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From genes to circuits and behaviors

Neuropeptides expand the coding potential of the nervous system

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Neuropeptide signaling remodels the composition of a chemosensory circuit and shapes behavior in *Caenorhabditis elegans*. We reported that the ASE left (ASEL) salt sensory neuron uses a proprotein convertase, BLI-4, to cleave the insulin-like peptide INS-6. INS-6 peptides are released from the ASEL neuron in response to large, but not small changes in salt stimuli. Fast INS-6 signaling functionally transforms the AWC olfactory sensory neuron into an interneuron in the neural circuit for high salt. This new circuit configuration potentiates behavioral attraction to high salt. Here, in the context of genes, circuits, and behaviors, we discuss the diverse modes of neuropeptide processing and signaling, which expand the coding potential of the nervous system. First, neuropeptide processing and release genes prepare insulin peptides to signal in the nervous system. Second, this neuropeptide signaling diversifies the communication of neural circuits and introduces circuit-level flexibility. Finally, the resulting multisensory neurons and circuits drive finely tuned behavioral choices.

Keywords: neuropeptide, insulin, proprotein convertase, neural circuit, sensory neuron, salt, multisensory

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Introduction

Neuropeptides, including insulins, have numerous roles in modulating neural circuits and regulating the execution of diverse behaviors.[1-4] In the nervous system, insulin signaling regulates synapse development,[5] the signal to noise ratio of olfactory sensory neuron activity,[4] gustatory and olfactory learning behaviors,[2,6,7] and many other circuit functions. Recently, we reported a novel sensory context-dependent and insulin neuropeptide-mediated remodeling of a chemosensory neural circuit in *Caenorhabditis elegans*.[8] We defined an unexpected role for the processing and release of insulin-like peptides in regulating the composition of the neural circuit that drives behavior in response to changes in salt. Upon high salt stimulation, the insulin-like peptide INS-6, which is processed by the proprotein convertase BLI-4, is released from the ASEL salt sensory neuron (**Fig. 1C**).[8] The release of mature INS-6 peptides depends on the *C. elegans* homolog of calcium-dependent secretion activator, UNC-31, which is a key regulator of neuropeptide-containing dense core vesicle secretion (**Fig. 1C**).[9,10] INS-6 binds the DAF-2 insulin receptors on the AWC olfactory sensory neuron, activating this cell (**Fig. 1C**).[8] Exciting the AWC neuron in this manner recruits AWC and its target interneurons to the high salt circuit and specifically potentiates attraction behavior toward high salt.[8]

In this commentary, we divide our discussion about the significance and context of our novel findings into three sections focusing on genes, circuits, and behaviors. At the level of genes, we discuss our findings about novel roles for several genes in the processing and release of insulins in the nervous system and speculate about the generality of these molecular mechanisms. At the level of circuits, we describe how neuropeptide signaling adds a dynamic new language of communication to the nervous system, and we highlight

