Invertebrate Biology

Invertebrate Biology 130(4): 313–324.

DOI: 10.1111/j.1744-7410.2011.00242.x

Conservation of a *Tritonia* Pedal peptides network in gastropods

Michael J. Baltzley,[1,2,3,a] Allison Sherman,[4,5] Shaun D. Cain,[4,6] and Kenneth J. Lohmann[1]

[1] Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, USA
[2] Biology Department, St. Mary's College of Maryland, St. Mary's City, Maryland 20686, USA
[3] Present address: Biology Department, Western Oregon University, Monmouth, Oregon 97381, USA
[4] Friday Harbor Laboratories, University of Washington, Friday Harbor, Washington 98250, USA
[5] Present address: Department of Biological Sciences, California State University, East Bay, Hayward, California 94542, USA
[6] Department of Biology, Eastern Oregon University, La Grande, Oregon 97850, USA

Abstract. Adults of the nudibranch mollusc *Tritonia diomedea* crawl using mucociliary locomotion. Crawling is controlled in part by the large Pedal 5 (Pd5) and Pedal 6 (Pd6) neurons that produce *Tritonia* Pedal peptides (TPeps). TPeps elicit an increase in ciliary beat frequency, thereby increasing crawling speed. In adults of *T. diomedea*, an extensive network of TPep-containing neurites adjacent to the basement membrane of the pedal epithelium delivers TPeps to the ciliated cells. In this study, we show that diverse nudibranchs all have a pattern of TPep-like immunoreactivity similar to that of *T. diomedea*, with thin tracts of TPep-like immunoreactive (TPep-LIR) neurites projecting to the epithelial layer. We also show that members of two non-nudibranch gastropod species have a pattern of TPep-innervation similar to that of the nudibranchs. In addition, we characterized two pairs of motor neurons in adults of the nudibranch *Armina californica* that are possible homologues of the Pd5 and Pd6 cells in *T. diomedea*. Activity in one of these pairs, the Pedal Peptidergic Dorsal 1 (PPD1) cells, was correlated with mucociliary locomotion. The second pair, the Pedal Peptidergic Ventral 1 cells, shared synchronous synaptic input with the PPD1 cells, a pattern consistent with the shared synaptic input of the *T. diomedea* Pd5 and Pd6 cells. These findings suggest that the roles of the Pd5 and Pd6 cells as mucociliary motor neurons in nudibranchs are conserved evolutionarily. Additionally, the extensive network of TPep-LIR neurites seen in the foot of *T. diomedea* appears likely to be a common feature among gastropods.

Additional key words: *Armina*, TPeps, mucociliary, locomotion, crawling

Members of many species of nudibranch molluscs, or sea slugs, crawl primarily using mucociliary propulsion (Agersborg 1922; Audesirk 1978a; Crow & Tian 2003). Mucociliary crawling in members of *Tritonia diomedea* Bergh 1894 is mediated in part by the bilaterally symmetrical Pedal 5 (Pd5) and Pedal 6 (Pd6) neurons, which are located on the dorsal surface of the pedal ganglia (Wang et al. 2003; Cain et al. 2006). Pd5 and Pd6 are large neurons (300–500 μm in diameter) which produce *Tritonia* Pedal peptides (TPeps). These cells project to the foot of the animal, where an extensive network of TPep-like immunoreactive (TPep-LIR) neurites adjacent to the basement membrane of the epithelium delivers TPeps to the ciliated cells (Willows et al. 1997; Cain et al. 2006). Similar TPep-LIR cells have been identified in the pedal ganglia of diverse nudibranchs, but the function of these cells has not been established (Croll et al. 2001; Newcomb & Katz 2007; Baltzley & Lohmann 2008).

Although putative Pd5 and Pd6 homologues have been identified in members of other species of nudibranchs based on TPep-LIR, size, and location, these cells do not necessarily control mucociliary locomotion. For example, the Pedal 7 (Pd7) cells in individuals of *T. diomedea* are large, TPep-producing neurons located on the dorsal surface of

[a]Author for correspondence.
E-mail: baltzlem@wou.edu

the pedal ganglia adjacent to Pd5 and Pd6. However, unlike the Pd5 and Pd6 cells, the Pd7 cells innervate the anterior body wall, oral veil, and mouth, rather than the foot, and are therefore unlikely to control crawling (Wang et al. 2004). Similarly, there are a number of TPep-LIR cells in the cerebral, pleural, and buccal ganglia of *T. diomedea* that, based on location and morphology, are unlikely to be involved in locomotion (Beck et al. 2000).

TPep-like peptides have also been localized in the central nervous system and adjacent to the foot epithelium in adults of the pulmonate gastropod *Cornu aspersum* (Müller 1774) (formerly known as *Helix aspersa*) (Pavlova & Willows 2005). While members of *C. aspersum* have an extensive network of TPep-LIR neurites adjacent to the foot epithelium, the animals crawl using muscular contractions of the foot rather than mucociliary propulsion (Pavlova & Willows 2005). Because TPep-LIR has been observed at a number of different ciliated epithelia in both *T. diomedea* and *C. aspersum* (Gaston 1998; Pavlova & Willows 2005), it is possible that the presence of TPep-like peptides at ciliated epithelia is highly conserved across the gastropods. Thus, the Pd5 and Pd6 neurons may have evolved a specialized role in mucociliary locomotion in *T. diomedea*, whereas the homologues of Pd5 and Pd6 may have a different role in other gastropods.

In this study, we examined the large TPep-LIR neurons of the nudibranch *Armina californica* Cooper 1863, which are likely homologous to the Pd5 and Pd6 cells in members of *T. diomedea* (Baltzley & Lohmann 2008). We also examined the pedal epithelium of members of nine other species of nudibranchs, representing all four nudibranch suborders, as well as two non-nudibranch gastropods, *Phyllaplysia taylori* Dall 1900 and *Calliostoma ligatum* Gould 1849, to determine if the network of TPep-LIR neurites is conserved across the nudibranchs and possibly more widely in the gastropods. We found that the presence of TPep-LIR neurites adjacent to the pedal epithelium is a widespread trait in nudibranchs. We also found TPep-LIR neurites adjacent to the pedal epithelium of *P. taylori* and *C. ligatum*, indicating that this TPep network may be a general feature in all gastropods. Finally, in adults of the nudibranch *A. californica*, we characterized the activity of the PPD1 and PPV1 neurons. The results are consistent with the hypotheses that the PPD1 and PPV1 cells are mucociliary motor neurons and are homologous to the Pd5 and Pd6 cells in *T. diomedea*.

Methods

Animal collection

Members of the species shown in Figure 1 were examined in this study; that figure also shows their phylogenetic relationships. All specimens were collected within the state of Washington, USA. Adults of *Tritonia diomedea* and *Dendronotus iris* Cooper 1863 were trawled from Bellingham Bay at depths of ~30 m. Adults of *Armina californica* were collected from Dash Point State Park and Seahurst Park by SCUBA divers. Members of *Melibe leonina* Gould 1852 were collected by hand in shallow areas off Shaw Island, and adults of *Dirona pellucida* Volodchenko 1941 (also known as *D. aurantia*) and *Archidoris montereyensis* Cooper 1862 were collected from the floating dock of the University of Washington's Friday Harbor Laboratories. Members of all other species were collected around San Juan Island by various researchers at the Friday Harbor Laboratories. All animals were maintained in flow-through seawater tanks.

