Discovery of leucokinin-like neuropeptides that modulate a specific parameter of feeding motor programs in the molluscan model, *Aplysia*

Guo Zhang[1]*, Ferdinand S Vilim[2]*, Dan-Dan Liu[1]*, Elena V Romanova[3]*, Ke Yu[1], Wang-Ding Yuan[1], Hui Xiao[1], Amanda B Hummon[3], Ting-Ting Chen[1], Vera Alexeeva[2], Si-Yuan Yin[1], Song-An Chen[1], Elizabeth C Cropper[2], Jonathan V Sweedler[3], Klaudiusz R Weiss[2], Jian Jing[1,2]

From [1]State Key Laboratory of Pharmaceutical Biotechnology, Collaborative Innovation Center of Chemistry for Life Sciences, Jiangsu Engineering Research Center for MicroRNA Biology and Biotechnology, Advanced Institute for Life Sciences, School of Life Sciences, Nanjing University, Nanjing, Jiangsu, China, [2]Department of Neuroscience and Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, New York, United States of America, [3]Beckman Institute for Advanced Science and Technology and Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois, United States of America

* These authors contributed equally to this work.

Running title: *Aplysia* leucokinin-like neuropeptides and feeding modulation

To whom correspondence should be addressed: Jian Jing, Ph.D., School of Life Sciences, Nanjing University, Nanjing, Jiangsu 210046, China, Telephone: 025-8968-0241; FAX: 025-8968-0241; E-mail: jingj01@live.com

Current address for Dr. A.B. Hummon: Department of Chemistry and Biochemistry, 251 Nieuwland Science Hall, University of Notre Dame, Notre Dame, IN 46556

Keywords: Leucokinin, feeding, *Aplysia*, molluscs, arthropods, single cell mass spectrometry, neuromodulation

ABSTRACT

A better understanding of neuromodulation in a behavioral system requires identifying active modulatory transmitters. Here, we used identifiable neurons in a neurobiological model system, the mollusc *Aplysia*, to study neuropeptides, a diverse class of neuromodulators. We took advantage of two types of feeding neurons, B48 and B1/B2, in the *Aplysia* buccal ganglion that might contain different neuropeptides. We performed a representational difference analysis (RDA) by subtraction of mRNAs in B48 *versus* mRNA in B1/B2. The RDA identified an unusually long (2025 amino acids) peptide precursor encoding *Aplysia* leucokinin-like peptides (ALKs, *e.g.*, ALK-1 and ALK-2). Northern blot analysis revealed that, compared with other ganglia, *e.g.,* the pedal-pleural ganglion, ALK mRNA is predominantly present in the buccal ganglion, which controls feeding behavior. We then used *in situ* hybridization and immunohistochemistry to localize ALKs to specific neurons, including B48. MALDI-TOF MS on single buccal neurons revealed expression of 40 ALK precursor-derived peptides. Among these, ALK-1 and ALK-2 are active in the feeding network; they shortened the radula protraction phase of feeding motor programs triggered by a command-like neuron. We also found that this effect may be mediated by the ALKs-stimulated enhancement of activity of an interneuron, which has previously been shown

to terminate protraction. We conclude that our multipronged approach is effective for determining the structure and defining the diverse functions of leucokinin-like peptides. Notably, the ALK precursor is the first verified nonarthropod precursor for leucokinin-like peptides with a novel, marked modulatory effect on a specific parameter (protraction duration) of feeding motor programs.

In both invertebrates and vertebrates, neuropeptides are the most diverse class of neuromodulators (1-4). This makes it challenging to identify the full complement of peptides that modulate a particular network. Much progress along these lines has, however, been made in experiments that have investigated peptidergic neurotransmission in experimentally advantageous model organisms. The present study is conducted in one such preparation, the mollusc *Aplysia californica*.

A number of bioactive neuropeptides have been identified in *Aplysia* using biochemical techniques to purify substances, molecular biological tools to characterize neuropeptides and their precursors. In particular, many peptides that have been characterized are present in connectives and identified neurons, including motoneurons, of the feeding network (5-13). This network has been extensively characterized, e.g., sensory neurons, motoneurons, and interneurons have been identified (14-25). The *Aplysia* feeding network is, therefore, ideally suited for functional studies of peptidergic modulation. In this report, we demonstrate that it contains leucokinin-like peptides.

Previous studies of the leucokinin family of peptides have been primarily conducted in arthropods. Members of this family were initially purified from the cockroach *Leucophaera maderae* using biochemical methods (26-30). Subsequently, leucokinin-like peptides were identified in a number of other insect species (31). Insect leucokinins have been studied in physiological experiments with most studies focusing on their myoactivity in the hindgut and increasing diuretic activity via the insect Malpighian tubules (31-33). Further, more recent work has demonstrated that leucokinin signaling pathways play a role in regulating feeding behavior in *Drosophila* (34). Despite this progress, there are still major gaps in knowledge. For example, bioinformatic studies have predicted the amino acid (aa) sequences for leucokinin precursors in non-arthropods (35,36). However, these gene products have not been experimentally verified. It is therefore not clear how extensive the leucokinin family actually is in metazoa. Also, the descriptions of actual expression patterns of leucokinins in the nervous systems and their functional roles are unavailable in non-arthropods, and are poorly understood even in some arthropod species (37). Importantly, although neuropeptides can alter feeding responses by modulating the feeding central pattern generator (CPG) that controls the feeding movements (2-4,38), no prior studies have examined the effects of leucokinins on any feeding CPG, and this is one of our main objectives in this work.

Although there have been relatively few leucokinin-like peptides predicted in non-arthropod species, Cox et al 1997 (39) did biochemically identify a single leucokinin-like peptide in *Lymnaea*. This suggested that leucokinin-like peptides could be present in *Aplysia*, which, like *Lymnaea*, is a gastropod mollusc. To determine whether there are novel peptides, such as leucokinins, in *Aplysia*, we performed representational difference analysis (RDA) (11,40,41) by subtraction of mRNAs in two types of identified neurons in the feeding circuit. This is a nontargeted approach that can potentially identify any mRNA that is unique to one neuron. RDA in various forms has been successfully utilized in other model systems including vertebrates (42,43).

Using RDA, we identified an *Aplysia* leucokinin-like peptide (ALK) precursor. The precursor that we identify is one of the longest neuropeptide precursors characterized to date. Additionally, we mapped the distribution of ALK-containing neurons in the *Aplysia* CNS using immunohistochemistry and *in situ* hybridization, and demonstrated that ALKs are indeed expressed in single neurons using single cell MALDI-TOF

mass spectrometry. Finally, we showed that ALKs are bioactive in the *Aplysia* feeding network, where they modulate a specific parameter of feeding motor programs partly through a direct action on an identified element of the feeding CPG, interneuron B64. Thus, ALK precursor is the first verified non-arthropod leucokinin precursor. Together with previous work in *Drosophila* (34), these results suggest that the leucokinins may play an important role in regulating feeding behavior.

RESULTS

Identification of ALK precursor by RDA, cDNA library screening and RACE and bioinformatics

RDA takes advantage of the fact that two types of neurons, i.e., a tester and a driver, may express different complements of mRNA which may code proteins including neuropeptide precursors. To identify neuron specific proteins, cDNA is amplified and the cDNA from one neuron is subtracted from the cDNA of the second neuron. This technique is non-targeted, meaning that it can potentially identify any proteins/peptides that are present in the tester, but not in the driver, and has been used successfully to identify several novel proteins in *Aplysia* (11,40,41).

In the current work, we used buccal motoneuron B48 (14,44) as a tester and two large neurons B1/B2 as a driver. The first round of subtraction between B48 and B1/B2 allowed abundant RNAs that are unique to B48 to appear as prominent difference bands between 500bp and 750bp (Fig. 1). In some cases, one round of subtraction permits identification of clones that contain unique products (11,41). In other cases (40) and in the present study, this was not the case. Therefore, a second round of subtraction was performed by hybridizing the subtracted cDNA from the first round with the driver's DNA. In addition, cDNAs corresponding to peptide precursors known to be expressed by the tester neuron B48 (i.e., myomodulin cDNA) were added to exclude these cDNAs. Subcloning the products from the second subtraction produced many colonies that contained unique products (as determined by differential screening). The difference clones obtained from B48 were sequenced to reveal an open reading frame that contained partial sequences of several hundred bps. Sequences were considered partial because at the 5' end, there was no start codon, and at the 3' end, there was no poly "A" addition site.

In order to obtain a complete sequence, we used the RDA fragments to screen a poly T bluescript library and then used RACE to extend the sequence. We successfully obtained a clone with 6591 bp, that contained a complete protein of 2025 aa in length (Fig. 2). The gene sequence has been deposited into the NCBI database (GenBank accession number: MF664476). The precursor is predicted by NeuroPred (http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py) (45,46) to code a number of putative neuropeptides, some of which appear in multiple copies. Some of these sequences are homologous to the insect leucokinins and have an amidated C-terminal pentapeptide motif: FXXWX-amide. Consequently, we putatively named the peptides *Aplysia* leucokinin-like peptides (ALK). We named the most abundant ALK on the precursor ALK-1. Its sequence is PAFHSWSamide and it is present in 20 copies. There are 7 copies of ALK-2 (PAFHAWSamide), and two copies of PAFSAWSamide (Fig. 2). There is only one copy of the 13 other amidated peptides (see Table S2). A comparison of ALK-1 and ALK-2 with leucokinin-like peptides in various species is shown in Table 1. Leucokinin-like peptides in insects typically have an F amino acid residue near the N-terminus and have a C-terminal WGamide. Gastropod molluscs differ in that the C-terminal is WSamide. In addition, we compared some selected leucokinin-like peptide precursors, which are listed in Table S1. For these precursors, we generated a sequence alignment (Fig. S1) and a hypothetical phylogenetic tree (Fig. S2).

While ALK preproprotein has a structure characteristic of the neuropeptide prohormone, encodes numerous putative peptides, and has a priming methionine, its signal peptide cannot be identified reliably. Using PrediSi

(http://www.predisi.de/) prediction (47), there is a 57% chance of signal peptide cleavage site between Y[50] and A[51] in the preproprotein (Table S1). Our MS analysis later indicate that a peptide detected closest to the N-terminus was S[75]-V[98]. Therefore, the exact length of the ALK signal peptide remains unknown.

We searched the NCBI database (including the EST database), and found several short matching nucleotide sequences ranging from 218-781 bp that matched 20% of the ALK sequence in the beginning and the end portions of the precursor. These short sequences are EST deposits from an earlier *Aplysia* genome project (48). We then searched a recently completed *Aplysia* EST database (http://www.*Aplysia*genetools.org/) which has become a more popular source for *Aplysia* sequences. Indeed, we found several long matching sequences that showed more than 99% similarity with the ALK mRNA sequence in the following ranges: 37-990 (comp80863_c0_seq1); 1119-5215 (comp127554_c0_seq3), 5216-6578 (comp88691_c3_seq1) (Fig. S3). Overall, these three sequences matched 81.52% of ALK, providing independent support for the ALK precursor sequence. The search results are consistent with the idea that there is a single *Aplysia* gene that encodes leucokinin-like peptides. However, we cannot rule out the possibility that an additional gene exists.

Distribution of ALK mRNA and immunoreactivity in the Aplysia CNS

We performed a Northern blot analysis to determine the distributions of ALK mRNA in specific ganglia of *Aplysia*. Fig. 3 shows that ALK mRNA is predominantly expressed in buccal ganglia. ALK mRNA is ~ 8 kb in length (of which ~ 6kb is the coding sequence), which is in the similar range with the long mRNA (6591bp) that we identified. ALK mRNA was not detected in other ganglia. It is likely that this is due to the fact that the overall mRNA level in these ganglia was too low to be detected.

To map ALK positive neurons and their processes in the CNS of *Aplysia*, we used two methods: *in situ* hybridization performed on whole mounts obtained from small size animals (~10 gm) (n = 3 for each ganglion), and immunohistochemistry performed on whole mounts obtained from larger animals (> 90 gm) (n = 3 for each ganglion). Smaller animals are preferred for *in situ* hybridization experiments as they tended to generate less background. For immunohistochemistry experiments, we raised a polyclonal antibody against ALK-1. The specificity of the antibody was demonstrated by preabsorption experiments which abolished staining (Fig. S4*B*). We detected ALK positive neurons in all central ganglia, i.e., in the buccal ganglion (Fig. 4*A, B*), the cerebral ganglion (Fig. 4*C, D*), the pleural-pedal ganglion (Fig. 5*A-D*), and the abdominal ganglion (Fig. 5*E, F*). Notably, the patterns of cell body staining by both *in situ* hybridization and immunostaining are similar, providing further support for the specificity of the antibody we generated.