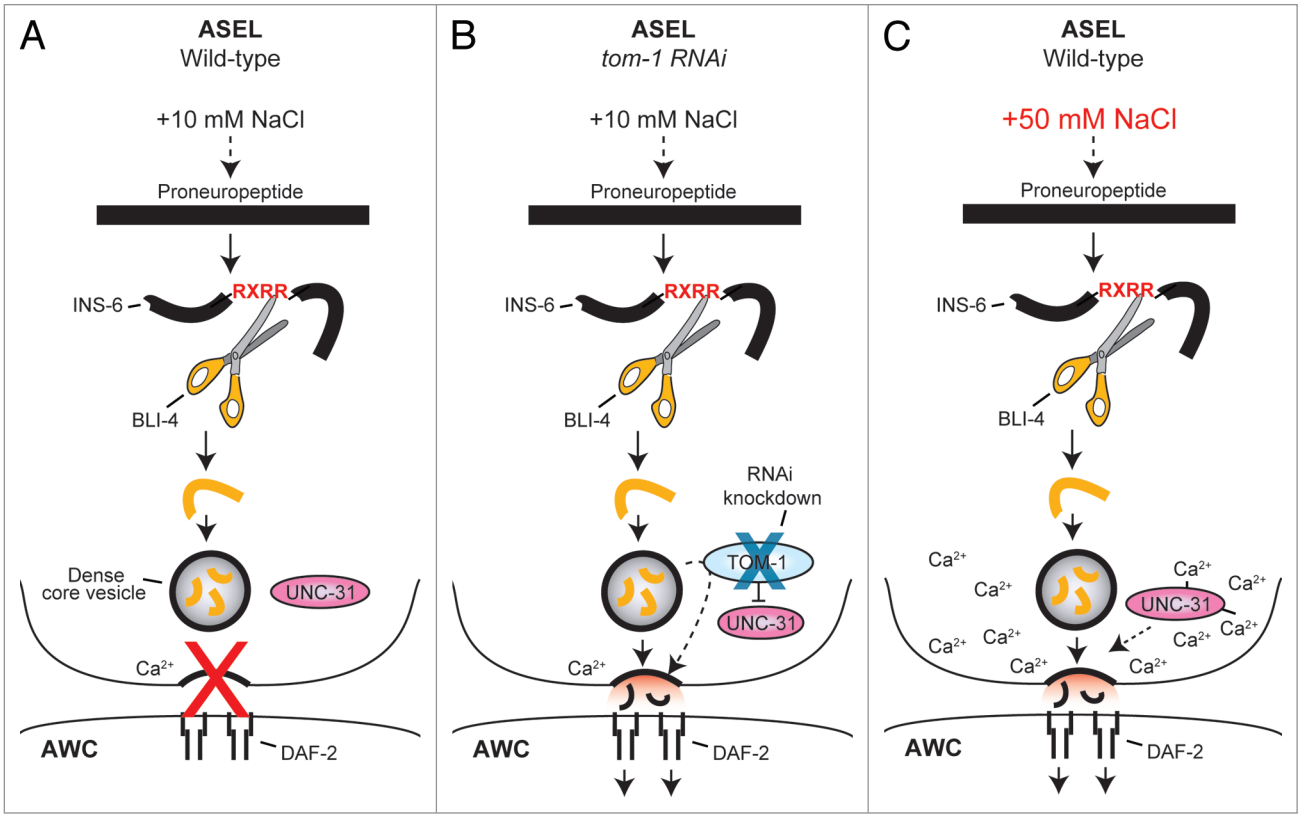

Figure 1. Genes: insulin neuropeptide processing and release machinery. (**A–C**) Proneuropeptides are processed to generate mature neuropeptides. The proprotein convertase BLI-4 recognizes and cleaves the pro-insulin-like peptide INS-6 at an RXRR motif to generate mature INS-6, in the ASEL sensory neuron.[8] (**A**) Low +10 mM NaCl stimulation is insufficient to release INS-6 containing dense core vesicles in wild-type animals.[8] (**B**) In ASEL neuron-specific *tom-1* RNAi transgenic animals, an inhibitor of dense core vesicle release is removed. +10 mM NaCl stimulation in these transgenics results in the release of INS-6-containing dense core vesicles from the readily releasable pool, even in the absence of a large increase in calcium. INS-6 signals through the DAF-2 insulin receptor on AWC neurons.[8] (**C**) In wild-type animals, high +50 mM NaCl stimulation causes a large increase in calcium levels, which leads to UNC-31-dependent release of INS-6-containing dense core vesicles.[8]

the intriguing spatial and temporal constraints that expand the neurotransmitter vocabulary. Finally, at the level of behaviors, we discuss how individual neurons may act in flexible neural circuits to direct behavior toward multiple stimulus modalities across species, in *C. elegans, D. melanogaster,* and *M. musculus*.

