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Precision of the Pacemaker Nucleus in a Weakly Electric Fish: Network Versus Cellular Influences

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Moortgat, Katherine T., Theodore H. Bullock, and Terrence J. Sejnowski. Precision of the pacemaker nucleus in a weakly electric fish: network versus cellular influences. *J. Neurophysiol.* 83: 971–983, 2000. We investigated the relative influence of cellular and network properties on the extreme spike timing precision observed in the medullary pacemaker nucleus (Pn) of the weakly electric fish *Apteronotus leptorhynchus.* Of all known biological rhythms, the electric organ discharge of this and related species is the most temporally precise, with a coefficient of variation (CV = standard deviation/mean period) of 2×10^{-4} and standard deviation (SD) of 0.12–1.0 μs. The timing of the electric organ discharge is commanded by neurons of the Pn, individual cells of which we show in an in vitro preparation to have only a slightly lesser degree of precision. Among the 100–150 Pn neurons, dye injection into a pacemaker cell resulted in dye coupling in one to five other pacemaker cells and one to three relay cells, consistent with previous results. Relay cell fills, however, showed profuse dendrites and contacts never seen before: relay cell dendrites dye-coupled to one to seven pacemaker and one to seven relay cells. Moderate (0.1–10 nA) intracellular current injection had no effect on a neuron's spiking period, and only slightly modulated its spike amplitude, but could reset the spike phase. In contrast, massive hyperpolarizing current injections (15–25 nA) could force the cell to skip spikes. The relative timing of subthreshold and full spikes suggested that at least some pacemaker cells are likely to be intrinsic oscillators. The relative amplitudes of the subthreshold and full spikes gave a lower bound to the gap junctional coupling coefficient of 0.01–0.08. Three drugs, called gap junction blockers for their mode of action in other preparations, caused immediate and substantial reduction in frequency, altered the phase lag between pairs of neurons, and later caused the spike amplitude to drop, without altering the spike timing precision. Thus we conclude that the high precision of the normal Pn rhythm does not require maximal gap junction conductances between neurons that have ordinary cellular precision. Rather, the spiking precision can be explained as an intrinsic cellular property while the gap junctions act to frequency- and phase-lock the network oscillations.

INTRODUCTION

The electric organ, whose timing precision was first quantified decades ago (Bullock 1970; Bullock et al. 1972), remains the most precise known biological pacemaker, but the mechanism of its extreme precision has yet to be elucidated. The electric organ is commanded by the medullary pacemaker nucleus (Pn), whose neurons in the in vivo Pn fire synchronously with a coefficient of variation (CV = SD/mean period) as low as 6×10^{-4}, in turn driving the electric organ to produce a signal with CV = 2×10^{-4}, which gives SDs in the submicrosecond range (Moortgat et al. 1998b). Most other pacemaking systems have CVs in the range of 10^{-2} to 10^{-1}, with circadian rhythms slightly better with CVs of 2–5 $\times 10^{-3}$ (see Table 1). We investigated the relative roles of network electrotonic coupling and intrinsic cell properties in setting the extreme spike timing precision observed in one species of weakly electric fish, *Apteronotus leptorhynchus.*

The weakly electric fish electrolocates using its electric organ discharge, detecting its own electric field which is modified by the surrounding environment, with electroreceptors along its body. The timing and amplitude of the electric field at the electroreceptors are the key information the fish has to make electrosensory discriminations (Heiligenberg 1991). The precise timing of the electric organ is commanded by the Pn. Neurons of the Pn fire in synchrony, with each cell firing every cycle of the 500- to 900-Hz oscillation, even when its inputs and outputs are cut (Dye 1988; Meyer 1984).

The Pn is a network of 100–160 neurons that develop from the same rhombomeres as other brain stem pacemakers, including the inferior olive and VOR circuitry (Bass and Baker 1997). The Pn contains neurons of two types: pacemaker cells (25–30 μm soma diameter), which remain intrinsic to the nucleus, and relay cells (60–70 μm soma diameter) (Dye and Heiligenberg 1987; Elekes and Szabo 1985), whose somata lie in the nucleus and whose axons project down the spinal cord (Ellis and Szabo 1980) to the electromotor neurons [electric organ in *Apteronotus* (Bennett et al. 1967a)]. These two neuron types occur in a ratio of ~4:1, respectively, in adult fish (Elekes and Szabo 1985), although the number of pacemaker cells increases linearly with a fish's length without a corresponding increase in relay cells (Dye and Heiligenberg 1987). The input impedance of the pacemaker and relay neurons is ~3 and 1–4 MΩ, respectively (Dye 1991; Juranek and Metzner 1998). The pacemaker cells converge onto relay cells, which in turn send their axons down the spinal cord to command the precise firing of the electric organ, with each relay cell spike corresponding to one cycle of the electric organ discharge. Evidence for a third neuron type have been reported (Turner and Moroz 1995) but was not mentioned in earlier electron microscopic studies (Elekes and Szabo 1985; Ellis and Szabo 1980). What role this third neuron type could have in the Pn oscillations remains unclear.

Among Pn neurons of *Apteronotus leptorhynchus,* the species studied here, gap junctions are the sole synaptic commu-

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TABLE 1. *Coefficient of variation measured in various pacemaking systems*

System	CV	Period	Reference
EOD of weakly electric fish	0.0002	1–2 ms	Bullock 1970; Moortgat et al. 1998b
Weakly electric fish Pn cell	0.0006 – 0.002	1–2 ms	Here and Moortgat et al. 1998b
Circadian rhythm	0.002 – 0.005	1 day	Enright 1980
Aplysia pacemaker neuron	0.01 – 0.08	0.1 – 4 s	Junge and Moore 1966
Avian MVN neurons	0.01 – 0.1	20 – 100 ms	du Lac and Lisberger 1995
Rat respiration	0.04 – 0.09	0.4 – 0.5 s	Sammon et al. 1993
Heart ECG	0.03	0.7 – 0.9 s	Gustafson et al. 1978
Cat spinal motoneuron	0.10	80 – 130 ms	Calvin and Stevens 1967
Bullfrog sciatic nerve	0.10 – 0.37	50 – 200 ms	Hagiwara 1954

CV, coefficient of variation; EOD, electric organ discharge; Pn, pacemaker nucleus; MVN, medial vestibular nucleus; ECG, electrocardiogram.

nication, and occur at large axosomatic and axoaxonic club endings (Dye and Heiligenberg 1987; Elekes and Szabo 1985). Each neuron appears to contact only a small fraction of the total number of neurons, similar to the coupling reported in *Hypopomus pinnicaudatus* (Dye and Heiligenberg 1987; Moortgat and Keller 1995; Spiro 1997). Gap junctions are present in abundance throughout the adult gymnotid electrosensory and electromotor pathways (Bennett et al. 1967a; Yamamoto et al. 1989) and are thought to be important in time coding (Carr et al. 1986) and synchronization. It has been proposed that gap junctions among Pn neurons could also play a role in reducing the CV of spike timing below the intrinsic or natural CV of isolated neurons.

We investigated the network and cellular basis of spike timing precision in the in vitro Pn. We provide evidence that the precision of the pacemaker nucleus (Pn) in an electric fish could derive from cellular rather than network properties, and that the frequency- and phase-locking is modulated by gap junction coupling.

Earlier versions of this work were included in a PhD thesis and a conference abstract (Moortgat 1999; Moortgat et al. 1998a).

METHODS

Male and female *Apteronotus leptorhynchus* were obtained under the common name "brown ghost" from a commercial fish supplier. Fish were kept in aquaria whose water was maintained in temperature (26.0–28.0°C), pH (7.0–8.0), and resistivity (5–15 kΩ-cm). Fifty-six fish of 11–20 cm body length were used in this study.

The dissection procedure was similar to that of previous studies (Dye 1988; Meyer 1984; Spiro 1997). Each fish was cold anesthetized, and the brain was rapidly removed into cold artificial cerebral spinal fluid (ACSF; in mM: 124 NaCl, 2 KCl, 1.25 KH_2PO_4, 1.1 $MgSO_4$, 1.1 $CaCl_2$, 16 $NaHCO_3$, and 10 D-glucose), which had been oxygenated and pH adjusted to 7.4. Tissue 1 mm rostral and caudal, and ~2 mm dorsal to the Pn's ventral surface was cut away with a scalpel. The remaining tissue block, including the whole Pn, was pinned in a silicone elastomer (Slygard) well and the meninges pulled away. The tissue was continuously perfused with oxygenated ACSF.