Consistent with Northern blot data, the heaviest labeling was observed in the buccal ganglion. Particularly well labeled was a cluster of dorsal-lateral cells that can be seen in both the rostral and caudal surfaces near the roots of the esophageal nerve (EN) and the buccal nerve 1 (Fig. 4*A2, B2*). Some neurons in this location project their axons in the cerebro-buccal connective (CBC). To determine whether this is the case for the ALK positive neurons, we backfilled the buccal end of the CBC (Fig. 6*B1*) and processed preparations for immunohistochemistry (Fig. 6*B2*). Within the limits of our resolution, 12 of the 13 ALK immunoreactive neurons were doubled labeled and therefore their axons are present in the CBCs. Note that the giant, largest B1/B2 neurons on the caudal surface (Fig. 4*B*), used as the driver for RDA, were negative in both *in situ* hybridization and immunohistochemistry experiments. This also indicates that the antibody we used is specific.

In the cerebral ganglion, we observed ALK positive neurons scattered on the dorsal surface, and within the G and E clusters of the ventral surface. Some neurons in the E cluster may be cerebral-buccal interneurons (CBIs) (21,49-51). In

immunostaining, we observed axons of at least some of these neurons that appeared to continue out of the anterior and posterior tentacular nerves (AT and PT) (Fig. 4*C2, D2*). Additionally numerous immunoreactive axons were present in CBC (Fig. 4*A2, B2, D2*). These results are consistent with the idea that ALKs play a role in feeding as feeding motoneurons and pattern-generating interneurons are located in the buccal ganglion, and feeding higher-order interneurons (i.e., CBIs) are located in the cerebral ganglion (52-54).

Other ganglia, including the pleural-pedal and abdominal ganglion, did show staining of some scattered cells, but the overall staining was relatively weak. Moreover, immunostaining showed little staining of axons.

Because the buccal motoneuron B48 was the original source of the tester material used for RDA, we performed double-labeling experiments to determine whether B48 is ALK immunoreactive (n = 3). We physiologically identified B48 neurons, injected carboxyfluorescein (Fig. 6*A1*), then performed immunohistochemistry (Fig. 6*A2*). B48 was indeed ALK immunopositive.

Mass spectrometric analysis of prohormone processing and posttranslational modifications (PTMs)

MALDI-TOF MS is well suited for profiling neuropeptides from small samples such as individual *Aplysia* neurons (55,56). Here we isolated eight individual buccal neurons from left and right dorsal-lateral clusters expressing ALK mRNA and showing immunoreactivity, and profiled them using MALDI-TOF MS to determine the processing of the ALK precursor and the structure of the final peptides expressed. We detected a total of 40 peptides that had masses that matched *in-silico* predicted (45,46) ALK precursor-derived peptides. The most intense signal corresponded to the amidated form of ALK-1, which is present in 20 copies on the prohormone, at m/z 830.402 ±10 ppm (Fig. 7 and Table S2). Another amidated peptide, ALK-2, was also detected at high intensity. In total, 16 predicted amidated peptides and 24 linker peptides have been detected (Table S2). Among the 16 amidated peptides, all but one (DSPRMFAFNSLS[Amide]) have a C-terminal pentapeptide motif (FXXWX-amide) (see Discussion) with the last aa being an S, G, A, or N, and thus are classified as ALKs (Fig. 2). Isotopic pattern fit has been calculated to verify assignment of select peptides with close molecular weights and to resolve their overlapping isotopic clusters in the mass spectrum (PAFSPWS-NH2, m/z 790; PAFSAWN-NH2, m/z 791; PAFHSWS-NH2, ALK-1, m/z 830; PAAFHAWS-NH2, m/z 885; PAFHSWSG, m/z 888; PRFHAWS-NH2, m/z 899; LAAFHAWS-NH2, m/z 901). Interestingly, among detected peptides, four are presumably processing intermediates (Table S2).

We also performed MALDI-TOF MS analysis of the CBC to determine whether it contains ALK precursor-derived peptides, i.e., whether ALK precursor-derived peptides are present in cerebral-buccal and/or buccal-cerebral interneurons. These two types of interneurons play an important role in the generation of feeding motor programs (16-18,22,49). We did indeed detect ALK precursor-derived peptides, including ALK-1 and ALK-2, in the CBC. These data are consistent with the idea that the ALKs may influence feeding.

Modulatory actions of ALKs in the feeding circuit

Feeding in *Aplysia* has both appetitive and consummatory phases (57). Since food is ingested during the consummatory phase, it has been the subject of much research (49,58,59). Consummatory behaviors are mediated by the radula, which protracts and retracts and opens and closes. Behaviors are ingestive if the radula closes during retraction (to pull food into the buccal cavity), and are egestive if the radula closes during protraction (which pushes food out) (58,59).

Previous studies have shown that fictive feeding or feeding motor programs, that are an *in vitro*

representation of consummatory feeding, can be elicited in the isolated CNS by stimulation of a command-like interneuron, CBI-2 (14,16-18,20 ,22,49,60). CBI-2 evokes motor programs through its excitatory actions on pattern-generating interneurons in the buccal ganglion, such as B34, which in turn provide inputs to other pattern-generating neurons. Pattern generating interneurons drive motoneurons in the buccal ganglion, which ultimately produce the behavior.

In the isolated CNS, radula protraction is monitored by recording extracellularly from the I2 nerve, which contains axons of protraction motoneurons (15). Radula retraction can be indirectly monitored by recording intracellularly from protraction interneurons, i.e., they are hyperpolarized during retraction. Radula closing is monitored by recording intracellularly from the B8 motoneurons. Motor programs are ingestive when B8 is predominately active during retraction, and are egestive when B8 is active during protraction, and intermediate when B8 is active during both protraction and retraction.

We sought to determine whether the ALKs are bioactive in the feeding circuit. We tested both ALK-1 and ALK-2 and found that their effects were similar, so we pooled the results from ALK-1 and ALK-2 together. In the first set of experiments, we examined whether the ALKs can modulate parameters of motor programs elicited by CBI-2. We elicited a single-cycle of a motor program by stimulating CBI-2 at 8-10 Hz. The inter-stimulation interval was 1.5 min. One such experiment is shown in Figure 8*A1-4*. Under control conditions (Fig. 8*A1*), CBI-2 stimulation elicited an ingestive motor program, i.e., B8 was predominantly active during retraction. When the preparation was superfused with 10^{-6} M ALK (Fig. 8*A2*), and 10^{-5} M ALK (Fig. 8*A3*), there was a concentration dependent shortening of protraction (Fig. 8*A5*, $F(3, 21) = 40.93$, $p < 0.001$, $n = 8$). Other phases of the motor program were not significantly altered (i.e., there was no effect on retraction duration (Fig. 8*A6*, $F(3, 21) = 0.63$, $p > 0.05$, $n = 8$), or on B8 activity during protraction (Fig. 8*A7*, $F(3, 21) = 1.68$, $p > 0.05$, $n = 8$) or retraction (Fig. 8*A8*, $F(3, 21) = 0.79$, $p > 0.05$, $n = 8$).

Similar to most rhythmic behaviors, *Aplysia* feeding behavior is controlled by a CPG. In a subsequent set of experiments, therefore, we sought to determine whether the ALKs shorten the duration of the protraction phase via modulation of the CPG. Specifically, we examined the effects of ALKs on the excitability of the CPG element, interneuron B64. Previous studies have shown that B64 acts as a terminator of protraction and thus as a shortener of protraction (15,61). We found that the ALKs increased B64 excitability in a concentration-dependent manner (Fig. 8*B*, $F(3, 21) = 12.97$, $p < 0.001$, $n = 8$). In addition, the ALKs decreased B64 threshold to spike (Fig. 8*C*, $F(3, 6) = 10.37$, $p < 0.01$, $n = 3$).

DISCUSSION

Here, we used RDA and RACE to discover a novel, unusually long neuropeptide precursor that encodes *Aplysia* leucokinin-like peptides (ALKs). Below, we discuss our approach, and evolutionary and functional aspects of leucokinin-like peptides.

Utility of our approach and properties of the ALK precursor

It is most notable that the ALK precursor is the first verified and functionally characterized leucokinin-like precursor outside of arthropods (see Figs. S1 and S2). Importantly, RDA proved to be critical for the identification of this protein. Querying the NCBI public depositories with ALK precursor, we did not find any leucokinin precursors from other animals. This is not surprising given the fact that shared motifs (Table 1), that interact with their cognate receptor, are short, i.e., around 5 amino acids. Large sections of leucokinin-like prohormones are often species specific (see Figs. S1 and S2). Thus, the homology of the full length prohormone to well-studied species is not long enough to offer accurate neuropeptide gene annotation by similarity across species. In addition, searches of NCBI EST (48) and of a recently completed, a more complete transcriptome database (*Aplysia* gene tools) (Fig. S3) did come up with some

matching sequences, but they were all shorter pieces, none of them encoding the complete ALK precursor. Absence of the ALK precursor in any currently available database reinforces the utility of our RDA-based approach in identifying long sequences like ALK precursor.

RDA is a non-targeted approach to identify potentially novel peptide precursors that are present in motoneuron B48, but not in neurons B1/B2. RDA generated incomplete transcripts. Our subsequent usage of RACE enabled us to generate the complete precursor, i.e., the unusually long ALK precursor. By contrast, all currently identified/predicted leucokinin-like peptide precursors, including several unverified molluscan leucokinin-like peptide precursors (Fig. S1, and Table S1) range from 97 to 347 aa. At present, it is not clear why the ALK precursor is so long. This may, however, become clearer as additional leucokinin-like peptide precursors are identified in other molluscs, including other *Aplysia* species. Regardless, it is likely that the ALKs play critical regulatory roles in *Aplysia*, given that a number of copies of ALK-1 and ALK-2 can be expressed and secreted through a single translation.

We had difficulty in determining the signal peptide for the ALK precursor. Perhaps, the occurrence of numerous basic and acidic amino acid residues in the predicted amino terminus of the prohormone precursor made it less likely to form hydrophobic alpha-helix (H-region) in the center of a signal peptide. In fact, the exact length and location of the cleavage site of the signal peptide cannot be confidently predicted by prediction tools (47,62) (Table S1). We found a similar challenge with the leucokinin-like peptide precursor of *Lucilia cuprina* (Table S1) where signal peptide cleavage is predicted at a low 71% probability. This suggests that signal peptide predictions may be challenging for some neuropeptide precursors. Regardless, the presence of ALKs in the CNS is indicated by our immunostaining and direct mass spectrometric measurement. The latter experiments detected multiple processed peptides in individual neurons and in the CBC. Thus, our data indicate that ALK transcript is expressed, translated, and post-translationally processed like a typical neuropeptide prohormone. Moreover, numerous processed peptides undergo further PTM, i.e., amidation of C-terminus, which often renders biological activity to mature peptides.

Evolutionary relationship of leucokinin-like peptides

To compare peptides, we selected a number of identified and predicted leucokinins from other species (Table 1). We included peptides from molluscs, annelids, and nematodes. Some of the included peptides are predicted in silico from putative precursors, and some of the predicted precursors are incomplete. Leucokinin-like peptides in arthropods (mostly insects) and Annelids share an amidated C-terminal pentapeptide motif called FXXWG-amide. In nematodes, the motif becomes FXXWA-amide. In molluscs, leucokinin-like peptides showed some variability in the last amino acid. In most cases (including *Lymnaea* (39) and *Aplysia*), this motif is FXXWS-amide. In fact, the *Lymnaea* leucokinin and ALK-1 only differ by a single amino acid. Other motifs, such as FXXWG-amide, FXXWA-amide are observed. Indeed, *Aplysia* also express a FXXWG-amide peptide (PAFHAWGamide) and a FXXWA-amide peptide (AGFAPWAamide), and both are present in single copies (Fig. 2 and Table S2). Thus, the ALK precursor encodes peptides matching most motifs currently identified for leucokinin-like peptides, which is perhaps not surprising given its unusually long sequence. This also suggests that ALK precursor may reflect the structure of an ancient leucokinin-like peptide prohormone.

Initially, leucokinins were thought to be orthologues of the vertebrate tachykinins (which include substance P) (e.g., (34)). Tachykinins have a somewhat similar C-terminal pentapeptide motif, FXGLM-amide. The similarity between the *Drosophila* leucokinin receptor (Lkr) and vertebrate tachykinin receptors appears to support this notion (63). However, the *Drosophila* genome encodes several other peptides with a C-terminal motif (FXGXR-amide) that are more

similar to tachykinins than leucokinins (31,64). More recently, two extensive bioinformatic studies of a large number of verified and predicted neuropeptide precursors and G-protein coupled receptors (GPCRs) (35,36) have demonstrated that tachykinin orthologues are present in both protostomes and deuterostomes (see Dataset S1 of (36)). On the other hand, leucokinin orthologues are only present in protostomes (including arthropods and molluscs) (35,36,65). Thus, it is likely that leucokinin orthologues are not orthologues of the tachykinins and are not even present in vertebrates.

In protostomes, most of the known leucokinins, and their precursors, are from arthropods. The ALK precursor is the first complete, structurally and functionally characterized precursor that is not present in an arthropod. The two bioinformatics studies mentioned above (35,36) did, however, identify *in-silico* leucokinin-like precursors that are non-arthropod. Some of these are incomplete transcripts. Others are complete and include precursors from the annelid *Malacoceros fuliginosus*, the mollusc *Tritonia diomedea*, and the nematode *Trichinella spiralis* (Fig. S1 and Table S1, some of the predicted leucokinin-like peptides are listed in Table 1).

