Title: Synaptic and membrane conductance underlie phase transition in a simple half-center oscillator.

Running Head: Dynamic properties within a half-center oscillator

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Abstract (250/250 words)

Rhythmically active neural circuits often contain reciprocally inhibitory modules that act as half-center oscillators. In half-center oscillators, alternating burst discharges require a mechanism to transition activity from one phase to the next, which requires particular synaptic and membrane properties. Here we found that active membrane properties of specific neurons and the temporal dynamics of particular synapses both contribute to the production of a stable rhythmic motor pattern in the swim central pattern generator (CPG) of the nudibranch mollusc, *Dendronotus iris*. This CPG is composed of only four neurons that are organized into two competing modules of a half-center oscillator. Each module is composed of a Swim Interneuron 2 (Si2) and the contralateral Swim Interneuron 3 (Si3). Si2 and Si3 each inhibit their own contralateral counterparts; however, the S2 contralateral synapses have a more negative reversal potential, making them more effective at hyperpolarizing the Si2 and the electrically coupled Si3 of the other module. Si3 rebounds first from inhibition due to a hyperpolarization-activated slow inward current. Si3 excites the Si2 in its module through both chemical and electrical synapses. An Si2-evoked slow inhibitory synaptic potential in Si3 suppresses its firing, terminating the burst generated by the module. Using dynamic clamping, we showed that the magnitude of the slow inhibition sets the periodicity of the oscillator. Thus, the network-driven oscillation is produced by each module rebounding from inhibition, maintaining the burst through self-excitation, and then terminating its burst through a buildup of slow synaptic inhibition, thereby releasing the other module from inhibition.

Keywords: Rhythmogenesis; Gastropod; Locomotion; voltage clamp, Invertebrate

Introduction (561 words)

Since the original proposal of the half-center oscillator (HCO) concept by Bown (1911), reciprocal inhibition between two modules has been considered to be the fundamental architecture of network oscillators (Grillner and El Manira 2015; Kiehn 2016; Moulton et al. 2013). However, reciprocal inhibition by itself is not sufficient to maintain stable rhythmic bursting. In many systems, endogenous or conditional bursting properties contribute to maintaining the alternation of half-center oscillators (Arshavsky et al. 1986; Cymbalyuk et al. 2002; Kiehn et al. 1996; Li et al. 2010; Reith and Sillar 1998; Selverston and Miller 1980; Wallen and Grillner 1987). However, a complete mechanistic understanding of network-driven oscillations in a central pattern generator (CPG) is lacking. Here we found that synaptic and membrane properties of identified neurons within each HCO module account for rhythmic bursting activity in the CPG underlying swimming in the nudibranch mollusc, *Dendronotus iris*.

An HCO is composed of a pair of modules that exhibit spiking or bursting activity in alternation through reciprocal inhibition. The reciprocal inhibition is crucial for alternating bursts; however, the network also requires a dynamic property that terminates ongoing excitation in each module so that it can either be released from inhibition or escape from it, and thereby trigger phase transition to the other module (Arbas and Calabrese 1987a; Friesen 1994; Friesen and Stent 1978). Active membrane properties of neurons, such as "sag" depolarization and post-inhibitory rebound, have been shown to be involved in the transition of activity by allowing the postsynaptic neurons to escape from inhibition or firing once released from inhibition (Arbas and Calabrese 1987b; Arshavsky et al. 1985; Dethier et al. 2015; Li and Moulton 2012). Studies using computer models also demonstrated the role of the post-inhibitory rebound in escaping from inhibition (Daun et al. 2009; Perkel and Mulloney 1974; Wang and Rinzel 1992).

Another mechanism that assists the activity transition is self-inhibition within each half-center module, which releases the other module from inhibition (Friesen 1994). However, there have been few reports on such self-inhibition playing an essential role in the HCO. In this study,

we found a new mechanism of the phase transition. A slow inhibitory synapse within each half-center acts as a feedback inhibition that terminates its own burst to prompt the phase transition to the other half of the HCO.

Dendronotus iris swims by rhythmically flexing its body alternately from left to right (Sakurai et al. 2011). The rhythmic motor pattern originates from the swim CPG, which consists of only two pairs of interneurons, Si2 and Si3 (Fig. 1A,B) (Sakurai and Katz 2016). Both Si2 and Si3 form reciprocally inhibitory synapses with their contralateral counterparts, forming an HCO (Fig. 1B). The neuronal components (Si2 and Si3) of each HCO module (α and β) are contralateral to each other in the brain; that is, the contralateral Si2 and Si3 fire in phase with each other as a module (Fig. 1Ci). A strong excitatory synapse from Si3 to Si2 and electrical coupling mediates their synchronous firing in each module (Fig. 1Cii). In this study, we investigated the mechanisms underlying the transition of activity between the two modules of the swim CPG. We found that the two neurons in the module each contribute differently to burst initiation and termination on each cycle. Specifically, the combined action of a slow synapse and active membrane properties provide dynamic features needed for this half-center oscillator to function.

Materials and Methods

Animal collection, maintenance, and dissection

Specimens of *Dendronotus iris* (Mollusca; Gastropoda; Nudibranchia), 6-20 cm long, were collected on the Pacific coast of North America by Monterey Abalone Company (Monterey, CA) and Living Elements Ltd (Delta, BC, Canada). The animals were kept in artificial seawater tanks at 10-12°C with a 12:12 light/dark cycle.