Genes: A novel molecular mechanism for processing and releasing insulin peptides

Our work identified unexpected requirements for peptide processing and release genes in the nervous system. Neuropeptides are processed post-translationally in multiple steps: Proneuropeptides are cleaved by proprotein convertases and then further modified by a variety of other enzymes.[11] A family of kex2/subtilisin-like proprotein convertases, which cleave these proneuropeptides, is conserved across eukaryotes.[12] Four convertase family members have been identified in *C. elegans*: *egl-3, bli-4, kpc-1,* and *aex-5*.[13] Prior to our study, it was hypothesized that these enzymes might cleave the 40 pro-insulin-like peptides in *C. elegans* to produce mature insulins; however, no genetic, proteomic, or physiology experiments supported this theory.[11,14] Our research demonstrates that the insulin-like peptide INS-6 is produced through a BLI-4 proprotein convertase-dependent cleavage step in the ASEL sensory neurons (**Fig. 1**).[8] In ASEL neurons, we confirmed that BLI-4 recognizes an RXRR motif in INS-6 (**Fig. 1**). This is the first description of a role for BLI-4 in the nervous system. We speculate that BLI-4 also cleaves other neuropeptides. Given that the BLI-4 cleavage site is highly conserved (RXRR or RXKR),[13] a molecular genetic approach would be ideal for determining the identity of the other peptides that are also processed by this convertase.

The genes involved in releasing mature insulins in the nervous system were predicted to be similar to those involved in the release of other neuropeptides; however, this had not been confirmed experimentally. UNC-31, the homolog of calcium-dependent secretion activator, is a key regulator of dense core vesicle priming, docking, and secretion.[9,10] Our results suggest that INS-6 peptides (along with other insulin-like peptides) are released from dense core vesicles in an UNC-31-dependent manner[8] (**Fig. 1**). Interestingly, this insulin release from ASEL neurons activates AWC neurons only when the animals experience sufficiently large changes in salt, ≥ 20 mM NaCl (**Fig. 1**). These large changes in salt cause significantly larger magnitude and longer duration calcium transients in the ASEL neurons than

smaller changes in salt (10 mM NaCl).[8] Together, these findings suggest that insulin-containing dense core vesicles are only released when sufficiently high levels of calcium mobilization are achieved in the presynaptic neuron (**Fig. 1C**). In contrast, we hypothesize that the lower levels of calcium in ASEL neurons resulting from low salt stimulation are only enough to release small, clear synaptic vesicles containing small neurotransmitters such as glutamate. These results suggest that dense core vesicle release occurs only when high, global levels of calcium have been reached after strong stimulation, thereby supporting a theory that has long been proposed[15,16] and extending it to also include insulin-containing vesicles. Additionally, our results provide the first illustration of circuit and behavior level consequences of the different calcium levels triggered by varying sensory stimuli (**Fig. 1**). Nevertheless, it cannot be ruled out that very low levels of dense core vesicle release do occur with lower intensity salt stimulation. In this scenario, the few peptides from these vesicles are simply insufficient to bind a high level of receptors on the postsynaptic AWC neuron or cause a detectable increase in the AWC calcium signal.

Furthermore, the requirement for global calcium to produce dense core vesicle release can be bypassed. TOM-1, the *C. elegans* homolog of Tomosyn, acts upstream of UNC-31 to inhibit neurotransmission[17] (**Fig. 1B**). If this brake on neurotransmission is removed, then dense core vesicles can be released even in the absence of high, global calcium. We found that TOM-1 RNAi-knockdown, specifically in ASEL neurons, is sufficient to trigger dense core vesicle release in response to low (10 mM) salt stimulation[8] (**Fig. 1B**). Therefore, this manipulation also activates the post-synaptic AWC neurons. Importantly, this result indicates that insulin peptides are present in a readily releasable pool. High calcium levels or upstream signaling simply function as the switches that permit exocytosis of the insulin-containing dense core vesicles from this readily releasable pool.

