**Title:** Expression patterns and behavioral effects of conopressin and APGWamide in the nudibranch *Berghia stephanieae*

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## Abstract:

The highly conserved oxytocin/vasopressin family of nonapeptides plays many roles across the animal kingdom, from osmoregulation to reproductive physiology. We investigated the expression patterns and pharmacological effects of the gastropod ortholog of this peptide, conopressin, along with another peptide involved in gastropod reproduction, APGWamide, in the nudibranch *Berghia stephanieae*. A brain transcriptome was used to identify and annotate the gene sequences for the peptides and one conopressin receptor. *In-situ* hybridization chain reaction showed that many neurons in the brain expressed these peptides. However, the peptide genes were co-expressed by only three neurons, which were in the right cerebral ganglion, the same side on which the reproductive organs are located. A conopressin receptor (BSCPR1) was expressed in a prominent population of APGWamide expressing neurons. Placing animals in a solution containing the APGWamide peptide caused minimal behavioral changes. However, exposure to conopressin reduced locomotion, increased gut contractions, and caused voiding at high concentration. The genes for these peptides and BSCPR1 were expressed in cells in the digestive system. BSCPR1 was also expressed by a line of neurons on the anterior portion of the radula and would be contacted during feeding. APGWamide-expressing neurons were found in the genital ganglion. All three genes expressed in cells on sensory appendages. These results are consistent with the conopressin playing a variety of roles in the brain and the body and being involved in both reproduction and digestion. This study sheds light on the function of this ancient nonapeptide in a new-to-neuroscience invertebrate species.

## Keywords:

Vasopressin, oxytocin, peptide receptor, egestion, reproductive behavior

## Highlights:

- Gene sequences for conopressin, APGWamide, and a conopressin receptor were identified in a nudibranch brain transcriptome and labeled with in-situ hybridization chain reaction.
- Conopressin was expressed by fewer neurons in the brain than APGWamide, with co-localization seen in only 3 lateralized neurons, suggesting a role in reproduction.
- Bath application of conopressin reduced locomotion and caused gut contractions, with egestion in recently fed animals, whereas APGWamide had no effect.
- Conopressin and conopressin receptor were also expressed in the gut, reflecting their role in digestion.

## 1. Introduction

The oxytocin/vasopressin family of nonapeptides is ancient, displaying strong sequence conservation across all of bilaterians [1]. In invertebrates, there is only one copy of the gene for this peptide family rather than the two named peptides seen in vertebrates, oxytocin and vasopressin [2]. In gastropod molluscs, it has been called conopressin. There are differential patterns of expression of these peptides and their receptors in specific brain regions and tissues in many organisms, which has functional significance [3]. Here we investigated the localization and behavioral actions of conopressin and another important molluscan reproductive peptide, APGWamide in a nudibranch.

Conopressin has a variety of effects in gastropods. In *Lymnaea stagnalis,* conopressin affects vas deferens contractions [4] similar to the effects of another neuropeptide, APGWamide [5]. Together these neuropeptides play a direct role in *L. stagnalis* reproduction, with APGWamide and conopressin working antagonistically to modulate activity in the vas deferens and penis musculature, and being secreted by neurons in the brain [5]. Both peptides are co-localized in a region of the brain associated with reproduction, and occasionally co-expressed by the same neurons [6–8]. The role of conopressin is not limited to the physiology of semen release; in the land snail *Theba pisana,* conopressin is expressed in the dart sac, where it functions as an allo-hormone that increases paternity rates when darts are successfully injected into mates [9]. In *Aplysia californica*, conopressin has effects across non-reproductive bodily systems, modulating sensory neurons on the siphon [10] and respiratory behaviors [11].

Conopressin receptors have not been studied as extensively in gastropods. Sequences for two conopressin receptors (LSCPR1 and LSCPR2) have been found in *L. stagnalis* [12]. Receptors for conopressin have also been identified in *T. pisana* [9] and most recently in *A.*

*californica* [13]. They have only been localized to specific neurons in the brain of *L. stagnalis* where they were expressed by neurons across all ganglia, but most densely in the right lobes associated with reproduction [14]. Quantitative PCR studies indicate that LSPCR1 might also be expressed by peripheral tissues in *L. stagnalis* [14]

Here we focused on the nudibranch gastropod *Berghia stephanieae*, which we are developing as an accessible laboratory species. Neurons in the central ganglion of *B. stephanieae* were recently characterized with single cell sequencing [15] and a high quality genome was recently published [16]. Here, we identified the gene sequences for conopressin, APGWamide, and a conopressin receptor, then created probes for in-situ hybridization chain reaction and located the neurons and cells expressing these genes in the brain and body of the nudibranch. Lastly, we used bath application of conopressin and APGWamide to examine their effects on various behaviors of *B. stephanieae*.

## 2. Materials and methods

### 2.1 Animals

Live specimens of the nudibranch, *Berghia stephanieae*, were obtained commercially from Salty Underground. They were maintained and bred in artificial seawater (ASW, Instant Ocean) on a 12:12 LD cycle with aeration provided by an air stone. Animals were fed live *Exaiptasia diaphana* anemones (Carolina Biological) once every other day, and kept in groups of 10-30 individuals, observed to be mating and laying eggs frequently. Mature adults of 3-6 months of age were used for behavioral experiments and for labeling brain tissues. Large juveniles 6-8 weeks post-hatching were used for labeling peripheral tissues due to reduced autofluorescence.

## 2.2 Molecular identification of APGWamide, conopressin, and a conopressin receptor

To identify the nucleotide sequences for the genes that code for APGWamide, conopressin, and the *B. stephanieae* conopressin receptor (BSCPR1), a recent *B. stephanieae* reference transcriptome [15] was searched using BlastP [17] with the amino acid sequences known from *Lymnaea stagnalis*, as well as other molluscs as queries (supplemental table 1). The major features of each of these proteins including signal peptides and transmembrane domains were annotated using InterProscan [18]. Because the BSCPR1 sequence was partial, missing several transmembrane domains, we identified an orthologous conopressin receptor in a neural transcriptome of a related nudibranch *Melibe leonina* (accession number SRX1889794) using the same methods. We then used MUSCLE [19] to construct a multiple sequence alignment between known sequences in molluscs (for APGWamide) as well as other invertebrates and vertebrates (for conopressin and the conopressin receptor), with Jalview [20] to curate the alignment. IQtree [21] was used to build phylogenetic trees based on maximum likelihood methods, and iTOL [22] was used for tree curation and visualization.