Neural activity was monitored with a combination of sharp intracellular (9–30 MΩ) and local field potential electrodes (300—500 kΩ). Amplified voltage signals were either directly digitized (National Instruments ATMIO 16E2) at a rate of 20–200 kHz, or passed to a Schmitt trigger circuit. The Schmitt trigger (Getting Instruments, Iowa City, IA) has independently adjustable hysteresis center and width to allow measurement of individual cycle periods within the detection (±50 ns) of the data acquisition board.

To reveal neuron morphology and dye coupling, some neurons were filled intracellularly with Neurobiotin (2% in 3 M KCl, Vector Laboratories, Burlingame, CA). This tracer was injected ionotophoretically with depolarizing current (0.5–2.0 nA) for 30–120 min. Tissue was then fixed in 4% paraformaldehyde for 1–3 days and processed as described elsewhere (Wong 1997).

We aimed to reduce gap junctional conductance with bath application of gap junction blockers. The three agents used were halothane vapor (2.5–5.0% in 95% O_2-5% CO_2) (Peinado et al. 1993; Wojtczak 1985), octanol (1–5 mM, from 1 M stock in DMSO, Sigma) (Johnston et al. 1980), and carbenoxolone (100 μM to 1 mM, Sigma) (Draguhn et al. 1998).

RESULTS

The spiking precision observed in neurons of the in vitro Pn, an intact nucleus with all inputs and outputs cut, matched that seen in neurons in the in vivo preparation. Namely, the distribution of cycle-by-cycle periods (interspike intervals, Fig. 1*A*) for single cells tested was Gaussian (χ^2 tests were significant) with a minimum half-width of 1.2 μs, corresponding to a coefficient of variation (CV = standard deviation/mean period) of 7.0×10^{-4}, similar to minimum values seen in vivo (Moortgat et al. 1998b). CV values, observed during intracellular recordings, ranged from this minimum to $\sim 25 \times 10^{-4}$ in 40 neurons from 17 nuclei (Fig. 1*B*), with no apparent correlation between a cell's CV and its frequency at room temperature. The pacemaker and relay cells, which were distinguishable visually (when somata lay on the Pn surface) and sometimes from spike shapes (see for example, Fig. 4*A*), did not appear to differ in the distribution of their mean CV values (Fig. 1*B*).

Because the pacemaker cells contact other pacemaker cells and relay cells, simultaneously recorded neurons could have correlations in the cycle-by-cycle variability about the mean period, correlations that could act to increase the CV. If the variability were correlated between cells, we would expect a distinct peak in the covariance. We would also expect that the root sum of the squared CV of two neurons would be greater than the CV of the cycle-by-cycle period difference. In the eight neuron pairs for which cycle-by-cycle periods were simultaneously recorded (Fig. 1*C*), the CVs were additive, and we found no clear peak in the covariance (Fig. 1*D*) and thus conclude that the cycle-by-cycle variability is independent between neurons.

Because the precision of Pn cell firing was maintained when all Pn inputs and outputs are cut, the spiking precision must be intrinsic to the Pn; either due to network connections, single cell properties, or a combination of the two. We first characterized the numbers and strengths of electrotonic connections

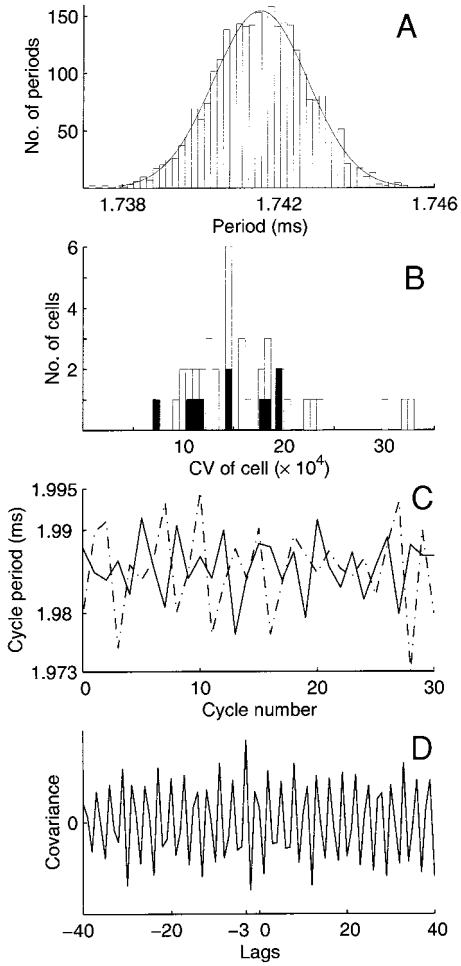

FIG. 1. Precision and statistics of pacemaker nucleus (Pn) oscillations in vitro match those seen in vivo. *A*: the histogram of interspike (cycle) periods from an individual in vitro neuron fit a Gaussian distribution with width determined by the SD = 1.2 μs, corresponding to a coefficient of variation (CV) of 7.0×10^{-4}. *B*: the histogram of CV values compiled from 38 neurons from 17 in vitro Pns. ■, relay cell CVs; □, pacemaker cells or neurons of unknown type. Minimum CV values matched the minima observed in vivo (Moortgat et al. 1998b). Cycle periods (*C*) of 2 neurons simultaneously recorded intracellularly did not clearly covary (*D*). The cross covariance was calculated over 500 cycles of the 2 neurons' cycle periods. The peak normalized covariance (correlation coefficient) is 0.34 at a lag of −3 cycles. The measured lag between the 2 neurons could be shifted by up to ~10 cycles, depending on the data acquisition delay between the 2 neurons. This, however, has no effect on the form of the cross covariance function.

and then modulated their strengths without altering the number of electrotonic connections.

Neuron morphology and dye coupling between neurons

Previous intracellular horseradish peroxidase (HRP) injections into a number of pacemaker cells and one relay cell revealed their cellular morphology and the number of axosomatic contacts made (Dye and Heiligenberg 1987). We have extended these anatomic studies by intracellularly injecting the tracer Neurobiotin into multiple pacemaker and relay cells. Neurobiotin, unlike HRP, crosses gap junctions, thus allowing us to assess not only physical proximity but also functional gap coupling. The functional gap junctions permit staining in coupled neurons, which are then readily recognized and typed, even when coupling occurs between processes. These latter contacts have been documented as axoaxonic gap junctions in electron microscopy studies (Ellis and Szabo 1980) but were not counted in the HRP studies.

Only one cell per Pn was injected with Neurobiotin. Three of six stained pacemaker cells were filled darkly in tissue with low enough background staining to evaluate dye coupling to other pacemaker cells and relay cells (Table 2). The filled pacemaker cells were dye coupled to one to five other pacemaker cells and one to three relay cells.

The morphology of pacemaker cells stained with Neurobiotin and viewed as fixed and histologically processed tissue was consistent with previous studies (Dye and Heiligenberg 1987; Elekes and Szabo 1985). A few additional pacemaker cells were injected with Lucifer yellow, which did not cross gap junctions in these neurons, and observed in the live tissue under combined infrared and fluorescent light using DIC optics. A striking observation was the abundance of large-diameter (~6–9 μm) processes coursing throughout the Pn [also noticed in electron microscopy (Elekes and Szabo 1985)]. These processes were found to be the pacemaker cells' primary axons and their multiple, equally large diameter branches. The axon narrowed slightly at branch points, but within ~5 μm regained its initial diameter in both new branches. These large pacemaker cell axons appear to take up a large percentage of the Pn volume.

In five relay cells, dye injection resulted in staining of one to seven pacemaker and one to seven other relay cells. Unlike previously observed contacts, none of these contacts appeared to stem from the filled relay cell's soma or axon but rather from its dendrites, one of which is shown in Fig. 2*A* coming in close proximity to a dye-filled pacemaker axon. This result was not reported in previous electron microscopic or electrophysiological studies, but is not contradicted by them. To confirm that the dye coupling from the relay cell dendrites did not result from the "shish kebob artifact," in which multiple cells absorb dye through cell damage along the electrode track (Spray and Bennett 1985), we injected neurons (1 per Pn) that lay on the brain surface and were the only cell recorded in that Pn. Still, dye coupling was observed. In addition, dye was localized to the soma and axon initial segment of some dye-coupled neurons that were on opposite sides of the Pn from the directly stained neuron. Thus our data suggest electrotonic coupling at the relay cell dendrites.

