Spike-Firing Resonance in Hypoglossal Motoneurons

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INTRODUCTION

Respiratory motoneurons that are active during inspiration are driven by synaptic inputs characterized by a depolarizing envelope on top of which oscillatory amplitude fluctuations occur (reviewed by Funk and Parkis 2002). Hypoglossal motoneurons (HMs) receive inspiratory-phase-related synaptic input and their output in the postnatal mouse in vitro is characterized by inspiratory-phase synchronous oscillatory firing recorded from the hypoglossal (XII) nerve that is in the 20- to 40-Hz range (Sebe et al. 2006). The function of these short timescale synchronous oscillations remains unclear, but they have been shown to increase the output firing rate of phrenic motoneurons and to increase spike-timing precision (Parkis et al. 2003). In general, synchronous oscillatory firing depends on an intricate interplay between synaptic excitation and inhibition, electrical coupling, and intrinsic membrane properties (reviewed by Sejnowski and Paulsen 2006). It is not known whether the intrinsic properties of HMs are tuned to maintain synchronicity when stimulated with time-varying inputs. We intracellularly recorded from HMs in an in vitro brain stem slice preparation from juvenile mice. Cells were held at or near spike threshold and were stimulated with steady or swept sine-wave current functions (10-s duration; 0- to 40-Hz range). Peristimulus time histograms were constructed from spike times based on threshold crossings. Synaptic transmission was suppressed by including blockers of GABAAergic, glycinergic, and glutamatergic neurotransmission in the bath solution. Cells responded to sine-wave stimulation with bursts of action potentials at low (<3- to 5-Hz) sine-wave frequency, whereas they phase-locked 1:1 to the stimulus at intermediate frequencies (3-25 Hz). Beyond the 1:1 frequency range cells were able to phase-lock to subharmonics (1.2, 1.3, or 1.4) of the input frequency. The 1:1 phase-locking range increased with increasing stimulus amplitude and membrane depolarization. Reliability and spike-timing precision were highest when the cells phase-locked 1:1 to the stimulus. Our findings suggest that the coding of time-varying inspiratory synaptic inputs by individual HMs is most reliable and precise at frequencies that are generally lower than the frequency of the synchronous inspiratory oscillatory activity recorded from the XII nerve.

METHODS

Experimental procedures

In vitro experiments were performed on nonrhythmically active medullary slice preparations from Swiss–Webster mice (P10–P16). Mice were anesthetized with isoflurane and killed by decapitation in accordance with the regulations of the University of Washington Institutional Animal Care and Use Committee. The brain stem was removed and 300-μm transverse slices containing the hypoglossal motor nucleus were cut. Slices were incubated for 1 h at 37°C before recording. For intracellular recordings slices were transferred to a perfusion chamber mounted on the stage of an upright Zeiss microscope (Axoscope) equipped with infrared differential interference contrast (DIC) optics. The perfusion solution (1–2 ml/min) contained (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO₄, 1 NaH₂PO₄, 26.2 NaHCO₃, 11 d-glucose, and 2.5 CaCl₂, gassed with 95% O₂-5% CO₂ to maintain a pH of 7.4. The temperature in the chamber was maintained at 32 ± 1°C. To reduce background synaptic noise, all recordings were done in the presence of blockers of α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA)–mediated glutamatergic [6,7-dini-
troquinoxaline-2,3-dione (DNQX), 10 μM, GABAergic [2-(3-carbonylpropyl)-3-aminoo-6-methoxyphenyl]-pyridazinum bromide (SR95531), 0.5 μM), and glycine (strychnine, 1 μM) synaptic transmission.

To assess the firing-resonance properties of neurons it was essential that stable recordings could be maintained for long periods of time to permit examination of responses to repeated stimuli under a range of experimental conditions. We used two different patch solutions to maximize recording stability based on either K-glucuronate (Kgluc) or K-methylsulfate (Kmeth). The composition of the Kgluc solution was (in mM): 150 K-meth, 2 HEPES, 2 Mg-ATP, 0.1 K-EGTA, and 15 K-methylsulfate (Kmeth). The composition of the Kmeth solution was: 115 K-gluc, 25 KCl, 1 MgCl$_2$, 9 NaCl, 10 HEPES, 3 K$_2$-ATP, 1 Na-ATP, and 0.2 K-EGTA. The pH of both solutions was adjusted to 7.3 with KOH. For whole cell patch recordings electrodes were pulled from thin-walled borosilicate glass to a resistance of 3–5 MΩ.

The location of the hypoglossal motor nucleus (nucleus XII) was determined as described in previous publications from this laboratory (Bayliss et al. 1992; Viana et al. 1993). The densely packed multipolar cells that together form the XII nucleus were identified under high power using infrared DIC optics. These cells could be easily distinguished from neurons in the adjacent dorsal motor nucleus of the vagus by their large cell bodies and their ability to fire repetitively in response to depolarizing current pulses. To confirm this cell-identification process, several cells were stained intracellularly with biocytin (data not shown). Electrodes were advanced onto visually identified HM neurons, using positive pressure; after formation of a gigaohm seal the cell membrane was ruptured by applying brief suction to the patch.