Before the surgery for brain isolation, the animal was anesthetized by injecting 0.33 M magnesium chloride solution (20-50 mL) into the body cavity, and an incision was made through the body wall near the esophagus. Then all nerve roots exiting the brain were severed and the brain (Fig. 1A) consisting of the cerebral ganglia, pleural ganglia, and pedunculopontine ganglia were isolated by severing the esophagus. The isolated brain was pinned with the dorsal side up on the bottom of a Sylgard-lined dish and perfused at a rate of 0.5 ml/min with normal saline (mM: 420 NaCl, 10 KCl, 10 CaCl₂, 50 MgCl₂, 11 D-glucose, 10 HEPES, pH 7.6) or artificial seawater (Instant Ocean, Mentor, OH). The brain was chilled down to 4°C to, and the connective tissue and the brain sheath were removed with forceps and fine scissors. After desheathing, the temperature was raised to 10°C for electrophysiological experiments.

Electrophysiology

Intracellular recordings were performed using 15-35 MΩ glass microelectrodes filled with a solution containing 2 M potassium acetate and 0.2 M potassium. Each electrode was connected to the headstage of the Axoclamp 2B amplifier (Molecular Devices, Sunnyvale, CA). The output signals of the amplifier were digitized at a sampling frequency of >2 kHz using a 1401Plus or Micro1401 A/D converter (Cambridge Electronic Design, Cambridge, UK). Data acquisition and analysis were performed with Spike2 software (CED, Cambridge, UK) and SigmaPlot (Jandel Scientific, San Rafael, CA).

To investigate the effects of depolarization or hyperpolarization of the swim interneurons on the swim rhythm, a positive or negative current pulse (from -4 nA to 4 nA) was injected directly into the neurons via bridge-balanced microelectrodes. In some experiments, spontaneous neuronal activity was suppressed by applying high divalent cation (Hi-Di) saline, which raises the threshold for neuronal spiking. The composition of Hi-Di saline (in mM) was 285 NaCl, 10 KCl, 25 CaCl₂, 125 MgCl₂, 11 D-glucose, 10 HEPES, pH 7.6. The reversal potentials of synaptic potentials and the strengths of electrical connections were measured by injecting a steady current under discontinuous current-clamp mode or through an additional microelectrode placed in the same neuron. In some experiments, calcium-free Hi-Di saline was used to block chemical synaptic transmission. In low calcium Hi-Di saline, CaCl₂ was replaced by MgCl₂.

Dynamic clamp

To artificially enhance or counteract the Si2-evoked slow IPSPs in Si3, we performed dynamic clamping with the software StdpC (Kemenes et al. 2011). Each Si3 was impaled with two microelectrodes, one for measuring the membrane potential and the other for injecting current (see Fig. 10Ai). Every time Si2 generates an action potential that exceeded 0 mV, the amount of current injected into the postsynaptic Si3, I_{stim} , was calculated using a first-order kinetics model of the release of neurotransmitter (Destexhe et al. 1994; Kemenes et al. 2011; Sharp et al. 1996):

$$I_{syn} = g_{syn} S(t) [V_{syn} - V_{post}(t)], \quad (\text{eq. 1})$$

where $S(t)$ is the instantaneous synaptic activation, g_{syn} is the maximum synaptic conductance, V_{syn} is the reversal potential (-70 mV) of the synapse. To counteract Si2-evoked synaptic potentials, a negative value was used for g_{syn} .

The instantaneous activation, $S(t)$, is given by the differential equation:

$$\left(1 - S_{\infty}(V_{\text{pre}})\right) \tau_{\text{syn}} \frac{dS}{dt} = \left(S_{\infty}(V_{\text{pre}}) - S(t)\right), \quad (\text{eq. 2})$$

where

$$S_{\infty}(V_{\text{pre}}) = \begin{cases} \tanh\left[\frac{V_{\text{pre}} - V_{\text{thresh}}}{V_{\text{slope}}}\right] & \text{if } V_{\text{pre}} > V_{\text{Thresh}} \\ 0 & \text{otherwise} \end{cases} \quad (\text{eq. 3})$$

S_{∞} is the steady-state synaptic activation, and τ_{syn} is the time constant for synaptic decay. The Si2-evoked inhibitory postsynaptic current recorded in Si2 under voltage clamp was 0.94 ± 0.2 sec (mean $\pm$ SD, N=5). In this study, τ_{syn} was set to 1.0 sec. V_{pre} is the presynaptic membrane potential of Si3 and V_{thresh} is the threshold potential for the release of neurotransmitter; it was set to the level of 50% height of the smallest Si3 action potentials. The synaptic slope parameter of the activation curve (V_{slope}) was set to 25 mV. g_{syn} was varied between -1000 and 1000 nS.

Statistics

Statistical comparisons were performed using SigmaPlot ver. 12.5 (Jandel Scientific, San Rafael, CA) for Student's *t*-test, paired *t*-test, and Kruskal-Wallis one-way ANOVA on Ranks with all pairwise Multiple Comparison Procedures. Shapiro-Wilk test was used to assume normality of data structure. In all cases, $P < 0.05$ was considered significant. For *t*-tests, a two-tailed *P* value was used. Results are expressed as the mean $\pm$ standard deviation (SD).

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Results

Si2-evoked IPSPs have a more hyperpolarized reversal potential than those of Si3

In the *Dendronotus* swim CPG, two types of neurons, Si2 and Si3, each form inhibitory reciprocal synapses with the contralateral analog. To compare the strength of these synapses, we suppressed spontaneous activity using high divalent cation (Hi-Di) saline, which raises the spike threshold and thereby minimizes spontaneous and polysynaptic activity. We found that the reversal potentials of these inhibitory synapses differ (Fig. 2); the average reversal potential of Si2-to-Si2 IPSPs was -73.9 ± 8.1 mV ($N = 5$; Fig. 2Ai, B), whereas the Si3-to-Si3 IPSP reversal potential was -46.4 ± 3.9 mV ($N = 4$; Fig. 2Aii, B). This indicates that, near the resting potential (about -50 mV), the inhibitory synapse of Si2 would greatly hyperpolarize the postsynaptic Si2, whereas the inhibitory synapse of Si3 would hardly change the membrane potential of the postsynaptic Si3 but only increase the membrane conductance. This also suggests that the inhibitory synapses between the Si3 pair differ from that of the Si2 pair in the ionic basis of their postsynaptic currents.