The morphology of relay cells was found to be more complex, with more profuse and more finely branching dendrites than previously reported. The dendrites sometimes tapered, only to increase in diameter again more distally from the relay

TABLE 2. *Number of dye-coupled cells when a pacemaker or relay cell is injected with Neurobiotin*

		Dye-Coupled Cell		
Injected Cell	*n*	Pacemaker	Relay	Total
Pacemaker	3, 7	1–5 (2.3)	1–3 (2.7)	2–13 (5.3)
Relay	5	1–7 (4.4)	1–7 (3.6)	2–14 (8.0)

Range of dye-coupled cells in *n* dye injections in each cell type is given, along with the mean value in parentheses. For pacemaker cells, the last column incorporates our data as well as the number of contacts made by 4 pacemaker cells described in Dye (1987), to give $n = 7$.

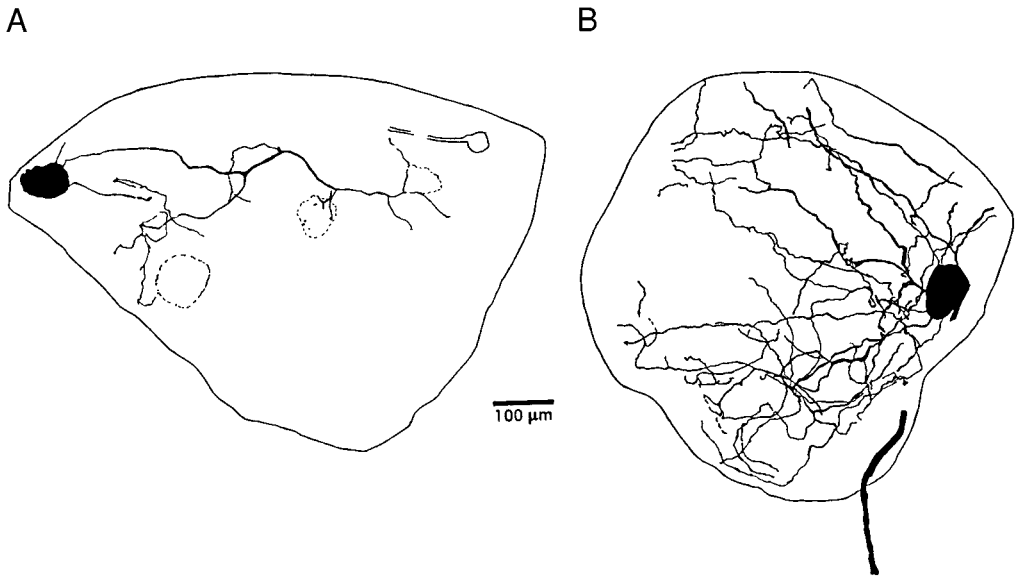

FIG. 2. Camera lucida reconstructions of dye-injected relay cells. *A*: the relay cell was injected with Neurobiotin while the smaller distant pacemaker cell soma and axon (*top right*) showed dye coupling, probably through the relay cell dendrite that came in close proximity to the pacemaker cell axon. Shown here are the dendrites within 2 100-μm sections. This relay cell was counted as contacting 1 relay and 3 pacemaker cells. Dendrites from the filled relay cell also approached 3 other relay cell somata (dotted perimeters). *B*: an injected relay cell had profuse dendrites that extended ~390, 390, and 550 μm in the lateral, dorsoventral, and rostrocaudal dimensions, respectively. Shown here are the dendrites within 3 100-μm sections. This relay cell was counted as contacting 2 pacemaker and 2 relay cells. These transverse sections are shown ventral side up.

soma (Fig. 2*A*), and the finest dendrites showed swellings or "beads." The dendrites of two relay cells, one from a 12.9-cm-long female and one from a 12.4-cm-long male, were particularly well stained and cover, respectively, ~100% and 68% of the lateral, 100% and 62% of the dorsal-ventral, and 61% and 53% of the rostral-caudal extent of Pn. One of these neurons (the former) is drawn in Fig. 2*B*. If we assume that each relay cell's dendrites are similarly extensive, the branches of different relay cells are highly overlapping. The overlaps may be crucial to the prepacemaker nuclei's ability to rapidly and simultaneously modulate all relay cells' frequency, which is thought to be modulated primarily at relay cell dendrites (Heiligenberg et al. 1996).

Electrophysiological measures of coupling strength across gap junctions

To unveil the effect of gap junctions on the firing of each neuron, we need to know not only the number of contacts, but also their strengths. The strength of coupling between a pair of neurons can be quantified by the coupling coefficient, defined as the ratio of the voltage deflection in one cell to that in another cell that is injected with a constant current. We hypothesized that the coupling coefficient between pairs of neurons making direct contact would be large because, despite relatively sparse connections (low numbers of contacts), the frequency and phase are tightly locked between all Pn neurons.

Coupling coefficient

Because the contacts are sparse, we expected only a subset of coupling coefficients to be nonzero. We were initially surprised that the coupling coefficients measured between every one of 26 neuron pairs, including both pacemaker and relay cells, were not significantly different from zero. These coupling coefficients were calculated from the deflection of the minimum voltage during 0.5–2.0 s of constant current injections (0.1–1.5 nA). Coupling coefficients were not observed at either long or short time constants. Likewise, current injection in one cell changed the spike amplitude in that cell but not in any simultaneously recorded neuron.

We recorded spike amplitudes to test whether poor space clamping or membrane voltage fluctuations could explain the lack of measurable coupling coefficient. A poor space clamp could allow a somatic current injection to dissipate through leak conductances in the cell membrane, resulting in a much smaller current at the distant gap junction contact at the end of the axon. The low spike amplitude, often recorded at the soma (as confirmed visually during the experiment and in some cases after Neurobiotin staining), and its unusual decay with increased membrane potential suggest that the injected current did indeed leak. First, in the hundreds of intracellular recordings we made, the recorded spike amplitude had a maximum of ~40 mV, but was more typically in the range of 15–30 mV. The low spike amplitudes were not indicative of poor recordings, because they occurred with stable membrane potentials of approximately −75 to −65 mV in recordings that lasted from tens of minutes to 7 h. Second, when currents were injected, the peak voltage of the spike was not fixed (Fig. 3*A*, ±0.3 nA sine wave). Also, the spike amplitude (Fig. 3*B*) decreased with depolarizing current at a lower rate than the minimum membrane potential increased (Fig. 3*C*) and showed hysteresis with membrane potential. These results indicate that current was not injected in electrotonic proximity to the spike initiation zone.

In our recordings, neurons did not have a fixed initial membrane potential, as required for measuring coupling coefficient. Rather, the neurons' active spikes clouded their passive responses to the gap junction drive. An active spike in a presynaptic neuron causes a substantial voltage drive across the gap

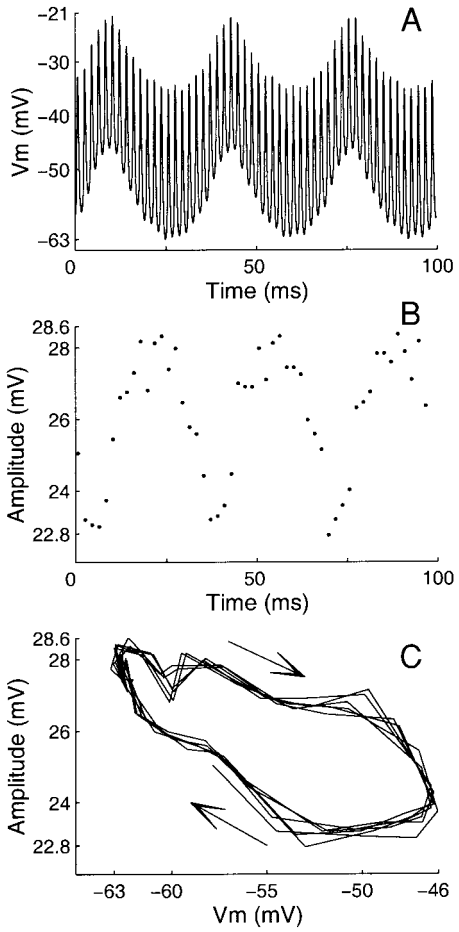

FIG. 3. Intracellular response of a pacemaker cell soma to a moderate sinusoidal current injection (±0.3 nA). *A*: membrane voltage (V_m) during injection of the sinusoidal currents. *B*: spike amplitude during the current injection decreased with depolarization and increased with hyperpolarization, but by only ~⅓ of the baseline membrane voltage changes. *C*: the spike amplitude and voltage showed a relative clockwise hysteresis, a sort of cellular memory of previous voltage and spike amplitude states. This allows 2 amplitudes for any membrane voltage and vice versa, a feature reported previously in vivo (Moortgat et al. 1998b) and confirmed here. The range of spike amplitude and membrane voltages is indicated on the axes.

junction to a phase-lagged postsynaptic neuron (Fig. 4*A*). The voltage drive is as large as the amplitude of a spike, or ~40 mV. Thus the effect of a small somatic current injection, like those we applied, whose amplitude has decayed along the long axon, may be overwhelmed by the drive of the presynaptic spike and the subsequent postsynaptic spike response.