To avoid intracellular dialysis of the cell with the internal patch solution we also recorded a subset of neurons with “sharp” microelectrodes. Intracellular recording microelectrodes were pulled from thick-walled borosilicate glass to a final resistance of 40–80 MΩ. First, under visual guidance, the electrodes were placed on the surface of the slice over the hypoglossal motor nucleus and gradually advanced into the slice using a micromanipulator (Sutter Instrument). We continuously monitored bridge balance while the electrodes were advanced by injecting a 5-ms hyperpolarizing current pulse into the electrode. On an increase in electrode resistance we attempted to gain access to the cell interior by briefly ringing the electrode capacitance using the “buzz” control of the amplifier (Axoclamp 2A). We did not find any systematic differences between the firing properties or stability of cells recorded with either patch solution or with sharp or whole cell patch-based techniques (data not shown), and results from all three recording techniques were therefore pooled in this study. We checked for changes in firing pattern over time by repeating the same stimulus at the beginning and end of each trial. We also checked the firing rate in response to DC depolarization at regular intervals during the experimental protocol. We aborted or excluded any recordings where significant changes occurred in firing responses over time.

**Analysis of spike-firing resonance**

Cells were driven by computer-generated sinusoidal current injections using the external command input of an intracellular amplifier (Axoclamp 2A), operating in bridge mode, through the use of the Clampex 8 software program (Axon Instruments). The same electrode was used for membrane voltage recording and current injections. Sine-wave current injections were superimposed on a DC offset current whose magnitude was adjusted such that the membrane potential of the recorded HM cell was near or at spike threshold. At this membrane potential most neurons fired spontaneously at a low rate. Use of the DC current offset allowed us to use relatively small amplitudes for sine-wave current injections (<0.6 nA). In pilot experiments we found that large-amplitude stimuli were detrimental to the long-term stability of the cells. In HMs current injections of this magnitude typically give rise to membrane potential fluctuations between 5 and 30 mV (see Fig. 1). We stimulated the cells with either steady sine-wave currents (10-s duration) whose frequency was varied stepwise between 2 and 20 Hz or sinusoidal currents of slowly and linearly varying frequency (ZAP functions). The ZAP current stimulus is described by: \( I(t) = I_0 \sin(2\pi f_0 t) \times f(t) \), where \( f(t) = f_0 + (f_m - f_0) \times (t/T) \). The time-dependent frequency of the current \( f(t) \) was increased from \( f_0 = 0 \) Hz to \( f_0 = 20 \) Hz (2 Hz/s) or from \( f_0 = 10 \) Hz to \( f_m = 40 \) Hz (3 Hz/s) over a total time period of \( T = 10 \) s. In some cells we also tested the reversed protocol (reverse ZAP) where frequency was varied from 20 to 0 or 40 to 10 Hz. Each sine wave or ZAP function was repeated at least twice. Firing responses in 15-s-long digitized data files, including the 10-s period of sine-wave stimulation, were digitized at 2 kHz (Clampex) and detected with an amplitude window discriminator, using Neuromatic software (J. Rothman) written for IgorPro (WaveMetrics). Detection threshold was set along the upstroke of the action potential at about 20 mV below spike peak and the time at which the spike occurred was stored with a precision of 500 μs. In addition, in all cells we also injected with short (5-ms) and long (1,000-ms) DC current pulses to characterize action potential characteristics and firing properties at resting membrane potential, respectively. These recordings were digitized at 20 kHz and analyzed using Clampex/Clampfit (Axon Instruments).

**Measurement of reliability and spike-timing precision**

Reliability and spike-timing precision analysis was performed with custom-written functions implemented in IgorPro. To quantify the reliability of firing (i.e., the likelihood that a cell will fire an action potential at a certain time relative to the onset of the stimulus), the cells were stimulated with repeated ZAP current stimuli (10 trials). The times of occurrence of spikes from the start of the stimulus \( (T = 0) \) were collected in 3-ms-wide bins and a peristimulus time histogram (PSTH) was constructed from the number of spikes that fell within each bin. Only bins that contained more than one spike were used for analysis. Reliability was expressed as the number of spikes/bin divided by the number of trials. Highest reliability (1 spike/bin/stimulus) was obtained if the cell always fired a spike at the same latency (i.e., fell within the same bin) at each trial. This reliability measure is sensitive to both the presence of a spike (i.e., firing probability) and spike-timing precision. A low reliability might therefore be due to either a low probability of spike firing or/and low precision. To examine the probability of spike firing we calculated the average number of spikes per sinusoidal cycle and the trial-to-trial variability of the number of spikes/cycle, expressed as the coefficient of variability (CV = SD/mean). Spike-timing precision was examined using phase angle, phase error, and spike jitter of single-trial responses to steady sine-wave stimuli. Phase angle was calculated from the time of the nadir of the trough of the input current time to the time of the first spike in the following half-cycle. The phase error is the SD of this spike latency expressed in degrees, whereas jitter is the SD of spike latency expressed in milliseconds. At the onset of steady sine-wave stimuli, during the first depolarizing half-cycle, the cells often fired more action potentials than in the remainder of the sine-wave stimulus. For this reason the first three-quarter cycle until the first trough in the sine wave was ignored in the spike-timing precision analysis. Values for phase angle, phase error, and jitter were averaged over all remaining cycles of a given 10-s steady sine-wave stimulus. The cells were either entrained by the stimulus or fired irregularly. To quantify the regularity of firing we adopted the modified coefficient of variation \( (CV_m) \) proposed by Holt et al. (1996): \( CV_m = 2 \times \text{abs}[(I_{t+1} - I_t)/(I_{t+1} + I_t)] \). This measure of variability for an interval at \( t = i \) seconds compares only two adjacent spike intervals \( I_{t+1} \) and \( I_t \). A perfectly regular spike train has a \( CV_m \) of 0, whereas spikes occurring randomly (Poisson process) have a \( CV_m \) of 1.
FIG. 1. Response of hypoglossal (XII) motoneurons (HMs) to steady sine-wave stimulation. A: representative examples of firing response of a HM to intracellular sine-wave stimulation (2–20 Hz). For 3 selected stimulus frequencies (2, 10, and 20 Hz; 10-s stimulus duration; 300-pA amplitude) the injected current (bottom) and corresponding membrane voltages (top) are shown (spikes are truncated at the top). In this neuron and in all subsequent experiments cells were depolarized to a membrane potential at or near firing threshold by DC current injections prior to the sine-wave current injections. Two sine-wave cycles from the traces on the left are shown on an expanded timescale on the right. B: graphs of the average number of spikes/sine-wave cycle (B1) and instantaneous firing frequency (B2) as a function of sine-wave stimulation frequency for the cell in A. Dotted straight lines mark a perfect 1:1 correspondence between input and output frequency. Instantaneous firing frequency during cycles when the cell fired more than one spike/cycle represent instantaneous firing during the burst averaged over all cycles in the stimulus. Note that this cell fires one spike/cycle and is able to faithfully code the sine-wave frequency of the input signal for frequencies between 8 and 16 Hz (≈1:1 phase-locking range). Inset (B1) shows membrane voltage responses of this cell to positive and negative DC current pulses recorded at rest.
Statistics