We speculate that other invertebrate species as well as mammals use homologs of the *C. elegans* insulin processing and release genes that we described. For example, insulin, insulin-like growth factors (IGF-1 and IGF-II), and the related relaxin ligands in other species also require post-translational processing,[18] which could be performed by proprotein convertases of the BLI-4 family. There is evidence that pro-IGF-II is processed by a proprotein convertase, PC4, whose catalytic domain shares significant homology with *C. elegans* BLI-4.[13,19] This processing generates mature IGF-II that functions in the development of the human placenta.[19] If IGF-II processing is abnormally reduced, then normal development is seriously impaired.[19] Taken together, the conservation of the peptide processing machinery indicates that processing events play crucial roles in generating mature insulins capable of signaling in animals and people.

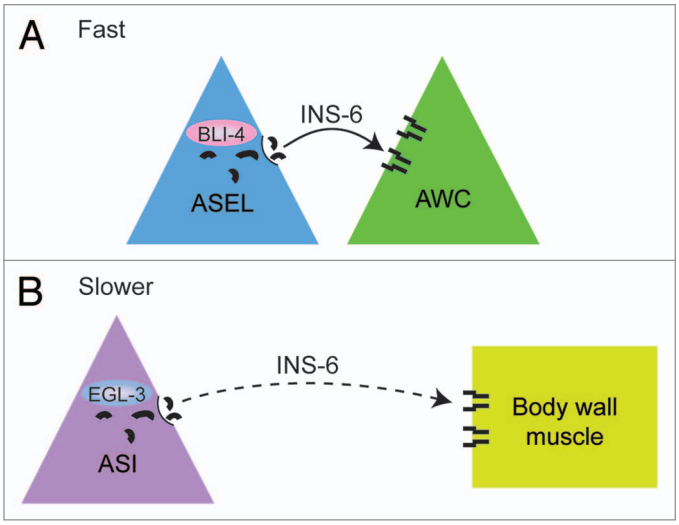

Figure 2. Circuits: neuropeptide processing, release, and downstream signaling may influence the bioactivity of insulin peptides in neural circuits. (**A**) INS-6 peptides are processed by the proprotein convertase BLI-4 in ASEL sensory neurons.[8] INS-6 released from ASEL acts as a fast neurotransmitter, signaling to nearby AWC neurons in less than a second.[8] (**B**) INS-6 peptides are processed by the proprotein convertase EGL-3 in ASI sensory neurons.[5] Insulins from ASI function as slow, long-range signals to distant body wall muscles.[5]

Circuits: A neuropeptide language diversifies neural circuit communication

Insulins and other neuropeptides function as neuromodulators, co-transmitters, and independent transmitters.[20] The diverse functions of neuropeptides acting at different spatial and temporal scales are still being appreciated. However, it is clear that peptide signaling adds an additional nuanced language for encoding dynamic experiences and driving complex behaviors.[21] Whether a particular peptide signals locally and rapidly (< 1 s) to relay information in a neural circuit or globally and over longer developmental time periods cannot be predicted simply by the peptide identity.[5,8]

Neuropeptide processing, release, and downstream signaling mechanisms are likely to play a role in determining the bioactivity of peptides (**Fig. 2**). A recent study showed that INS-6 peptides released from ASI sensory neurons play a crucial role in shaping synapses at the neuromuscular junction[5] (**Fig. 2**). Furthermore, this study showed that a different proprotein convertase, EGL-3, processes INS-6 in ASI neurons[5] (**Fig. 2**). Taken together with our findings, these results indicate that the same ligand can act on two different timescales: in less than one second to recruit AWC neurons into the salt circuit when released from ASE neurons[8] and over multiple hours to shape neuromuscular synapses during development when released from ASI neurons[5] (**Fig. 2**). We suggest three possible mechanisms that could explain these interesting dual roles for INS-6 peptides.