## 2.3 Behavioral observations and analysis of bath-applied conopressin and APGWamide

We utilized commercially available synthetic forms of gastropod conopressin and APGWamide (Phoenix pharmaceuticals). For behavioral assays, the neuropeptides were dissolved in artificial seawater to the concentrations indicated in the results. Animals were placed individually in one well of a 4-well plate on top of a light board and video recorded from above using a webcam (Logitech 615), for one hour in ASW to establish a baseline of behaviors. Conopressin or APGWamide was added to create a final concentration of $10^{-6}$ M and the video recording was continued for another hour. We chose $10^{-6}$ M as the concentration because studies in *Aplysia californica* [11] and *Lymnaea stagnalis* [5] showed that micromolar concentrations

had strong and consistent effects in reduced preparations for electrophysiological experiments. Because our study used bath-application on an intact individual and targeted behavioral effects, we decided that this high concentration would be where we would be most likely to see any behavioral effect at all. We used another group of *B. stephanieae* to determine dosage response to conopressin in a series from $10^{-7}$ M to $10^{-5}$ M.

All videos of individuals were manually scored for duration of active movement behavior ("exploration") using the event logging software BORIS [23]. We defined exploration as locomoting at a steady pace with cephalic appendages extended and cerata relaxed. We noticed increased defecation and egestion during assays involving conopressin, so these behaviors were then scored as well.

**2.4 Statistical analysis of behavioral observations**

We used R software for all statistical comparisons [24]. To compare bath application of APGWamide, conopressin, or the null, saline, we subtracted the first hour of baseline "exploration" from the second treatment hour and then divided by the baseline amount to get a percentage change in exploration for each individual. We compared these three groups using a one-way ANOVA for independent samples. For our conopressin dosages experiment, we compared the percentage of time spent exploring out of one hour of observation in the same groups of animals in a repeated measures ANOVA. Number of voiding events, also measured across conopressin dosages, were compared across groups also using a repeated measures ANOVA. All data visualization consisted of boxplots generated first in R, which were edited in Adobe Illustrator.

**2.5 In-situ hybridization chain reaction**

To visualize expression of the genes for APGWamide, conopressin, and BSCPR1, we used *in-situ* hybridization chain reaction (HCR) [25]. Procedures were the same as previously used in *B. stephanieae* [15,26]. Briefly, tissues were first dissected and then fixed in 4% paraformaldehyde. Short DNA probe pairs complementary with the gene sequences were generated by Molecular Instruments (for APGWamide and conopressin) or via a custom python script which were manufactured by IDTDNA as oligopools (for BSCPR1). These probes were applied to the fixed tissue, and incubated for 16-24 hours at 37° C. This protocol has been found to be robust to degradation across many different organisms when applied to fixed tissues [25]. Probes for conopressin and BSCPR1 were used at 1 µL / 100 µL in hybridization buffer, for a final concentration of 0.01 M, whereas the probe for APGWamide was used at a final concentration of 0.001 M. The next day, samples were washed and then hairpins conjugated with fluorophores were added for 1 day (conopressin or APGWamide) or 2 days (BSCPR1), incubated in the dark at room temperature. In co-labeling experiments where APGWamide and BSCPR1 were multiplexed, the amount of hairpin solution used for APGWamide was halved (from 2 µL to 1 µL in 100 µL of amplification buffer) to account for differences in brightness driven by differences in expression level. After the HCR process, samples were incubated in DAPI at 1:1000 in 5X SSCT (saline sodium citrate tween) for 1 hour, then washed and mounted to slides in Vectashield mounting medium.

**2.6 Imaging and image quantification**

Fluorescence micrographs were taken using 10x or 20x objectives on a Zeiss 710M or a Nikon A125R scanning confocal microscope. ZenBlack software (Zeiss) or NIS-Elements (Nikon), respectively, was used to acquire the images. Raw z-stacks were then processed into s using FIJI [27].

## 3. Results

### 3.1 Identification of genes for APGWamide, conopressin, and a conopressin receptor

The gene for the APGWamide preprohormone in *B. stephanieae* was 215 amino acids in length, with a signal peptide and 8 repeats of the bioactive APGW, each flanked by proteolytic "KR" sequences and terminal glycines, as in other gastropod APGWamides (Fig. 1A, supplemental table 2). The number of APGW repeats varied, as seen in a multiple sequence alignment between *B. stephanieae* and several other gastropods; *B. stephanieae* lacked the first bioactive APGW repeat, having only eight repeats compared with nine in the other species shown (Figure 1A). In a molluscan gene tree of APGWamide, the *B. stephanieae* sequence grouped with other gastropods, away from bivalves (Figure 1B).

The sequence for the conopressin preprohormone was 243 amino acids in length and similar in structure to reported invertebrate sequences, with a signal peptide, the bioactive nonapeptide, and a long domain coding for the neurophysin co-factor (Figure 1C, supplemental table 2). Conopressin for *B. stephanieae* grouped with other molluscan sequences in a gene tree (Figure 1D).