Si2 evokes a larger inhibitory effect on the opposite module than Si3.

During the swim motor pattern, both Si2 and Si3 receive rhythmic synaptic inhibition from their contralateral counterparts. To better evaluate the contributions of Si2 and Si3, we stimulated each independently (Fig. 3). A train of action potentials in Si2 produced a brief hyperpolarization and a delayed depolarization in the Si2 and Si3 of the opposite module (Fig. 3A). The depolarization led to a burst discharge in Si3. Similar post-inhibitory rebound discharges in Si3 were seen in all preparations examined (100%, $N = 28$). In 68% of preparations, the contralateral Si2 also showed the post-inhibitory rebound discharge ($N = 19$ of 28), which were all triggered by the Si3-evoked EPSPs (Fig. 3A, asterisks).

In contrast, a similar train of action potentials in Si3 evoked only small synaptic potentials in Si2 and Si3 of the opposite module. Rebound firing in the contralateral Si3

occurred in 69% of preparations (N=11 of 16), which induced the ipsilateral Si2 firing in 43.8% of preparations (N = 7 of 16). Thus, an Si2 spike train induced a rebound discharge of Si3 in the opposite module more frequently than the Si3-induced discharge. These results show that the inhibitory synaptic inputs from Si2 not only provide inhibition to the other half-center module but also induce an excitatory response in Si3 via post-inhibitory rebound excitation.

The difference in the size of the evoked inhibition was more pronounced in Hi-Di saline (Byrne et al. 1978; Liao and Walters 2002; Sakurai and Katz 2003; Sakurai et al. 2011), which raises spike threshold and thereby minimizes spontaneous and polysynaptic activity (Fig. 4). A 10Hz spike train in the L-Si2 produced a 10mV hyperpolarization of the R-Si2 (N=21, Fig. 4A). The L-Si3 was also hyperpolarized because it is electrically coupled to the R-Si2 (Sakurai and Katz 2016). The mean amplitude of the IPSP evoked by a 10-Hz spike train of Si2 was -12.3 ± 1.6 mV (N = 7) in the contralateral Si2 and -7.0 ± 2.0 mV (N = 6) in the ipsilateral Si3 (Fig. 4C). In contrast, a 10Hz spike train in the R-Si3 produced just a small hyperpolarization in both neurons of the other module (N = 19, Fig. 4B); the mean IPSP amplitudes were only -2.0 ± 1.9 mV (N = 5) in the contralateral Si3 and 1.4 ± 1.0 mV (N = 7) in the ipsilateral Si2 (Fig. 4C). Thus, Si2 evoked larger inhibitory potentials than Si3 in neurons of the other module.

Si3, not Si2, exhibits a post-inhibitory rebound

Immediately after the Si2 stimulation, the membrane potential of the contralateral Si2 in the other module slowly returned to its original resting potential (R-Si2 in Fig. 4A), while Si3 not only recovered more quickly but also showed a rebound depolarization that exceeded the original membrane potential (L-Si3 in Fig. 4A, asterisk). Neither neuron exhibited rebound depolarization when Si3 was stimulated (Fig. 4B). The rebound depolarization induced by Si2 onto the ipsilateral Si3 in the other module was significantly larger than any other synapses (Fig. 4D, $P < 0.05$ by Kruskal-Wallis one-way ANOVA on Ranks with all pairwise multiple Comparison Procedures). The post-inhibitory rebound depolarization was largest in Si3 than Si2.

Si3 exhibited a larger post-inhibitory rebound depolarization following hyperpolarizing current steps than Si2 (Fig. 5). In normal saline, injection of a hyperpolarizing current pulse (1-4 nA, 4 sec) into Si2 produced only a hyperpolarizing potential change with little or no spike discharges afterward (Fig. 5A, left). In contrast, a hyperpolarizing current pulse in Si3 caused a post-inhibitory rebound discharge upon its termination of the pulse (Fig. 5A, right). The number of spikes after the current injection pulse was significantly larger in Si3 than Si2 (Fig. 5B; $P < 0.001$ by Two-way repeated-measures ANOVA with Holm-Sidak method, $N = 9-12$ for Si2 and $N = 8-10$ for Si3). When the spike threshold was raised by Hi-Di saline, Si3 showed a depolarizing overshoot when released from the hyperpolarizing current injection (Fig. 5C). This rebound potential was significantly larger in Si3 than Si2 (Fig. 5D; $P < 0.001$ by Two-way repeated-measures ANOVA with Holm-Sidak method, $N = 20-23$ for Si2 and $N = 19-21$ for Si3).

Si3 exhibits a hyperpolarization-activated inward current

In addition to the rebound excitation, both Si2 and Si3 exhibited a "sag" potential during the hyperpolarizing current injection (Fig. 5A, C). Although the difference in the magnitude of this sag potential was less prominent than the rebound potential, there was a significant difference in the responses caused by a -4 nA current pulse (Fig. 5E; $P < 0.001$ by Two-way repeated-measures ANOVA with Holm-Sidak method, $N = 23$ for Si2 and $N = 21$ for Si3).

Under voltage clamp, Si3, but not Si2 exhibited a hyperpolarization-activated inward current (Fig. 6). Step-wise hyperpolarizing changes of membrane potential did not produce a noticeable current response in Si2, whereas in Si3 it evoked a slow inward current (Fig. 6A). The amplitude of the inward current increased in proportion to the hyperpolarizing voltage steps (Fig. 6B).

