Animal-to-animal variability of connection strength in the leech heartbeat central pattern generator

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Roffman RC, Norris BJ, Calabrese RL. Animal-to-animal variability of connection strength in the leech heartbeat central pattern generator. J Neurophysiol 107: 1681–1693, 2012. First published December 21, 2011; doi:10.1152/jn.00903.2011.—The heartbeat central pattern generator (CPG) in medicinal leeches controls blood flow within a closed circulatory by programming the constrictions of two parallel heart tubes. This circuit reliably produces a stereotyped fictive pattern of activity and has been extensively characterized. Here we determined, as quantitatively as possible, the strength of each inhibitory synapse and electrical junction within the core circuit of the heartbeat CPG. We also examined the animal-to-animal variability in strengths of these connections and, for some, determined the correlations between connections to the same postsynaptic target. The core CPG is composed of seven bilateral pairs of heart interneurons connected via both inhibitory chemical synapses and electrical junctions. Fifteen different connections within the core CPG were measured for strength using extracellular presynaptic recordings and postsynaptic voltage-clamp recordings across a minimum of seven individuals each, and the animal-to-animal variability was characterized. Connection strengths within the core network varied three to more than sevenfold among individuals (depending on the specific connection). The balance between two inputs onto various postsynaptic targets was explored by within-individual comparisons and correlation across individuals. Of the seven comparisons made within the core CPG, three showed a clear correlation of connection strengths, while the other four did not. We conclude that the leech heartbeat CPG can withstand wide variability in connection strengths and still produce stereotyped output. The network appears to preserve the relative strengths of some pairs of inputs, despite the animal-to-animal variability.

For neuronal networks to reliably process sensory information or program motor output, they must produce reliable, albeit plastic, output. Over the past decade, we have come to realize that reliable network output can result from networks in which the intrinsic membrane properties (maximal conductances) of the neurons and the strengths of the synaptic connections can show two- to fivefold animal-to-animal variability. For networks to reliably produce fictive motor patterns, they must produce reliable, albeit plastic, output. Over the past decade, we have come to realize that reliable network output can result from networks in which the intrinsic membrane properties (maximal conductances) of the neurons and the strengths of the synaptic connections can show two- to fivefold animal-to-animal variability (Goaillard et al. 2009; Gunay et al. 2008; Gunay et al. 2009; Marder and Goaillard 2006; Prinz 2010; 2007; Prinz et al. 2004; Swenson and Bean 2003; 2005).

This new understanding of neuronal networks has been paced in large part by theoretical (Prinz 2010; 2007; Prinz et al. 2004) and experimental studies (Grashow et al. 2010; Schulz et al. 2006; Schulz et al. 2007; Tobin et al. 2009) in the crustacean stomatogastric nervous system (STN). Consisting of fewer than 30 neurons, the stomatogastric ganglion, the core of the STN, comprises three distinct yet interacting networks, each of which is a central pattern generator (CPG) (Marder and Bucher 2007). The pyloric CPG has been particularly well studied; in vitro it produces a highly similar and reliable three-phase motor program (Bucher et al. 2005). Seeming to belie this reliability is a two- to fivefold animal-to-animal variation in intrinsic membrane (Schulz et al. 2006; Schulz et al. 2007) and synaptic parameters (Goaillard et al. 2009). Similarly models of the pyloric network can produce similar and reliable output with very different combinations of intrinsic membrane and synaptic parameters (Prinz 2010; 2007; Prinz et al. 2004). These studies imply that, to understand fundamentally a neuronal network, we must gather as much as possible complete data from individuals, because networks from different individuals, while functionally indistinguishable, may have different mechanistic underpinnings at the level of membrane currents and synaptic connections. The reaction in the modeling community has ranged from a continued pursuit of “ideal parameter sets” or sticking to averaged data for parameters to evolutionary algorithms for generating multiple functional models for testing or brute force parameter variation generating, in some cases, millions of functional models (Marder 2011; Marder and Taylor 2011). The situation is clearly still fluid, but it seems apparent that experimental studies that document the extent of parameter variation in the living system and potential correlations among these parameters are needed across a variety of networks.

CPGs are good choices to broaden the studies of network parameters and their variation begun in the crustacean STN, because they produce reliable fictive motor patterns in isolation, which present interesting complexities in coordination. Well-studied CPGs include those that control locomotion, breathing, and chewing (Garcia et al. 2011; Kiehn et al. 2010; Lund and Kolta 2006). Invertebrate CPGs remain among the best studied of all neuronal networks, owing to their unique experimental accessibility (Selverston 2010). We have exploited the heartbeat CPG of medicinal leeches to study successfully how animal-to-animal variation in the strength of synaptic output of the CPG onto motoneurons impacts their coordination (Norris et al. 2011; Wright and Calabrese 2011). Here we extend that type of analysis to the heartbeat CPG itself. We determined the strength of each accessible inhibitory synaptic and electrical connection in the core CPG, established mean and median responses, and tested for significant differences in connection strengths across animals. We further determined the variability in the strength of each of these connections, establishing the range of strength values in the

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population. Finally, we explored potential correlations among connection strengths impinging on single postsynaptic targets or emerging from the divergent connections of single presynaptic neurons. Achieving these goals has suggested strength differences and correlations that may be necessary for proper network function, established averaged data for parameterizing canonical models, and established parameter ranges and correlations for brute force model analysis.