TPep immunolabeling

A piece of tissue (~1 cm^2) from the middle of the foot was placed in filtered seawater (100 μm mesh

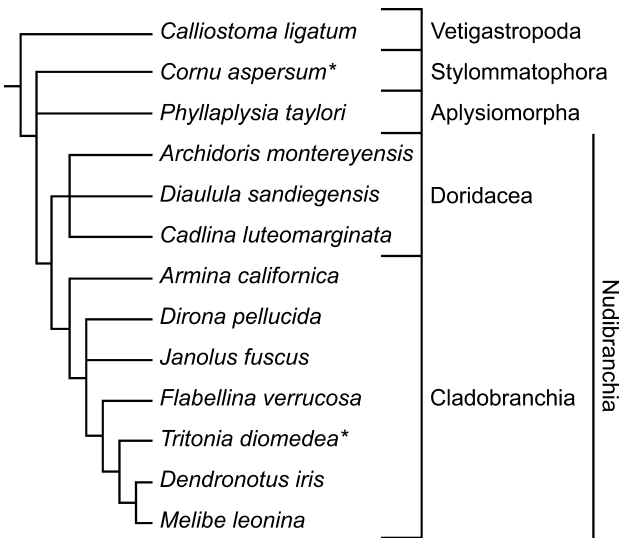

Fig. 1. Phylogeny of species examined for TPep-like immunoreactivity near the ciliated pedal epithelium. Asterisks indicate species previously examined in other studies (*Cornu aspersum*: Pavlova & Willows 2005; *Tritonia diomedea*: Cain et al. 2006). The phylogeny is based on morphological and molecular characters (Wägele & Willan 2000; Wollscheid-Lengeling et al. 2001; Grande et al. 2004).

size, FSW) at 4°C for 24–48 h. This kept the tissue viable, but allowed the epithelium to secrete much of its mucus. Tissue samples were then fixed at 4°C for 5–12 h in 4% paraformaldehyde in FSW. They were subsequently rinsed in FSW, then in phosphate-buffered saline (PBS). Tissue samples were embedded in a gel of 1.5% low melting point agarose in PBS and then cut in transverse sections 100–200 μm thick using a Vibratome Series 1000.

The central ganglia and foot tissue were fluorescently immunolabeled for TPeps using a previously described protocol (Beck et al. 2000; Baltzley & Lohmann 2008). All TPep-immunolabeling was imaged with a Bio-Rad Radiance confocal microscope and captured using Bio-Rad LaserSharp2000 software (Bio-Rad, Hercules, CA, USA). Brightness and contrast of all images were adjusted in Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA, USA). The TPeps are a trio of peptides (TPep-PLS, TPep-PAR, and TPep-NLS), each 15 amino acids in length, that differ at positions 1, 8, and 12 (Lloyd et al. 1996). We used an antiserum to TPep-NLS provided by A.O.D. Willows. The secondary antibody was labeled with Alexa Fluor 647 (Molecular Probes, Eugene, Oregon, USA). As an autofluorescence control, tissue was incubated without the secondary antibody. To control for non-specific labeling by the secondary antibody, tissue was incubated without the TPep-NLS antiserum. Finally, to control for non-specific labeling by our primary antibody, we added 100 μmol L^{-1} TPep-NLS to our primary antibody solution 24 h before incubation. None of these control tissues showed any TPep-like immunolabeling.

Crawling stimulus

Salt solutions applied externally to adults of *T. diomedea* cause an increase in mucociliary locomotion (Audesirk 1978a). To determine if a salt solution elicits a similar response in adults of *A. californica*, 0.5 mL of 3 mol L^{-1} KCl was applied to the dorsal posterior body wall of intact, stationary animals in a flow-through seawater tank. The time and distance each animal crawled along the bottom and sides of the tank were measured until the animal reached the surface of the water.

Intracellular and extracellular recordings

In semi-intact whole animal preparations of *A. californica*, we recorded intracellularly from a TPep-LIR cell that resembles the Pd5 cell of *T. diomedea* in terms of size and location within the central ganglia. The preparation used was similar to that commonly used for *T. diomedea* (Willows et al. 1973; Cain et al. 2006). Intracellular recordings were made using glass microelectrodes (8–15 MΩ resistance) back-filled with 10 mmol L^{-1} Alexa Fluor 488 hydrazide in 200 mmol L^{-1} KCl, and then filled with 3 mol L^{-1} KCl. The Pd5-like cell was impaled and allowed to recover for at least 30 min. The cell membrane potential was monitored using an A-M Systems Neuroprobe Amplifier (A-M Systems, Carlsborg, WA, USA).

Extracellular recordings of Pedal Nerve 3 (PdN3) of adults of *A. californica* were made using suction electrodes. In *T. diomedea*, PdN3 projects from the lateral margin of the pedal ganglion and contains a neurite of Pd5. Voltage was monitored using an A-M Systems Differential AC Amplifier. The signals from the amplifiers were digitized using a CED 1401 digitizer and analyzed with Spike2 software (Cambridge Electronic Designs, Cambridge, UK).

For each animal, an experimental trial and a control trial were attempted. In the experimental trial, a solution of 0.5 mL of 3 mol L^{-1} KCl with fluorescein was applied to the dorsal, posterior body wall to elicit a crawling response. The fluorescein was added to visualize the location of the KCl solution in the recording chamber and to confirm that the solution was swept toward the water outflow tube and away from the incision. For each animal, a random number generator was used to determine whether the experimental trial or the control trial was performed first. For both experimental and control trials, the trial did not begin until the action potential frequency of the neuron was <0.10 Hz for 5 min. In experimental trials, the stimulus was applied 10 min after the start of the trial. In control animals, the animal was left untouched. The protocol was then repeated with either the control or experimental trial (whichever trial was not performed first).

Results were analyzed using a repeated-measures ANOVA with time as a within-subject variable (1st 10 min vs. 2nd 10 min) and stimulus (none vs. 3 mol L^{-1} KCl) as a between-subject variable (PASW Statistics 18.0, IBM, Armonk, NY, USA). We performed *post hoc* paired t-tests to compare (1) the mean action potential frequency in the first 10 min of the control trials to the mean frequency in the subsequent 10 min, and (2) the mean action potential frequency in the first 10 min of the experimental trials to the 10 min after stimulus application. We performed an independent t-test to compare the first 10 min of the control trial to the first 10 min of the experimental trial. Using the

Bonferroni correction, our criterion for significance for the *post hoc* t-tests was $p<0.0167$.

Neuroanatomy

After completing the paired control and experimental treatments, Alexa Fluor 488 hydrazide was injected into the Pd5-like cells. The central ganglia were then fixed in 4% paraformaldehyde for 6–12 h. Tissue was immunolabeled with an antibody to TPep-NLS to confirm that the cells we were recording from were in fact TPep-LIR neurons. We used a similar dye injection protocol to visualize the morphology of other TPep-LIR cells.

Pedal Nerve 2 (PdN2) and PdN3 were dissected to determine their gross areas of innervations. In members of *T. diomedea,* these nerves contain neurites of Pd5. We removed the viscera and incubated the animal in 1% methylene blue for 2 h at 4°C. The nerves were followed from the pedal ganglia to the foot musculature. We dissected the nerves from the foot musculature until the branches were too small to visualize. Additional methylene blue incubations were used as needed.

