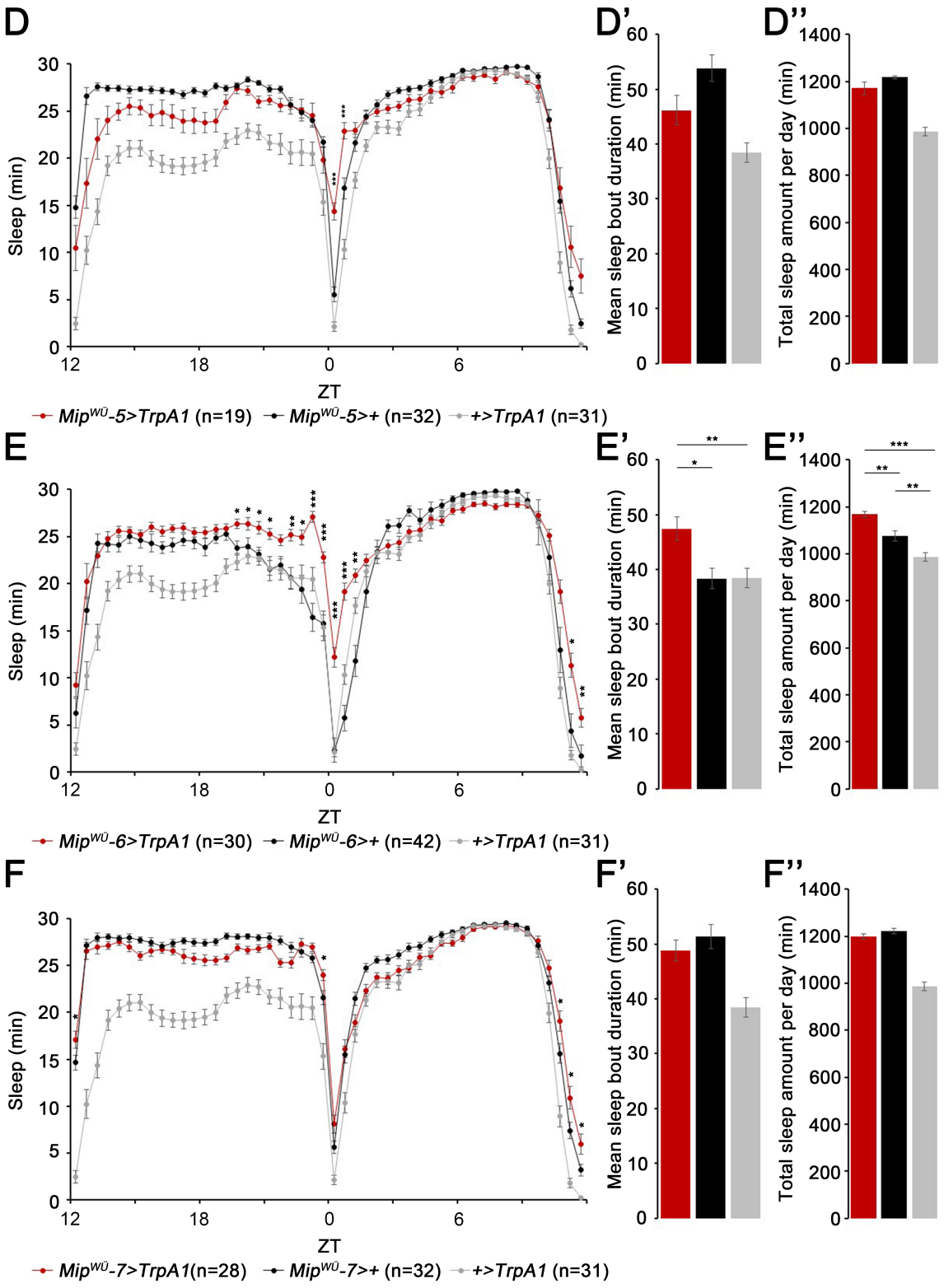

D
D'
D''
Sleep (min)
ZT
12
18
0
6
Mean sleep bout duration (min)
Total sleep amount per day (min)
Mip^WÜ-5>TrpA1 (n=19)
Mip^WÜ-5>+ (n=32)
+>TrpA1 (n=31)
E
E'
E''
Mip^WÜ-6>TrpA1 (n=30)
Mip^WÜ-6>+ (n=42)
+>TrpA1 (n=31)
F
F'
F''
Mip^WÜ-7>TrpA1(n=28)
Mip^WÜ-7>+ (n=32)
+>TrpA1 (n=31)

Fig 21. Thermogenetic activation of MIP^{WÜ} cells resulted in varying sleep states. Thermoactivation of MIP^{WÜ}-2 (A) or 3 (B) cells reduced sleep time in the siesta and sleep bout duration (A' and B'). (C) Thermogenetic activation of MIP^{WÜ}-4 cells increased sleep time in the evening and during the dark phase, whereas it decreased sleep time during the siesta. The sleep bout duration (C') and total sleep amount (C") of *Mip^{WÜ}-4>TrpA1* flies were not significantly different compared to the control. (D) Thermogenetic activation of MIP^{WÜ}-5 cells just increased sleep duration at ZT 0, but did not affect sleep bout duration (D') or total amount of sleep (D") compared to the control. (E) Thermogenetic activation of MIP^{WÜ}-6 cells increased sleep time in the late of dark phase, in the morning and in the evening, and the sleep bout duration (E') and total sleep amount (E") of *Mip^{WÜ}-6>TrpA1* flies were significant longer than the control. (F) Thermogenetic activation of MIP^{WÜ}-7 cells increased sleep duration slightly at ZT11, but did not affect sleep bout duration (F') or total amount of sleep (F") compared to the control. $*$ p $\leq$ 0.05. $**$ p $\leq$ 0.01, $***$ p $\leq$ 0.001, n.s. not significant.

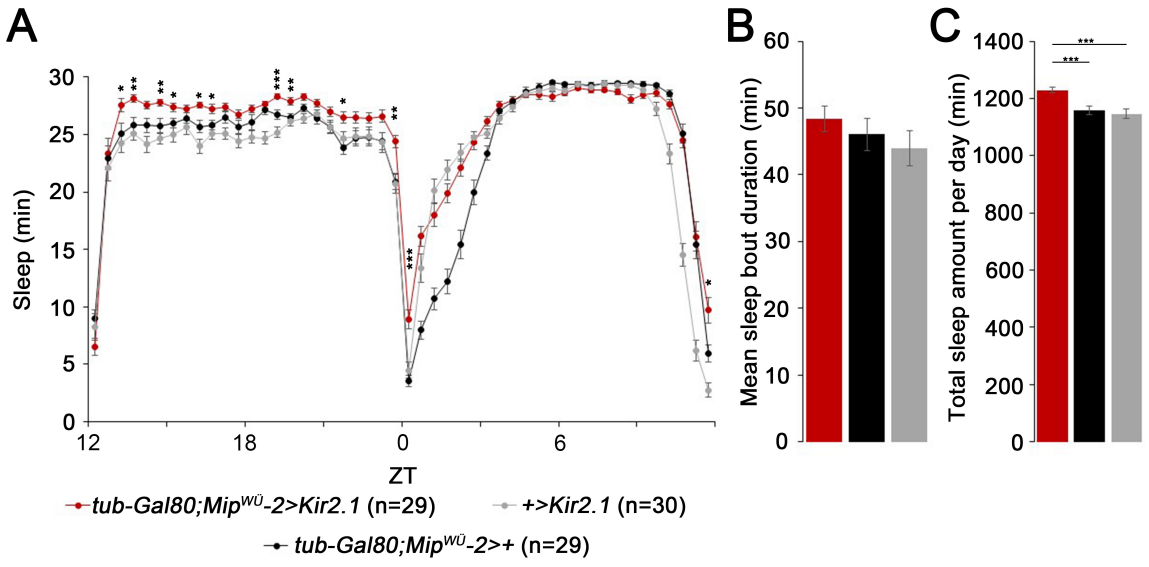


Fig 22. Conditional silencing of MIP^{WÜ} cells by ectopic expression of the inward rectifying K$^+$ channel Kir2.1 increased sleep during the dark phase. At 30°C, the averaged sleep profile over 24h of *tub-Gal80ts;Mip^{WÜ}-2>Kir2.1* experimental flies showed increased sleep duration during the dark phase. Total sleep amount but not the mean sleep bout duration of experimental flies was significantly higher than in the control. $*$ p $\leq$ 0.05. $**$ p $\leq$ 0.01, $***$ p $\leq$ 0.001.

2.4 Genetic silencing of MIP^{KR} cells using *Mip^{KR}-Gal4* considerably reduces food intake.

Manipulation of MIP$^{W\ddot{U}}$ cells did not affect food intake (Fig 19 and 20), which is not consistent with the results of Min et al. (2016). They found that activation of MIP^{KR} cells decreased food intake while silencing increased food intake and body weight. The IAM neurons played a critical role in this respect. GFP was also detected in the IAM neurons of our *Mip$^{W\ddot{U}}$>GFP* flies (Fig 17) showing that the IAM neurons are also included in the *Mip$^{W\ddot{U}}$-Gal4* expression pattern. In order to address the driver line-specific effects, and to test their *Mip^{KR}-Gal4* line and compare the effect to our observed results, we requested the *Mip^{KR}-Gal4* line. Jongkyeong Chung kindly provided us the *Mip^{KR}-Gal4* line. I followed the protocol in Min et al. (2016) using *UAS-TNT* to silence MIP^{KR} cells constitutively, and *UAS-TrpA1* to activate MIP^{KR} cells conditionally. Besides a CAFE assay, I also performed the automated FLIC assay (Ro et al. 2014) to test for a possible role of MIP^{KR} cellls in food consumption.

First I measured food intake of *Mip^{KR}-Gal4>TNT* flies in the CAFE assay at 25°C. Food consumption of *Mip^{KR}-Gal4>TNT* flies was significantly lower than that of controls (Fig 23A). However, at 29°C I did not observe a significant difference of food consumption in *Mip^{KR}-Gal4>TrpA1* flies compared to the control (Fig 23B) when MIP^{KR} cells were thermogenetically activated. In the FLIC assay, the electrical signals induced by physical contacts between individual flies and liquid food were recorded, and were displayed as representative feeding plots (Fig 24C). After checking the feeding behaviour patterns of each fly, signals higher than 40 (Fig 24C, dotted lines) were considered as ingestion. Asterisks indicated "tasting" or "touching" behaviour of flies. *Mip^{KR}-Gal4>TNT* flies were raised at 25°C, and then assayed over a period of two days after one day acclimation in the FLIC assay. Silencing of MIP^{KR} cells in *Mip^{KR}-Gal4>TNT* decreased ingestion behaviour, especially appearing during the time of the evening activity (Fig 24A). *Mip^{KR}-Gal4>TNT* flies showed a tendency towards a reduced total ingestion over two days compared to the control (Fig 24B). Due to a high evaporation of liquid sugar solution at 29°C, the FLIC assay could not be used to test food intake of *Mip^{KR}-Gal4>TrpA1* and the control flies.

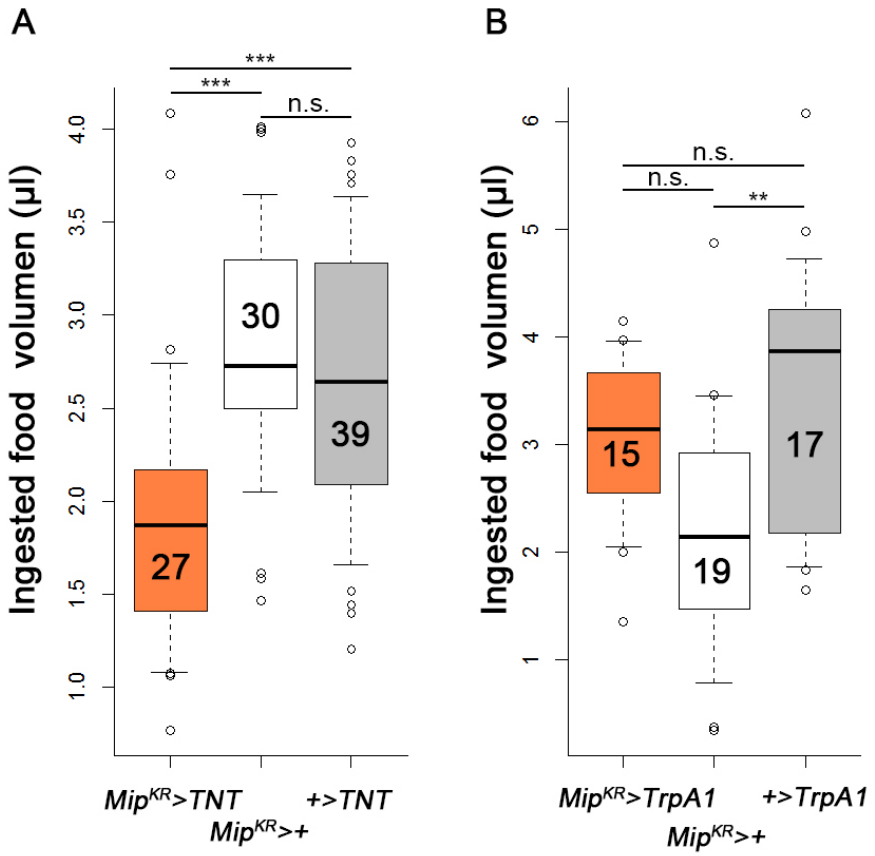


Fig 23. Effect of MIP^{KR} cell manipulation on food intake. (A) Constitutive silencing of MIP^{KR} cells by expression of *UAS-TNT* resulted in reduced food intake. *Mip^{KR}>TNT* flies consumed significantly less food than controls. (B) Thermogenetic activation of MIP^{KR} cells did not affect food intake. At 29°C, food consumption was comparable between *Mip^{KR}>TrpA1* and control flies. Numbers in each box indicate the amount of tested flies. $**$ p $\leq$ 0.01, $***$ p $\leq$ 0.001, n.s. not significant.