Statistical comparisons were performed using the t-test, with significance set at $P < 0.05$. Results are expressed as means ± SE.

RESULTS

For this study we obtained stable recordings from a total of 34 HMs. All neurons had stable resting membrane potentials and overshooting action potentials. Firing characteristics, determined from intracellular injection of DC depolarizing current pulses, were similar to those described in the XII nucleus in neonatal rat, including incrementing, decrementing, and mixed incrementing–decrementing firing patterns (Viana et al. 1995). As observed previously, cells showed hyperpolarizing membrane voltage “sag” of varying magnitude in response to hyperpolarizing current pulses (Fig. 1B1, inset) and in some cells rebound spikes were seen after cessation of the pulse (data not shown; see Viana et al. 1993).

Response of HMs to sine-wave stimulation

After the firing properties of the recorded HMs at rest were established we depolarized the cells to a membrane potential near or at firing threshold by DC current injection. We stimulated the cells with 10-s-long sine-wave current inputs (Fig. 1), superimposed on this steady DC current injection. This protocol was chosen to simulate the synaptic drive that HMs receive during inspiration, consisting of a depolarizing envelope on top of which membrane potential oscillations are superimposed (Parkis et al. 2003; Smith and Denny 1990). In addition, by bringing the cells to firing threshold we were able to reduce the amplitude of the sine-wave current, which greatly improved the stability of our recordings (see METHODS). All cells studied fired only during the depolarizing half-cycle of the sine current stimulus. We used two types of sine-wave stimulation protocols: one in which sine frequency was varied in steps (steady sine-wave stimulation) and another in which sine-wave frequency was varied continuously (swept sine wave or “ZAP” stimulation). Most cells in this study were stimulated with both protocols.

