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Running title: Consistent Dynamics in the Pyloric Network

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Keywords: pyloric network, lobster, central pattern generator, burst, A-current, synaptic connectivity

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Consistent Dynamics Suggests Tight Regulation of Biophysical Parameters in a Small Network of Bursting Neurons

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Abstract

The neuronal firing patterns in the pyloric network of crustaceans are remarkably consistent among animals. While this characteristic of the pyloric network is well-known, the biophysical mechanisms underlying the regulation of the systems output are receiving renewed attention. Computer simulations of the pyloric network recently demonstrated that consistent motor output can be achieved from neurons with disparate biophysical parameters among animals. Here we address this hypothesis by pharmacologically manipulating the pyloric network and analyzing the emerging voltage oscillations and firing patterns. Our results show that the pyloric network of the lobster stomatogastric ganglion maintains consistent and regular firing patterns even when entire populations of specific voltage-gated channels and synaptic receptors are blocked. The variations of temporal parameters used to characterize the burst patterns of the neurons as well as their intraburst spike dynamics do not display statistically significant increase after blocking the transient K-currents (with 4-aminopyridine), the glutamatergic inhibitory synapses (with picrotoxin) or the cholinergic synapses (with atropine) in pyloric networks from different animals. These data suggest that in this very compact circuit the biophysical parameters are cell-specific and tightly regulated.

Keywords: pyloric network, lobster, central pattern generator, burst, A-current, synaptic connectivity

Introduction

Generating functional spatiotemporal motor patterns does not require large numbers of neurons and complex synaptic connectivity. Invertebrate central pattern generators (CPGs) are among the best examples of compact neural circuits in which a small number of neurons produce temporally

organized and fine-tuned motor rhythms. Besides the high efficiency of such circuits, the consistency and regularity of their activities is also remarkable. As one of the most extensively studied CPGs, the pyloric circuit of the crustacean stomatogastric nervous system produces a characteristic and robust burst pattern with well-regulated phase relationships between the bursts of the neurons (Miller, 1987; Selverston, 2005). A detailed analysis of the animal-to-animal variability has recently brought new attention to the problem (Bucher et al., 2005). Not only are the overall activity patterns very similar among different animals, but also the voltage waveforms of the individual neurons. The high degree of consistency between activity patterns is fundamental to the identifiability of the component neurons in such neural systems, a property, that in turn, has been a cornerstone of the success in establishing the synaptic connectivity of invertebrate microcircuits.

If the firing patterns produced by small neuronal circuits are quantitatively similar among different animals, does it automatically imply that the biophysical properties of the component neurons and their synapses are consistent? Do similar voltage waveforms and burst phase-relationships assume they are produced by similar populations and densities of voltage-gated channels and receptors? The genes and developmental factors could produce a functional nervous system in two different ways. Individual neurons and synapses would have consistent properties because either they are identical biophysically, i.e. they contain similar distributions of specific ion channels or they have combinations of different biophysical parameters which nevertheless are balanced to produce the equivalent function. This second possibility has been recently suggested by authors who performed an extensive set of computer simulations of a reduced pyloric network (Prinz et al., 2004). These simulations showed that similar network outputs can be achieved by neuronal circuits with widely different biophysical parameters in their components. In this respect, the properties of individual neurons and synapses are not necessarily fine-tuned, but instead can vary over a wide range among different animals. The most direct experimental verification of this hypothesis would be to measure the individual currents for each identified neuron directly in a large number of animals. Alternatively the expression levels of each channel protein could be measured using molecular biological techniques although this does not reveal functionality. Both of these approaches have been used previously in the crustacean stomatogastric system. A survey of channel types in a single neuron (the IC) of the pyloric subsystem indicated that there was a two-fold to five-fold variability in the density of three separate K^+ currents (Golowasch et al., 1999). Similar interanimal variability in the abundance of three different potassium mRNAs was found in isolated LP neurons (Schulz et al., 2006).

In this paper we perform a simple experimental test of the above idea that single channel density is variable. We also examine synaptic variability between identified neurons in the pyloric network. We block entire populations of specific voltage-gated channels and synaptic receptors in the pyloric circuit and then analyze the emerging voltage waveforms and firing patterns. The logic behind these

experiments is that if a particular voltage-gated current such as I_A has a *different* maximal conductance for an identifiable cell such as the pyloric dilator (PD) in each animal, then the cell must adjust the parameter values of all other conductances so as to balance and compensate for each different I_A value. This is obviously necessary if the neuron is to perform consistently both in its role as a component of a network and in its response to neuromodulators. Blocking I_A in PD neurons from different animals therefore would change the voltage output of each PD because the remaining conductances would not have the balance necessary to produce the typical voltage waveform of the cell (Fig.1). If the hypothesis were correct each neuron would be expected to behave differently. Similarly, if one single class of neurotransmitter were blocked, the correct balance between the remaining synapses would be disrupted. Large animal-to-animal variations in the motor output as well as in the voltage waveforms of single neurons therefore would be expected even if the compensatory mechanisms included network-level balancing. To examine this question, we have pharmacologically isolated PD neurons and selectively blocked their I_A channels with 4-aminopyridine. After blocking, an analysis of the resulting waveforms indicates a consistent change in PD neurons from different animals. Similarly, blocking either glutamatergic or cholinergic synapses also shows consistent changes in the pattern between animals.

Materials and Methods

Animals. Panulirus interruptus were obtained from Don Tomlinson Commercial Fishing (San Diego, CA). The animals were kept in aerated and circulated seawater at 15-16 °C. Prior to dissection the animals were anesthetized by packing them in ice for 40 min.

Solutions. The standard *Panulirus* physiological saline we used was composed of (in mM) 483 NaCl, 12.7 KCl, 13.7 CaCl2, 10 MgSO4, 4 NaSO4, 5 HEPES, and 5 TES; pH was set to 7.40. For the biophysical manipulation of cellular properties we blocked transient potassium currents with 4-aminopyridine (4-AP, 4 mM). A reduced configuration of the pyloric CPG was achieved by applying picrotoxin (PTX, 8 µM) in the bath. Here, fast glutamatergic inhibitory interconnections were blocked (Bidaut, 1980) while the slow cholinergic synapses were left operational. In another set of experiments we applied atropine (2 mM) to block cholinergic inhibitory connections (Marder and Eisen, 1984) and to obtain another synaptic configuration of the circuit. Near complete pharmacological isolation of the LP and PY neurons were achieved by combined application of PTX and atropine.

