Systems/Circuits

Parallel Evolution of Serotonergic Neuromodulation Underlies Independent Evolution of Rhythmic Motor Behavior

Joshua L. Lillvis and Paul S. Katz
Neuroscience Institute, Georgia State University, Atlanta, Georgia 30303-5030

Neuromodulation can dynamically alter neuronal and synaptic properties, thereby changing the behavioral output of a neural circuit. It is therefore conceivable that natural selection might act upon neuromodulation as a mechanism for sculpting the behavioral repertoire of a species. Here we report that the presence of neuromodulation is correlated with the production of a behavior that most likely evolved independently in two species: *Tritonia diomedea* and *Pleurobranchaea californica* (Mollusca, Gastropoda, Opisthobranchia, Nudipleura). Individuals of both species exhibit escape swimming behaviors consisting of repeated dorsal-ventral whole-body flexions. The central pattern generator (CPG) circuits underlying these behaviors contain homologous identified neurons: DSI and C2 in *Tritonia* and As and A1 in *Pleurobranchaea*. Homologs of these neurons also can be found in *Hermissenda crassicornis* where they are named CPT and C2, respectively. However, members of this species do not exhibit an analogous swimming behavior. In *Tritonia* and *Pleurobranchaea*, but not in *Hermissenda*, the serotonergic DSI homologs modulated the strength of synapses made by C2 homologs. Furthermore, the serotonin receptor antagonist methysergide blocked this neuromodulation and the swimming behavior. Additionally, in *Pleurobranchaea*, the robustness of swimming correlated with the extent of the synaptic modulation. Finally, injection of serotonin induced the swimming behavior in *Tritonia* and *Pleurobranchaea*, but not in *Hermissenda.* This suggests that the analogous swimming behaviors of *Tritonia* and *Pleurobranchaea* share a common dependence on serotonergic neuromodulation. Thus, neuromodulation may provide a mechanism that enables species to acquire analogous behaviors independently using homologous neural circuit components.

Introduction

Behaviors are produced by neural circuits whose outputs depend upon the neuronal and synaptic properties of their components. Neuromodulation can alter those properties and thereby select a particular functional output (Harris-Warrick and Marder, 1991; Katz, 1999; Katz and Calin-Jageman, 2008). Thus, species differences in behavior might be caused by differences in neuromodulation (Arbas et al., 1991; Katz and Harris-Warrick, 1999). Conversely, similar neuromodulatory actions in disparate species might underlie independent evolution of analogous behaviors (Young and Wang, 2004).

Tritonia diomedea and *Pleurobranchaea californica* are two sea slug species within the monophyletic clade Nudipleura (Mollusca, Gastropoda, Opisthobranchia) (Waegele and Willan, 2000; Bouchet et al., 2005) that exhibit similar escape swimming behaviors consisting of alternating dorsal and ventral whole-body flexions (Willows, 1967; Gillette et al., 1991). The central pattern generators (CPGs) underlying swimming contain homologous identified neurons. Included is a set of three serotonergic neurons, named $DSI_{A\text{-}C}$ (DSI) in *Tritonia* (Getting et al., 1980) and $As_{1–3}$ (As) in *Pleurobranchaea* (Jing and Gillette, 1999). The CPGs also contain homologous peptidergic neurons called C2 in *Tritonia* (Getting, 1977; Taghert and Willows, 1978) and A1 in *Pleurobranchaea* (Jing and Gillette, 1995). As previously shown (Getting et al., 1980; Jing and Gillette, 1999), these neurons exhibit similar bursting activity during swim motor patterns (Fig. 1). Despite the similarities in the behavior and neural mechanisms, phylogenetic evidence suggests that the dorsal-ventral swimming behaviors of *Tritonia* and *Pleurobranchaea* evolved independently from nonrhythmic neural circuitry present throughout the Opisthobranchia (Katz et al., 2001; Newcomb et al., 2012).

In *Tritonia*, the serotonergic DSI modulates C2 synaptic strength (Katz et al., 1994; Katz and Frost, 1995b). This intrinsic neuromodulation appears to be necessary for the production of the swimming behavior; the serotonin (5-HT) receptor antagonist methysergide, which blocks this synaptic modulation (Katz and Frost, 1995a), also blocks the swimming behavior and the swim motor pattern in the isolated brain (McClellan et al., 1994). Moreover, blocking G-protein coupled signaling in C2, which appears to mediate DSI modulation of C2 synaptic strength, also inhibits the swim motor pattern (Clemens and Katz, 2003). 5-HT

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Correspondence should be addressed to Dr. Paul S. Katz, Neuroscience Institute, Georgia State University, P.O. Box 5030, Atlanta, GA 30303-5030. E-mail: pkatz@gsu.edu.
J.L. Lillvis' present address is Research Institute of Molecular Pathology, Dr. Bohrgasse 7, A-1030 Vienna, Austria.
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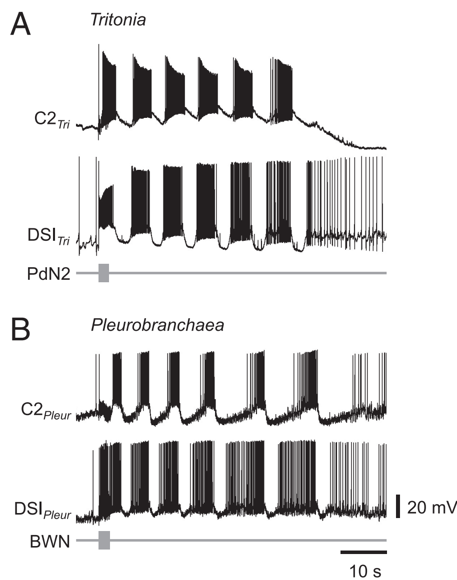

Figure 1. Examples of dorsal-ventral swim motor patterns in isolated *Tritonia* and *Pleurobranchaea* brains. Simultaneous intracellular recordings from DSI and C2 show rhythmic bursting that is characteristic of the swim motor patterns in *Tritonia* (***A***) and *Pleurobranchaea* (***B***). The motor patterns were elicited by stimulating pedal nerve 2 (PdN2, gray bar) in *Tritonia* (***A***) and the body wall nerve (BWN, gray bar) in *Pleurobranchaea* (***B***). Examples are previously unpublished recordings.

induces the swimming behavior and the swim motor pattern in the isolated brain (McClellan et al., 1994). Finally, modeling studies suggest that the enhancement of C2 synaptic strength is necessary for the production of rhythmic output (Calin-Jageman et al., 2007).

Here, we sought to determine whether the DSI homolog and 5-HT played a similar role in the independently evolved swimming behavior of *Pleurobranchaea*. We found that the DSI homolog modulated the synaptic strength of the C2 homolog and that serotonergic neuromodulation was necessary for swim production. Furthermore, the extent of this modulation correlated with the robustness of swimming. We found that this modulation was absent in a species that does not exhibit the behavior, *Hermissenda crassicornis*. Finally, we found that injection of 5-HT induced the swimming behavior in *Pleurobranchaea*, but not in *Hermissenda*. These results suggest that serotonergic neuromodulation is responsible, at least in part, for the ability of shared neural circuitry to generate the rhythmic motor pattern in certain species.