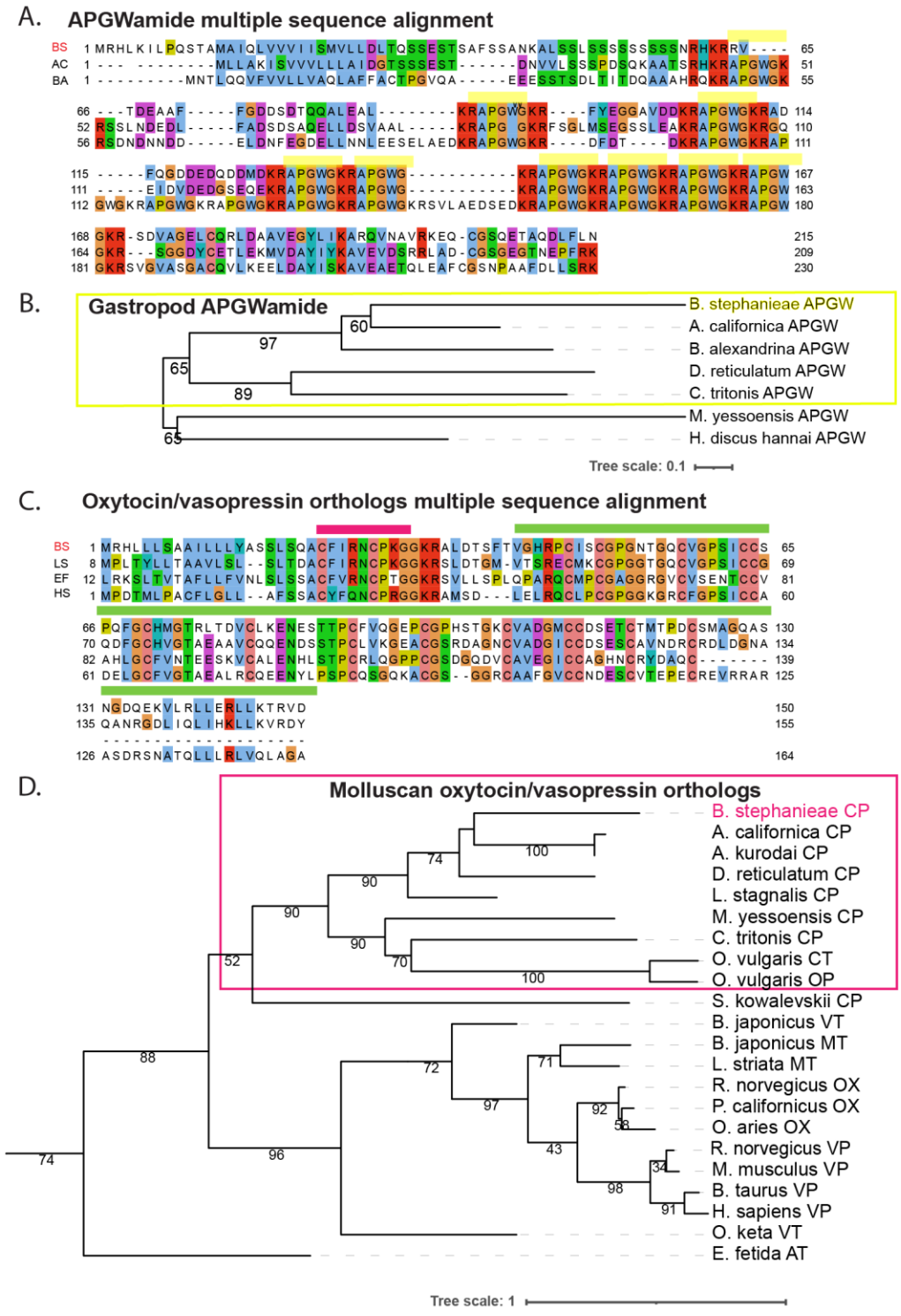


**Figure 1: Molecular identification of conopressin and APGWamide peptides in *Berghia stephanieae*. A)** Multiple sequence alignment of the predicted amino acid sequences of APGWamide in *Berghia stephanieae* (BS) and two other gastropods: *Aplysia californica* (AC) and *Biomphalaria alexandria* (BA). Amino acids are highlighted to show level of conservation across species. Yellow bars over the amino acid sequences indicate the APGW motif of the

bioactive peptide. **B)** Gene tree of molluscan APGWamides, showing the placement of the BS sequence with other gastropod sequences (yellow box). **C)** Multiple sequence alignment of the amino acid sequences of conopressin in BS, *Lymnaea stagnalis* (LS), *Eisenia fetida* (EF), and *Homo sapiens* (HS). The pink bar shows the oxytocin/vasopressin/conopressin orthologous nonapeptide; the green bar indicates the conserved neurophysin domain. **D)** Gene tree of the oxytocin/vasopressin/conopressin orthologs across taxa. Molluscs including *Berghia stephanieae* are grouped in a magenta box. Abbreviations of oxytocin/vasopressin orthologs are as follows: CP: conopressin, CT: cephalotocin, OP: octopressin, VT: vasotocin, MT: mesotocin, OX: oxytocin, VP: Vasopressin, AT: Annetocin

The identity of the *Berghia stephanieae* conopressin receptor (BSCPR1) gene was more ambiguous than the genes for the peptides. A BlastP search resulted in a sequence that, when annotated by InterProscan, was found to be a partial G-protein coupled receptor (GPCR), lacking the full seven transmembrane domains that are necessary for function (supplemental table 2). We used BlastP to find the full-length ortholog conopressin receptor (CPR) in the transcriptome of a related nudibranch, *Melibe leonina* (supplemental table 2). The *M. leonina* CPR sequence aligned well with both the *B. stephanieae* sequence and the original *Lymnaea stagnalis* receptor gene (LSCPR1) at each of the included transmembrane domains, illustrating the truncated nature of the *B. stephanieae* sequence. (Figure 2A). The absence of a full sequence in *B. stephanieae* might be due to low coverage in the transcriptome and we therefore conclude that this transcript represents a *B. stephanieae* conopressin receptor. It also groups well with other gastropod CPRs (Figure 2B).