In response to steady sine-wave stimulation (varied stepwise between 2 and 20 Hz) HM neurons showed several distinct firing patterns depending on the input frequency ($n = 14$). At low input frequencies (generally <5 Hz) cells fired multiple spikes/cycle (i.e., “burst” mode), at intermediate frequency cells fired one spike/cycle, whereas at higher frequencies occasional cycle skipping occurred (Fig. 1A). By plotting the number of spikes per sine-wave cycle against frequency it was apparent that there was a range of input frequencies where the cells fired exactly one spike/cycle and this frequency range we called the 1:1 phase-locking range (Fig. 1B1). Instantaneous firing frequency was highest with low input frequencies when the cells fired in “burst” mode (Fig. 1B2). At intermediate sine frequencies the firing frequency of the cells faithfully followed the input frequency (1:1 phase-locking range; Fig. 1B2). Beyond the maximum firing frequency at which the cells were able to phase-lock 1:1 to the stimulus (i.e., the “critical” or maximum frequency, $F_{\text{max}}$) cells were unable to fire every cycle and the firing frequency of the cells was less than the input frequency. All 14 cells in this group showed the three distinct firing ranges (“burst” mode, phase-locking, and cycle skipping), but the sine frequency at which these firing patterns occurred varied from cell to cell. To study the firing patterns to sine-wave stimulation in more detail we stimulated a group of HMs ($n = 19$ cells) with ZAP functions. Firing patterns obtained with ZAP stimulation showed many similarities compared with those obtained with steady sine-wave stimulation, including “burst” firing, 1:1 phase-locking, and cycle skipping as shown by the representative cell in Fig. 2. A and B (same cell as in Fig. 1). When we plotted instantaneous firing frequency ($f$) against time ($t$), the resulting $f$–$t$ relationships clearly showed these distinct regions of firing responses. In “burst” mode (region “a” in Fig. 2A1) the cells fired more than one spike/cycle in a relatively high frequency burst during the depolarizing half-cycle of the sine wave. The length of the silent period between bursts (i.e., the reciprocal of the inter-burst frequency) reflected the frequency of the sine-wave input. At intermediate ZAP frequencies (between 7 and 21 Hz in the example shown in Fig. 2) the neurons abruptly transitioned from “burst” firing to firing one spike/cycle. Between this minimum frequency ($F_{\text{min}}$) and the maximum frequency ($F_{\text{max}}$) HMs coded the frequency content by phase-locking 1:1 to the input stimulus (Fig. 2A, region b). Beyond $F_{\text{max}}$ irregular or chaotic firing occurred due to occasional cycle skipping (Fig. 2A2, region c). At even higher ZAP frequency additional phase-locking regimes were revealed, often separated by regions of irregular firing (Figs. 2A2 and 3). Most cells were able to fire every second (1:2), every third (1:3), and sometimes every fourth (1:4) cycle, phase-locked to subharmonics of the fundamental frequency of the ZAP stimulus (Figs. 2B and 3). On average the minimum input frequency ($F_{\text{min}}$) for 1:1 phase-locking was 4.5 ± 0.36 Hz ($n = 19$ cells). The maximum frequency for 1:1 phase-locking ($F_{\text{max}}$) in this group of cells was 11.4 ± 0.93 Hz. Although in individual cells $F_{\text{min}}$ and $F_{\text{max}}$ were influenced by stimulus amplitude (see Fig. 4), average $F_{\text{min}}$ and $F_{\text{max}}$ values were not correlated with stimulus amplitude (average stimulus amplitude, 253 ± 22 pA; range 100–400 pA) in this group of HMs ($n = 19$ cells; Pearson correlation, $P > 0.05$). In addition, the maximum and minimum frequencies and width of the frequency range where HMs phase-locked 1:1 to the ZAP stimulus were not correlated with the input resistance of the cells (Pearson correlation, $P > 0.05$).

In individual cells in this group the size of the phase-locking range and the median ZAP frequency at which 1:1 phase-locking occurred were highly variable (Fig. 2C). In general, cells with wider phase-locking ranges also phase-locked at higher median input frequencies (Fig. 2D). Furthermore, the peak frequency achieved when the cells fired in “burst” mode correlated well with the median frequency for 1:1 phase-locking (Fig. 2D). These data suggest that the intrinsic firing properties of HMs constitute an important factor in determining phase-locking characteristics of individual cells. The minimum and maximum firing for the 1:1 phase-locking range were not different when steady-state sine wave or ZAP sine stimuli were compared in individual cells ($n = 9$ cells; $t$-test, $P > 0.05$).

These results show that the frequency information present in a time-varying input stimulus is encoded by precisely timed spikes when HMs phase-lock to the stimulus, indicating that in this frequency range they function as temporal encoders. The frequency range where this occurs varies from cell to cell.
Phase-locking region depends on DC depolarization and stimulus amplitude

Firing resonance in HMs depended on the level of DC membrane depolarization (Fig. 3A). When the cells were hyperpolarized from spike threshold the phase-locking regions became smaller and at high-input frequencies the cells stopped firing altogether (Fig. 3A1). Both the minimum and maximum frequencies of the 1:1 phase-locking range were influenced by the level of depolarization. On average, the minimum frequency for 1:1 phase-locking ($F_{\text{min}}$) increased by 2.1 ± 0.20 Hz and $F_{\text{max}}$ by 5.8 ± 1.03 Hz for a +10-mV membrane depolarization in the voltage range between rest and spike threshold ($n = 4$ cells; Fig. 3B). Since $F_{\text{max}}$ increased more than $F_{\text{min}}$, the 1:1 phase-locking range was expanded by 68% in this group of cells by this manipulation. DC depolarization also resulted in an expansion of the regions of irregular or chaotic firing that flanked phase-locking regions (Fig. 3A3, arrows).

Stimulus amplitude also influenced the shape of the $f$–$t$ relationship (Fig. 4A). With increased stimulus amplitude the instantaneous firing frequency and the frequency range over which the cells fired in “burst” mode increased. Peak instantaneous firing frequency in “burst” mode increased from 15.0 ± 1.7 to 22.2 ± 2.6 Hz for an increase in ZAP amplitude from 200 to 300 pA ($P < 0.05$, paired $t$-test, $n = 7$ cells). Increasing stimulus amplitude also shifted the 1:1 phase-locking region to higher frequencies (Fig. 4A). Mean $F_{\text{min}}$ and mean $F_{\text{max}}$ increased by 1.32 ± 0.47 Hz and 2.04 ± 0.44 Hz, respectively, for a 100-pA increase in ZAP amplitude from 200 to 300 pA (both increases were significant at $P < 0.05$, paired $t$-test, $n = 9$ cells; Fig. 4C). Although $F_{\text{max}}$ increased more on average than $F_{\text{min}}$, the resulting 11% increase in width of the 1:1 phase-locking range by increasing stimulus amplitude by 100 pA was not significant ($P > 0.05$). Increasing stimulus amplitude also increased the irregular firing regions at the transition between phase-locking regions (Fig. 4A).
example shown in Fig. 4, the cell was able to smoothly switch between 1:1 and 1:2 phase-locking with a 100-pA peak-to-peak amplitude stimulus (Fig. 4B, top trace), but fired irregularly between 1:1 and 1:2 phase-locking when the ZAP stimulus amplitude was doubled (Fig. 4B, bottom trace). These results indicate that the spike-firing rate carries information about the stimulus amplitude when the input frequency is low and the cells fire in “burst” mode (so-called rate coding). The timing of individual spikes carries information about the frequency content of the stimulus when the cells are in “phase-locking” mode (“temporal coding”). In this mode a change in spike-firing frequency requires a change in input frequency. The amplitude of the input stimulus influences at what frequency the switch from rate to temporal coding occurs and also the frequency range for phase-locking and cycle skipping.