Materials and Methods

Animal collection and maintenance. T. diomedea specimens were obtained from Living Elements. *P. californica* and *H. crassicornis* specimens were obtained from Monterey Abalone. All species are simultaneous hermaphrodites. Animals were maintained in artificial seawater (Instant Ocean) tanks at a fixed 12:12 light/dark cycle. *Tritonia* individuals were maintained at 11 ± 1°C. *Pleurobranchaea* and *Hermissenda* individuals were maintained at 13 ± 1°C.

In vivo tests of swimming. To test *Pleurobranchaea* for swimming, a 10–30 V (~1.6–5 mA) shock was applied for <5 s to the dorsal body wall. A reflex withdrawal response was reliably observed in response to this stimulus, sometimes accompanied by the initiation of dorsal-ventral swimming, which began with a dorsal body flexion. A dorsal flexion followed by a ventral flexion and a return to a dorsal flexion was defined as a swim cycle. Upon arriving in the laboratory *Pleurobranchaea* individuals were tested for swimming about every day until they were used for electrophysiological experiments. *Tritonia* swimming was elicited by applying a 5 M NaCl salt solution to the dorsal body wall, which reliably induces swimming. *Tritonia* also reliably swims in response to a 10 V electric shock (Mongeluzi et al., 1998), but the salt stimulus is more consistent with other studies in the literature. *Pleurobranchaea* rarely swam in response to the salt stimulus. *Hermissenda* was stimulated by applying a 5 M NaCl salt solution to the dorsal body wall, which can induce rhythmic left-right flexions (Lillvis et al., 2012). Electric shock did not cause *Hermissenda* to swim.

To test for the effect of methysergide or 5-HT on swimming, size-matched individuals were selected and stimulated to swim every 30 min. Animals were injected with a 5% of total animal volume 10 mg/L methysergide (dissolved in 1:1000 injection volume of DMSO) solution in artificial seawater. The total volume of the animal was determined by seawater displacement. The 10 mg/L measurement was based on the total volume plus the 5% injection volume. Fast Green (0.2%) was added to the solution to ensure that the injection spread throughout the body cavity. Control injections consisted of DMSO (1:1000 injection volume) with Fast Green (0.2%) in artificial seawater. For 5-HT, animals were injected with a 5% of total volume 20 mM 5-HT solution in artificial seawater. Fast Green (0.2%) was added to ensure the injection spread throughout the body cavity. Control injections consisted of artificial seawater with Fast Green (0.2%) at 5% of total body volume.

Dissection. Individual *Tritonia* were anesthetized by cooling. *Pleurobranchaea* and *Hermissenda* were anesthetized by injection of 0.33 M $MgCl_2$ into the body cavity. A cut was made on the dorsal surface of the body wall near the buccal mass. The brain, consisting of the cerebral, pleural, and pedal ganglia, was removed by cutting all nerve roots. The brain was transferred to a Sylgard-lined dish, where it was superfused at a rate of ~1.0 ml/min with saline (in mM): 420 NaCl, 10 KCl, 10 $CaCl_2$, 50 $MgCl_2$, 11 D-glucose, and 10 HEPES, pH 7.5.

Connective tissue surrounding the brain was manually removed with forceps and fine scissors while maintaining a temperature of ~4°C to reduce neuronal activity. The temperature was raised to 10–11°C for *Tritonia* and 13–14°C for *Pleurobranchaea* and *Hermissenda* electrophysiological experiments.

Neuron identification and nomenclature. To simplify the nomenclature, we will use the *Tritonia* neuron names and include a subscript for the species (*Tri*, *Pleur*, and *Herm*) if there is ambiguity as to which species it refers. If there is no subscript, then the property can be assumed to be common to all homologs or the species is clearly defined in context.

C2 is conspicuous and relatively easy to identify visually across species (Lillvis et al., 2012). In *Tritonia*, C2 (www.neuronbank.org/Tri0002380) was identified preliminarily using soma position, size, and white appearance. Upon impaling C2 with a microelectrode, its characteristic resting activity, synaptic input, and response to a nerve stimulus further aided identification. In *Pleurobranchaea*, the axon projection of $C2_{Pleur}$ (A1, www.neuronbank.org/Ple0002601) was confirmed by monitoring the contralateral anterior cerebral-pedal connective and the larger of the pedal-pedal commissures (PP2) (see Newcomb et al., 2006 for nerve nomenclature), which in this case is the anterior pedal-pedal commissure, via extracellular recordings. In *Tritonia* and *Pleurobranchaea*, C2 identity could be confirmed by its involvement in the dorsal-ventral swim motor pattern. Finally, in experiments where C2 identity was not certain, the soma was filled with the biotinylated tracer biocytin (2.5% dissolved in 0.75 M KCl) (Invitrogen) or Neurobiotin (2–4% dissolved in 0.75 M KCl) (Vector Laboratories) and processed for FMRFamide and/or Small Cardioactive Peptide B (SCP_B) immunoreactivity (see below). The axon projection in conjunction with FMRFamide and SCP_B immunoreactivity uniquely identifies C2 across species (Lillvis et al., 2012).

There are three DSIs found in a cluster in a characteristic location in each species (Getting et al., 1980; Jing and Gillette, 1999; Tian et al., 2006; Newcomb and Katz, 2007). Upon impaling DSI with a microelectrode, it shows characteristic spiking activity, action potential shape, and synaptic input that distinguish it from neighboring neurons. In *Tritonia*, DSI (www.neuronbank.org/Tri0001043) was identified preliminarily by soma position, size, and its mottled dark pigmentation. In *Pleurobranchaea*, the axon projection of DSI (As, www.neuronbank.org/Ple0002605) could be confirmed by monitoring the contralateral anterior cerebral-pedal connective. In *Tritonia* and *Pleurobranchaea*, DSI

could be uniquely identified simply by its involvement in the dorsal-ventral swim motor pattern. The DSI soma was filled with the biotinylated tracer biocytin or Neurobiotin after the experiment and processed for 5-HT immunohistochemistry (see below). Soma position and axon projection in conjunction with 5-HT immunoreactivity uniquely identify DSI across species (Newcomb and Katz, 2007), allowing the homolog in *Hermissenda* (CPT, www.neuronbank.org/Her0002693) to be identified. 5-HT immunoreactivity was also used to confirm DSI identity in all *Tritonia* and *Pleurobranchaea* preparations in which identity was not confirmed by involvement in the swim motor pattern.

Immunohistochemistry. Biocytin or Neurobiotin processing and immunohistochemistry procedures were identical to those reported in Lillvis et al., 2012. Briefly, after fixation, brains were washed and incubated in one or more of the following primary antisera: rabbit anti-FMRFamide (Immunostar, lot #719001) or anti-serotonin (Invitrogen, lot #541317) antiserum diluted 1:1000 and/or mouse monoclonal anti-SCP_B (courtesy of Stephen Kempf) diluted 1:20 in antiserum diluent (0.5% Triton X-100, 1% normal goat serum, and 1% BSA in PBS (50 mM Na_2HPO_4 in 140 mM $NaCl_2$, pH 7.2). Streptavidin–AlexaFluor-594 conjugate (1:50–1:200, Invitrogen) was also added to visualize the biotinylated tracer. Brains were then washed and incubated in goat anti-rabbit and/or goat anti-mouse antiserum conjugated to AlexaFluor-488, AlexaFluor-594 (Invitrogen), or DyLight 405 (Jackson ImmunoResearch Laboratories) diluted 1:100 in antiserum diluent. Brains were then washed, dehydrated, and mounted on a slide to visualize soma immunohistochemistry and axon projection.