Together, these results suggest that Si2 and Si3 have distinct membrane properties. The hyperpolarization-activated slow inward current in Si3 likely underlies the sag depolarization and subsequent rebound depolarization, which produces the post-inhibitory rebound discharge

in Si3 and consequently induces the phase transition from one module of the half center to the other.

Si2 provides a burst-limiting mechanism within each half-center module

We previously demonstrated that strong excitatory coupling between Si2 and the contralateral Si3 via an excitatory synapse and electrical coupling causes them to fire together as a half-center module (Sakurai and Katz 2016). Here we found that Si2 also evokes a slow inhibitory synaptic action on Si3 (Fig. 7A). Although the Si2-to-Si3 electrotonic connection contributes to enhanced spiking within the module, the excitation was always overwhelmed by delayed inhibition that suppressed spiking in Si3 (Fig. 7A,B).

When the spike threshold was increased in Hi-Di saline, an Si2 spike train produced a one-for-one electrotonic EPSPs in the contralateral Si3 (Fig. 7Bi). With increased spike frequency, a slow hyperpolarizing potential counteracted the electrotonic EPSPs and produced an overall hyperpolarization (Fig. 7Bi, 10 Hz).

The application of Ca^{2+} -free saline blocked this hyperpolarization without changing the electrotonic EPSPs (Fig. 7Bii, C). Subtracting traces in Ca^{2+} -free saline (Fig. 7Bii) from those in Hi-Di saline (Fig. 7Bi) revealed the Ca^{2+} -sensitive component of the Si2-evoked postsynaptic potential in Si3 (Fig. 7Biii). These results suggest that this slow inhibition was mediated by chemical neurotransmitter release.

Under voltage clamp, a spike train in Si2 produced a slow inhibitory postsynaptic current (IPSC) in the contralateral Si3 (Fig. 8A). The amplitude of the slow IPSC was outwardly rectifying. The reversal potentials were variable across individuals ranging from -60 to -80 mV (Fig. 8B). The time constant of the falling phase of the slow IPSP was 0.94 ± 0.2 sec ($N = 5$). During the Si2 spike train, the membrane conductance of Si3 increased in all three preparations examined from an average of 80.7 ± 15.3 nS to 141 ± 22.1 nS ($P = 0.039$ by paired t -test, $N = 3$). The outward rectification of the slow IPSC was not likely due to a change in background membrane

conductance via opening or closing of voltage-gated channels because the input resistance of each cell was constant throughout the voltage range examined (Fig. 8C). At present, it is unclear how such apparent outward rectification was brought about. The variability in the reversal potential and the non-linearity of the I-V relationships could also be an experimental artifact caused by poor space clamping, suggesting that the synapse is located contralateral to the Si3 soma.

Feedback inhibition from Si2 to Si3 contributes to burst termination

To determine the role that the inhibition from Si2 to the contralateral Si3 plays in rhythmic burst generation, we examined the effect of suppressing spikes in Si2 during the swim motor pattern (Fig. 9). When unperturbed, the intra-burst spike frequency of Si3 showed an initial peak and a subsequent steep decline during each burst (Fig. 9A,B, burst *a*). Suppression of spiking in Si2 by hyperpolarizing current injection increased the duration of Si3 bursts immediately (Fig. 9A,B burst *b*). The duration of the Si3 burst during the swim motor pattern increased significantly from 2.7 ± 0.9 sec to 4.1 ± 1.1 sec when the Si2 in its module was hyperpolarized (Fig. 9Ci; $P < 0.001$ by two-tailed paired t-test, $N=12$). The duty cycle of Si3 burst increased significantly from 0.54 ± 0.09 to 0.67 ± 0.13 with the Si2 hyperpolarized (Fig. 9Cij; $P < 0.001$ by Wilcoxon Signed Rank Test, $N = 12$). The Si2 hyperpolarization also caused a significant increase in the number of Si3 spikes from 19.5 ± 4.9 to 28.4 ± 9.3 ($P < 0.001$ by two-tailed paired t-test, $N = 12$) (Fig. 9C3). These results suggest that Si2 has two opposing actions on the Si3 in its module: 1) it enhances the Si3 burst through an electrotonic synapse, and 2) it slowly decreases Si3 firing through a chemical inhibitory synapse.

Dynamic clamp manipulation of slow inhibitory synapses revealed its role in the burst frequency of the swim motor pattern

The results suggest that the slow inhibition from Si2 to Si3 in each module decreases Si3 firing and hence decreases the excitatory drive on Si2. We hypothesized that the self-inhibition within the module helps to terminate the burst and transition to activation of the other module. To test this, we artificially enhanced or reduced the magnitude of the inhibition from Si2 to Si3 in both modules during a swim motor pattern using the dynamic clamp technique. The amplitudes and time courses of the Si2-evoked synaptic currents were calculated in real-time (Fig. 10Ai, see Materials and Methods) and injected into Si3 through the recording electrode in response to spikes in the contralateral Si2. While the dynamic clamp was engaged, an Si2 burst set off a slow negative current injected into Si3, boosting the slow inhibitory synapse, which shortened the Si3 burst (Fig. 10Aii). Spikes in the postsynaptic Si3 also produced spike-like downward surges in the injected current because the spikes moved the membrane potential away from the reversal potential of the slow IPSC (-70 mV).

Augmenting the amplitude of the slow inhibitory synaptic conductance at the Si2-to-Si3 synapse shortened the Si3 burst duration, thereby decreasing the burst period (Fig. 10B,D). In contrast, counteracting the inhibition with an inverted conductance reduced the Si2-to-Si3 inhibition and extended Si3 bursts, thereby increasing the burst frequency (Fig. 10C,D). Thus, the feedback inhibition from Si2 to Si3 within the module plays a crucial role in maintaining the periodicity of the half-center oscillator.

