Neuropeptides as Regulators of Behavior in Insects

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Abstract

Neuropeptides are by far the largest and most diverse group of signaling molecules in multicellular organisms. They are ancient molecules important in regulating a multitude of processes. Their small proteinaceous character allowed them to evolve and radiate quickly into numerous different molecules. On average, hundreds of distinct neuropeptides are present in animals, sometimes with unique classes that do not occur in distantly related species. Acting as neurotransmitters, neuromodulators, hormones, or growth factors, they are extremely diverse and are involved in controlling growth, development, ecdysis, digestion, diuresis, and many more physiological processes. Neuropeptides are also crucial in regulating myriad behavioral actions associated with feeding, courtship, sleep, learning and memory, stress, addiction, and social interactions. In general, behavior ensures that an organism can survive in its environment and is defined as any action that can change an organism's relationship to its surroundings. Even though the mode of action of neuropeptides in insects has been vigorously studied, relatively little is known about most neuropeptides and only a few model insects have been investigated. Here, we provide an overview of the roles neuropeptides play in insect behavior. We conclude that multiple neuropeptides need to work in concert to coordinate certain behaviors. Additionally, most neuropeptides studied to date have more than a single function.

INTRODUCTION

Ever since the groundbreaking work of Karl von Frisch (136), who deciphered the meaning behind the waggle and round dance performed by scouting bees, it has become gradually clearer that insects are not mere automatons, but rather complex organisms with intricate behaviors. Though obvious today, insects were not always considered to be highly evolved organisms with a sophisticated central nervous system (CNS). Linnaeus (86), the father of taxonomy, had classified insects as having no brain. Despite their small brain size, however, insects are not mindless critters. Humans possess approximately 100 billion neurons. Insects, on average, survive with 100,000 times fewer nerve cells. Yet, as Chittka & Niven (31) argued, bigger brains are not necessarily better.

Even with a tiny brain, insects are capable of very complex behavior. Honey bees conduct at least 59 distinct behavioral actions, more than is known for any rodent and approximately half of what a human child or bottlenose dolphin is capable of (29, 31). Insects can learn rules and generalize them across sensory modalities. Their sensory systems often match or surpass that of humans. Moreover, insects seem to grasp concepts such as above/below (9), sameness/different (48), numerosity (the ability to distinguish between sets of more or fewer items) (23), and counting (33, 104). They can solve complex problems (10, 40, 162), recognize faces of members of their own species (38, 119), display Pavlovian behavior (113, 141), show pessimistic or optimistic tendencies (i.e., be "down") (12), and even be "heartbroken" (120).

There is increasing compelling evidence that these behaviors, whether of complex cognitive character or merely basic locomotive, are in some way regulated by specific signaling molecules known as neuropeptides, small proteins released by neurons and/or neurosecretory cells. Neuropeptides can act as neurotransmitters, hormones, neuromodulators, and growth factors. They are processed from their larger, inactive precursors by enzymes, i.e., proprotein convertases or furins that recognize specific cleavage sites. After cleavage, carboxypeptidases E or D remove the mono- or dibasic amino acids, and in many cases, PAM (peptidylglycine-alpha-amidating monooxygenase) or its invertebrate ortholog catalyzes the conversion of the carboxyterminal glycine into an amidated residue (45). Mature, bioactive neuropeptides are formed while their precursors are transported within secretory granules from the Golgi to the cell membrane, where they are released. Note, however, classical neurotransmitters are produced at the synaptic terminus and are released only in situ. Neuropeptides act on target cells, usually through interaction with specific membrane receptors, known as G protein–coupled receptors (GPCRs). These GPCRs usually connect to an intracellular protein kinase cascade or second messengers such as Ca^{2+} or cAMP. Because of these cascades, neuropeptides can exert a variety of effects. This is in stark contrast to aminergic neurotransmitters, which usually alter only the excitability of their target cells.

ORIGIN OF NEUROPEPTIDES

Insects possess a wide range of neuropeptides, some of which display a high resemblance to vertebrate neuropeptides such as neuropeptide F (NPF), tachykinins (TKs), or sulfakinins (SKs). Other insect neuropeptides such as proctolin or eclosion hormone are unique to arthropods (102). The common ancestors of metazoans, choanoflagellates and filastereans, already possessed a wide variety of gene families controlling multicellularity and development. The ancient origin of neuropeptides dates back to at least 600 Mya, as they are found not only in Cnidaria, supposedly the oldest animals with a nervous system and which include polyps, jellyfish, corals, and anemones (106, 127), but also in Ctenophora (97) and Placozoa (121). According to recent phylogenetic analyses, the latter two families might be basal to Cnidaria (57). Placozoans are quite simple, soft-bodied animals, lacking three germ layers, neurons, synapses, muscles, and a true gut, but they can coordinate cells to move or contract (62). Neuropeptides might therefore be even older.

