The Whisking Rhythm Generator: A Novel Mammalian Network for the Generation of Movement

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Cramer NP, Li Y, Keller A. Whisking rhythm generator: novel mammalian network for the generation of movement. J Neurophysiol 97: 2148–2158, 2007. First published January 3, 2007; doi:10.1152/jn.01187.2006. Using the rat vibrissa system, we provide evidence for a novel mechanism for the generation of movement. Like other central pattern generators (CPGs) that underlie many movements, the rhythm generator for whisking can operate without cortical inputs or sensory feedback. However, unlike conventional mammalian CPGs, vibrissa motoneurons (vMNs) actively participate in the rhythmogenesis by converting tonic serotonergic inputs into the patterned motor output responsible for movement of the vibrissae. We find that, in vitro, a serotonin receptor agonist, α-Me-5HT, facilitates a persistent inward current (PIC) and evokes rhythmic firing in vMNs. Within each motoneuron, increasing the concentration of α-Me-5HT significantly increases the both the magnitude of the PIC and the motoneuron’s firing rate. Riluzole, which selectively suppresses the Na+ component of PICs at low concentrations, causes a reduction in both of these phenomena. The magnitude of this reduction is directly correlated with the concentration of riluzole. The joint effects of riluzole on PIC magnitude and firing rate in vMNs suggest that the two are causally related. In vivo we find that the tonic activity of putative serotonergic premotoneurons is positively correlated with the frequency of whisking evoked by cortical stimulation. Taken together, these results support the hypothesized novel mammalian mechanism for movement generation in the vibrissa motor system where vMNs actively participate in the rhythmogenesis in response to tonic drive from serotonergic premotoneurons.

INTRODUCTION

The ability to move allows an organism to adapt and respond to its environment. Indeed, a primary goal in processing sensory inputs is to select an appropriate motor act in response to those inputs. Often the patterned activation of muscles required to generate a movement is produced subcortically by neural elements that can operate without patterned inputs from higher brain centers or sensory feedback. In mammals, these critical components of motor networks, or central pattern generators (CPGs), are typically composed of groups of interneurons that generate rhythmic drive to motoneurons that innervate the target muscles (Arshavsky et al. 1997; Stein et al. 1997). The motoneurons themselves are not considered part of the rhythm-generating network (Orlovsky et al. 1999). Here, using the rat vibrissa motor system, we provide evidence for an alternative mechanism for the generation of movement in mammals where the motoneurons are involved in the rhythmogenesis.

Rats explore their environment by rhythmically palpating objects with their mystacial vibrissae (Brecht et al. 1997; Vincent 1912; Welker 1964). These rhythmic movements, or whisking, persist after sensory denervation (Welker 1964), cortical ablation (Gao et al. 2003; Semba and Komisaruk 1984), or decerebration (Lovick 1972), suggesting that the whisking motor pattern is produced subcortically by a CPG. We demonstrated previously that serotonin (5-HT) drives vibrissa motoneurons (vMNs) at whisking frequencies and that infusion of 5-HT receptor antagonists onto vMNs suppresses voluntary whisking (Hattox et al. 2003). On the basis of these results, we hypothesized that 5-HT is both necessary and sufficient for rhythmic whisking and that, in this system, the motoneurons are part of the rhythm-generating network. The experiments described here were designed to test this hypothesis.

In spinal (Perrier and Hounsgaard 2003) and trigeminal (Hsiao et al. 1998) motoneurons, 5-HT acts as a potent facilitator of inactivation-resistant inward currents. First discovered in cat spinal motoneurons (Schwindt and Crill 1980), these persistent inward currents (PICs) modulate the firing frequencies of motoneurons (Heckman et al. 2003; Schwindt and Crill 1982). Thus by activating and amplifying PICs, 5-HT is reported to modulate firing in motoneurons (Harvey et al. 2006b; Heckman et al. 2005; Hounsgaard and Kiehn 1989; Houns-gaard et al. 1988; Hsiao et al. 1998). Here, we test the hypothesis that 5-HT is sufficient to generate rhythmic firing in vMNs through a graded facilitation of PICs. In addition, we test the prediction that the tonic output from serotonergic premotoneurons is positively correlated with whisking frequencies. Our findings support the hypothesis that vMNs generate the rhythmicity of the whisking motor pattern in response to a graded facilitation of a PIC by 5-HT. Furthermore, they suggest that, in this system, there exists a novel mammalian mechanism for movement generation.