Electrophysiology. Intracellular recordings were obtained using 10–80 MΩ glass microelectrodes filled with 3 M KCl connected to Axoclamp 2B amplifiers (Molecular Devices). Extracellular suction electrode recordings were obtained by drawing individual nerves into polyethylene tubing filled with saline connected to a differential AC amplifier (model 1700, A-M Systems). Both intracellular and extracellular recordings were digitized (>1 kHz) with a 1401 Plus using Spike2 software (Cambridge Electronic Design).

In the isolated brain, body wall nerve stimulation was used to activate sensory neurons in a standard protocol (Frost and Katz, 1996). Pedal nerves 2 or 3 (PdN2/PdN3) were used in *Tritonia* and *Hermissenda* (Newcomb et al., 2006). The body wall nerve was used in *Pleurobranchaea* (Jing and Gillette, 1999). The stimulus parameters were systematically varied in an effort to thoroughly test whether the DSI and C2 homologs were capable of producing the bursting activity that underlies dorsal-ventral swimming (1–5 s, 5–20 ms pulses, 5–20 Hz, 5–50 V).

High divalent cation (HiDi) saline, which raises the threshold for spiking and reduces spontaneous neural firing, was used for all modulation experiments. The composition of HiDi saline was (in mM): 285 NaCl, 10 KCl, 25 $CaCl_2$, 125 $MgCl_2$, 11 D-glucose, and 10 HEPES, pH 7.5. All drugs used in the modulation experiments were dissolved in HiDi saline. Superfusion rate for these experiments was ~1 ml/min.

In *Tritonia*, DSI modulation of C2 is presynaptic (Katz and Frost, 1995b); therefore, neurons in the pedal ganglion that are postsynaptic targets of C2 were used as convenient monitors of the modulation. The following standard stimulus protocol was used to test DSI modulation of C2 synapses. C2, DSI, and a pedal target neuron were impaled with intracellular microelectrodes. Two electrodes were inserted into the postsynaptic target of C2: one electrode monitored the membrane potential and the other electrode was used to inject current to hold the membrane potential near −70 mV to prevent action potential firing. C2 was stimulated intracellularly to fire four action potentials every 2 min (four 20 ms pulses at 20 Hz). DSI was stimulated intracellularly to fire 50 action potentials (fifty 20 ms pulses at 5 Hz) that ended 3 s before every other C2 stimulus.

For 5-HT bath-application experiments, C2 was stimulated to fire four action potentials every 2 min. After baseline postsynaptic potential amplitude was established, 100 μM 5-HT (Sigma Aldrich) was superfused into the recording dish. For experiments testing whether methysergide blocked DSI modulation, the DSI modulation of C2 protocol was used; 50 μM methysergide (dissolved in DMSO at 1:1000 total volume; Sigma Aldrich) was superfused into the recording dish. For the experiment testing whether methysergide blocked the *Pleurobranchaea* swim motor pattern in the isolated brain, a body wall nerve was stimulated every 10 min in saline; 50 μM methysergide (in 1:1000 DMSO) in saline was superfused into the recording dish.

Data acquisition, analysis, and statistics. Data acquisition and analysis were performed with Spike2 software (Cambridge Electronic Design) and Sigma Plot (Jandel Scientific). The amplitudes of the synaptic potentials were measured by subtracting the baseline membrane potential before C2 stimulation from the peak membrane potential after C2 stimulation. The percentage of DSI modulation was calculated by subtracting the amplitude of the C2-evoked synaptic potential immediately before a DSI spike train (C2 alone EPSP) from the amplitude of the C2-evoked EPSP when C2 was stimulated immediately after the DSI train (DSI + C2 EPSP) and dividing by the mean amplitude of the EPSP when C2 was stimulated alone as shown in this formula:

$$\%\text{DSI mod.} = \left(\frac{\text{DSI} + \text{C2 EPSP} - \text{C2 alone EPSP}}{\text{mean C2 alone EPSP}}\right) * 100$$

To determine whether a relationship between swim strength and the extent of DSI_{Pleur} modulation of $C2_{Pleur}$ was present, the average percentage of DSI_{Pleur} modulation from each experiment was plotted against the number of swim cycles exhibited in *Pleurobranchaea* on the day of the experiment.

The percentage modulation caused by 5-HT bath application was calculated by dividing the average of 10 consecutive synaptic amplitudes at the time of the maximum effect of 5-HT by the average of the 10 consecutive baseline synaptic amplitudes immediately before 5-HT application.

The effect of control and methysergide injections on *Pleurobranchaea* swimming was tested using a one-way repeated measures ANOVA with *post hoc* pairwise multiple comparisons using the Holm-Sidak method. Pairwise comparisons of the percentage of DSI modulation in swimming and nonswimming *Pleurobranchaea* were made using a Student's *t* test. Pairwise comparisons of the percentage of 5-HT modulation in swimming and nonswimming *Pleurobranchaea* were made using a Mann–Whitney rank-sum test as the data failed the Kolmogorov–Smirnov test of normality. Pairwise comparisons of the effect of methysergide on DSI modulation in *Pleurobranchaea* were made using paired Student's *t* tests. Comparisons of the amplitude of C2-evoked EPSPs when C2 was stimulated alone to when C2 was stimulated after DSI in all species were made using paired Student's *t* tests. Comparisons of the amplitude of C2-evoked EPSPs in saline and in 5-HT were made using paired Student's *t* tests or Wilcoxon signed rank tests when the data failed the Kolmogorov–Smirnov test of normality. Comparisons of the percentages of DSI and 5-HT modulation across all groups were made using a one-way ANOVA with *post hoc* pairwise multiple comparisons using the Holm-Sidak or Dunn's method. Dunn's method was used when comparing the results of 5-HT bath application where the data failed the Kolmogorov–Smirnov test of normality. In all cases where data failed the test of normality, the median values of each species were compared. Results are expressed as the mean ± SEM unless stated otherwise. In all cases, $p < 0.05$ was considered significant.

Results

DSI and serotonin enhance C2 synaptic strength in *Tritonia*

We replicated previous results in *Tritonia* (Katz et al., 1994; Katz and Frost, 1995a,b) by demonstrating that DSI_{Tri} enhanced the strength $C2_{Tri}$-evoked synapses. Using a standard stimulus protocol (Materials and Methods), DSI_{Tri} increased the amplitude of $C2_{Tri}$-evoked synaptic potentials by an average of 107.4 ± 26.2% ($n = 5$). The average amplitude of the $C2_{Tri}$-evoked EPSP when $C2_{Tri}$ was stimulated alone was 3.2 ± 1.2 mV compared with 5.9 ± 1.9 mV when $C2_{Tri}$ was stimulated after DSI, a significant increase (paired Student's *t* test, $t_{(4)} = -3.32$, $p = 0.029$; Fig. 2*A*). We also replicated prior results showing that bath application of 5-HT mimics the DSI_{Tri} modulation (Katz et al., 1994; Katz and Frost, 1995a). Here, 100 μM 5-HT increased the size of $C2_{Tri}$-evoked synaptic potentials by 137.0 ± 8.0% ($n = 2$; Fig. 2*B*). The average amplitude of the $C2_{Tri}$-evoked EPSP in HiDi

saline was 3.0 ± 2.1 mV compared with 7.0 ± 4.7 mV in 100 μM 5-HT ($n = 2$).