First, we speculate that properties of the neurons that release INS-6 ligands regulate the functions of these neuropeptides. It is possible that ASE and ASI neurons

have differing release probabilities for INS-6 leading to these two functions. For example, in the adult, ASE neurons likely release INS-6 acutely. Upon high salt stimulation, ASE-released INS-6 immediately modifies the composition of the salt neural circuit by recruiting AWC neurons.[8] These INS-6 peptides function as fast, locally acting transmitters, much like classical small molecule and amino acid neurotransmitters. In contrast, ASI neurons of young, larval stage animals may release INS-6 tonically to influence the development of neuromuscular junctions. This sustained release of INS-6 peptides from ASI could influence neuromuscular synapses over multiple hours.[5]

A second possibility for the dual functions of INS-6 is that there is specificity in peptide processing mechanisms. Since EGL-3 and BLI-4 proprotein convertases cleave the same site in the INS-6 pro-peptide, it is unlikely that mature INS-6 peptides with different sequences are released from ASI and ASE neurons.[5,8] However, it is possible that the proprotein convertases associate with specific downstream processing components leading to differences in how INS-6 peptides are modified in these two neurons. For example, EGL-3-processed INS-6 could be amidated, while BLI-4-processed INS-6 may not be modified in the same manner. Previous results have shown that amidated peptides are more stable and can therefore exert their influence over longer timescales.[11] This difference in stability could explain the long-lasting effects of ASI-released INS-6 when compared with INS-6 peptides released from ASE neurons. This prediction of differential processing of the mature INS-6 from ASI and ASE neurons can be easily tested. We suggest that a mass spectrometry-based approach is ideal for probing the status (amidation, acetylation, etc.) of the mature INS-6 peptides that are specifically released from the ASI or ASE neurons, respectively.

A third possibility is that the distance to and nature of the receiving cells enable INS-6 to have these dual functions. In one instance, ASE and AWC neurons are in close proximity to each other and share direct synapses.[22] INS-6 signaling between these two neurons is rapid and serves to modify the target neuron (AWC). Furthermore, the target neuron (AWC) is competent to process and respond to this rapid INS-6 signal. In a second instance, ASI-released INS-6 peptides have to travel many cell diameters in order to influence distant motor neurons and their synapses on the body wall muscle[5] (**Fig. 2**). Moreover, the developing muscle targets might also retain the ability to sense INS-6 over many hours. Additionally, the downstream signaling pathways in the receiving cells may play a crucial role in transducing INS-6 signals over different timescales. For example, the rapid ASE-AWC communication is unlikely to involve transcription, while the slower ASI-motor neuron INS-6 signaling could modify the transcription of target genes involved in synapse formation. Finally, neuropeptide degradation may also contribute to the timescale of peptide actions. NEP-1, a *C. elegans* proteolytic enzyme of the neprilysin family, could degrade neuropeptides before they reach their targets.[23] Varying degradation rates across developmental stages or preferential degradation of unstable peptides (which lack amidation) could differentially restrict peptide actions. These mechanisms could enable related peptides to have divergent effects on near and distant targets.

We suggest that properties of the releasing and receiving neurons constrain the actions of all neuropeptides. Peptides could function either as local and fast acting or long-range and slower signals. Peptides with these different temporal and spatial ranges may also be processed in different ways. We speculate that similar mechanisms exist to regulate the bioactivity of neuropeptides in other species. Taken together, neuropeptides may signal across many temporal and spatial scales to introduce plasticity into neural circuits. These wide-ranging signaling mechanisms reveal an additional layer of complexity in the neuronal communication for coordinating developmental processes, processing sensory information, and driving flexible behaviors.