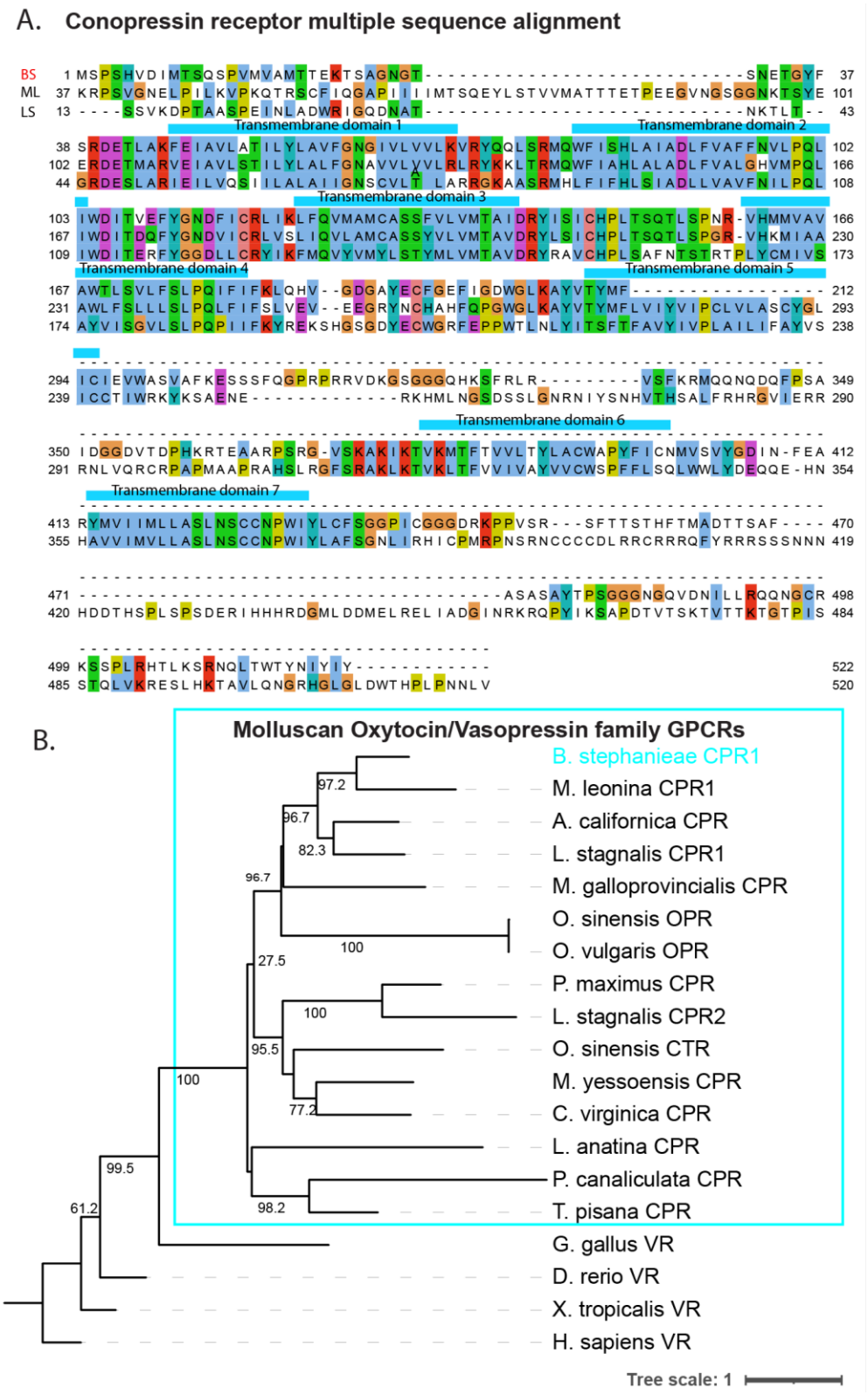


**Figure 2: Molecular identification of a conopressin receptor, BSCPR1, in *Berghia stephanieae*. A)** Multiple sequence alignment of conopressin receptors (CPRs) in three gastropods: BS, *Melibe leonina* (ML), and *Lymnaea stagnalis* (LS). Transmembrane domains are

numbered 1-7 and shown with blue bars above the sequences. **B)** Gene tree of vasopressin receptors (VR) and CPR orthologs across taxa. Molluscan CPRs group together (blue box). Abbreviations of oxytocin/vasopressin receptor orthologs are as follows: CPR: conopressin receptor, CTR: cephalotocin receptor, VR: vasopressin receptor

### 3.2 Localization of APGWamide, conopressin, and BSCPR1 in the brain

Neurons expressing APGWamide were labeled using *in-situ* hybridization chain reaction (HCR; N = 10 samples). Somata expressing APGWamide were found in the rhinophore ganglion (*rhg*), cerebral ganglion (*ceg*), pedal ganglion (*pdg*), pleural ganglion (*plg*), and buccal ganglion (*bcg*) (Figure 3A). The RNA expression levels were generally high as judged by the signal-to-noise ratio of the fluorescence and by the presence of signal in the axons projecting from the somata. The most prominent neurons were medially located, bilaterally on the dorsal face of the *ceg*.

Neurons expressing conopressin were present in the *rhg* and *ceg*, but not the *plg* or *bcg* (N = 16 samples, Figure 3B). There were three conopressin-expressing neurons asymmetrically located only in the right *ceg*, suggesting a potential role in reproduction because the reproductive organs are also on the right side in nudibranchs. In the distal lateral quadrant of the *rhg*, hundreds of very small (< 5 um) neurons expressed conopressin at low levels (Figure 3C). Each pedal ganglion had one large neuron with two visible neurite projections (Figure 3D). A cluster of small conopressin expressing neurons was also located only in the left pedal ganglion, near the connective to the cerebral ganglion (Figure 3B).

Co-expression of APGWamide and conopressin is a hallmark of neurons in the brain of other gastropods that directly control male aspects of reproduction [7]. In *B. stephanieae*, only three neurons in the right *ceg* consistently expressed both conopressin and APGWamide (Figure

3Ei and 3Eii). These neurons lack counterparts in the left *ceg*, suggesting that they might be involved in reproduction.

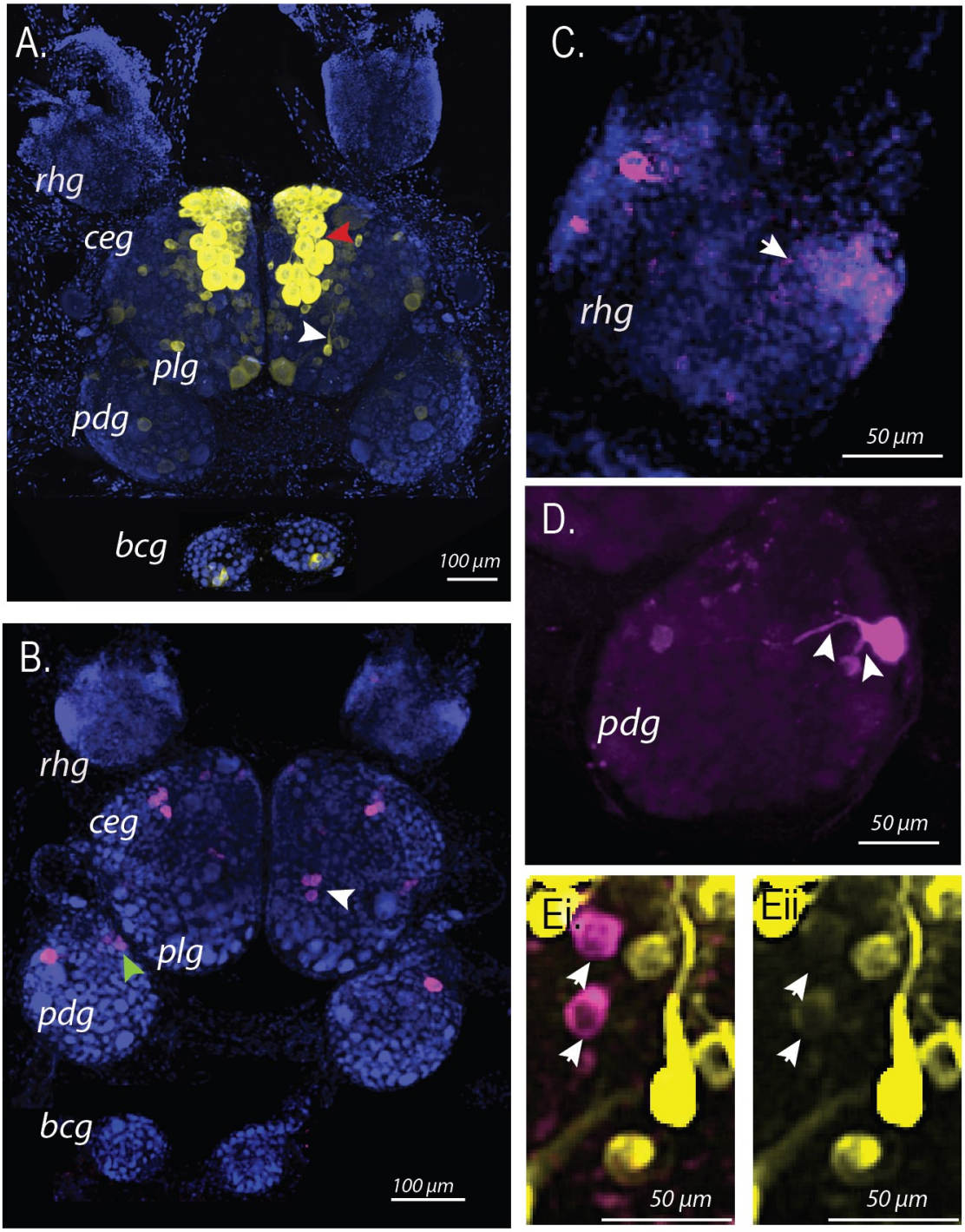


**Figure 3: Localization of the genes for APGWamide and conopressin in the neurons of the nudibranch brain. A)** APGWamide expression across the ganglia of the brain, with the most prominent and largest expression in the dorsal *ceg* (red arrowhead) and some neurons with