METHODS

In vitro slice preparation

We cut 500-μm-thick coronal brain stem slices from P13 to P18 Sprague-Dawley rats (i.e., after the onset of voluntary whisking Welker 1964). During the isolation of the brain stem and cutting of slices, the tissue was submersed in ice-cold modified artificial cerebrospinal fluid (ACSF) in which sucrose was substituted for NaCl. The sucrose-ACSF was composed of (in mM) 248 sucrose, 26 NaHCO3, 1.25 NaH2PO4, 3 KCl, 5 MgSO4, 1 CaCl2, and 15 glucose. We allowed the slices to recover for 1 h in a holding chamber containing warmed normal ACSF (32°C), aerated with 95% O2-5%
CO₂. The normal ACSF was composed of (in mM) 124 NaCl, 25 NaHCO₃, 5 N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 3 KCl, 1.3 MgSO₄, 2.0 CaCl₂, and 15 glucose. For recording, we transferred individual slices to a recording chamber and continuously perfused the slice with normal ACSF (3–4 ml/min).

Whole cell recordings

Visually guided whole cell patch-clamp recordings were made from motoneurons in the lateral subdivision of the facial nucleus with the use of near-infrared differential interference-contrast microscopy. This region contains motoneurons innervating the intrinsic muscles of the vibrissae (Hattox et al. 2003; Klein and Rhoades 1985; Sembu and Egger 1986). Recordings were obtained with an EPC-10 amplifier (HEKA Instruments), digitized at 20 kHz with an A/D board (ITC-18; Instrutech, Great Neck, NY) using PatchMaster software (HEKA), and stored on PC. The impedance of patch electrodes was 3–5 MΩ. The intracellular recording solution contained (in mM) 120 K-glucconate, 10 KCl, 10 HEPES, 1 MgCl₂, 2.5 MgATP, 0.2 Tris-GTP, 0.1 bis-(O-aminophenoxy)-N,N,N',N'-tetraacetic acid (BAPTA), and 5.2 biocytin (pH adjusted was 7.3 with KOH).

The following pharmacological agents were obtained from Sigma-Aldrich (St. Louis, MO): d(-)-2-amino-5-phosphonopentanoic acid (AP5; applied at 50 μM to the perfusate), 6-cyano-7-nitroquinoline-2,3-dione disodium (CNQX; 20 μM), gabazine (10 μM), tetrodotoxin (TTX; 1 μM), α-Me-5HT (1–60 μM), and riluzole (2–5 μM).

Cells were filled with biocytin (Molecular Probes, Eugene, OR) through the recording pipette and slices were fixed overnight in a buffered solution containing 4% paraformaldehyde. To visualize labeled cells, slices were reacted with the Vectastain ABC kit (1:1000; Vector Labs, Burlingame, CA) and 3–3’ diaminobenzidine (DAB; 0.5 mg/ml), ura H₂O₂ (0.3 mg/ml), and CoCl₂ (0.2 mg/ml) in 0.05 M Tris buffer containing 0.5 M NaCl.

Analysis of PICs

The magnitude and activation voltage of PICs in vMNs were determined using a slow triangular voltage ramp command. The ascending phase of the voltage command consisted of a linearly increasing ramp (speed: 8–16 mV/s) for 5 s. The descending phase returned the membrane potential to 90 mV at the same rate. For each cell, we selected the fastest single ramp speed that minimized the occurrence of breakthrough action potentials in all conditions (control and drug).

All analyses were performed on leak-subtracted currents, generated by subtracting the predicted ohmic leak currents from the measured currents. The leak currents were determined by multiplying the subthreshold membrane conductance (i.e., the membrane conductance prior to activation of voltage gated channels) by the voltage command. We monitored each cell’s series resistance throughout the experiments and rejected from analysis cells in which the series resistance changed by >10%.