Although there was hysteresis in the number of spikes/cycle when forward and reverse stimuli were compared in the same cell (forward ZAP stimuli overall elicited more firing; see Fig. 6), we found that the range for 1:1 phase-locking was similar for these types of stimuli. $F_{\text{min}}$ and $F_{\text{max}}$ for forward and reverse ZAP stimuli were not significantly different ($n = 10$ cells; paired $t$-test, $P > 0.05$).

**Reliability and spike-timing precision**

Synchronicity of firing of assemblies of neurons depends critically in the ability of the cells to fire with high reliability and precision to repeated stimuli. We examined these properties in HMs by repeatedly stimulating HMs with ZAP stimuli. Such an experiment is illustrated in Fig. 5. This cell was
repeatedly (10 trials) stimulated with a 0–20 Hz ZAP function. From the rasterplots (Fig. 5, A and B) and superimposed f–t curves (Fig. 5C) it is clear that this cell fired consistently at a specific sine-wave cycle from trial to trial from the beginning of the stimulus to the end of the 1:1 phase-locking range, but less consistently afterward. This is further demonstrated in Fig. 5D, which shows that the variability in the average number of spikes/cycle (as measured by the coefficient of variation) is smallest when the cell phase-locks 3:1 (burst mode) or 1:1 to the stimulus. Repeatability from trial to trial did not have the same frequency profile as reliability, however. Although the cell in Fig. 5 fired three spikes at every trial at the first cycle of the stimulus (i.e., high repeatability), it did so with low temporal precision (Fig. 5B, left). When the cell was entrained 1:2 the opposite was true, it now fired with high precision but low repeatability because it did not always fire in the same cycle from trial to trial (Fig. 5B, right). Low precision at low ZAP frequencies and low repeatability at high frequencies resulted in roughly bell-shaped reliability curves (Fig. 5E). Reliability (which depends on both the presence and timing of a spike and was expressed as the number of spikes/bin/stimulus; see METHODS) peaked when the cell phase-locked 1:1 to the stimulus at intermediate input frequencies. Experiments similar to those shown in Fig. 5 were performed in eight other HMs. Peak reliability, as determined from cubic spline fits to PSTH data, was found at an average input frequency of 8.68 ± 1.38 Hz (n = 9 cells; ZAP current peak-to-peak amplitude between 150 and 400 pA). Peak reliability was 0.82 ± 0.05 spike/bin/stimulus and in all but one of these neurons peak reliability occurred within the 1:1 phase-locking region. We found that spike reliability curves were similar when repeated steady sine-wave stimuli were compared with repeated ZAP stimuli in the same neuron (n = 3 cells; data not shown).

In three HMs we compared spike reliability as determined from repeated forward (0–20 Hz) or reverse (20–0 Hz) ZAP stimuli. Although HMs fired on average more spikes/cycle with a forward compared with a reverse ZAP stimulus, especially at low sine frequencies (compare Fig. 6, A1 and A2), peak reliability was achieved in the frequency range where the cells phase-locked 1:1 with both types of stimuli (Fig. 6, A and B).

We tested the effect of stimulus amplitude on spike reliability in two HMs. In both cells overall spike reliability increased when stimulus amplitude was increased (see example in Fig. 6C) and the frequency range of high spike reliability was expanded.

In summary, the low reliability at low- and high-input frequencies suggests that HMs act as a band-pass filter and not as a low-pass filter, with highest reliability in the intermediate 1:1 phase-locking range. Spike reliability and the frequency where reliability is highest are functions of stimulus amplitude.