Phase shift with moderate current injection

Because measuring electrotonic coupling proved difficult with the traditional measure, we sought other ways of detecting coupling. We looked for phase and frequency shifts between a neuron injected with current and another simultaneously recorded neuron. Normally, any two neurons in the Pn fired at the same frequency with a fixed phase lag (Fig. 4*A*). The phase lag was smaller between cells of the same type than different types (Fig. 4*B*) (Dye 1988). If a neuron responds to current injection with either a change in phase or frequency, then a similar simultaneous shift in the second neuron would suggest coupling between the two neurons. We found that current injections into one neuron did not alter the phase of the simultaneously recorded neuron (relative to its original phase) in any of the 26 pairs we recorded. However, an injected neuron's phase relative to another neuron (or its own original phase) was linearly advanced with depolarizing current and delayed with hyperpolarizing current (Fig. 4*C*, slope = −0.55%/nA, r^2 = 0.99). These results further confirm that the current injections in one cell's soma were not reaching the other recorded cell.

Skipped spikes during massive current injection

A neuron's firing frequency was immune to somatic injection of at least ±10 nA. Even with this large current injection, a neuron continued to fire with every cycle of the collective Pn oscillations. How could large current injections have no effect on pacemaking frequency when the Pn neurons can be driven

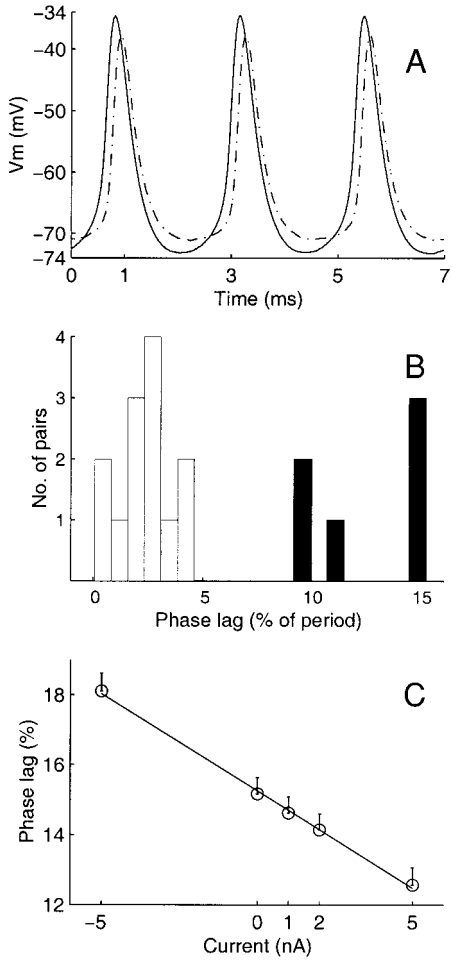

FIG. 4. Phase-locked firing of neuron pairs. *A*: simultaneous intracellular voltage recordings of a pacemaker and a relay cell show the difference in the 2 cell types' spike waveforms. The pacemaker cell (—) has a slowly increasing potential lacking in the relay cell (– · –). The recorded amplitude of the spikes was typical for the pacemaker cell, but particularly large for a relay cell that in many recordings had 8- to 15-mV spikes. *B*: the phase lag for 19 pairs of simultaneously recorded neurons is plotted. Shaded bars indicate pacemaker-relay cell pairs; open bars indicate same-type cell pairs, as determined from the intracellular waveforms. *C*: phase lag is labile to intracellular current injection (slope −0.55%/nA, r^2 = 0.99). Mean phase lag is marked with a circle, the SD with a bar. The phase lag changed asymmetrically to 5-nA hyperpolarizing and −5-nA depolarizing currents, with a time average phase advance of 2% and phase delay of 3.5%, respectively.

to a range of frequencies by higher-order brain centers, as shown in many previous studies (Dye 1988; Heiligenberg et al. 1996; Spiro 1997; see Heiligenberg 1991 for review)? We sought to answer this question by injecting even larger currents. A strong gap junction drive to each neuron (which could bring the neuron to threshold and act as a current shunt) combined with the large electrotonic distance between the gap junctions and the recording electrode could result in the lack of response to the ±10 nA current injections. If so, such injections at the soma might not be sufficient to overcome the combined gap junction inputs at the axon initial segment and at the soma, and larger currents would be required to alter the firing rate of the neuron in the intact Pn.

Intermediate current injections (10–14 nA) caused an alternation in the voltage amplitude from cycle to cycle. In one example, the spike amplitude was ~20% higher in one cycle than the succeeding cycle, and the time intervals were constant between the oscillations (Fig. 5*B*). All current injections caused an immediate, substantial drop in membrane voltage, followed by a slower voltage decay with time constant ~0.5 s (Fig. 5*C*), too large to be the membrane time constant of the single neuron, but possibly the time constant for slowly charging the entire Pn. The alternation in spike amplitudes began sooner, sometimes within the stimulus artifact time. During the time of slower voltage decay, the low-amplitude oscillations were distinguishable from the full amplitude oscillations, but both changed amplitude over 1 s.

When neurons were instead injected with massive hyperpolarizing step currents of 15–25 nA, not only the spike amplitude but also the spike timing was affected. The full spikes that persisted arrived at roughly integer multiples of the preinjection cycle period, but the neuron no longer spiked with every cycle of the Pn oscillation (Fig. 5*A*). That is, the hyperpolarized neuron no longer had full amplitude oscillations locked 1:1 with the Pn oscillation, but rather had a full amplitude oscillation followed by a low-amplitude oscillation, usually locked 1:2 with the Pn oscillation. Concomitant with the alternation of amplitudes was an alternation in the peak-to-peak interval times of the oscillations (Fig. 5*A*). The intervals varied by ±0.05 ms (4%) around the cell's mean spike period before current injection.

Frequency locking between an injected neuron and the rest of the Pn could take on values of 1:1 and 1:2, and also much lower integer ratios that could change in time during the current injection. One neuron receiving 20 nA hyperpolarization, for example, went from normal firing (firing with every network oscillation) to skipping 1 spike in 16 network oscillations (1:16, Fig. 6*A3*), back to normal spiking, and then to skipping in a ratio of 1:23 (Fig. 6*B3*). When this neuron, which had a pacemaker cell waveform, skipped a spike, the membrane voltage still oscillated with a mean amplitude of 3.7 mV (range 0.5–7 mV). The ratio of the skipped to full spike amplitude can be taken as an estimate of the coupling coefficient (see discussion). The cycle period varied slightly and took on a new value during the cycle after every subthreshold oscillation (Fig. 6, *A2* and *B2*). The membrane voltage continued to hyperpolarize in the repolarizing phase (trough) after each spike during the 2-s current injection (Fig. 6*C*).