The leech heartbeat CPG. The leech heartbeat system has been described in detail (Kristan et al. 2005; Norris et al. 2006; Norris et al. 2007a; b; Norris et al. 2011; Wenning et al. 2011), so we briefly summarize here. Blood flow in the medicinal leech is driven by the rhythmic constriction of a bilateral pair of heart tubes, with one heart tube constricting with a rear-to-front pattern (i.e., peristaltic), while the other heart tube constricts nearly synchronously along most of its length (i.e., synchronous) (Wenning et al. 2004a; Wenning and Meyer 2007). The heart tubes receive excitatory input from an ipsilateral member of a pair of segmental heart (HE) motoneurons, located in each midbody segmental ganglion, 3–18, of the ventral nerve cord (Maranto and Calabrese 1984a; b). The firing pattern of the heart motoneurons (i.e., the fictive motor pattern) is bilaterally asymmetric, with motoneurons on one side firing with a rear-to-front progression, while those on the other side fire nearly synchronously with appropriate side-to-side coordination of these two firing patterns (Norris et al. 2007b; Wenning et al. 2004b). The heart motoneurons are controlled and coordinated by the heartbeat CPG through rhythmic inhibitory drive (period 5–12 s).

Nine pairs of identified segmental heart (HN) interneurons (plus one pair of unidentified identity) compose the CPG. The core CPG consists of seven pairs of these interneurons located in the first seven midbody ganglia of the nerve cord and are indexed by ganglion number and body side [HN(L,1)–HN(R,7)]. The functional classification and web of inhibitory synaptic and electrical connections of the interneurons of the core CPG are shown in Fig. 1. Rhythmic activity in the network is paced by a highly interconnected timing network consisting of coordinating [HN(1) and HN(2) interneurons] and oscillator interneurons [HN(3) and HN(4) interneurons]. The firing pattern of the interneurons [and particularly of the front [HN(3) and HN(4)] and the middle [HN(6) and HN(7)] premotor interneuron] of this core CPG has been extensively characterized (Norris et al. 2006, 2011) and is also bilaterally asymmetric like that of the heart motoneurons and with appropriate side-to-side coordination. [The HN(3) and HN(4) interneurons have a double role as premotor interneurons and oscillator interneurons.] The asymmetry in firing patterns is not permanent; however, there are regular (every 200–400 s) side-to-side switches in the peristaltic and synchronous patterns in the heart interneurons that underlie the changes in both the motor pattern and the rhythmic constriction patterns of the heart tubes. These switches in coordination are modulated by the HN(5) switch interneurons, which link the timing network to the middle premotor interneurons by bilateral inhibitory connections. Only one of this pair of interneurons is rhythmically active at a time; the other is silent. Premotor interneurons and motoneurons on the side of the active switch interneuron are coordinated synchronously, while those on the on the side of the silent switch interneuron are coordinated peristaltically.

The heartbeat CPG has been modeled in detail (Hill et al. 2001; Hill et al. 2002; Jezzini et al. 2004), and a recent complete model of the core CPG (Weaver et al. 2010) identified the strength of the ipsilateral electrical connections from the oscillators interneurons and of the bilateral inhibitory connections onto the middle premotor interneurons as important parameters for proper coordination in the network. This realization prompted measurement of the average strength of these connections across animals and identified key differences in strength.

Animal-to-animal variability in the phasing of the leech heartbeat CPG is relatively small (Norris et al. 2006; Norris et al. 2011). Based on this stereotypy, it appears that the maintenance of appropriate phase relationships between network components may be functionally important. Establishing the variability in connection strengths and their correlations in the CPG will be important in understanding this maintenance.

**METHODS**

**Animals and solutions.** Leeches (Hirudo sp) (Siddall et al. 2007) were obtained from commercial suppliers (Leeches USA, Westbury, NY and Biopharm, Charleston, NC) and maintained in artificial pond water at 16°C. After the animals were anesthetized on ice, chains of ganglia consisting of the head brain to at least midbody ganglion 8 were dissected in cold saline and were pinned (ventral surface up) in 60-mm Petri dishes lined with Sylgard (Dow Corning, Midland, MI). Ganglia in which heart interneurons were to be recorded were desheathed using fine scissors or microscalpels.

The preparation was superfused continuously with normal leech saline containing the following (in mM): 115 NaCl, 4 KCl, 1.8 CaCl₂, 10 glucose, and 10 HEPES buffer, adjusted to pH 7.4 with NaOH, at 1–2 ml/min (bath volume 6–8 ml). Experiments were performed at room temperature. Heart interneurons were identified based on soma size and location in the ganglion and ultimately identified by their

![Fig. 1. Circuit diagram illustrating the synaptic connections among the heart (HN) interneurons that compose the core network of the heartbeat central pattern generator (CPG). Lines indicate cell processes, small colored/black circles indicate inhibitory synaptic connections, and diodes indicate electrical connections. One HN(5) interneuron is grayed out to signify that only one of these interneurons is active in bursts at a time, corresponding to the two coordination modes of the CPG. Cells with similar input and output connections and function are lumped (share a circle). The color scheme for HN interneurons is maintained in all circuit diagrams and to label all voltage/ current recordings from that neuron, e.g., red color denotes HN(1) and HN(2); the coordinating interneurons and all other HN interneurons have unique colors. In all figures, interneurons are indexed by body side and midbody ganglion numbers HN(L,1) to HN(R,7).](http://jn.physiology.org/doi/abs/10.1152/jn.00903.2011)
To provide uniformity in preparations, every effort was made to keep dissection time to less than 1 h and to minimize the number of ganglia desheathed. A total of 171 animals were used in this study.

