Neuropeptides in modulation of *Drosophila* behavior: how to get a grip on their pleiotropic actions

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Short title: Pleiotropic *Drosophila* peptides

Abstract

Neuropeptides constitute a large and diverse class of signaling molecules that are produced by many types of neurons, neurosecretory cells, endocrines and other cells. Many neuropeptides display pleiotropic actions either as neuromodulators, co-transmitters or circulating hormones, while some play these roles concurrently. Here, we highlight pleiotropic functions of neuropeptides and different levels of neuropeptide signaling in the brain, from context-dependent orchestrating signaling by higher order neurons, to local executive modulation in specific circuits. Additionally, orchestrating neurons receive peptidergic signals from neurons conveying organismal internal state cues and relay these to executive circuits. We exemplify these levels of signaling with four neuropeptides, SIFamide, short neuropeptide F, allatostatin-A and leucokinin, each with a specific expression pattern and level of complexity in signaling.

Key words: Neuropeptides; peptide hormones; neuromodulation; behavior orchestration

Highlights:

- Many neuropeptides display pleiotropic functions
- Neuropeptide actions can be circuit-specific or orchestrating across the organism
- Pleiotropic neuropeptide actions can occur spatially or temporally

Introduction

Recent studies of *Drosophila* neuropeptide signaling often highlight roles of small subpopulations of peptidergic neurons in modulation of specific aspects of behavior or physiology. In extreme cases, one or two pairs of neurons are described with significant impacts on a behavior, such as for instance four SIFamide producing neurons that orchestrate appetitive behavior [1], and one pair of neurons expressing leucokinin (LK) that are required for metabolic regulation of sleep [2]. This is intriguing since it suggests specific roles of single types of neurons utilizing one neuropeptide. However, most neuropeptides are produced by larger and more diverse populations of cells, and are therefore likely to contribute to more diverse and complex modulatory output [3]; one such example is short neuropeptide F (sNPF) (see [3-6]). This causes problems when analyzing neuropeptide and neuropeptide receptor mutants or activating/inactivating a peptidergic neuron population using promoter-Gal4 lines. The likely outcome of such analysis is a compound behavioral phenotype that is the sum of outputs of the individual neurons in different circuits. Although this might be relevant when the set of peptidergic neurons act together to orchestrate specific behaviors, it can confound analysis if the neurons have divergent roles and the neuropeptide acts as a local neuromodulator or co-transmitter with distributed circuit-specific functions. Another layer of complexity is added when a neuropeptide additionally acts systemically as a hormone released by neurosecretory cells or by enteroendocrine cells (EECs) of the intestine. In the latter case the peptide may target intestinal cells, the central nervous system (CNS), and other tissues to ensure nutrient-dependent inter-organ

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communication [7-9]. Furthermore, a neuropeptide can play very different roles depending on stage in life cycle.

In this review, we focus on the different layers of neuropeptide signaling in (1) orchestrating physiology and behavior, and in (2) diverse distributed functions, where subsets of peptidergic neurons have distinct local actions. We have selected four neuropeptides that represent distinct distribution patterns, and which illustrate different layers of neuromodulation: SIFamide (SIFa), short neuropeptide F (sNPF), allatostatin-A (Ast-A), and leucokinin (LK).

Hierarchical organization of peptidergic neuronal systems

Peptidergic neurons rarely act independently, but rather integrate modulatory effects of other peptidergic systems. However, this integration is not balanced. In the one extreme, a specific neuropeptide is expressed in numerous locally restricted neurons, where it acts circuit-specifically and performs executive modulation (e. g. sensitization) at specific synapses. Other neuropeptides mediate a context-specific influence (e. g. satiety or hunger) in several locations, sometimes recruiting executive modulation within individual compartments (Fig. 1). Context-dependent neuropeptide signaling can also be integrated into orchestrating peptidergic systems that act on yet another spatio-temporal level. Such systems commonly constitute few, widely arborizing neurons that convey basal states (e. g. metabolic status or arousal) across many circuits. These global states feed back into the more dynamic context-specific systems, but can also set thresholds at the executive modulatory level. Most neuropeptides in *Drosophila* are likely to act on multiple targets since they are expressed in more than one major cell type such as CNS interneurons of different types, neurosecretory cells, intestinal EECs, peripheral sensory neurons, or even in fat body cells (reviewed in [3,6]) (Fig. 2; Supplemental Table 1).