Effects of pharmacological gap junction blockers

We next tested the importance of the observed gap junction inputs in setting the extremely low CV of spike timing. We compared the effects of bath application of halothane (1–5% vapor), carbenoxolone (100 μM to 1 mM), and octanol (1–5

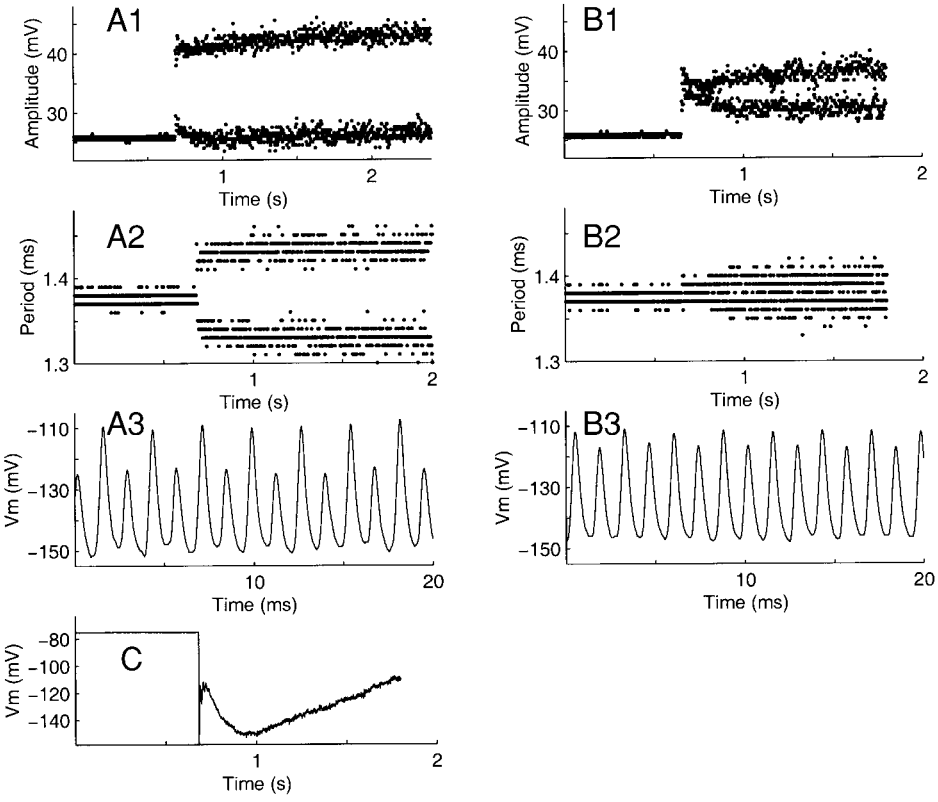

FIG. 5. Massive intracellular current injection into a Pn neuron, probably a relay cell, caused spike amplitude and, with increased current, cycle period to vary bimodally. *A1*: during a −23-nA current injection, the spike amplitude switched between 2 values, whereas in *A2* the cycle period remained relatively fixed. *A3*: the amplitude variation was evident in the intracellular membrane potential. *B1*: during a −21-nA current injection into the same cell, both the spike amplitude and the cycle period (*B2*) varied bimodally, with long periods followed by shorter ones, and vice versa. *B3*: the intracellular membrane potential, from which the amplitude and period were calculated are plotted here. Note that the voltage usually reached its minimum after a small "spike," but not after a large spike. This did not seem to be the case in *A3*. *C*: membrane voltage (measured at the trough of the oscillation) responded slowly, with at least 2 time constants, to the injected current.

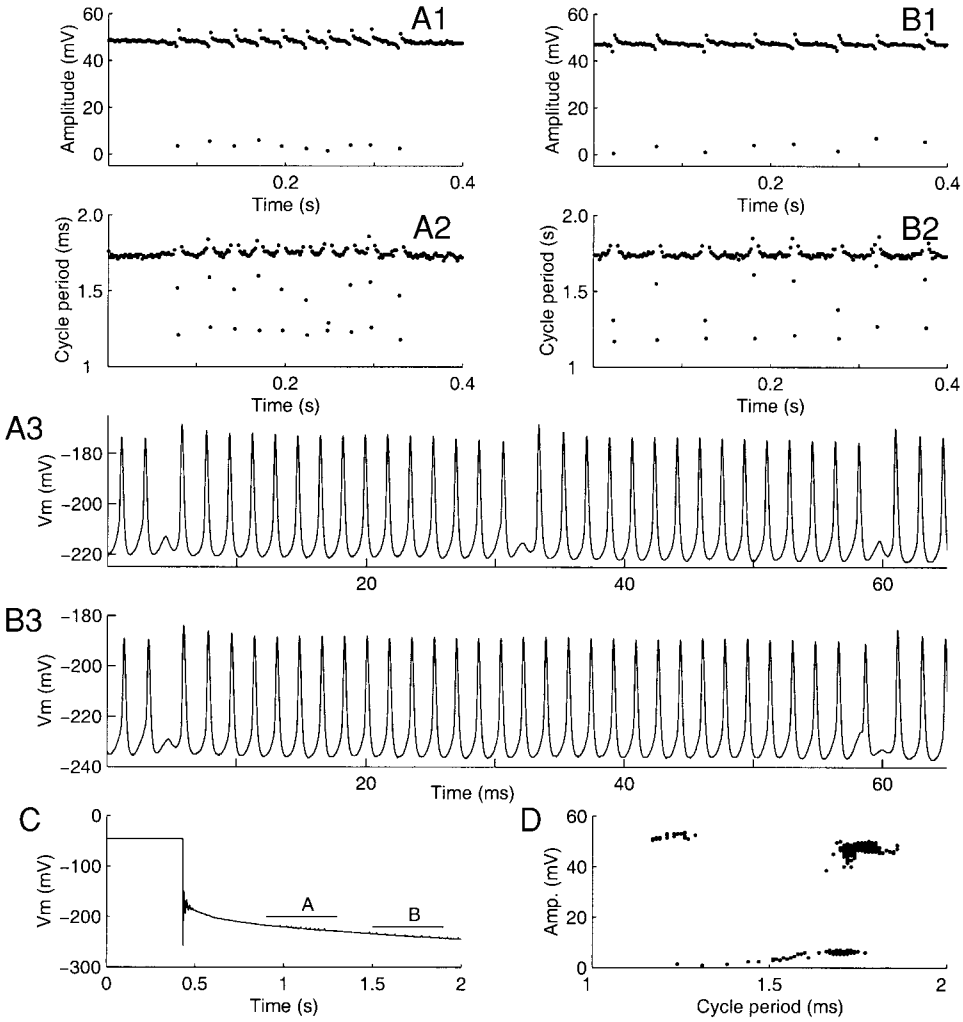

FIG. 6. Intracellular injection of −20 nA caused another neuron, probably a pacemaker cell, to skip spikes but remain locked to the Pn oscillation. The spike amplitudes (*A1* and *B1*) and periods (*A2* and *B2*) corresponding to voltage traces (*A3* and *B3*) occurred, respectively, early and late during the current injection. The timing, and thus the amplitude of the subthreshold oscillation varied with reference to the spikes. *C*: the membrane potential (measured at the trough of the membrane oscillation) during the 2-s recording is plotted, with bars indicating the segments represented in *A* (0.9–1.3 s) and *B* (1.5–1.9 s). The membrane voltage response to the current injection had a slow time component. *D*: spike amplitude vs. cycle period.

mM), three putative gap junction blockers (halothane: Peinado et al. 1993; Wojtczak 1985; octanol: Johnston et al. 1980; carbenoxolone: Draguhn et al. 1998). Of primary interest were their effects on the CV of interspike periods. We found that the CV, measured intracellularly, remained at its initial low value, sometimes decreasing slightly (up to 20%) during application of halothane (Fig. 7*A*, $n = 4$) or carbenoxolone ($n = 2$) over 30–40 min, or sometimes up to 100 min, of sustained application of medium or high drug concentrations. This relative constancy in the CV occurred during substantial frequency decay elicited by all three drugs. In fact, the frequency decreased by up to 50% in halothane (Fig. 7*A*), 20% in carbenoxolone, and 50% in octanol. Application of the blockers also shifted the phase lag between simultaneously recorded neurons, sometimes even changing its sign (Fig. 7*B1*). The phase lag returned to its original value when the drugs were washed out.

After prolonged and continuous application of a high drug concentration, the spike amplitude decreased to a point beyond which no further spikes were elicited (Fig. 7, *B2* and *B3*). As the spike amplitude decreased, the measured CV increased. This CV increase could simply be due to lower ratio of biological signal to electrical noise rather than to major changes in the gap junction strength. The minimum membrane potential (during the repolarization phase after a spike) remained within 5–10 mV of its original in all 11, 6, and 2 trials in, respectively, halothane, carbenoxolone, and octanol.

Spiking frequency decreased, apparently at the same rate in all Pn neurons, reaching a dose-dependent minimum frequency. Continued drug application reduced the spike amplitude such that all neurons appeared to stop firing simultaneously in halothane (Fig. 8*A*). That is, once one neuron stopped firing in a halothane-treated Pn, all others were also silent. In contrast, neurons in an octanol- or carbenoxolone-treated Pn stopped firing minutes apart. Neurons on the ventral surface of the Pn (which is the ventral surface of the brain stem) stopped firing while about two or three neurons deeper in the Pn continued firing for 1–3 min, although with reduced amplitude and often with 1:2 locking in the spike amplitude (Fig. 8*B*).