Diverse functions of leucokinin-like peptides: roles in feeding

Prior to our study, functional studies of leucokinin-like peptides have been almost entirely conducted in insects. An exception, however, is research that has been conducted in *Lymnaea*. The GPCR for the *Lymnaea* leucokinin has been identified, and one biochemically isolated *Lymnaea* leucokinin (i.e., lymnokinin) exerts effects via this receptor (39). The lymnokinin distribution in the CNS has, however, not been determined using either *in situ* hybridization or immunohistochemistry.

In insects, although earlier work focused on the roles of leucokinin-like peptides in the digestive system and fluid secretion (31,34), a more recent study conducted in *Drosophila* demonstrated that leucokinin signaling pathways regulate meal size, indicating a major role in feeding (34). This is the first and only functional study of the role of the leucokinins in feeding.

In the present work, we provide multiple lines of evidence that clearly show that ALKs play a marked role in *Aplysia* feeding (Fig. 8*D*). First, Northern blots, *in situ* hybridization, immunohistochemistry and MALDI-TOF MS all demonstrate that the ALKs are predominantly located in the buccal ganglion, which directly controls feeding. Immunostaining also shows the ALKs are present in the CBC, which links the buccal ganglion to the cerebral ganglion (which also contains feeding circuitry). Thus, the ALKs are present in feeding neurons and processes.

Second, we directly demonstrate that ALKs modulate one specific parameter of the protraction-retraction sequence of consummatory feeding, i.e., they shorten protraction in motor programs evoked by a command-like neuron in the isolated CNS. Previous studies have demonstrated that identical patterns of motor activity can be recorded from both behaving animals (59,66) and *in vitro* ganglia (17,60). Thus, the *in vitro* effects of ALKs likely translate to effects on consummatory feeding behavior. In addition, we identified a potential mechanism by which ALKs shorten protraction because ALKs enhance the excitability and decrease the spiking threshold of an identified interneuron (B64). This neuron is normally active during retraction and serves to terminate protraction (15,61). An increase in its excitability and a decrease of threshold would therefore be expected to shorten protraction.

Future studies may identify the ALK positive neurons that release ALKs and shorten protraction. For example, it would be of interest to identify the ALK positive neurons that send their axons to the CBCs. Regardless of which neurons are the sources of ALKs, the current study and the work on *Drosophila* (34) both support the idea that leucokinin-like peptides play a critical role in various aspects of consummatory feeding. Importantly, in both *Drosophila* and *Aplysia*, leucokinin-like peptides do not prevent the

execution of feeding behavior or the generation of motor programs. Rather, they exert modulatory actions. Specifically, our study suggests that ALKs may promote faster consumption of food because a shorter protraction is likely to result in a shorter cycle period. In contrast, in *Drosophila*, leucokinins function to reduce meal size and increase meal frequency, possibly by enhancing a gut distension signal. The *Drosophila* work used a gross measure of feeding, i.e., the amount of food intake, but did not monitor the duration of individual feeding movements. Consequently, it is presently unclear how the feeding effects in the two species may be related. Importantly, to the best of our knowledge, our findings represent the first demonstration of modulation of the feeding CPG by leucokinin-like peptides as this has not been previously demonstrated in any animals, including arthropods.

In addition, given the presence of a number of ALK positive neurons in the buccal and cerebral ganglia, it is likely that ALKs may also play a role in other aspects of feeding, including regulation of meal size and frequency as is the case in *Drosophila*, which would be another interesting subject for future studies. In this context, it is notable that *Aplysia* Neuropeptide Y plays a role during the transition from hunger to satiation, and reduces meal size (67). Effects of *Aplysia* Neuropeptide Y are exerted by reconfiguring the feeding CPG and converting ingestive programs to egestive activity. In contrast, if ALKs also reduce meal size, it is unlikely they do so by reconfiguring the feeding circuit. This is suggested by the fact that we demonstrate that patterns of B8 activity during protraction and retraction did not change (Fig. 8*A7-8*). This indicates that program type was not altered.

Finally, wide distributions of ALKs in the *Aplysia* CNS also suggest that ALKs may have broader functions in the modulation of other behavioral networks. Although not as prominent, ALK positive neurons are present in the pleural-pedal and abdominal ganglia, which mediate other behaviors, such as locomotion and visceral functions. Indeed, some other roles of leucokinins in insects been reviewed recently (68).

In summary, we used RDA to identify ALK mRNA. We mapped the distribution of ALKs, and characterized proteolytic processing and PTM of ALK using single-cell mass spectrometry. Finally, we demonstrated a novel role of ALKs in modulating a movement parameter of feeding motor programs. Importantly, we identified a CPG element as a target of leucokinin modulation that may carry out leucokinin action on the duration of the protraction phase of feeding responses. ALK precursor represents a first verified precursor for leucokinin-like peptides outside of arthropods. Our functional studies of ALK in the feeding system taken together with previous work in *Drosophila* establish that leucokinin-like peptides play a regulatory role in different aspects of feeding, supporting their diverse functions in protostomes. Moreover, our multidisciplinary approach based on RDA is also likely to be useful in other model systems in which specific neurons are identifiable.

EXPERIMENTAL PROCEDURES

Animals — Experiments were performed on *Aplysia californica* (10-300 g), which are purchased from Marinus Scientific (Long Beach, CA) and the Aplysia Research Facility (Miami, FL). *Aplysia* are hermaphroditic (i.e., each animal has reproductive organs normally associated with both male and female sexes). Animals were kept in an aquarium containing aerated and filtered artificial seawater (Instant Ocean, Aquarium Systems Inc., Mentor, OH) at 14-16 °C until use. The animal room was equipped with a 24 h light-dark cycle with light period from 6:00 am to 6:00 pm. Prior to dissection, animals were anesthetized by injection of isotonic 333 mM $MgCl_2$ (about 50% of body weight) into the body cavity.

Reagents and peptides — All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. We synthesized two *Aplysia* leucokinin-like peptides: ALK-1

(PAFHSWSamide) by ChinaPeptides Co., Ltd., ALK-2 (PAFHAWSamide) by PeptidoGenic research Co., Inc. The peptide standards for mass spectrometry (MS) calibration were supplied by Bruker Daltonics (Bremen, Germany).

RDA procedures—Previously, we modified the RDA approach (69,70) to identify novel peptides from single identified neurons in the *Aplysia* CNS, as described in detail elsewhere (11).

Briefly, the entire procedure can be divided into three steps (Fig. 1): (1) Isolation of two types of identified cells where the first is the cell of interest, or "tester," whose proteins are to be identified. The second cell, or "driver," is used to subtract sequences that are shared with the tester. The rationale behind this RDA approach is that the tester contains one or more proteins not present in the driver. In this study, the buccal motoneuron B48 of the feeding network was the tester, whereas buccal neurons B1/B2 were the drivers. Since any peptides unique to B48 can potentially be identified, this method can be considered a non-targeted approach for peptide identification. Multiple cells from each cell type were collected in a solution of ice-cold 50% propylene glycol and 1.2 M NaCl in diethylpyrocarbonate-treated H_2O and stored at -80°C. (2) Amplification of the cDNA from the RNA of the tester and the driver was done as described previously (71). (3) RDA (69,70) with the amplified cDNA of the tester and the driver was performed. The cDNAs of the driver and tester were digested with DpnII and the driver cDNA was ligated to R-Bam adaptors, while the tester cDNA was ligated to N-Bam adaptors. DpnII was used to cut cDNA with an average length of 256 bp that contain overhangs to facilitate ligation of adapters for PCR and to clone amplified fragments, because the overhangs are complementary to BamHI digested plasmids. The driver DNA was amplified with biotinylated R-Bam primer and then hybridized with unamplified tester cDNA. Here, we modified the RDA method by including a physical subtraction based on biotinylated driver primers, streptavidin incubation, and phenol/ chloroform extraction (72). This procedure removed the driver and with it any tester cDNA that is complementary and bound to it. We then performed the final amplification with tester N-Bam primer to further enrich the protein sequences that were unique to the tester.

Cloning—Standard molecular techniques were used except where noted. The RP p32 labeled fragments (incomplete mRNA transcripts) derived from the above RDA procedure were used to screen poly T Bluescript library. The isolated clones were subcloned and sequenced. Then RACE (rapid amplification of cDNA ends) was performed to obtain the complete sequence of ALK precursor. RACE includes the following steps. First, total RNA isolated from the buccal ganglion were reverse transcribed to cDNA. Next, 5' RACE and 3' RACE were implemented using gene specific primers.

Sequence comparison—We compared leucokinin-like peptides (Table 1) and ALK precursor and 16 leucokinin-like peptide precursors (Figs. S1 and S2). For peptide comparison in Table 1, we generated a frequency plot of each aa (aligned from c-terminus) using a Weblogo software (http://weblogo.berkeley.edu/logo.cgi) with default parameters except that bitmap resolution was changed to 300. The larger the character for each aa is, the higher frequency of the aa to appear in the group of the peptides.

For precursor comparison in Figs. S1 and S2, the 16 precursor sequences were downloaded from NCBI protein database (https://www.ncbi.nlm.nih.gov/protein) (Table S1). We generated the sequence alignment (Fig. S1) using BioEdit Software. We used MEGA7 software (http://mega.co.nz) to generate a phylogenetic tree. First, the 17 sequences were aligned by ClustalW using default parameters. Second, the alignment result was used to perform phylogenetic analysis (Fig. S2) with Construct/Test Maximum Likelihood Tree, of

which, a phylogeny was chosen using Bootstrap method. The other parameters were set as default.

Antibodies—Antibodies were generated to ALK-1 (PAFHSWSamide). The peptide antigens were prepared as described in details previously (9,73,74). Briefly, the antigen was made by coupling ALK-1 to BSA (Sigma, catalog #A0281) using 1-ethyl-3-(dimethylaminopropyl)carbodiimide (EDC) (Sigma, catalog #E7750), and then purified. For each antigen, two male Sprague Dawley rats (250-300 g; Taconic Farms) were immunized by intraperitoneal injections. At days 21 and 42, the rats were boosted by intraperitoneal injections. Animals were killed by decapitation at 49 d, and the blood was harvested and processed for serum. Sera were aliquoted, frozen, and lyophilized or stored at 4°C with EDTA (25 mM final concentration) and thimerosal (0.1% final concentration) added as stabilizers. For antibodies that produced immunostaining, specificity was confirmed by pre-incubation overnight of the primary antibodies with the corresponding synthetic peptide, i.e., ALK-1 (100 μM), which abolished the staining (Fig. S4).

Northern blot analysis—Northern blot analysis was performed as described previously (11,73,74). Briefly, RNA was isolated from homogenized pooled ganglia using the acid–phenol method (75). RNA from each ganglion type (buccal, cerebral, pleural, pedal, and abdominal) was fractionated separately using denaturing agarose gels (1.5%) and transferred to nylon membranes (Biodyne B; Invitrogen). The RNA was immobilized with Stratalinker UV (Stratagene) and visualized by staining with 0.02% methylene blue in 0.3 M Na-acetate, pH 5.5. After washing out the excess stain with DEPC-treated water, the blot was scanned to document the amount of RNA transferred from each lane. After destaining with 1% SDS, 1 mM EDTA, and 50 mM Na_3PO_4, pH 7.2, the blot was prehybridized for 1 h at 50°C using 50% formamide, 10% dextran sulfate, 7% SDS, 10 mM EDTA, 50 μg/ml salmon sperm DNA, and 250 mM Na_3PO_4, pH 7.2. Heat-denatured, random-primed (New England Biolabs), [^{32}P] dCTP-labeled cDNA probe (ALK precursor [4848-5235 bp], 388 bp in length) was added, and hybridization was continued overnight at 50°C. Blots were washed twice for 15 min at room temperature with 2 X standard saline phosphate EDTA (SSPE) and 0.1% SDS, washed for 60 min at 50°C with 0.1 X SSPE and 0.1% SDS, and exposed to film. Autoradiographs and methylene blue-stained blots were scanned into Photoshop (Adobe Systems) and compiled into figures.

In situ hybridization—*In situ* hybridization was performed as described previously (40,71). Ganglia were digested with 1% protease type IX (Sigma-Aldrich) in10 ml of ASW for 3 h at room temperature (with rocking) to facilitate the removal of the sheath. After digestion, ganglia were washed with ASW and fixed overnight at 4°C with 4% paraformaldehyde (Electron Microscopy Sciences) in PBS. Ganglia were then washed, desheathed, and dehydrated in an ascending ethanol series. After rehydration in a descending ethanol series, e ganglia were prehybridized for 6 h and then hybridized overnight at 50°C in hybridization buffer (50% formamide, 5 mM EDTA, 5 X SSC, 1 X Denhardt's solution, 0.1% Tween 20, and 0.5 mg/ml yeast tRNA) containing 2 μg/ml digoxigenin-labeled cRNA probes made from a partial clone from RDA, which corresponded to ALK precursor [4848-5235 bp] (388 bp in length). After washout of the probes, ganglia were then incubated overnight at 4°C with a 1:200 dilution of alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Molecular Biochemicals) in PBS containing 0.1% Tween 20 (PBT), 0.2% bovine serum albumin (BSA) (catalog #A0281; SigmaAldrich), and 1% normal goat serum (NGS). After washes with PBT to remove unbound antibody, ganglia were washed with detection buffer (100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween 20, 1 mM levamisol, and 100 mM Tris-HCl, pH 9.5) and developed with 4.5 μl of nitroblue tetrazolium and 3.5 μl of 5-bromo-4-chloro-3-indolyl phosphate (Roche Molecular Biochemicals) in 1 ml of detection buffer. The

staining reaction was monitored visually and stopped by washing with PBT when the level of staining was adequate. The stained ganglia were observed and photographed using a fluorescence microscope (Nikon) with epiillumination against a white background. Photographs were taken with a Nikon CoolPix 990 digital camera, imported into Photoshop, and compiled into figures.