Four types of peptidergic neuron systems that illustrate pleiotropic actions and layers of neuromodulation

SIFamide. In *Drosophila,* SIFamide is produced by a unique set of four neurons

with cell bodies in the pars intercerebralis (PI) and wide arborizations in most regions of the brain and thoracico-abdominal ganglia (TAG) [10]. Downregulation of SIFamide signaling in male flies results in increased mating attempts, also with other males, and in females it increases the responsiveness to males [10]. Activation of SIFamide signaling inhibits reproductive behavior by action on *fruitless* expressing neurons that are required for sex-specific behavior [11]. More recently, Martelli et al. [1] showed that the SIFamide neurons (SIFaN) coordinate and weigh satiety (anorexigenic) signals generated by neurons producing myoinhibitory peptide [12] and hunger (orexigenic) signals from Hugin-pyrokinin neurons to regulate appetitive behavior (Fig. 3a). The output of the SIFaN in hungry flies acts at several levels: it enhances odor responses of olfactory projection neurons in the antennal lobe, increases sugar responses in gustatory neurons, and increases acute food intake [1] (Fig. 3a,b). The SIFaN may additionally be regulated by brain neurons producing sNPF, corazonin, insulin-like peptides and sulfakinin, based on proximity of their neuronal processes [1] (Fig. 3a). These are peptides known to affect feeding and metabolism [13-16]. The SIFaN also promote sleep; ablation of these neurons shortened baseline sleep and sleep-bout length [17]. Interestingly, SIFaN execute the sleep-promoting function via SIFamide receptor-expressing neurons in the PI [17], possibly the diuretic hormone 44-expressing neurons [18]. Thus, the four SIFaN are central in integrating and relaying multiple peptidergic signals that coordinate the internal nutritional state with sensory inputs to modulate systems regulating food search, feeding, reproductive behavior and sleep [1,11,17,18].

Short neuropeptide F. sNPF is a multifunctional neuropeptide, mainly involved in short range signaling. It is produced by several hundred neurons in the CNS, and in over thousand Kenyon cells of the mushroom bodies (MB) [19,20]. Expression of sNPF is in subsets of chemosensory cells of the antennae and maxillary palps, in two sets of lateral neurosecretory cells (LNCs) and many types of small interneurons in the visual system, central complex, clock system and other brain regions [19,21,22]. In many neurons, sNPF is colocalized with classical

neurotransmitters, or with other neuropeptides, and acts as a co-transmitter [5,6,23-25].

In the LNCs, sNPF acts locally within the brain to regulate activity in insulin-producing cells (IPCs), and probably not as a circulating hormone [21]. In the MBs, sNPF presynaptically sensitizes Kenyon cells and acts as a co-transmitter of acetylcholine in odor-associated memory formation [24,26], and in the antennal lobe glomerulus DM1 to presynaptically sensitize cholinergic olfactory sensory neurons (OSNs) [23,27] (Fig. 3b). The *Drosophila* clock system employs sNPF as a co-transmitter in the s-LN_v neurons [22]. The s-LN_vs release sNPF, together with pigment-dispersing factor (PDF), to sequentially inhibit other clock neurons and thereby provide a delay in the network that ensures phase-setting of the neuronal activity (entrainment) [25]. Furthermore, specific sNPF interneurons of the central complex modulate aspects of explorative walking [28]. Finally, it has been shown that sNPF regulates sleep [29,30], modulates taste receptors [31], nociception in larvae [32], and induces food ingestion in larvae and adults [4,14,33].