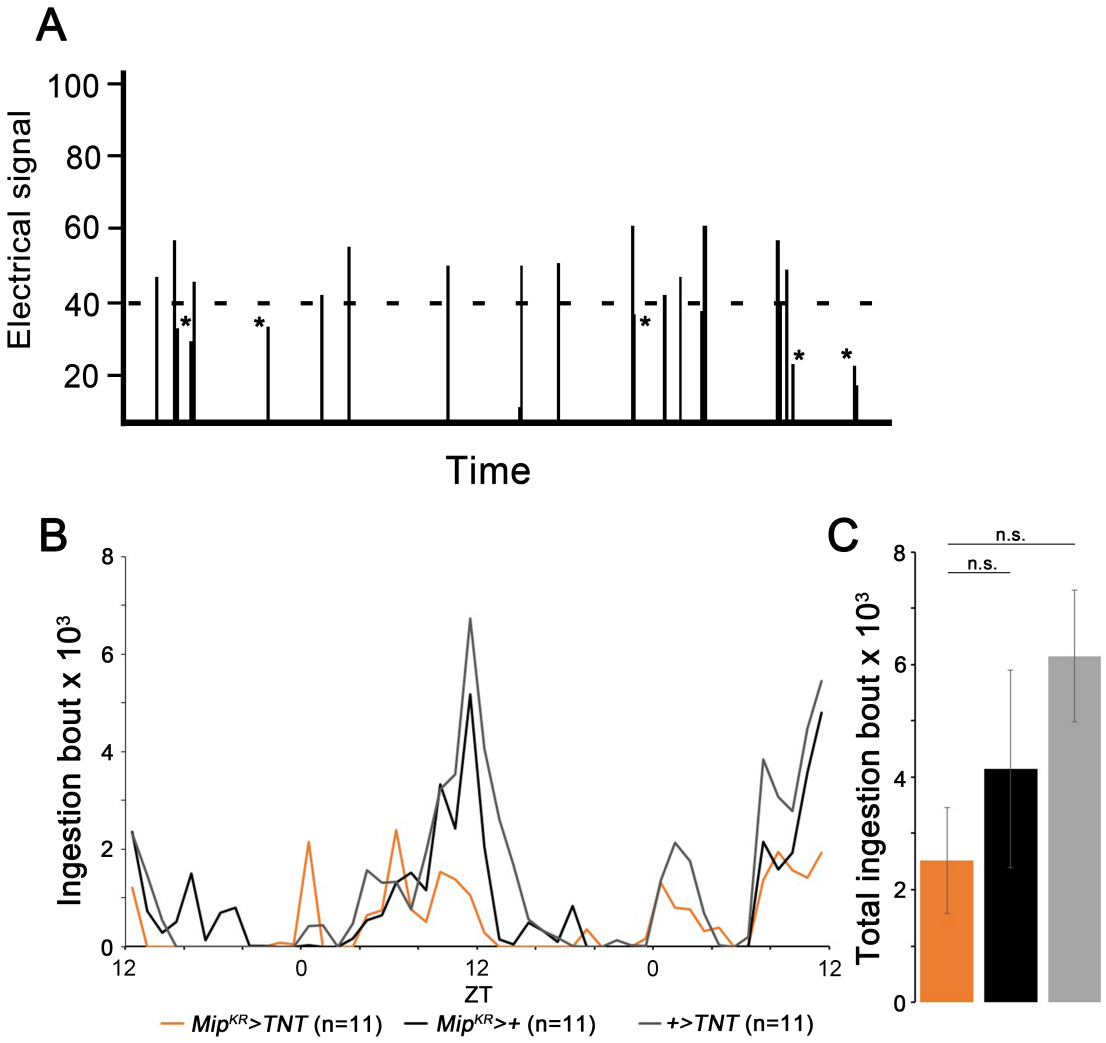


Fig 24. Effect of *UAS-TNT*-mediated silencing of MIP^{KR} cells on feeding in the FLIC assay. The electrical signals induced by physical contact between individual flies and liquid food over an hour were displayed as representative plots (A). (A) The signal higher than 40 (dotted line) was considered as ingestion behaviour. Asterisks indicated possibility of "tasting" or "touching". (B) Average ingestion bouts over two days of $Mip^{KR}>TNT$ flies and the controls. (C) showed the total ingestion bouts over two days calculated from (B), n.s. not significant.

2.5 Thermogenetic activation of MIP^{KR} cells in *Mip^{KR}-Gal4* strongly reduces sleep

In the last sections I showed that silencing of MIP^{KR} cells in *Mip^{KR}-Gal4* results in reduced food consumption, whereas manipulation of MIP$^{W\ddot{U}}$ cells does not affect food intake (Fig 19 and 20). I next asked whether manipulation of MIP^{KR} cells can affect sleep or locomotor activity, and employed the DAM system to record locomotor activity. Silencing of MIP^{KR} cells in *Mip^{KR}-Gal4>TNT* flies reduced sleep in the later half of the dark phase,and promoted sleep during the time of the morning activity (Fig 25A). The average sleep bout duration of *Mip^{KR}>TNT* flies, but not total sleep per day, was longer than in the controls (Fig 25B and 25C). Next, I thermogenetically activated MIP^{KR}

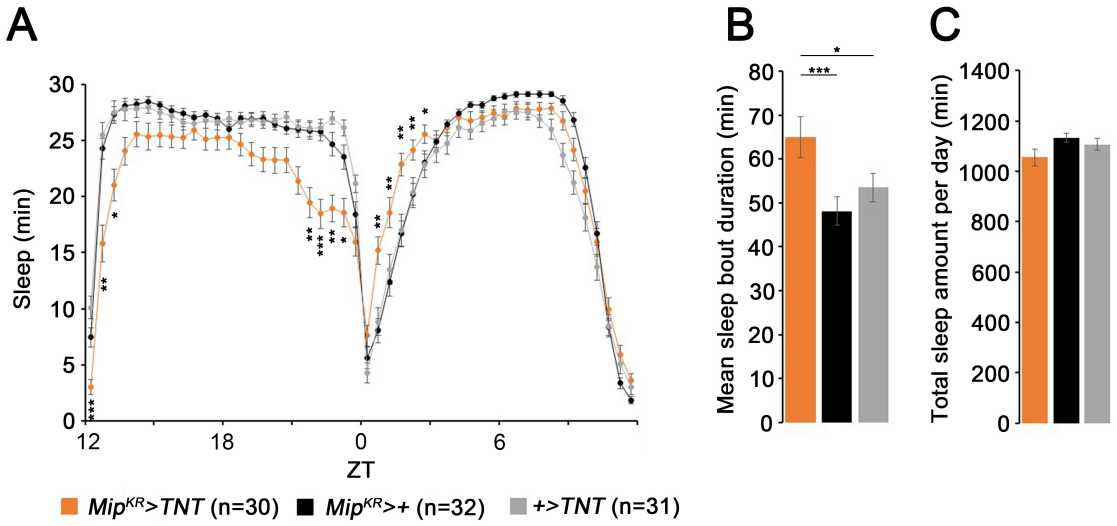


Fig 25. Effect of expression of *UAS-TNT* in MIP^{KR} cells on sleep. Constitutive expression of TNT induced slight reduction of sleep during the dark phase, and an increase in sleep especially during the time of the morning activity. The average of sleep bout duration but not total sleep amount was significantly increased by TNT expressiong. $*$ p $\leq$ 0.05. $**$ p $\leq$ 0.01, $***$ p $\leq$ 0.001.

cells in *Mip^{KR}-Gal4>TrpA1* flies. At 29°C, but not at 22°C, thermogenetic activation of MIP^{KR} cells strongly reduced sleep compared to the control (Fig 26A and 26D). *Mip^{KR}>TrpA1* flies rarely slept during the dark phase. At 29°C, the sleep bout duraton and total sleep amount were both significant lower than in the controls (Fig 26E and 26F), which was not observed at 22°C (Fig 26B and 26C).

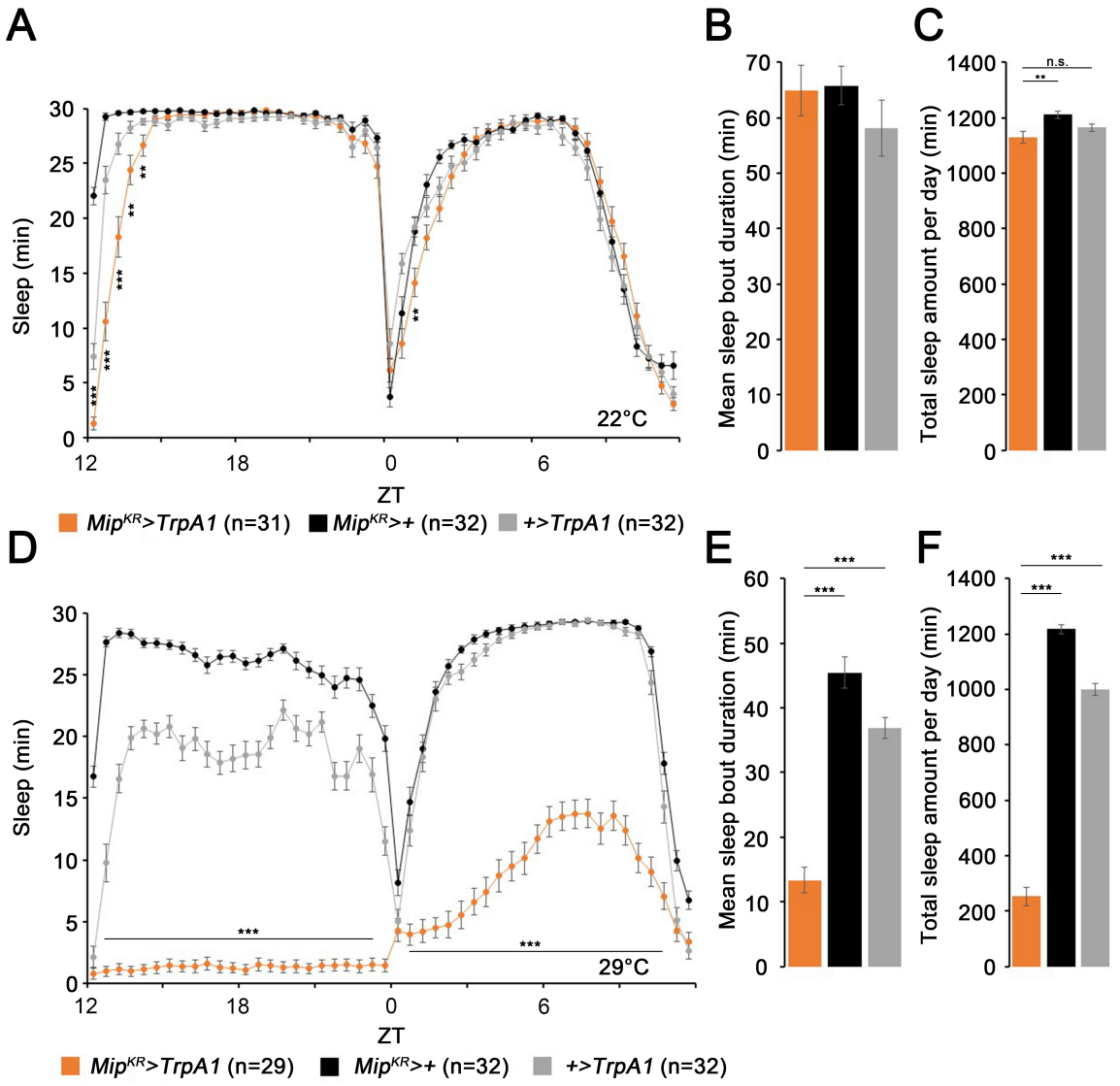


Fig 26. Thermogenetic activation of MIP^{KR} cells strongly reduced sleep. At 22°C, *Mip^{KR}>TrpA1* male flies did not sleep less than controls. Activation of the TrpA1 channel at 29°C resulted in strongly decreased sleep in *Mip^{KR}>TrpA1* flies. The flies almost did not sleep during the dark phase. Both the average sleep bout duration and the total amount of sleep per day were significantly reduced. $**$ $p \leq 0.01$, $***$ $p \leq 0.001$, n.s. not significant.

3 Discussion

My results show that manipulation of MIP$^{W\ddot{U}}$ cells in *Drosophila* using different *Mip$^{W\ddot{U}}$-Gal4* drivers did not affect feeding. Using the *Mip^{KR}-Gal4* driver, Min et al. found that food intake and body weight were regulated by MIP^{KR} cells, which could be traced to MIP signalling in *Drosophila* (Min et al. 2016). Flies consumed more food when MIP^{KR} cells were constitutively silenced by *UAS-TNT* and food consumption was decreased when MIP^{KR} cells were conditionally activated by *UAS-TrpA1*. Based on these results, Min and colleagues suggested that MIP^{KR} cells via MIP signalling serve states of satiety in an anorexigenic pathway and speculated that the IAM neurons are the critical neurons to regulate food intake and body weight. In contrast, however, my results with specific and restricted *Mip$^{W\ddot{U}}$-Gal4* drivers show that neither conditional silencing by *UAS-Kir2.1* (Fig 20) nor thermogenetic activation by *UAS-TrpA1* (Fig 19) of MIP$^{W\ddot{U}}$ cells affected food intake, although IAM neurons were included in the *Mip$^{W\ddot{U}}$-Gal4* (Fig 17B, red arrows) expression pattern. One possible explanation for the discrepancy between my results and that of Min et al. is that a large amount of unspecific MIP immunonegative cells are detected in *Mip^{KR}-Gal4* (Fig 18B), which are also genetically manipulated during experiments and might influence feeding behaviour in a way unrelated to MIP signalling. *Mip$^{W\ddot{U}}$-Gal4* lines are much more specific and restricted, and include most MIP-IR neurons besides IAM neuron, while some MIP-IR cells such as ALS and SGS neurons are not included (Fig 18A). Thus, it is possible that the increased food intake phenotype can be traced to MIP signalling from ALS and/or SGS cells. This might be the reason why I could not detect decreased food intake when MIP$^{W\ddot{U}}$ cells were thermogenetically activated.

I also tested the *Mip^{KR}-Gal4* driver. However constitutive silencing of MIP^{KR} cells (*Mip^{KR}>TNT*) reduced food ingestion and thermoactivation of MIP^{KR} cells (*Mip^{KR}>TrpA1*) did not affect food ingestion, which are not consistent of Min et al. (2016)'s research. Table 10 shows the recipes of standard medium for raising and the food recipes for the CAFE assay. Compared to the protocol used in Min et al. (2016), I raised the Korean flies on different standard medium and applied the food with different concentration of sucrose and yeast in the CAFE assay. The effect of the ingested food types in the larval and early adult stages on the results of the CAFE assay is not known. In my CAFE assay, food with 3.6 fold higher concentration of yeast extract was applied. Probably, MIPs play a role in the regulation of yeast (protein) ingestion or protein homoeostasis. Different food types could be one reason why different phenotypes were observed.

Table 10. Recipes of standard medium and for the CAFE assay.