Preparation. The stomatogastric nervous system containing the stomatogastric ganglion (STG) and the anterior commissural and esophageal ganglia was separated from the stomach (Mulloney and Selverston, 1974) and pinned in a silicone elastomer-lined Petri dish. Nerves interconnecting the anterior centers as well as the output motor nerves of the STG were left intact. The connective sheath

of the stomatogastric ganglion was removed using sharp forceps to facilitate access to the somata of the neurons. The STG was enclosed in a small well made of petroleum jelly that served as a separate perfusion chamber of ~ 2 ml volume. 4-AP, PTX and atropine were applied to the STG while the anterior ganglia were bathed in normal physiological saline.

Electrophysiology. Intracellular voltage traces were measured using Neuroprobe 1600 amplifiers (A-M Systems Inc., Carlsborg, WA) in bridge mode. Microelectrodes were filled with 3 M K-acetate plus 0.1 M KCl solution with a resistance of 12-15 MΩ. Cell identification was achieved by visual inspection of the voltage waveforms and by comparing intracellular membrane potential traces with simultaneous extracellular recordings from the output nerves. Extracellular signals were measured using an A-M 1700 differential AC amplifier (A-M Systems).

Data acquisition. Voltage traces of the pyloric neurons were acquired and digitized at 20 kHz by a computer equipped with a PCI-MIO-16E4 data acquisition board (National Instruments, Austin, TX) and running the DASYLab 6.0 program (Datalog GmbH, Germany). Acquisition protocols (worksheets) were written by the authors. Action potential (spike) occurrences were detected in real-time by calculating the first time-derivative of the intracellular membrane potential and observing the local maxima of the derivative time series. The arrival times of spikes of each recorded neuron were saved sequentially into separate ASCII files.

Data analysis. Detailed quantitative analysis was performed using the spike trains $\{t_i\} = \{t_1, t_2, t_3,\ldots t_N\}$, where $t_1 < t_2 < t_3 \ldots$ are successive spike arrival times available for each neuron recorded. Timing of the spikes relative to the preceding one was characterized by the interspike interval (ISI): $ISI_i = t_{i+1} - t_i$. ISI sequential graphs were constructed by plotting interspike intervals ISI_i as a function of the elapsed time (t_i). Relations between successive interspike intervals were examined by ISI return maps also called joint interspike interval plots (Szücs et al., 2003). Here, ISI_{i+1} was scatter-plotted against ISI_i for each $i <= N - 2$. To characterize the local density of the return maps we calculated joint-interval probability density distributions by convolving the points in the return maps with a two-variable Gaussian kernel (half-width: 0.75 ms, resolution: 0.1 ms) (Sanderson and Kobler, 1976; Szücs et al., 2003). The probability density distributions are plotted in grayscale. (The probability density distributions appear in a color-coded form in the online version. Gradually warmer colors indicate a higher local density of points.) Zero-density areas are painted as a light-gray background for better visualization and separation of the non-empty areas. Spike density functions (SDFs) were calculated by convolving the spike trains with a Gaussian kernel (45 ms half-width, 7.5 ms sampling resolution). The SDFs contained clearly separated peaks at the positions of the bursts and zero values during the interburst periods. Intraburst spike density envelopes were obtained by overlapping and averaging a minimum of 100 successive peaks (bursts) in selected stationary sections of the SDFs. The mean and standard deviations of the overlapping SDF sections were calculated. The

regularity and periodicity of the burst oscillations was characterized by Fourier amplitude spectra. The Fourier amplitude spectra were calculated from 100 s sections of the spike density time series before and during the application of drugs. Fourier amplitudes were calculated at 1.5 mHz resolution. All spike time analysis was performed with our own software (http://inls.ucsd.edu/~attila/OS3.html) .

Results

The effects of pharmacological manipulation of the intrinsic cellular properties and the synaptic connectivity were analyzed on different time scales. We acquired long time series (1000 s and more) of intracellular voltage waveforms and the spike times of the neurons. We calculated parameters of the burst timing/phasing as well as the spike timing within the bursts. The pyloric dilator (PD, N=12/21/11 in 4-AP/PTX/atropine experiments) and the lateral pyloric (LP, N=6/13/8) neurons were the ones whose responses we studied in most detail.

Effect of 4-AP on the overall burst pattern

4-aminopyridine is known to be an effective and specific blocker of the I_A current in stomatogastric neurons (Graubard and Hartline, 1991). When applied at a concentration of 4 mM, 4-AP clearly sped up the pyloric oscillation (Fig. 2). The burst frequency was increased by 52.6 ± 10.8% (mean ± S.D., N=12). This is in agreement with earlier results (Tierney and Harris-Warrick, 1992) and with the provisional expectation that removal of a transient hyperpolarizing current should cause a more rapid depolarization of the pyloric neurons, hence, more vigorous bursting. Concurrent with the increase in burst frequency, the membrane potential waveforms of the neurons appeared to become slightly irregular and less periodic. The PY neurons displayed the greatest variations in their bursts, producing a variable number of spikes in the successive cycles (Fig. 2B, PY trace). Does this indicate the overall disruption of the pyloric rhythm? The instantaneous burst frequency as a function of the elapsed time is displayed on Fig. 3B. As this plot clearly shows, 4-AP not only increased the mean burst frequency but it also induced great variations in the successive points. Further analysis showed, however, that the increased variations were not caused by the overall disruption of the pyloric activity. 4-AP affects not only the neurons of the pyloric network but all those located in the stomatogastric ganglion, including the gastric mill CPG. Thus, 4-AP also enhances the gastric activity, which, in turn, affects the activity of the pyloric neurons, too. There are multiple synaptic interactions between the two CPGs (Mulloney, 1977) and the boosting of the gastric mill activity induces characteristic effects in the pyloric neurons (Russell and Hartline, 1982; Clemens et al., 1998; Thuma and Hooper, 2002). The PY neurons display the most apparent gastric modulation but the LP and to a lesser degree the PD

neurons are also modulated (Thuma et al., 2003). Hence, the increased variations in the burst cycle periods are due to the augmented gastric modulation. A closer look at the burst frequency time series reveals that the variations are not irregular, rather they appear as rhythmic fluctuations (Fig. 3B, zoomed panels). This is a typical effect of the slower gastric activity on the faster pyloric rhythm (Clemens et al., 1998). Moreover, Fourier analysis of the spike density time series clearly shows that the regularity of the pyloric oscillation is well preserved under the action of 4-AP. The Fourier-spectra are dominated by discrete and sharp peaks and the broad-band baseline remains at a low amplitude. As the gastric input becomes stronger under the action of 4-AP, the peaks at the location of the gastric frequency (f_g=0.18 Hz) and the two satellites ($f_p \pm f_g$) on both sides of the main pyloric peak (f_p=1.56 Hz) increase in amplitude (Fig. 2E). These data show that elimination of the A-current in the entire pyloric circuit does not disrupt the rhythmic bursting, but instead shifts the temporal parameters of the burst pattern. We note that washout of the drug reverses the effect in 10-20 min (not shown). All these effects of 4-AP were highly reproducible among preparations.