It is likely that most of these functions of sNPF are regionalized and independent, although some of the sNPF signaling is associated with increasing appetitive behavior, food search and food intake. The local sNPF action in the olfactory system to strengthen attractive food odor responses is under influence of nutritional state (hunger) and insulin signaling, which regulates sNPF receptor expression in specific OSNs [23] (Fig. 3b). This local executive sNPF neuromodulation is hence recruited by a higher-level regulatory system (IPCs) that is state dependent (Fig. 1, 3b). Possibly, sNPF signaling in MBs and other neuronal compartments is likewise regulated by nutritional state and insulin, which thereby coordinate some of the sNPF functions.

Allatostatin-A. Despite the name, the four Ast-A peptides have no allatostatic role in *Drosophila*, but act as neuromodulators and hormones in regulation of food search, sugar reward, feeding, metabolism and sleep [33-38]. A diverse set of cells express the Ast-A peptides. There are about 22 Ast-A interneurons in the

midbrain, and a large number in the optic lobes [39,40]. In the TAG, there are approximately 14 pairs of Ast-A neurons, three of which innervate the hindgut; four neuroendocrine cells are associated with dorsolateral TAG nerve roots [35,39]. Furthermore, Ast-A peptides are produced by EECs in the midgut.

Constitutive activation or inhibition of *all* Ast-A neurons inhibit food intake and responsiveness to sugar, but have no effect on metabolism or energy stores [37]. Interestingly, Ast-A mediates satiation via MB circuits [33,38]. Ast-A neurons inhibit specific dopaminergic input neurons, which in turn inhibit specific MB output neurons, resulting in suppressed food search behavior [33]. Two sets of Ast-A expressing cells, the brain PLP neurons and gut EECs, act together to reduce feeding and promote sleep [35]. The brain neurons are in turn targets of PDF-expressing clock neurons and potentially the EECs are autonomous nutrient sensors. The PLP neurons probably use Ast-A to target several types of neurons in the posterior superior protocerebrum that regulate sleep [34], and also interact with IPCs and adipokinetic hormone producing cells to mediate satiety [35,36]. Furthermore, Ast-A from EECs may act directly on the intestine to modulate peristalsis, or ion and nutrient transport. Thus, the EECs and the clock-controlled PLP neurons use Ast-A to ensure an energy-saving sleep state with low digestive activity and metabolism [35].

Leucokinin. In *Drosophila,* LK induces secretion in renal tubules *in vitro,* as well as regulates clock output, metabolism-related sleep, acute feeding, water balance, and plays roles in modulating tolerance to desiccation and ionic stress [2,41-46]. There are two pairs of LK producing brain neurons, LHLK and SELK, and 11 pairs of neurosecretory cells, ABLK, in the TAG (Fig. 4). A few recent studies have sought to determine the functions of each of these three groups of cells [2,45,47].

The ABLKs, but not the other LK neurons, colocalize diuretic hormone 44 (DH44) and it was shown that knockdown of each peptide specifically in these neurons affect responses to desiccation, starvation and ionic stress [47]. However, only LK-knockdown affected water content in flies, whereas only

DH44-RNAi influenced food intake. Thus, the same set of neurons can release two peptide hormones that have some common targets (renal tubules), but each may also act on separate ones [47]. Presumably the roles of the two peptides of the ABLKs are to coordinate functions of several targets in response to a physiological trigger.

While the ABLKs, but not brain neurons, are associated with regulation of water balance and responses to desiccation [45], the question is what are the roles of LHLKs and SELKs. The LHLKs are associated with the effects of LK signaling in regulation of locomotor activity, sleep and metabolism [2], whereas the subesophageal SELKs may be responsible for modulation of acute food ingestion (see [41,45]). Some of the effects of LK signaling are indirect via action on the brain IPCs, which express the LK receptor, LKR [2,45]. The LHLKs are essential for starvation-mediated changes in sleep and it was shown that LKR expression in IPCs is required for mediating this phenotype [2]. In summary, the three types of LK neurons appear to each perform distinct functions, but together they may link metabolic state to regulation of water balance, activity and sleep (Fig. 4).