Cnidarian neuropeptides induce settling behavior in sponge larvae (144). Sponges are considered a sister lineage to true animals and contain a rich repertoire of rhodopsin-type GPCRs. Our analysis of the genome of the sponge *Amphimedon queenslandica* and several sponge transcriptomes reveals several hypothetical neuropeptides, separated within their precursors by typical dibasic cleavage sites as well as amidation sites. None of these mined neuropeptides displays any recognizable homologies to known neuropeptides, but cross-lineage conservation of neuropeptides is often limited to a few residues. Nevertheless, the sponge genome also harbors well-conserved processing enzymes, such as PAM (XP_003388865.2), furins (XP_011405480, XP_011404107, XP_003387614), proprotein convertases (XP_011405715, XP_011405715, XP_011404106, XP_011405930, XP_011407660), and carboxypeptidase D (XP_003384134), which are required for cleavage and maturation of neuropeptides from their precursors. Even the genome of the choanoflagellates encodes homologs of neuropeptide signaling and GPCR genes. All these findings suggest that neuropeptide signaling had already occurred in the progenitors of metazoans and choanoflagellates (41) and thus predates the emergence of neuronal tissue. Early neural systems might have been neuropeptidergic rather than aminergic, because ctenophores do not use serotonin, acetylcholine, dopamine, noradrenaline, adrenaline, octopamine, histamine, or glycine as neurotransmitters, suggesting that these amines were recruited later in evolution.

NEUROPEPTIDES AND INSECT BEHAVIOR

Sensory stimuli detected by GPCRs elicit stereotypical animal behavior comprising combinations of different motor patterns, which can vary depending on the external environment or internal states. The recruitment of appropriate motor programs as a result of changing conditions is an essential aspect of animal behavior. How sensory cues or internal states are perceived by the brain and subsequently transformed into appropriate decisions and actions is poorly understood. Neuromodulators such as neuropeptides or monoamines reconfigure the dynamics of neuronal circuits to change output motor patterns (11). To modulate neural circuits, they also change the activity of the composing neurons or the synaptic efficacy of the neural connections or engage in other yet-unknown mechanisms.

How neuropeptides change the information flow within neuronal circuits remains largely elusive. Insects are ideal model organisms for studying the underlying molecular mechanism of hardwired innate behavioral responses as well as of behaviors acquired by learning and memory. Here, we review the state of the art on the roles neuropeptides play in various insect behaviors. Although neuropeptides also have roles in physiological processes, including growth and development, digestion, energy homeostasis, water balance, and metabolism, among others, these fall outside the scope of this review. We also do not include the effects of neuropeptides on ecdysis behavior, as there is an excellent recent review on this topic (145).

Neuropeptides in Feeding Behavior

Similar to mammals, insects consume food for its reward value. In *Drosophila*, conserved dopamine and NPF signaling mediate the circuit mechanism (140). Feeding behavior can be subdivided into discrete parts. First, an animal experiences a lack of energy that is interpreted as hunger. The animal starts to forage for food. Once a food source is detected, the animal initiates feeding and eats until a satiety signal urges it to disengage from the food. The amount of food an insect ingests depends on multiple factors, such as age, nutritional state, environment, and activity. Thus, numerous neuropeptides affect this behavior.

The amount of ingested food is easy to measure and is therefore often used to monitor the effect of neuropeptides. For instance, SKs, which resemble the vertebrate gastrin/cholecystokinin

peptides, inhibit food intake in several insects, including cockroaches, bugs, locusts, and flies, possibly by stimulating gut contractions (8). Injection of SKs significantly decreases food uptake in fifth-instar locusts (142, 161). In cockroaches, SK-induced reduction in food intake reaches as much as 84% (89). In line with this, RNA interference (RNAi) of SK increases consumption in crickets and the red flour beetle *Tribolium castaneum* (93, 160). In *Drosophila*, drosulfakinins (DSKs) induce satiety, possibly by affecting transcription of insulin-like peptides. DSK RNAi in insulin-producing cells (IPCs) results in increased food uptake and causes aberrant food-choice behavior (123). The effects of SK on feeding might be regulated through short neuropeptide F (sNPF). The SK receptor is highly expressed in the corpora cardiaca, an organ that in several insects also contains sNPF (21, 36, 94, 107). sNPF is a potent orexic peptide, abundantly expressed in the CNS (81, 101). In fruit flies, overexpression of sNPF increases food consumption, resulting in bigger flies, whereas loss-of-function mutants display the opposite phenotype (54, 81). Interfering with sNPF does not, however, affect the behavioral switch from feeding to wandering larvae. In the silkworm, injection of sNPF results in accelerated onset of feeding (99), suggesting a role in feeding initiation.