DSI and serotonin enhance C2 synaptic strength in *Pleurobranchaea*

We found that using the same stimulus protocol as in *Tritonia*, DSI_{Pleur} increased the amplitude of $C2_{Pleur}$-evoked synapses by 112.4 ± 23.7% ($n = 10$) (Fig. 2*C*). The average amplitude of the $C2_{Pleur}$-evoked EPSP when $C2_{Pleur}$ was stimulated alone was 2.7 ± 0.7 mV compared with 5.0 ± 1.1 mV when $C2_{Pleur}$ was stimulated after DSI_{Pleur}, a significant increase (paired Student's *t* test, $t_{(9)} = -3.45$, $p = 0.007$).

Additionally, bath application of 100 μM 5-HT increased the amplitude of $C2_{Pleur}$-evoked synaptic potentials by an average of 68.5 ± 16.1% ($n = 21$) (Fig. 2*D*). The average amplitude of the $C2_{Pleur}$-evoked EPSP in HiDi saline was 4.3 ± 0.8 mV compared with 6.1 ± 0.9 mV in 100 μM 5-HT, a significant increase (paired Student's *t* test, $t_{(20)} = -3.76$, $p = 0.001$). Thus, *Pleurobranchaea*, which responds to an electric shock with a swimming behavior that is analogous to that of *Tritonia*, exhibited similar neuromodulation intrinsic to the swim CPG.

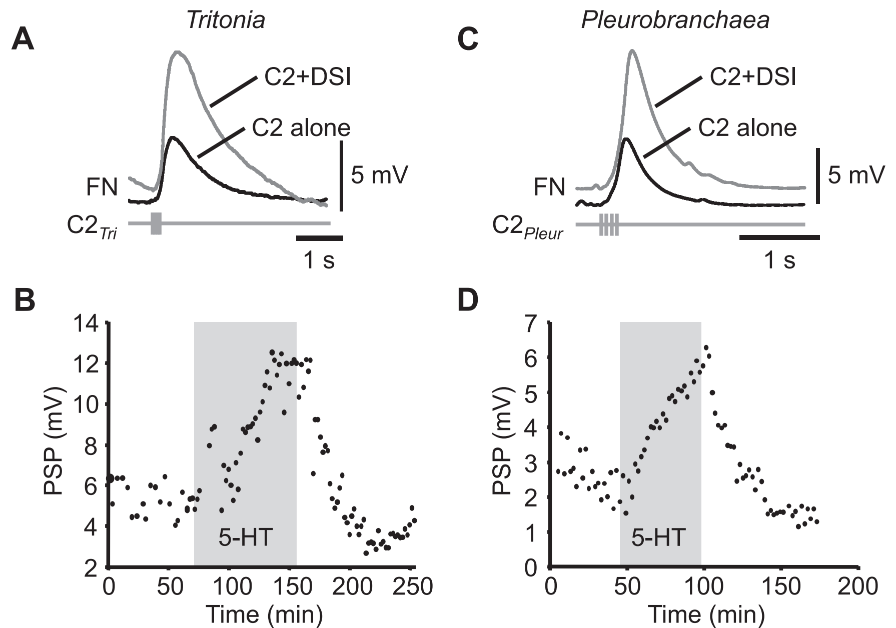

Figure 2. DSI and 5-HT modulated C2-evoked synaptic potentials in *Tritonia* and *Pleurobranchaea*. ***A***, ***C***, Stimulating C2 (bottom gray lines) elicited an EPSP (black trace) in a postsynaptic follower neuron (FN) in *Tritonia* (***A***) and *Pleurobranchaea* (***C***). Stimulating DSI 3 s before the C2 stimulation increased the amplitude of the C2-evoked EPSP (gray trace). Traces are vertically offset from each other for better visibility. ***B***, ***D***, Bath applying 100 μM 5-HT increased the amplitude of C2-evoked EPSPs in *Tritonia* (***B***) and *Pleurobranchaea* (***D***). The amplitude of the C2-evoked EPSPs was plotted over time.

However, some experiments showed markedly reduced levels of DSI_{Pleur} and serotonergic neuromodulation compared with others and thus reduced the average modulatory effect. This encouraged an investigation of the possible causes for the variable results.

The *Pleurobranchaea* swimming behavior exhibits variability

Unlike *Tritonia*, which reliably produces the rhythmic motor pattern *in vivo* in response to starfish tube feet (Willows, 1967), concentrated NaCl solution (Getting, 1976), or electric shock (Mongeluzi et al., 1998), *Pleurobranchaea* individuals exhibit heterogeneity in their propensity to swim (Jing and Gillette, 1995, 1999). In our hands, electric shock was the most reliable trigger for *Pleurobranchaea* swimming. Even so, 18 of 61 (30%) individual *Pleurobranchaea* tested failed to produce a single swim cycle in response to electric shock even when stimulated repeatedly. Of the animals that did swim in response to electric shock, the average number of swim cycles observed by each animal was 3.1 with an average coefficient of variation of 0.80 ($n = 43$). The propensity to swim varied daily; on some days, a given animal would swim, but on other days, it would not (Fig. 3). This variability allowed a natural experiment in which we could test whether the strength of swimming correlated with the extent that DSI_{Pleur} modulated $C2_{Pleur}$ synapses.

The extent of neuromodulation is related to the duration of swimming in *Pleurobranchaea*

We found that animals that did not swim on the day of testing tended to exhibit little or no modulation (Fig. 4*A*). The difference was not the result of a problem with DSI_{Pleur} stimulation; DSI_{Pleur}-evoked EPSPs followed one-for-one with DSI_{Pleur} spikes regardless of the number of swim cycles expressed ($n = 5$ for nonswimmers, $n = 5$ for swimmers; Fig. 4*B*). The extent of DSI_{Pleur} modulation of $C2_{Pleur}$ synapses correlated with the maximum number of swim cycles observed on the day of testing with the maximum modulation near 200% (Fig. 4*C*). For animals that swam fewer than two cycles (which we considered nonswimmers), DSI_{Pleur} caused only a 61.4 ± 26.8% increase in $C2_{Pleur}$-evoked EPSP amplitude ($n = 5$), whereas there was a 163.4 ± 22.3% increase in $C2_{Pleur}$-evoked EPSP amplitude in individuals that swam two or more cycles ($n = 5$). This difference between the levels of modulation in swimmers compared with nonswimmers was significant (Student's *t* test, $t_{(8)} = 2.92$, $p = 0.019$). In nonswimming *Pleurobranchaea*, the average amplitude of the $C2_{Pleur}$-evoked EPSP when $C2_{Pleur}$ was stimulated after DSI_{Pleur} (4.4 ± 1.7 mV) was not significantly different from when $C2_{Pleur}$ was stimulated alone (3.2 ± 1.3 mV) (paired Student's *t* test, $t_{(4)} = -1.82$, $p = 0.144$). In swimming *Pleurobranchaea*, however, the average amplitude of the $C2_{Pleur}$-evoked EPSP when $C2_{Pleur}$ was stimulated after DSI_{Pleur} was 5.6 ± 1.6 mV, which was significantly larger than the average amplitude of 2.2 ± 0.6 mV when $C2_{Pleur}$ was stimulated alone (paired Student's *t* test, $t_{(4)} = -3.46$, $p = 0.026$). The lack of modulation in nonswimmers appears to be caused by a decreased sensitivity to 5-HT. In animals that did not swim, bath-applied 5-HT caused little or no modulation of $C2_{Pleur}$ synaptic strength (Fig. 4*D*). The mean value of the increase in $C2_{Pleur}$-evoked EPSP amplitude resulting from 5-HT was 32.3 ± 20.1% (median, 15.4%) in nonswimmers ($n = 11$) compared with 101.4 ± 20.8% (median, 92.0%) in swimmers ($n = 10$) (Mann–Whitney rank-sum test, $t_{(19)} = 71.00$, $p = 0.007$). The effect of 5-HT on $C2_{Pleur}$-evoked EPSP amplitude was not significant in nonswimming *Pleurobranchaea* but was significant in swimming individuals. In nonswimming individuals, the average amplitude of the $C2_{Pleur}$-evoked EPSP in HiDi saline was 5.4 ± 1.3 mV (median of 4.7 mV) compared with 6.5 ± 1.4 mV (median of 6.0 mV) in 100 μM 5-HT (Wilcoxon signed rank test, $Z(10) = 1.69$, $p = 0.102$). Whereas, in swimming *Pleurobranchaea*, the average amplitude of the $C2_{Pleur}$-evoked EPSP in HiDi saline was 3.0 ± 0.7 mV compared with 5.7 ± 1.4 mV in 100 μM 5-HT (paired Student's *t* test, $t_{(9)} = -4.89$, $p < 0.001$).