Conclusions and future perspectives

Our examples above support that peptidergic signaling displays several layers of complexity and therefore analysis of mutants alone is not sufficient to uncover the functional roles of neuropeptides. Furthermore, the analysis of single pairs, or small groups of peptidergic neurons probably reveals only a portion of the total function of a peptide. Thus, to uncover all functional aspects of a given neuropeptide one must examine all the different neuron types producing it and assess whether they operate in orchestrating or distributed local functions.

Despite functional pleiotropy, it is remarkable that in many cases certain functions of neuropeptides are conserved over evolution. For example Ast-A and its vertebrate ortholog galanin regulate feeding, sleep and hormone release [34,35,37,48].

A major challenge is to generate a peptidergic connectome in *Drosophila* since neuropeptides are known to also act through extrasynaptic (paracrine) signaling (see [5,49]). Therefore, there may be peptidergic targets that are not in synaptic contact with the "sender neurons". A recent technique, *trans*-Tango, has been devised to enable light microscopical connectomics analysis [50]. So far this technique has not been successfully used for exclusively peptidergic neurons, and it seems less likely that paracrine partners can be revealed with the present version of *trans*-Tango, unless they are also connected by conventional synapses.

Another challenge in insect neuropeptide studies is the lack of data on the cellular distribution of neuropeptide receptors. With a few exceptions, we have no information on the location of receptor protein. Fluorophore-labeled neuropeptides seem promising to identify receptor-binding sites that are accessible to neurohormones [51]. Using Gal4-driven GFP does not reveal the subcellular localization of the receptor, and in most cases there is no independent evidence that the GFP-labeled neurons produce the receptor protein. Single cell transcriptome databases can contribute to identify candidate receptor-expressing neurons [52], and targeted receptor-RNAi followed by specific functional assays can be performed to support expression. As an example, RNAi for LK- and sNPF-receptors targeted to IPCs resulted in physiological phenotypes indicative of regulation of these cells by the corresponding neuropeptides [2,13,45]. Single cell transcriptomics can also be utilized to generate predictions of testable neuromodulatory networks [53]. Recent techniques that recruit redesigned GPCRs that specifically label or modify a target cell, like Tango and *trans*-Tango [50,54], CRISPR ChaCha [55], and $GRAB_{DA}$ [56], or visualize receptor activation dynamics [57] could provide promising platforms to target modulatory signaling within neuronal sub-circuits, to come closer to an integrative physiological understanding of the role of neuropeptides at the organismal level.

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Figure legends

Fig. 1. Distributed and global actions of neuropeptides.

The four neuropeptides sNPF (grey), SIFa (yellow), Ast-A (blue) and LK (orange) act in both independent and overlapping compartments, despite their differing cell number (compartments: MB, mushroom body; OL, optic lobe; AL, antennal lobe; CX, central complex; PLP/LH, posterolateral protocerebrum/lateral horn; SEZ/TAG, subesophageal zone/thoracico-abdominal ganglia). While sNPF seems to operate autonomously in each local circuit, the activity of each of the other neuropeptides can be synchronized at an organismal level across sites of action. Thus, a neuropeptide functions on various levels in the organism, either to perform an executive role in a local circuit or to mediate different contexts (i.e. thirst, hunger, sex drive, circadian rhythm). In some cases, context-mediating neuropeptides may recruit other neuropeptides.

Fig. 2. Peptide distribution patterns in *Drosophila*.