Discussion

In this study, we identified the synaptic and membrane conductances that contribute to the stable alternations underlying the *Dendronotus* swim motor pattern. Specifically, we found that slow inhibitory synaptic connection within each module acts as a self-limiting mechanism to halt the excitatory synaptic drive. This, in turn, causes a transition of excitation from one side to

the other. Moreover, the neurons that receive the slow synaptic inhibition exhibit membrane properties that cause post-inhibitory rebound firing that is triggered following release from inhibition by the other module.

The actual sequence of activity in the *Dendronotus* swim CPG circuit is as follows (Fig. 11): Si2 and the contralateral Si3 in Module α fire synchronously through excitatory synapses and electrical coupling (a). As Si2 exerts a slow synaptic inhibition on Si3, the spike frequency of Si3 gradually decreases, lessening the excitatory drive to Si2 (b). Concurrently Module α Si2 inhibits its contralateral counterpart in Module β . Cessation of spiking in Module α leads to post-inhibitory rebound of Si3 in Module β . The firing of the Module β neurons suppresses the activity of the Module α , and hence the transition of excitation occurs (c). Slow inhibitory feedback in Module β weakens the Si3 activity until Module α rebounds (d).

The role of active membrane properties the swim CPG

Si3 neurons, but not Si2 neurons, showed post-inhibitory rebound when it was released from hyperpolarization or after a train of IPSPs evoked by the other half-center module. The post-inhibitory rebound property is present in many neural circuits with the half-center configuration; it plays a crucial role in the maintenance of rhythmic neural activity by prompting the transition of activity phases from one side to the other (Angstadt et al. 2005; Arshavsky et al. 1998; Dethier et al. 2015; Li and Moulton 2012; Nagornov et al. 2016; Pirtle and Satterlie 2007; Pirtle et al. 2010; Roberts et al. 2008; Rodriguez et al. 2013). For example, in tadpoles, the firing of excitatory descending interneurons is triggered by a post-inhibitory rebound. The rebound occurs when the membrane is held in a depolarized state by sustained excitatory input and then hyperpolarized by inhibitory input (Li et al. 2007; Roberts et al. 2008). Computer models of the half-center oscillator have demonstrated the contribution of a post-inhibitory rebound in phase transitions (Daun et al. 2009; Dethier et al. 2015; Wang and Rinzel 1992).

Before the rebound discharge, both Si2 and Si3 neurons showed a sag potential that gradually depolarizes the membrane. This time-dependent change in membrane potential may

contribute to the escape of these neurons from synaptic inhibition by the contralateral half-center module (Arbas and Calabrese 1987b; Pirtle et al. 2010). It has been well acknowledged that a hyperpolarization-activated inward current and a low-threshold T-type calcium current contribute to rebound responses in both vertebrates and invertebrates (Angstadt et al. 2005; Dethier et al. 2015). The contribution of hyperpolarization-activated sodium current (Nadim et al., 1995) and persistent sodium current (McCrea and Rybak 2008; Tazerart et al. 2007; Zhong et al. 2007; Ziskind-Conhaim et al. 2008) to the sag potential has also been shown. These observations suggest that sag potentials and post-inhibitor discharges contribute substantially to the transition of activity in the Dendronotus swim CPG. Interestingly, of the two cell types that make up the half-center, the post-inhibitory rebound property was found only in Si3 but not in Si2. It is currently unclear which ion permeability underlies the sag and the rebound discharge in Si3 neurons. In the previous study, we have shown that the central command neuron, Si1, makes synapses onto Si3 and potentiates the Si3 synaptic strength (Sakurai and Katz 2019). The modulatory effects of Si1 on the membrane excitability of Si3 are of interest and remain to be investigated in the future.

Differentiation of the role inhibitory synaptic actions onto the contralateral module

Although both Si2 and Si3 have reciprocal inhibitory synapses with their contralateral counterparts, as is typical for a half-center oscillator, the results of this study show that their synaptic properties are different. Specifically, the Si2-evoked IPSPs in the contralateral Si2 had a reversal potential at a more hyperpolarized level than that of Si3, which results in a larger IPSP than the Si3-Si3 synapse. The Si2-evoked IPSPs in the contralateral Si2 caused a hyperpolarization in Si3 in the same module via electrical coupling, which leads to a post-inhibition rebound firing in the Si3. In contrast, Si3-evoked IPSPs had a reversal potential near the resting potential, and therefore, their inhibitory effect is not due to membrane hyperpolarization but to shunting inhibition caused by an increase in membrane conductance (Paulus and Rothwell 2016), which has little effect on the voltage-dependent channels that

underlie the induction of rebound discharge. As described later, Si2 also provides inhibitory feedback to Si3 within the same half-center module. Thus, the inhibitory synapse of Si2 is responsible for stopping the activity of Si3 in the same module while triggering rebound firing of Si3 in the contralateral module. This is in stark contrast to the excitatory synapse of Si3, which triggers the excitation of Si2. These facts indicate that there is a functional differentiation between the inhibitory synaptic functions of Si2 and Si3 neurons, which is reflected in their ability to induce a post-inhibitory rebound.

The role of self-inhibition in the rhythmogenesis

The key finding of this study is the discovery of a synaptic mechanism that serves as a brake on self-excitation within each module of the half-center circuit. In a previous study, we described that Si2 and Si3 are strongly linked by excitatory synapses and electrical connections in each half-center of the *Dendronotus* swim CPG. The resulting synchronized activity is essential for maintaining half-center oscillator activity (Sakurai and Katz 2016). Here, we found that Si2 also inhibits Si3 and that this slow synaptic action acts as feedback inhibition to limit the burst duration.