Starvation increases the response toward stimuli as distinct as food odorants, repellents, and pheromones. Both sNPF and CCHamide modulate these starvation effects. Expression of sNPF in numerous olfactory receptor neurons in the antenna and maxillary palps of adult *Drosophila melanogaster* suggests a role in adult food-seeking behavior (24). Indeed, upon starvation, elevated sNPF levels as well as increased expression of sNPF receptor (sNPFR) are seen in the olfactory receptor neurons in the antennal lobes of flies, suggesting that sNPF is at least partly responsible for starvation-dependent food-seeking behavior (111). Constitutive activation of the insulin receptor blocks this effect, showing that high insulin levels act as a satiety signal by blocking elevated sNPFR expression. In the cockroach *Periplaneta americana*, sNPF titers rise dramatically upon four weeks of starvation. Moreover, sNPF activates the locomotor program in fed animals, whereas crustacean cardioactive peptide (CCAP) inhibits this activity in starved cockroaches (95).

Ko and coworkers (73) showed that sNPF enhances attraction to odors in starved flies and that TK suppresses the activity of neurons wired for aversion in hungry flies. Their studies show that both sNPF and TK have a stimulatory effect on feeding. Nonetheless, in some insects, sNPF has negative effects on feeding. For instance, in the mosquito *Aedes aegypti*, injection of sNPF-3 reduces host-seeking behavior (84). In *Schistocerca gregaria*, sNPF injection decreases food uptake, whereas RNAi has the opposite effect (36, 37). In the red imported fire ant, starvation causes a decrease in sNPF expression (30). Because the evolutionary distance between these insects is large, the switch to an inhibitory role of sNPF on feeding most likely occurred multiple times.

NPF is also involved in feeding behavior in insects. NPF is structurally related to vertebrate neuropeptide Y, well known for its ability to increase food intake (32). In *D. melanogaster*, NPF overexpression induces continuous feeding in larvae and increases tolerance to noxious food (150, 151). NPF is also involved in appetitive memory: Stimulation of NPF-expressing neurons mimics food deprivation, whereas blocking *npfr1*-containing neurons suppresses memory performance in hungry flies (74). Besides the brain, NPF is found in midgut cells of larvae and adults and in the suboesophageal ganglion (SOG), further implying a role in feeding (13, 20). Wang and coworkers (140) showed that brief presentation of appetitive odors urge fed larvae to feed impulsively. Deficiencies in NPF signaling blocks this feeding by disrupting dopamine-mediated olfactory processing. This is in line with the observation that silencing NPF neurons or NPFR abolishes food-odor attractiveness, whereas enhanced NPF activity displays an increased attraction to aversive odors and noxious food (16, 151). NPFR1 neuroactivity might be mediated by the insulin/insulin-like growth factor signaling pathway (IIS), as upregulation of this pathway suppresses attraction to noxious food

(151). In *A. aegypti*, NPF is also present in the brain and midgut of females; titers are highest before and 24 h after a blood meal (125). A direct correlation between NPF injection and increased food uptake and weight gain is also seen in *S. gregaria*, where NPF knockdown results in weight loss (132).