Changes in the spike dynamics of the PD neurons

At the single cell level, 4-AP induced characteristic effects in the burst oscillations. While all neurons became more depolarized, the two PD neurons showed the most interesting changes. In *Panulirus*, the PD neurons can be easily identified by visually inspecting the shape (envelope) of their bursts. The two PDs and the electrically coupled AB neuron produce accelerating type bursts. Here, the rising (onset) phase of the bursts is less steep than their termination phase (Fig. 3C). This is a distinguishing characteristics of the pacemaker neurons in a normal bursting preparation. 4-AP dramatically changed this dynamics. With the A-currents blocked, the PD-bursts began with a very steep onset while the termination became slower. The intraburst spike frequency accurately followed the time course of the membrane voltage envelope. By convolving the arrival times of the spikes with a Gaussian kernel function we obtained a smooth estimate of the local intraburst firing frequency called the spike density function. Bursts corresponded to well-separated peaks in the SDF. Superimposing and averaging these peaks we obtained the mean intraburst spike density envelope shown in Fig. 3D and G for the normal bursting PD neuron and for the one with blocked A-currents, respectively. To describe the overall shape and symmetry of bursts of pyloric neurons we introduced a new statistical parameter. This parameter is called the burst shape factor (BSF) and it is calculated by integrating the intraburst spike density envelope before and after its maximum (t_{max}) and dividing the difference between the two areas by the total area (note that the areas closely approximate the number of spikes within the burst):

$$BSF = \frac{a_2 - a_1}{a_2 + a_1} \quad \text{where}$$

$$a_1 = \int_{t_1}^{t_{max}} SDF(t)dt \quad \text{and} \quad a_2 = \int_{t_{max}}^{t_2} SDF(t)dt$$

t_1 and t_2 mark the times where the peak in the SDF (burst envelope) starts and ends, respectively. The BSF parameter is normalized in a way so that it ranges from –1 to +1 corresponding to fully accelerating type to fully decelerating type bursters, respectively. BSF values are calculated for as many as 100 successive bursts in the time series (before and after drug application) and their mean and standard deviation are obtained. As expected from the visual inspection of the burst waveforms of the PD neuron, the sign of its burst shape factor flipped in the 4-AP experiment (Table 1).

Another method of analyzing the spike dynamics of the pyloric neurons is the joint interspike interval map or ISI return map. As we have shown earlier, the ISI return map of the normal bursting PDs is a *V*-shaped form with characteristic clustering indicating gradually decreasing ISIs as the burst develops (ISI signature) (Szücs et al., 2003). Remarkably, the typical ISI signature of the PD was transformed into a slanted comet shaped form when the A-currents were blocked (Fig. 4, see also supplementary Movie 1). Remarkably, this latter ISI return map had the same shape as that of the LP neuron in normal conditions. Therefore, the PD neurons, when their A-currents are blocked, produce bursts very similar to those of the LP neuron. As earlier voltage clamp studies showed, the PD neuron displays far stronger A-currents than the rest of the pyloric circuit, including the LP (Tierney and Harris-Warrick, 1992). It is therefore not unexpected to find the strongest 4-AP effect in the PD. On the other hand, the slow rising of the PD burst in normal conditions is now clearly explained, as being due to the burst onset delaying action of the A-current. Hence, we find clear evidence here that the intraburst spike dynamics of the PD neuron strongly depend on the PD's intrinsic biophysical properties, including the strength of the A-current.

Effects of removal of fast inhibitory connections

Another set of experiments we performed were aimed at the pharmacological manipulation of the network connectivity. These experiments also resulted in consistent and reproducible changes in the pyloric activity. Picrotoxin (PTX) at 8 μM concentration blocks a considerable portion of the circuit interconnections, the ones using glutamate as the neurotransmitter (Marder and Eisen, 1984). Hence, PTX entirely removes fast inhibitory inputs to the neurons. This treatment greatly reduces the connectivity of the pyloric network. Since the slow cholinergic connections remain functional, the circuit still receives synaptic inputs from the two PD neurons and from the single VD neuron. At the same time, the pacemaker group, including the anterior burster (AB) neuron and the two PDs, no

longer receive synaptic inputs from the rest of the network. (Except weak electrical connections from the ventricular dilator VD neuron.)

The effects of PTX at the network level were less dramatic than those of 4-AP. While the burst frequency of the motor rhythm was virtually unchanged, PTX appeared more effective in changing the phasing of bursts (Table 1). As a general characteristic of the PTX-effect, the burst oscillations of all the pyloric neurons became smoother (Fig. 5). Pre-burst IPSPs in the PD neurons (from the LP) were abolished similarly to late-burst IPSPs in the LP neuron (from the PYs). The burst envelope and intraburst spike density of the PD neuron slightly changed after removing the glutamatergic inputs (Fig. 6). The ISI signature of the PD neuron became less clustered and, at the same time, more compact (Movie 2). This effect shows some similarities to that of 4-AP. When the PD neuron (normally) receives potent IPSPs from the LP neuron, its pre-burst membrane potential becomes more hyperpolarized than without the inhibition. Hence, the A-current becomes more de-inactivated and its impact on the rising phase of the PD burst becomes stronger. Consequently, we find further evidence here that the strength and kinetics of the A-current play a key role in shaping the PD burst.

The near-synchronization of the LP and PY neurons is one of the most striking effects of PTX. The LP and PY neurons are all glutamatergic and mutually inhibiting each other in normal conditions. The late phase of the LP burst is strongly affected by the IPSPs arriving from the PY neurons. Once the mutual inhibition between the LP and the PYs are blocked, they start bursting more in-phase. It is noteworthy that the PY neurons display stronger A-currents than the LP neuron (Tierney and Harris-Warrick, 1992). Therefore, the burst onset is naturally slower for the PY neuron than for the LP neuron.