Standard Medium				**CAFE Assay**			
KR		**WÜ**		**KR** (m/v)		**WÜ** (m/v)	
Dextrose	70g	Golden Syrup	45g	Sucrose	5%	Sucrose	5.4%
Cornmeal	35.28g	Cornmeal	147.5g	Yeast extract	1%	Yeast extract	3.6%
Propionic acid	4.67ml	Diamalt	48g			BPB	0.03%
Yeast	50g	Soybean meal	10g				
Tegosept	7.33ml	Cernovis	18.5g				
Agar	5.06g	Nipagin	2.5g				
Water	1L	Agar	6.25g				
		Water	1L				

Energy homoeostasis and sleep-wakefulness circuits are two very important systems. Disruption of energy homeostasis, e.g. during starvation, can result in unusual locomotor behaviour and affect the sleep-wake circuit, which in turn affects food intake and energy storage. Activation of MIP^{WÜ} cells using *Mip^{WÜ}-Gal4* drivers (Fig 21) resulted in changes in the sleep status, which showed no coherent pattern. Thermoactivation of MIP^{WÜ}-2 and 3 cells increased sleep duration, *Mip^{WÜ}-5>TrpA1* and *Mip^{WÜ}-6>TrpA1* flies showed reduced sleep duration and thermoactivation of MIP^{WÜ}-4 cells increased sleep duration in the evening and the dark phase whereas reduced sleep duration in the siesta (Fig 21). The six *Mip^{WÜ}-Gal4* lines were generated with the same promoter construct injected into *Drosophila* embryos by P-element. The line-specific changes in the sleep status are therefore likely due to different genomic insert positions. In addition, thermoactivation of MIP^{KR} cells strongly reduced sleep duration (Fig 26), which suggests that the reduction of the sleep duration either could be traced to MIP signalling from ALS and SGS neurons which are not included in *Mip^{WÜ}-Gal4* lines or is induced by the activation of unspecific MIP immunonegative cells.

Chapter one pointed out the importance of circadian clock which is modulated by several peptides and influences locomotor behaviour, feeding and sleep (Lee et al. 2006, Hermann et al. 2012, Shang et al. 2013, Hermann-Luibl et al. 2014, Oh et al. 2014, Kunst et al. 2014, Yao & Shafer 2014). In the Madeira cockroach *Rhyparobia maderae*, injection of MIPs delays the circadian locomotor activity at the beginning of subjective night (Schulze et al. 2013) and in the German cockroach *Blattella germanica*, injection of MIPs decreased food intake (Aguilar et al. 2006). My confocal imaging results show that a pair of ICLI neurons innervates the GNG and projects to the dorsal posterior

protocerebrum. 10 categories of gustatory neurons aborising in the GNG and 9 in TAG are mapped in *Drosophila* (Kwon et al. 2014). The LMIo neurons locate close to the LNvs, arborize in the medulla and project to the dorsal lateral protocerebrum, which resembles the anatomy of PDF-expressing LNs (Helfrich-Förster et al. 2007). SPL interneurons locate in the superior lateral protocerebrum and arborize in the posterior superior protocerebrum, which as we mentioned in chapter one is the location of sleep-relevant dopaminergic neurons (Liu et al. 2012, Ueno et al. 2012). Although, none of the clock genes has been demonstrated in LMIo neurons (Kolodziejczyk & Nässel 2011), SPR was detected in the s-LNvs and l-LNvs, thus PDF neurons might be a downstream target of MIP-producing cells in *Drosophila* (Oh et al. 2014). Based on the expression pattern, MIPs carrying the gustatory signal from the GNG or mechanical signal from peripheral neurons in TAG might be transferred via ICLI neurons further to the dorsal part of the brain or via LMIo to LNvs affecting PDF, in order to regulate feeding and sleep, which suggests that MIPs might play a role involved in neuronal circuits to modulate feeding and sleep associated with the circadian clock.

MIPs are characterized by the sequence W(X_6)Wa, which is similar to a part of mammalian galanin. This sequence-similarity explains why galanin-like immunostaining patterns in blowflies correspond to MIP antiserum labelled neurons in *Drosophila* (Nässel & Winther 2010). Galanin is a brain-gut peptide (Rattan & Tamura 1998, Crawley 1999). As mentioned in chapter one, AstA receptors are homologous to the galanin receptor; galanin and its receptor have pleiotropic effects on food intake, locomotor activity, sleep-wake circuit and the control musculature in different species (Woods et al. 2014, Lang et al. 2015). Investigating the effects of AstA and MIPs can improve the understanding of diversity and function of brain and gut peptides in insects, and contributes to the evolutionary understanding of Ast/galanin signalling in the control of food intake and sleep in animals.

III. Metabolic labelling and quantification of *Drosophila* neuropeptides and peptide hormones

1 Introduction

Regulatory peptides are signalling molecules used in intercellular communication. Regulatory peptides are released from CNS or endocrine system through the regulated secretory pathway and then activate specific G protein-coupled receptors (GPCRs) in neurons or peripheral target cells outside the nervous system to induce many physiological or behavioural effects (Strand 1999, Burbach 2011, van den Pol 2012). In order to understand the functions of regulatory peptides in modulating neuronal circuits or the endocrine system, we try to obtain information about the timing and amount of peptide hormone release. The most direct way to obtain these information is to quantify peptide titres in the circulation of animals. In vertebrates, typically a dialysis probe is implanted to monitor pulsatile release of peptide, from which released peptide can be extracted and timing of released peptide can be analysed (Robinson & Justice 2013). *Drosophila* is a good genetic model organism to investigate peptide functions. *Drosophila* haemolymph is analogous to mammalian blood and circulates in the interior of body. Yet, due to the small size of *Drosophila* compared to vertebrate animals, it is much more difficult to monitor peptide in haemolymph directly (Fastner et al. 2007, Zeng et al. 2016), not to mention to implant a dialysis probe by surgery. The combination of liquid chromatography (LC) with mass spectrometry (MS) potentially permits to evaluate the production and release of peptides in *Drosophila* (Pauls et al. 2014). In the CNS, there are in principle two different pools of peptides. One pools consist of intracellular peptides stored in large dense-core vesicles (LDCV), the other pool consists of the released extracellular peptides. Using MS in CNS extracts cannot distinguish between the amount of stored and released peptides. Quantitative real-time polymerase chain reaction (qRT-PCR) is a useful laboratory technique to quantify the mRNA of a given peptides, which could serve as a semiquantitative proxy reflecting peptide production. Considering that the rate of peptide production and release might not be constant, the following formula can be used to estimate the release of peptide:

$A(t) = A_0 + \int_0^t v_\mathrm{p}(t)dt - \int_0^t v_\mathrm{r}(t)dt$

(from timepoint 0 to t, A: total amount of peptides in tissue, v_p: rate of peptides produced, v_r: rate of peptide released).

To be able to quantify the total amount of peptides by MS, flies are fed with yeast grown in medium that contained either light (^{14}N) or heavy (^{15}N) stable nitrogen. The metabolic incorporation of heavy or light isotope into cells labels all amino acids and hence leads to either heavy or light

peptides. The stable heavy isotope-label atom (^{15}N) increases the mass of peptides, which can be detected by mass spectrometry. Light (^{14}N) labelled tissue dissected at timepoint t_0 and heavy (^{15}N) labelled tissue dissected at timepoint t_x (or also light (^{14}N) labelled tissue dissected at timepoint t_x and heavy (^{15}N) labelled tissue dissected at timepoint t_0) are pooled and then analysed by MS. By calculating light-to-heavy peptide ratio (^{14}N/^{15}N) for each identified peptide, we can obtain the relative quantitative information of total peptide amount A between t_0 and t_x. To estimate the amount of peptide production, qRT-PCR can be used to analysed the mRNA levels of correlated peptides. Finally, according to the formula,the quantitative information of released peptides from t_0 to t_x can be calculated. For example, if the relative amount of peptides between two timepoints (t_0 and t_x) is unchanged, while the peptide mRNA level is increased from t_0 to t_x, then it is reasonable to assume that physiological condition during this period (from t_0 to t_x) induces the release of peptide and the amount of released peptides can be assessed by measuring mRNA as a semiquantitative proxy using qRT-PCR.

After optimizing the protocol for metabolic labelling, I carried out a quantitative analysis of peptides before and after eclosion as a test. 110 peptides in the nervous system and 5 peptides in the gut were detected. The levels of peptides e.g. LPAISHYTH (eclosion hormone (EH)), LPAISHYT (EH) and AYRKPPFNGSIFa (SIFamide (SIFa)) were decreased after eclosion. Then, I applied the metabolic labelling in *Drosophila* adult, which were either fed *ad libitum* or starved for 24 hrs, and analysed the release of AstA, MIPs and their receptors. In the mRNA level, my results showed that in the brain *AstA* mRNA level in the 24 hrs starved flies was increased compared to in the *ad libitum* fed flies, whereas in the gut the *AstA* mRNA level was decreased. Starvation induced the reduction of *Mip* mRNA level in the brain and gut. For the receptors, in the brain *Dar-1* mRNA level in the starved flies was increased while *Dar-2* and *Spr* mRNA levels were decreased compared to in the *ad libitum* fed flies. In the gut, *Dar-2* and *Spr* mRNA levels were increased and *Dar-1* mRNA level was unchanged in the starved fed flies compared to in the *ad libitum* fed flies. Unfortunately, due to technical problems I was unable to analyses the metabolic labelled peptides during the course of this thesis.

2 Results

2.1 Quantitative peptidomics based on metabolic labelling before and after eclosion

At first, I carried out a quantitative analysis of peptides before and after eclosion as a proof-of-principle. Wild type Canton-S flies were raised on either ^{15}N or ^{14}N labeled-yeast sugar solution. Brain, thoracic-abdominal ganglia (TAG) and gut were dissected either from pharate adults which were close to eclosion, or from freshly eclosed flies with unexpanded wings. ^{15}N labelled tissue of pharate adults and ^{14}N labelled tissue of freshly eclosed flies, or ^{14}N labelled tissue of pharate adults and ^{15}N labelled tissue of freshly eclosed flies were pooled into a low-binding tube (Table 11). Extracted peptides were analysed by nanoLC-MS/MS on an orbitrap mass spectrometer by Jens Vanselow (AG Schlosser, Virchow Center of the JMU).

Table 11. Samples of pooled ^{14}N- and ^{15}N-labelled tissues.

Samples	Tissues
Sample 1	Brain and TAG of pharate adult (^{14}N) + Brain and TAG of eclosed flies (^{15}N)
Sample 2	Brain and TAG of pharate adult (^{15}N) + Brain and TAG of eclosed flies (^{14}N)
Sample 3	Gut of pharate adult (^{14}N) + Gut of eclosed flies (^{15}N)
Sample 4	Gut of pharate adult (^{15}N) + Gut of eclosed flies (^{14}N)

In total, we detected 110 peptides originating from 31 different prepropeptides in the nervous system samples (either in Sample 1 or 2), of which 44 peptides from 14 prepropeptides were detected in both sample 1 and 2 (Fig 27A). In gut samples, we detected 18 peptides originating from 9 prepropeptides (either in sample 3 or 4), of which 5 peptides from 3 prepropeptides were detected in both sample 3 and 4 (Figure 27C). The levels of a peptide associated with the eclosion hormone (EH) precursor (LPAISHYTH and LPAISHYT) and SIFamide (SIFa) AYRKPPFNGSIF were decreased after eclosion (Fig 27A and 27B). After eclosion, the peptide level of LPAISHYTH was 7-8 times and LPAISHYTH was about 3 times lower than before eclosion (Pauls et al. 2014) (Fig 27B). Other peptide levels were increased after eclosion except for ion transport peptide (ITP) (LPHNHNL), Neuropeptide-like precursor 1 (NPLP1) (LQSAPSTHRDPK, NLGALKSSPVHGVQQ, NVAAVARYNSQHGHIQRAGAE and YNSQHGHIQRAGAE), showed conflicting results (Fig 27A and 27C). This result suggests that

the change of peptide levels can be quantified by metabolic labelling in *Drosophila*.

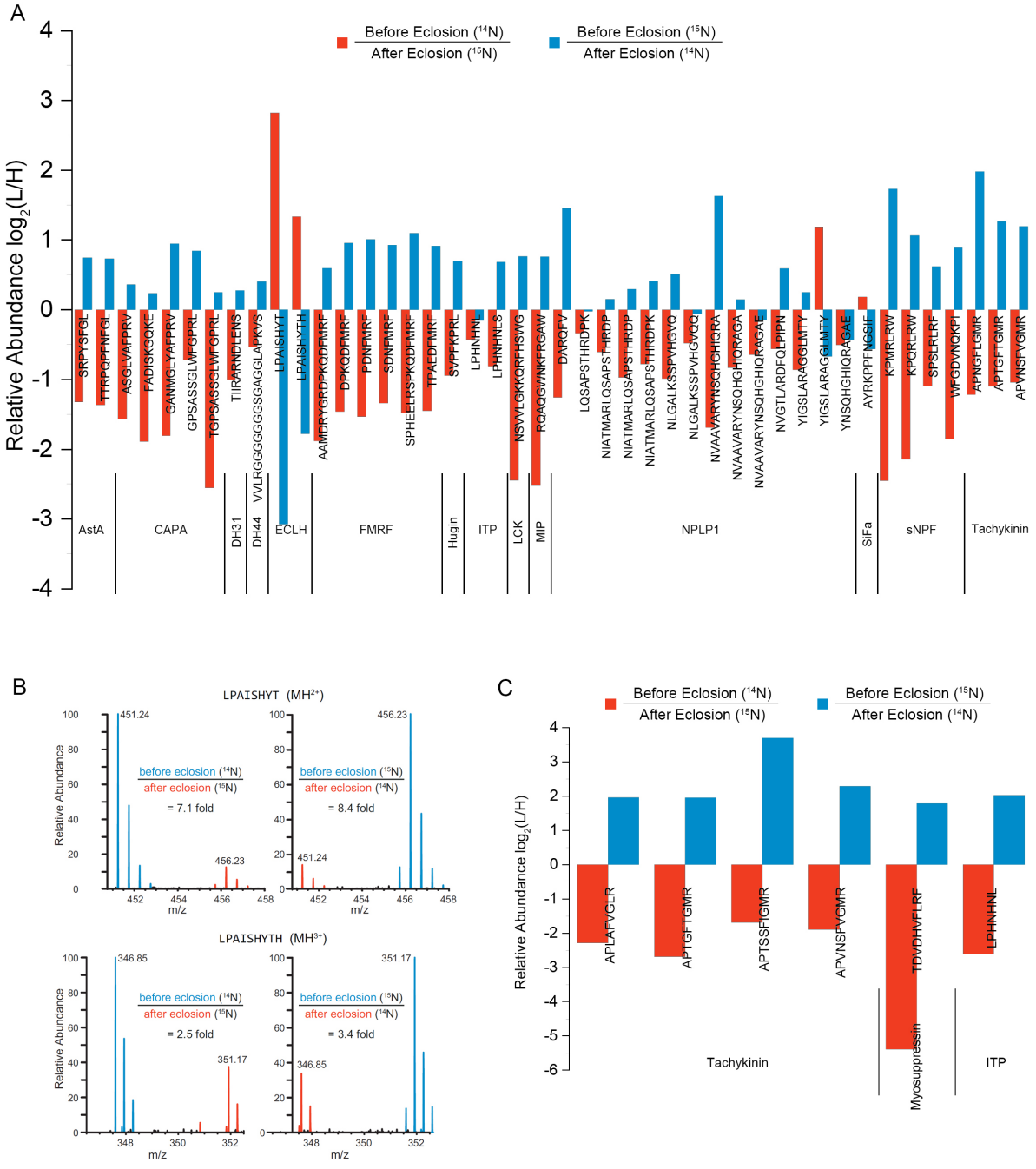


Fig 27. Quantitative peptidomics based on metabolic labelling in *Drosophila*. Quantification of peptidomics in *Drosophila* CNS (A) and gut (C). After eclosion, LPAISHYTH (EH) LPAISHYT (EH) and AYRKPPFNGSIFa (SIFa) are decreased. The other peptides show increased peptide levels after eclosion. (B) Quantification of the spacer peptide LPAISHYT and LPAISHYTH from the EH precursor before and after eclosion. After eclosion, LPAISHYT (7.1 or 8.4 fold) and LPAISHYTH (2.5 or 3.4 fold) are decreased (Pauls et al. 2014).