Results

TPep immunolabeling

All gastropod species had TPep-LIR neurites in the foot tissue near the ciliated epithelium. In most species, there was an extensive network of TPep-LIR neurites near the epithelium, and small tracts of TPep-labeling in the connective tissue and musculature. This pattern was observed in *Dirona pellucida* (*n*=3; Fig. 2), as well as in specimens of *Archidoris montereyensis* (*n*=3), *Cadlina luteomarginata* McFarland 1966 (*n*=5), *Diaulula sandiegensis* Cooper 1862 (*n*=2), *Flabellina verrucosa* Sars 1829 (*n*=4), and *Janolus fuscus* O'donoghue 1924 (*n*=3; Fig. 3A–E). In members of *J. fuscus*, the TPep-LIR neurites appeared to penetrate into the epithelial layer.

In *Melibe leonina* (*n*=4) and *Dendronotus iris* (*n*=3), the TPep-LIR neurites that projected to the ciliated pedal epithelium did not appear to branch extensively (Fig. 3F–I). In members of other species, tracts of TPep-LIR neurites were observed along the basement membrane of the pedal epithelium, but this pattern was not observed in *M. leonina* and *D. iris*. Members of the two non-nudibranch species examined, *Phyllaplysia taylori* (*n*=3) and *Calliostoma ligatum* (*n*=3), had a network of TPep-LIR neurites similar to the majority of the nudibranchs (Fig. 4). In specimens of *Armina californica*, there was again an extensive network of TPep-LIR neurites along the basement membrane of the foot epithelium and small tracts of TPep-labeling in the connective tissue and musculature (Fig. 5A). As previously reported, the central ganglia of *A. californica* had three large TPep-LIR cells on the dorsal surface of the pedal ganglion and four large TPep-LIR cells on the ventral surface (Baltzley & Lohmann 2008). Here we designated the cells on the dorsal surface Pedal Peptidergic Dorsal cells 1, 2, and 3 (PPD1, PPD2, and PPD3; Fig. 5B). The four largest cells on the ventral surface we designated Pedal Peptidergic Ventral cells 1, 2, 3, and 4 (PPV1, PPV2, PPV3, and PPV4). Based on dye injection, the PPD1 cell in both pedal ganglia appeared to have a primary neurite that bifurcated and sent a branch into PdN2 and PdN3 (Fig. 5C). In addition to the two large processes that projected out PdN2 and PdN3, small branches split off from the primary neurite in the neuropil. The PPV1 cells had a primary neurite that bifurcated

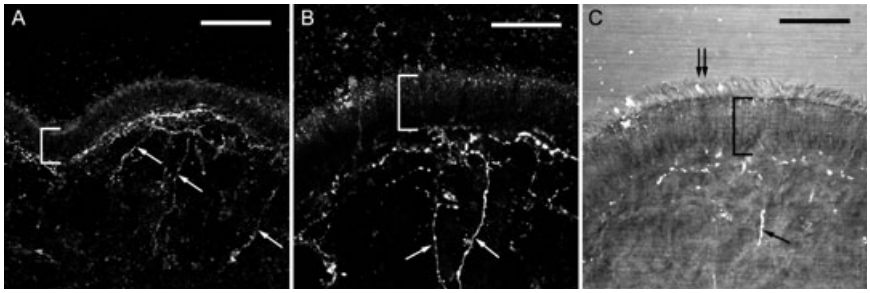

Fig. 2. TPep-like immunolabeling in foot tissue of *Dirona pellucida*. **A.** Confocal micrograph of a transverse section of the foot immunolabeled for TPep-NLS. The epithelial layer is shown with the bracket. Individual neurites, indicated by single arrows, project towards the epithelium and then run along the base of the epithelium. Scalebar=100 μm. **B.** Another view of a transverse section of the foot immuolabeled for TPep-NLS, with bracket and single arrows indicating epithelium and neurites, respectively. Scalebar=50 μm. **C.** Confocal micrograph of the same section as in (b), overlaid on transmitted light micrograph. The bracket shows the epithelial layer, and the single arrow shows a neurite that projects to epithelial layer. Double arrows indicate cilia on surface of the epithelium. Scalebar=50 μm.