Immunohistochemistry — Immunohistochemistry was performed as described previously (67,74). The tissue was fixed in a buffer (4% paraformaldehyde, 0.2% picric acid, 25% sucrose, and 0.1 M NaH_2PO_4, pH 7.6), for either 3h at room temperature or overnight at 4°C. All subsequent incubations were done at room temperature. The tissue was washed with PBS, and was permeabilized and blocked by overnight incubation in blocking buffer (10% normal goat serum, 2% Triton X-100, 1% BSA, 154 mM NaCl, 50 mM EDTA, 0.01% thimerosal, and 10 mM Na_2HPO_4, pH 7.4). The primary antibody was diluted 1:250 in blocking buffer and incubated with the tissue for 4-7 d. The tissue was then washed twice per day for 2-3 d with washing buffer (2% Triton X-100, 1% BSA, 154 mM NaCl, 50 mM EDTA, 0.01% thimerosal, and 10 mM Na_2HPO_4, pH 7.4). After washing, the tissue was incubated with a 1:500 dilution of secondary antibody (lissamine rhodamine goat anti-rat; Jackson ImmunoResearch) for 2-3 d and then washed again two times with washing buffer for 1 d and four times with storage buffer (1% BSA, 154 mM NaCl, 50 mM EDTA, 0.01% thimerosal, and 10 mM Na_2HPO_4, pH 7.4) for 1 d. We observed and photographed the tissue under fluorescence microscope.

For double labeling of physiologically identified B48 cells with ALK immunohistochemistry, neurons were identified based on morphology and electrophysiological characters and injected with carboxyfluorescein (76). For double labeling with backfills, CBCs of live buccal ganglia were cut and the buccal ends of the CBCs were incubated overnight in a Vaseline well containing biocytin. This allowed biocytin transport to the somata of neurons with axons in the CBC. Ganglia were then fixed, and processed with avidin-fluorescein so that somata were green. Tissues were also processed for immuocytochemistry as described above.

Mass spectrometric analysis of peptides — To characterize ALKs in the *Aplysia* CNS, we used matrix-assisted laser desorption/ionization (MALDI) time-of flight (TOF) MS on individual ALK-expressing neurons from the buccal ganglion and the cerebrobuccal connectives (CBCs). Buccal hemi-ganglia were treated with 1% protease IX in ASW with antibiotics (100 units/mL penicillin G, 100µg/mL streptomycin, and 100 µg/mL gentamicin) for 45 min at 34°C and then desheathed. Individual buccal neurons were manually isolated according to the ALK *in situ* hybridization staining map using electrolytically sharpened tungsten needles. Isolated neurons were transferred one by one onto a stainless steel MALDI sample plate (Bruker Daltonics, Bremen, Germany) using homemade plastic micropipettes filled with deionized water. Excess liquid was aspirated from the sample plate and 0.5 µl of saturated DHB matrix (DHB: 2, 5-dihydroxybenzoic acid, 20 mg/ml deionized water) was applied onto the sample spots. Peptide profiles were measured using ultrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a Smartbeam II frequency tripled Nd:YAG solid state laser. Mass spectra were manually acquired in positive reflectron mode and calibrated externally using Bruler Peptide Mix II standards. Signals from 600-1800 laser shots fired at 2000 Hz frequency at multiple locations within each sample spot were summed into representative cell spectrum. Obtained mass spectra were processed using flexAnalysis 3.4 software (Bruker Daltonics).

To aid interpretation of the MS results, we generated a library of putative peptides encoded by the ALK prohormone using the Neuropred prediction tool (http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py) (45,46). When predicted masses of select peptides were very similar and

signals in the mass spectrum were expected to overlap, the theoretical isotopic ratios of the accurate mass formula for each of the conflicting peptides was compared with the experimentally determined isotopic patterns using the Simulated Isotopic Pattern function in flexAnalysis 3.4, and assignment was based on the best fit. Location of the putative signal peptide cleavage site was inferred from PrediSi (http://www.predisi.de/) model (47).

In the single cell MS experiments (Fig. 7), endogenous processed peptides were identified using a peptide mass fingerprint (PMF) approach (77). The PMF approach is effective when numerous peptides are encoded by a specific prohormone and are detected experimentally. We verified that the masses of experimentally detected peptides were within the acceptable margin of error from theoretical masses of predicted peptides derived from the ALK precursor. In other words, we fingerprinted ALK precursor-derived peptides by detecting the predicted processed peptides. The fact that we detected 40 peptides from the ALK prohormone (Table S2) increased the confidence of our assignments. The PMF method is accepted in protein characterization studies and its accuracy is determined by both the mass assignment errors and the number of predicted peptides that were detected (both parameters are included in Table S2).

Electrophysiology—Intracellular and extracellular recordings techniques were utilized as described previously (13,25,40,78,79). Briefly, ganglia were desheathed, transferred to a recording chamber containing 1.5 mL of artificial seawater (ASW, 460mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 55 mM $MgCl_2$, and 10 mM HEPES, pH 7.6), continuously perfused at 0.3mL/min, and maintained at 14-17°C. Peptides, ALK-1 or ALK-2, were dissolved in ASW immediately before each physiological test, and the peptide/ASW solution was perfused into the recording chamber. Some experiments were also performed in a high divalent (HiDi) saline (368 mM NaCl, 8 mM KCl, 13.8 mM $CaCl_2$, 115 mM $MgCl_2$, and 10 mM HEPES, pH 7.6), which increases the spiking threshold of neurons and therefore curtails polysynaptic influences. Intracellular recordings were obtained using 5-10 MΩ sharp microelectrodes filled with an electrolyte (0.6 M K_2SO_4 plus 60 mM KCl). Extracellular recordings were acquired from polyethylene suction electrodes. Grass S88 and WPI Pulsemaster A300 stimulators were used to provide timing signals.

To test the effects of ALK-1 or ALK-2 on the feeding circuit, we performed experiments in the cerebral and buccal ganglia. The buccal ganglion innervates the feeding organ (radula). Feeding motor programs were elicited by stimulation of command-like neuron CBI-2 at 8-10 Hz, and were monitored by cyclic bursts in the I2 nerve of the buccal ganglion (17,80). Electrophysiological recordings were digitized on line using AxoScope software (version 10.7, Molecular Devices, LLC, Sunnyvale, CA), and were plotted by CorelDraw (version X7, Corel Corporation, Ottawa, ON, Canada). Bar graphs are plotted with Prism (version 5, GraphPad Software, La Jolla, CA). Data are expressed as mean ± S.E. All statistical tests (e.g., repeated measures one-way analysis of variance) were performed using Prism. When the data showed significant effects in analysis of variance, further individual comparisons were performed with a Bonferroni's correction.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions:

Conceived and designed the experiments: GZ, FSV, EVR, KRW, JVS and JJ. Performed the experiments and analyzed the data: GZ, FSV, DL (Dan-dan Liu), EVR, KY, WY (Wang-ding Yuan), HX, ABH, TC (Ting-ting Chen), VA, SY (Si-yuan Yin), SC (Song-an Chen), ECC, KRW, JVS, JJ. Wrote the paper: GZ, FSV, EVR, ECC, JVS, KRW, JJ.

REFERENCES

1. Strand, F. L. (1999) *Neuropeptides: Regulators of Physiological Processes*, MIT Press, Cambridge
2. Nusbaum, M. P., and Blitz, D. M. (2012) Neuropeptide modulation of microcircuits. *Curr. Opin. Neurobiol.* **22**, 592-601
3. Taghert, P. H., and Nitabach, M. N. (2012) Peptide neuromodulation in invertebrate model systems. *Neuron* **76**, 82-97
4. Marder, E., O'Leary, T., and Shruti, S. (2014) Neuromodulation of circuits with variable parameters: single neurons and small circuits reveal principles of state-dependent and robust neuromodulation. *Annu. Rev. Neurosci.* **37**, 329-346
5. Cropper, E. C., Lloyd, P. E., Reed, W., Tenenbaum, R., Kupfermann, I., and Weiss, K. R. (1987) Multiple neuropeptides in cholinergic motor neurons of *Aplysia*: evidence for modulation intrinsic to the motor circuit. *Proc. Natl. Acad. Sci. U S A* **84**, 3486-3490
6. Cropper, E. C., Price, D., Tenenbaum, R., Kupfermann, I., and Weiss, K. R. (1990) Release of peptide cotransmitters from a cholinergic motor neuron under physiological conditions. *Proc. Natl. Acad. Sci. U S A* **87**, 933-937
7. Church, P. J., and Lloyd, P. E. (1991) Expression of diverse neuropeptide cotransmitters by identified motor neurons in *Aplysia*. *J. Neurosci.* **11**, 618-625
8. Li, L., Moroz, T. P., Garden, R. W., Floyd, P. D., Weiss, K. R., and Sweedler, J. V. (1998) Mass spectrometric survey of interganglionically transported peptides in *Aplysia*. *Peptides* **19**, 1425-1433
9. Sweedler, J. V., Li, L., Rubakhin, S. S., Alexeeva, V., Dembrow, N. C., Dowling, O., Jing, J., Weiss, K. R., and Vilim, F. S. (2002) Identification and characterization of the feeding circuit-activating peptides, a novel neuropeptide family of *Aplysia*. *J. Neurosci.* **22**, 7797-7808
10. Proekt, A., Vilim, F. S., Alexeeva, V., Brezina, V., Friedman, A., Jing, J., Li, L., Zhurov, Y., Sweedler, J. V., and Weiss, K. R. (2005) Identification of a new neuropeptide precursor reveals a novel source of extrinsic modulation in the feeding system of *Aplysia*. *J. Neurosci.* **25**, 9637-9648
11. Jing, J., Sweedler, J. V., Cropper, E. C., Alexeeva, V., Park, J. H., Romanova, E. V., Xie, F., Dembrow, N. C., Ludwar, B. C., Weiss, K. R., and Vilim, F. S. (2010) Feedforward compensation mediated by the central and peripheral actions of a single neuropeptide discovered using representational difference analysis. *J. Neurosci.* **30**, 16545-16558
12. Vilim, F. S., Sasaki, K., Rybak, I. A., Alexeeva, V., Cropper, E. C., Jing, J., Orekhova, I. V., Brezina, V., Price, D., Romanova, E. V., Rubakhin, S. S., Hatcher, N. G., Sweedler, J. V., and Weiss, K. R. (2010) Distinct mechanisms produce functionally complementary actions of neuropeptides that are structurally related but derived from different precursors. *J. Neurosci.* **30**, 131-147
13. Bai, L., Livnat, I., Romanova, E. V., Alexeeva, V., Yau, P. M., Vilim, F. S., Weiss, K. R., Jing, J., and Sweedler, J. V. (2013) Characterization of GdFFD, a D-amino acid-containing neuropeptide