2.2 mRNA Expression of *AstA*, *Mip* and their receptors under fed and food-deprived conditions

Chapter One and two described that activation of Allatostatin A (AstA) cells reduces food intake which can be traced to AstA signalling (Fig 8), and that myoinhibiotory peptides (MIPs) are possibly involved in regulating food intake (Fig 23) (Min et al. 2016). I am interested in whether and how AstA, MIPs and their receptors regulate *Drosophila* food intake, when adult flies are either fed *ad libitum* or starved for 24 hrs. I applied metabolic labelling to obtain the quantitative information of total amont of peptides. According to the constructed formula, the amount of released peptides can be assessed, if the amount of produced peptides is known. mRNA levels of correlated peptides can be as a proxy reflecting production. Therefore, *AstA*, *Dar-1*, *Dar-2*, *Mip* and *Spr* mRNA levels of *ad libitum* fed and food-deprived flies were measured via qRT-PCR. 20 four days old *Canton-S* flies were transferred into vials with normal food (fed) or 2% agarose (food deprivation). After 24 hours, brains, thoracic-abdominal ganglion (TAG) and guts were dissected, of which the mRNA was isolated and reverse transcribed into cDNA. qPCR was performed to compare mRNA levels of *AstA*, *Dar-1*, *Dar-2*, *Mip* and *Spr* between fed and food-deprived flies.

In the brain, 24 hours starvation increased *AstA* mRNA levels 1.34 fold and *Dar-1* mRNA levels 1.27 fold while it decreased *Dar-2* mRNA levels 1.48 fold. *Mip* and *Spr* mRNA levels of food-deprived flies were decreased around 1.45 fold compared to fed flies (Fig 28A).

In the TAG, starvation reduced *AstA* mRNA levels 1.16 fold and increased the mRNA levels of *Dar-1* and *Dar-2* 1.26 fold and 1.39 fold, respectively. *Mip* mRNA level of food-deprived flies was decreased 1.5 fold. In contrast, the *Spr* mRNA level was increased 1.28 fold (Fig 28B) compared to fed flies.

In the gut, *AstA* and *Mip* mRNA levels of food-deprived flies were much lower (5 fold and 6 fold, respectively) than in fed flies. *Dar-1* mRNA levels were similar between food-deprived and fed flies, only a starvation-induced increase of 1.01 fold was observed. *Dar-2* mRNA levels of food-deprived flies were increased 1.28 fold and *Spr* mRNA levels also increased 1.23 fold compared to fed flies (Fig 28C).

Unfortunately, due to technical problem I was unable to analyse the metabolic labelled peptides during the course of this thesis to obtain the relative quantitative imformation of the peptides between fed and food starved flies.

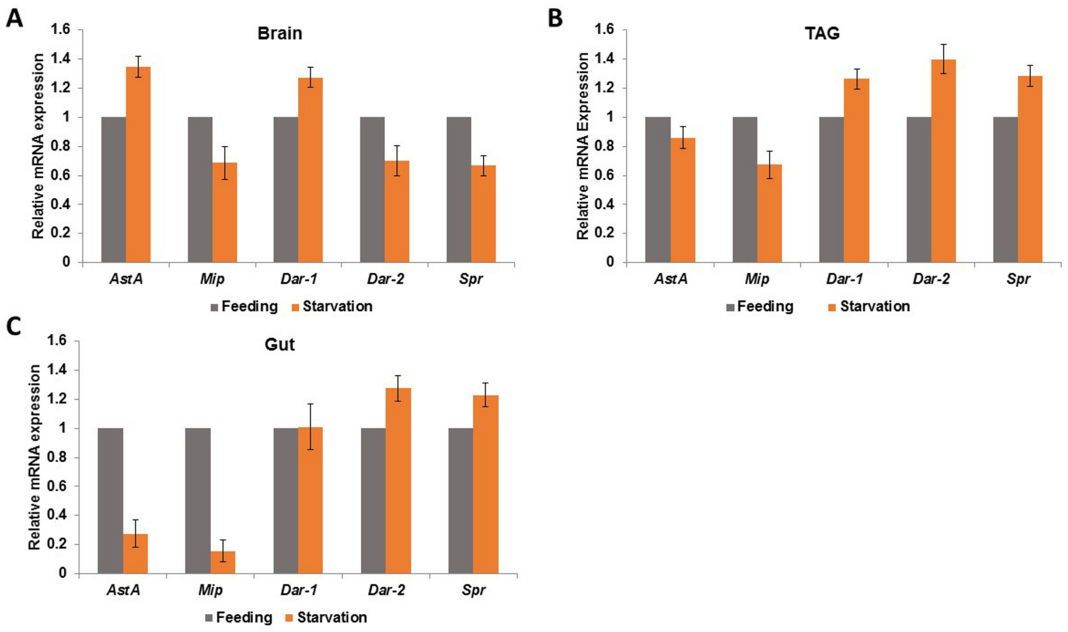


Fig 28. Relative mRNA expression of *AstA*, *Mip* and their receptors under fed and food-deprived conditions. *AstA*, *Mip* and their receptors mRNA expression levels in the brain (A), TAG (B) and gut (C) of food-deprived flies compared to fed flies. (A) In the brain, *AstA* and *Dar-1* mRNA expression is increased while *Dar-2 Mip* and *Spr* mRNA expression are decreased. (B) In the TAG, *AstA* and *Mip* mRNA expression is decreased, while their receptor mRNA expression were all increased. (C) In the gut, *AstA* and *Mip* mRNA expression are strongly decreased. *Dar-1* mRNA expression is comparable between the fed and food-deprived status. *Dar-2* and *Spr* mRNA expression are increased. Five biological replicates were measured in three technical replicates.

3 Discussion

In this study I describe a method to quantify the relative abundance of peptides, which in combination with qRT-PCR can be used to assess peptide release in different physiological or behavioural conditions. The mRNA levels can be used as a proxy reflecting peptide production. However, whether the encoded peptides are released is not clear. To visualize location and release of peptides, previous work used a transgenic construct, containing a fusion of atrial natriuretic peptide and green fluorescent protein (*UAS-ANF-GFP*) (Rao et al. 2001). A decrease in detected GFP intensity suggests release of tagged neuropeptide. Here, I obtained the quantitative information of peptides by using metabolic labelling and constructed a formula:

$$A(t) = A_0 + \int_0^t v_{\mathrm{p}}(t)dt - \int_0^t v_{\mathrm{r}}(t)dt$$

Based on the formula, the amount of released peptides can be calculated. At first, I tested the method in eclosion. During calculation of the ratio between the amount of identified peptides before and after eclosion, I found that after eclosion the amount of most peptides is increased (Fig 27). However, EH and SIFa are decreased suggesting EH and SIFa are released during eclosion, no matter whether and how many EH and SIFa are produced in eclosion. This is in line with the finding by McNabb & Truman (2008) that showed that the intensity of EH inmmunostaining is lower after eclosion suggesting EH is massively released. The reduction of SIFa suggests a new and unexpected role for SIFa during eclosion.

I am interested in the role of AstA and MIPs under food-deprived conditions. The metabolic labelling method was applied to aquire the quantitative imformation of the released peptides under food-deprived conditions. The mRNA levels reflect the production of peptides, and we can estimate the amount of released peptide. The qRT-PCR result shows that starvation increased *AstA* mRNA levels in the brain, whereas it reduced *AstA* mRNA levels in the TAG and gut. This suggests that AstA expressed in the brain, TAG and gut may have differential effects under food-deprived conditions. When flies are starved, increased *AstA* mRNA levels in the brain (Fig 28A) suggest an upregulation of AstA production, and increased *Dar-1* mRNA levels in the brain (Fig 28A) indicate upregulation of receptors suggesting in response to increased release of AstA in the brain. This suggests that upon starvation, AstA signalling is increased in the brain in order to promote sleep (Fig 11) via Dar-1 as an adaptive response to save energy during the absence of food. In the gut, the mRNA level of *AstA* was strongly decreased under food-deprived condition suggesting AstA in EECs can probably acquire the

information of nutrition, because EECs reach the gut lumen with apical extensions (Veenstra 2009, Veenstra et al. 2008) and express gustatory receptors (Park & Kwon 2011). Thus, AstA-expressing EECs potentially send nutritional signal from the gut to brain via Dar-2. Dar-2 is expressed in the adipokinetic hormone (AKH) - producing cells in the corpus cardiacum (CC) (Hentze et al. 2015) and according to the FlyAtlas (http://flyatlas.org/atlas.cgi), Dar-2 is mainly expressed in the gut (Chintapalli et al. 2007) suggesting Dar-2 might be a gut receptor, and CC cells might be the target cells of AstA releseased from EECs. When flies are starved, reduced *Dar-2* mRNA levels in the brain (Fig 28A) reflect reduction of receptors in response to downregulation of *AstA* from the EECs (Fig 28C) in order to reduce the release of AKH and then increase starvation resistance to live longer (Hentze et al. 2015).

In the brain, the *Mip* mRNA level is decreased upon starvation suggesting reduced production of MIPs. The decreased *Spr* mRNA level indicates downregulation of produced receptors in response to decreased release of MIPs. Oh et al. (2014) suggests that downregulation of *Mip* and *Spr* reduces the duration of sleep in *Drosophila*. This suggests that MIPs and SPR are potentially able to promote foraging behaviour in the absence of food. In the gut, the mRNA levels of *AstA* and *Mip* were strongly reduced during starvation suggesting reduced production of AstA and MIPs. Yet, starvation increased the mRNA levels of *Dar-2* and *Spr* reflecting increased production of Dar-2 and SPR. Due to the increased Dar-2 and SPR, AstA and MIPs from the brain or stored in the gut may be released into the gut during starvation.Unfortunately, due to technical problem I was unable to quantify peptide amounts during the course of this thesis. Thus, at current, I am unable to estimate the release rates of AstA and MIPs during fed conditions or at starvation.

Appendix

Supplementary

Chapter 1: Allatostatin A signalling in Drosophila regulates feeding and sleep and is modulated by PDF.

S1 Text. Recipe for standard *Drosophila* medium and primers.

5.9 kg corn semolina was mixed with 34 L water, boiled for 3 min, and then constantly and slowly stirred for 4 h while cooling down. The next day, 6 L water, 1.8 kg malt extract, 1.8 kg sugar beet molasses, 0.4 kg soy flour, 0.74 kg yeast powder and 0.25 kg agar-agar were added, and the mixture was boiled for 3 min under constant stirring. When the medium had cooled down to $\sim$ 80°C, 0.1 kg methyl-4-hydroxybenzoate (nipagin) was intermixed.

Primer sequences used for the amplification of the AstA promoter region

1.03 kB promoter fragment, $AstA^{1X}$:
sense 5'-GCGCAATTGATGGCTATTTCCCAGCTCCT-3'
antisense 5'-GCCGGATCCAGAGGTTCCGCGGACTAAAT-3'

2.05 kB promoter fragment, $AstA^{2X}$:
sense 5'-GCGCAATTGAGTAGAAGCTGCGCCAGAAG-3'
antisense 5'-GCCGGATCCAGAGGTTCCGCGGACTAAAT-3'

2.74 kB promoter fragment, $AstA^{3X}$:
sense 5'-GCGCAATTGGGGAAAAATCTCCGAAAACC-3'
antisense 5'-GCCGGATCCAGAGGTTCCGCGGACTAAAT-3'

Occasionally, we noticed several additional somata with weak AstA IR in the CNS. Since these cells were not included in the *AstA^{34}-Gal4* expression pattern and projections were not labelled, we did not analyse them further.

S1 Table. Expression patterns of *AstA^{34}-Gal4* and *tsh-Gal80;AstA^{34}-Gal4*.

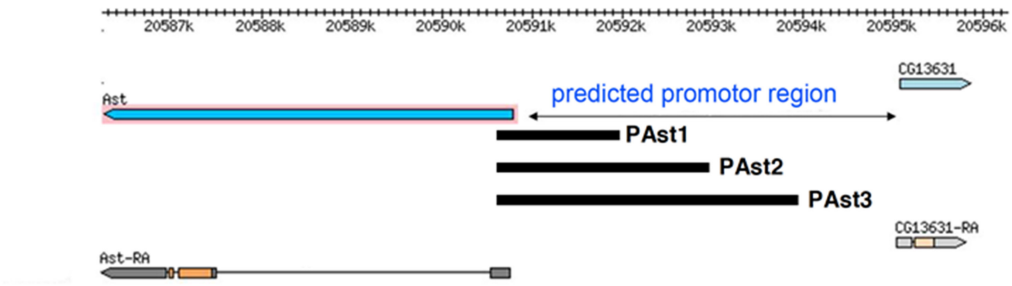
Organ	GFP expression *AstA^{34}-Gal4*	GFP expression *tsh-Gal80;AstA^{34}-Gal4*	AstA immunoreactivity*	GPF or LacZ expression *AstA^{1}-Gal4*#
central brain	2-3 per hemisphere in the posterior lateral protocerebrum	+	+ (PLP cells)	+
	2–4 per hemisphere in the lateral cell body rind (LCBR cells)	+	-	-
optic lobes	small number of cells in the medulla	+	+	+
TAG	3 pairs of abdominal cells at the posterior end of the TAG (innervate the gut)	-	+ (DLAa§ cells in abdominal neuromeres)	+
peripheral NS	2 pairs of cells on segmental nerves exiting the thoracic part of the TAG	-	+ (peripheral cells on the wing and haltere nerves)	+
gut	EECs in the posterior midgut	+	+	+

*AstA IR detected in this study, as well as from Yoon & Stay (1995) and Santos et al. (2007), which are consistent with our findings. For nomenclature, see Yoon & Stay (1995).