Extracellular and intracellular recording techniques. We used conventional electrophysiological procedures for leech neurons described in Norris et al. (2007a; b). For extracellular recordings from presynaptic heart interneurons, we used suction electrodes filled with normal saline. Electrodes were pulled from borosilicate glass (1 mm outer diameter, 0.75 mm inner diameter, A.M. Systems) on a Flaming/Brown micropipette puller (P-97, Sutter Instruments, Novato, CA) and placed in a suction electrode holder (E series, Warner Instruments, Hamden, CT). To ensure a tight fit between the cell and electrode, the electrode tips had a final inner diameter of \( \frac{2}{0.11011} \), approximately the diameter of a heart interneuron’s soma. The electrode tip was brought in contact with the cell body, and light suction was applied using a syringe until the entire cell body was inside the electrode. Extracellular signals were monitored with a differential A.C. amplifier (model 1700, A-M Systems, Carlsborg, WA) at a gain of 1,000 with the low- and high-frequency cut-off set at 100 and 1,000 Hz, respectively. Noise was further reduced with a 60-Hz notch filter. A second amplifier (model 410, Brownlee Precision, Santa Clara, CA) amplified the signal appropriately for digitization. The HN(5) switch interneurons are very difficult to identify and record extracellularly because their somatic spikes are very small (\( \frac{5}{0.11011} \) mV). To aid our search, we always monitored an easily identified and recorded front premotor interneuron. Signal-to-noise ratios were often poor for the switch interneuron recordings, necessitating offline filtering so that the spikes could be easily discerned and detected.

For intracellular voltage and voltage-clamp recordings from postsynaptic heart interneurons, we used sharp intracellular electrodes (\( \frac{2}{0.11011} \)–30 M\( \Omega \)) filled with 2 M KAc, 20 mM KCl) and an Axoclamp-2B amplifier (Axon Instruments, Union City, CA) operating in discontinuous current-clamp or discontinuous single-electrode voltage-clamp mode with a sample rate of 2.5–2.8 kHz. The electrode potential was monitored to ensure that it settled during each sample cycle. Output bandwidth was 0.3 kHz. Voltage-clamp gain was set at a minimum of 8 nA/mV. The voltage-clamp holding potential (\( V_{\text{Hold}} \)) for spike-triggered averaging of spontaneous inhibitory postsynaptic current (IPSCs) in interneurons was \(-45 \) mV and for all continuous records shown in the figures. The voltage-clamp \( V_{\text{Hold}} \) for spike-triggered averaging of spontaneous spike-mediated coupling currents (CCs) was \(-55 \) mV. The minimum input resistance threshold required before voltage-clamping any heart interneuron was set at 60 M\( \Omega \) [a Table 1. Summary data from all recordings

<table>
<thead>
<tr>
<th>Presynaptic Interneuron</th>
<th>Postsynaptic Target Currents, pA</th>
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<tbody>
<tr>
<td></td>
<td>HN(3)</td>
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<tr>
<td>HN(1)</td>
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<tr>
<td>Type</td>
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<tr>
<td>Mean</td>
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<tr>
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<td>( R_e ), ( M\Omega )</td>
<td>62–91</td>
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<tr>
<td>HN(2)</td>
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<tr>
<td>Type</td>
<td>IPSC</td>
</tr>
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<td>Median</td>
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<td>IPSC</td>
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<td>( R_e ), ( M\Omega )</td>
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<tr>
<td>HN(5)</td>
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<tr>
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<tr>
<td>( R_e ), ( M\Omega )</td>
<td>67–87</td>
</tr>
</tbody>
</table>

HN, segmental heart interneurons; SD, standard deviation; CV, coefficient of variation; N, no. of animals; \( R_e \), input resistance; IPSC, inhibitory postsynaptic current; CC, coupling current.
value previously used in this system to ensure accurate measurements (Norris et al. 2011). The range of input resistances in the recordings presented here was nearly twofold: 61–112 MΩ (Table 1). At the end of each experiment, the electrode was withdrawn from the neuron, and only data in which the electrode potential was within ±5 mV of ground were included. Thus all V_Hold values were accurate within ±5 mV.

Data were digitized (5-kHz sampling rate) using a digitizing board (Digi-Data 1200 Series Interface, Axon Instruments, Foster City, CA) and acquired using pCLAMP software (Molecular Devices, Sunnyvale, CA) on a personal computer (Gateway, Irvine, CA).

Offline data analysis. Spike detection and IPSC/spike-mediated CC averaging were performed offline using custom-made MATLAB software (Mathworks, Natick, MA); see Norris et al. (2006; 2007a; b; 2011) for more details. For each spike-triggered average, we used at least 10 spike bursts and ignored the first 5 spikes in each burst, except when recording the HN(1) or HN(2) interneurons as the presynaptic neuron, where we used all the spikes in these bursts because there were so few of them. The average strength of a synaptic connection was defined as the amplitude of the largest peak/trough of the spike-triggered average IPSC or spike-mediated CC measured from the preceding baseline current. Thus, although the IPSCs were recorded as outward currents and the CCs were measured as inward currents, they are both reported as having positive amplitudes.