We tested whether the frequency decrease could be due to a decrease in excitatory drive. Namely, halothane, known to depress glutamate transmission in some preparations (MacIver et al. 1996), might be blocking glutamatergic synapses from glia or from axons arriving from higher order centers onto both cell types (Dye et al. 1989; Heiligenberg et al. 1996). However, 30 min of bath application of 2-amino-5-phosphonovaleric acid (APV; 100 μM) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μmM), which are *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-

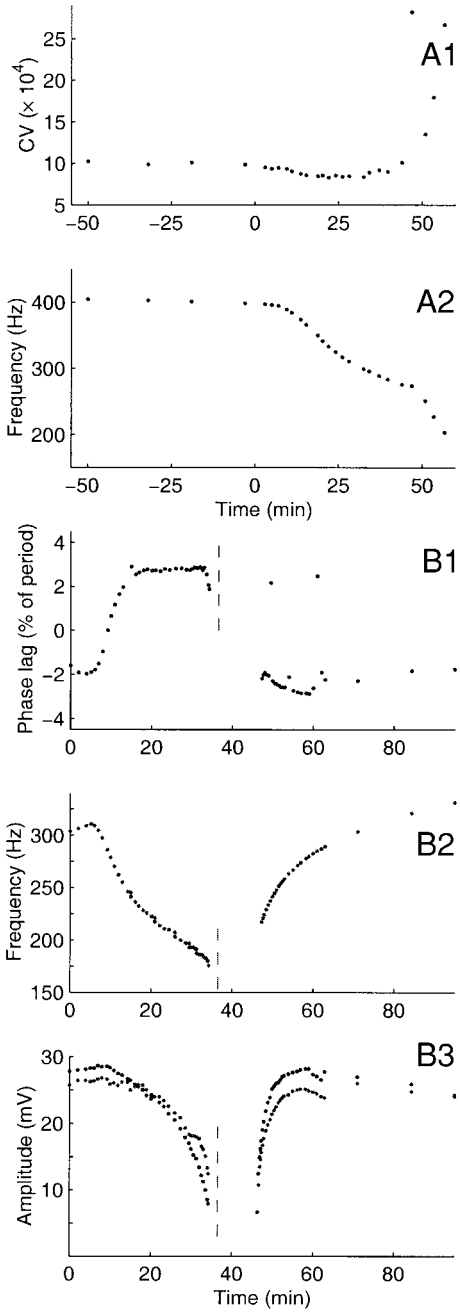

FIG. 7. Bath application of a gap junction blocker left a neuron firing with its initial precision, but at a new phase and frequency. *A1*: the CV of interspike intervals remained constant or decreased slightly (increases precision) during application of halothane (5% starting at *time* = 0 min). *A2*: the frequency, however, simultaneously decreased by 50%. *B1*: the phase lag between simultaneously recorded neurons shifted continuously, even changing sign, with ongoing bath application of halothane (starting at *time* = 0 min), and returned to original values with drug wash out (starting at *time* = 36.5 min, marked with dashed vertical line). Concurrently, the frequency (*B2*) and later the spike amplitude (*B3*) decreased at the same rate in both neurons until the oscillations stopped entirely throughout the Pn. Oscillations returned with drug wash out.

methyl-4-isoxazolepropionic acid (AMPA) antagonists, respectively, did not alter the firing frequency or its CV, and, on addition of halothane, did not alter halothane's ability to decrease the Pn frequency (Fig. 9). Therefore halothane does not seem to decrease frequency by modulating glutamatergic synapses.

Other parameters that could affect precision of spike timing

Because pharmacological reduction of network coupling within the range tested did not alter the firing precision of Pn neurons, the precision must either have a highly nonlinear dependence on coupling, or be largely a cellular property. We considered whether other manipulations known to change cellular spike frequency also alter the spike timing precision, or CV. Increasing the aquarium water temperature from 20 to 30°C did not affect the CV of the electric organ discharge in two *A. leptorhynchus* (B. Keeley and K. T. Moortgat, unpublished observations), although the frequency changed with a Q_{10} of 1.5 (Enger and Szabo 1968).

We found no correlation between female fish's body length and the CV of their electric organ discharge, although body length and number of pacemaker cells in the Pn are positively correlated (Dye and Heiligenberg 1987). Two large males who chirped repeatedly had particularly high CV of the electric organ discharge ($CV = 20 \times 10^{-4}$), even when not chirping.

Although the CV of the fish's electric organ discharge varies spontaneously, as well as in response to behavioral stimuli (Moortgat et al. 1998b), we found no such CV modulations in the 10-s intracellular recordings made in the isolated in vitro Pn.

DISCUSSION

We have shown that the *A. leptorhynchus* Pn retains its extreme precision of firing in vitro. We have quantified the number of functional gap junctions between pacemaker and relay cells, and measured and modulated their strength.

We estimated the number of neurons electrotonically coupled to an intracellularly dye-filled neuron by the number of dye-coupled neurons, as done in rat neocortex (Kandler and Katz 1998; Peinado et al. 1993). The extent of dye coupling to pacemaker and relay cells varied widely, due to natural variation in the number of contacts and total number of Pn neurons, as well as inconsistent dye uptake and transport. Indeed, dye appeared to travel more completely into some neurons, sometimes even better in one process or type of process than others of the directly injected neuron. For example, one relay cell showed a well-stained soma and axon, but only part of one dendritic process was obviously filled. Dye coupling was not symmetrical: the number of relay cells dye coupled to a pacemaker cell was slightly smaller than the number of pacemaker cells dye coupled to a relay cell. Some of the differences in dye uptake and transport could result from larger currents for longer durations in some cells, as well as the difficulty of fully filling with dye the huge volume, including, for the pacemaker cell, a 30-μm-diam soma and 7- to 10-μm-diam axon of 1 mm length and multiple branches, a volume that can be even larger for the relay cell. Because many of these factors act to reduce the number of dye-coupled neurons, we tend to bias our estimate of the number of contacts toward the higher values.

In a typical adult Pn of 150 neurons, in a ratio of 4:1 pacemaker to relay cells (Ellis and Szabo 1980), the pacemaker cells contact a maximum of 4% (5/120) of other pacemaker cells and 10% (3/30) of relay cells. The relay cells in turn are in electrotonic contact with 6% (7/120) pacemaker cells and

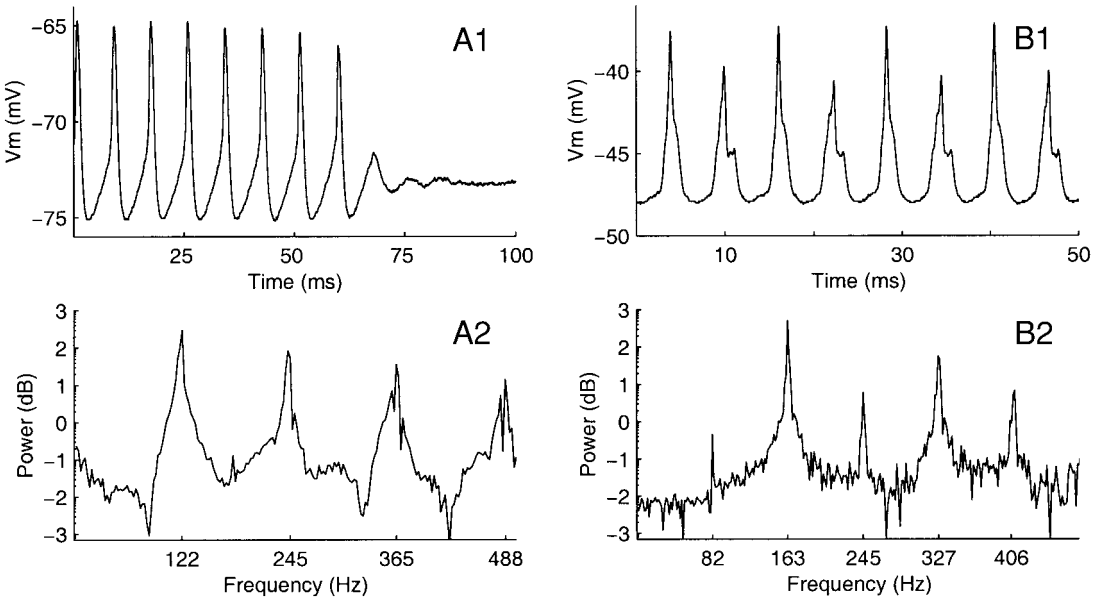

FIG. 8. Halothane and octanol had different time courses of shutting down the Pn oscillations. *A1*: under halothane, the final intracellular oscillations before Pn shutdown had an exaggerated pacemaker potential, and low amplitude, and stopped simultaneously in all cells. *A2*: power spectrum of the waveform over 400 ms of the last oscillations in halothane. *B1*: under octanol, a few neurons in an otherwise silent Pn continued to show low-amplitude oscillations of unusual waveform, such as the one shown here. *B2*: despite the distorted waveform, the power spectrum over 600 ms has a single dominant frequency at 163 Hz with a subharmonic at 82 Hz. The frequency of peaks in both power spectra are indicated along the abscissa.