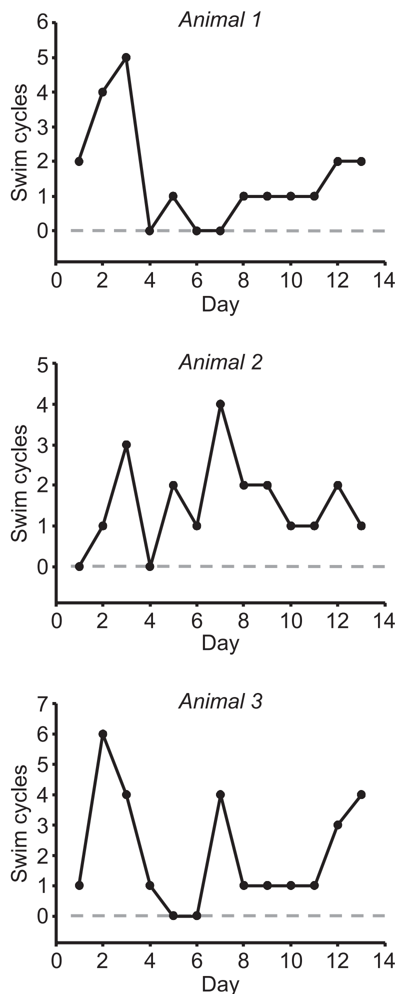

Figure 3. Individual *Pleurobranchaea* varied daily in their ability to produce a swim. Three representative examples of the daily variability in the swimming behavior of individual *Pleurobranchaea* specimens. The number of swim cycles elicited from an electrical stimulus to the body wall changed over a period of days.

Methysergide reduces the extent of neuromodulation

In *Tritonia*, the 5-HT receptor antagonist methysergide reduces the modulation of $C2_{Tri}$-evoked synaptic strength by 5-HT and DSI_{Tri} (Katz and Frost, 1995a). We found that, in *Pleurobranchaea*, applying 50 μM methysergide reduced the extent of the DSI_{Pleur} modulation of $C2_{Pleur}$ synaptic strength (Fig. 5). In this set of experiments, DSI_{Pleur} enhanced $C2_{Pleur}$ synaptic strength by an average of 108.8 ± 18.4% before applying methysergide. Methysergide reduced the enhancement to 26.7 ± 11.3%, a significant reduction (paired Student's *t* test, $n = 5$, $t_{(4)} = 3.46$, $p = 0.026$). Thus, in both *Tritonia* and *Pleurobranchaea*, methysergide inhibits receptors responsible for DSI modulatory actions.

Methysergide blocks *Pleurobranchaea* swimming

It was previously shown that injection of methysergide into the body cavity of *Tritonia* inhibited swimming (McClellan et al., 1994). We observed a similar effect in *Pleurobranchaea* (Fig. 6, ■). For these experiments, baseline swimming in response to electric shock was measured for at least 4 trials. Any individual that did not show consistent swimming in response to this stimulus during the baseline tests was removed from the experiment. The latency to the effect of methysergide varied across individuals, but on average the number of swim cycles was significantly reduced from baseline measurements by 2 h after the injection (one-way repeated-measures ANOVA, Holm-Sidak pairwise comparisons, $n = 8$, $F_{(14,94)} = 8.5$, $p < 0.001$). The next day, there was a recovery of swimming behavior in response to electric shock. Control vehicle injections never showed a change in the number of swim cycles (one-way repeated-measures ANOVA, Holm-Sidak pairwise comparisons, $n = 6$, $F_{(13,63)} = 0.48$, $p = 0.927$) (Fig. 6, ○).

Whereas the isolated *Tritonia* brain preparation reliably produces a swim motor pattern in response to electrical stimulation of a body wall nerve, we rarely encountered an isolated *Pleurobranchaea* brain preparation that repeatedly and reliably produced a swim motor pattern for the time that it would take to test methysergide. However, in one such case, bath application of methysergide also blocked expression of the swim motor pattern, which recovered after washout of the antagonist.

5-HT lowers the threshold for *Pleurobranchaea* swimming

In *Tritonia*, injection of 5-HT *in vivo* reliably evokes dorsal-ventral swimming (McClellan et al., 1994). We repeated these experiments and confirmed that 5-HT injection into the body cavity of *Tritonia* caused vigorous swimming ($n = 2$), whereas control seawater injections did not ($n = 2$). In *Pleurobranchaea*, 5-HT injection induced weak dorsal and ventral flexions ($n = 4$). Additionally, the threshold for swim induction was greatly reduced; in *Pleurobranchaea*, electrical stimulation of the skin generally is required to evoke swimming, but after 5-HT injection simply touching the animal triggered vigorous dorsal-ventral swims in three of the four animals tested. No behavioral effects were observed from control seawater injections into *Pleurobranchaea* ($n = 4$).

In summary, as in *Tritonia*, DSI_{Pleur} modulation of $C2_{Pleur}$ was present and serotonergic modulation was necessary for *Pleurobranchaea* to produce a swim in response to electrical stimulation. Moreover, individual variation in swimming correlated with the extent that DSI_{Pleur} modulated $C2_{Pleur}$ synapses. These results suggest that serotonergic modulation within the CPG circuit is necessary for expression of the dorsal-ventral swimming behavior and its neural basis in *Pleurobranchaea*.

DSI and serotonin modulation of C2 synapses in *Hermissenda*

The vast majority of species in the Nudipleura do not swim (Newcomb et al., 2012). We tested whether DSI and serotonergic modulation were present in *H. crassicornis*, a species that does not produce dorsal-ventral swimming (Lillvis et al., 2012). Nerve stimuli that evoke swim motor patterns in the isolated brains of *Tritonia* and *Pleurobranchaea* (Fig. 1) did not elicit rhythmic bursting in *Hermissenda* as monitored in the homologs of the CPG neurons, $C2_{Herm}$ and DSI_{Herm} ($n = 29$) (Fig. 7*A*).