In a number of cases (n = 35) where the “presynaptic” cell was not recorded and the correct postsynaptic currents were clearly identifiable, we hand-measured the “postsynaptic” currents using Clampfit 9.0 software (Molecular Devices, Sunnyvale, CA). The difference between the baseline and peak value was measured for each postsynaptic current in a burst (minus the first 5) over 10 individual bursts. The average postsynaptic current amplitude was used as the measure of synaptic strength. At each connection, input resistance was correlated with synaptic strength, and variability within the recording was measured to assess potential experimental artifacts.

When we compare the two types of connections (chemical inhibitory synapses and excitatory electrical junctions), we compare currents measured at the specified V_Hold (inhibitory chemical connections V_Hold = −45 mV and excitatory electrical junctions V_Hold = −55 mV). We infer that this comparison reflects the relative efficacy of the connection, but on an unknown absolute scale.

In all electrophysiological records, heart (HN) interneuron traces were identified by midbody ganglion number and body side using an indexing scheme HN(L,1)–HN(R,7) and using the color scheme shown in Fig. 1.
**Statistics.** Means are presented ± standard deviation (SD), and in some cases the coefficient of variation was calculated. Current measurements were subjected to single-factor ANOVA to determine significant differences between connections. $F$ statistic, degrees of freedom (df), and $P$ are reported. Where appropriate, post hoc testing was done with Tukey’s honestly significant difference test. In cases where ANOVA was not appropriate, we performed paired $t$-tests (two-tailed). For all tests, $P < 0.05$ was the criterion for significant difference.

**RESULTS**

We systematically analyzed the strengths of all the inhibitory synaptic and electrical connections of core heartbeat CPG by considering each subcircuit of the network one at a time (Fig. 1). Thus we first analyzed the connections within the timing network, then between the timing network and the switch interneurons, and finally from the switch interneurons and the timing network to the middle premotor interneurons. In the subcircuits illustrated in figures that follow, the connections analyzed are black, while those that are not are grayed out.

**Timing network: connections of the coordinating interneurons onto the oscillator interneurons.** To determine the strength and variability across individuals of each inhibitory synaptic connection from a coordinating interneuron to a postsynaptic oscillator interneuron, we recorded extracellularly from one of the HN(1) or HN(2) coordinating interneurons. We then voltage-clamped one or both (serially) of the ipsilateral HN(3) or HN(4) oscillator interneurons, recording spontaneous IPSCs for a minimum of 10 burst cycles in $n = 30$ total preparations (Fig. 2).

![Fig. 3. Comparing the connections of the coordinating interneurons onto the oscillator interneurons with those of the oscillator interneurons onto their half-center partners (contralateral homologs). A1: the presynaptic ipsilateral HN(L,2) coordinating interneuron and contralateral HN(R,3) oscillator interneuron were recorded extracellularly, and the postsynaptic HN(L,3) oscillator interneuron was voltage clamped ($V_{Hold} = -45$ mV). A2: the presynaptic ipsilateral HN(L,2) coordinating interneuron and contralateral HN(R,4) oscillator interneuron were recorded extracellularly, and the postsynaptic HN(L,4) oscillator interneuron was voltage clamped ($V_{Hold} = -45$ mV). B1: spike-triggered averages from the HN(2) to HN(3) and HN(3) to HN(3) synapse within a single individual are overlaid to emphasize the difference in strength. This difference in strength is also seen in voltage recordings (not shown). B2: spike-triggered averages for the HN(2) to HN(4) and HN(4) to HN(4) inhibitory synaptic connections within a single individual are overlaid to illustrate the difference in strength between them. This difference in strength is also seen in voltage recordings (not shown). Delays are negative for IPSCs evoked by coordinating interneurons because their spike initiation sites are located in segmental ganglion 4, whereas their recording sites are in their home ganglion (Masino and Calabrese 2002b). C: corresponding strength measurements (IPSC amplitude in pA) from the ipsilateral coordinating interneuron and the contralateral oscillator interneuron for the HN(3) oscillator interneuron ($C_1$) and for the HN(4) oscillator interneuron ($C_2$) in individual animals are linked with a single line to illustrate that the strength of these connections covaries in individuals and are significantly correlated.
Figure 2A illustrates typical voltage-clamp recordings of an HN(3) oscillator interneuron (Fig. 2A1) and an HN(4) oscillator interneuron (Fig. 2A2) with simultaneous extracellular recordings of the ipsilateral HN(2) or HN(1) coordinating heart interneuron, respectively. Figure 2B shows the complete data set from such recordings as spike-triggered averages of IPSCs. Figure 2B and Table 1 illustrate the approximately fivefold variability in the strengths of these synapses across individuals. Synaptic strength was not correlated with input resistance at any of these synapses (P > 0.05). On average, the connection from the HN(2) coordinating interneuron to the HN(3) oscillator interneuron was stronger than the connection from the HN(2) coordinating interneuron to the HN(4) oscillator interneuron (P < 0.05, unpaired t-test).