#Hergarden et al. (2012)

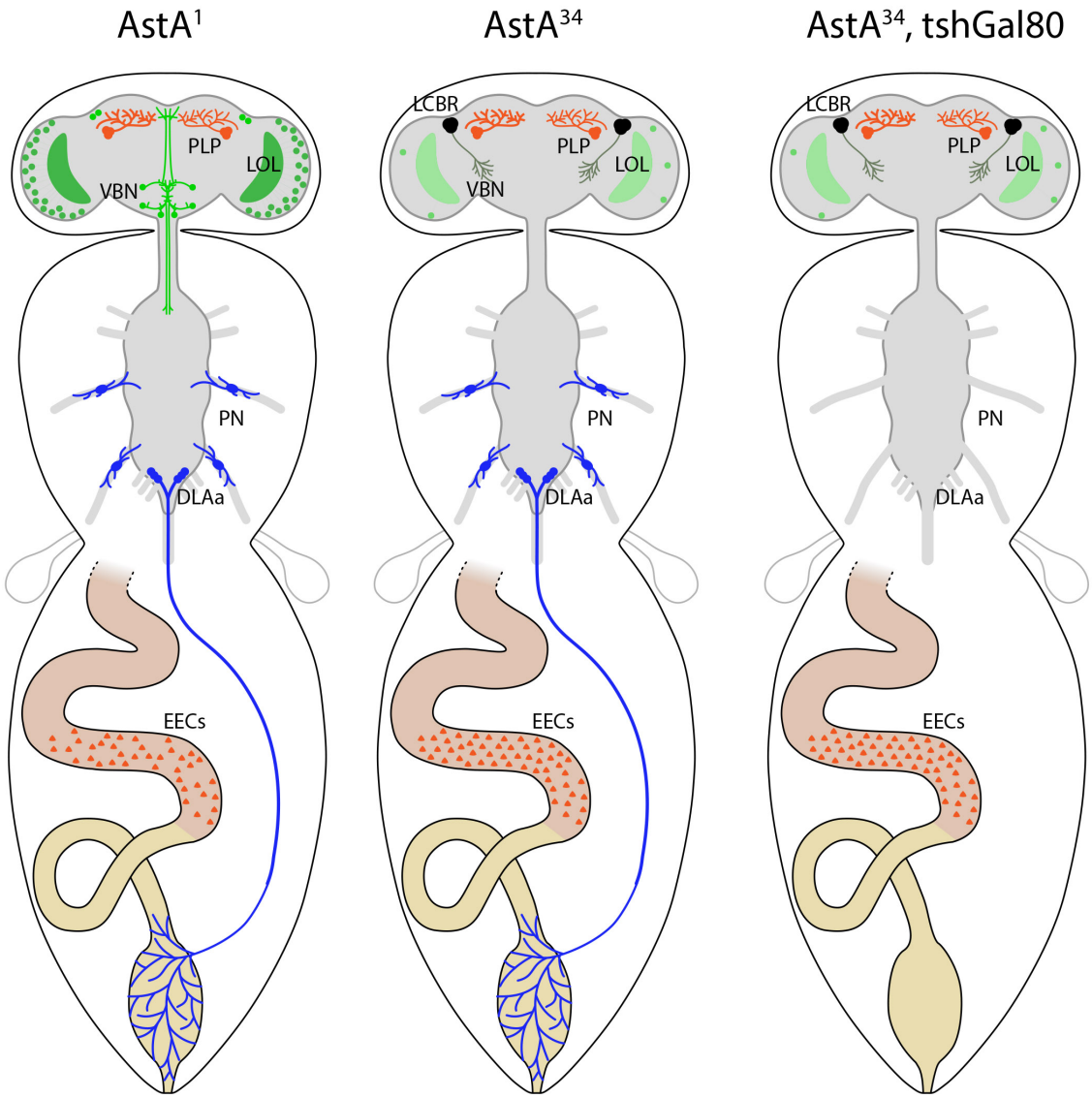
§The designation DLAa (dorsolateral abdominal a) neurons seems not fully accurate because part of their somata lie centrally or slightly ventrally within the adult thoracico-abdominal ganglion (TAG).

DLAa dorsolateral abdominal a, EECs enteroendocrine cells, LCBR lateral cell body rind, NS nervous system, PLP posterior lateral protocerebrum, TAG thoracico-abdominal ganglion, VG ventral ganglion, VMA ventromedial abdominal.

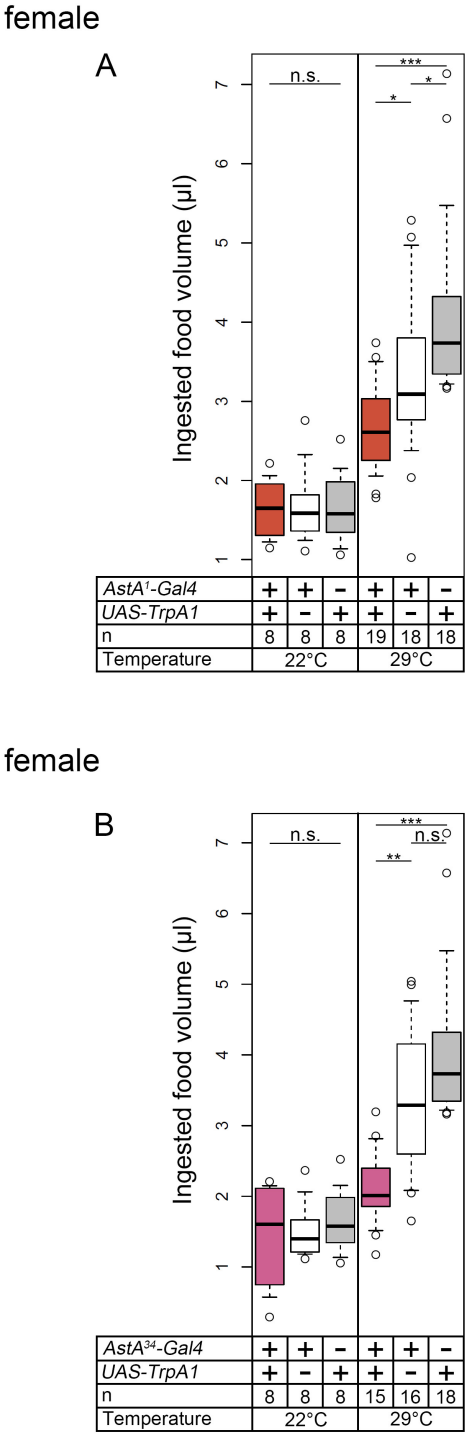


		CNS			midgut		
transgene	line#	AstA-LacZ overlap	AstA-LacZ false negatives	AstA-LacZ false positives*	AstA-LacZ overlap	AstA-LacZ false negatives	AstA-LacZ false positives
pAstA1	1	-	+	-	-	+	+
	2	-	+	-	-	+	+
	3	-	+	-	-	+	-
	4	-	+	-	-	+	+
	5	-	+	-	-	+	-
pAstA2	1	+	+	+	+	+	+
	2	+	+	+	+	-	-
	3	+	+	-	+	-	-
	4	+	+	+	+	+	+
	5	+	+	+	+	+	-
	6	+	+	-	+	+	+
	7	+	+	-	+	-	-
	8	+	+	-	+	-	-
	9	-	+	-	-	+	+
	10	+	+	-	+	+	+
pAstA3	1	+	+	-	+	+	-
	3	+	+	-	+	-	-
	4	+	+	-	+	-	-
	5	+	+	-	+	-	-
	6	-	+	-	+	+	-
	7	+	+	-	+	-	-
	8	+	+	-	+	-	-
	9	+	+	-	+	-	-
	10	+	+	+	+	+	-

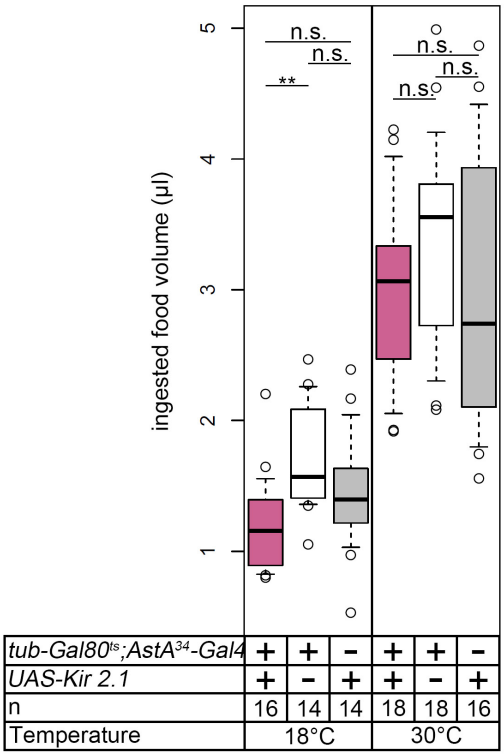
S1 Fig. *UAS-LacZ* expression pattern in the larval CNS in relation to AstA immunostaining for the different generated *AstA-Gal4* lines. AstA-LacZ overlap: *Gal4-UAS-LacZ* expressing cells are AstA-immunpositive, AstA-LacZ false positives: *Gal4-UAS-LacZ* expressing cells are not AstA-immunopositive, 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'-GCGCAATTGATGGCTATTTCCCAGCTCCT-3'
5'-GCCGGATCCAGAGGTTCCGCGGACTAAAT-3'
pAstA2:
5'-GCGCAATTGAGTAGAAGCTGCGCCAGAAG-3'
5'-GCCGGATCCAGAGGTTCCGCGGACTAAAT-3'
pAstA3:
5'-GCGCAATTGGGGAAAAATCTCCGAAAACC-3'
5'-GCCGGATCCAGAGGTTCCGCGGACTAAAT-3'



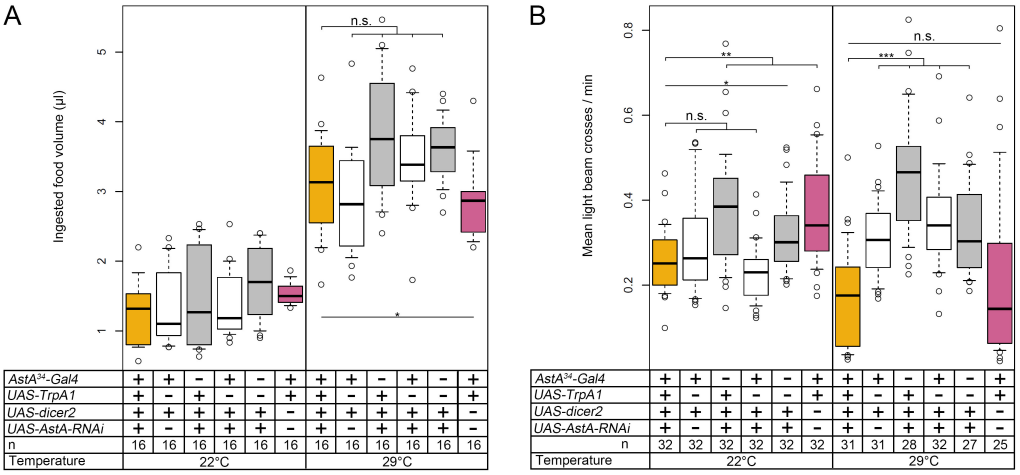
S2 Fig. Schematic summary of the expression pattern of the two *AstA-Gal4* drivers used, in conjunction with *tsh-Gal80*. AstA neurons in the posterior lateral protocerebrum (PLP cells) are in red, the dorsolateral abdominal AstA neurons (DLAa) in the thoracico-abdominal ganglion and the peripheral neurons (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.



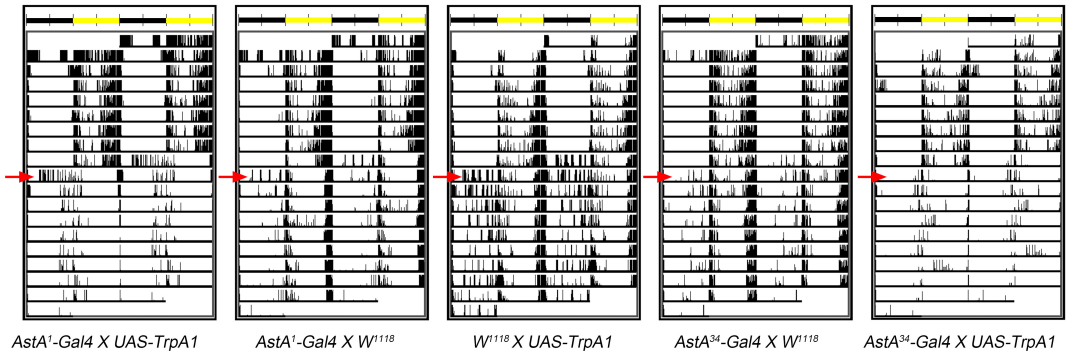
S3 Fig. Thermogenetic activation of the AstA cells of female adults resulted in reduced food intake. (A) *AstA*^{1}>*TrpA1*. (B) *AstA*^{34}>*TrpA1* and respective controls. $*$ p $\leq$ 0.05. $**$ p $\leq$ 0.01, $***$ p $\leq$ 0.001.



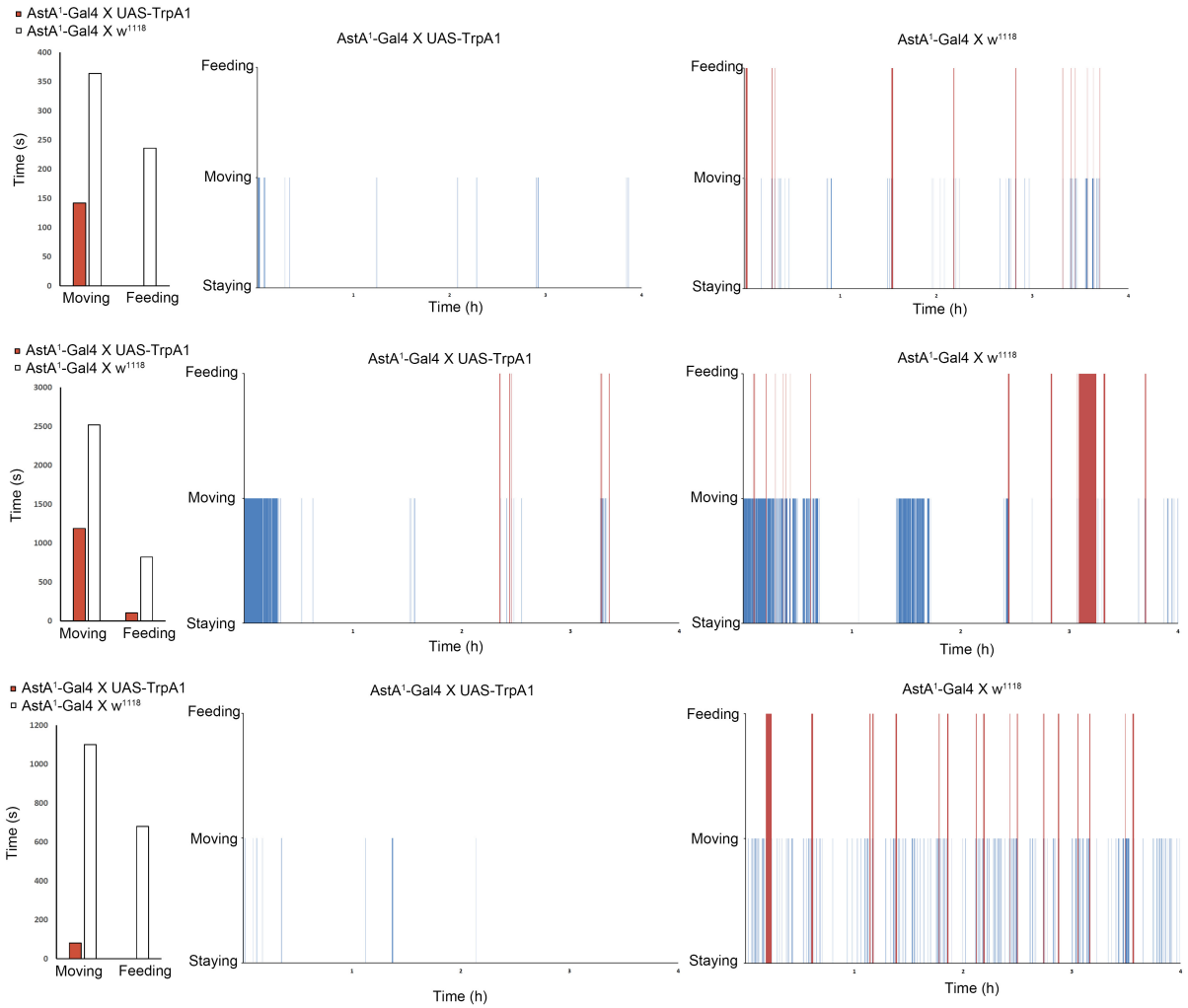
S4 Fig. Conditional silencing of AstA^{34} cells by temperature-dependent expression of Kir2.1 did not alter food consumption: *tubGal80ts;AstA^{34}>Kir2.1* flies with silenced AstA cells at 29°C and with normally active AstA cells at 18°C consumed the same amount of food than the controls.