Allatostatins (ASTs) and allatotropin (AT), two neuropeptide families initially discovered in cockroaches for their roles in regulating the release of juvenile hormone from the corpora allata, are also involved in feeding behavior. Insects can have one type of AT and three types of ASTs (type A, B, and C). Injections of AT suppress feeding in the fall armyworm *Spodoptera frugiperda*, increase mortality in larvae, and reduce life span in adults. Combined injections of AT and C-type ASTs have similar effects (103). Curiously, in a different moth *Lacanobia oleracea*, AT has no discernable effect on feeding or growth, while AST injections result in reduced feeding, decreased growth, and increased mortality (7), an effect also seen in the tomato aphid (90), underscoring that neuropeptides can acquire distinct functions in separate species. In the silkworm, injection of AT or an AT-like peptide promotes latency to feed (99). AST-A from the cockroach *Blattela germanica* inhibit food consumption (2). In *Drosophila*, activation of the AST-A-expressing neurons inhibits feeding in starved adults without mimicking satiety, as the metabolic changes accompanying a state of satiety are not promoted (52). Activation of NPF-expressing neurons suppresses this inhibitory effect on feeding, suggesting a link between NPF and AST-A. Unfortunately, it is not clear which other neuropeptides are released from these neurons. In addition, RNAi of *Drosophila* AST-A or its receptor induces a strong reduction in larval foraging in the presence of food (138). These effects may be regulated through insulin-like peptides (DILPs) and adipokinetic hormone (AKH). *Drosophila* AST-A receptor (DAR-2) is present on AKH- and DILP-expressing cells of the corpora cardiaca. Silencing DAR-2 results in phenotypes comparable with DILP and AKH disruption. Insulin has a well-established role in feeding and energy metabolism in insects, and meddling with the insulin pathway invariably affects feeding, growth, and life span. For instance, pan-neuronal overexpression of *dilp2* and *-4* reduces hunger-driven foraging in larvae (151). Disrupting IIS in Kenyon cells (important neurons in mushroom bodies) also results in decreased food acquisition (163). Starvation in flies leads to higher preferences for caloric sugars. This can be mimicked by mutating *dilp2* and *-3* and blocking the insulin receptor (124). The NPF/IIS signaling cascade may mediate a survival strategy that enables starving larvae to adapt to unfavorable conditions. NPFR1 is required for engaging in risk-prone food acquisition in deleteriously cold temperatures, whereas decreased expression of the insulin receptor in the same cells was sufficient to induce high-risk feeding in fed larvae (85). This is consistent with the observation that downregulating IIS in NPFR neurons induces feeding in fed larvae (151). In honey bees, downregulation of IIS also biases them toward protein-rich food (139).

One of the most potent inhibitors of food intake in *B. germanica* is myosuppressin. In high doses, it inhibits food uptake up to 75% (1). When myosuppressin is injected in *Spodoptera littoralis*, feeding is drastically inhibited in larvae (135), whereas injecting it into *Bombyx* causes a prolonged latency period to the first bite. Contrarily, TK1 and -2 and sNPF1–3 reduce this period (100). Indeed, TK receptor RNAi leads to a decrease in body weight (51). TK is important in odor-based searching behavior of fruit flies. Several olfactory neurons contain high TK levels. RNAi of the peptide or receptor impairs odor preference toward indifference. In contrast, overexpression increases responsiveness to specific odorants (56, 148). Injection of TKs also results in diminished olfactory responses in electroantennograms (63). Leucokinins, neuropeptides with some resemblance to TKs, are also important in regulating feeding. Mutations in leucokinin and its receptor in fruit flies results in an increase of meal size, but owing to a decrease in meal frequency, they do not affect body weight (3). Blockade of leucokinin release diminishes the preference for sweetness and increases responsiveness to aversive bitter tastes (88).

The insect *hugin* gene is homologous to vertebrate neuromedin U and encodes neuropeptides designated as pyrokinins. *Hugin*-expressing neurons, which reside in the SOG, make contact with gustatory receptor cells, suggesting they relay taste information. When flies are transferred to a new food source, they tend to wait for a certain period before feeding. This initiation depends on the quality of the previous food source and hunger state of the flies. Blocking transmission of *hugin*-expressing neurons causes adult flies to have completely filled crops after only 5 min, whereas control flies need 180 min to fill their crop (92). Melcher & Pankratz (92) concluded that *hugin* neurons regulate feeding initiation.

Two other neuropeptides directly involved in feeding behavior are hypertrehalosemic hormone (HrTH) and CCHamide. HrTH belongs to the AKH family and primarily evokes higher carbohydrate concentrations in the hemolymph. Additionally, in the blow fly *Phormia regina*, HrTH inhibits the crop lobe muscles (pump 5) while also stimulating pump 4, which is involved in pushing fluids out of the crop. Thus, HrTH is likely involved in pumping carbohydrates from crop to midgut (126). The recently discovered CCHamide-2 augments feeding in blow flies (55). This is consistent with the reduced nutrient uptake and impaired locomotive activity caused by disruption of this gene in *Drosophila* (109). CCHamide-2 activates IPCs, and disrupting its expression or that of its receptor CCHa2R reduces expression levels of *dilp2* and *-5* (112). Silencing CCHa1R in Or59b-expressing olfactory neurons results in an abolished starvation effect (43). Other neuropeptides including proctolin, FMRFamides, capa, ion transport peptide (ITP), CCAP, diuretic hormones (DHs), antidiuretic peptides, orcokinin, neuropeptide like peptide 1 (NPLP1), NVP-like peptide, ITG peptide, GPA2/GPB5, corazonin, and AKH have an effect on the salivary glands, gut, digestion, diuresis, or energy metabolism (8), but no direct link to feeding behavior has been observed.