The timing of spikes within individual cycles varied with input frequency when the cells were stimulated with steady sine-wave stimuli (Fig. 7). Spiking occurred early, during the rising phase of the sine-wave stimulus, when the input sine frequency was low (phase advance; Fig. 7A2). The phase advance gradually decreased with increasing input frequency until a delay of zero (= “preferred” frequency) was reached. At even higher input frequency spikes were delayed with respect to the sine-wave stimulus (phase delay). The preferred fre-
FIG. 5. Reliability and precision are a function of sine-wave frequency. A: rasterplots marking the occurrence of spikes for each trial of a neuron that was repeatedly stimulated with ZAP current pulses (10 trials; 0–20 Hz; 10-s duration; 400-pA amplitude). Before ZAP stimulation began the cell was depolarized to just below threshold membrane voltage by injection of steady depolarizing current (+150 pA) and kept at this potential for the duration of the experiment. Rasterplots were aligned at the start of the stimulus (T = 0 s). The ZAP current stimulus is shown at the bottom. B: superimposed membrane voltage trajectories (top) and the corresponding rasterplot section (middle) and ZAP current stimulus (bottom) shown on an expanded timescale at the times indicated by a, b, and c in A. Note the trial-to-trial jitter in the spike timing when the cells fired 3 spikes/cycle (3:1) at a, whereas when the cell fired 1:2 at c it did not always fire at the same cycle. C: superimposed f–t plots (10 trials) constructed from the rasterplot in A. Dashed straight line shows the frequency of the ZAP stimulus as a function of time. Note that the minimum frequency for 1:1 phase-locking varies little from trial to trial, but that the maximum frequency is much more variable. D: average number of spikes per cycle (red trace; left axis) and the corresponding coefficient of variation (black trace; right axis) plotted against cycle number of the ZAP stimulus for the cell shown in A. Note that the cell always fired a spike in every trial for cycles where the cell phase-locked 3:1 or 1:1 to the stimulus (CV = 0), and that the variability of the number of spikes/cycle is high when the cell fired fewer than one spike/cycle at frequencies higher than the 1:1 phase-locking range. The absence of data points between cycles 90 and 95 is due to the off-scale CV values. E: peristimulus time histogram (PSTH) constructed from the number of spikes/bin/stimulus for the response of the cell in A to repeated ZAP stimuli. Spikes were binned (3-ms bin width) using the rasterplots shown in A and the number of spikes that were collected in each bin, divided by the number of trials, were expressed as a function of time (bottom axis) and ZAP frequency (top axis). Only bins with more than one spike were counted. The contour of the histogram was fitted with a peak-normalized cubic spline function. Note that although this cell fired with high reliability when it fired 3:1 (see B, section a), spike-timing precision was such that few spikes fell in the same bin at this ZAP frequency. Spike-timing precision is highest for the frequency range where this cell was able to phase-lock 1:1 to the stimulus (2–12 Hz) and peaked near the middle of this range at 8 Hz (arrow). When the cell phase-locked 1:2 to the stimulus, between 15 and 20 Hz, spike-timing precision was high, but repeatability was low (B, section c), resulting in a decrease in reliability at these input frequencies.
In this study we examined whether HMs show firing resonance in response to time-varying sinusoidal inputs. We found that HMs are capable of encoding these types of stimuli into highly reproducible spike trains, depending on the sine-wave frequency. Over a limited range of frequencies (3–25 Hz) HMs were able to phase-lock 1:1 to the sine-wave stimulus. HMs function as rate encoders at low sine frequency, where they fire in “burst” mode, and as temporal encoders at higher sine frequencies when they phase-lock to the stimulus. In phase-locking mode the pattern and timing of the spikes carry information about the frequency of the input sine-wave current. Our results show that spike-timing precision and reliability were greatest in the phase-locking range. Areas of low reliability flanked the phase-locking range, indicating that HMs act as band-pass filters.

Spike-timing reliability and precision have been shown to be frequency dependent in other neuronal types in the brain (Fellous et al. 2001; Leung and Yu 1998; Pike et al. 2000; Schreiber et al. 2004b). The range of frequencies where precision and reliability are highest in HMs is similar to the preferred frequency range in hippocampal (Leung and Yu 1998; Pike et al. 2000) and cortical pyramidal cells (Fellous et al. 2001), but lower than that in hippocampal and cortical interneurons (Fellous et al. 2001; Pike et al. 2000). The preferred frequency is a function of the properties of the ionic currents responsible for action potential generation, ISI duration, and spike-frequency adaptation (Fuhrmann et al. 2002; Schreiber et al. 2004b). Differences in the composition of ion channels are most likely responsible for differences in preferred frequencies among neuronal cell types. For instance, slow potassium channels have been shown to be important for setting the preferred frequency (Fellous et al. 2001; Schreiber et al. 2004b). In modeling studies (Schreiber et al. 2004b) it was found that a higher peak conductance for slow potassium channels, such as the muscarinic potassium channel (K_{M}) or a calcium-dependent potassium channel (K_{Ca}), decreased the preferred frequency of cortical neurons (but increased spike firing in this study we examined whether HMs show firing resonance in response to time-varying sinusoidal inputs. We found that HMs are capable of encoding these types of stimuli into highly reproducible spike trains, depending on the sine-wave frequency. Over a limited range of frequencies (3–25 Hz) HMs were able to phase-lock 1:1 to the sine-wave stimulus. HMs function as rate encoders at low sine frequency, where they fire in “burst” mode, and as temporal encoders at higher sine frequencies when they phase-lock to the stimulus. In phase-locking mode the pattern and timing of the spikes carry information about the frequency of the input sine-wave current. Our results show that spike-timing precision and reliability were greatest in the phase-locking range. Areas of low reliability flanked the phase-locking range, indicating that HMs act as band-pass filters.

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reliability). In contrast, an increase in sodium conductance, such as the fast (Na_f) and persistent (Na_p) sodium currents, increased the preferred frequency (but decreased reliability). These conductances and their kinetics also influenced the width of the 1:1 phase-locking range in these studies. It is conceivable that the wide ranges of preferred frequencies and widths of phase-locking ranges that we found in our study are a reflection of the heterogeneity in density and/or kinetics of these conductances among hypoglossal motoneurons. Neuromodulation of ion channels can alter the preferred frequencies and increase
spike-timing precision and reliability by adapting the intrinsic oscillatory properties of neurons to facilitate entrainment of these cells to certain input frequencies (Lawrence et al. 2006).