To explore the balance of inhibitory connections onto a single oscillator interneuron, we made simultaneous recordings from a single postsynaptic target, either an HN(3) or an HN(4) oscillator interneuron, and two presynaptic partners: the ipsilateral HN(2) coordinating interneuron and the contralateral HN(3) or HN(4) oscillator interneuron correspondingly (Fig. 3, A and B). In n = 5 individual animals, we compared the strength of both inhibitory inputs onto the HN(3) oscillator interneuron (Fig. 3C1). Measurements made from the same individual are connected with a solid line. The strength of these two inputs was correlated across individuals (R^2 = 0.93, P < 0.01), with the inhibition from the coordinating interneuron being slightly, but not significantly, stronger than the inhibition from the contralateral oscillator interneuron (P > 0.05, paired t-test). In n = 9 individual animals, we compared the strength of both inhibitory inputs onto the HN(4) oscillator interneuron (Fig. 3C2). Again, measurements from the same individual are connected with a solid line. The two synaptic strengths were also correlated across individuals (R^2 = 0.52, P < 0.005), with the inhibition from the contralateral oscillator interneuron being stronger than the inhibition from the HN(2) coordinating interneuron (P < 0.005, paired t-test). The balance of synaptic strength appeared to be maintained at these synapses, despite wide variability among individuals.

The timing network: interconnections of the oscillator interneurons. To determine the strength of the HN(3) and HN(4) interneurons’ inhibitory synaptic connections with their contralateral homologs, we recorded extracellularly from one of the oscillator interneurons and then voltage-clamped the postsynaptic contralateral homolog in that preparation, recording...
spontaneous IPSCs for a minimum of 10 interneuron burst cycles in a total of $n = 30$ preparations.

Figure 4A illustrates a typical voltage-clamp recording of the postsynaptic HN(3) oscillator interneuron and an extracellular recording of the presynaptic, contralateral HN(3) oscillator interneuron. Figure 4C (left) and Table 1 illustrate the variability across individuals observed in the strength of the inhibitory synapses in these two half-center oscillators. Synaptic strength was not correlated with input resistance at either of these synapses ($P > 0.05$). There was no significant difference in the strength of the synapse between the HN(3) oscillator interneurons and the synapse between the HN(4) oscillator interneurons, ($P > 0.05$, two-tailed, unpaired $t$-test).

To determine the strength of the electrical connection from the HN(3) oscillator interneuron to the HN(4) oscillator interneuron, we recorded extracellularly from the HN(3) oscillator interneuron ($n = 7$) and then voltage clamped the ipsilateral HN(4) oscillator interneuron (Fig. 4B). Figure 4C (right) and Table 1 illustrate the nearly fivefold range in coupling strength seen between individuals in this electrical connection. Synaptic strength was not correlated with input resistance at this connection ($P > 0.05$).

The HN(4) oscillator interneurons allowed us another interesting comparison of the balance of two different connections onto a single postsynaptic target across individuals. The HN(4) oscillator interneuron receives an electrical connection from the ipsilateral HN(3) oscillator interneuron, as well as an inhibitory input from the contralateral HN(4) interneuron. We compared $n = 5$ individuals and found no correlation between these two connections with the same postsynaptic target across individuals (Fig. 5). In three individuals, the synaptic strengths were nearly equivalent, with the electrical connection slightly stronger than the inhibitory synapse. In one individual, the electrical connection was nearly eight times stronger than the inhibitory connection, and in the final individual the inhibitory connection was nearly twice as strong as the electrical connection.

The switch interneurons: connections of the oscillator interneurons onto the switch interneurons. To assess the strength and variability of synaptic connections in this part of the circuit, we recorded extracellularly from the presynaptic HN(3) and HN(4) oscillator interneurons either separately [HN(3) $n = 6$, HN(4) $n = 7$], or together ($n = 2$) and voltage clamped the postsynaptic HN(5) switch interneuron, recording the IPSCs elicited by spikes in the oscillator interneuron (Fig. 6A). Figure 6B and Table 1 illustrate the nearly sixfold range in variability in the strength of the HN(3) and HN(4) to HN(5) synapses. Input resistance was not correlated with synaptic strength at either of these synapses ($P > 0.05$). No difference was seen in the synaptic strength of the HN(3) and HN(4) oscillator interneuron inputs onto the HN(5) switch interneurons when the switch interneuron was in the active vs. the inactive state ($P > 0.05$ $n = 12$ paired $t$-test), nor was there any significant difference in synaptic strength between the HN(3) to HN(5) and HN(4) to HN(5) synapses ($P > 0.05$, $n = 6$, paired $t$-test).

Middle premotor interneurons: connections of the switch interneurons and oscillator interneurons onto the middle premotor interneurons. In a previous study (Weaver et al. 2010), our laboratory quantified the strength of the connections of the HN(5) switch interneurons and the HN(3) and HN(4) oscillator interneurons onto the middle premotor interneurons and reported average data across animals. We found that, for both the HN(6) ($n = 11$) and the HN(7) ($n = 14$) middle premotor interneurons, their inhibitory input from the contralateral HN(5) switch interneuron was significantly smaller than that from the ipsilateral HN(5) switch interneuron, and that this was the case in every individual examined ($n = 25$ animals). We also found that there were no significant differences in the strengths of any of the electrical connections from the HN(3) and HN(4) oscillator interneurons onto the ipsilateral middle premotor interneurons ($n = 28$ animals). We have expanded this dataset by adding data from $n = 4$ animals for a total of $n = 14$ HN(6) and $n = 17$ HN(7) voltage-clamp recordings, reanalyzed the data, and, most importantly, explored the animal-to-animal variability.