It is obvious from these examples that manipulations of a single neuropeptide can cause similar effects for different peptides. Moreover, because many neuropeptides are coreleased, it is very likely the entire cocktail of neuropeptides modulates or regulates the final sum of behavioral actions. Data have to be interpreted with care, because elimination or high concentrations of a particular peptide can provoke drastic phenotypes.

Neuropeptides in Reproductive Behavior

Courtship in fruit flies comprises a series of well-defined steps such as chasing, avoidance, dancing, rejection, and copulation, all of which involve intense locomotive activity. When virgin fruit fly females are confronted with a courting male, they usually readily mate. However, their behavior drastically changes after mating. They are no longer receptive, fend off approaching males, and start to lay up to 80 eggs per day (77). This switch in behavior is primarily regulated by a single peptide present in male sperm called sex peptide (SP) (87). Females mated with SP-deficient males show higher fitness and higher lifetime reproductive success, suggesting that SP comes at a high cost for females (146). The SP receptor, also designated as the myoinhibitory peptide receptor, is widely expressed in the CNS, but expression in *doublesex* neurons is (necessary and) sufficient for mediating postmating behavior (157). Injection of *Drosophila* SP into *Helicoverpa armigera* moths also inhibits pheromone biosynthesis by suppressing calling behavior and expression of the myoinhibitory peptide receptor (50).

A well-known neuropeptide important for mating behavior in moths is PBAN (pheromone biosynthesis-activating neuropeptide). Knockdown in females results in decreased male attractiveness (79), as PBAN stimulates production of pheromones needed to attract mates. Repellent gustatory pheromones, by contrast, function as potent suppressors of male courtship behavior. When a male fruit fly mates with a female, it leaves behind an antiaphrodiasic pheromone that

deters the next male from mating with the female. A cluster of 8–10 neurons in the SOG mediates this decision through release of TKs (118).

Other neuropeptides involved in mating behavior include natalisin (59) and NPF. NPF is upregulated by mating and decreases upon sexual deprivation in male flies. Peculiarly, rejected flies have a higher preference for alcohol. Downregulation of NPF has a similar effect in mated flies, whereas activation of NPF neurons decreases alcohol preference in virgin males, thereby mimicking a mated state (120). Ablation of male-specific NPF neurons or NPF knockdown results in reduced male courtship behavior (80).

Males usually prolong their mating duration in the presence of competitors to increase the chance of successful gene transfer. In *Drosophila*, this effect requires the presence of both NPFR1 and pigment-dispersing factor (PDF) in four small ventrolateral neurons as well as the PDF receptor and NPF in two dorsolateral neurons (70). NPF neurons are also necessary for detecting the female sex pheromone (47), the production of which is under influence of IIS. Females with increased insulin production are more attractive to males, whereas reduced IIS has the opposite effect (78). In addition to stimulating vitellogenesis, oocyte growth, and ecdysteriogenesis, NPF injections promote copulation behavior and fertility in male *S. gregaria* (114, 132). PDF fly mutants also show an increased frequency of remating compared with wild-type flies (76). In addition, PDF-expressing small ventrolateral neurons contribute to male sex-drive rhythm, which is disrupted when PDF is not present (46). A remarkable change in phenotype is seen with reduction or absence of SIFamide in fruit flies. Males start vigorously courting both males and females, whereas females are extremely receptive (129). A similar effect is seen when the copper transporter ATP7 is knocked down in SIFamide-expressing neurons. This causes a decrease in mature amidated neuropeptides, probably owing to a lack of functional peptidylglycine-α-hydroxylating mono-oxygenase (PHM), the insect equivalent of PAM, which contains copper. Flies affected in this manner display male-male courtship behavior (115).

Drosophila females also exhibit a characteristic sequence of behaviors when they lay their eggs. They prefer to lay them in medium that does not contain high sucrose levels. The few *dilp7* neurons found in the CNS send projections to the SOG and female reproductive tract. Interestingly, females with hyperactivated *dilp7* neurons show no ovipositor motor programs. Elevation of just *dilp7*, however, causes flies to lay more eggs on the undesirable sucrose medium (156). The neuropeptide amnesiac (AMN) also influences whether flies will lay eggs: When it is lacking, flies display impaired decision-making behavior and do not distinguish between the unfavorable sucrose medium and the much preferred caffeine medium for laying their eggs. Expression of *amn* in two dorsal-pair median neurons completely rescues this effect (149). Males also coordinate mating length during copulation with the transfer of sperm and seminal fluid, which is blocked and copulation lengthened upon silencing of four abdominal ganglion interneurons containing corazonin (128).