observable neurites (white arrowhead). **B)** Conopressin expression across the ganglia of the brain, with an asymmetrical cluster of three in the right *ceg* (white arrowhead) and an asymmetric cluster of >10 in the left *pdg* (green arrowhead). **C)** Conopressin is expressed in many small neurons in the lateral rhinophore ganglion (white arrowhead). **D)** A very large, lateral neuron in the pedal ganglion shows two neurites (arrowheads). **E)** A trio of neurons located only in the right *ceg* and not the left co-express APGWamide and conopressin (arrowheads to two of them). In all panels, APGWamide is yellow, conopressin is magenta, and DAPI is blue.

Many neurons also consistently and strongly expressed BSCPR1 (Figure 4A). BSCPR1 was expressed by more neurons than was conopressin, but fewer than those expressing APGWamide. BSCPR1-expressing neurons were in all ganglia of the brain, including the buccal ganglia, unlike conopressin. Two very large neurons in the left *plg* and one in the right *plg* were present in all samples (N = 6).

Co-expression of BSCPR1 with APGWamide or conopressin was not common, occurring in only a small number of neurons in specific regions of the brain. BSCPR1 was always expressed in the large ventral medial APGWamide-expressing neurons (Figure 4B). Conopressin-expressing neurons in the pedal ganglion sometimes co-expressed BSCPR1 (Figure 4C, N = 2 out of 4).

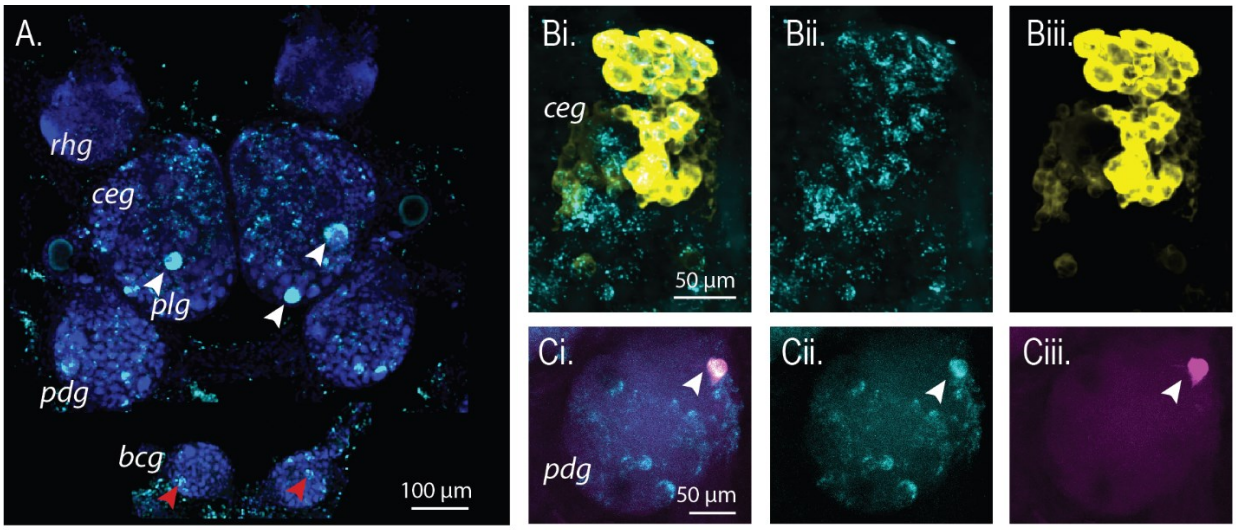


**Figure 4: Localization of the genes for conopressin receptor BSCPR1 in the neurons of the nudibranch brain. A.** Conopressin receptor expression (cyan) in all ganglia of the brain,

including in very large neurons in the *plg* (white arrowheads) and neurons in the *bcg* (red arrowheads). **B.** The medial, ventral APGWamide neuronal clusters (yellow) consistently express BSCPR1 (cyan). **C.** Sometimes the large conopressin expressing neuron (magenta) in the *pdg* (arrowheads) expressed BSCPR1 (cyan). In all panels, APGWamide is yellow, conopressin is magenta, conopressin receptor is cyan, and DAPI is blue.

### 3.3 The behavioral effects of APGWamide and conopressin peptides

In the presence of conopressin versus normal seawater or APGWamide, there were several significant behavioral effects, including a decrease in locomotion and an increase in egestion (vomiting) or defecation behaviors. A percentage representing the change in movement between the first baseline hour and the second treatment hour was calculated for each individual, and a one-way ANOVA showed that these values significantly differed between the conopressin, seawater, and APGWamide groups ($df = 2$, $F = 8.58$, $p = 0.0013$, Figure 5A). The percent decrease in exploration did not differ between the untreated and APGWamide treated groups (one-way ANOVA, post-hoc Tukey's test, $P>0.05$), but in the presence of bath-applied conopressin, *Berghia stephanieae* reduced its exploratory behavior (Figure 5A, N =12), with the difference significantly different from untreated animals and those treated with APGWamide (one-way ANOVA, post-hoc Tukey's test, $P<0.01$).

When viewing video playback, it was observed that conopressin-exposed individuals exhibited body contractions with cerata and oral tentacles raised and/or twisted versus relaxed postures of animals not dosed with conopressin (Figure 5B). When different dosages of conopressin were applied, exploration was significantly suppressed at all concentrations of conopressin tested ($10^{-7}$ – $10^{-5}$ M, Figure 5C). Instances of defecation or egestion/vomiting ("voiding") increased with increasing concentrations of conopressin (Figure 5D), although only significantly at the highest dosage. In trials with individuals that had been fed in the previous 3 hours, egestion/vomiting occurred strongly and almost immediately when exposed to $10^{-5}$ M

conopressin (as in supplemental video 1). The major observable changes at these high concentrations were a shortening of the body, raising of cerata, and pumping of the mouth visible via bobbing of the head. At times, contractions were very strong and characterized by rhythmic movements traveling from the bobbing head through the body and up through the cerata. The head also twisted back and forth during the stronger contractions, with this series of events often ending in a defecation or vomiting event, followed by a return to a quiescent but tensed state, as in figure 5B.