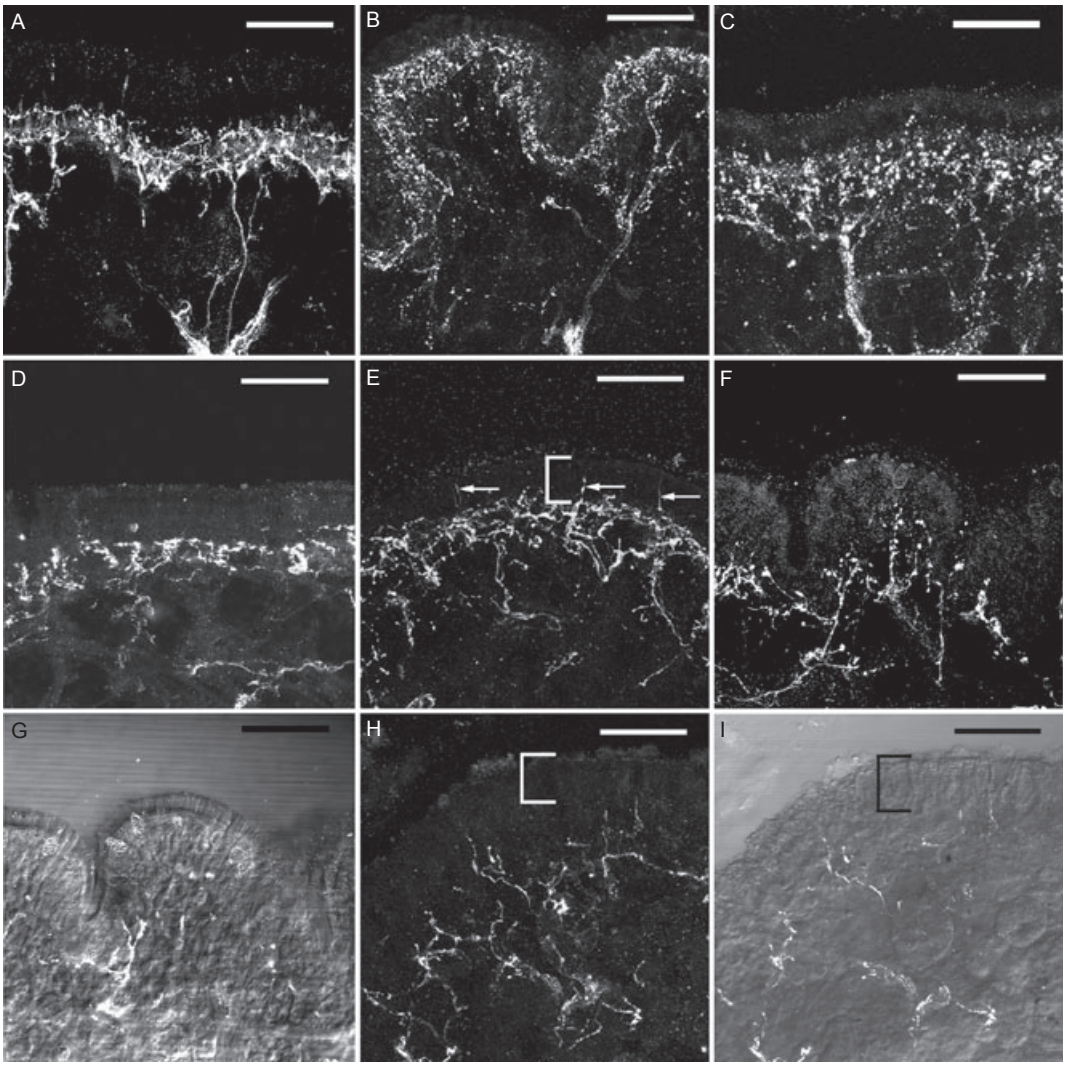

Fig. 3. TPep-like immunolabeling in foot tissue of various nudibranchs. Each confocal micrograph is of a transverse section of foot tissue immunolabeled for TPep-NLS. In each species, TPep-like immunoreactive (TPep-LIR) neurites project to the epithelium, and there is extensive TPep-like immunolabeling adjacent to the base of the epithelium. **A.** *Archidoris montereyensis*. Scalebar=50 μm. **B.** *Cadlina luteomarginata*. Scalebar=75 μm. **C.** *Diaulula sandiegensis*. Scalebar=50 μm. **D.** *Flabellina verrucosa*. Scalebar=50 μm. **E.** *Janolus fuscus*. The epithelial layer is marked with the bracket. Individual neurites, indicated by single arrows, project into the epithelial layer. Scalebar=75 μm. **F.** *Melibe leonina*. Scalebar=50 μm. **G.** *M. leonina*. Confocal micrograph of the same section as in (f), overlaid on transmitted light micrograph. Scalebar=50 μm. **H.** *Dendronotus iris*. Bracket indicates epithelium. Scalebar=75 μm. **I.** *D. iris*. Confocal micrograph of the same section as in (h), overlaid on transmitted light micrograph. Scalebar=75 μm.

and sent a branch into PdN1 and PdN2 (Fig. 5D). The PPD2 cell, located on the dorsal surface near PdN3, had a primary neurite that appeared to project towards the base of PdN2 and PdN3. The PPD2 cells also had multiple processes that branched off the primary neurite and projected towards the neuropil of the pedal ganglion.

Gross dissection indicated that PdN2 of *A. californica* innervated the ipsilateral foot adjacent and anterior to the brain (n=4). Dissection showed that three branches split off PdN3 before the nerve and all the branches entered the foot musculature. After entering the musculature, PdN3 ran the entire length of the foot posterior to the brain and numerous branches extended towards the ipsilateral body wall. The innervation area was not observed to cross the mid-body line.

Response to salt stimulus

When 0.5 mL of 3 mol L^{-1} KCl was applied to the posterior end of intact, stationary individuals of

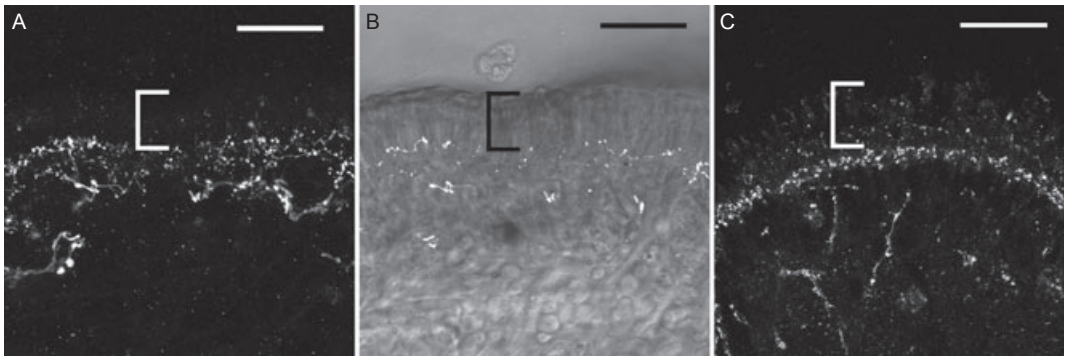

Fig. 4. TPep-like immunolabeling in foot tissue of *Phyllaplysia taylori* and *Calliostoma ligatum*. **A.** Confocal micrograph of a transverse section of the foot of *P. taylori* immunolabeled for TPep-NLS. Bracket indicates epithelium. Scalebar=25 μm. **B.** Confocal micrograph of the same section as in (a), overlaid on transmitted light micrograph. Scalebar=25 μm. **C.** Confocal micrograph of a transverse section of the foot from *C. ligatum* immunolabeled for TPep-NLS. Scalebar=50 μm.

A. californica, the animals initially contracted the dorsal body wall. The dorsal body wall slowly relaxed and the animals began to crawl between 31 and 138 s after stimulus application (mean ± SEM=68 ± 13 s, n=8; Fig. 6A). The mean crawling speed was 5.5 ± 0.5 cm min^{-1}. All animals crawled for at least 10 min before they reached the water surface.

To determine the rate of action potentials in the right PPD1 cell before and after the application of an experimental salt stimulus, the action potential frequency was averaged over the 10 min prior to stimulus application. This baseline frequency was then compared to the average action potential frequency over a 10-min period after stimulus application. Because the intact animals did not start crawling until 31–138 s after the salt solution was applied, the action potential frequency was averaged over the 10-min period beginning 2 min after the stimulus was applied.

Repeated-measures ANOVA showed a significant effect of time (p=0.01) on the rate of action potentials in the PPD1 cell. There was also a significant interaction between time and stimulus (p=0.003), but no significant main effect of stimulus (p=0.11). When 0.5 mL of 3 mol L^{-1} KCl was applied to the posterior end of a semi-intact preparation of *A. californica*, the average rate of action potentials in the right PPD1 cell increased from 0.04 ± 0.01 Hz to 0.14 ± 0.03 Hz (n=10; *post hoc* t-test: p=0.006; Fig. 6B,C). If the salt solution was not applied to the posterior end of the animal, the average rate of action potentials was not significantly different from the baseline condition (n=8; *post hoc* paired t-test: p=0.03; $\alpha = 0.0167$). There was no difference between the average action potential frequency in the experimental and control animals before the stimulus was applied (*post hoc* t-test: p=0.54). To control for the effects of adding KCl to the bath, we also ran several trials in which 0.5 mL of 3 mol L^{-1} KCl was added to the bath next to the anterior end of the animal, ~3 cm away from the incision. In these trials, the average action potential frequency was 0.06 ± 0.03 Hz (n=3) before the solution was added and 0.04 ± 0.02 Hz after the solution was added.

To determine if the PPD1 neurite in PdN3 was carrying efferent or afferent information, we recorded intracellularly from PPD1 and extracellularly from the ipsilateral PdN3. Spontaneous action potentials were detected extracellularly in PdN3 after the action potential was detected in the cell soma (Fig. 6D).

We performed simultaneous recordings of right and left PPD1. The response of left PPD1 to 3 mol L^{-1} KCl applied to the dorsal body wall was similar to the response of right PPD1 (Fig. 7). The rate of action potentials in left PPD1 increased from 0.01 ± 0.01 Hz (n=3) to 0.09 ± 0.06 Hz (n=3). Right and left PPD1 received different synaptic input and rarely had synchronous action potentials.

We also performed simultaneous recordings of the ipsilateral PPD1 and PPV1 cells as well as the ipsilateral PPD1 and PPD2 cells. The ipsilateral PPD1 and PPV1 cells receive synchronous synaptic input and have similar activity patterns (Fig. 8A,B). The ipsilateral PPD1 and PPD2 show periods of simultaneous excitation, but often have different activity patterns (Fig. 8C).