One typical set of behaviors that is involved in both feeding behavior and reproduction is aggression. Aggression can ensure an individual has a better chance of survival if food is sparse or when multiple males wish to mate with the same female. Activation of the *Tk* gene in fruit flies results in higher intermale aggression. Surprisingly, the Tk^+ neurons were present only in males (6). A subset of these neurons also express fru^+, a transcription factor important in courtship (153). Activation of the Tk^+/fru^+ neurons also results in male-male aggression (154). In the presence of a female and absence of a male, activation of these neurons initiates courtship behavior, showing that courtship and intermale aggression are distinct circuits (6, 28). The convergence of aggression and sex within a common neuropeptidergic pathway suggests that the choice to fight or court is modulated by TK release, which in part is triggered by external stimuli such as pheromones. The biogenic amine octopamine also influences aggression (and mating behavior). Octopamine-regulated aggression

in flies is modulated through DSK. In addition, DSK overexpression also induces hyperactivity (147). Interestingly, in mice cholecystokinin, the vertebrate homolog of DSK also regulates aggression (165). Genetic silencing of NPF also increases fly aggression (35). This is peculiar, as NPF RNAi inhibits courtship behavior (see above), which is usually related to aggression.

Neuropeptides in Learning and Memory

Similar to other animals, insects can learn via training and can store information that may help them in future situations. One of the most studied neuropeptide genes involved in learning and memory is *amn*. AMN has not been biochemically isolated and seems to be dipteran specific. *Amn* mutants show aberrant behavior in memory assays such as olfactory conditioning and shock reactivity (44, 108). Expression of *amn* in two dorsal-paired medial neurons is sufficient for rescuing *amn* mutants (34, 137). Wild-type flies that learn to avoid dangerous odors will subsequently take into account both the temporal factor and the level of disadvantage. *Amn* mutants make choices only on the basis of the temporal factor, completely ignoring any danger (158).

AMN also affects taste discrimination between two different concentrations of sucrose (98). Long-term memory in odor-avoidance behavior is also affected in *amn*-mutant larvae whose memory length is reduced by half (69). Interestingly, the retention time of aversive and appetitive memory differs: Aversive conditioning forms short-time memories, whereas appetitive conditioning forms stable memories. Both require an intact *amn* gene (27, 159). AMN is also necessary to form cold-induced anesthesia-sensitive memory, as shown via Pavlovian associative memory tests (82). The *amn* gene is also involved in nonassociative learning. Female flies usually avoid laying their eggs in alcohol-rich medium. In the presence of endoparasitoid wasps, female flies suppress their oviposition rate to protect their offspring. However, when ethanol-rich medium is in close proximity, female flies will actively lay eggs (fruit fly larvae are quite resistant to concentrations of up to 10% ethanol), and this preference is maintained for days after the wasps are removed. Flies lacking *amn* do not remember the wasp exposure and do not maintain their altered oviposition behavior after removal of the wasps (64).

Interestingly, RNAi of NPF or NPFR1 also increases the oviposition preference for ethanol, regardless of the presence of wasps, whereas overexpression has the opposite effect. Thus, wasp visual perception may cause a decrease in NPF signaling (65). As mentioned above, stimulation of NPF neurons also promotes memory performance in satiated flies (74). This is remarkable, as robust appetitive memory formation usually requires flies to be hungry (75). *Drosophila* larvae are able to associate odors with a fructose reward, which means they can be trained to choose between odors after conditioning. This appetitive memory formation is strongly impaired when NPF-expressing neurons are artificially activated. Moreover, activation of just one of the six NPF-expressing neurons in the larval brain is sufficient to interfere with appetitive memory. Aversive memory formation is not affected (110). Olfactory memory also requires the *snpf* gene to be active in the Kenyon cells of the mushroom bodies. Knockdown of *snpf* or its receptor in Kenyon cells outside the mushroom bodies results in decreased sugar-rewarded olfactory memory (72). In honey bees, injection of ASTs decreases appetitive olfactory learning (131).

Neuropeptides Involved in Stress and Addiction

NPF, which is involved in all behaviors discussed so far, also plays a role in stress and addiction. Food-deprived insects may be forced to forage in dangerous conditions to ensure their survival. Hungry flies will search for food in hostile cold temperatures. Yet, lowering NPF in hungry larvae causes them not to feed in cold environments. NPF overexpression causes even fed larvae to feed on deleterious nutrients (85). *Npfr1* is expressed in a subset of *painless* neurons. *Painless* (*pain*)

codes for a transient receptor potential channel that is involved in aversive responses to thermal, chemical, and mechanical stressors. *Npfr1* knockdown in *pain* neurons abolishes larval aversion to sugar. By contrast, *npfr1* overexpression in these neurons blocks sugar-stimulated channel activity. Therefore, the NPF signaling pathway may have antinociceptive functions (152).