that functions as an extrinsic modulator of the *Aplysia* feeding circuit. *J. Biol. Chem.* **288**, 32837-32851
14. Church, P. J., and Lloyd, P. E. (1994) Activity of multiple identified motor neurons recorded intracellularly during evoked feedinglike motor programs in *Aplysia*. *J. Neurophysiol.* **72**, 1794-1809
15. Hurwitz, I., and Susswein, A. J. (1996) B64, a newly identified central pattern generator element producing a phase switch from protraction to retraction in buccal motor programs of *Aplysia californica*. *J. Neurophysiol.* **75**, 1327-1344
16. Hurwitz, I., Kupfermann, I., and Susswein, A. J. (1997) Different roles of neurons B63 and B34 that are active during the protraction phase of buccal motor programs in *Aplysia californica*. *J. Neurophysiol.* **78**, 1305-1319
17. Jing, J., and Weiss, K. R. (2001) Neural mechanisms of motor program switching in *Aplysia*. *J. Neurosci.* **21**, 7349-7362
18. Jing, J., and Weiss, K. R. (2002) Interneuronal basis of the generation of related but distinct motor programs in *Aplysia*: implications for current neuronal models of vertebrate intralimb coordination. *J. Neurosci.* **22**, 6228–6238
19. Evans, C. G., Jing, J., Rosen, S. C., and Cropper, E. C. (2003) Regulation of spike initiation and propagation in an *Aplysia* sensory neuron: gating-in via central depolarization. *J. Neurosci.* **23**, 2920-2931
20. Hurwitz, I., Kupfermann, I., and Weiss, K. R. (2003) Fast synaptic connections from CBIs to pattern-generating neurons in *Aplysia*: initiation and modification of motor programs. *J. Neurophysiol.* **89**, 2120-2136
21. Jing, J., Cropper, E. C., Hurwitz, I., and Weiss, K. R. (2004) The construction of movement with behavior-specific and behavior-independent modules. *J. Neurosci.* **24**, 6315-6325
22. Jing, J., and Weiss, K. R. (2005) Generation of variants of a motor act in a modular and hierarchical motor network. *Curr. Biol.* **15**, 1712-1721
23. Sasaki, K., Brezina, V., Weiss, K. R., and Jing, J. (2009) Distinct inhibitory neurons exert temporally specific control over activity of a motoneuron receiving concurrent excitation and inhibition. *J. Neurosci.* **29**, 11732-11744
24. Sasaki, K., Cropper, E. C., Weiss, K. R., and Jing, J. (2013) Functional differentiation of a population of electrically coupled heterogeneous elements in a microcircuit. *J. Neurosci.* **33**, 93-105
25. Wu, J. S., Wang, N., Siniscalchi, M. J., Perkins, M. H., Zheng, Y. T., Yu, W., Chen, S. A., Jia, R. N., Gu, J. W., Qian, Y. Q., Ye, Y., Vilim, F. S., Cropper, E. C., Weiss, K. R., and Jing, J. (2014) Complementary interactions between command-like interneurons that function to activate and specify motor programs. *J. Neurosci.* **34**, 6510-6521
26. Holman, G. M., and Cook, B. J. (1983) Isolation and partial characterization of a second myotropic peptide from the hindgut of the cockroach, Leucophaea maderae. *Comp. Biochem. Physiol. C.* **76**, 39-43
27. Holman, G. M., Cook, B. J., and Nachman, R. J. (1986) Primary structure and synthesis of a blocked myotropic neuropeptide isolated from the cockroach, Leucophaea maderae. *Comp. Biochem. Physiol. C.* **85**, 219-224
28. Holman, G. M., Cook, B. J., and Nachman, R. J. (1986) Primary structure and synthesis of 2 additional neuropeptides from *Leucophaea-Maderae* - members of a new family of Cephalomyotropins. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology* **84**, 271-276
29. Holman, G. M., Cook, B. J., and Nachman, R. J. (1987) Isolation, primary structure, and synthesis of Leukokinin-V and Leukokinin-Vi - myotropic peptides of *Leucophaea-Maderae*.

Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology **88**, 27-30

30. Holman, G. M., Cook, B. J., and Nachman, R. J. (1987) Isolation, primary structure and synthesis of Leukokinin-Vii and Leukokinin-Viii - the final members of this new family of cephalomyotropic peptides Isolated from head extracts of *Leucophaea-Maderae*. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology* **88**, 31-34
31. Nassel, D. R. (2002) Neuropeptides in the nervous system of Drosophila and other insects: multiple roles as neuromodulators and neurohormones. *Prog. Neurobiol.* **68**, 1-84
32. Hayes, T. K., Pannabecker, T. L., Hinckley, D. J., Holman, G. M., Nachman, R. J., Petzel, D. H., and Beyenbach, K. W. (1989) Leucokinins, a new family of ion transport stimulators and inhibitors in insect Malpighian tubules. *Life Sci.* **44**, 1259-1266
33. Coast, G. M., Holman, G. M., and Nachman, R. J. (1990) The diuretic activity of a series of cephalomyotropic neuropeptides, the achetakinins, on Isolated malpighian tubules of the house cricket, *Acheta-Domesticus*. *J. Insect Physiol.* **36**, 481-488
34. Al-Anzi, B., Armand, E., Nagamei, P., Olszewski, M., Sapin, V., Waters, C., Zinn, K., Wyman, R. J., and Benzer, S. (2010) The leucokinin pathway and its neurons regulate meal size in Drosophila. *Curr. Biol.* **20**, 969-978
35. Jekely, G. (2013) Global view of the evolution and diversity of metazoan neuropeptide signaling. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 8702-8707
36. Mirabeau, O., and Joly, J. S. (2013) Molecular evolution of peptidergic signaling systems in bilaterians. *Proc. Natl. Acad. Sci. U. S. A.* **110**, E2028-2037
37. Nassel, D. R., and Homberg, U. (2006) Neuropeptides in interneurons of the insect brain. *Cell Tissue Res.* **326**, 1-24
38. Cropper, E. C., Friedman, A. K., Jing, J., Perkins, M. H., and Weiss, K. R. (2014) Neuromodulation as a mechanism for the induction of repetition priming. *Curr. Opin. Neurobiol.* **29**, 33-38
39. Cox, K. J., Tensen, C. P., Van der Schors, R. C., Li, K. W., van Heerikhuizen, H., Vreugdenhil, E., Geraerts, W. P., and Burke, J. F. (1997) Cloning, characterization, and expression of a G-protein-coupled receptor from Lymnaea stagnalis and identification of a leucokinin-like peptide, PSFHSWSamide, as its endogenous ligand. *J. Neurosci.* **17**, 1197-1205
40. Jing, J., Alexeeva, V., Chen, S. A., Yu, K., Due, M. R., Tan, L. N., Chen, T. T., Liu, D. D., Cropper, E. C., Vilim, F. S., and Weiss, K. R. (2015) Functional characterization of a vesicular glutamate transporter in an interneuron that makes excitatory and inhibitory synaptic connections in a molluscan neural circuit. *J. Neurosci.* **35**, 9137-9149
41. Romanova, E. V., Sasaki, K., Alexeeva, V., Vilim, F. S., Jing, J., Richmond, T. A., Weiss, K. R., and Sweedler, J. V. (2012) Urotensin II in invertebrates: from structure to function in *Aplysia californica*. *PLoS One* **7**, e48764
42. Dong, X., Han, S., Zylka, M. J., Simon, M. I., and Anderson, D. J. (2001) A diverse family of GPCRs expressed in specific subsets of nociceptive sensory neurons. *Cell* **106**, 619-632
43. Pfisterer, P., Ehlermann, J., Hegen, M., and Schorle, H. (2002) A subtractive gene expression screen suggests a role of transcription factor AP-2 alpha in control of proliferation and differentiation. *J. Biol. Chem.* **277**, 6637-6644
44. Evans, C. G., Rosen, S., Kupfermann, I., Weiss, K. R., and Cropper, E. C. (1996) Characterization of a radula opener neuromuscular system in *Aplysia*. *J. Neurophysiol.* **76**, 1267-1281
45. Southey, B. R., Amare, A., Zimmerman, T. A., Rodriguez-Zas, S. L., and Sweedler, J. V. (2006) NeuroPred: a tool to predict cleavage sites in neuropeptide precursors and provide the masses of the resulting peptides. *Nucleic Acids Res* **34**, W267-272

46. Southey, B. R., Sweedler, J. V., and Rodriguez-Zas, S. L. (2008) A python analytical pipeline to identify prohormone precursors and predict prohormone cleavage sites. *Front Neuroinform* **2**, 7
47. Nielsen, H., Engelbrecht, J., von Heijne, G., and Brunak, S. (1996) Defining a similarity threshold for a functional protein sequence pattern: the signal peptide cleavage site. *Proteins* **24**, 165-177
48. Moroz, L. L., Edwards, J. R., Puthanveettil, S. V., Kohn, A. B., Ha, T., Heyland, A., Knudsen, B., Sahni, A., Yu, F., Liu, L., Jezzini, S., Lovell, P., Iannucculli, W., Chen, M., Nguyen, T., Sheng, H., Shaw, R., Kalachikov, S., Panchin, Y. V., Farmerie, W., Russo, J. J., Ju, J., and Kandel, E. R. (2006) Neuronal transcriptome of *Aplysia*: neuronal compartments and circuitry. *Cell* **127**, 1453-1467
49. Rosen, S. C., Teyke, T., Miller, M. W., Weiss, K. R., and Kupfermann, I. (1991) Identification and characterization of cerebral-to-buccal interneurons implicated in the control of motor programs associated with feeding in *Aplysia*. *J. Neurosci.* **11**, 3630-3655
50. Perrins, R., and Weiss, K. R. (1998) Compartmentalization of information processing in an *Aplysia* feeding circuit interneuron through membrane properties and synaptic interactions. *J. Neurosci.* **18**, 3977-3989
51. Sasaki, K., Due, M. R., Jing, J., and Weiss, K. R. (2007) Feeding CPG in *Aplysia* directly controls two distinct outputs of a compartmentalized interneuron that functions as a CPG element. *J. Neurophysiol.* **98**, 3796-3801
52. Cropper, E. C., Evans, C. G., Hurwitz, I., Jing, J., Proekt, A., Romero, A., and Rosen, S. C. (2004) Feeding neural networks in the mollusc *Aplysia*. *Neurosignals* **13**, 70-86
53. Jing, J. (2009) Command systems. in *Encyclopedia of Neuroscience, volume 2* (Squire, L. R. ed.), Academic Press, Oxford, UK. pp 1149-1158
54. Jing, J., Cropper, E. C., and Weiss, K. R. (2017) Network functions of electrical coupling present in multiple and specific sites in behavior-generating circuits. in *Network Functions and Plasticity: Perspectives from Studying Neuronal Electrical Coupling in Microcircuits* (Jing, J. ed.), Academic Press, London, UK. pp 79-107
55. Romanova, E. V., Aerts, J. T., Croushore, C. A., and Sweedler, J. V. (2014) Small-volume analysis of cell-cell signaling molecules in the brain. *Neuropsychopharmacology* **39**, 50-64
56. Comi, T. J., Do, T. D., Rubakhin, S. S., and Sweedler, J. V. (2017) Categorizing cells on the basis of their chemical profiles: progress in single-cell mass spectrometry. *J. Am. Chem. Soc.* **139**, 3920-3929
57. Kupfermann, I. (1974) Dissociation of the appetitive and consummatory phases of feeding behavior in *Aplysia*: a lesion study. *Behav. Biol.* **10**, 89-97
58. Morton, D. W., and Chiel, H. J. (1993) The timing of activity in motor neurons that produce radula movements distinguishes ingestion from rejection in *Aplysia*. *J. Comp. Physiol. A* **173**, 519-536
59. Morton, D. W., and Chiel, H. J. (1993) In vivo buccal nerve activity that distinguishes ingestion from rejection can be used to predict behavioral transitions in *Aplysia*. *J. Comp. Physiol. A* **172**, 17-32
60. Morgan, P. T., Jing, J., Vilim, F. S., and Weiss, K. R. (2002) Interneuronal and peptidergic control of motor pattern switching in *Aplysia*. *J. Neurophysiol.* **87**, 49-61
61. Wu, J. S., Due, M. R., Sasaki, K., Proekt, A., Jing, J., and Weiss, K. R. (2007) State dependence of spike timing and neuronal function in a motor pattern generating network. *J. Neurosci.* **27**, 10818-10831
62. Petersen, T. N., Brunak, S., von Heijne, G., and Nielsen, H. (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Methods* **8**, 785-786