Behaviors: Multisensory neurons and circuits drive finely tuned behavioral choices

Across species, multimodal neurons exist, which are activated by changes in smell, taste, temperature, and other sensory experiences. These multisensory neurons are uniquely positioned to signal that global environmental conditions are improving or worsening and to direct behavior. In *C. elegans*, a subset of sensory neurons respond directly to multiple modalities.[24] Neurotransmission recruits other *C. elegans* neurons to circuits that encode several senses.[8] AWC neurons were first identified as olfactory sensory neurons.[25] However, recent work has shown that AWC neurons are also active under particular salt, temperature, and sex pheromone conditions[8,24,26,27] (**Fig. 3A**). Excited AWC neurons may release glutamate and neuropeptides, exclusively or in combination, to recruit particular downstream effector circuits, thereby shaping behavioral outputs[1,8,28] (**Fig. 3A**).

Insulin-mediated recruitment of the AWC neurons into the high salt circuit is critical for strongly exciting downstream AIA interneurons.[8] AWC uses neuropeptides, not glutamate, to signal high salt to AIA interneurons.[8] In the future, it will be interesting to determine the identity of these AWC-released, UNC-31-dependent neuropeptides. Perhaps these neuropeptides from AWC also activate or inhibit other interneurons. For instance, AWC neuropeptides may recruit the AVA neurons to the high salt circuit, even though these neurons are not targets of the ASEL neurons and so may not participate in the low salt circuit.[22] Together, the interneurons and motor neurons downstream of the AWC neurons potentiate chemotaxis behavior toward high concentrations of salt.[8] This circuit configuration may expand the upper end of the dynamic range of salt-guided behaviors. This could promote additional salt consumption and improved ion homeostasis for animal survival. Moreover, flexible, AWC circuits may integrate information about salt conditions with all other AWC-detected sensory modalities to regulate attraction behaviors (**Fig. 3A**).

AWC neurons may express the receptors necessary for directly sensing temperature and sex pheromones.[24,27] AWC neurons release glutamate to activate downstream interneurons and promote migration to warmer temperatures.[28] In neural circuits for temperature, AWC neurons may

integrate temperature information with all of the other environmental conditions that they detect (**Fig. 3A**). AWC neurons could then fine-tune a behavioral program that was initiated by the broadly tuned primary thermosensory neurons, AFD (**Fig. 3A**).[24] The same principle may apply to AWC's role in sex pheromone detection and male mating.[27] AWC may contribute a global view of the sensory environment to shape male mating behavior, which was initiated by other sensory and male-specific neurons.[27] Together, these results demonstrate a general organizing principle: individual multisensory neurons can participate in overlapping circuits that drive behaviors directed toward multiple sensory modalities.

Multisensory neurons and circuits have been found in many species with significantly larger and more complex nervous systems than *C. elegans*.[29-31] However, to maximize the coding potential of their compact nervous system, *C. elegans* sensory neurons themselves may actually perform the integration or other computations that are typically reserved for higher order neurons of flies (*D. melanogaster*) and mammals. For instance, information from peripheral olfactory sensory neurons and gustatory neurons are integrated in flies by the dorsal paired medial (DPM) neurons in the central nervous system, for appropriate behavior in some memory tasks (**Fig. 3B**).[31] The neuropeptide AMN, a homolog of the mammalian pituitary adenylate cyclase-activating peptide, is required acutely for this multisensory behavior (**Fig. 3B**).[31] Another study showed that insulin and short neuropeptide F signaling modulates flies' olfactory sensitivity and food-search behavior based on global satiety signals.[32] Neuropeptides have also been shown to be important for various integrative behaviors in mammals.[33] For example, oxytocin peptides from the hypothalamus alter the release probability of neurons in the nucleus accumbens and have effects on multisensory social behaviors.[33] In summary, animals of all species integrate diverse sensory cues with state-dependent information to drive motor patterns that will improve the animal's quality of life. As the above examples show, neuropeptide signaling plays an important role in shaping the multisensory neural circuits and their motor outputs.