Discussion

In all of the nudibranchs investigated, TPep-LIR neurites projected to the ciliated pedal epithelium

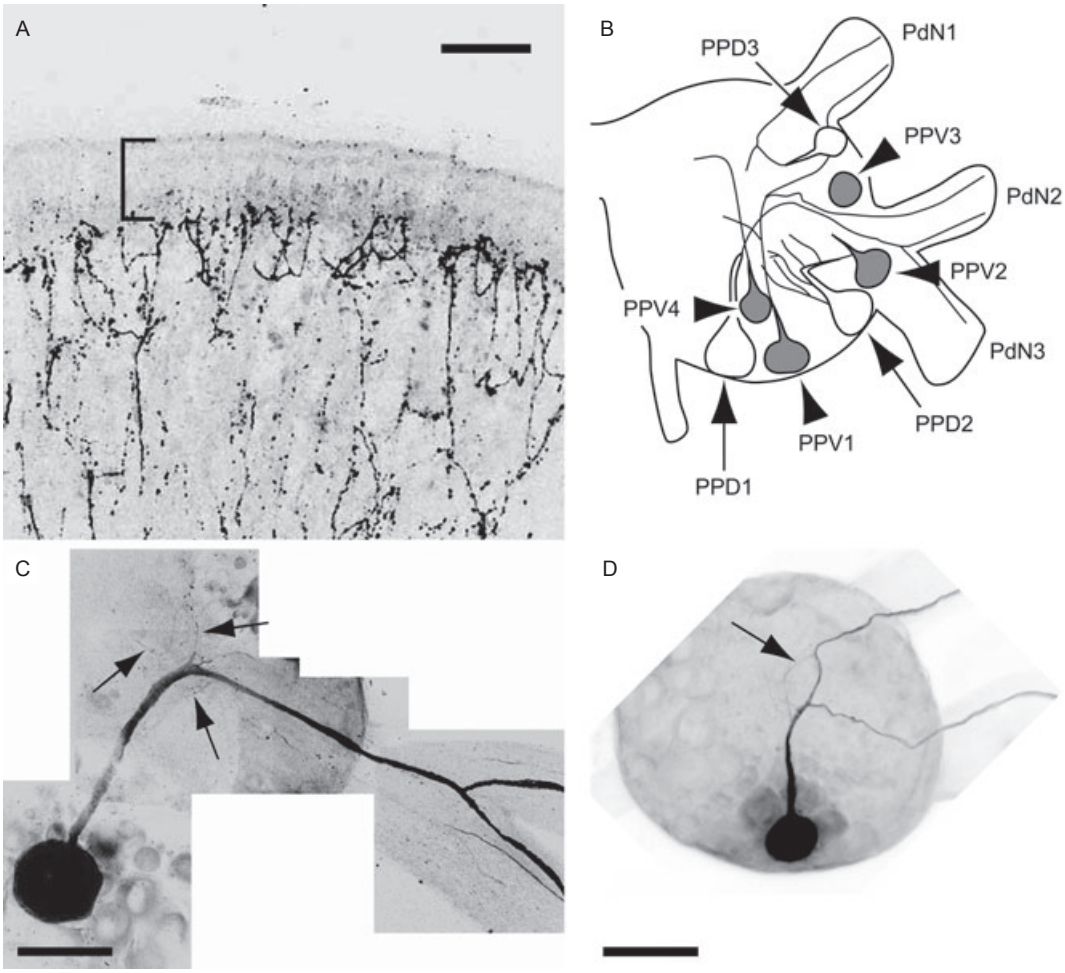

Fig. 5. Identification of TPep-LIR tissue in the foot and central nervous system of *Armina californica*. **A.** Confocal micrograph of a transverse section of *A. californica* foot tissue immunolabeled for TPep-NLS. The epithelial layer is indicated with the bracket. Scalebar=50 μm. **B.** Schematic diagram of the TPep-LIR neurons in the pedal ganglia. Arrows indicate the large TPep-LIR neurons on the dorsal surface of the ganglion, and arrowheads the large TPep-LIR neurons on the ventral surface. The white cells are the Pedal Peptidergic Dorsal cells (PPD1, PPD2 and PPD3), and the grey cells are the Pedal Peptidergic Ventral cells (PPV1, PPV2, PPV3 and PPV4). The ganglia, nerves and cell bodies were traced in Adobe Illustrator. The projection patterns of neurites were determined using NIH Image J. **C.** Dye injection of a PPD1 cell. A primary neurite projects from the cell body into the neuropil. There is extensive branching within the neuropil. Several small branches are indicated by arrows. The primary neurite bifurcates into two large branches. One branch projects into the ipsilateral PdN2 while the other branch projects into the ipsilateral PdN3. Scalebar=150 μm. **D.** Dye injection of a PPV1 cell. The primary neurite of PPV1 bifurcates into two large branches and several smaller branches. One small branch is indicated by the arrow. One large branch projects into the ipsilateral PdN1 while the other large branch projects into the ipsilateral PdN2. Scalebar=200 μm.

(Figs. 2, 3). In members of most species, the neurites branched near the pedal epithelium, resulting in an extensive network of TPep-LIR neurites adjacent to the base of the epithelium. In individuals of two species, *Melibe leonina* and *Dendronotus iris* (Fig. 3), TPep-LIR neurites projected to the epithelium, but did not appear to branch extensively. In adults of the aplysiomorph *Phyllaplysia taylori* and the prosobranch *Calliostoma ligatum* (Fig. 4), the TPep-LIR neurites formed an extensive network similar to that found in the nudibranchs. These results indicate that the presence of a TPep-like neuropeptide in neurites adjacent to the pedal epithelial layer is a widespread feature in gastropods, and may be a basal feature of the gastropod nervous system.

TPeps act as cilio-excitatory neuropeptides on pedal, salivary duct, and esophageal cilia of members of *Tritonia diomedea* (Willows et al. 1997; Gaston 1998). The conserved function of TPeps on different epithelial surfaces, combined with the