23% (7/30) other relay cells. This coupling is quite sparse compared with the all-to-all coupling often estimated in models of tightly synchronized oscillatory systems.

We found that relay cell dendrites were intricately branched and could reach across the full dorsal-ventral and medial-lateral extent, and ~60% of the rostral-caudal extent of the Pn. This dendritic arbor exceeds estimates from the one relay cell fill previously described (Dye and Heiligenberg 1987) and may provide multiple sites of contact with the widely distributed inputs (Heiligenberg et al. 1996) from higher centers. These higher centers may require multiple contact sites to effect their strong and rapid modulations of the Pn frequency (Dye 1987; Kawasaki and Heiligenberg 1990; Keller et al. 1991; Metzner 1993), something that proved difficult to do with somatic current injection into a single neuron (Fig. 3).

Dendritic gap junctions onto relay cells were previously reported in other species of electric fish (*Sternopygus* and *Steatogenys*) but were thought to originate only from pacemaker cell axons (Bennett et al. 1967b). Electrotonic coupling between relay cells, which was physiologically supported in these species, was hypothesized to occur indirectly through pacemaker cell terminals (Bennett et al. 1967b). In the species studied here, *A. leptorhynchus*, axosomatic and axoaxonic electrotonic coupling had been reported (Elekes and Szabo 1985). We provide the first evidence in this species that an additional site of coupling occurs: axodendritic connections between pacemaker and relay cells. Additionally we show that relay cells are directly dye coupled to other relay cells, often through connections from one relay cell's soma to another's dendrite. Thus electrotonic spread between relay cells can occur directly. This may facilitate coordination between relay cells when higher order inputs bypass the pacemaker cells and send commands directly to the relay cells (Heiligenberg et al. 1996; Kawasaki and Heiligenberg 1989). Similarly, dendritic

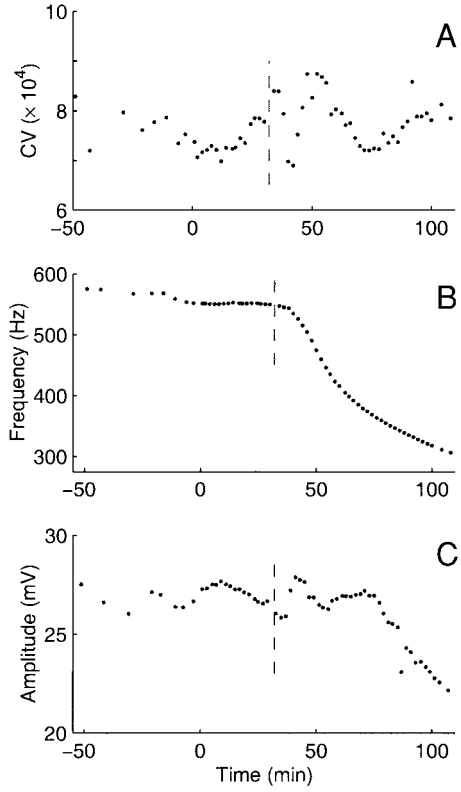

FIG. 9. Effects of halothane were not blocked by a cocktail of glutamate antagonists. The glutamate antagonists 2-amino-5-phosphonovaleric acid (APV) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were bath applied in combination (starting at *time* = 0 min) before and during halothane application (starting at *time* = 32 min, marked with vertical dashed line). *A*: the cocktail did not alter spiking precision or frequency, and (*B*) did not interfere with halothane's effects on frequency, suggesting that halothane is not acting through glutamate receptors.

gap junctions are thought to be involved in synchronization of spiking in the inferior olive (review in DeZeeuw et al. 1998) and in the developing visual cortex (Kandler and Katz 1998).

How can higher order centers drive Pn cells to fire at higher frequencies while intracellular injection of up to 10 nA has no effect on firing frequency? The main reason may be that the higher order centers synaptically modulate many cells simultaneously, while we inject current into only one or two cells at a time. Also, the location of the inputs may be crucial. Boutons of chemical synapses, thought to arise from the higher order centers, cover the dendrites, somata, and axon hillock (Elekes and Szabo 1985). Even when these synaptic inputs are silent, our current injections must overcome gap junction inputs. Gap junctions from one pacemaker cell contact other pacemaker cells primarily at the axon initial segment, but also on a short dendrite, and the soma. Likewise, the axon initial segment of the relay cell receives particularly dense axoaxonic gap junction innervation, which is considered "the morphological correlate of a synchronizing function" (Elekes and Szabo 1985). Likewise, axoaxonic gap junctions in a model of another system, the hippocampus, were shown sufficient to synchronize high-frequency oscillations between pyramidal neurons (Draguhn et al. 1998; Traub et al. 1999).

The limited effect of current injections to the pacemaker and relay cell somata may also arise from a long electrotonic distance to the action potential initiation zone. Indeed, the small somatic spike amplitude, particularly in the relay cell, indicates just this. Similarly small somatic action potentials can be seen in other systems when the spike initiation zone lays distant from a neuron's soma, such as in the molluscan photoreceptors (Alkon and Fuortes 1972). Another reason to believe the Pn cells' action potential initiation zones are distant from the soma is the small size of observed changes in spike amplitude with current injection. In the example of Fig. 3, the spike amplitude only decreased ~5.8 mV during a recorded membrane potential shift of ~17 mV. Thus the peak spike voltage, rather than being a fixed property of the spike, appears labile to membrane voltage at the electrode. We hypothesize that the small change in spike amplitude reflects a small membrane voltage deflection at the site of action potential initiation. The recorded membrane potential is local to the recording electrode, but does not indicate the true membrane potential at the axon initial segment. That is, the neuron is not well space clamped.

Lack of space clamping in a neuron will hinder traditional measures of coupling coefficient: the voltage deflection of a current-injected neuron will decay down the axon and may not alter the membrane potential of a coupled neuron. Even measuring coupling coefficients between neurons silenced with tetrodotoxin or 4-aminopyridine (Dye 1991; Smith and Zakon 1998) would fix membrane potentials of both cells at the recording sites, but still have a space-clamp problem. A space clamp could best be achieved with dual patch-clamp recording, in which the prejunctional axon and the postjunctional soma or axon are simultaneously recorded. The latter experiments have not yet been conducted for technical reasons.

We can, nevertheless, estimate the coupling coefficient. When massive current was injected in the soma, a neuron began to skip spikes in multiples of the Pn oscillation. The ratio of the subthreshold oscillation to the full spike amplitude can be taken as an estimate of the total coupling coefficient, the ratio of postsynaptic voltage to presynaptic voltage, if we assume that the presynaptic voltage has the amplitude of a full spike. For the data in Fig. 6 (probably a pacemaker cell recording), the ratio is ~0.08. Dividing by the maximum number of contacts to a pacemaker cell (Table 1) yields a coupling coefficient of ~0.01. Similar analysis of the data from a likely relay cell (Fig. 5) yields a post- to presynaptic voltage ratio of ~0.65, giving a coupling coefficient of ~0.05. Thus the larger subthreshold oscillations in Fig. 5 than in Fig. 6 may reflect larger numbers of gap junction contacts, higher coupling coefficient, and different cell types. Certainly the coupling coefficient among Pn neurons is significantly smaller than the 0.03–0.14 measured in mouse motoneurons (Rekling and Feldman 1997), or the 0.6–0.9 between pairs of cultured horizontal cells (Lasater and Dowling 1985). Our estimate of the coupling coefficients will vary slightly depending on the strength of injected current. Also, the true coupling coefficient between pacemaker and relay cells may be significantly higher if there are fewer contacts, or if presynaptic voltage is less than the amplitude of a full spike as recorded in the current-injected cell.