Among the various statistical parameters we calculated, we found the most significant changes in the LP-to-PD relative burst phase, which was shortened by 25 $\pm$ 13 % (N=11) under PTX. Other parameters, especially the gross burst parameters, were less affected by the treatment (Fig. 5, Table 1). At the same time, the burst shape factor of both the PD and the LP neurons was significantly different before and after the application of PTX. This is in agreement with the visual appearance of the burst waveforms. As revealed by Fourier-analysis of the spike density functions, the regularity of the pyloric oscillation is not affected by blocking the glutamatergic connections (Fig. 5D and E).

Effects of removal of slow cholinergic connections

The pacemaker group of the pyloric network can be synaptically isolated from the rest of the circuit by applying PTX. This is because the AB/PD group receives chemical inhibition only from the LP neuron, which is glutamatergic. Nonetheless, the pacemaker neurons, periodically bursting under PTX, still impose a common order on the other pyloric neurons because the two PD neurons are cholinergic

and they inhibit several neurons in the circuit. A combination of blockers of glutamatergic and cholinergic connections would therefore uncouple the pacemaker group and the rest of the network. Atropine is known to block cholinergic synapses in the system including the key PD-to-LP and PD-to-PY connections (Marder and Eisen, 1984). First we studied the effects of 2 mM atropine on the pyloric circuit to observe the effect of blocking cholinergic connections alone, then we applied PTX and atropine together to see the neurons' activity under near complete pharmacological isolation.

Atropine, similarly to PTX, did not disrupt the regularity of the pyloric oscillation. The pacemaker neurons, as well as the other pyloric cells kept bursting but with slightly different phases and spike frequencies in their bursts (Fig. 7). While atropine alone did not block glutamatergic connections, the rapid glutametergic IPSPs were still less discernible than in control conditions. The reason for this might be that the intraburst spike frequency increased in most neurons and the glutamatergic synapses became more depressed. At the same time, the overall burst frequency slightly increased, too. Synaptic depression therefore might have decreased the amplitude of the single IPSPs and resulted in smoother voltage oscillations in the neurons. The burst shape of the LP neuron changed notably. While the LP did not receive cholinergic inhibition from the PD neuron, it was still inhibited by the glutamatergic AB neuron. A small dip in the rising phase of the LP's burst envelope is seen just before the first spikes and just when the PD and AB neurons fire (Fig. 7B), a clear indication of the inhibition from the AB. The LP-to-PD relative burst phase changed significantly under the action of atropine: it increased by 48 $\pm$ 24 % (N=11). This effect is the opposite of that observed with picrotoxin, where the phase moved in the opposite direction. Moreover, Fourier analysis of the spike density data confirmed that the regularity of the pyloric rhythm did not deteriorate under the action of atropine (Fig. 7D and E).

What is the degree of variation among preparations?

Comparing the voltage waveforms and spike time sequences of the pyloric neurons from different animals we found consistent changes under the action of the 3 drugs tested in our experiments. The changes in the burst frequency, in the phase-relationships between the neurons as well as in the burst shape and interspike interval pattern of the individual neurons were very reproducible and cell-specific. We calculated 11 different statistical parameters to characterize the overall burst pattern as well as the activity of single neurons in control and after manipulation of biophysical properties and synaptic connectivity. These parameters (mean $\pm$ S.D.) and the significance of changes in their mean values and variances are shown in Table 1. We ran one-way ANOVA tests to test the significance of changes in the mean. 4-AP had a strong impact on most burst parameters we calculated (Table 1, 4-AP effects). At the same time, the duty cycle of the LP neuron as well as its burst shape factor were

not notably affected by the drug. This weak effect might be in part a consequence of the low amount of I_A in the neuron (Tierney and Harris-Warrick, 1992). At the same time, the same parameters for the PD neuron show strong changes after 4-AP application. Hence, blocking the A-currents in the preparation results in cell-specific changes in the neurons' voltage output and firing patterns. In contrast to 4-AP, PTX was less effective in changing the burst temporal parameters of the PD neuron but the LP neuron displayed strong changes in its duty cycle and burst shape factor. Clearly, the removal of glutamatergic synaptic inputs from the PY neurons, mostly arriving during the termination phase of the bursts, had a strong impact on the LP neuron's activity. Atropine affected the phasing of the bursts (LP and PD) stronger than the intraburst parameters.

As a key observation related to the animal-to-animal variability of activity patterns, the standard deviations of the statistical parameters are consistently small and very similar before and after the application of the drugs. Brown-Forsythe tests of the population variances revealed no significant differences (N.S.) before and after the manipulation of biophysical parameters and synaptic connectivity. Conclusively, neither the manipulation of intrinsic cellular properties nor that of synaptic connectivity increase the animal-to-animal variability of the pyloric motor output.

Blocking both glutametergic and cholinergic connections

In the last experiment using a combined application of the drugs, we first perfused the preparation with 8 µM PTX to block all glutamatergic connections and then we added 2 mM atropine into the perfusion chamber. We note that the effect of atropine was reversible and ~10 min of washout nicely recovered the normal activity of the pyloric neurons. The PTX effect was not reversible. This experiment was important to demonstrate the effectiveness of the two drugs in blocking their specific target synapses. Since the pacemaker group of the pyloric circuit is the main source of the regular burst rhythm, complete removal of the inhibition from both the AB and the PD neurons would be expected to result in desynchronization of the follower neurons, i.e. the LP and PY neurons. This is exactly what we observed after the application of atropine into the PTX-containing saline (Fig. 8). The LP-to-PD relative phase is one of the quantitaive measures characterizing the degree of synchronization between the pacemaker group and the LP neuron. In normal conditions or with PTX in the bath, the LP-to-PD phase is stable and shows only minor fluctuations along the time series and among animals (see Table 1). The LP-to-PD phase started fluctuating widely as the effect of atropine set in (Fig. 8C). Not only the timing of LP burst onset relative to that of the PD neuron displayed great cycle-to cycle variations, but the burst duration, too. We note, that computer algorithms detecting bursts in the firing pattern of such 'irregular' neurons face difficulties when attempting to calculate the correct onset and termination times of bursts and, therefore, the LP-to-PD phases. This is even more problematic for the PY neurons, where the normal bursting activity changed entirely into a complex

mixture of high-frequency spiking and bursting under the combined action of PTX and atropine (Fig. 8B). Following the initial observations of the firing patterns and relative phases we performed Fourier analysis of the spike density functions from the PD and the LP neurons. This method is far more sensitive in detecting synaptic actions of the pacemaker group on the follower cells (entrainment) than the calculation of relative phases. Fourier-amplitude spectra clearly showed that the LP neuron became independent from the PD neuron as atropine removed the remaining cholinergic inhibition (the AB input was already blocked by PTX). The Fourier spectrum of the LP neuron developed from a narrow peaked distribution (Fig. 8D) into a broad-band one (Fig. 8E). We note that even a weak influence (driving) from the pacemaker group through polysynaptic interactions would have produced a narrow peak in the Fourier spectrum of the LP at the exact position of the PD's burst frequency (f_p). The total loss of synchronization between the LP and the PD neurons and the disappearance of the peak at the pacemaker frequency in the LP's Fourier spectrum are strong indications of the potency of both PTX and atropine in blocking their target synaptic connections.