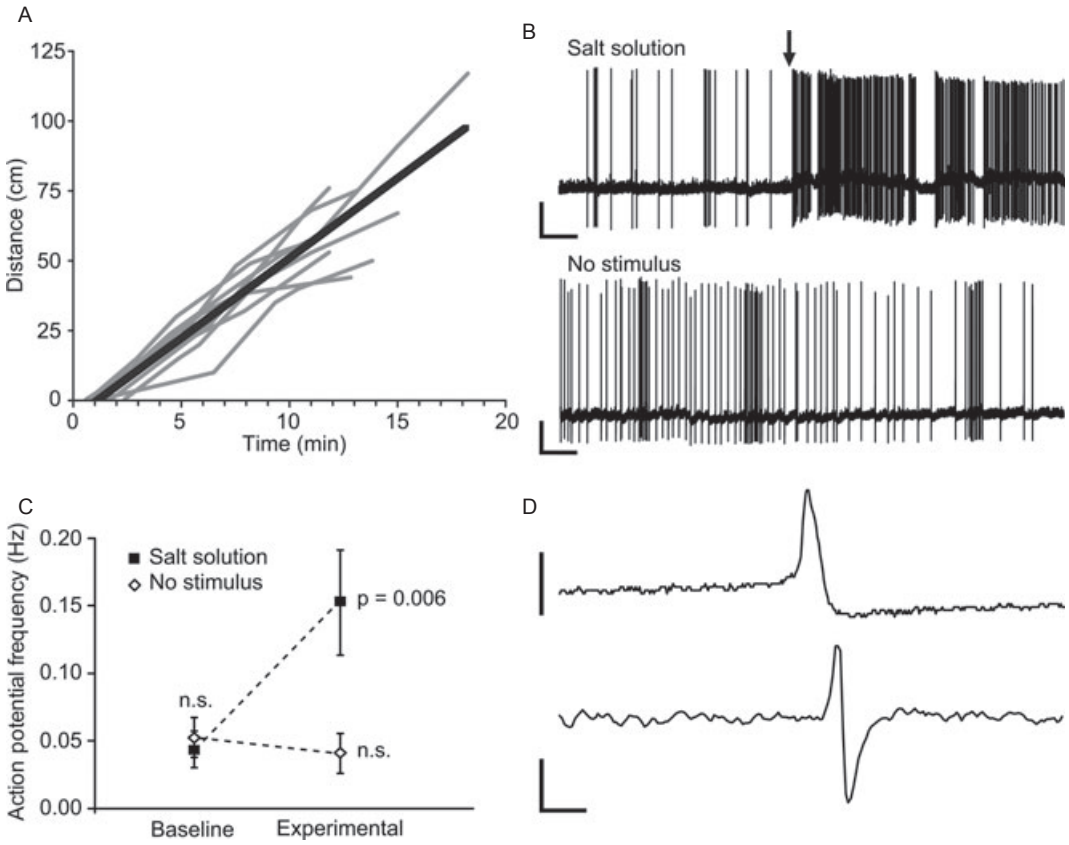

Fig. 6. Behavioral and cellular response to noxious stimulus. **A.** Crawling response of intact *Armina californica* to the application of 0.5 mL of 3 mol L^{-1} KCl to the dorsal, posterior body wall. The black line represents the average response of intact *A. californica* to application of KCl (n=8). Gray lines represent the response of individual animals. Animals were not crawling prior to stimulus application. The stimulus was applied at 0 min. **B.** Response of the right PPD1 neuron to 3 mol L^{-1} KCl. Top panel is a representative intracellular recording from a right PPD1 neuron. The arrow indicates when the stimulus was applied to the animal. Scalebars=20 mV, 2 min. Bottom panel is a representative intracellular recording from a right PPD1 neuron during a control trial when no stimulus was applied. Scalebars=20 mV, 2 min. **C.** Average action potential frequency over 10 min in the right PPD1 cell. In the 'No stimulus' trials, the animal was left undisturbed. In the 'Salt solution' trials, a 3 mol L^{-1} KCl solution was applied to the posterior, dorsal tip of the body wall. Error bars represent SEM. p values are from *post hoc* t-tests (see Results). **D.** Propagation of spontaneous action potentials in the right PPD1 cell. The top trace is a single action potential in the PPD1 cell. The bottom trace is a spike triggered average of the voltage recording from PdN3 after each action potential in the PPD1 neuron. The trace is an average of ~500 action potentials in PPD1. The delay between detection of the action potential in the soma and in PdN3 is ~10 ms. Top trace, scalebar=50 mV. Bottom trace, scalebars=10 μV, 20 ms.

presence of TPep-LIR adjacent to the pedal epithelium of all gastropods investigated, suggests that TPep-like neuropeptides may have a conserved role in ciliary beating. Most gastropods appear to have a ciliated pedal epithelium (Clark 1964), so TPeps may modulate ciliary beating even in species that do not use the pedal cilia for locomotion. For example, adults of the two non-nudibranchs examined here, *P. taylori* and *C. ligatum*, crawl using muscular waves. Additionally, TPep-LIR neurites are present adjacent to the pedal epithelium of *Cornu aspersum*, a terrestrial snail that also crawls using muscular waves (Pavlova & Willows 2005). However, in members of these species, TPeps might affect mucus secretion or muscular contractions instead of ciliary beating.

Because TPep-LIR neurites exist in the feet of gastropods that crawl using muscular contractions, homologues to the Pd5 and Pd6 cells of *T. diomedea* do not necessarily function in mucociliary locomotion in other nudibranch species. Here, we examined the physiology of the putative Pd5 and Pd6 homologues in members of *Armina californica*. The PPD1 cells of *A. californica* resemble the Pd5

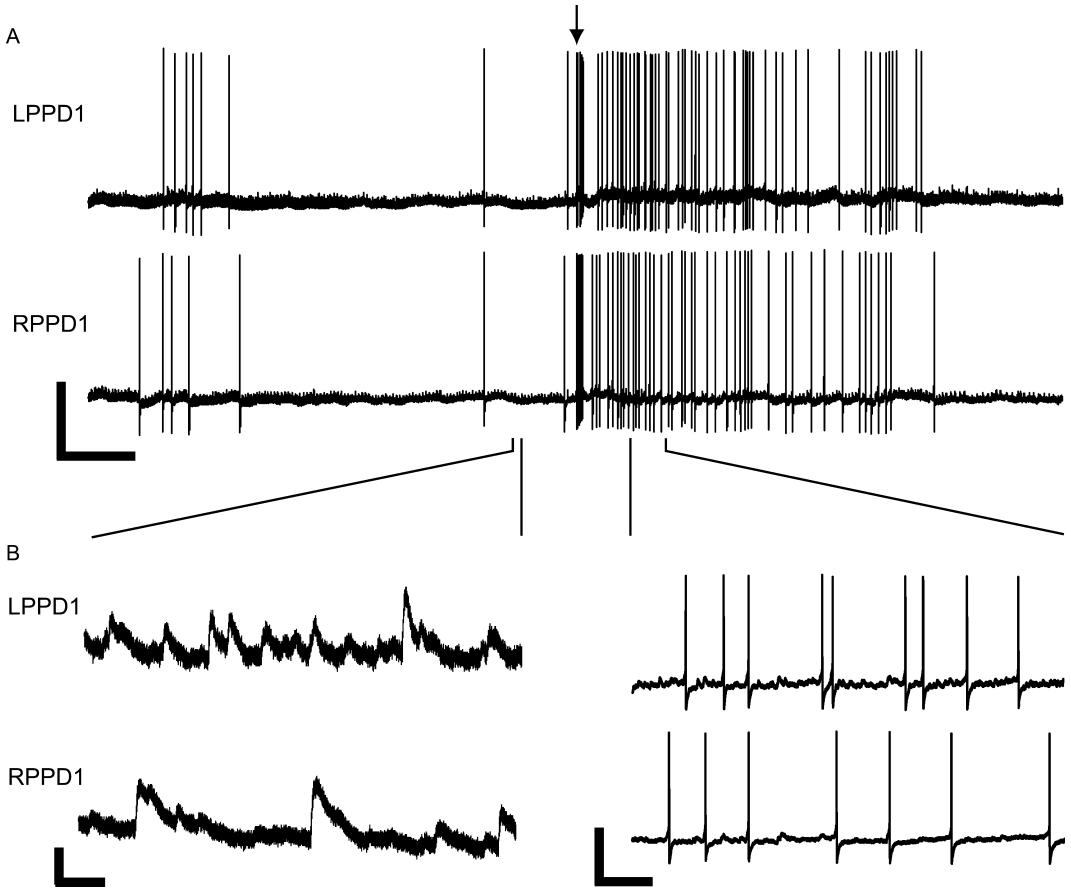

Fig. 7. Simultaneous intracellular recording from contralateral PPD1 cells. **A.** Applying a noxious stimulus to the dorsal surface results in a similar response in both left and right PPD1. The arrow indicates when the stimulus was applied to the animal. Scalebars=40 mV, 100 s. **B.** Left traces show a higher magnification of part of the trace in (a), showing postsynaptic potentials in both left and right PPD1. The contralateral PPD1 neurons do not appear to have synchronous synaptic input. Scalebars=2 mV, 1 s. The right traces show a higher magnification of part of the trace in (a), showing action potentials in left and right PPD1. The contralateral neurons have similar firing patterns but do not have synchronous action potentials. Scalebars=40 mV, 5 s.