Phase-locking depends on input frequency, stimulus amplitude, and level of depolarization

When HMs were brought close to firing threshold, spike firing was entrained by the sine-wave input, depending on sine-wave frequency. At low input frequency HMs fired highly repeatable, stereotypical “bursts” of action potentials during the depolarizing phase of the sine-wave cycle (i.e., more than one spike/cycle). This burst firing mode is most suitable to reliably evoke a postsynaptic response when the timing of the spikes is not important, since spike-timing precision in burst mode was low. This study and those by others (Carandini et al. 1996; Hunter and Milton 2003; Schreiber et al. 2004b) show that small increases in the input frequency can lead a neuron to shift from coding spike rate (burst mode) to coding spike timing (phase-locking mode). Phase-locking was most prevalent at intermediate and high-input frequencies, but this frequency range varied from cell to cell. Phase-locked responses could result in the firing of exactly one spike/cycle (1:1 mode), or in one spike for each n cycle(s) of the sine-wave stimulus (with n being an integer number ≥2). This phase-locked firing mode was characterized by high temporal precision. Phase-locking did not always result in high reliability since the cells did not always fire in the same cycle when cycle skipping was present (1:n mode), similar to the results obtained by Hunter and Milton (2003). Thus the 1:1 phase-locking region is optimized for encoding input signals with both high reliability and high precision.

The range of frequencies where the cells phase-locked depended on the mean level of depolarization and the amplitude of the sinusoidal inputs. The relationship between reliability, membrane depolarization, stimulus amplitude, and input frequency has been explored in several types of neurons (Beierholm et al. 2001; Fellous et al. 2001; Hunter and Milton 2003; Smith et al. 2000). In general, these studies show that the most robust areas of high reliability are found when the cell phase-locks 1:1 to the stimulus using intermediate relative stimulus amplitude. Small-amplitude input fluctuations are unreliable because under these conditions noise becomes the most important determinant of spike timing (Hunter and Milton 2003). The preferred frequency range increased with increasing amplitude of the input stimulus, similar to results obtained by others (Carandini et al. 1996; Fellous et al. 2001), but large-amplitude voltage fluctuations eventually lead to cell damage and decreased reliability (Fellous et al. 2001). Thus intermediate-amplitude signals in the band-pass frequency range delivered when the neurons are depolarized to near spike threshold will be transmitted more reliably than those outside the band-pass frequency range or when the cells are hyperpolarized.

Several studies have suggested that firing resonance emerges from subthreshold resonance properties (Erchova et al. 2004; Hutcheon et al. 1996; Schreiber et al. 2004a), but others have not found such a clear correlation (Brumberg and Gutkin 2007; Carandini et al. 1996; Haas and White 2002; Nowak et al. 1997). These discrepancies might be explained in part by differences in subthreshold resonance properties between cell types (Erchova et al. 2004; Haas and White 2002). In resonant cells, the amplitude of their membrane potential responses to time-varying subthreshold currents peaks at the resonance frequency, whereas nonresonant cells act as low-pass filters. In resonant cells firing occurs preferentially at the subthreshold resonance frequency when they are stimulated with a supra-threshold stimulus. Subthreshold resonance has been described...
in HMs that peaks at 4 Hz (Nguyen et al. 2004). Our own results indicate that firings in HMs are either not resonant or only weakly resonant in the subthreshold range (unpublished observations). HMs therefore act predominantly as low-pass filters in the membrane voltage range between rest and spike threshold for input frequencies \( >3-5 \) Hz. This property is responsible for the cessation of firing at high frequencies that was observed in many HM neurons when they are moved away from spike threshold by DC current injection (see Fig. 3A1).

The frequency range where firing occurs can be widened by increasing the stimulus amplitude or by membrane depolarization. At depolarized membrane potentials small-amplitude currents can more easily generate spikes and the cell fires at higher input frequencies despite low-pass filtering of the input signal (Hutcheon et al. 1996). The firing resonance range can also be increased by the addition of stochastic noise to the sine-wave input in thalamocortical neurons (Reinker et al. 2004). Whether this is also the case in HMs remains to be established.

**Relationship between spike jitter, phase, and input frequency**

Our results show that HMs show phase advance at low-frequency stimulation and phase delay at high frequency, separated by the preferred frequency at which no phase shift occurs. Similar phase-frequency curves have been described for thalamic relay neurons (Smith et al. 2000), regular spiking cortical neurons (Carandini et al. 1996), and hippocampal CA neurons (Leung and Yu 1998). The mechanism behind this property has been examined in modeling studies (Fuhrmann et al. 2002) and has been explained as an interplay between two opposing mechanisms: spike-frequency adaptation and the time constant of firing rate dynamics. Firing rate dynamics causes a neuron to delay its response to stimulation and dominates at high frequency. Spike-frequency adaptation causes the response to a sustained input to gradually decrease with time. The slow rising phase with low-frequency sinusoidal stimulation leads to accumulation of adaptation and thus the firing probability will be higher before the peak of the sinusoidal stimulus than after, leading to a phase advance. At the preferred frequency (phase = zero) these two mechanisms balance each other (Fuhrmann et al. 2002).