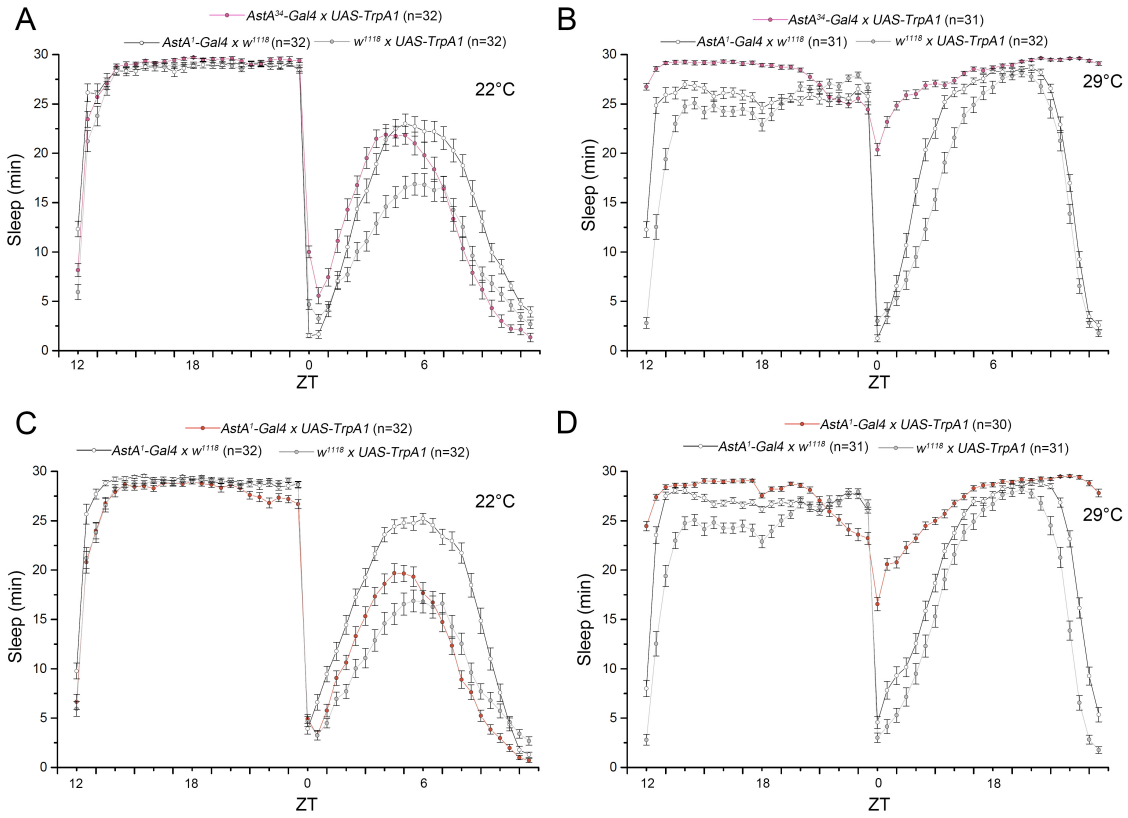
S5 Fig. AstA RNA-interference rescued reduced food intake but not locomotor activity in flies with thermogenteically activated AstA cells. At 22°C, food intake (A) and locomotor activity (B) of *AstA*^{34}*>TrpA1* flies did not show significant differences to controls. At 29°C, thermogenetic activation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



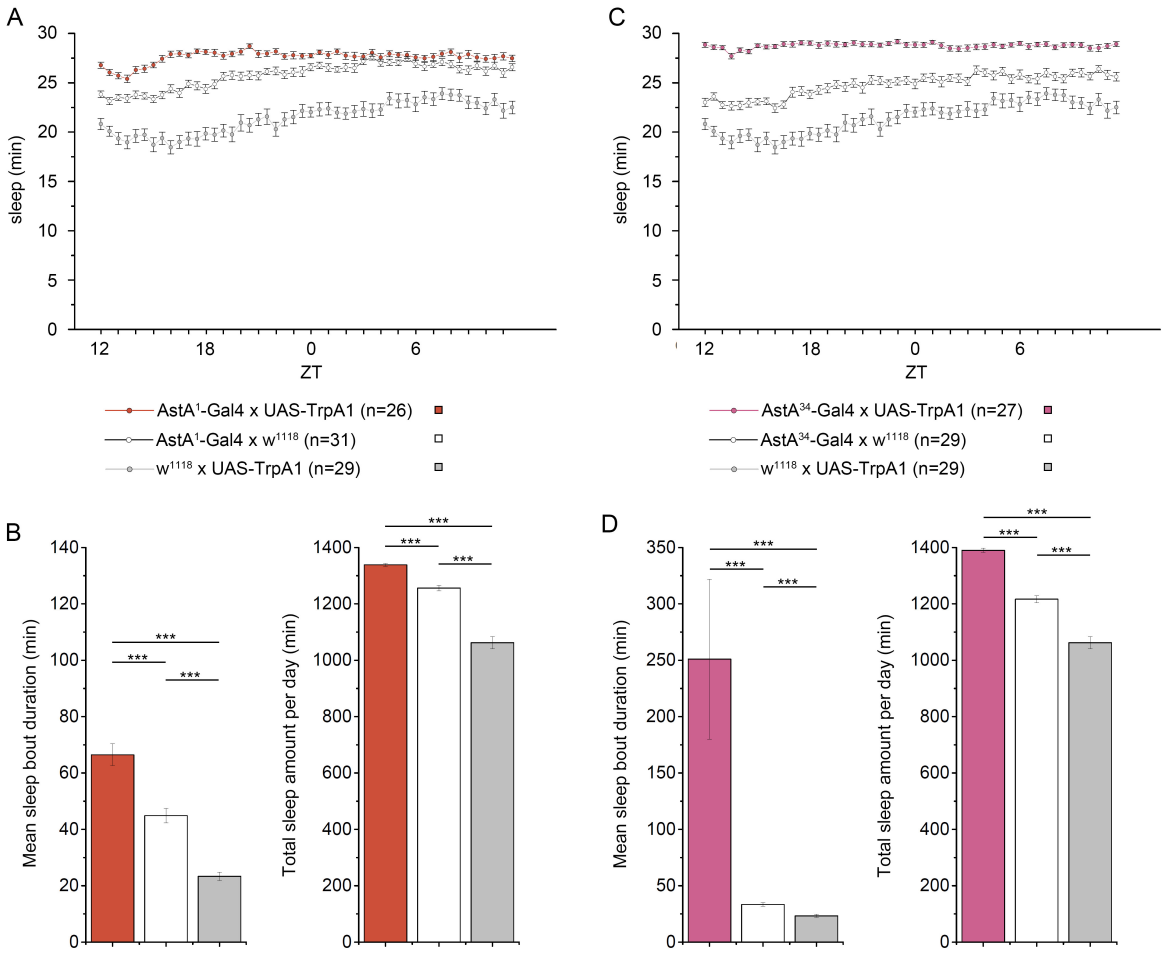
S6 Fig. Thermogenetic activation of AstA cells resulted in a strongly inhibited locomotion. Examples of double-plotted single fly actogramms underlying the results shown in Fig 4. Flies were initially kept at 22°C, then temperature was raised to 29°C at the time point indicated by a red arrow.



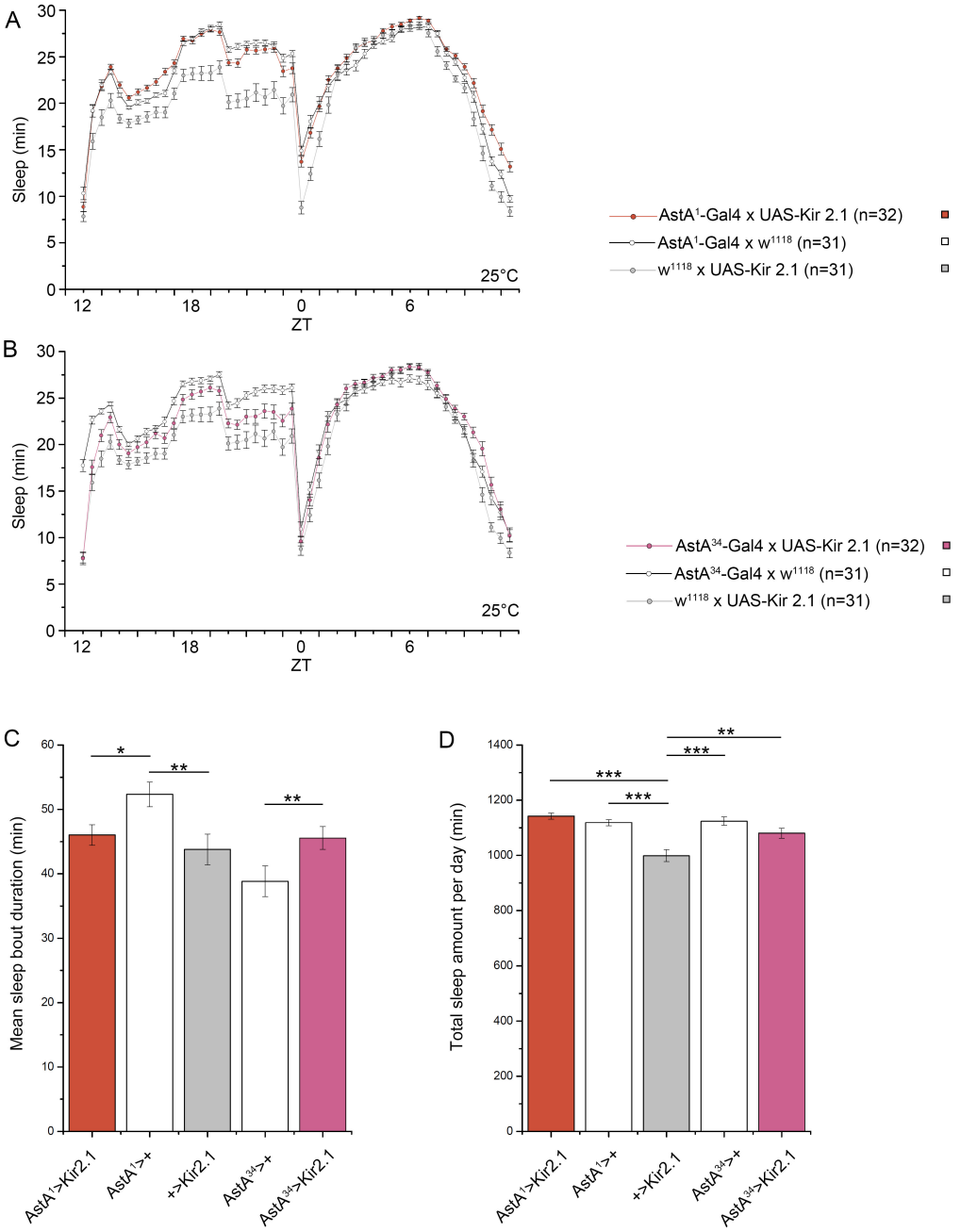
S7 Fig. Thermogenetic activation of AstA^1 cells reduced food intake and locomotion in the CAFE assay. Individual flies were filmed for 4 hours in a modified CAFE assay (three flies per genotype). Behaviour was categorized as "not moving", "moving" and "feeding". *AstA^1>TrpA1* flies with activated AstA^1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