The synaptic inputs to the LP and PY neurons were nearly completely blocked during the PTX & atropine application. Not surprisingly, the firing patterns of such neurons showed remarkable similarities to those obtained from the LP and PY neurons under complete synaptic isolation, i.e. when presynaptic neurons were photoinactivated. As we and other groups observed earlier, synaptically isolated LP neurons display complex and irregular bursting (Bal et al., 1988; Elson et al., 1999) with signs of low-dimensional chaotic dynamics (Rabinovich et al., 1997). The gross parameters used above to characterize the activity of pyloric neurons commonly display great variations along the time series of the LP neurons' voltage waveform. This is a natural consequence of their irregular behavior and the neurons' high sensitivity to slight changes in the neurochemical environment (initial conditions). It is therefore useful to compare not only the mean values of the temporal parameters among preparations but also their variances. We analyzed LP neurons from 5 preparations displaying irregular but stationary bursting under the combined application of PTX and atropine. The mean intraburst ISI of such virtually isolated neurons was 20.2 $\pm$ 3.8 ms; their mean burst cycle period was 0.48 $\pm$ 0.07 s; the duty cycle was 0.385 $\pm$ 0.072; the intraburst spike frequency was 58.5 $\pm$ 7.4 s^{-1}. The variances (S.D.s) of the same parameters were 12.5 $\pm$ 1.3 ms; 0.12 $\pm$ 0.08 s; 0.120 $\pm$ 0.057; and 12.1 $\pm$ 3.2 s^{-1}. These data show that even though the LP neurons from different preparations all display irregular firing patterns and naturally they appear different from each other, their long-term behavior is still similar. In this aspect, the irregular dynamics of LP neurons is consistent among preparations with both glutamatergic and cholinergic synapses blocked. We note that the PY neurons all displayed tonic spiking with small dips in their membrane potential in irregular intervals under such conditions (Fig. 8B).

Discussion

We used pharmacological tools to manipulate the intrinsic biophysical properties and synaptic connectivity of the neurons in the pyloric CPG of the lobster. Complete block of the A-currents of the neurons did not disrupt the pyloric oscillation, instead, different preparations responded with very consistent changes in their burst patterns. We also found a clear relationship between the amount of A-current and the intraburst spike dynamics of the neurons. Complete removal of fast inhibitory connections in the pyloric circuit had a low impact on the burst parameters, however, the changes were again highly consistent among animals.

Consistency of the motor patterns under pharmacological manipulation

A recent modeling study of the pyloric system suggests that the entire neural circuit can produce similar firing patterns even if the parameters of the intrinsic voltage-dependent conductances and the synaptic connections vary over a wide range (Prinz et al., 2004). This hypothesis was supported by direct experimental evidence on the variability of the K-currents in the LP and IC neurons of the crab pyloric network (Liu et al., 1998; Golowasch et al., 1999; Golowasch et al., 2002). According to the model there are many equally acceptable combinations of cellular and synaptic parameters that can produce equivalent firing patterns. One particular voltage-gated current can be very strong in an identified neuron from one animal but be very weak in the same type of neuron from a different animal. The desired network output would then come about as a result of proper combinations of cellular and synaptic properties, i.e., strong hyperpolarizing currents would be balanced by strong depolarizing currents. This kind of balancing requires compensatory mechanisms not only at the network level but also in the individual neurons. Developing muscle cells in *Xenopus* for example compensate for the overexpression of exogenous Na^+ channels by upregulating the expression of at least two endogenous K^+ channels (Linsdell and Moody, 1994). One way to see if such a possibility exists for the pyloric system is to remove one of the functionally important currents and observe the subsequent behavior of the neurons. If there are different combinations of currents present in each neuron, removing one single current such as I_A would lead to heterogeneous membrane responses in different animals. Each voltage-activated ionic current contributes to the observed voltage output of the neuron and complete block of a particular conductance would unmask the random variations in the remaining conductances.

The transient potassium current I_A has long been known to be one of the most important voltage-gated currents in shaping the voltage output of single neurons as well as the patterned activity of circuits containing those neurons (Tierney and Harris-Warrick, 1992; Greenberg and Manor, 2005).

Neurons with such fast potassium currents would be expected to display strong changes in their behavior following blocking of the I_A. Therefore if we assume that the same type of neuron from different animals may indeed have very different maximal conductances for I_A, then the compensatory currents, e.g., the H-current which has been suggested by others (MacLean et al., 2003), will also vary over a wide range. A total blocking of an identified cell's A-current in different preparations should result in populations of neurons with zero conductance in I_A but with very different conductances in the compensatory type voltage-gated currents. Accordingly, the activity pattern of single pyloric cells as well as the overall pyloric pattern should vary widely among different preparations reflecting the great variations in the remaining voltage-gated conductances. The same reasoning would apply to the animal-to-animal variability of synaptic properties.

In fact, 4-AP produces very similar effects among different preparations. These effects are consistent and reproducible not only in the gross network parameters but also in the ones characterizing the spike patterning of single neurons. Criteria for acceptance of the validity of computer models of such oscillatory neural circuits are often based on their power to reproduce the gross burst patterns observed in the real systems. While not questioning the fact that the gross network parameters characterize the functionally most important aspects of the motor output, we emphasize that the burst envelope and the spike time-based parameters are closely related to intrinsic biophysical properties of the neurons and also help in understanding their synaptic interactions. As shown by a recent comprehensive study, the gross burst parameters and especially the mean phase-relationships are very similar among different animals (Bucher et al., 2005). We reported earlier that the temporal patterns of spikes within bursts of the pyloric neurons are also cell-specific and consistent among animals (Szücs et al., 2003). The similarity of burst oscillations is actually one of the reasons why the identification of the pyloric neurons is straightforward for the experienced neurophysiologist. The shape (envelope) of the bursts and the patterns of spikes within the bursts strongly depend on the intrinsic properties of the neurons. Accordingly, consistency of these parameters among different animals and their reproducible alterations after biophysical manipulation of the circuit suggests the consistency of the cellular properties of the neurons. We note that the experiments we performed with the channel- and receptor blockers result in conditions in the pyloric circuit that never occur during the lifetime of the animal in nature. Clearly, total loss of an important voltage-gated current or an entire population of inhibitory synapses is something the system is not likely 'prepared for'. We do not expect that there are still built-in compensatory mechanisms that can make proper adjustments and result in very consistent motor patterns in pyloric CPGs built from very different components when an important ionic current is suddenly knocked out in the whole system. This latter possibility, although not ruled out by the present data alone, would require extremely quick repair of the damaged pyloric system (within minutes) and effective re-balancing of the remaining populations of voltage-gated channels.