Disrupting NPF/NPFR1 neurons in adults also confers resistance to ethanol sedation (143). *Amn* mutants show increased initial sensitivity to ethanol (17) and are less responsive to noxious heat stimulation. Thus, AMN is critical for thermal nociception (4). Similar to NPF, reducing corazonin in *crz*-expressing neurons lowers ethanol-sedation sensitivity (91). This is possibly regulated by alcohol dehydrogenase, which is found in lower levels in *crz* mutants (116). Corazonin may be the ortholog of vertebrate GnRH, which is directly regulated by stress hormones (26). Similarly, most *crz* neurons have receptors for DH31 and DH44, two diuretic neuropeptides that are orthologous to the mammalian stress hormones, calcitonin and corticotropin-releasing factor (61). Ablation of *crz*-expressing neurons confers resistance to various stressors such as starvation, high salt concentration, and paraquat-containing food to induce oxidative stress as measured by survival (164). Knocking down DH44 or its receptor increases desiccation tolerance, whereas leucokinin receptor knockdown augments resistance to starvation (22).

A set of dorsolateral peptidergic neurons communicating with IPCs is also involved in stress responses. These cells express sNPF and corazonin, and knockdown of either increases resistance to starvation (68). Three neuropeptides, ITP, sNPF, and *Drosophila* TK (DTK), are coexpressed in five pairs of large protocerebral neurosecretory cells. Flies with reduced levels of DTK or sNPF, but not ITP, in these cells have difficulty surviving in dry and foodless environments (66). Targeted knockdown of DTKR, dilp5, or the insulin receptor in the principal cells of the Malphigian tubules also confers resistance to desiccative, oxidative, and nutritional stress (122). Recently, Terhzaz and colleagues showed that yet another neuropeptide, capa, also renders fruit flies more resistant to desiccation when knocked down and, additionally, lengthens recovery time after cold stress (130). Many organisms experience augmented sleep after stressful events such as infection or heat shock. In *Drosophila*, FMRFamide modulates this stress-induced sleep. Knockdown of this neuropeptide or its receptor causes reduced sleep after infection or heat stress (83). A particular FMRFamide (DPKQDFMRFamide) also enhances the escape response from intense light exposure. Both FMRFamide and dromyosuppressin receptors are required for this behavior (71).

Circadian Rhythms, Sleep, and Wakefulness

Sleep is an essential behavior that is conserved widely across animals. Sleep loss is detrimental to memory and learning performance as well as to health in general. Support for the hypothesis that the basic molecular mechanisms of sleep have been evolutionarily conserved across animal species is compelling. One of the most well-conserved pathways for sleep regulation is the circadian clock, composed of transcriptional feedback loops that were discovered in *D. melanogaster* (see 14, 39). Intrinsic rhythms in clock-pacemaker neurons are coordinated by PDF and propagate to multiple downstream circuits to orchestrate behavioral rhythms. For instance, the leucokinin circuit connects these neurons to brain areas that regulate locomotive activity and sleep (25). Other neuropeptides involved in sleep and wakefulness regulation include ITP, sNPF, calcitonin-gene related peptide, AMN, and SIFamide, which all have sleep-promoting functions (39, 53, 117). PDF promotes arousal in *Drosophila*. Information on neuropeptidergic modulation of sleep in other insects is scarce. In *Tribolium*, orcokinin-A and -B RNAi resulted in longer death feigning (thanatosis) (58), a behavioral defense mechanism to avoid attacks by jumper spiders (96). Orcokinin may, thus, be involved in inducing the "awakened" state in beetles.

Social Behavior

The social structure of honey bees is an ideal model for understanding how neuropeptides regulate social behavior. Although several peptidomics studies have revealed neuropeptide signatures associated with particular behaviors (19, 49), including labor division, causal evidence is scarce. Changes in IIS are associated with social behavior (5). In locusts, high levels of pyrokinin peptides are correlated with solitary behavior, whereas ITP may be associated with gregarious behavior (133). Removal of dead nest mates or necrophoretic behavior is thought to limit the potential spread of pathogens within a social insect colony and is a common behavior in many ant species. Application of pyrokinins enhances this behavior (42).