cells of *T. diomedea* in several ways. Both are pairs of large, TPep-LIR cells on the posterior margin of the dorsal surface of each pedal ganglion (Fig. 5C); both also have major branches in PdN2 and PdN3, which innervate the foot (Lohmann et al. 1991; Cain et al. 2006). As in *T. diomedea*, the action potentials detected in PdN3 represent efferent information from the PPD1 cell of *A. californica* (Fig. 5). The activity of the PPD1 neurons is associated with stimulus-induced crawling in individuals of *A. californica* (Figs. 6, 7), consistent with the function of the Pd5 cells in *T. diomedea* (Popescu & Frost 2002). Likewise, the *A. californica* PPV1 is a large, TPep-LIR cell in each pedal ganglion with a similar projection pattern and activity pattern as the Pd6 cell in *T. diomedea* (Figs. 5D, 8A). Based on these similarities, we conclude that the *A. californica* PPD1 and PPV1 are mucociliary motor neurons homologous to the *T. diomedea* Pd5 and Pd6 cells.

Similar considerations have led to the identification of putative Pd5 and Pd6 homologues in 15 species of nudibranch (Croll et al. 2001; Newcomb & Katz 2007; Baltzley & Lohmann 2008). Based on nudibranch phylogenies (Wägele & Willan 2000; Wollscheid-Lengeling et al. 2001), if the Pd5 and Pd6 cells of *T. diomedea* and the PPD1 and PPV1 cells of *A. californica* are motor neurons that control mucociliary locomotion, then their homologues in the rest of the Cladobranchia are likely to be mucociliary motor neurons as well (Fig. 1). The Doridacea is the sister-taxon to the clade containing *A. californica* and *T. diomedea*; thus, although it is plausible that the Pd5- and Pd6-homologues in this group are also mucociliary motor neurons, this prediction is less certain.

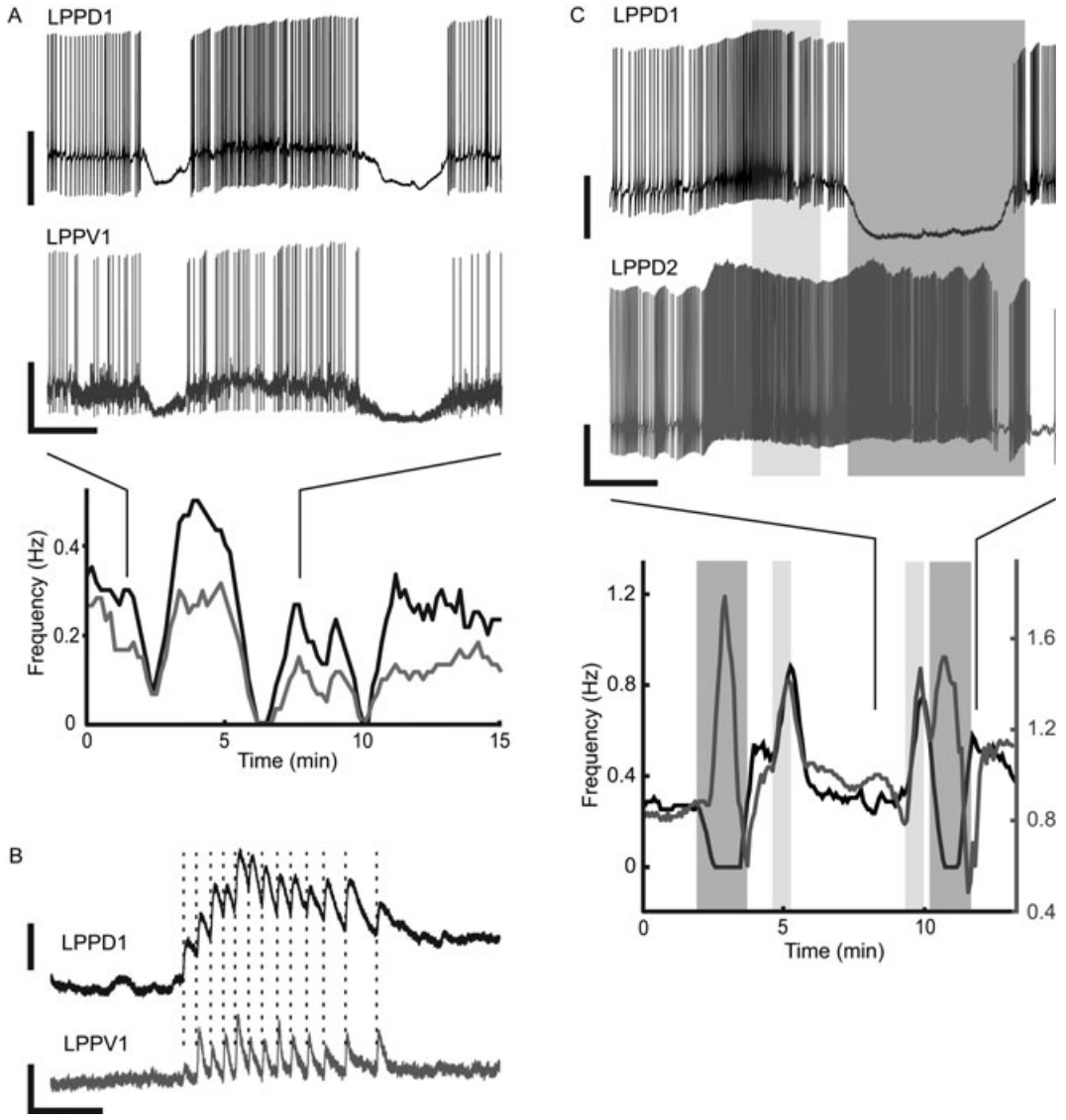

Fig. 8. Simultaneous intracellular recording from TPep-LIR neurons in the pedal ganglia of *Armina californica*. **A.** The top panel is a representative recording of ipsilateral PPD1 and PPV1 showing similar activity patterns. Scalebars=20 mV, 60 s. The bottom panel shows the average spike frequency of PPD1 (black) and PPV1 (grey) (bin size=60 s). **B.** A representative recording showing synchronous postsynaptic potentials in the ipsilateral PPD1 and PPV1. Scalebars=1 mV, 2 s. **C.** Simultaneous intracellular recording from ipsilateral PPD1 and PPD2 cells. The top panel is a representative recording of PPD1 and PPD2. Scalebars=20 mV, 30 s. The bottom panel is the average spike frequency of PPD1 (black) and PPD2 (grey) (bin size=60 s). The light stippling indicates periods where the instantaneous firing frequency of PPD1 and PPD2 were simultaneously increasing. The dark stippling indicates periods where the PPD1 was inhibited while PPD2 was excited.

While the PPD1 and PPV1 cells of adults of *A. californica* are likely mucociliary motor neurons, the function of the PPD2 cells is unclear. Like PPD1, the PPD2 cells have a neurite that projects towards the foot via PdN2 or PdN3. PPD1 and PPD2 have periods of simultaneous excitation, but often have contrasting patterns of excitation and inhibition (Fig. 8C). It is possible that the PPD2 cells innervate foot muscle or mucus-secreting cells rather than the ciliated cells in the pedal epithelium. TPeps have been shown to affect muscle contractions in other molluscs (Malyshev et al. 1999). Because foot posture, mucus secretion, and ciliary beating are all components of crawling, motor neurons that target these different effector cells may be expected to have similar synaptic input, but

would not necessarily have identical activity patterns.