Our results show that spike jitter generally decreases with increasing sine frequency. Studies in neocortical neurons have shown that spike jitter is less for high-frequency than that for low-frequency input (Mainen and Sejnowski 1995; Nowak et al. 1997). The mechanism behind this observation is not known, but it is has been suggested that increased timing precision at high stimulus frequencies is related to the time that the neuron spends near spike threshold. The time spent near threshold is brief for high- than that for low-frequency stimulation. Therefore at high-frequency stimulation there is only a small probability that random fluctuations in membrane potential will exceed spike threshold and thus spike jitter is reduced (Nowak et al. 1997).

Regions of phase-locking, flanked by regions of irregular firing when HMs were driven with a time-varying stimulus, have also been described in supragranular pyramidal cells under similar conditions (Brumberg 2002; Brumberg and Gutkin 2007). Together this firing pattern forms a so-called devil’s staircase. The regions of irregular firing are influenced by stimulus amplitude and level of depolarization and they are characterized by low precision and reliability. Our studies suggest that there exists an optimal level of depolarization and stimulus amplitudes where in response to sine-wave stimuli irregular firing in HMs can be largely avoided. If the oscillation frequency or the power of the oscillations change (for some examples of this behavior see Sebe et al. 2006), HMs will have to adjust their encoding to maintain phase-locking to the stimulus. A widest possible phase-locking range with “smooth” transitions between 1:1 and 1:n phase-locking ranges will ensure that HMs will be able to maintain synchrony over a wide range of time-varying inputs while avoiding regions of irregular firing. The 1:1 phase-locking region is most robust in the presence of noise (Hunter and Milton 2003), but whether in vivo synaptic noise stabilizes firing in HM neurons remains to be investigated.

**Importance of HM spike timing and precision for synchronous oscillation in HM output**

The motoneurons in the hypoglossal nucleus are part of the brain stem inspiratory network. From studies performed in *in vitro* preparations in neonatal or juvenile rodents it is known that inspiratory motoneurons are driven by a slow depolarizing envelope on top of which fast (20- to 50-Hz) membrane potential oscillations are superimposed (Funk and Parkis 2002; Parkis et al. 2003). Our results indicate that with time-varying synaptic input in this frequency range HMs operate in the phase-locking mode. Short-timescale inspiratory-phase oscillations in this frequency range recorded from the hypoglossal nerve during these inspiratory bursts are characterized by clusters of action potentials that occur at a regular interval separated by periods of little or no spike firing (Sebe et al. 2006), suggesting that HMs fire synchronously. In phase-locking mode groups of HMs are able to produce precise and reliable spike output, which would transform common oscillatory synaptic input into synchronous volleys of spikes. High reliability of spike timing enhances synchronization of groups of functionally related neurons activated by a common synaptic input (Usrey and Reid 1999).

The function of inspiratory-phase synchronous oscillations in the brain stem is not known. Inspiratory-phase synchronous oscillations are not required for respiratory rhythm generation, but they are important in shaping the pattern of inspiratory discharge. At the motoneuron level, oscillations increase the input–output efficiency of and the precision of spike timing (Parkis et al. 2003). Oscillations provide for a mechanism that avoids accumulation of spike jitter, as occurs with DC stimuli (Mainen and Sejnowski 1995), by alternating spiking and nonspiking periods. Membrane hyperpolarization between half-cycles allows for recovery from inactivation of voltage-activated ion currents involved in spike generation. Phrenic motoneurons tend to fire within a couple of milliseconds from the short-timescale oscillatory peaks in inspiratory synaptic drive (Parkis et al. 2003). This timescale is similar to the minimum in spike jitter that we found in this study when the cells phase-lock to the stimulus (see Figs. 7 and 8). The DC current injection that we used in our study mimics the membrane depolarization of the slow depolarizing inspiratory envelope, but it did not account for the increased membrane conductance accompanying this excitatory synaptic drive. It is possible that the increased conductance—and associated decrease in mem-
brane time constant—will improve spike-timing precision and shift the optimum frequency for phase-locking to input frequencies higher than those we found in our quiescent, synaptically isolated slice preparation.

Similar to what has been found in spinal interneurons (Beierholm et al. 2001), it appears that the combination of long- and short-timescale synaptic inputs to HMs during inspiration reliably drive the cells with high timing precision. The depolarizing envelope during inspiration brings the cells close to spike threshold, so that high reliability can be achieved with relatively small amplitude membrane voltage fluctuations (similar to a very low frequency sine-wave input stimulus). The high-frequency oscillation on top of this depolarizing envelope serves to give high spike reliability and precision.

Power spectra of inspiratory oscillations recorded in vivo from motor nerves in adult animals show two peaks in the 20- to 50- and 50- to 150-Hz ranges (Richardson and Mitchell 1982; Smith and Denny 1990). Single-fiber recordings from the phrenic nerve during inspiratory activity showed that the discharge frequency of individual motoneurons is lower than that of the whole nerve during short-timescale oscillations in vivo (Richardson and Mitchell 1982). In these studies it was found that single fibers sometimes skipped cycles, firing every second, third, or fourth cycle. It is possible that individual HMs in vivo phase-lock to subharmonics of the fundamental oscillation frequency, skipping cycles and firing at a lower frequency than the population fundamental. We hypothesize that, even though spikes are not elicited at every cycle in individual HMs, the high-frequency input signal still serves to synchronize neurons by reducing jitter and improving spike timing. It would therefore be of interest to establish the firing resonance properties of individual HMs during inspiration in vivo and to relate these to the motor output of the XII nucleus as a whole.

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