In the original concept of the half-center oscillator, it was originally thought that the reciprocal inhibition between two half-center modules was essential for alternating rhythm generation and that endogenous rhythmogenesis in each module is not required (Brown 1911). However, without a mechanism that prompts the transition of activity from one side to the other, the module in one side would remain active for an extended period of time while the other side is inhibited. Friesen (1994) suggested that one potential mechanism that could provide a dynamic property to the half-center oscillator is spike frequency adaptation which limits the duration of excitation in one module, ensuring that a pair of mutually inhibitory neurons acts as an alternating oscillator rather than a bistable switch. However, few such circuits have been reported. Instead, neural circuits with a half-center configuration often are capable of generating endogenous bursting upon isolation (Arshavsky et al. 1986; Cymbalyuk et al. 2002; Selverston and Miller 1980). Similar

conditional pacemaker properties also have been found in each half of the locomotor circuit in the spinal cord (Kiehn et al. 1996; Li et al. 2010; Reith and Sillar 1998; Wallen and Grillner 1987). When the left and right modules of the spinal swimming circuit are separated in lamprey, each module exhibits rhythmic bursting activity at a faster rhythm than normal (Grillner 2003). If each half-center possesses endogenous rhythmogenesis, then the role of mutual inhibition between half-centers is limited to ensuring coordinated bursts in alternation (Ausborn et al. 2018; Hagglund et al. 2013). A counterexample to this was reported by Moulton et al. (2013), in which they showed that the onset of rhythmic activity did not occur instantaneously when unilateral activity is momentarily inhibited but took some time, suggesting that some neuroplasticity may be involved in the experimentally induced onset of unilateral automaticity (Moulton et al. 2013). In *Dendronotus*, it is unlikely that each module of the swim CPG has rhythmogenesis, because no oscillatory activity was seen when the activity of one side was suppressed by current injection (Sakurai and Katz 2016). The Si3 pair alone cannot generate oscillatory activity even though they were depolarized by current injection. Thus, the *Dendronotus* swim CPG is a unique example of a network oscillator in half-center configuration, where each half-center has no rhythmogenesis. Instead, the recurrent feedback synapse within each half-center module, the escaping from inhibition by sag depolarization, and subsequent post-inhibitory rebound are essential for rhythm generation by prompting the activity transfer from one side to the other.

Comparisons to the Melibe swim CPG

Another nudibranch, *Melibe leonina*, swims with rhythmic left-right body flexions like *Dendronotus*, and the details of its swim circuits have been well-elucidated (Sakurai et al. 2014; Sakurai et al. 2011; Watson et al. 2002). The swim CPG of *Melibe* consists of four pairs of interneurons, each forming a half-center configuration with reciprocal inhibition similar to *Dendronotus*, but it has more complex synaptic interaction networks than that of *Dendronotus* (Sakurai et al. 2014). In *Melibe*, multiple synaptic inputs acting in succession are essential for the transition of activity from one side of the swim CPG to the other. In the *Melibe* swim CPG, the primary half-center kernel, consisting of Si1 and Si2, generates alternating burst discharges. The burst discharge in one side of the primary half-center kernel is terminated by the inhibitory synaptic input from the secondary half-center kernel, Si3, which fires with a 25% delay from the

primary kernel. On the other hand, it is the inhibitory synaptic input of Si2 and Si4 in the first kernel that triggers and terminates the group discharge of Si3 in the second kernel and triggers the transition of activity. Meanwhile, Si1, Si2, and Si4 of the primary kernel trigger and terminate the burst discharge of Si3, and hence trigger a transition of activity in the secondary kernel. Thus, despite showing similar swimming behaviors and have some homologous neurons, the mechanisms underlying the generation of the motor pattern are substantially different between these two nudibranch species. In *Melibie*, the primary and secondary half-center kernels promote the transition of each other's activity, which contrasts with that of *Dendronotus*, in which burst discharge is terminated by a feedback mechanism within each side of a single half-center kernel.

Conclusion

The present study demonstrated how a transition of excitation from one side to the other in a simple CPG with a half-center configuration. The transfer of excitation is prompted through the combined action of feedback inhibitory synapses and active membrane properties within each half-center module: the self-termination of a burst by the recurrent inhibition, the strong inhibitory input from the other half-center, escape from hyperpolarization by the sag potential, and the rebound discharge upon release from inhibition. Thus, even in the half-center oscillator, which is said to be the simplest building block of the neural circuit, many functional factors overlap and act to produce a stable rhythm. It is of interest how two different neural circuit mechanisms diverged from their common ancestor.

Figure Legends

Figure 1.

The central pattern generator (CPG) underlying the swimming behavior of *Dendronotus iris*. **A.** Schematic drawing of the cell body locations and the axonal projections of the swim CPG neurons, Si2 (blue) and Si3 (red), in the *Dendronotus* brain, showing Cerebral, Pleural, and two Pedal (proximal and distal) ganglia based on Sakurai and Katz (2016). Si2 and Si3 have their cell bodies in the proximal pedal ganglion. They each project an axon to the contralateral pedal ganglion through one of the two pedal-pedal commissures. Only L-Si2 and R-Si3 axon projections are depicted. Each module of the half-center oscillator that constitutes the swimming circuit is composed of Si2 and Si3 with cell bodies on opposite sides. **B.** The previously published connectivity of the *Dendronotus* swim CPG. Lines terminating in triangles indicate excitatory synapses, filled circles inhibitory synapses. Resistor symbols indicate electrical connections. We named each half-center module, module α and β . Module α consists of left Si2 and right Si3, and module β consists of right Si2 and left Si3. **C.** The swim motor pattern recorded intracellularly from all four swim interneurons (**Ci**). Si2 and Si3 in the same module burst together via an excitatory synapse and an electrical connection. The two modules exhibit alternating bursts. A portion of the traces indicated by the dotted box is enlarged in **Cii**.