63. Radford, J. C., Davies, S. A., and Dow, J. A. (2002) Systematic G-protein-coupled receptor analysis in *Drosophila melanogaster* identifies a leucokinin receptor with novel roles. *J. Biol. Chem.* **277**, 38810-38817
64. Siviter, R. J., Coast, G. M., Winther, A. M., Nachman, R. J., Taylor, C. A., Shirras, A. D., Coates, D., Isaac, R. E., and Nassel, D. R. (2000) Expression and functional characterization of a *Drosophila* neuropeptide precursor with homology to mammalian preprotachykinin A. *J. Biol. Chem.* **275**, 23273-23280
65. Bauknecht, P., and Jekely, G. (2015) Large-scale combinatorial deorphanization of *Platynereis* neuropeptide GPCRs. *Cell Rep* **12**, 684-693
66. Lum, C. S., Zhurov, Y., Cropper, E. C., Weiss, K. R., and Brezina, V. (2005) Variability of swallowing performance in intact, freely feeding *Aplysia*. *J. Neurophysiol.* **94**, 2427-2446
67. Jing, J., Vilim, F. S., Horn, C. C., Alexeeva, V., Hatcher, N. G., Sasaki, K., Yashina, I., Kupfermann, I., Sweedler, J. V., and Weiss, K. R. (2007) From hunger to satiety: reconfiguration of a feeding network by *Aplysia* neuropeptide Y. *J. Neurosci.* **27**, 3490-3502
68. Schoofs, L., De Loof, A., and Van Hiel, M. B. (2017) Neuropeptides as regulators of behavior in insects. *Annu. Rev. Entomol.* **62**, 35-52
69. Lisitsyn, N., Lisitsyn, N., and Wigler, M. (1993) Cloning the differences between two complex genomes. *Science* **259**, 946-951
70. Hubank, M., and Schatz, D. G. (1994) Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic Acids Res* **22**, 5640-5648
71. Vilim, F. S., Alexeeva, V., Moroz, L. L., Li, L., Moroz, T. P., Sweedler, J. V., and Weiss, K. R. (2001) Cloning, expression and processing of the CP2 neuropeptide precursor of *Aplysia*. *Peptides* **22**, 2027-2038
72. Sive, H. L., and St John, T. (1988) A simple subtractive hybridization technique employing photoactivatable biotin and phenol extraction. *Nucleic Acids Res* **16**, 10937
73. Fujisawa, Y., Furukawa, Y., Ohta, S., Ellis, T. A., Dembrow, N. C., Li, L., Floyd, P. D., Sweedler, J. V., Minakata, H., Nakamaru, K., Morishita, F., Matsushima, O., Weiss, K. R., and Vilim, F. S. (1999) The *Aplysia* mytilus inhibitory peptide-related peptides: identification, cloning, processing, distribution, and action. *J. Neurosci.* **19**, 9618-9634
74. Furukawa, Y., Nakamaru, K., Wakayama, H., Fujisawa, Y., Minakata, H., Ohta, S., Morishita, F., Matsushima, O., Li, L., Romanova, E., Sweedler, J. V., Park, J. H., Romero, A., Cropper, E. C., Dembrow, N. C., Jing, J., Weiss, K. R., and Vilim, F. S. (2001) The enterins: a novel family of neuropeptides isolated from the enteric nervous system and CNS of *Aplysia*. *J. Neurosci.* **21**, 8247-8261
75. Chomczynski, P., and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159
76. Jing, J., Vilim, F. S., Wu, J. S., Park, J. H., and Weiss, K. R. (2003) Concerted GABAergic actions of *Aplysia* feeding interneurons in motor program specification. *J. Neurosci.* **23**, 5283-5294
77. Yates, J. R., 3rd, Speicher, S., Griffin, P. R., and Hunkapiller, T. (1993) Peptide mass maps: a highly informative approach to protein identification. *Anal. Biochem.* **214**, 397-408
78. Livnat, I., Tai, H. C., Jansson, E. T., Bai, L., Romanova, E. V., Chen, T. T., Yu, K., Chen, S. A., Zhang, Y., Wang, Z. Y., Liu, D. D., Weiss, K. R., Jing, J., and Sweedler, J. V. (2016) A D-amino acid-containing neuropeptide discovery funnel. *Anal. Chem.* **88**, 11868-11876
79. Yang, C. Y., Yu, K., Wang, Y., Chen, S. A., Liu, D. D., Wang, Z. Y., Su, Y. N., Yang, S. Z., Chen, T. T., Livnat, I., Vilim, F. S., Cropper, E. C., Weiss, K. R., Sweedler, J. V., and Jing, J. (2016) *Aplysia* locomotion: Network and behavioral actions of GdFFD, a D-amino acid-containing neuropeptide. *PLoS One* **11**, e0147335

80. Hurwitz, I., Neustadter, D., Morton, D. W., Chiel, H. J., and Susswein, A. J. (1996) Activity patterns of the B31/B32 pattern initiators innervating the I2 muscle of the buccal mass during normal feeding movements in *Aplysia californica*. *J. Neurophysiol.* **75**, 1309-1326
81. Struck, T. H., Paul, C., Hill, N., Hartmann, S., Hosel, C., Kube, M., Lieb, B., Meyer, A., Tiedemann, R., Purschke, G., and Bleidorn, C. (2011) Phylogenomic analyses unravel annelid evolution. *Nature* **471**, 95-98
82. Holman, G. M., Nachman, R. J., and Wright, M. S. (1990) A strategy for the isolation and structural characterization of certain insect myotropic peptides that modify the spontaneous contractions of the isolated cockroach hindgut. in *Chromatography and isolation of insect hormones and pheromones* (McCaffery, A. R., and Wilson, I. D. eds.), Plenum, New York. pp 195-204
83. Schoofs, L., Holman, G. M., Proost, P., Vandamme, J., Hayes, T. K., and Deloof, A. (1992) Locustakinin, a novel myotropic peptide from *Locusta-Migratoria*, isolation, primary structure and synthesis. *Regul. Pept.* **37**, 49-57
84. Clottens, F. L., Meola, S. M., Coast, G. M., Hayes, T. K., Wright, M. S., Nachman, R. J., and Holman, G. M. (1993) Characterization of an antiserum against an achetakinin-I analog and its use for the localization of culekinin depolarizing peptide-Ii in the mosquito, *Culex-Salinarius*. *Regul. Pept.* **49**, 145-157
85. Veenstra, J. A. (1994) Isolation and identification of three leucokinins from the mosquito Aedes aegypti. *Biochem. Biophys. Res. Commun.* **202**, 715-719
86. Blackburn, M. B., Wagner, R. M., Shabanowitz, J., Kochansky, J. P., Hunt, D. F., and Raina, A. K. (1995) The isolation and identification of 3 diuretic kinins from the abdominal ventral nerve cord of adult *Helicoverpa-Zea*. *J. Insect Physiol.* **41**, 723-730
87. Terhzaz, S., O'Connell, F. C., Pollock, V. P., Kean, L., Davies, S. A., Veenstra, J. A., and Dow, J. A. T. (1999) Isolation and characterization of a leucokinin-like peptide of *Drosophila melanogaster*. *J. Exp. Biol.* **202**, 3667-3676
88. Radford, J. C., Terhzaz, S., Cabrero, P., Davies, S. A., and Dow, J. A. (2004) Functional characterisation of the Anopheles leucokinins and their cognate G-protein coupled receptor. *J. Exp. Biol.* **207**, 4573-4586
89. Mitreva, M., Jasmer, D. P., Zarlenga, D. S., Wang, Z., Abubucker, S., Martin, J., Taylor, C. M., Yin, Y., Fulton, L., Minx, P., Yang, S. P., Warren, W. C., Fulton, R. S., Bhonagiri, V., Zhang, X., Hallsworth-Pepin, K., Clifton, S. W., McCarter, J. P., Appleton, J., Mardis, E. R., and Wilson, R. K. (2011) The draft genome of the parasitic nematode Trichinella spiralis. *Nat. Genet.* **43**, 228-235

FOOTNOTES

The work was supported by the National Natural Science Foundation of China (Grants 31671097, 31371104, J1103512, and J1210026), Award Nos. RO1 NS066587, RO1 NS070583, and RO1 NS031609 from the National Institute of Neurological Disorders and Stroke, Award Number P30 DA018310 from the National Institute on Drug Abuse, and National Science Foundation of the United States (Award No. CHE 16-067915).

FIGURE LEGENDS

FIGURE 1. RDA procedure used to identify the ALK precursor. Illustrated is the RDA between a buccal motoneuron, B48, and B1/B2 neurons. The results of the subtractions performed are shown in the gels at the bottom. Bottom left, The results of the first subtraction performed. Lane 1 is a blank subtraction. Lanes 2 and 3 are additional control subtractions using the B1 or B2 neurons subtracted against themselves, respectively. These lanes only show a weak smear with some faint bands. Lane 4 is a B48 that was subtracted against neurons B1 and B2. This lane shows some intense bands that do not appear in the control subtractions. M, Marker lane showing cDNA masses in base pairs. Bottom right, Lane 5 shows the results of a second round of subtraction. One difference clone obtained from B48 was sequenced to reveal an open reading frame that coded for an incomplete ALK transcript. The complete transcript was subsequently obtained with RACE (see Results).

FIGURE 2. Schematic diagram of the ALK precursor, which is composed of 2025 aa. Illustrated are the approximate locations of a putative signal peptide, 16 amidated peptides (shown in colors) and 24 nonamidated peptides (shown in gray) (See Table S2). Among the 16 amidated peptides, all but one (DSPRMFAFNSLS[Amide], shown in light blue) have a C-terminal pentapeptide motif (FXXWX-amide) with the last aa being an S, G, A, or N, and are classified as ALKs. Note that several peptides at three locations have overlapping sequences, indicating alternative cleavage sites.

FIGURE 3. Northern blot gel of ALK mRNA in the *Aplysia* CNS. *In situ* hybridization of total RNA isolated from the different *Aplysia* ganglia with an ALK cDNA probe. Northern analysis only detected ALK mRNA in the buccal ganglion. ALK mRNA in other ganglia was below the level of detection. ALK mRNA is about 8 Kb in length. Abbreviations: Bu, buccal ganglion; Cb, cerebral ganglion; Pl: pleural ganglion; Pd, pedal ganglion; Ab, abdominal ganglion.

FIGURE 4. Distribution of ALK-positive neurons and fibers in the buccal and cerebral ganglia (whole mounts). *A, B*, Rostral (*A*) and caudal (*B*) buccal ganglia. Note the immunoreactive axons in the CBC. *C, D*, Dorsal (*C*) and Ventral (*D*) cerebral ganglia. 1, *in situ* hybridization (*in situ*); 2, immunohistochemistry (immuno.); 3, composite drawings of ALK neurons. *In situ* hybridization and immunohistochemistry reveal that the majority of ALK neuropeptides are located in the buccal ganglion. Note the staining of G cluster and E cluster neurons on the cerebral ventral surface. Darker shades of grey indicate more intense staining. Scale bar: 500 μm (in *A1* and *C1*). Buccal abbreviations: CBC, Cerebrobuccal connective; N1, nerve 1; N2, nerve 2; N3, nerve 3; SN, salivary nerve; EN, esophageal nerve; RN, radula nerve. Cerebral

abbreviations: UL, Upper labial nerve; PT, posterior tentacular nerve; ON, optic nerve; AT, anterior tentacular nerve; LL, lower labial nerve; CBC, cerebrobuccal connective; CPe, cerebropedal connective; CPl, cerebropleural connective.

FIGURE 5. Distribution of ALK-positive neurons and fibers in the pleural and pedal ganglia and the abdominal ganglion (whole mounts). *A-D*, Left dorsal (*A*), left ventral (*B*), right dorsal (*C*), right ventral (*D*) pedal ganglia. *E, F*, Dorsal (*E*) and ventral (*F*) abdominal ganglia. 1, *in situ* hybridization (*in situ*); 2, immunohistochemistry (immuno.); 3, composite drawings of ALK neurons. Darker shades of grey indicate more intense staining. Scale bar (in *A1* and *E1*): 500 μm. Pleural-pedal abbreviations: Rt, Right; L, pleural ganglion; E, pedal ganglion; LE, pleuropedal connective; EE, pedal commissure; EC, cerebropedal connective; LC, cerebropleural connective; LA, pleuroabdominal connective; E5, posterior tegumentary nerve (P5); E6, anterior parapodial nerve (P6); E9, posterior pedal nerve (P9). Abdominal abbreviations: LC, Left pleuroabdominal connective; RC, right pleuroabdominal connective; VN, vulvar nerve; BN, branchial nerve; STN, spermathecal nerve; PN, pericardial nerve; GN, genital nerve; SN, siphon nerve. For simplicity, not all nerves in the pleural and pedal ganglia were drawn.

FIGURE 6. ALK is present in B48 and several buccal neurons that project to the CBC. *A1*, B48 injected with carboxyfluorescein. *A2*, immunostaining with an antibody to ALK-1. The figure shows that B48 contains the ALKs. The buccal ganglion (one hemi-ganglion) was photographed from the caudal surface. Scale bar: 100 μm. *B1*, Backfill of CBC using biocytin (processed with avidin-fluorescein). *B2*, immunostaining with an antibody to ALK-1. Some cells are double-labeled. The buccal ganglion was photographed from the rostral surface. Scale bar: 500 μm.

FIGURE 7. Processing of the ALK prohormone as detected by MALDI-TOF MS in individual buccal neurons. The main panel shows a representative spectrum over a wide mass range from one of the eight neurons; inserts show zoom-in views into mass range segments from spectra obtained on different neurons because not all reported peptides were detected in a single spectrum (see Table S2). Detected peptides are labeled according to their numerical order in Table S2.

FIGURE 8. ALKs modulate feeding motor programs elicited by CBI-2 partly through their actions on the interneuron B64. *A*, ALKs shorten protraction duration in a concentration-dependent manner. *A1-4*, A representative example; A single cycle of a motor program was elicited by simulating CBI-2 at 10 Hz until the end of the protraction phase, detected by the sharp synaptic inhibition of interneuron B34 and the abrupt ending of I2 nerve activity. Protraction phase (open bar) is defined by the activity in the I2 nerve. Retraction phase (filled bar) is defined by a period of hyperpolarization of B34 after the protraction phase is terminated and also by a period of high-frequency activity of the radula closing motoneuron B8. Upon wash, protraction duration returned to its control value. *A5*, group data on protraction duration. In contrast the ALKs had no significant effect on the duration of retraction (*A6*), or B8 activity during either protraction (*A7*) or retraction (*A8*). *B,C*, ALKs increase B64 excitability (*B1*) and decrease B64 threshold to spike (*C1*). *B2, C2*, group data. Bars in *B1* and *C1* denote current injections in B64. Recordings in *A* were made in ASW, whereas recordings in *B* and *C* were made in high divalent saline. Bonferroni *post hoc* tests: *$p<0.05$; **$p<0.01$; ***$p<0.001$. Error bars indicate SEM. *D*, A schematic drawing of the *Aplysia* cerebral and buccal ganglia, illustrating the locations of the neurons recorded from, and the

source (ALK neurons, red) and target (B64, green) of ALKs. Cerebral ganglion: neurons on the ventral surface are shown at left, dorsal at right. Buccal ganglion: neurons on the rostral surface are shown at left, caudal at right. Nerve abbreviations as in Fig. 4

Table 1. Comparison of leucokinin-like neuropeptides in representative species.