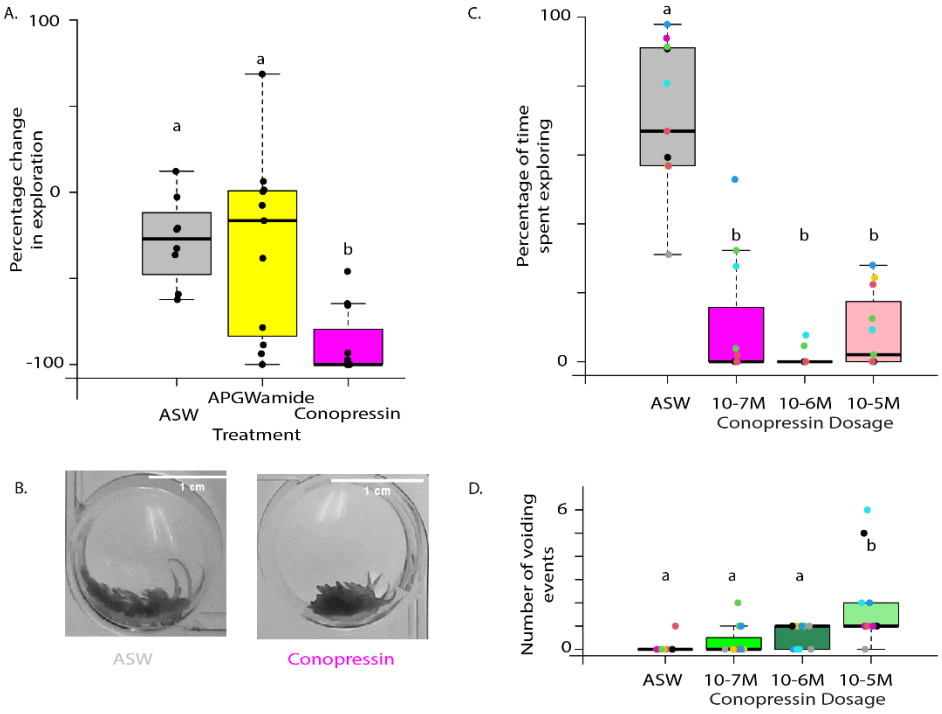


**Figure 5: Behavioral impacts of bath applied APGWamide and conopressin. A.** Three groups of *B. stephanieae* were tested, first for their behavior for 1 hour in artificial seawater (ASW), then after saline, APGWamide, or conopressin was added. Shown is the percentage decrease in exploration behavior. In all groups, exploration behavior decreased, with this decrease significantly differing from saline treated animals only after conopressin treatment (one-way ANOVA; df=2, F=8.58, p=0.0013, Tukey's Test; p<0.01). Lowercase letters show significantly different groups. **B.** Exploration behavior in ASW versus conopressin treatments – note the relaxed versus contractile bodily postures. **C.** Conopressin's effect on exploration was tested across a range of dosages, showing that exploration behavior was suppressed across all concentrations in this range (repeated measures ANOVA; df=3, F= 69.11, p<0.0001, Tukey's Test, p<0.01 for comparisons between ASW and each of the dosages). Colors of dots show the same individual across treatments. Lowercase letters show significantly different groups **D.** With

increasing dosages of conopressin, there was an increase in defecation or egestion, "voiding", events, with one individual voiding 6 times within one hour at the highest concentration tested. The means were significantly different only at the highest concentration (repeated measures ANOVA; df=3, F=7.24, p= 0.0009, Tukey's Test, $p<0.05$ for comparisons between $10^{-5}$ M and all other treatments). Colors of dots show the same individual across treatments. Lowercase letters show significantly different groups.

### 3.4 Localization of APGWamide, conopressin, and BSCPR1 in peripheral tissues

All three genes were expressed by cells outside of the brain. Neurons expressing APGWamide were found extensively across the peripheral epithelial surfaces, including on the cephalic sensory appendages (Figure 6Ai), and the foot. Many of these neurons had obvious dendritic projections to the epithelial surface. Conopressin-expressing neurons were found in many of the same areas, with putative sensory neurons on the rhinophore, oral tentacle (Figure 6Aii) and foot expressing the gene. However, no peripheral neurons co-expressed the two peptides.

Peptide expression was also visualized on the reproductive organ complex on the right side of the nudibranch's body. APGWamide labeling extended into axons that projected through body wall nerves, especially on the right side (Figure 6B). A right-side asymmetrical cluster of large neurons outside of the cerebral ganglion expressed APGWamide (Figure 6B, seen in N = 3 samples). Conopressin was not found in nerves projecting to this area or neurons present in this location. This is likely a dispersed genital ganglion, set along the genital nerve, as it is near the lateralized reproductive organs of *Berghia stephanieae*. A similar ganglion has been noted in *Aplysia californica* [28], though not the presence of APGWamide expressing neurons.

Conopressin was extensively expressed in the cells lining the full length of the gut (Figure 6C); APGWamide was not expressed in the gut itself. Conopressin expression extended into each of the cerata (Figure 6D), novel appendages of aeolid nudibranchs that contain

diverticula of the gut and sequester nematocysts from cnidarian prey [29]. APGWamide was expressed along the outer layers of the structure, whereas conopressin expression was confined to deeper layers. The cerata themselves contained multiple types of conopressin expressing cells. Those with the most distinct morphology occurred at the distal cnidosac, where nematocysts are stored (Figure 6D).

BSCPR1 expressing cells had a more restricted expression pattern. They were on the base of the oral tentacles and there was a distinct line of BSCPR1 neurons with sensory dendrites along the anterior of the radula, near the "teeth" (Figure 6E). Cells expressing BSCPR1 also lined the gut, immediately adjacent to the conopressin expressing cells (Figure 6C). Unlike in the central ganglia, the peptides and BSCPR1 were not co-expressed in cells in the periphery.