The analysis of variations in the voltage output of pyloric neurons under the manipulation of biophysical parameters is clearly an indirect approach to characterize the consistency of such parameters among animals. Direct measurements of the voltage-activated ionic currents have been already performed by several groups and these studies provided important information on the cell-specificity and consistency of biophysical properties in the pyloric circuit. These measurements of the maximal conductances and other kinetic parameters in various pyloric neurons (including the PD, LP and PY cells) revealed relatively small animal-to-animal variations (S.D./mean < 0.3) in the properties of several voltage-gated currents including the delayed rectifier K-current (Golowasch and Marder, 1992; Kloppenburg et al., 1999; Gruhn et al., 2005); the A-current (Hartline et al., 1993; Harris-Warrick et al., 1995); and the high-threshold Ca-current (Johnson et al., 2003). Nevertheless, three- to four-fold variations have been also reported in the maximal conductances of the K-currents, including the inferior cardiac (IC) neuron (Golowasch et al., 1999) and the LP neuron (Schulz et al., 2006). While this degree of vatiation may be accurate, none approach the 8-fold variations for 1-spike bursters described by Golowasch (Golowasch et al., 2002) or the all-or-none type variations (0 vs. 5 nS conductances for the same current) described by Prinz (Prinz et al., 2004).

Physiological factors at least partially responsible for animal-to-animal variations of biophysical parameters have been already documented. These include state-dependent modulations of voltage-gated currents as well as synaptic connections of the pyloric network. It has been reported for *Panulirus* LP neurons that the maximal conductance of their K-currents, H-currents as well as their input resistance depended on their prior activity state and the frequency of sensory inputs arriving to the pyloric network (Nargeot, 2003). Furhermore, great variations in the parameters of K-currents of IC neurons were found to be correlated with their prior activity state and the duration of intracellular stimulation the authors used to depolarize the IC neurons (Golowasch et al., 1999). It is therefore appealing to suggest that great animal-to-animal variations in the measured biophysical parameters of pyloric neurons are at least partially caused by different preceding activity levels in the system. Also, frequent transient synaptic inputs to the pyloric circuit from extrinsic networks may shift the voltage-gated properties of these neurons when compared to stationary and periodically bursting preparations.

Neuromodulation and pharmacological dissection of neural networks

The drugs we used in the current study affected only specific populations of membrane channels. Endogenous neuromodulators of the pyloric system, such as monoamines or peptides, adjust the maximal conductance and the kinetics of the voltage-gated currents in a more complex manner (Harris-Warrick et al., 1992; Kloppenburg et al., 1999). It is known that neuromodulators often regulate a range of various types of ionic channels and receptors and that their action is target-specific

(Johnson et al., 1995; Peck et al., 2001; Swensen and Marder, 2001; Johnson et al., 2003). Therefore, they can sculpt different functional configurations from the same pyloric circuit (Weimann et al., 1997; Harris-Warrick et al., 1998; Swensen and Marder, 2001; Marder and Thirumalai, 2002). Despite the diversity and complexity of neuromodulatory actions in the pyloric system, these substances produce highly consistent changes in different animals (Flamm and Harris-Warrick, 1986; Harris-Warrick, 1991; Harris-Warrick et al., 1992; Szücs et al., 2005). The reliable and robust actions of endogenous neuromodulators also suggest that there is a high degree of biophysical consistency of the pyloric network. If there were disparate biophysical parameters in the target neurons, it would be surprising to achieve consistent reponses to neuromodulators concurrently while affecting a range of voltage-gated and synaptic conductances differentially.

Considering all of these arguments, we suggest that the pyloric circuit is composed of neurons with tightly adjusted biophysical properties and that there probably are not several-fold random variations in the properties of voltage-gated or synaptic currents of the same type of neurons from different animals. We suggest that the natural variations observed in the normal bursting neurons as well as in the biophysically manipulated ones likely reflect the differences in their morphological properties (Bucher et al., 2005) and the spatial distribution of their voltage-gated channels. Besides, state-dependent alterations and chemical neuromodulation of the circuits contribute to the observed animal-to-animal variations in the measured biophysical parameters. In addition to natural variablity of intrinsic and synaptic properties of the pyloric circuit, one faces difficulties in assessing the biophysical parameters of the neurons due to complex morphology, weak space-clamp (Graubard and Hartline, 1991; Johnson et al., 2003), poor correlation between membrane capactiance and surface area (Hartline et al., 1993) and problems related to incomplete current isolation. The contribution of such experimental factors to the observed biophysical variability is difficult to estimate.

Generation and operation of voltage-gated channels are metabolically costly events in the cells' life (Wong-Riley, 1989; Ames, 2000). Hence, it is reasonable to expect that neurons adjust their biophysical properties in a way that they can perform the desired function reliably and meet constraints of energy-efficiency at the same time (Balasubramanian and Berry, 2002). We suggest that there is, indeed a target or optimal set of biophysical parameters for the pyloric network, and that under natural conditions these networks spontaneously develop into the target configurations.

Other systems

While the pyloric network of the crustacean nervous system is one of those producing a particularly reproducible and stationary motor output, other small neuronal circuits are known to display far greater variations among animals. As a remarkable example, striking animal-to-animal variations have been described in the synaptic properties of identified cardio-respiratory neurons in the *Lymnaea*

nervous system (Magoski and Bulloch, 2000). As it turns out, even the functional classification of the synapses is problematic for some cell-pairs, having either EPSPs or IPSPs between the same identified neurons from different animals. Notwithstanding these differences, the neural circuits containing these variable cell-pairs produce behaviorally consistent output. Clearly, the number of interacting cellular and synaptic components is far lower in the pyloric circuit than in the molluscan cardiorespiratory network. Besides, a continuous operation of rhythmic and coordinated muscle activity of the lobster pylorus requires reliable generation of the neuron bursts. There is likely a strong relation between the animal-to-animal variability of single neuron properties and the complexity of the neural assembly. Compact networks with low redundancy in their components are likely fine-adjusted, and their biophysical parameters are optimized to achieve a specific function with high reliability. More complex neural networks with hundreds or thousands of neurons are more likely to show greater variations in their cellular and synaptic properties.