Figure 2.

The inhibitory synaptic potentials generated by Si2 and Si3 have different reversal potentials. **A.** Schematic diagrams of synaptic connections in the swim CPG and reversal of Si2-to-Si2 (**Ai**) and Si3-to-Si3 (**Aii**) synaptic potentials. Spikes in the presynaptic L-Si2 and L-Si3 and the resulting postsynaptic potentials in the contralateral R-Si2 (**Ai**) and L-Si3 (**Aii**) were recorded in Hi-Di saline. One electrode was inserted into L-Si2 (**Ai**) or R-Si3 (**Aii**) to record the presynaptic action potentials, while two electrodes (one for voltage recording and the other for current injection) inserted into R-Si2 (**Ai**) or L-Si3 (**Aii**), respectively. Positive or negative current was injected into the postsynaptic cell (R-Si2 or L-Si3) to change its membrane potential. The numbers on the left side of the traces indicate the membrane potential, and ten traces were overlaid for each

voltage level. Traces were triggered at the peak of presynaptic action potentials. **B.** The relationships between the amplitude of the IPSPs and the membrane potential of the postsynaptic cell are shown. The values used are derived from the data shown in **A**. The white circles indicate the Si2-evoked IPSPs in the contralateral Si2, and the gray circles are the Si3-evoked IPSPs in the contralateral Si3.

Figure 3.

A Si2 spike train causes a post-inhibitory rebound discharge in Si3 of the contralateral module. **A.** A spike train of about 10 Hz in Si2 produced hyperpolarizing responses in Si2 and Si3 of the other module, followed by post-inhibitory discharges in Si3. From top to bottom, the circuit schematic, membrane potential traces (R-Si2, L-Si3, and L-Si2), and an instantaneous spike frequency plot are shown. Recorded neurons are drawn in black in the top schematic. A current pulse of 4 nA was injected into L-Si2 to evoke a train of spikes at around 10 Hz. **B.** A current pulse of 3 nA was injected into R-Si3 to evoke a train of spikes at around 10-14 Hz, which produced only small hyperpolarization in the neurons of the other module but evoked no rebound discharge.

Figure 4.

A Si2 spike train causes a sag depolarization and a post-inhibitory rebound depolarization in postsynaptic Si3. **A.** A 10-Hz spike train in Si2 in module α produced hyperpolarization of both Si2 and Si3 in module β . After the stimulation, the membrane potential of the postsynaptic Si2 slowly recovered back to the resting potential, whereas Si3 showed a faster recovery and a rebound depolarization (asterisk), which lasted for 10 to 15 sec. The spike train was evoked by injecting repetitive current pulses (10 nA, 20 msec) at 10 Hz. **B.** A 10-Hz spike train in Si3 in module α produced hyperpolarization of both Si2 and Si3 in module β . The amplitude of hyperpolarization was smaller than those evoked by Si2 in A, and there was no rebound depolarization after the stimulus. The prefixes “c” and “i” for Si2 and Si3 in parentheses stand for “contralateral” and “ipsilateral”. **C.** Graph comparing the amplitudes of the Si2- and Si3-

evoked hyperpolarization in the neurons (*c*Si2, contralateral Si2; *i*Si3, ipsilateral Si3; *c*Si3, contralateral Si3; *i*Si2, ipsilateral Si3) in the other module. **D.** Graph comparing the amplitude of the post-inhibitory rebound depolarization.

Figure 5

Si3 showed remarkable sag and a rebound depolarization. **A.** Membrane potential responses (upper traces) of Si2 and Si3 to a hyperpolarizing current pulse (-4 nA for 4 sec, lower trace) in normal saline. **B.** Graph comparing the numbers of spikes in Si2 (black) and Si3 (gray) evoked after the hyperpolarizing current pulses (-1, -2, -3, and -4 nA). Each bar represent mean $\pm$ SD (Si2, N = 9-12; Si3, N = 7-10). The asterisk indicates significant difference (see text). **C.** Membrane potential responses (upper traces) of Si2 and Si3 to a hyperpolarizing current pulse (lower trace) in Hi-Di saline. The traces are overlaid. **D.** Graph comparing the amplitude of rebound depolarization in Si2 (black) and Si3 (gray) after the hyperpolarizing current injection (-1 to -4 nA, 4 sec). The amplitude represents the height from the resting membrane potential to the peak of the rebound depolarization. The asterisk indicates significant difference (see text). **E.** Graph showing the amplitude of sag depolarization in Si2 (black) and Si3 (gray) during the hyperpolarizing current injection (-1 to -4 nA, 4 sec). The amplitude represents the change in membrane potential from the bottom to the most depolarized potential during the stimulation. The asterisk indicates significant difference (see text).

Figure 6

Si3 showed a hyperpolarization-induced inward current. **A.** Membrane current responses (upper traces) and command voltage pulses (bottom traces). The membrane potential of Si2 or Si3 were held at -50 mV under voltage clamp and then were stepped down to more hyperpolarized potentials (from -60 to -90 mV). Each neuron was impaled with two electrodes (one for voltage recording and the other for current injection). All voltage-clamp experiments were performed in

Hi-Di saline. **B.** The amplitudes (nA, mean $\pm$ SD; Si2, N=5; Si3, N = 8) of membrane current are plotted against the command voltage values (mV).