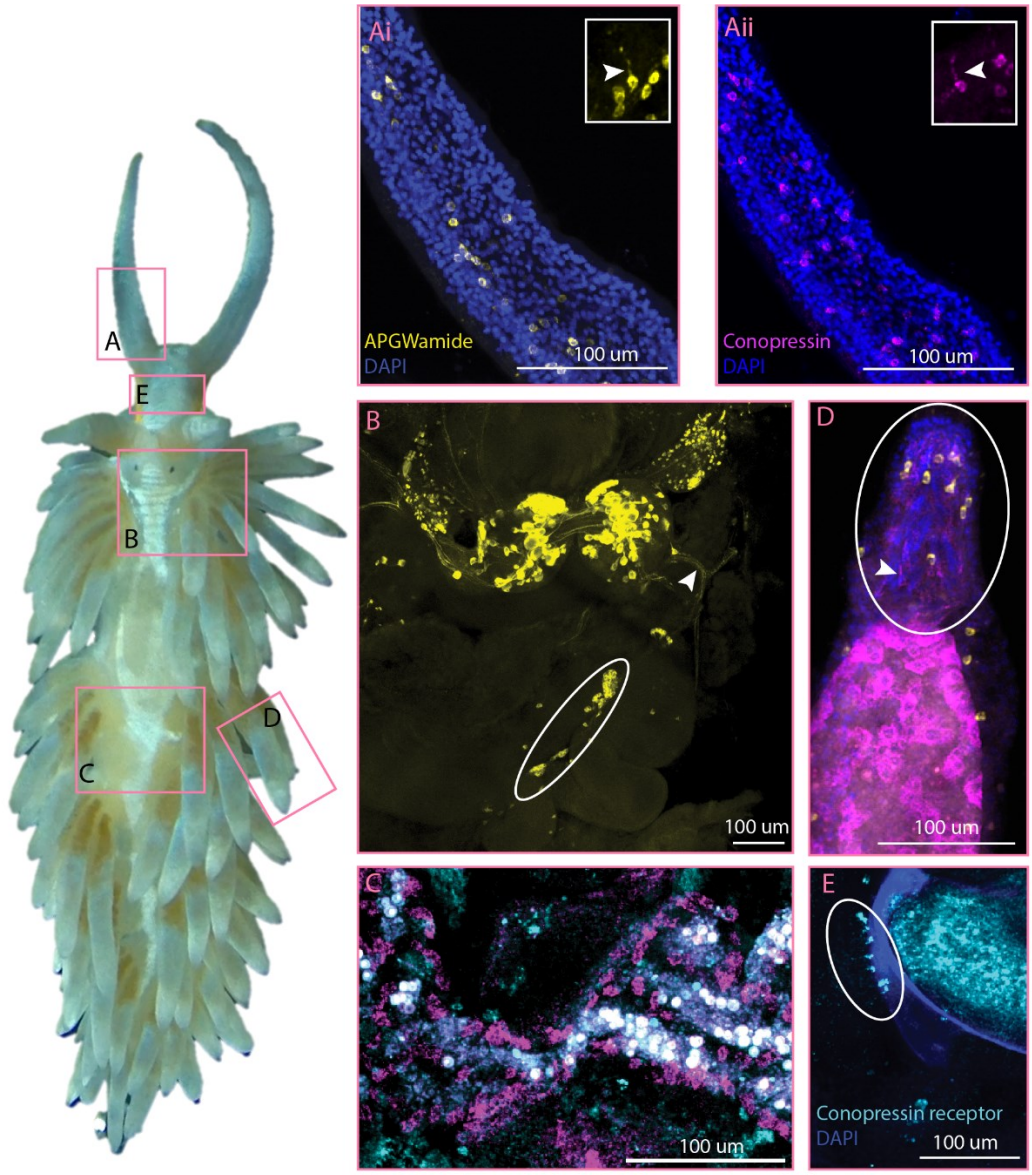


**Figure 6: Localizing APGWamide, conopressin, and BSCPR1 expression in the body. A.** Dense expression of i) APGWamide and ii) conopressin was seen on the oral tentacles, in putative sensory neurons (insets showing suspected dendritic projections through the epithelium, white arrowheads). **B.** The reproductive organs are located on the right side of the body, innervated by a nerve that leaves the right pedal ganglion (white arrowhead). Along this nerve are clusters of large APGWamide-positive cells, which are likely part of the peripheral genital ganglion (white circle). **C.** Conopressin and its receptor are shown in the digestive gland (gut) of the nudibranch in adjacent populations lining the structure. White circular structures are dinoflagellates from recent feeding on anemones. **D.** Conopressin expression continues into the cerata, novel nudibranch appendages, and at the distal tip of the cerata is the cnidosac (white circle), for storing and releasing nematocyst stinging cells for defense. Conopressin is expressed in a cell type with unique morphology in the cnidosac (arrowhead) versus that seen in the lower cerata and digestive gland, which possess larger, likely non-neuronal cells. APGWamide

expressing neurons (yellow) also occur on the surface of the cnidosac. **E.** BSCPR1 is expressed in a line of neurons on the front of the radula (white circle), likely contacting anything coming through the mouth and esophagus. In all panels, APGWamide is yellow, conopressin is magenta, conopressin receptor is cyan, and DAPI is blue.

## 4. Discussion

The nudibranch *Berghia stephanieae* expresses conopressin, a highly conserved oxytocin/vasopressin ortholog, as well as at least one conopressin receptor. Sequence homology for the APGWamide and conopressin genes was extremely high, as is characteristic across molluscs. Only one conopressin receptor was found in the transcriptome of *B. stephanieae* based on a homology search. In *Lymnaea stagnalis*, there are at least two conopressin receptors. Newly reported BSCPR1 is most similar in sequence to LSCPR1, rather than the other *Lymnaea* receptor, LSCPR2 [12]. LSCPR1 has a more specific binding affinity for conopressin, while LSCPR2 is thought to be more similar to the more broadly responding ancestral receptor between vertebrates and invertebrates and is more promiscuous in binding other nonapeptides beyond conopressin. In *L. stagnalis*, LSCPR1 is reported only from the brain and vas deferens, not the skin, using RT-PCR to measure levels of mRNA [12]. In *B. stephanieae,* we found BSCPR1 expressed in the brain, gut, and radula. Future studies could determine whether *B. stephanieae* has any other conopressin receptors expressed in different locations.

Expression patterns of APGWamide and conopressin in *Berghia stephanieae* differed from other gastropods. In *B. stephanieae,* conopressin was rarely co-localized with the functionally related molluscan reproductive peptide APGWamide, although together these peptides have marked homologous reproductive lobes in other gastropods [5,7]. APGWamide was expressed by the largest number of neurons, across all ganglia of the brain. These neurons were diverse in size, ranging from 5 microns in diameter in the rhinophore ganglia to greater

than 50 microns in diameter in the cerebral ganglia. Such widespread expression is reminiscent of the widespread expression of APGWamide reported in *A. californica*, which indicates that the peptide might be a general neuromodulator rather than having a specific role in reproduction [30].

Conopressin was expressed in fewer total neurons of the brain of *Berghia stephanieae* than in other species, with none observed in the pleural or buccal ganglia. There was some asymmetry in location of conopressin neurons, with a cluster of small neurons seen only in the left pedal, and a prominent cluster of three neurons only seen in the lower right cerebral ganglion. On the right side of the brain, the same side as the reproductive organs, these three are also the only neurons that co-express APGWamide. This possibly marks them as homologous with reproductive clusters and lobes seen in other gastropods [7,8], which generally contain tens or hundreds more neurons than we saw in this nudibranch. In these earlier studies, traditional *in situ* hybridization techniques were used across these gastropods to verify expression patterns at the level of the mRNA, thus are directly comparable to the patterns we observed. The smaller number of neurons that co-express conopressin and APGWamide is likely biological. However, this does not mean that they do not interact. Alternatively, in *B. stephanieae*, 20 large APGWamide-expressing neurons in both the right and left ganglia on the ventral side of the cerebral ganglia express the conopressin receptor (Figure 4B). It may be that these two peptides interact indirectly to affect reproduction rather than being co-released by the same neurons. An indirect signaling system based on an invertebrate oxytocin/vasopressin ortholog has been found in the brain of the flour beetle, *Tribolium castaneum* [31].