CONCLUSIONS AND FUTURE PERSPECTIVES

Synchronous light signaling of thousands of fireflies, procession marches of caterpillars, trap building or maintenance of mushroom farms by ant species, electric navigation in bumble bees, and swarm formation in locusts are examples of extraordinary and complex insect behaviors. What is the molecular basis of such complex behaviors, and how can variations in behaviors be explained from an evolutionary point of view? Driven by sensory information, neuropeptide signaling affects hardwired neuronal circuits. The neuropeptidergic state adds an extra layer to the regulation of behavioral output: It modulates the functional output of neuronal circuits and shapes that output over time by altering the circuit composition, activity, or dynamics.

One of the routes of behavioral evolution may involve novel actions by conserved genes. For example, prothoracicotropic hormone, a neuropeptide that controls the developmental transition from juvenile stage to sexual maturation, also modulates light-avoidance behavior through its receptor Torso in *Drosophila* larvae (155). This photophobicity may ensure that wandering larvae maintain a preference for darkness, a condition that is required for successful pyrokinin-controlled pupariation (134).

Another example concerns the role of vasopressin and oxytocin in social behavior, pair bonding, and parental care. This role may have originated from their physiological functions. Reproduction would have been the driving force to use oxytocin to regulate parental care. Urine concentration under vasopressin control would have been the antecedent of scent marking and territorial behavior in males. Recent studies contest this hypothesis and state that oxytocin's role in cognition does not derive from its physiological role, but instead probably derived from its ancient function in associative learning (15), a function that more likely originated when animals started to move to explore environments for food and mates, behaviors that require decision making and experience-based memory. As such, behavioral outputs evolved early in evolution.

In spite of the vast increase in insect neuropeptide research, only a small number of neuropeptides have so far been associated with behavioral outputs. The recent introduction of Crispr-Cas9 technology in insect research is likely going to change this. Most insect neuropeptides studied to date are implicated in several distinct behaviors and thus able to integrate information from various cues to different behavioral outputs. AMN and NPF, for instance, modulate feeding, reproductive, learning, and stress behavior.

Species-specific sensory cues and variations in spatial and temporal expression of neuropeptides and their receptors explain the phenotypic diversity in behavioral output. Also, most neuropeptidergic cells copackage neuropeptides into the same vesicles and thus release them simultaneously. Different neuronal cells can transcribe different subsets of neuropeptides. Additionally, neuropeptide transcripts can be differentially spliced, and neuropeptides can be differentially sorted in the same cell. If you consider the sheer number of neuropeptides (most animals express more than 100 neuropeptides), picturing the multiplicity of this network and the variation it can generate

is not difficult (18). Studying their function, however, is not easy because one or two neuropeptides are usually tested at a time to observe their effects, even though possibly dozens are released simultaneously. The action of a single neuropeptide modulator is likely different from its action when in the presence of another (18). Thus, in the best version of the standard paradigm, the role of a particular neuropeptide will invariably be oversimplified.

Some neuropeptides can activate two or more related receptors. The same receptor molecule may couple to different G proteins in different types of cells, leading to different outcomes. Ultimately, this results in an extremely complex neuropeptide network that is largely situated in interneurons, connecting sensory and motor neurons.

Certain behaviors need multiple neuropeptides to work in unison to bring a behavioral response to a successful conclusion. For instance, for ecdysis to work properly in *Manduca sexta*, five neuropeptides are needed in sequential order (pre-ecdysis-triggering hormone, ecdysis-triggering hormone, eclosion hormone, CCAP, bursicon). In addition, other neuropeptides such as kinins, corazonin, myoinhibitory peptides, sNPF, and FMRFamides may play a role as well (67, 145). Park and coworkers (105) showed that the *Drosophila* larval CNS harbors 24 different neuropeptides, each with a unique expression pattern and showing little overlap, with some cells expressing two to three different neuropeptides. This is in stark contrast to studies in the snail *Lymnaea stagnalis* where single-cell mass spectrometry allowed detection of 17 distinct neuropeptides in one single neuron (60). In the near future, this technique could potentially be used to target single neurons in the insect brain to provide us with a detailed map of neuropeptide contents of neurons and thus improve our understanding of neuronal networks.

Some neuropeptides and/or their receptors are absent in particular insect species. Other neuropeptides occur in multiple variants within a single species. Several studies indicate that neuropeptides and their receptors, if not hindered by structural constraints as with oxytocin, can rapidly coevolve, resulting in the elimination of clear structural similarities among evolutionarily related neuropeptides. The birth-and-death evolution as well as coevolution is not uncommon for neuropeptide genes and their receptors and explain the enormous behavioral variation in various environmental contexts.

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