Phylum	Species	Peptide name	Amino acid sequence
Mollusca	*Aplysia* (this paper)	ALK-1	PA**F**HS**WS**-amide
		ALK-2	PA**F**HA**WS**-amide
Mollusca	*Tritonia diomedea* (NCBI Accession: EV289917.1)	Leucokinin I	*PS**F**HA**WS**-amide
		Leucokinin II	*QS**F**AA**WA**-amide
Mollusca	*Lymnaea stagnalis* (39)	Lymnokinin	PS**F**HS**WS**-amide
Mollusca	*Lottia gigantea* (36)	Leucokinin I	**AA**F**AA**WG**-amide
		Leucokinin II	**AP**F**SV**WN**-amide
Mollusca	*Idiosepius paradoxus* (36)	Leucokinin I	**K**F**SP**WA**-amide
Annelida	*Malacoceros fuliginosus* (81) (NCBI Accession: FR764292.1)	Leucokinin I	*A**F**SP**WG**-amide
Annelida	*Capitella teleta* (36)	Leucokinin I	**A**F**SA**WG**-amide
Arthropoda	*Leucophaera maderae* (27)	Leucokinin I	DPA**F**NS**WG**-amide
		Leucokinin II	DPG**F**SS**WG**-amide
Arthropoda	*Acheta domesticus* (82)	Achetakinin I	SGAD**F**YP**WG**-amide
		Achetakinin II	AY**F**SP**WG**-amide
Arthropoda	*Locusta migratoria* (83)	Locustakinin	A**F**SS**WG**-amide
Arthropoda	*Culex salinarius* (84)	CDP I	NP**F**HS**WG**-amide
		CDP II	NNANV**F**YP**WG**-amide
Arthropoda	*Aedes aegypti* (85)	Leucokinin I	NSKYVSKQK**F**YS**WG**-amide
		Leucokinin II	NPFHAY**F**SA**WG**-amide
Arthropoda	*Helicoverpa zea* (86)	Helicokinin I	Y**F**SP**WG**-amide
		Helicokinin II	VR**F**SP**WG**-amide
Arthropoda	*Drosophila melanogaster* (87)	Leucokinin	NSVVLGKKQR**F**HS**WG**-amide
Arthropoda	*Anopheles* (88)	Leucokinin I	DTPRYVSKQK**F**HS**WG**-amide
		Leucokinin II	NP**F**HS**WG**-amide
		Leucokinin III	NTAQV**F**YP**WG**-amide
Nematode	*Trichinella spiralis* (89)	Leucokinin	*K**F**YA**WA**-amide
Nematode	*Caenorhabditis elegans* (35,36)	Leucokinin	**KQ**F**YA**WA**-amide

Notes: *Predicted; **Predicted and the precursor is incomplete;

Additional peptides included for the frequency plot above but not in the table for clarity:

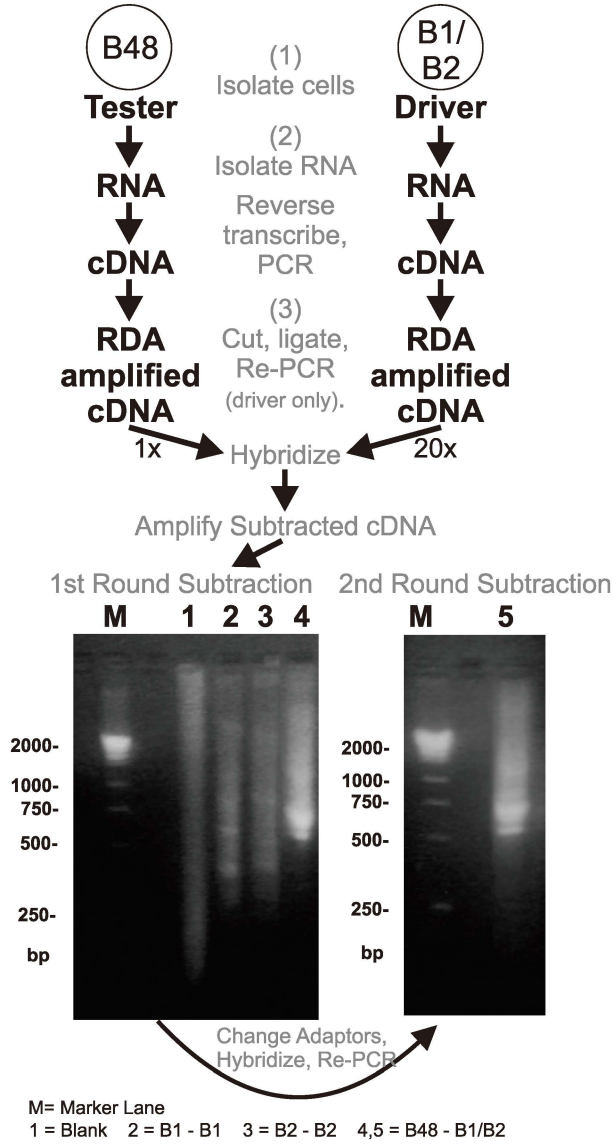
Leucophaera maderae: Leucokinin III (DQGFNSWG-amide), Leucokinin IV (DASFHSWG-amide) (28); Leucokinin V (GSGFSSWG-amide), Leucokinin VI (pESSFHSWG-amide) (29); Leucokinin VII (DPAFSSWG-amide), Leucokinin VIII (GADFYSWG-amide) (30).

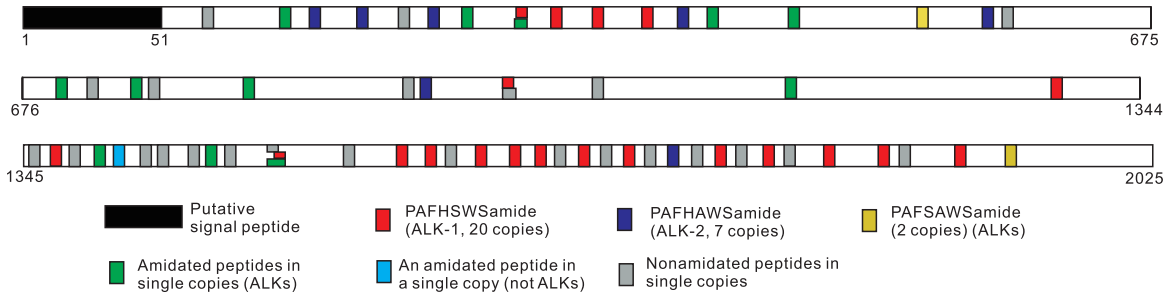
Acheta domesticus: Achetakinin III (ALPFSPWG-amide), Achetakinin IV (NFKFNPWG-amide), Achetakinin V (AFHSWG-amide) (82).

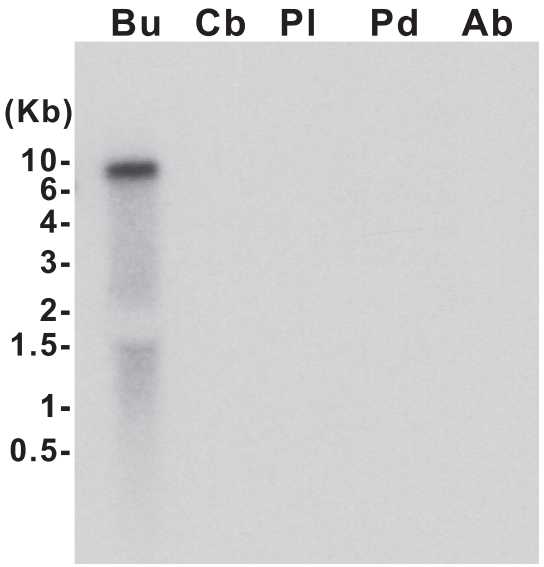
Culex salinarius: CDP III (TKYVSKQFFSWG-amide) (84).

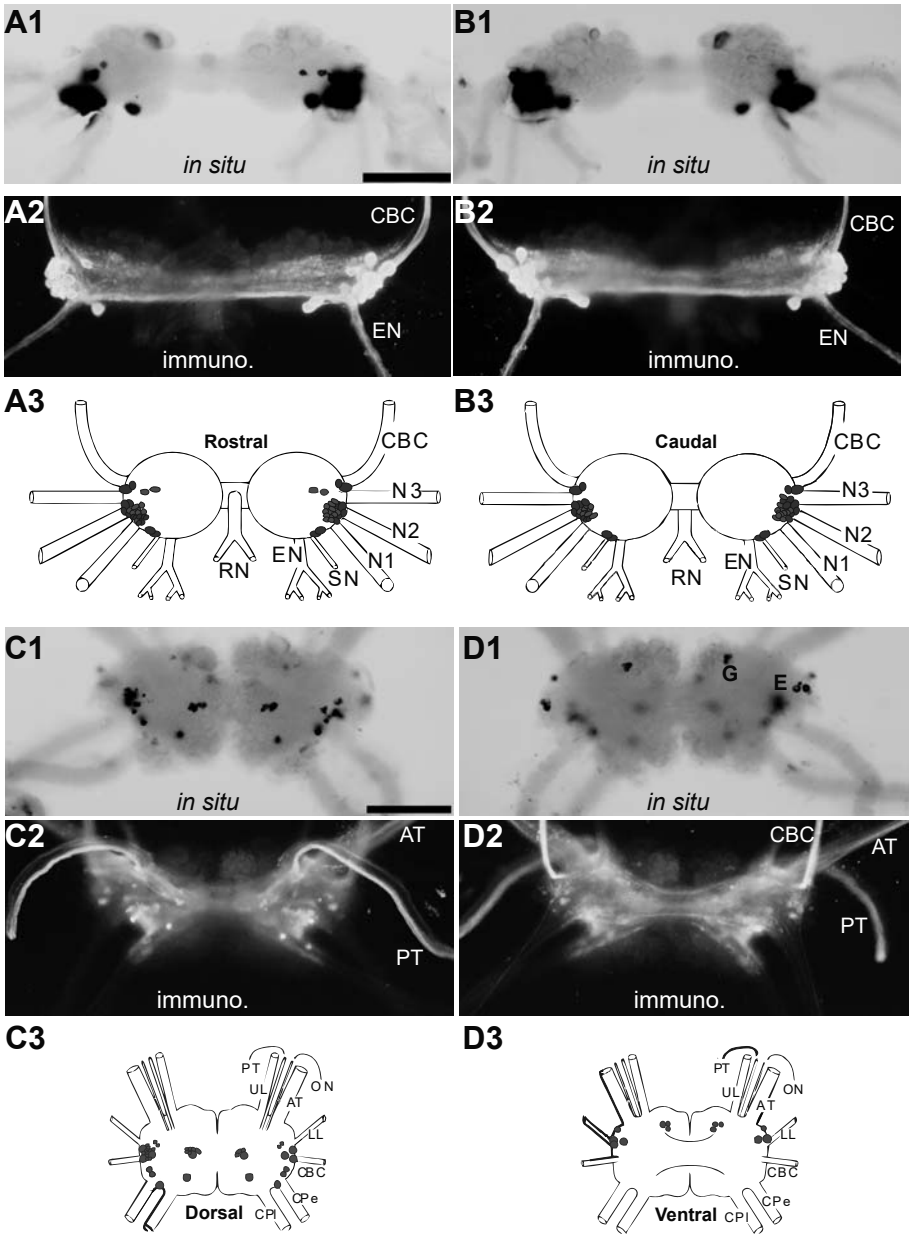
Aedes aegypti: Leucokinin III (NNPNVFYPWG-amide) (85).