Figure 7

A spike train in Si2 evokes a slow inhibitory synaptic potential in Si3 in the same half-center module. **A.** An Si2 spike train inhibited ongoing spiking activity of Si3. Traces show the membrane potential activity of Si2 and Si3 and the injected current traces. Tonic spiking in Si3 was evoked by injecting 1-nA current pulse (lower traces), during which an intense spiking was evoked in Si2 by injecting 2-nA current pulse (upper traces). The spike train in Si2 inhibited spiking of Si3 in the same module. The schematic on the left shows synaptic interactions between Si2 and Si3 within a half-center module. **B.** A spike train of Si2 (upper trace) produced complex membrane potential responses of Si3 (lower trace) in the same half-center module, part of which was mediated by chemical transmitter release. Trains of action potentials in R-Si2 were evoked by injecting repetitive current pulses (7-15 nA, 20 msec) at 2, 5, and 10 Hz in Hi-Di saline. The Si2 spikes produced sharp electrotonic EPSPs in L-Si3, which overrode on a slow hyperpolarizing potential (**B1**). Ca^{2+} -free saline blocked the slow hyperpolarizing potential, leaving the electrotonic EPSPs relatively unchanged (**B2**). The waveforms of B2 minus the waveforms of B1 are shown in **B3**, revealing a slow hyperpolarizing component that was largely blocked by the Ca^{2+} -free saline. **C.** Graphs showing the amplitude of electrotonic EPSPs (upper graph) and the slow hyperpolarizing potentials (slow IPSPs; lower graph) recorded in Hi-Di saline (black bars) and in Ca^{2+} -free Hi-Di saline (white bars). Ca^{2+} -free saline largely diminished the slow IPSPs but had little effect on the electrotonic EPSPs.

Figure 8

The Si2-evoked slow IPSP in the contralateral Si3 is mediated by an outward current. **A.** Example traces of the synaptic currents at different holding potentials. Spike trains were evoked in R-Si2 by injecting repetitive current pulses (10 nA, 20 msec) at 10 Hz. Seven traces of the Si2

spike trains are overlaid and shown at the top, whereas the slow IPSPs in L-Si3, which was voltage-clamped at various potential levels, are shown below. The holding potentials (from -90 mV to -30 mV) are shown on the left side of the trace. **B.** The relationship between the amplitudes of the slow IPSPs and the holding potential of the postsynaptic Si3. The IPSC became outward current with more depolarized holding potential. With more depolarized holding potentials, the amplitudes of the slower IPSCs becomes larger. **C.** Si3 did not exhibit voltage-dependent current but was passive, and the IV relationship was linear with the holding potentials used in this voltage-clamp experiment.

Figure 9

Suppression of the Si2 spike resulted in a prolonged Si3 burst. **A.** Simultaneous intracellular recording of an Si2 pair and L-Si3. A plot of instantaneous spike frequency is shown at the bottom. From the time indicated by the arrow, a hyperpolarizing current of -4 nA was applied to suppress the action potential of R-Si2. During hyperpolarization, only trains of EPSPs evoked by the L-Si3 spikes were seen in R-Si2. The bursts before and during the hyperpolarizing current injection are boxed by blue (a) and red (b) dotted lines. The spike frequency during the period is indicated by the same color in the plot below. **B.** A comparison of bursts before and during the hyperpolarizing current injection. Traces **a** and **b** show the waveforms with a faster timescale of bursts indicated by the boxes in **A**. The dotted lines indicate 0 mV. Plots of the instantaneous spike frequencies of two bursts are shown below. **C.** Graphs of burst duration (C1), burst duty cycle (C2), and the number of Si3 spikes per burst before (blue) and immediately after (red) the suppression of the Si2 spikes by hyperpolarizing current injection. All three factors increased when the Si2 spikes were suppressed.

Figure 10

Artificial enhancement or suppression of slow inhibitory synaptic action of Si2 onto Si3 within each half-center module changed the burst period of CPGs. **A.** The experimental arrangement for dynamic clamp is shown (**A1**, see Materials and Methods for details). Every time the

membrane potential (V_m) of Si2 surpassed a specified threshold (50% of spike height), an artificial synaptic current (I_{stim}) was calculated by the computer and injected into the contralateral Si3 to boost the existing Si2-to-Si3 slow synapse (red). Traces in **A2** show the change in the burst before and during the application of dynamic clamp. The red arrow indicates the time of onset of dynamic clamping. The red arrow indicates the time when the dynamic clamp was started. The horizontal dotted lines show 0 mV. **B.** Simultaneous intracellular recording from all four neurons shows the effect of dynamic clamping (red bar). The synaptic boost caused by adding artificial synaptic conductance (500 nS) with the dynamic clamping accelerated the swim motor pattern by shortening the duration of bursts. **C.** Synaptic suppression by counteracting IPSPs with a negative postsynaptic conductance to Si3 extended the burst duration and consequently increased the burst period. **D.** Plot of burst periods against the conductance of artificial synapses from Si2 to Si3 in the same half-center module. Boosting the synaptic conductance reduced the burst period, whereas counteracting it extended the burst period.

Figure 11

Sequence of activities of the CPG neurons. **A.** The activity of a circuit neuron is divided into four parts (a, b, c, d): In phase a, Si3 in module α starts firing, and Si2, which is driven by Si3, starts firing a little later. The firing frequency of Si2 and Si3 in module α starts to decline in phase b. At some point, Si3 in module β escapes from inhibition and starts firing together with Si2 (phase c). As a result, the burst of module α ends and the activity switches. The activities of the module β neurons eventually decrease (phase d). **B.** Schematic diagram of the activity sequence of the CPG neurons. In phase a, Si2 and Si3 in module α fire together through an excitatory synapse and an electrical coupling. They inhibit module β through inhibitory synapses. The firing of Si2 gives inhibitory feedback to Si3, and the activity of both decreases (phase b), which weakens the inhibitory synaptic action on module β . Then, two neurons in module β fire together, inhibiting module α (phase c). Eventually, the activity of Si2 and Si3 in module β declines, becoming ready for the transition of excitation back to module α (phase d).

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