Venn diagram displaying patterns of distribution of neuropeptides in hormonal systems, interneurons and the intestine. Note that many peptides are part of all three systems, some in two, and relatively few are only hormonal or interneuronal. There appear to be no neuropeptides unique to the intestine. The peptides indicated in blue are also produced by efferent CNS neurons that innervate the intestine. Note that AKH, CRZ, DILP and DSK-expressing neurons supply axon terminations on the anterior intestine, but these are likely neurohemal areas. Peptides in brackets have not been demonstrated in adult structures. The peptide acronyms are available in Supplemental material Table 1. Note that some *Drosophila* peptides are missing in this figure due to lack of clear information.

Fig. 3. Peptide signaling acts at different levels.

(**a**) Some neuropeptides, like SIFamide, are produced by few neurons with arborizations widely spread throughout the brain. These four neurons are known to coordinate appetitive behavior, but also influence mating and sleep. The SIFa

neurons are under direct regulation by peptidergic satiety inputs (myoinhibitory peptide, MIP) and hunger inputs (Hugin-PK). SIFa neurons act on taste and olfactory neurons, as well as a number of neurons expressing the transcription factor Fruitless, known to regulate sex-specific behavior. They also act on neurosecretory systems in the pars intercerebralis that signal with diuretic hormone 44 and insulins, as well as specific neuronal circuits regulating sleep. It is also likely that the SIFa neurons receive inputs from neurons known to play roles in feeding and metabolism that produce corazonin (CRZ), DILPs, sulfakinin (DSK) and sNPF. (**b**) There are several levels of modulation of olfactory signals related to food attraction. Three channels responding to food odors from receptor (Or) to antennal lobe glomerulus (DM) are shown (red, blue and green). Two are modulated by nutrient-dependent insulin (DILP) signals (1 and 2), and a third by SIFa (3). The Or42b receptor neurons express sNPF and sNPF receptor (sNPFR), and in hungry flies low levels of DILPs upregulates sNPF expression (1) and thus the signal strength to higher order olfactory neurons increases and food search increases. Tachykinin (DTK) released from local neurons inactivates synaptic activity in Or85a neurons, and low levels of DILPs (in hungry flies) inhibit DTK action (2) leading to aversion for high concentrations of food odor (vinegar). SIFa neurons modulate food attraction via Or85a-DM3 (3) and under regulation of MIP and Hugin-PK. This figure was redrawn and altered from [58].

Fig. 4. Leucokinin (LK) signaling from a small set of neurons coordinates several functions related to feeding, metabolism, and stress. There are 22 LK producing neurons (LKn) in the CNS (LHLK, SELK and ABLK). Solid arrows indicate confirmed signaling pathways; dashed arrows indicate proposed ones (numbers refer to different pathways). The ABLKs are presumed to release LK into the circulation (via 3 and 4). By these hormonal routes LK may act on brain LK receptor-expressing IPCs (via 3) to affect insulin production/release and hence affect metabolism, stress responses and food intake and (via 4) act on peripheral targets such as gut, and renal tubules and thereby regulating water and ion balance and related stress responses [45] [47]. The SELKs may affect feeding

directly via motor neurons or indirectly via action on IPCs (2), ion transport peptide (ITP)-producing neurons (ITPn; 2) or unidentified brain neurons (red box). The LHLKs are glucose sensing and have been shown to activate IPCs (1) and thereby regulate metabolism-associated activity and sleep [2]. The signaling between IPCs and ITPn seem to be by DILP2 (see [59]) or and to other brain neurons possibly by colocalized classical neurotransmitters (CT). The LHLKs may regulate IPCs, which in turn activate ITPn and thereby affect central (sNPF and TK) and peripheral signaling (DILPs and ITP). Available data suggest that the three types of LK neurons act together to modulate a range of actions that together establish post-feeding homeostasis. This figure is updated and redrawn from [45] and incorporates findings from [2].

Supplemental material file.

Supplemental Table 1. Neuropeptides regulating behaviors in Drosophila

Peptide acronyms

Figure 1.