Figure Legends

Fig. 1 Schematic representation of two pyloric circuits with disparate intrinsic biophysical properties. CPG #1 and CPG #2 on the left both have the same type of neurons, but the populations of their specific voltage-gated ionic channels are different. Similarly, the strength of the synaptic connections are different in the two circuits. Circular sections with different colors represent 4 voltage-gated conductances in each cell. Although the cellular and synaptic properties of the two systems are different, their voltage output is similar, as a result of the hypothesized multi-level compensatory mechanisms and balancing. Blocking of a specific voltage-gated conductance would result the two circuits on the right. We suggest that the remaining unblocked populations of voltage-gated channels in the two circuits would produce dissimilar voltage dynamics.

Fig. 2. 4-AP increases the frequency of the pyloric rhythm and depolarizes the circuits' neurons. A typical 3-phase oscillation is observed in normal conditions (A). The voltage output of the PD, LP and PY neurons show characteristic changes after blocking their A-currents (B). Burst-to-burst variations in the neurons and particularly in the PY are due to strong gastric modulation of the pyloric rhythm. Horizontal markers here and in the following figures indicate the -60 mV level in the voltage waveforms. The Fourier-amplitude spectra of the spike density time series of the PD neuron are shown in D and E for the control and the 4-AP data, respectively. f_p is the pyloric burst frequency while f_g is the gastric frequency. F shows the mean pyloric burst frequency in control and during 4-AP application (N=12).

Fig. 3. 4-AP changes both the overall network activity in the pyloric CPG and the burst waveforms of the individual neurons. Interspike interval sequence and the instantaneous burst frequency of the PD neuron is shown in A and B, respectively. The application of 4-AP started at t=300 s. Zoomed parts of the burst frequency time series clearly show the gastric modulation of the pyloric rhythm. Note that 4-AP augments the gastric modulation and results in greater variations in the burst frequency time series. C and F display 6 consecutive bursts (overlapped) of the PD neuron in control conditions and when 4-AP is present. D and G are the intraburst spike density envelopes displayed as local mean firing frequencies and their standard deviations. Interspike intervals show a decreasing tendency along the burst of the PD neuron in normal conditions (E). With 4-AP, the shortest ISIs appear in the beginning of the bursts followed by gradually longer ISIs (H). This is in good agreement with the observed reshaping of the intraburst spike density envelope.

Fig. 4. The effect of 4-AP on the spike dynamics of the PD neuron is very characteristic and highly reproducible. Intraburst spike density envelopes and ISI signatures of PD neurons from three different preparations are displayed in control conditions (left) and after 4-AP application (right). All 3 PD neurons display similar 4-AP induced changes in their burst envelopes. Clustered and *V*-shaped ISI signatures on the left (control) are reshaped by 4-AP, and slanted, comet-shaped ISI return maps appear. These attractors are very similar to those of normally bursting LP neurons (Szücs et al., 2003). Washout of 4-AP always reverses the effect and the typical accelerating type bursts reappear (see also Movie 1).

Fig. 5. PTX shifts the burst phases of pyloric neurons but does not disrupt the regularity of the 3-phase motor rhythm. The voltage waveforms of the pyloric neurons become smoother as rapid IPSPs from glutamatergic neurons are blocked by PTX (A and B). At the same time, the intraburst spike number and duty cycle of the LP and PY neurons increase. The burst frequency of the rhythm barely changes after PTX application (Fourier spectra: D and E; mean burst frequencies: F, N=20). Note that the principal peak in the Fourier-spectrum (at f_p) remains very sharp after blocking the glutamatergic interconnections (E).

Fig. 6. Blocking the glutamatergic inhibitory connections has a small impact on the pyloric burst frequency and the voltage output of the pyloric dilator neurons. ISI sequence of the PD neuron is shown as a function of elapsed time in A. PTX application began at t=400 s. A moderate shift is visible in the intraburst ISIs. At the same time, the burst frequency is virtually unchanged by PTX (B). C and F each show 6 traces of overlapping bursts of the PD before and after the application of PTX. Note the removal of IPSPs in the PD in F. D and G show the corresponding intraburst spike density envelopes. ISIs tend to decrease along the bursts both in normal conditions and when PTX is applied

(E and H). The main difference between the control bursts and the ones after PTX is that the burst onset is steeper in the latter case (G) and intraburst ISIs are decreased (H).

Fig. 7. Atropine slightly intensifies the activity of pyloric neurons and shifts their burst phases. The voltage waveforms of the PD, LP and PY neurons become smoother and the intraburst firing frequency increases in the presence of atropine (A and B). The regularity of the pyloric oscillation is well preserved during the action of atropine as shown by sharp peaks in the Fourier-spectra of the PD neuron (D and E). The mean burst frequency of the pyloric circuit slightly increases (F, N=10).

Fig. 8. Combined application of PTX and atropine disconnects the LP and PY neurons from the pacemaker group and induces irregular voltage oscillations. A shows the voltage output of the 3 pyloric neurons in PTX solution as control. Irregular bursting appears in the LP and PY neurons after the addition of atropine (B). The synchronization of the LP and PY neurons to the pacemaker group is lost. The horizontal markers are -60 mV for the PD and LP neurons and -50 mV for the PY neuron. Relative burst phases of the LP-PD neurons and the LP-PY neurons are shown in C as functions of the elapsed time. Random burst phases appear 200 s after the introduction of atropine into the bath. Fourier amplitude spectra of the LP neuron are compared before (D) and during (E) the application of atropine. In control, a sharp peak at f_p dominates the spectrum indicating the LP's tight lock to the pacemaker rhythm. With atropine in the bath, the regularity of the LP's firing pattern is lost and a broad-band spectrum appears.