The conopressin receptor also has a widespread distribution across all ganglia of the brain. Perhaps most interesting is its co-localization in many APGWamide-expressing neurons,

as noted above. It is also co-localized within a large conopressin-expressing neuron in each of the pedal ganglia, indicating that this neuron in particular is likely auto-excitatory, with conopressin playing a role in feedback activity for that neuron. In addition, the conopressin receptor is expressed in specific neurons within the buccal ganglion. In other gastropods, specific neurons of the buccal ganglion coordinate ingestion and egestion behaviors [32], with specific peptides seen to contribute to switching between these behaviors [33]. The buccal neurons of *B. stephanieae* that express a conopressin receptor may be key neurons in the egestion behavior evoked by conopressin.

To examine how these peptides might interact outside of the brain, we also examined their expression peripherally . APGWamide and conopressin were both seen in large numbers of putative sensory neurons on the peripheral sensory appendages, the rhinophores and oral tentacles. A study reported APGWamide expressing sensory neurons in the olfactory organ of the fellow mollusc *Octopus vulgaris* [34], indicating either reproductive modulation of the sensory periphery, or, again, more widespread roles in signaling via these peptides. APGWamide was also noted in neurons in a peripheral genital ganglion similar to that seen in *A. californica* [28]. APGWamide has not yet been reported as a marker for that structure. The presence in the genital ganglion strongly indicates a reproductive function for APGWamide. We did not observe expression of conopressin or conopressin receptor in or near the area of the reproductive organs or genital ganglion, which differs from previous work in gastropods [4,9]. Fibers expressing both of these peptides are prominent in the vas deferens of *Lymnaea stagnalis* [5]. This difference may reflect the indirect nature of their interaction as now also seen in the neurons of the brain. It may indicate an increased role in reproductive physiology for APGWamide, perhaps with input coming from the genital ganglion, and a reduced role for conopressin, and conopressin instead

modulating digestive contractions. The lack of APGWamide and conopressin expressing cells on reproductive tissues may indicate that our *in-situ* hybridization reaction method could not capture the presence of fine fibers across these reproductive tissues, which was previously captured using immunohistochemistry and RT-PCR [5].

Instead, the most noted peripheral expression of conopressin and the conopressin receptor in *B. stephanieae* was associated with feeding and digestion. In nudibranchs, it is known that there are at least four different cell types in the gut: ciliated columnar cells throughout, with dense type A and type B tubule cells to absorb nutrients, and much sparser secretory cells [35]. Such cell type delineation has been enabled using electron microscopy to reveal ultrastructural details inside cells, which is beyond the scope of this study with *in-situ* hybridization in peripheral tissues labeling only the cell body immediately around the nucleus. Both conopressin and the conopressin receptor were seen to be expressed in cells throughout the gut, which winds through the body and into the cerata. Cerata are novel appendages that aeolid nudibranchs possess which sequester defensive stinging cells from the anemones they eat [29]. The presence of conopressin in the cerata and in the cnidosac indicates that the peptide may have been co-opted for some function in the novel cerata. In the lower ceras, cells expressing conopressin match those of the gut in their morphology. Different conopressin-expressing cell types occur distally in and around the cnidosac. APGWamide is also present in cells with neuronal morphology (e.g. dendritic projections) along the tip of the cnidosac. The conopressin receptor is not seen in the ceras or cnidosac; future studies could investigate whether *B. stephanieae* has additional conopressin receptors that may occur in the novel structure. The conopressin receptor we have identified was expressed by cells near the radula, however, which also implies conopressin plays a role in digestion and feeding behavior.

Our behavioral results align with our *in-situ* labeling, implying an expanded role for conopressin beyond reproductive behavior. Conopressin has a strong impact on the behavior of *B. stephanieae*, causing a cessation of movement, especially in individuals that recently fed. Application of the hormone induced whole body contractions that ultimately led to vomiting or defecation at high concentrations (supplemental video 1). We speculate that conopressin is released by the gut itself to modulate the guts' contractile activity, with auto-excitatory signaling noted to be evoked by conopressin in *L. stagnalis* [36] . In *Berghia stephanieae*, the conopressin receptor expressing cells also within the gut are the target of the secreted peptide, which could be secreted by conopressin cells in the gut or in the brain, allowing for more flexibility in evoking contractions in the gut. This would explain our results when bath-applying the peptide; we were seeing activation of the gut cells that express conopressin receptor. Increased voiding events, specifically urinary excretion have also been seen in arthropods including locusts and crickets, implying a role in water balance for their orthologs to conopressin [37]. Such a role is well known for mammalian vasopressin and supports wide-ranging effects of conopressin beyond reproduction in gastropods.

Another finding was that both defecation and vomiting were seen, though not in the same animal, indicating some state-dependence of conopressin. Combined with the observation of conopressin receptor in the buccal ganglia of the brain, and peripheral localization of conopressin and its receptor in the gut, cerata, radula, and oral tentacles, it becomes unsurprising that conopressin plays a role in digestive behavior of this nudibranch. It was recently reported that in starfish the orthologous peptide to conopressin induces fictive feeding [38]. In ants as well, the ortholog included in the oxytocin/vasopressin family affects foraging behaviors [39].

In contrast to conopressin, significant effects of bath applied APGWamide were not observed. It is possible an effect was too subtle to be seen with the resolution of our recording methodology. It is also possible that bath application of this peptide is not as effective as bath application of conopressin.

## 5. Conclusions

Here, we report that conopressin plays a more expansive role in digestion than previously observed in gastropods. Our evidence includes both localization of the genes in the brain and body as well as pharmacological experiments. Overall, the expression of this conserved nonapeptide and its receptors across various tissues and cell types is not well characterized in invertebrates. If expression patterns are determined in more invertebrate taxa alongside behavioral effects and sequence information, we may add to our understanding of how evolution has enabled one highly conserved nonapeptide sequence to become involved in such a wide range of pleiotropic functions.

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## Author contributions

CCT and PSK envisioned the study and designed the experiments. MNO and CCT identified the genes. MNO, KN, and CCT performed HCR labeling for the genes. MNO, KN, EK, and CCT performed behavioral experiments and analyses. CCT and PSK drafted and wrote the manuscript. All authors revised and approved of the manuscript.

## Competing Interests

The authors declare no competing interests.

## Data Accessibility

Raw data will be made available upon request. Full gene sequences used for probe making are included in the supplemental materials.

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