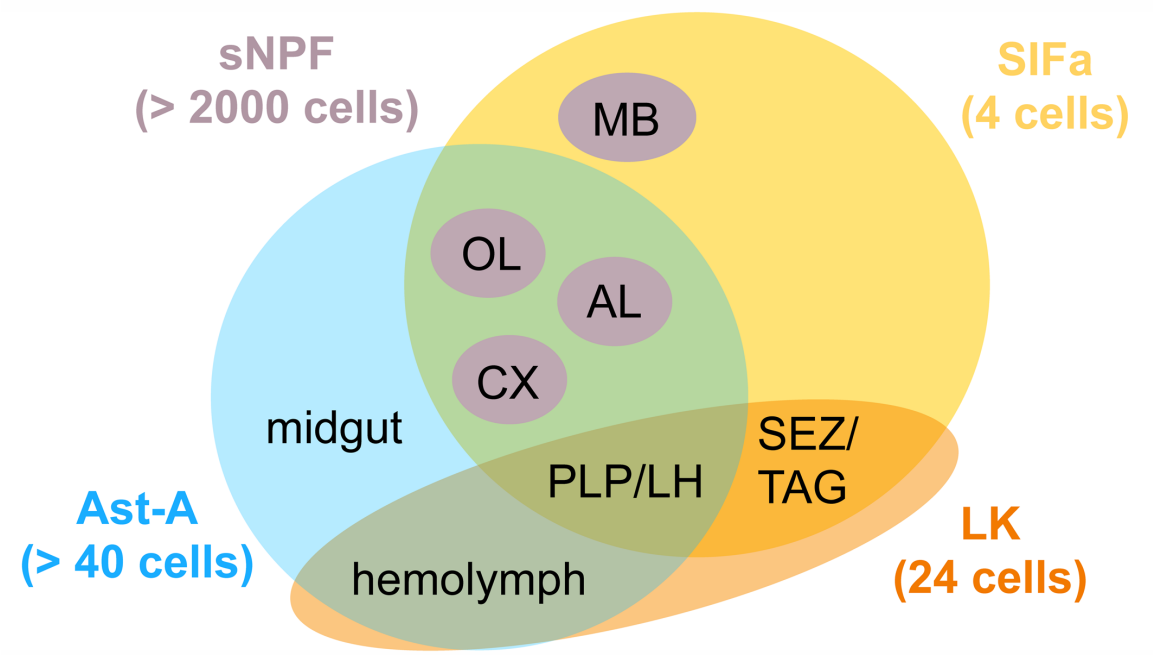

Figure 2.