The observation that the neural circuitry controlling pedal cilia in gastropods is highly conserved is not surprising due to the importance of ciliary beating in locomotion in members of this group. However, in addition to TPeps, serotonin also increases ciliary beating in phylogenetically diverse gastropods (Deliagina & Orlovsky 1990; Pavlova 1997; Braubach et al. 2006), suggesting that a serotonergic network may also be a highly conserved, basal feature of slugs and snails. In individuals of *T. diomedea*, the effects of the serotonin and TPep networks on mucociliary locomotion appear to be largely redundant. Like the Pd5 and Pd6 cells, the serotonergic Pedal 21 (Pd21) neurons increase ciliary beating on the pedal epithelium and produce crawling (Audesirk 1978b; Audesirk et al. 1979). Both TPeps and serotonin cause similar increases in ciliary beating of isolated pedal ciliated cells as well as mucociliary transport rates in isolated pieces of foot tissue (Willows et al. 1997). The Dorsal Swim Interneurons, which drive crawling following bouts of swimming, monosynaptically excite both the Pd21 and Pd5 cells (Popescu & Frost 2002). To determine whether the TPep and serotonin networks are complementary or redundant, Willows et al. (1997) suggested experiments to examine (1) whether there are interactions between serotonin and TPeps on ciliated cells, and (2) whether there are regional specializations in the types of ciliated cells present in the foot of *T. diomedea*. Such investigations may provide insight into why there appear to be two highly conserved networks that both function to increase mucociliary locomotion.

Acknowledgments. We thank J.H. Wang, R.C. Wyeth, C.M.F. Lohmann, J. Thompson II, and two anonymous reviewers for providing helpful feedback on the manucript. We thank the Center for Cell Dynamics at the Friday Harbor Laboratories (FHL) for help with confocal microscopy. In addition, we thank A.O.D. Willows for providing research space at the Friday Harbor Laboratories. This work was supported by an NSF Graduate Student Research Fellowship to M.J.B., a FHL Kohn Fellowship to M.J.B., a UNC Biology Wilson Fund Scholarship to M. J.B., and a PADI Aware Grant to M.J.B.

References

Agersborg HPK 1922. Notes on the locomotion of the nudibranchiate mollusk *Dendronotus giganteus* (O'Donoghue). Biol. Bull. 42: 257–266.

Audesirk G 1978a. Central neuronal control of cilia in *Tritonia diomedea*. Nature 272: 541–543.

——— 1978b. Properties of central motor neurons exciting locomotory cilia in *Tritonia diomedea*. J. Comp. Physiol. 128: 259–267.

Audesirk G, McCaman RE, & Willows AOD 1979. The role of serotonin in the control of pedal ciliary activity by identified neurons in *Tritonia diomedea*. Comp. Biochem. Physiol. 2C: 87–91.

Baltzley MJ & Lohmann KJ 2008. Comparative study of TPep-like immunoreactive neurons in the central nervous system of nudibranch mollusks. Brain Behav. Evol. 72: 192–206.

Beck JC, Cooper MS & Willows AOD 2000. Immunocytochemical localization of pedal peptide in the central nervous system of the gastropod mollusc *Tritonia diomedea*. J. Comp. Neurol. 425: 1–9.

Braubach OR, Dickinson AJ, Evans CC & Croll RP 2006. Neural control of the velum in larvae of the gastropod, *Ilyanassa obsoleta*. J. Exp. Biol. 209: 4676–4689.

Cain SD, Wang JH & Lohmann KJ 2006. Immunochemical and electrophysiological analyses of magnetically responsive neurons in the mollusc *Tritonia diomedea*. J. Comp. Phys. A 192: 235–245.

Clark RB 1964. Dynamics in Metazoan Evolution. Clarendon Press, Oxford.

Croll RP, Boudko DY & Hadfield MG 2001. Histochemical survey of transmitters in the central ganglia of the gastropod mollusc *Phestilla sibogae*. Cell Tissue Res. 305: 417–432.

Crow T & Tian L 2003. Interneuronal projections to identified cilia-activating pedal neurons in *Hermissenda*. J. Neurophysiol. 89: 2420–2429.

Deliagina TG & Orlovsky GN 1990. Control of locomotion in the freshwater snail *Planorbis corneus*: II Differential control of various zones of the ciliated epithelium. J. Exp. Biol. 152: 405–423.

Gaston MR 1998. Neuropeptide TPep action on salivary duct ciliary beating rate in the nudibranch mollusc *Tritonia diomedea*. Invert. Neurosci. 3: 327–333.

Grande C, Templado J, Cervera JL & Zardoya R 2004. Molecular phylogeny of euthyneura (Mollusca: Gastropoda). Mol. Biol. Evol. 21: 303–313.

Lloyd PE, Phares GA, Phillips NE & Willows AOD 1996. Purification and sequencing of neuropeptides from identified neurons in the marine mollusc, *Tritonia*. Peptides 17: 17–23.

Lohmann KJ, Willows AOD & Pinter RB 1991. An identifiable molluscan neuron responds to changes in earth-strength magnetic fields. J. Exp. Biol. 161: 1–24.

Malyshev AY, Norekian TP & Willows AOD 1999. Differential effects of serotonergic and peptidergic cardioexcitatory neurons on the heart activity in the pteropod mollusc, *Clione limacina*. J. Comp. Phys. A 185: 551–560.

Newcomb JM & Katz PS 2007. Homologues of serotonergic central pattern generator neurons in related nudibranch molluscs with divergent behaviors. J. Comp. Physiol. A. 193: 425–443.

Pavlova GA 1997. Effect of serotonin on locomotion of freshwater mollusc *Lymnaea stagnalis*. J. Evol. Biochem. Phys. 33: 529–533.

Pavlova GA & Willows AOD 2005. Immunological localization of *Tritonia* peptide in the central and peripheral nervous system of the terrestrial snail *Helix aspersa*. J. Comp. Neurol. 491: 15–26.

Popescu IR & Frost WN 2002. Highly dissimilar behaviors mediated by a multifunctional network in the marine mollusc *Tritonia diomedea*. J. Neurosci. 22: 1985–1993.

Wägele H & Willan RC 2000. Phylogeny of the nudibranchia. Zool. J. Linn. Soc. Lond. 130: 83–181.

Wang JH, Cain SD & Lohmann KJ. 2003. Identification of magnetically responsive neurons in the marine mollusc *Tritonia diomedea*. J. Exp. Biol. 206: 381–388.

———, 2004. Identifiable neurons inhibited by Earth-strength magnetic stimuli in the mollusc *Tritonia diomedea*. J. Exp. Biol. 207: 1043–1049.

Willows AOD, Dorsett DA, & Hoyle G 1973. The neuronal basis of behavior in *Tritonia*. I. Functional organization of the central nervous system.. J. Neurobiol. 4: 207–237.

Willows AOD, Pavlova GA & Phillips NE 1997. Modulation of ciliary beat frequency by neuropeptides from identified molluscan neurons. J. Exp. Biol. 200: 1433–1439.

Wollscheid-Lengeling E, Boore J, Brown W & Wägele H 2001. The phylogeny of Nudibranchia (Opisthobranchia, Gastropoda, Mollusca) reconstructed by three molecular markers. Org. Divers. Evol. 1: 241–256.