Although the relative amplitudes of the subthreshold oscillations and the spike peaks provide an estimate of the coupling coefficient, the relative timing of the two events provides evidence for the cell's intrinsic firing properties. Certainly we expect to find at least a subset of Pn neurons that are spontaneously active, because the Pn oscillates without external drive (Meyer 1984). The small precession in the pacemaker cell's cycle period around the Pn oscillation frequency during the massive current injection suggest that the pacemaker cell has an intrinsic firing frequency that is different from the Pn frequency (Chay et al. 1995; Winfree 1987). The cell's intrinsic frequency may normally be close to the Pn oscillation frequency, but may have been decreased by the massive current injection. The electric fish pacemaker neurons need not have the same firing frequency to synchronize: simulations have demonstrated synchrony between coupled oscillators of distributed intrinsic frequencies (Matthews and Strogatz 1990; Moortgat et al. 2000).

We next tested the importance of gap junctions in setting the extremely low CV of spike timing observed intracellularly in Pn cells. Our investigations mainly focused on decreasing the gap junctional coupling strengths with pharmacological agents that, in the ideal case, electrically dissociate neurons. Physical dissociation of the Pn, using techniques developed by Turner et al. (1995), resulted in live neurons with phase-bright somata and axon initial segments, but preliminary experiments yielded neurons that were electrically inactive (both spontaneously and with step current stimulation). The lack of electrical activity may reflect damage caused by the dissociation rather than the true nature of the normal neurons.

All 3 pharmacological gap junction blockers in 19 total trials decreased the spike frequency, demonstrating that the drugs are having some effect on the Pn. Still we did not know whether the drugs were affecting the gap junctions. Evidence that the drugs do indeed act on gap junctions was the consistency of responses to the three chemically different drugs: no drug caused an increase in the CV of a neuron's interspike period while the spikes maintained their full amplitude. Our preliminary results with high-pH ACSF, known to reduce gap junction conductance in some systems (Spray and Bennett 1985),

showed reversible frequency and spike amplitude decreases, similar to those reported here with other gap junction blockers.

Additional evidence that the gap junctions were closing comes with comparison to a realistic compartmental model of the Pn (Moortgat et al. 2000). The model is consistent with the biological spike waveform and frequency (Fig. 4*A*), the phase distribution observed in the Pn (Fig. 4*B*), and in a cell's phase response to current injection. The model predicts shifts in relative phase lag between neurons, even to the point of switching polarity, as gap junction coupling is decreased, as observed in the physiological data (Fig. 7*B1*). That the phases are still locked at all suggests that the drug is causing partial but possibly not full block while the spikes have their full amplitude. Another more general model also confirms that decreased gap junction conductance reduces firing frequency in neurons whose spike shape is similar to the Pn neurons (Chow and Kopell 2000). Other lines of evidence that the drug was taking its purported action, including a decreasing coupling coefficient or decreasing number of dye-coupled neurons, were considered but not pursued for technical reasons.

Although the three gap junction blockers had many consistent effects, they differed in the way their continued application stopped Pn oscillations. Although halothane seemed to stop oscillations in all neurons simultaneously, octanol and carbenoxolone left a few neurons oscillating deep in the nucleus while the surface neurons were silent. The halothane may diffuse more uniformly through the tissue, although this is not due to a lower formula weight (formula weights for halothane, octanol, and carbenoxolone are 197.4, 130.2, and 614.7, respectively). Another difference between the drugs was the reversibility of their effects: washing out halothane returned the Pn to its original synchronized firing state within minutes, with the phase lags returning to prewash values, whereas octanol and carbenoxolone were not entirely reversible, leaving only a subset of neurons firing asynchronously after hours of wash out. The ease of reversibility with halothane, and relatively greater difficulty with octanol, has been similarly observed in other systems (D. Spray, personal communication).

The Pn neurons stopped oscillating when a drug-dependent frequency was reached. The dependence of the minimum frequency on the drug suggests that frequency changes may not be mediated by gap junction closure, but by drug side effects. Halothane, for example, is known to enhance $GABA_A$-mediated inhibition (Pearce 1996), depress glutamate-mediated excitation (Perouansky et al. 1995, 1996), and inhibit Ca^{2+} and Na^{2+} channels (Franks and Lieb 1994), in addition to blocking gap junctions (Peinado et al. 1993; Wojtczak 1985). The first of these effects is not relevant to the *A. leptorhynchus* Pn, whose inputs are not GABA mediated but glutamatergic (Dye et al. 1989; Heiligenberg et al. 1996). We found that blocking the glutamatergic synapses did not decrease frequency, nor does it hinder halothane's ability to reduce Pn frequency. The possibility remains that halothane blocks the Pn neurons' Ca^{2+} and Na^{2+} channels, which is known to reduce the Pn firing frequency (Dye 1991; Smith and Zakon 1998). This hypothesis has not yet been tested experimentally but is supported by our realistic Pn model (Moortgat et al. 2000). In contrast, another model of gap junction–mediated oscillations did achieve ~40% frequency change with modulated gap junction conductance (Kepler et al. 1990).

In addition to modulating the strength of contacts between Pn neurons, we considered reducing their number by removing Pn neurons. Unfortunately, cutting the Pn in half with a razor or vibratome irreversibly silenced the Pn. More selective removal, killing one neuron at a time with Lucifer yellow injection followed by strong illumination (Miller and Selverston 1979), is feasible, but the data analysis is problematic. Functional removal of single neurons by hyperpolarization was not possible because even −25 nA did not silence a neuron.

Another way of assessing the importance of the number of Pn cells on their spike timing precision is to look at developmental changes in the CV of the electric organ discharge. Hagedorn et al. (1992) found in a related weakly electric fish, *Eigenmannia*, that the CV of the electric organ discharge decreases with increasing body length up to 1.5–2.0 cm, at which stage the Pn contains ~60 neurons, differentiated into two classes, as seen in the adult fish. The *A. leptorhynchus* Pn continues to develop more pacemaker cells with increasing body length even at the adult body lengths of 15–25 cm (Dye 1991). However, we did not observe a decrease in the CV of the electric organ discharge with increasing body length in the fish we studied (11–20 cm). Thus the spiking precision does not appear to be sensitive to the addition of neurons beyond a minimum number.

In summary, decreasing the gap junction strength, the number of gap junction inputs, or the number of Pn neurons did not alter the CV of the interspike period. Instead of dramatically decreasing CV, the gap junctions may be most involved in frequency and phase locking. Indeed, Dye (1991) showed that treatments thought to increase intracellular calcium broke up the frequency and phase locking between simultaneously recorded Pn neurons.

Taken with previous studies, our results indicate that the extreme spike timing precision in the *A. leptorhynchus* Pn could primarily be an intrinsic property of each neuron and may only minimally depend on the Pn network interactions. That is, the network may not act to dramatically increase the precision of otherwise sloppy neurons. The CV commonly reported for individual neurons is $\sim 10^{-1}$ to 10^{-2} (Table 1). This is the first suggestion that individual neurons could intrinsically have a CV as low as $\sim 7 \times 10^{-4}$, equivalent in this example cell to SD = 1.2 μs.

On the other hand, convergence of relay cells onto the electromotor neurons of the electric organ could explain the relatively small decrease in CV (increase in precision) between them. The minimum CV of the electromotor neurons, as measured outside the tail, is $\sim 2 \times 10^{-4}$ (Moortgat et al. 1998b), 3–4 times less than the minimum CV of relay cells. According to the law of large numbers, such a CV decrease would require a convergence of 9–16 independent relay cells onto each electromotor neuron. This prediction of convergence is consistent with anatomic data showing that each relay cell electrotonically innervates most or all the electromotor neurons (Ellis and Szabo 1980). We must assume that the electromotor neurons do not themselves add noise to the output; they too must be capable of extremely high precision spiking.

The CV of the fish's electric organ discharge varies spontaneously, as well as in response to behavioral stimuli. These changes may be mediated by a higher order nucleus, the prepacemaker nucleus (Moortgat et al. 1998b). We found no such CV modulations in the 10-s recordings made in vitro. However, we did observe that two large males (~20 cm) who

repeatedly modulated their electric organ frequency (in a glutamate-driven "chirp") had particularly high CV of the electric organ discharge (CV = 20×10^{-4}). The CV of Pn neurons, and hence of the electric organ discharge may be increased with active glutamatergic (AMPA) PPn inputs to their site of contact, the relay cells (Heiligenberg et al. 1996). Similarly, the CV of Pn neurons could be at its lowest with all inputs to the Pn silent. If so, application of AMPA to specific locations on the relay cell soma and dendrites would increase the interspike period CV.

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