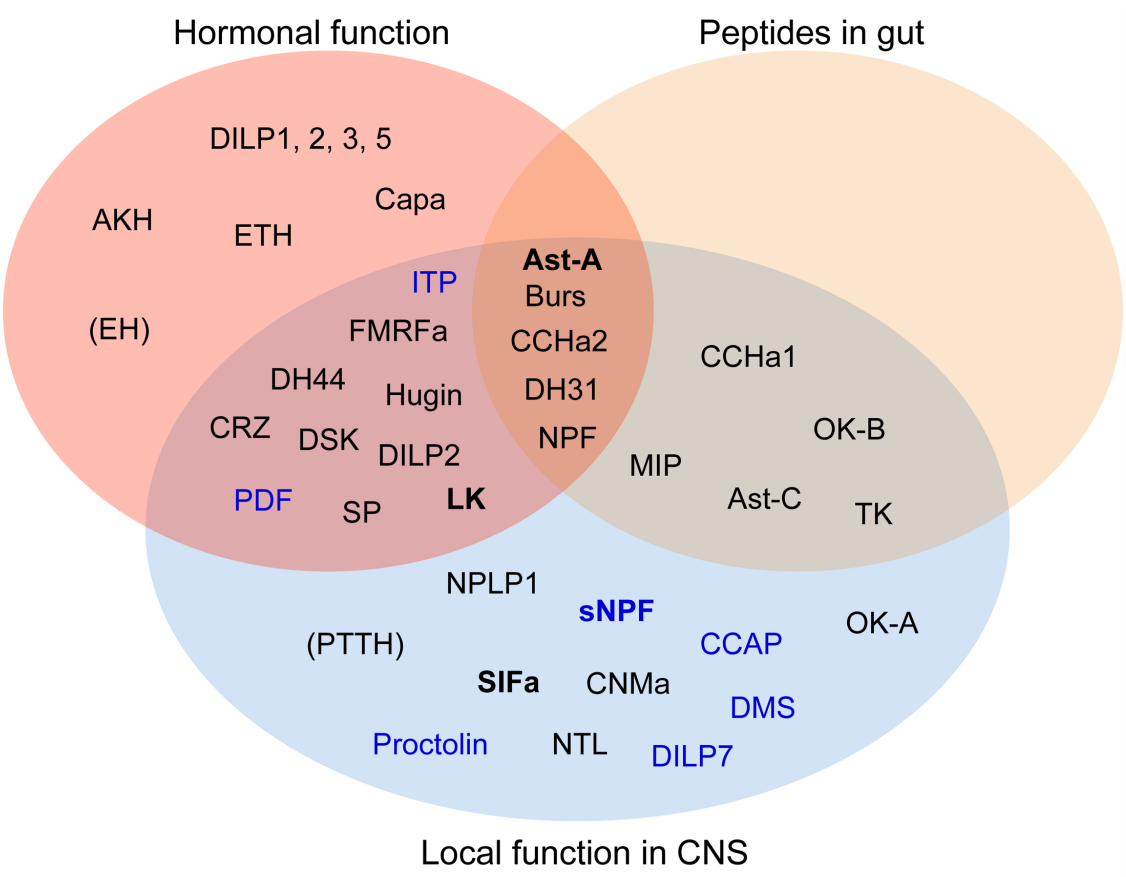

Figure 3.

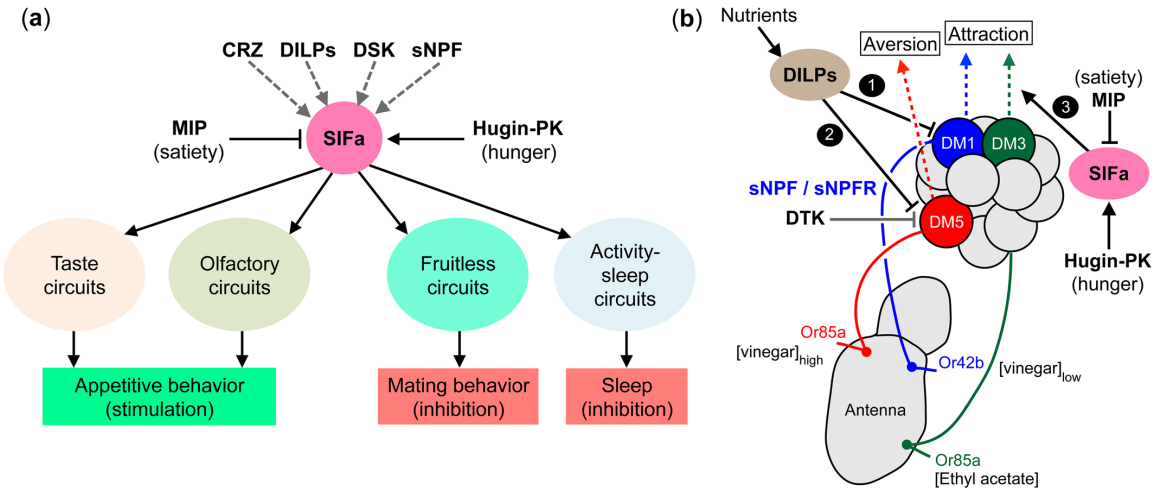

Figure 4.