To determine the strength of each inhibitory synaptic connection from a switch interneuron to a middle premotor interneuron (Fig. 7), we recorded extracellularly from one ($n = 25$) or both ($n = 4$) switch interneurons. When we recorded only one HN(5) switch interneuron, in 20 cases we were able to infer the synaptic strength of its contralateral homolog in the same middle premotor interneuron, during the recorded switch interneuron’s silent state, by manually measuring and averaging the spontaneous rhythmic IPSCs phase-locked with the activity of a simultaneously monitored oscillator interneuron’s CCs. When both HN(5) switch interneurons were recorded, direct comparisons of spike-triggered averaged IPSCs were made.

Figure 7B and Table 1 illustrate the wide variability seen in strength of the inhibitory synaptic connection from the switch interneurons onto the middle premotor interneurons [both ipsilateral (i) and contralateral (c)]. Synaptic strength was not correlated with input resistance at any of these synapses ($P > 0.05$).

As in our previous work, we found that all of these connections were significantly different in strength across animals.
single-factor ANOVA: $F = 6.50, \text{df} = 3.37, P < 0.001)$. All post hoc pairwise comparisons were significant to $P < 0.01$ with Tukey’s honestly significant difference. In particular, the strength of the inhibitory inputs onto a given middle premotor interneuron from the ipsilateral switch interneuron was stronger than from the contralateral switch interneuron. Because of this across-animal difference in connection strength, we explored whether all middle premotor interneurons always received stronger connections from the ipsilateral vs. the contralateral HN(5) interneuron. In 1/7 HN(6) middle premotor interneurons, the size of the inputs was comparable, and in 1/9 HN(7) middle premotor interneurons, the contralateral input was clearly larger; in all other cases the ipsilateral input was clearly larger.

To determine the strength of each electrical connection from an oscillator interneuron to a middle premotor interneuron (Fig. 8), we recorded extracellularly from one ($n = 23$) or both ($n = 12$) oscillator interneurons. We then voltage clamped the ipsilateral middle premotor interneurons, recording spike-mediated CCs for a minimum of 10 interneuron burst cycles. To assess the impact of switches in coordination mode on the spike-mediated CCs, continuous voltage-clamp measurements were made across a minimum of two switches, and synchronous and peristaltic coordination modes were compared. No significant difference in connection strength between coordination modes was found ($P = 0.05 n = 16$ paired, 2-tailed $t$-test).

In a single preparation, where both an HN(3) and an ipsilateral HN(4) oscillator interneuron and both the ipsilateral HN(6) and HN(7) interneurons were recorded, the strengths of the four electrical connections varied within a twofold range (Fig. 8B). These electrical connections varied in strength over a three- to fivefold range among individuals, however (Fig. 8C; Table 1). Synaptic strength was not correlated with input resistance at any of these synapses ($P > 0.05$). No significant difference was observed in the strength of these connections across animals ($P > 0.05$, two-way ANOVA).

As before, a comparison was made between multiple “pre-synaptic” partners onto the same “postsynaptic” target within individuals. In this case (Fig. 9), we compared the input of both the ipsilateral HN(3) and the ipsilateral HN(4) oscillator interneuron onto either an HN(6) ($n = 6$) or an HN(7) ($n = 6$) middle premotor interneuron. We observed that, although there is a trend for the connection from the HN(4) oscillator interneuron to be stronger than that from the HN(3) oscillator interneuron, there was no strict relationship, and no correlation

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**Fig. 6.** Connections of the oscillator interneurons onto the ipsilateral switch interneuron. A: both the presynaptic HN(L,3) and HN(L,4) oscillator interneurons were recorded extracellularly, and the HN(L,5) switch interneuron was voltage clamped ($V_{\text{hold}} = -45\ \text{mV}$). B1: strength and animal-to-animal variability of the inhibitory synaptic connection from the half-center oscillator interneurons onto the HN(5) switch interneuron (IPSC amplitude) are shown (in pA). Data are plotted as individual points (circles). B2: representative spike-triggered averages were chosen to illustrate the range of strengths observed (ultimate or penultimate points in the range chosen). For each individual spike-triggered average trace, the size (in pA; different scale from graph points) and delay (in ms) from the triggering spike (arrow at 0 ms) are given.
between the strengths of the two inputs in a single ‘postsynaptic’ target were detected ($P > 0.05$).

The balance between inhibitory and electrical input was examined in preparations where the ‘presynaptic’ input of both an oscillator interneuron and the ipsilateral switch interneuron were simultaneously recorded by voltage-clamping a middle premotor interneuron (Fig. 10). For the HN(3) to HN(6) connection and the HN(5) to HN(6i) connection ($n = 6$), there was no correlation between the CCs from the HN(3) oscillator interneuron and the IPSC from the ipsilateral HN(5) switch interneuron ($P > 0.05$). For the HN(3) to HN(7) connection and the HN(5) to HN(7i) connection ($n = 5$), there was a significant correlation, and in every case the IPSCs from the HN(5) switch interneuron were greater in amplitude than the electrical CC from the HN(3) oscillator interneuron ($R^2 = 0.58, P < 0.05$). These observations indicate that the balance of these two inputs maybe important for network function.

**DISCUSSION**

*Is all the variability in connections strength real?* Previous work on the pyloric network of the crustacean STN (Marder and Goaillard 2006; Schulz et al. 2006; Schulz et al. 2007) and on the inhibitory synaptic inputs to leech heart motoneurons (Norris et al. 2007a; Norris et al. 2011) has demonstrated a two- to fivefold animal-to-animal variability in intrinsic properties and/or in synaptic strengths, yet with functional circuit output. In this study, we uncovered an apparently much wider range of variability in synaptic strengths, with some connections in the core leech heartbeat CPG varying greater than sevenfold in strength across individuals. How much of this sizable variability is real, and how much is experimental artifact?