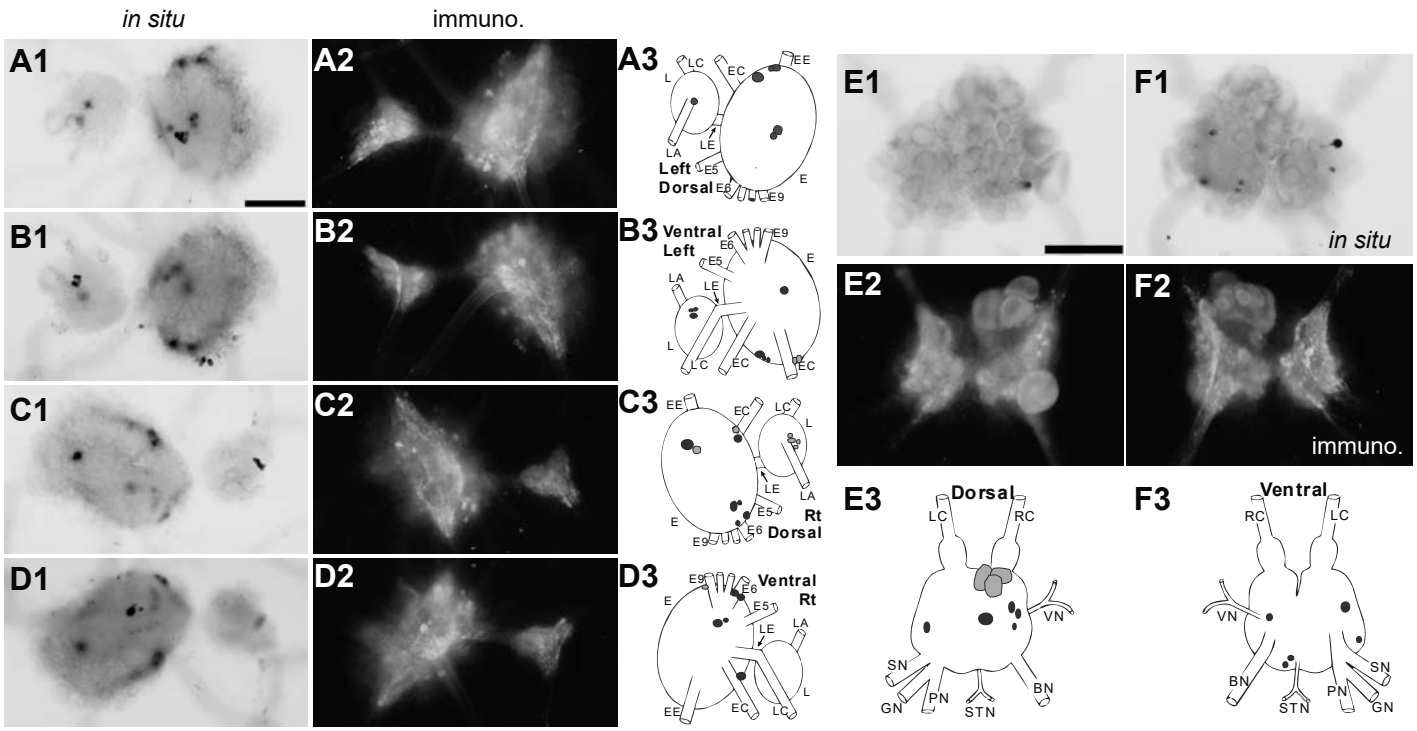
Helicoverpa zea: Helicokinin III (KVKFSAWG-amide) (86).

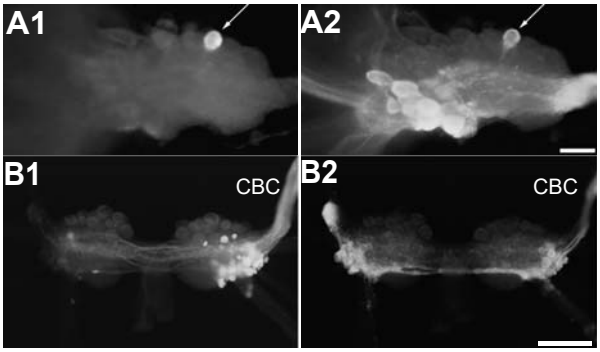

B48
Tester
RNA
cDNA
RDA amplified cDNA
1x
(1) Isolate cells
(2) Isolate RNA
Reverse transcribe, PCR
(3) Cut, ligate, Re-PCR (driver only).
B1/ B2
Driver
RNA
cDNA
RDA amplified cDNA
20x
Hybridize
Amplify Subtracted cDNA
1st Round Subtraction
2nd Round Subtraction
M 1 2 3 4
M 5
2000-
1000-
750-
500-
250-
bp
Change Adaptors, Hybridize, Re-PCR
M= Marker Lane
1 = Blank 2 = B1 - B1 3 = B2 - B2 4,5 = B48 - B1/B2

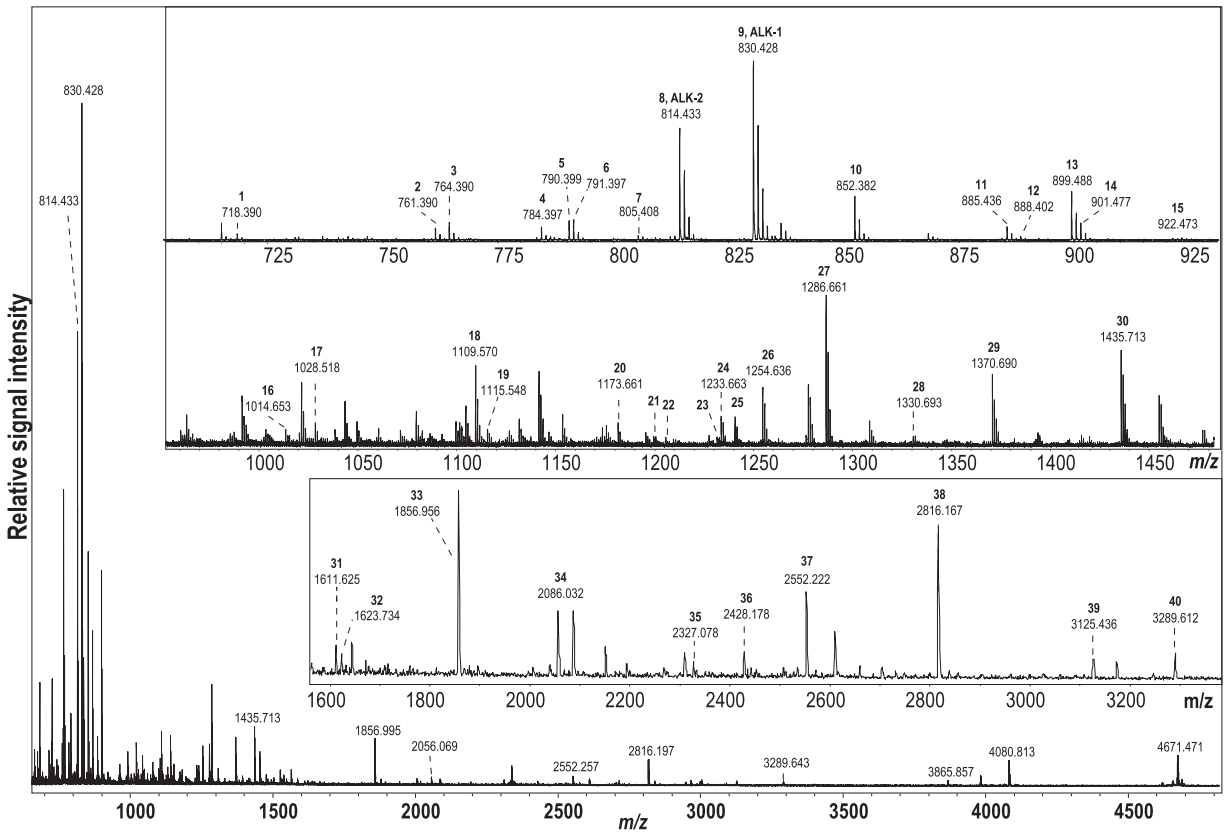

1
51
675
676
1344
1345
2025
Putative signal peptide
PAFHSWSamide (ALK-1, 20 copies)
PAFHAWSamide (ALK-2, 7 copies)
PAFSAWSamide (2 copies) (ALKs)
Amidated peptides in single copies (ALKs)
An amidated peptide in a single copy (not ALKs)
Nonamidated peptides in single copies

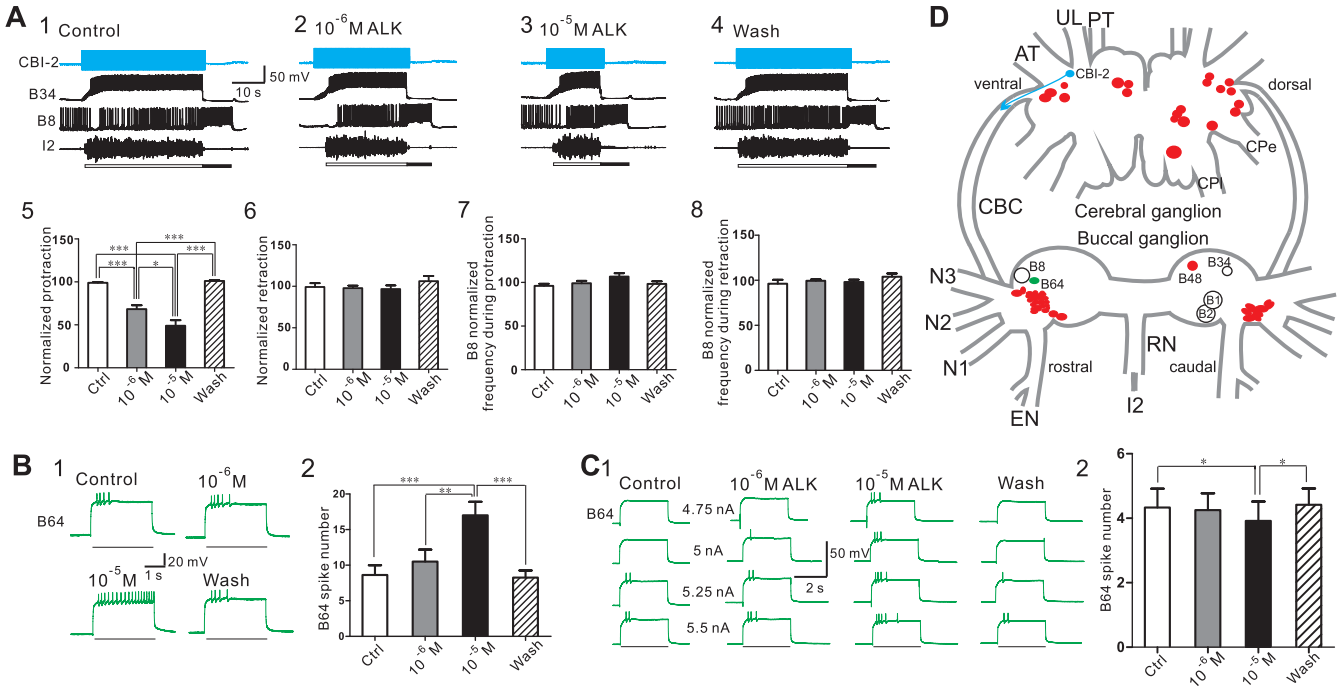

Bu
Cb
Pl
Pd
Ab
(Kb)
10-
6-
4-
3-
2-
1.5-
1-
0.5-


A1
in situ
B1
in situ
A2
CBC
EN
immuno.
B2
CBC
EN
immuno.
A3
Rostral
CBC
N3
N2
N1
EN
RN
SN
B3
Caudal
CBC
N3
N2
N1
EN
RN
SN
C1
in situ
D1
G
E
in situ
C2
AT
PT
immuno.
D2
CBC
AT
PT
immuno.
C3
PT
UL
AT
ON
LL
CBC
CPe
Dorsal
CPl
D3
PT
UL
AT
ON
LL
CBC
CPe
Ventral
CPl


in situ
immuno.
A1
A2
A3
LC
EC
EE
L
LA
LE
Left
Dorsal
E5
E6
E9
E
B1
B2
B3
Ventral
Left
C1
C2
C3
Rt
Dorsal
D1
D2
D3
Ventral
Rt
E1
F1
in situ
E2
F2
immuno.
E3
Dorsal
RC
VN
SN
BN
GN
PN
STN
F3
Ventral


A1
A2
B1
CBC
B2
CBC


Relative signal intensity
m/z
830.428
814.433
1435.713
1856.995
2056.069
2552.257
2816.197
3289.643
3865.857
4080.813
4671.471
1000
1500
2000
2500
3000
3500
4000
4500
9, ALK-1
830.428
8, ALK-2
814.433
1 718.390
2 761.390
3 764.390
4 784.397
5 790.399
6 791.397
7 805.408
10 852.382
11 885.436
12 888.402
13 899.488
14 901.477
15 922.473
725
750
775
800
825
850
875
900
925
16 1014.653
17 1028.518
18 1109.570
19 1115.548
20 1173.661
21
22
23
24 1233.663
25
26 1254.636
27 1286.661
28 1330.693
29 1370.690
30 1435.713
1000
1050
1100
1150
1200
1250
1300
1350
1400
1450
m/z
31 1611.625
32 1623.734
33 1856.956
34 2086.032
35 2327.078
36 2428.178
37 2552.222
38 2816.167
39 3125.436
40 3289.612
1600
1800
2000
2200
2400
2600
2800
3000
3200
m/z


A
1 Control
2 10-6 M ALK
3 10-5 M ALK
4 Wash
CBI-2
B34
B8
I2
50 mV
10 s
5
6
7
8
Normalized protraction
Normalized retraction
B8 normalized frequency during protraction
B8 normalized frequency during retraction
Ctrl
10-6 M
10-5 M
Wash
D
UL PT
AT
ventral
CBI-2
dorsal
CPe
CPl
CBC
Cerebral ganglion
Buccal ganglion
N3
N2
N1
B8
B64
B34
B48
B1
B2
RN
rostral
caudal
EN
I2
B
1
Control
10-6 M
B64
10-5 M
Wash
20 mV
1 s
2
B64 spike number
C1
Control
10-6 M ALK
10-5 M ALK
Wash
B64
4.75 nA
5 nA
5.25 nA
5.5 nA
50 mV
2 s
2
B64 spike number