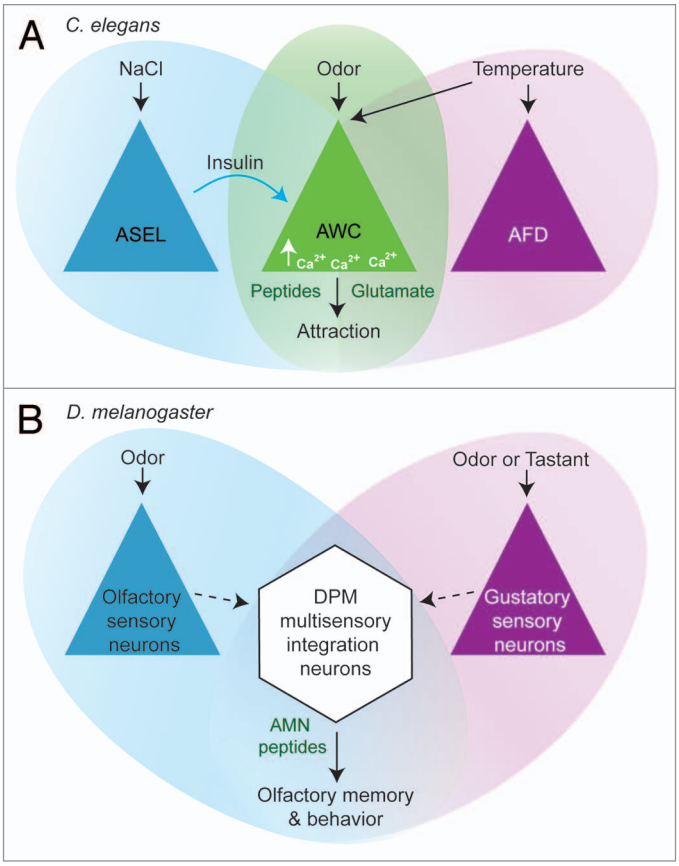

Figure 3. Behaviors: multisensory neurons integrate environmental cues to drive appropriate behaviors. (**A**) *C. elegans* AWC neurons are multisensory. AWC neurons sense and integrate information about odor together with salt information sent by the ASEL salt sensory neurons and temperature information that is also detected by the AFD thermosensory neurons.[8,24,25] AWC neurons then release neuropeptides and/or glutamate to downstream circuitry to drive behavioral attraction toward multiple sensory modalities.[1,8,28] (**B**) *D. melanogaster* olfactory and gustatory sensory neurons detect odorants and tastants in the environment and relay the sensory information to the dorsal paired medial (DPM) neurons, which function as multisensory integrators.[31] DPM neurons release AMN peptides to shape olfactory memory and behavior.[31]

Conclusions

Animals have neural circuits that enable them to process and respond appropriately to dynamic, multisensory features of their environments. At the level of genes, circuits, and behaviors, we have highlighted mechanisms that expand the coding potential of the nervous system. These principles of neural circuit flexibility and neuropeptide regulation, which were uncovered in *C. elegans,* are likely to be conserved in other species. Therefore, they present a major challenge to researchers embarking on large-scale brain activity mapping projects like the BRAIN initiative. Here, we summarize the key points:

(1) Genes: INS-6 neuropeptides are processed by a proprotein convertase, BLI-4. Mature insulins are released from dense core vesicles in an UNC-31-dependent manner, upon strong stimulation and a large rise in intracellular calcium.

(2) Circuits: Neuropeptides have spatial and temporal constraints on their bioactivity, which may result from their processing, release, and/or downstream signaling. The identity of an insulin neuropeptide is insufficient to predict its function. Moreover, the vast diversity of neuropeptide signaling mechanisms add a dynamic, new language to the nervous system.

(3) Behaviors: *C. elegans* sensory neurons integrate multisensory cues to drive

motor outputs that are appropriate for the global environment. Higher order neurons in *D. melanogaster* and mammals integrate multiple sensory stimuli with physiological needs to coordinate behavior. Neuropeptide signaling also fine tunes behavior by introducing flexibility to the multisensory neurons and circuits.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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