4-AP effect							
Parameter	Control	Treatment	Change	Mean diff.	F statistics mean (df)	Var. diff.	F statistics var. (df.)
Burst frequency	1.56±0.13	2.37±0.14	+52.6±10.8 %	***	189 (22)	N.S.	0.28 (22)
LP-to-PD phase	0.423±0.029	0.597±0.034	+41.7±9.0 %	***	152 (18)	N.S.	0.002 (18)
PD-to-LP phase	0.588±0.028	0.456±0.093	-22.6±13.6 %	**	12.9 (12)	N.S.	1.93 (22)
PD spike number	10.3±1.7	6.9±1.3	-32.4±5.6 %	***	26.6 (20)	N.S.	0.86 (20)
PD spike frequency	54.9±9.2	78.9±15.3	+43.6±11.0 %	***	19.8 (20)	N.S.	1.22 (20)
PD duty cycle	0.293±0.036	0.208±0.023	-28.8±6.4 %	***	48.3 (22)	N.S.	1.62 (22)
PD burst shape factor	-0.30±0.06	0.28±0.06	+0.58±0.06	***	428 (18)	N.S.	0.01 (18)
LP spike number	6.5±1.5	9.4±1.8	+52.0±46.1 %	*	9.51 (10)	N.S.	0.10 (10)
LP spike frequency	39.5±11.8	73.8±11.6	+95.5±38.1 %	***	25.8 (10)	N.S.	0.01 (10)
LP duty cycle	0.266±0.071	0.298±0.029	+18.3±30.6 %	N.S.	1.05 (10)	N.S.	3.87 (10)
LP burst shape factor	0.45±0.14	0.26±0.10	-0.20±0.23	N.S.	5.54 (6)	N.S.	0.14 (6)

PTX effect							
Parameter	Control	Treatment	Change	Mean diff.	F statistics mean (df)	Var. diff.	F statistics var. (df)
Burst frequency	1.76±0.24	1.80±0.20	+1.7±6.6 %	N.S.	0.10 (42)	N.S.	0.42 (42)
LP-to-PD phase	0.416±0.072	0.308±0.060	-25.0±12.7 %	***	14.4 (20)	N.S.	0.32 (20)
PD-to-LP phase	0.582±0.069	0.701±0.070	+21.0±11.2 %	***	16.7 (24)	N.S.	0.003 (24)
PD spike number	9.3±1.4	9.5±1.3	+2.4±7.1 %	N.S.	0.20 (40)	N.S.	0.08 (40)
PD spike frequency	56.3±9.2	61.2±10.5	+9.0±8.8 %	N.S.	2.59 (40)	N.S.	0.57 (40)
PD duty cycle	0.298±0.033	0.282±0.027	-5.0±6.3 %	N.S.	2.81 (38)	N.S.	0.32 (38)
PD burst shape factor	-0.26±0.05	-0.19±0.05	+0.07±0.04	***	14.3 (30)	N.S.	0.39 (30)
LP spike number	6.1±1.5	7.8±2.4	+28.0±21.0 %	*	4.86 (24)	N.S.	1.88 (24)
LP spike frequency	56.1±14.1	43.1±12.4	-21.7±16.4 %	*	6.16 (24)	N.S.	0.28 (24)
LP duty cycle	0.200±0.041	0.336±0.038	+71.3±25.5 %	***	76.1 (24)	N.S.	0.007 (24)
LP burst shape factor	0.20±0.13	0.49±0.06	+0.29±0.13	***	45.2 (20)	N.S.	1.67 (20)

Atropine effect							
Parameter	Control	Treatment	Change	Mean diff.	F statistics mean (df)	Var. diff.	F statistics var. (df)
Burst frequency	1.65±0.22	1.97±0.26	+19.7±11.9 %	**	8.8 (18)	N.S.	0.15 (18)
LP-to-PD phase	0.450±0.056	0.665±0.144	+47.6±24.4 %	***	21.3 (20)	N.S.	2.53 (20)
PD-to-LP phase	0.567±0.046	0.392±0.090	-30.7±16.5 %	***	27.2 (16)	N.S.	0.52 (16)
PD spike number	9.1±1.8	9.0±1.7	-0.5±6.9 %	N.S.	0.02 (20)	N.S.	0.11 (20)
PD spike frequency	52.5±9.5	69.1±11.6	+32.2±10.2 %	**	13.6 (20)	N.S.	0.40 (20)
PD duty cycle	0.292±0.032	0.252±0.042	-14.2±8.1 %	*	6.55 (20)	N.S.	0.62 (20)
PD burst shape factor	-0.25±0.06	-0.11±0.11	+0.14±0.08	**	13.7 (20)	N.S.	2.24 (20)
LP spike number	7.0±2.5	9.4±2.2	+40.3±24.2 %	*	5.48 (18)	N.S.	0.01 (18)
LP spike frequency	52.4±15.6	73.2±18.6	+43.5±27.8 %	*	5.87 (14)	N.S.	0.02 (14)
LP duty cycle	0.237±0.078	0.270±0.089	+18.4±34.9 %	N.S.	0.71 (16)	N.S.	0.05 (14)
LP burst shape factor	0.17±0.09	0.25±0.05	+0.09±0.07	*	4.98 (10)	N.S.	2.1 (10)

Table 1 Pharmacological manipulations of the intrinsic cellular properties and the network connectivity of the pyloric circuit induce consistent changes in different animals. ***, ** and * indicate significant differences in the means at $p<0.001$, $p<0.01$ and $p<0.05$ levels. N.S. means no significant differences ($p>0.05$). F values (with the degree of freedom in brackets) are shown both for the means and for the

variances. Changes for the burst shape factor are expressed as differences of the treatment and control values (not percentages).

Supplemental Movie 1 Application of 4-AP induces characteristic changes in the intraburst spike dynamics of the PD neuron. Each frame of the animation shows a joint interspike interval density map calculated from ~100 successive bursts in the time series. The movie starts with the normal ISI signature of the PD neuron. This *V*-shaped and clustered structure is transformed into a slanted and comet-shaped return map as the 4-AP blocks the A-current. Washout almost perfectly recovers the original structure as seen at the end of the animation. The 10 s duration time-lapse movie demonstrates the evolution of the ISI time series acquired in a 55 min experiment.

Supplemental Movie 2 PTX moderately affects the spike dynamics of the PD neuron. Successive sections of the ISI time series of the PD neuron were used to calculate the joint probability density maps. In the beginning of the movie the normal ISI signature of the PD neuron is seen. As PTX elicits its blocking effect on the glutamatergic connections and the LP-to-PD inhibition gets weaker, the well-separated clusters become fuzzy and less defined. No washout is recorded. The 10 s duration time-lapse movie demonstrates the evolution of the ISI time series acquired in a 43 min experiment.

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