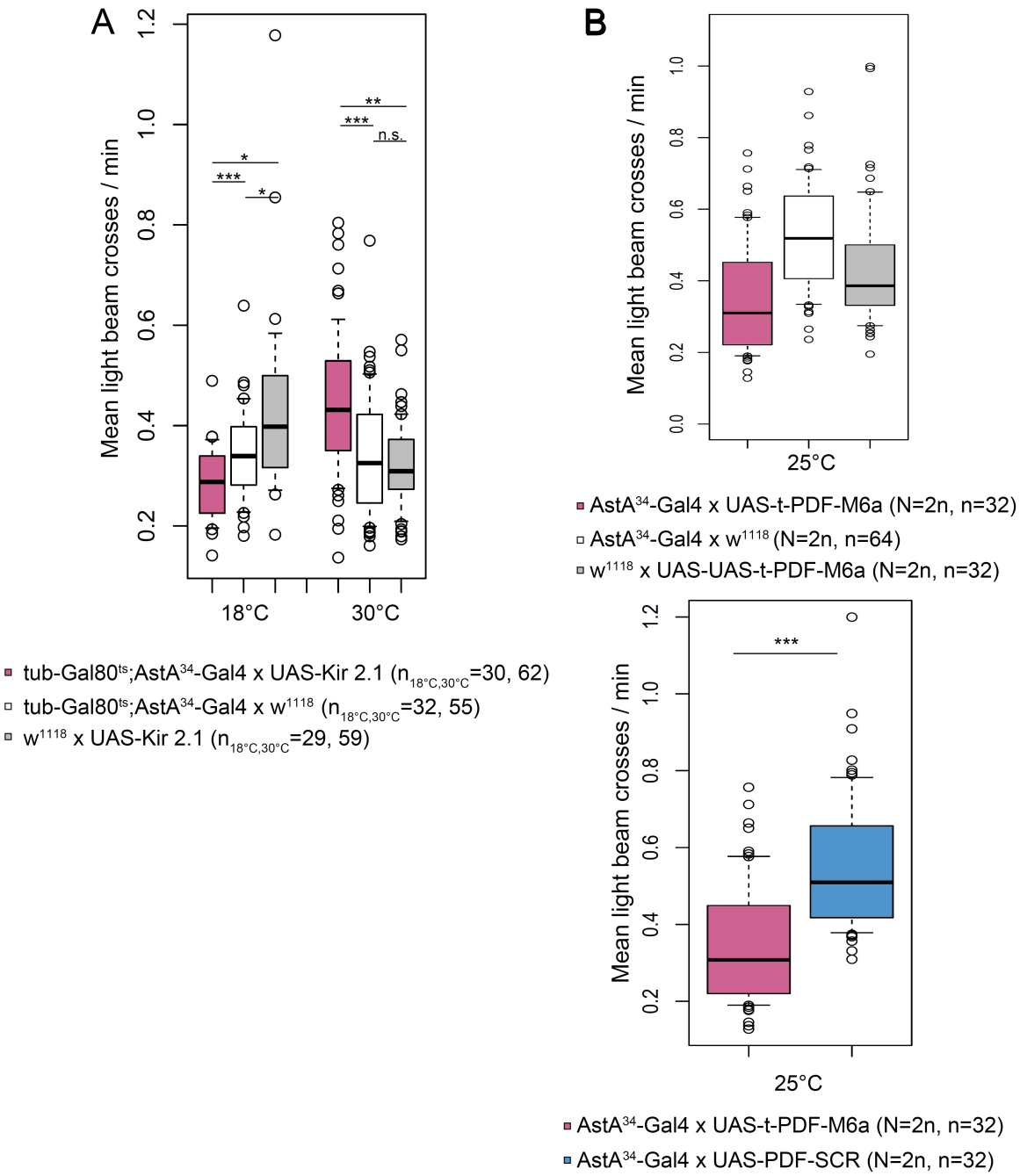
S8 Fig. Thermogenetic activation of AstA cells strongly promoted sleep also in female flies. At 20°C, *AstA*^{34}>*TrpA1* (top left) and *AstA*^{1}>*TrpA1* females (bottom left) did not sleep more than controls. Activation of the TrpA1 channel by 29°C resulted in increased sleep time of *AstA*^{34}>*TrpA1*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



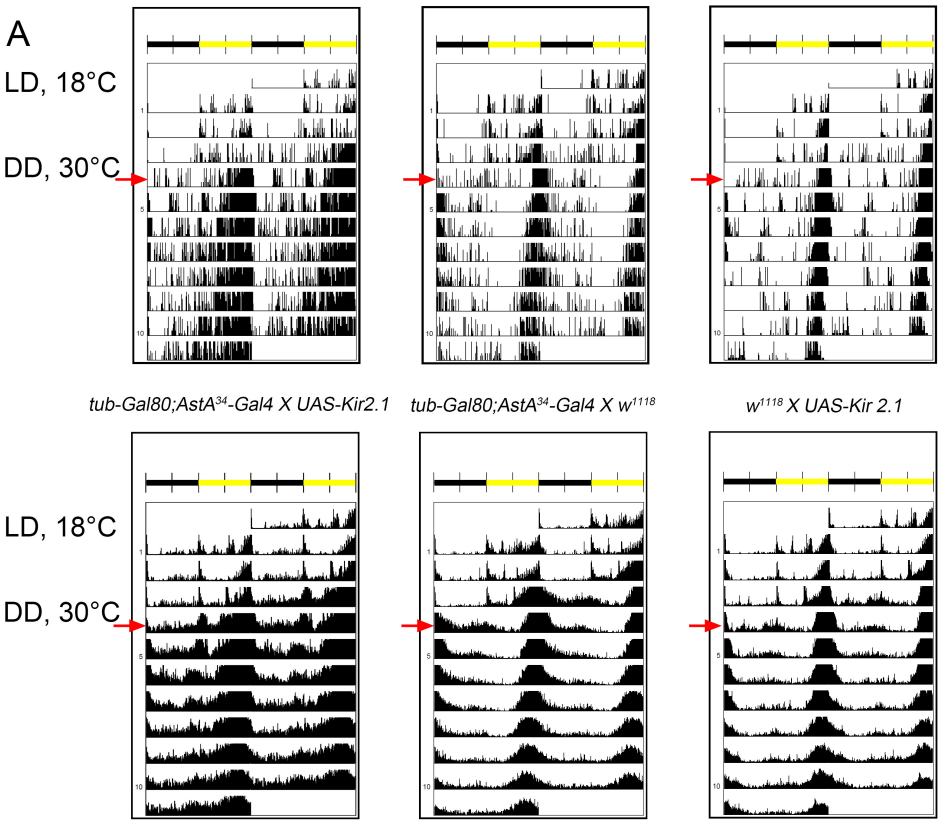
S9 Fig. Thermogenetic activation of AstA^{1} cells (A-B) or AstA^{34} cells (C-D) increased sleep under LL conditions known to impair the clock and to induce arrhythmicity.



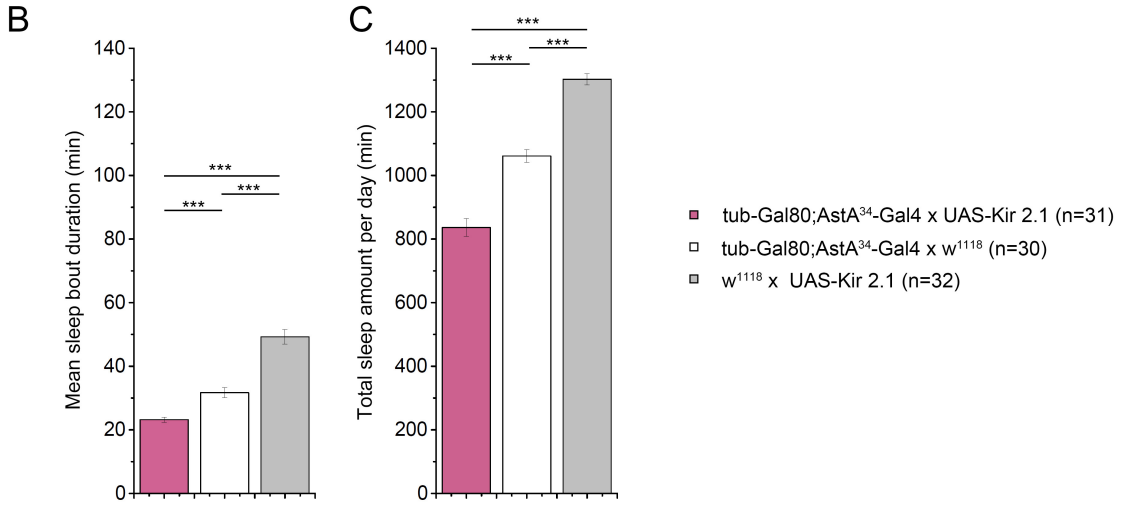
S10 Fig. Constitutive silencing of AstA cells by ectopic expression of Kir2.1 did not alter sleep behaviour in *AstA^{34}>Kir2.1* (A) and *AstA^{1}>Kir2.1* (B) flies. The mean sleep bout duration (C) and the total amount of sleep per day (D) of *AstA^{34}>Kir2.1* and *AstA^{1}>Kir2.1* is not significantly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



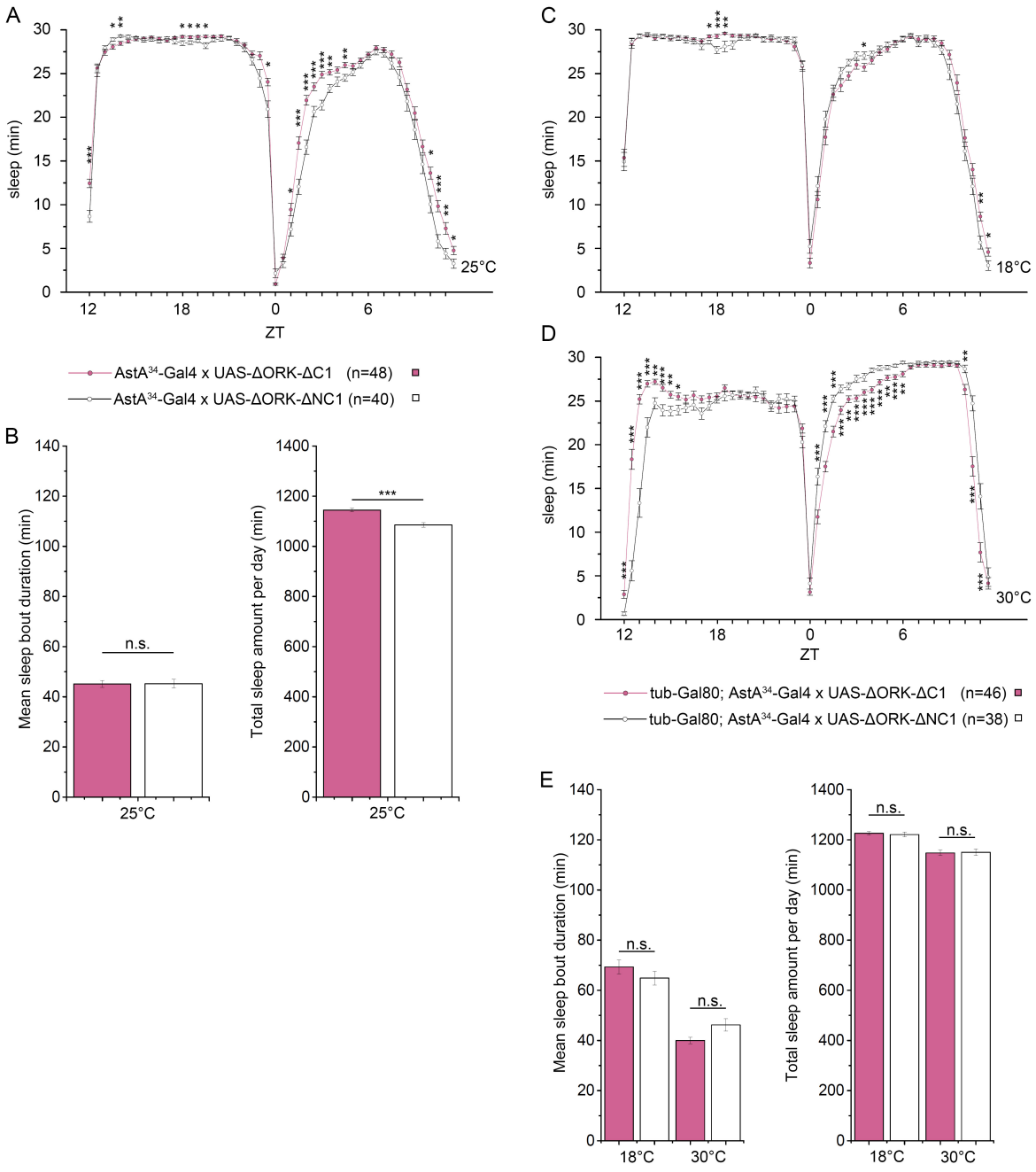
S11 Fig. Conditional silencing of AstA^{34} cells increases the mean locomotor activity. B) Ectopic expression of t-PDF in AstA^{34} cells decreases the mean locomotor activity.



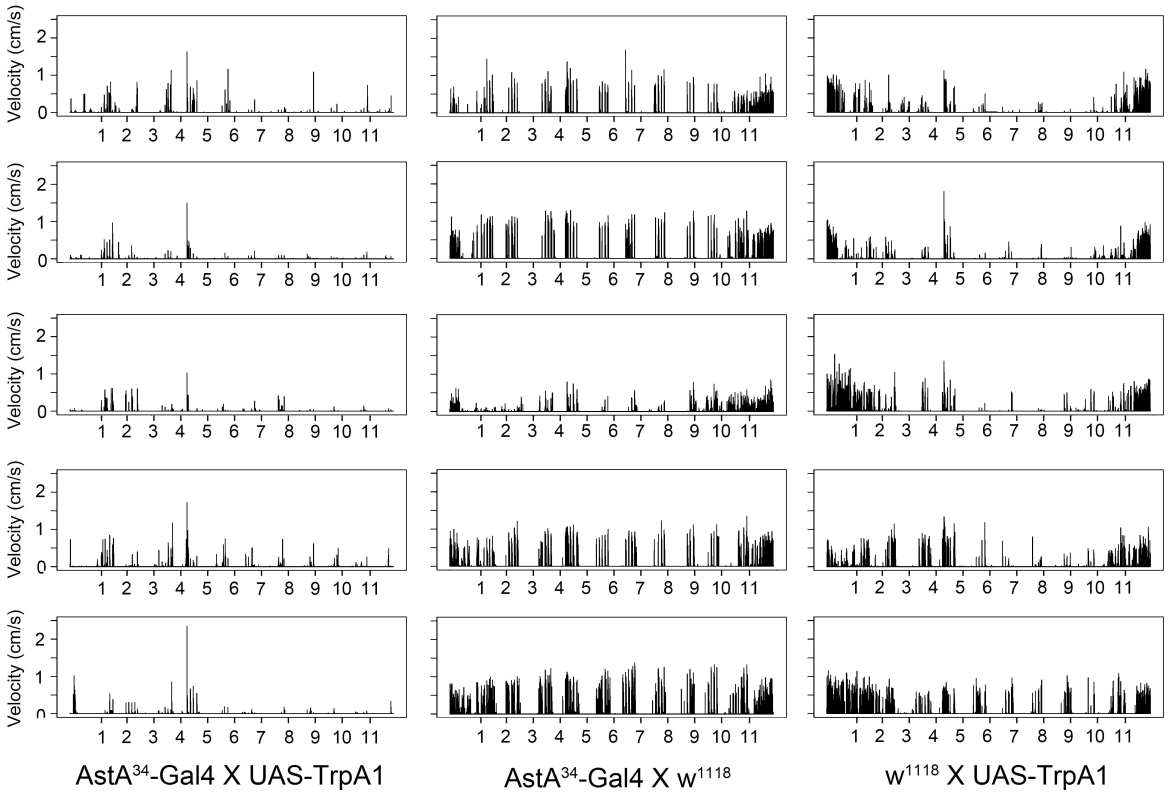
	n	Rhythmicity	Period (h)	SEM	Power	SEM
tub-Gal80;AstA^{34}-Gal4 x UAS-Kir 2.1	31	100%	23.78	0.09	243.62	25.54
tub-Gal80;AstA^{34}-Gal4 x w^{1118}	30	100%	23.54	0.06	368.81	42.35
w^{1118} x UAS-Kir 2.1	32	100%	24.02	0.07	319.96	34.67