Like its homologs in *Tritonia* and *Pleurobranchaea*, the *Hermissenda* DSI is serotonergic (Tian et al., 2006). However, using the same experimental protocol as in *Tritonia* and *Pleurobranchaea*, we found that DSI_{Herm} caused little increase in the amplitude of $C2_{Herm}$-evoked synaptic potentials. There was a minor increase of only 5.4 ± 1.8% ($n = 5$) (Fig. 7*B*). The mean PSP amplitude when $C2_{Herm}$ was stimulated alone was 13.1 ± 5.5 mV and 13.6 ± 5.6 mV when it was stimulated after DSI_{Herm} (paired Student's *t* test, $t_{(4)} = -2.78$, $p = 0.05$). The lack of an effect of DSI_{Herm} on $C2_{Herm}$ synaptic strength was not the result of the inability to stimulate DSI_{Herm}; DSI_{Herm}-evoked EPSPs could be observed in the $C2_{Herm}$ postsynaptic target ($n = 3$) (Fig. 7*C*).

The absence of modulation can be accounted for by an absence in the response to 5-HT. Bath application of 5-HT did not enhance $C2_{Herm}$ synaptic strength and actually caused a small reduction in most preparations (mean of −15.5 ± 6.9% and median of −6.4%, $n = 12$; Fig. 7*D*). The mean $C2_{Herm}$-evoked PSP in HiDi saline was 12.4 ± 3.0 mV (median of 9.4 mV) compared with 9.3 ± 2.3 mV (median of 6.8 mV) in 100 μM 5-HT (Wilcoxon signed rank test, Z(11) = −2.82, p = 0.002).

In *Hermissenda*, 5-HT injections did not induce dorsal-ventral swimming. Instead, 5-HT injection induced noticeable movement of cerata ($n = 5$) and in some cases induced rhythmic left-right flexions (n = 3 of 5). No behavioral effects were observed from control seawater injections ($n = 3$).

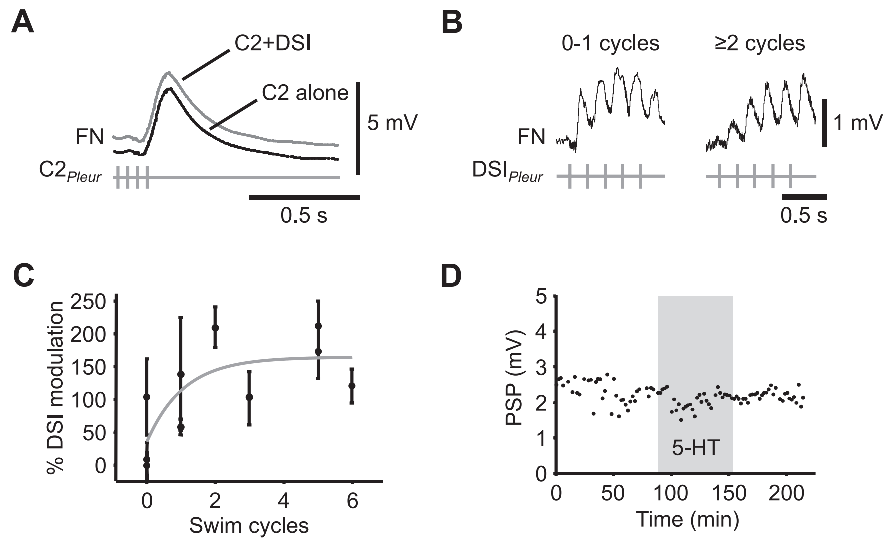

Figure 4. Individual variability in *Pleurobranchaea* swimming correlated with DSI and 5-HT modulation of C2-evoked synaptic potentials. ***A***, In an individual *Pleurobranchaea* that did not swim on the day of testing, there was little difference between the amplitudes of $C2_{Pleur}$-evoked EPSPs when $C2_{Pleur}$ was stimulated alone (black trace) and when $C2_{Pleur}$ was stimulated 3 s after DSI_{Pleur} (gray trace). Traces are offset vertically from each other for better visibility. ***B***, Examples showing the presence of DSI_{Pleur}-evoked one-for-one EPSPs in one individual that did not swim (left) and one that did (right). ***C***, The percentage modulation by DSI_{Pleur} correlated with the number of swim cycles. Each point represents one of 10 preparations (mean ± SD). Data were fit to the mean values by the equation $y = 36 + 151.7 * (x/0.9x)$ with an R^2 value of 0.58. ***D***, Example showing that bath application of 100 μM 5-HT did not increase the amplitude of $C2_{Pleur}$-evoked PSPs in an individual that did not swim on the day of testing.

Discussion

Similar neuromodulatory actions for similar behaviors

These experiments show that two species with independently evolved swimming behaviors share the same neuromodulatory actions, whereas a species that does not display the behavior lacks the neuromodulation. *Tritonia* and *Pleurobranchaea* both exhibit escape swimming responses consisting of dorsal and ventral body flexions (Willows, 1967; Gillette et al., 1991), whereas *Hermissenda* does not. In both *Tritonia* and swimming *Pleurobranchaea*, bath application of 5-HT or stimulation of the serotonergic DSI (As in *Pleurobranchaea*) enhanced the strength of synapses made by C2 (A1 in *Pleurobranchaea*). In contrast, in *Hermissenda*, the application of 5-HT or stimulation of DSI_{Herm} (CPT) did not modulate the strength of $C2_{Herm}$ synapses. *Tritonia* and *Pleurobranchaea* exhibited significantly more modulation by DSI (Fig. 8*A*) (one-way ANOVA, Holm-Sidak pairwise comparisons, $F_{(3,16)} = 9.46, p < 0.001$) and bath-applied 5-HT (Fig. 8*B*) (Kruskal-Wallis one-way ANOVA on ranks, Dunn's pairwise comparisons, H(3) = 25.58, $p < 0.001$) than did *Hermissenda*.

Serotonergic neuromodulation is necessary for swimming in both *Tritonia* and *Pleurobranchaea*. The 5-HT receptor antagonist methysergide blocked both the neuromodulation of C2 synaptic strength and the production of swimming in *Pleurobranchaea* as it does in *Tritonia* (McClellan et al., 1994; Katz and Frost, 1995a). 5-HT is also sufficient to trigger dorsal-ventral flexion swimming in both species, but not in *Hermissenda*. These results correlating the presence of serotonergic neuromodulation with species that swim with dorsal-ventral body flexions suggest that serotonergic neuromodulation plays a key role in the production of this behavior.