We took precautions to minimize experimental artifact, and some post hoc analysis was performed to detect systematic errors. For the voltage-clamp recordings, we used only those heart interneurons with an input resistance of at least 60 M$\Omega$. For each measurement, we assessed the strength over the duration of the recording used in the analysis and found no noticeable changes. In analyzing our data, we assessed the correlation across animals between connection strength and input resistance for each connection and found no correlation between the two ($P > 0.05$). These safeguards give us some confidence that the animal-to-animal variability observed is not due to experimental artifact. Moreover, the lack of correlations between connection strengths in single postsynaptic targets observed in some cases (Figs. 9 and 10) also argues that animal-to-animal variability is not experimental artifact. Still, our measurements of small currents, especially small CCs (where larger holding currents were required, and break away spiking more frequent) may have suffered from signal-to-noise problems in voltage clamp, and we must view the small end of our measurements of small currents, especially small CCs. In analyzing our data, we assessed the variability of the inhibitory synaptic connections across individuals. How much of this variability is real, and how much is experimental artifact?
that the connections assessed were functional, and thus the
variability reported can be considered as representative of the
variability that exists within the natural population. Both ge-
etric and experiential factors may have contributed to this
observed variation.

Timing network: coordinating interneuron and oscillator inter-
etron interconnections. The strength of the inhibitory con-
nnections from the HN(1) and HN(2) coordinating interneurons
onto the ipsilateral HN(3) and HN(4) oscillator interneurons
varied approximately fivefold among individuals (Fig. 2B).
When comparing the balance of inhibition onto an HN(3)
oscillator interneuron from the ipsilateral HN(2) coordinating
interneuron and the contralateral HN(3) oscillator interneuron,
we found a positive correlation between these connection
strengths (Fig. 3C1). On average, the strengths of the two
inputs were similar. When comparing the inhibition from the
ipsilateral HN(2) coordinating interneuron and the contralateral
HN(4) oscillator interneuron onto the HN(4) oscillator inter-
etron, we found a corresponding correlation (Fig. 3C2),
but the inhibitory input from the contralateral HN(4) oscillator
interneuron was significantly stronger than the inhibitory input
from the ipsilateral HN(2) coordinating interneuron.

The weaker input from the HN(2) coordinating interneuron
onto the HN(4) oscillator interneurons compared with the
inhibition from the HN(4) half-center partner (contralateral
homolog) could have interesting functional implications for the
network. The HN(4) half-center oscillator pair typically leads
the HN(3) half-center oscillator pair in phase in the intact core
CPG (Masino and Calabrese 2002a; b), and this strength
difference could help to explain how this phase difference is
generated. Modeling studies have shown that weak coordinat-
ing interneuron inhibition promotes a shorter period in that

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**Fig. 8.** Electrical connections of the oscillator inter-
etrons onto the ipsilateral middle premotor inter-
etrons. Both the presynaptic HN(R,3) and
HN(R,4) oscillator interneurons were recorded ex-
tracellularly, and the ipsilateral HN(R,6) (A1) or
HN(R,7) (A2) middle premotor interneuron was volt-
age clamped ($V_{	ext{hold}} = -55$ mV). B: sample spike-
triggered averages of all four of the electrical con-
nnections from a single individual are shown.

C1: strength (CC amplitude in pA) and animal-to-
animal variability of the electrical connections from
the oscillator interneurons onto the ipsilateral middle
premotor interneurons are shown. Data are plotted as
individual points (circles). C2: representative spike-
triggered averages were chosen to illustrate the range
of strengths observed (ultimate or penultimate points
in the range chosen).
onto an HN(4) oscillator interneuron is the critical factor in establishing the C1 to C1 connection and the inhibitory connection from the ipsilateral HN(5) to the HN(4) interneuron. Thus an HN(4) oscillator interneuron is a nexus point of four different connections.

Each of two HN(3) and HN(4) half-center oscillators have a reciprocal inhibitory connections and are linked to each other through inhibitory connections from the ipsilateral HN(1) and HN(2) coordinating interneurons and via an electrical connection (varies >10-fold in strength among individuals) from the ipsilateral HN(3) to the HN(4) interneuron. Thus an HN(4) oscillator interneuron is a nexus point of four different connections. We found no correlation between the strength of the electrical connection and the inhibitory connection from the half-center partner (contralateral homolog) in the HN(4) oscillator interneuron (Fig. 4C). If the total complement of input onto an HN(4) oscillator interneuron is the critical factor in balancing the strengths of its inputs, then this lack of correlation may be due to the incompleteness of our comparison, c.f. Norris et al. (2011).

Connections onto the HN(5) switch interneurons and onto the HN(6) and HN(7) middle premotor interneurons. The inhibitory synaptic inputs from the oscillator interneurons shape the bursting behavior of the switch interneurons and vary more than eightfold between individuals (Fig. 6B). In a small subset (n = 3) of individuals illustrated in Fig. 6B, the HN(3) and the HN(4) oscillator interneurons were recorded, while simultaneously voltage clamping the HN(5) switch interneuron, and in each case the inhibition from the HN(4) interneuron was slightly stronger than the inhibition from the HN(3) interneuron.