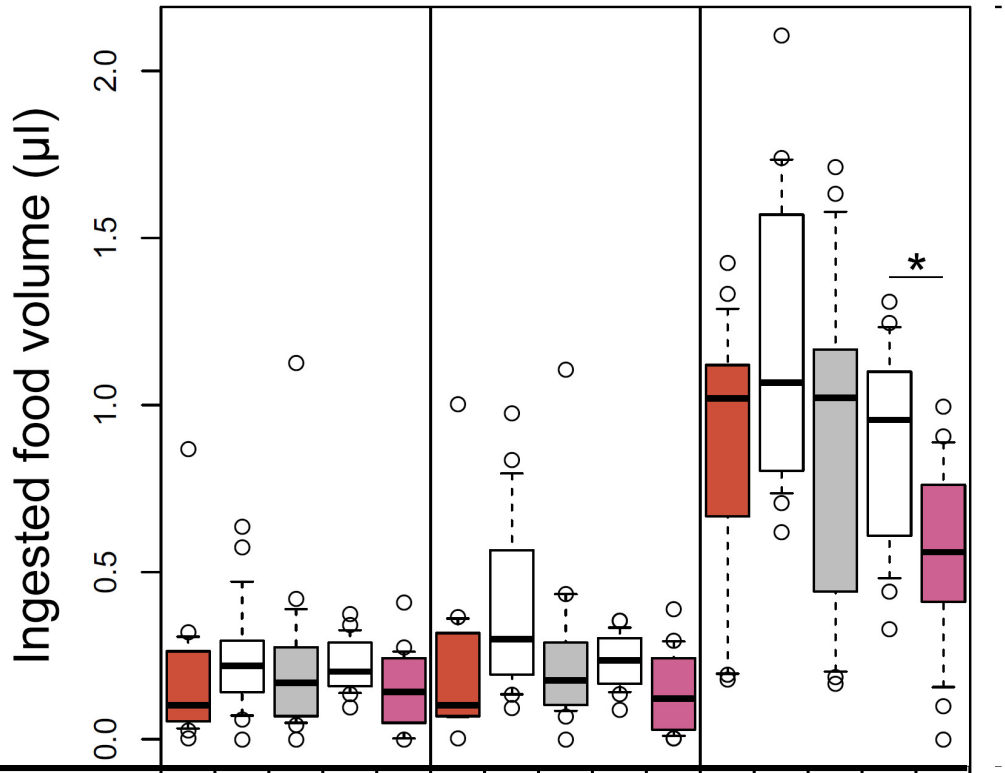
S12 Fig. Conditional silencing of AstA^{34} cells by *UAS-Kir2.1* decreased sleep in constant darkness (DD). (A) Actogramms of single flies show that locomotor activity increases during the subjective day and night upon silencing of AstA^{34} cells. Rhythmicity and period is not affected. Both the duration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



S13 Fig. The effect on sleep of constitutive (A-B) and conditional (C-E) silencing of AstA^{34} 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.

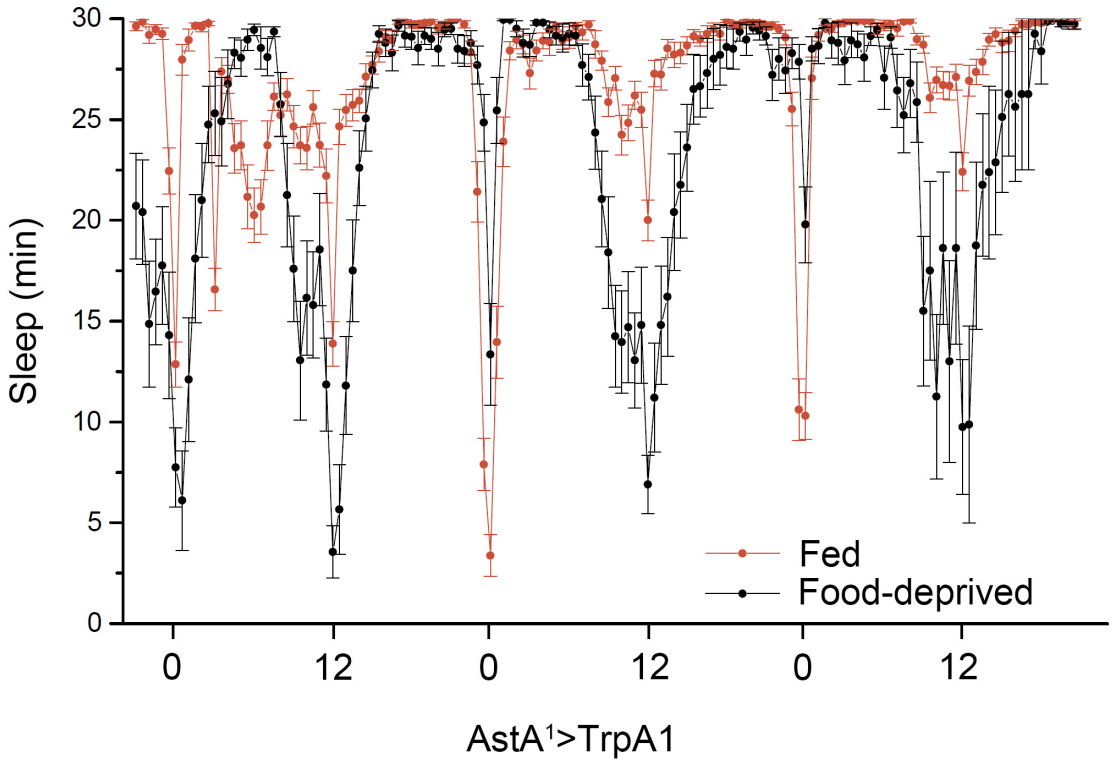


S14 Fig. Velocity of flies after mechanical arousal by a loudspeaker at 29°C (compare to Fig 7 which shows the average velocity of several flies). While *AstA*^{34}*>TrpA1* flies walked less (leading to a reduced average velocity), the maximum speeds when moving where not different to control flies,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

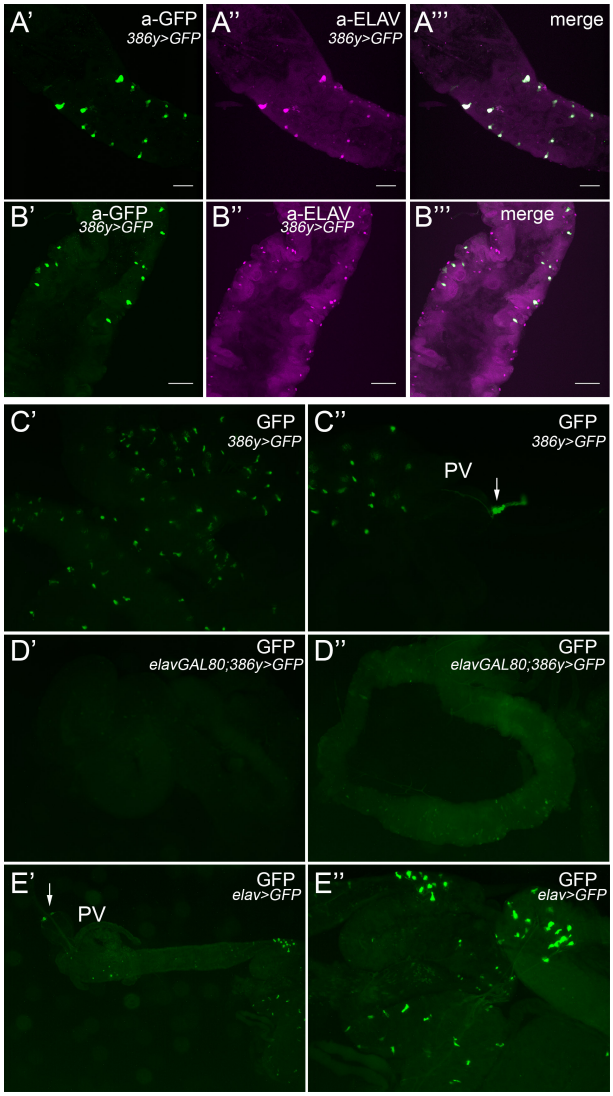


AstA^{1}-Gal4	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-
UAS-TrpA1	+	-	+	-	+	+	-	+	-	+	+	-	+	-	+
AstA^{34}-Gal4	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+
n	15	15	17	15	15	15	15	17	15	15	15	15	17	15	15
Time	0-3 h					0-6 h					0-24 h				

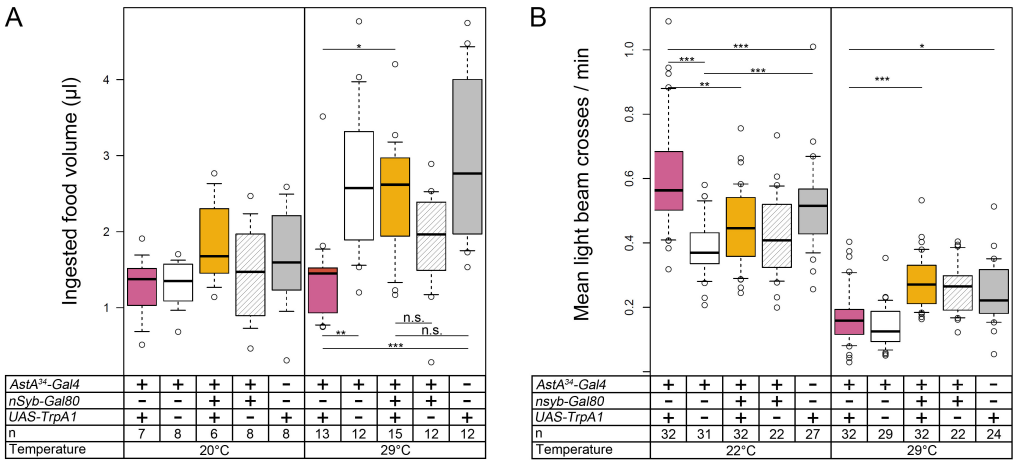
S15 Fig. Lack of a feeding rebound after releasing the activation of AstA cells. *AstA^{34}>TrpA1* and *AstA^{1}>TrpA1* flies were kept for 1 day at 22°C, then 2 days at 29°C in the CAFE assay. Afterwards, flies were put back again to 22°C, and food consumption was summed up for the first 3, 6 and 24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



S16 Fig. Starvation-induced locomotor hyperactivity in flies with thermogenetically activated AstA^1 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).



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.



S18 Fig. *nsyb-Gal80* rescued reduced food intake and locomotor activity. Thermogenetic activation of AstA^{34} 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^{34}>TrpA1* were not significantly different to controls, but significantly higher than *AstA^{34}>TrpA1* at 29°C. $*$ p $\leq$ 0.05. $**$ p $\leq$ 0.01, $***$ p $\leq$ 0.001.

S1 Movie. 3D rotation of the PLP neuron arborisations in the dorsal protocerebrum, anti-GFP staining in an adult $AstA^{34}>GFP$ fly.

S2 Movie. 3D rotation of AstA neuron immunoreactivity in the dorsal protocerebrum, same brain as in S1 Movie.

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

Buffers, Media and Substances

Haemolymph-like solution (HL3.1)	NaCl 128 mM, KCl 2 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 4 mM, sucrose 36 mM, HEPES 5 mM, pH 7.1
Minimal medium	1.7 g yeast nitrogen base without amino acids and ammonium sulfate (e.g.BD Difco 233520), 20 g sucrose (analysis grade, nitrogen-free), 1 g ^{15}N-labelled ammoniumsulfate or 1 g normal ammoniumsulfate, 1 l Aqua bidest
Phosphate-buffered saline (PBS)	8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 1 l Aqua bidest, pH 7.4
Yeast-Sugar solution	3.4 g yeast (labelled or unlabelled), 0.9 g sucrose, 7 ml water

Abbreviations

Abd	abdominal neuromere
Akh	adipokinetic hormone
AL	antennal lobe
aMe	accessory medulla
AstA	allatostatin A
AstB	allatostatin B or MIPs
BPB	bromophenol blue sodium salt
BR	brain
CA	corpora allata
CAFE	capillary feeder
Cas9	CRISPR associated protein 9
CB	central brain
CC	corpora cardiaca
CCK	cholecystokinin
CNS	central nervous system
CRISPR	clustered regularly interspaced short palindromic repeats
cv-c	*crossveinless-c*
DAM	*Drosophila* activity monitors
Dar-1	*Drosophila* allatostatin receptor 1
Dar-2	*Drosophila* allatostatin receptor 2
DCVs	dense-core vesicles
DD	constant darkness
DNs	dorsal neurons
dFB	dorsal fan shaped body
DFM	*Drosophila* feeding monitor
DH31	diuretic hormone 31
DH44	diuretic hormone 44
DILPs	*Drosophila* insulin-like peptides
Dop1R2	dopamine 1-like receptor 2

EECs	enteroendocrine cells
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
FB	fan shaped body
FLIC	fly liquid-food interaction counter
GFP	green fluorescent protein
GIRK	G-protein-gated inwardly rectifying potassium channels
GNG	gnathal ganglia
GPCRs	G-protein coupled receptors
gRNA	guide RNA
HL	haemolymph-like solution
HPLC	high performance liquid chromatography
IAM	interior anterior median
ICLI	inferior contralateral interneuron
ICV	Intracerebroventricular
IL	ileum
ITP	ion transport peptide
LNs	lateral neurons
IR	immunoreactivity
ITP	Ion transport peptide
JH	juvenile hormone
LC	liquid chromatography
LCBR	lateral cell body rind
LD 12:12	12:12 hours light-dark cycle
LMIo	lateral MIP-immunoreactive optic lobe
LN_ds	dorsolateral neurons
LNs	lateral neurons
LNs	local interneurons
LN_vs	ventrolateral neurons
LPNs	lateral posterior neurons
MB	mushroom body

MG	midgut
MIP	myoinhibitory peptide
MN	mesothoracic neuromere
MS	mass spectrometry
NPF	neuropeptide F
NPFR	neuropeptide F receptor
NPY	neuropeptide Y
OE	esophagus
OL	optic lobe
ORNs	Odorant receptor neurons
PAL	peptidyl α-hydroxyglycine-α-amidating lyase
PBS	phosphate-buffered saline
PDF	pigment dispersing factor
PER	proboscis extension reflex
PFA	paraformaldehyde
PHM	peptidylglycine-α-hydroxylating mono-oxygenase
PI	pars intercerebralis
PLP	posterior lateral protocerebrum
PN	prothoracic neuromere
PV	pyloric valve
R	rectum
rER	rough endoplasmic reticulum
ROIs	regions of interest
RV	rectal valve
SAM	superior anterior median
SIFa	SIFamide
SIFR	SIFamide receptor
SIP	superior intermediate protocerebrum
SK	sulfakinin
SLP	superior lateral protocerebrum
SMP	superior medial protocerebrum

sNPF	short neuropeptide F
sNPFR	short neuropeptide F receptor
SOG	suboesophageal ganglion
SP	signal peptide
SPL	superior posterior lateral
SPR	sex peptide receptor
TAG	thoracico-abdominal ganglion
TFA	trifluoroacetic acid
TTX	tetrodotoxin
ZT	zeitgeber time

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Curriculum Vitae

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Affidavit

I hereby confirm that my thesis entitled is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.

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