Although the results demonstrate that 5-HT plays a crucial role in the production of the swim in both *Tritonia* and *Pleurobranchaea*, this does not imply that only C2 synapses are involved. Methysergide and 5-HT likely act at many sites throughout the brain. For example, in *Tritonia*, DSI and 5-HT also modulate the synaptic strength of another swim CPG neuron (VSI) (Sakurai and Katz, 2003, 2009; Sakurai et al., 2006). The homolog of VSI has not

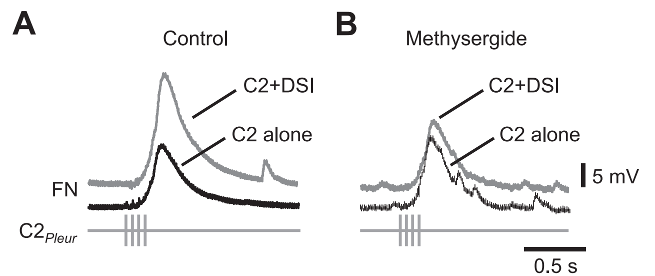

Figure 5. Methysergide blocked DSI modulation of C2 synapses in *Pleurobranchaea*. ***A***, Control: Stimulating $C2_{Pleur}$ elicited an EPSP (black trace) in a postsynaptic follower neuron (FN). Stimulating DSI_{Pleur} 3 s before the $C2_{Pleur}$ stimulation increased the amplitude of the $C2_{Pleur}$-evoked EPSP (gray trace). ***B***, In the presence of 50 μM methysergide, the EPSP elicited from $C2_{Pleur}$ alone (black trace) was similar to the control measurement, but the modulatory effect of DSI_{Pleur} was significantly reduced (gray trace). Traces are vertically offset from each other for better visibility.

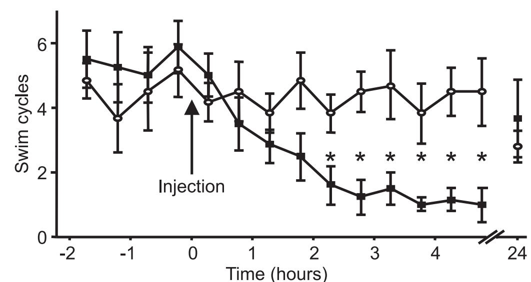

Figure 6. Methysergide inhibited the *Pleurobranchaea* swimming behavior. The average number of swim cycles (and SEM) is plotted over time. Injection of 10 mg/L methysergide (arrow) significantly reduced the number of swim cycles (■) (one-way repeated-measures ANOVA, Holm-Sidak pairwise comparisons, $n = 8$, $F_{(14,94)} = 8.54$, $p < 0.001$); the swim recovered overnight. Vehicle control injections (○) did not result in a reduction of swim cycles (one-way repeated-measures ANOVA, Holm-Sidak pairwise comparisons, $n = 6$, $F_{(13,63)} = 0.48$, $p = 0.927$).

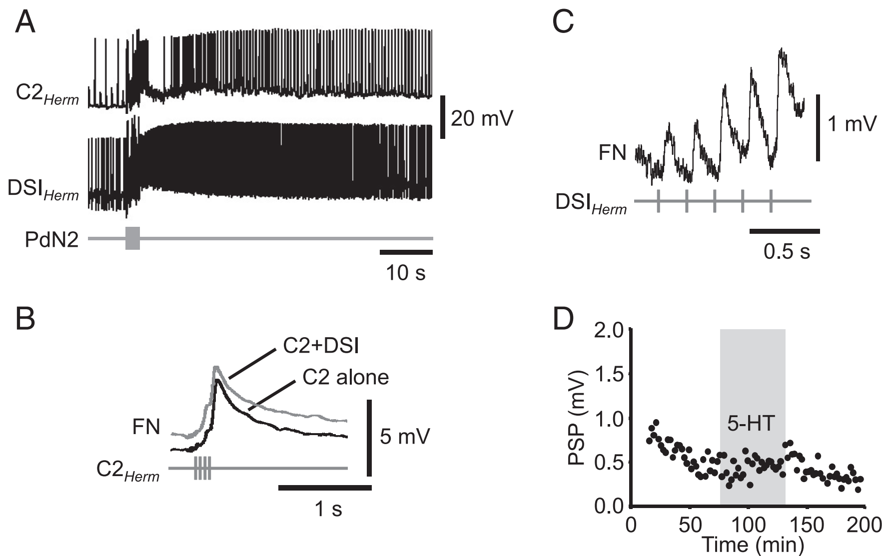

Figure 7. *Hermissenda* lacked the DSI and 5-HT modulation. ***A***, In *Hermissenda*, PdN2 stimuli (gray bar) elicited high-frequency spiking in DSI_{Herm} but no rhythmic bursting. PdN2 stimuli could also elicit high-frequency spiking in $C2_{Herm}$, and occasionally, a single burst, but no rhythmic bursting. ***B***, Stimulating $C2_{Herm}$ elicited an EPSP (black trace) in a postsynaptic follower neuron (FN). Stimulating DSI_{Herm} 3 s before the $C2_{Herm}$ stimulation did not increase the amplitude of the $C2_{Herm}$-evoked EPSP (gray trace). Traces are vertically offset from each other for better visibility. ***C***, The lack of an effect is not the result of failure of DSI_{Herm} spikes; DSI_{Herm} evoked one-for-one EPSPs in the FN. ***D***, Bath application of 100 μM 5-HT did not increase the amplitude of $C2_{Herm}$-evoked EPSPs.

been identified in *Pleurobranchaea*, so this site of modulation cannot be tested yet.

Here we have shown similar neuromodulatory actions underlie similar behaviors. Additionally, the results indicate that the lack of modulation in *Hermissenda* may be responsible the absence of the swim behavior. There are many examples of species differences in the presence of neuromodulatory transmitters or receptors, some of which have been shown to have implications for behavior (Katz and Harris-Warrick, 1999). For example, in amphibian embryos, species differences in neuromodulation account for differences in swimming (McLean and Sillar, 2004; Merrywest et al., 2004). Species variations in serotonergic neuromodulation have also been correlated with differences in sensitization in aplysiid molluscs (Marinesco et al., 2003; Hoover et al., 2006).

Parallel evolution of neuromodulation underlies independent evolution of behavior

Phylogenetic evidence suggests that *Tritonia* and *Pleurobranchaea* evolved dorsal-ventral flexion swimming independently (Newcomb et al., 2012). Both species belong to the monophyletic clade Nudipleura (Waegele and Willan, 2000; Bouchet et al., 2005), which consists of two monophyletic subclades: Pleurobranchomorpha (to which *Pleurobranchaea* belongs) and Nudibranchia (to which *Tritonia* and *Hermissenda* belong). *Tritonia* and *Hermissenda* can be further grouped together within the subsubclade, Cladobranchia (Bouchet et al., 2005; Pola and Gosliner, 2010). In total, there are ~3000 species of Nudipleura, the vast majority of which do not swim at all; only 17 species in seven genera have been reported to swim using dorsal-ventral flexions. The distribution of these species in the phylogenetic tree suggests that the behaviors evolved independently at least twice and possibly five times or more (Newcomb et al., 2012).

The results from this study show that the production of this independently evolved behavior in *Tritonia* and *Pleurobranchaea* relies on a neuromodulatory mechanism that is absent from at least one species that does not exhibit the behavior. Neurons homologous to DSI and C2 have been identified in several species that do not produce dorsal-ventral swimming (Tian et al., 2006; Newcomb and Katz, 2007; Lillvis et al., 2012), including more distantly related opisthobranchs, such as *Aplysia californica* (Jing et al., 2007) and *Clione limacina* (Panchin et al., 1995; Satterlie et al., 1995). Although these two neurons are present, it is not known whether DSI modulates C2 synaptic strength in any of these other species.