The middle premotor interneurons receive inhibition from both the ipsilateral HN(5) switch interneuron and the contralateral HN(5) switch interneuron, as well as an electrical connection from the ipsilateral HN(3) and HN(4) oscillator interneurons. Our modeling studies suggest that the balance between these opposing inputs in each of the middle premotor interneurons is critical for the proper phase relations for the peristaltic and synchronous coordination modes of the core CPG (Weaver et al. 2010). Our expanded analysis of these inhibitory connections here shows wide animal-to-animal variability, but still the ipsilateral HN(5) input to both the HN(6) and HN(7) middle premotor interneurons is significantly stronger (Fig. 7). In 2/16 cases, only 1 case an HN(6) and 1 case an HN(7) middle premotor interneuron] the contralateral input was comparable or larger; in all other cases, the ipsilateral input was clearly larger. It will be important in the future to hunt for other exceptional cases like these and determine the effect on middle premotor interneuron phasing.

Timing network: oscillator interneuron reciprocal connections. The reciprocal inhibitory connections between members of each half-center oscillator are critical in establishing the bursting behavior of the rest of the circuit and setting up side-to-side coordination in the core heartbeat CPG (Hill et al. 2001; Kristan et al. 2005). Still, each half-center oscillator is able to accommodate substantial variability in synaptic strength [~5-fold in HN(3) to HN(3) connection and ~10 fold in the HN(4) to HN(4) connection] (Fig. 4C). Across animals, we observed no significant difference in the strength of the HN(3)-to-HN(3) connection and the strength of the HN(4)-to-HN(4) connection (P > 0.05, unpaired t-test). Each of two HN(3) and HN(4) half-center oscillators have reciprocal inhibitory connections and are linked to each other through inhibitory connections from the ipsilateral HN(1) and HN(2) coordinating interneurons and via an electrical connection (varies >10-fold in strength among individuals) from the ipsilateral HN(3) to the HN(4) interneuron. Thus an HN(4) oscillator interneuron is a nexus point of four different connections. We found no correlation between the strength of the electrical connection and the inhibitory connection from the half-center partner (contralateral homolog) in the HN(4) oscillator interneuron (Fig. 4C). If the total complement of input onto an HN(4) oscillator interneuron is the critical factor in balancing the strengths of its inputs, then this lack of correlation may be due to the incompleteness of our comparison, c.f. Norris et al. (2011).
On average, the electrical connections onto the middle premotor interneurons in ganglia 6 and 7 were similar in strength (Fig. 8C). There were no significant differences in HN(6) and HN(7) middle premotor interneurons, nor between contralateral homologs, in the strength of these electrical connections across individuals ($P > 0.05$, t-test). Moreover, in individual “postsynaptic” HN(6) or HN(7) targets, there was no correlation between these two electrical inputs (Fig. 9).

We made a few comparisons of the electrical/inhibitory balance in the middle premotor interneurons to Fig. 10. When comparing the inhibitory input from the HN(5) switch interneuron onto the ipsilateral HN(6) middle premotor interneuron with the electrical connection from the HN(3) oscillator interneuron onto the HN(6) middle premotor interneuron, we observed no correlation. When comparing those same inputs onto the HN(7) middle premotor interneuron, however, there was a significant positive correlation, suggesting that balance of these two inputs may be important for network function.

Why aren’t connection strengths more tightly regulated across individuals? Modeling work in the pyloric network of the crustacean STN has suggested that, rather than connection strengths, it may be their ratios that are important in network function (Hudson and Prinz 2010). The importance of synaptic ratios is supported by the data presented here, where there is substantial variability in connection strengths among individuals, but there do appear to be some points (that we can measure and accurately compare) where the balance (ratio) of the two synapses appears to be maintained as indicated by significant correlations [for example, the HN(2) to HN(4) connection compared with the HN(4) to HN(6) connection or the HN(5) to HN(7) connection compared with the HN(3) to HN(7) connection (Figs. 3 and 10)].

In contrast to the idea of maintenance of synaptic-strength ratios, modeling work by Taylor et al. (2009) indicates that strong correlations between intrinsic membrane parameters may not be necessary for neurons to give rise to functionally similar outputs. Despite the fact that we observed significant strength correlations between several connection pairs, it is possible that these correlations are not necessary for functional network output, but rather a consequence of activity-dependent regulation. The compensatory mechanisms that presumably played a role in producing the variability in connection strengths may have obligatorily covaried certain synaptic strengths. This mechanistic linkage could create the appearance of important “balances”, when, in fact, these balances are not necessary for the functional network output.

How exact do connection strengths really need to be? LeMasson et al. (1993) and others [c.f., Edelman and Gally (2001)] have argued that it is not necessary to specify the exact number of ion channels or receptors that each neuron should express, either during development or over the lifetime of the neuron or animal. Rather, ongoing activity-dependent rules of various kinds can be used to modify receptor and channel numbers and distributions to maintain target circuit performance, despite ongoing channel and receptor turnover. These ideas have found strong experimental support and have led to the idea of homeostatic synaptic scaling, a form of synaptic plasticity that adjusts the strength of all of a neuron’s synapses up or down to stabilize firing (Turrigiano 2008). Although originally formulated for excitatory synapses, synaptic scaling may also apply to inhibitory inputs (Wenner 2011). This idea supports both our findings here that a specific set of connection strengths is not the important factor in a central pattern generating network, and that some key synapses on the same neuron may be regulated in a compensatory way.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