The fact that DSI and C2 are found in each member of the clade that has been studied suggests that they are ancestral features. Therefore, the apparent emergence of serotonergic neuromodulation of C2 synapses can be considered an example of parallel evolution, where homologous features undergo similar changes (Sanderson and Hufford, 1996; Wake et al., 2011; Pearce, 2012). Parallel evolution of brain mechanisms has been previously suggested to occur in the evolution of primate precision

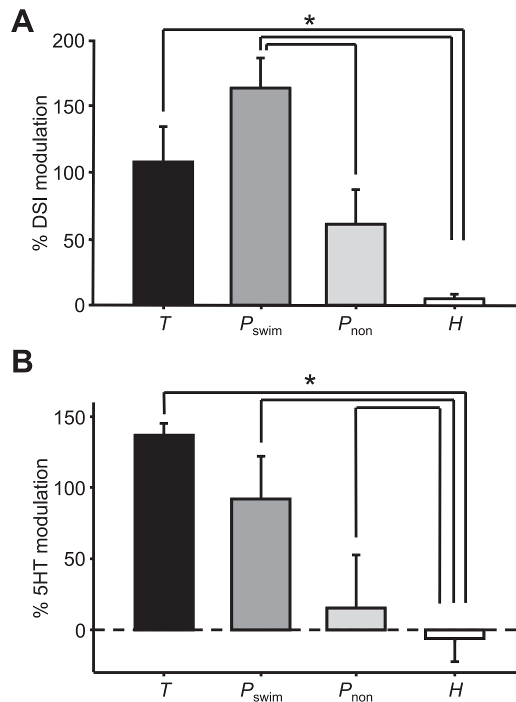

Figure 8. Summary of modulation results across species. ***A***, The average percentage increase in C2-evoked EPSPs produced by DSI in *Tritonia* (*T*) and swimming *Pleurobranchaea* (P_{swim}) was significantly greater than that in *Hermissenda* (*H*). Swimming *Pleurobranchaea* DSI_{Pleur} modulation percentage was also significantly greater than that of nonswimming *Pleurobranchaea* (P_{non}) (one-way ANOVA, Holm-Sidak *post hoc* pairwise comparisons, $n = 5$ for all species, $F_{(3,16)} = 9.46$, $p < 0.001$; *post hoc* comparisons: swimming *Pleurobranchaea* vs *Hermissenda*, $p < 0.001$; *Tritonia* vs *Hermissenda*, $p = 0.022$; swimming versus nonswimming *Pleurobranchaea*, $p = 0.018$). ***B***, The median percentage increase in C2 synapses in response to 5-HT bath application was significantly greater in *Tritonia* and both groups of *Pleurobranchaea* compared with *Hermissenda* (Kruskal-Wallis one-way ANOVA on ranks, Dunn's *post hoc* pairwise comparisons, $n = 2$ in *Tritonia*, 10 in swimming *Pleurobranchaea*, 11 in nonswimming *Pleurobranchaea*, 12 in *Hermissenda*, $H(3) = 25.58$, $p < 0.001$).

hand movements (Padberg et al., 2007) and butterfly color vision (Frentiu, et al, 2007).

The presence of DSI modulation of C2 synaptic strength may be a mechanism to transform a nonrhythmic network of neurons into a functional CPG. That is, there may be a circuit with the latent ability to form a swim CPG circuit when activated by this neuromodulatory signal. A similar latent circuit was described in voles; prairie vole (*Microtus ochrogaster*) males exhibit pair bonding behaviors that are absent from meadow voles (*Microtus pennsylvanicus*) (Young and Wang, 2004; Nair and Young, 2006). However, meadow voles can be transformed to exhibit pair bonding through viral expression of vasopression V1a receptors in ventral pallidum, an area that has high V1a receptor expression levels in prairie voles (Lim et al., 2004). Thus, meadow voles must have a latent ability to form a "pair bonding circuit" that is activated by the expression of V1a receptors in particular neurons.

Individual variability

Unlike individual *Tritonia*, which reliably swim in response to salt or electric shock stimuli (Mongeluzi et al., 1998), individual *Pleurobranchaea* exhibited daily fluctuations in the number of flexion cycles that they produced in response to electric shock. The extent of neuromodulation covaried with the maximum number of cycles on the testing day. Thus, there was a correlation, not just between species that exhibit the behavior and the presence of the modulation, but within a species in the extent of swimming and the extent of modulation. The extent of modulation from DSI in nonswimming *Pleurobranchaea* was not significantly different from that seen in *Hermissenda* (Fig. 8*A*).

We do not know the reason for the neuromodulatory and behavioral variability. It was previously reported that individual *Pleurobranchaea* differed in their tendency to swim (Jing and Gillette, 1995, 1999), but a longitudinal study to determine whether the same individuals consistently did or did not swim was not reported. The variability reported here was not related to the time of day or feeding state of the animal. Animals were housed in individual plastic containers within a larger aquarium; therefore, it was unlikely to be caused by interactions with each other.

The ability of a modulatory system to change the state of an animal and therefore the expression of a behavior is not unexpected; this is precisely the way that circulating hormones affect behavior. However, we are unaware of another situation in which neuron-to-neuron neuromodulatory actions covary with behavior.

Neuron multifunctionality, neuromodulation, and the evolvability of behavior

Neurons can be multifunctional; both DSI and C2 have other behavioral functions aside from being a part of the dorsal-ventral swim CPG. In *Tritonia*, *Pleurobranchaea*, and *Aplysia californica*, DSI can initiate crawling (Jing and Gillette, 2000; Popescu and Frost, 2002; Jing et al., 2008). In *Hermissenda*, DSI elicits nonrhythmic foot contractions (Tian et al., 2006). DSI also facilitates feeding in *Aplysia californica* (Jing et al., 2008) and *Pleurobranchaea* (Jing and Gillette, 2000), whereas C2 suppresses feeding motor patterns in *Pleurobranchaea* (Jing and Gillette, 1995). In *Clione limacina*, a species that swims with rhythmic wing-like flapping, DSI modulates the speed of the swim motor pattern (Panchin et al., 1995; Satterlie and Norekian, 1995).

In *Tritonia*, C2 may play a role in crawling (Snow, 1982). Such functions could be present in other species as well. Therefore, dynamic neuromodulation of C2 may allow it to participate in distinct behaviors at different times. Such sculpting of multifunctional neurons into different functional circuits has been well described in the stomatogastric nervous system of decapod crustaceans (Stein, 2009).

In this way, neuromodulation may be a mechanism that affects the evolvability of behavior as it can allow shared neural circuit components to produce various outputs (Katz, 2011). As discussed above, similar patterns of expression for the vasopressin receptor are hypothesized to underlie independent evolution of analogous social behavior (Young and Wang, 2004). Similar associations between nonapeptide receptor expression and social behavior have also been shown in fish, reptiles, birds, and other mammals, including humans (Donaldson and Young, 2008; Young, 2009; Goodson and Thompson, 2010). This suggests that different species have independently acquired similar neuromodulatory mechanisms to produce similar behavior. We propose such a mechanism here; *Tritonia* and *Pleurobranchaea* appear to have independently acquired a similar neuromodulatory mechanism using homologous neurons to produce a similar swim behavior. Thus, parallel evolution of neuromodulation may be a mechanism underlying the independent evolution of behavior.

Notes

Supplemental material for this article is available at http://neuronbank.org/wiki/. NeuronBank contains descriptions of identified neurons. This material has not been peer reviewed.

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