Surgical procedures

We used nine female Sprague-Dawely rats (225–270 g) for in vivo experiments. All procedures strictly adhered to Institutional and Federal guidelines. Under halothane (2.5%) anesthesia, a small incision was made in the facial skin, and a pair of bipolar EMG electrodes (76 μm Teflon-coated stainless steel wire) was tunneled subcutaneously into the deep intrinsic muscles. Prior to insertion, the electrode tips were bent into a hook to prevent shifts in location during movements. After infusion of local anesthetics to surgical sites, we performed craniotomies over the vibrissae motor cortex (vMCx) and the lateral paragigantocellularis (LPGi) nucleus. The exposed dura was kept from drying by using dental wax to build a saline filled well over the craniotomies. During recordings, rats were maintained at anesthetic level III-2, characterized by an ECoG frequency between 5 and 7 Hz, a respiratory rate of ~100 breaths/min, and an absence of a withdrawal reflex to noxious stimuli (paw or tail pinch) (Friedberg et al. 1999). As the threshold for evoking whisking depended on the depth of anesthesia, care was taken to maintain a consistent and stable level of anesthesia. Body temperature was maintained at 37°C with a servo-controlled heating blanket.

Cortical stimulation

We evoked rhythmic whisking through intracortical microstimulation (ICMS) of the rhythmic subregion (RS) of vMCx as in our previous study (Cramer and Keller 2006). Custom-made platinum-in-quartz electrodes (~20 μm tip) were lowered to a depth of 1.5 mm, corresponding to layer V of vMCx. The RS of vMCx was identified by applying low-intensity current pulses (50 monophasic pulses at 50 Hz, 200-μs pulse width, 25–250 μA) to the area of vMCx identified by Haiss and Schwarz (2005). Stimulation in this region evoked rhythmic vibrissae movements, whereas stimulation outside of this region either generated small vibrissal retractions or failed to produce movements. RS was reliably located at anteroposterior 1.3, mediolateral 1.3 relative to bregma, as previously documented (Cramer and Keller 2006; Haiss and Schwarz 2005).

Recording and analyses

We obtained extracellular recordings using a NeuroData IR183A amplifier (Cygnus Technology, Delaware Water Gap, PA), digitized at 40 kHz with an A/D board (ITC-18; Instrutech) using software custom written in IGOR (WaveMetrics, Lake Oswego, OR), and stored on PC. Recordings from putative serotoninergic premotoneurons were obtained from pulled borosilicate glass electrodes (~2 μm tip, 2–20 MΩ) stereotaxically guided to the rostral (juxtasial) LPGi, (anteroposterior 11.4 mm, lateralmedial 1.0 mm, relative to bregma).

To analyze the responses of putative premotoneurons to ICMS of vMCx, we isolated the action potential waveforms of each unit using Off-line Sorter (Plexon, Dallas, TX). The time stamps of individual units along with the corresponding stimulus triggers were imported into IGOR for further analyses.

We defined a premotoneuron response as significant if the number of action potentials fired during stimulation exceeded the baseline activity by 2 SD. The baseline activity for each cell was defined as the number of spikes fired during the 1-s interval prior to stimulation averaged over all trials. To measure response latency, we constructed peristimulus time histograms (PSTHs, bin size = 1 ms) from the premotoneuron timestamps and stimulus triggers. The onset latency was defined as the first occurrence of two consecutive bins that exceeded the baseline activity by 2 SD.

Boths of rhythmic whisking evoked by ICMS of vMCx were recorded using the implanted vibrissal EMG electrodes. EMG recordings were amplified (Model 1700 Differential AC Amplifier, A-M Systems, Carlsborg WA) sampled at 40 kHz with an A/D board (ITC-18, Instrutech), filtered (1 Hz to 5 kHz), and stored on a computer running software written in Igor. We defined the whisking frequency as the peak of the power spectral density generated from the rectified, filtered EMG data, sub-sampled to 500 Hz.

RESULTS

5-HT drive determines vMN firing rates

Using a brain stem slice preparation, we first tested the prediction that individual vMNs generate a range of firing frequencies in response to varying levels of serotonergic drive. The in vitro preparation is advantageous because it permits application of pharmacological agents with a greater accuracy
than can be achieved in vivo. To simulate endogenous serotonergic drive to vMNs, we bathed the serotonin type 2 (5-HT$_2$) receptor agonist, α-Me-5HT, to the brain stem slices. We focused on this receptor subtype because when activated, it produces rhythmic firing in vMNs, and its antagonist (metergolene) suppresses rhythmic whisking (Hattox et al. 2003). We examined whole cell recordings from 50 vMNs in this experiment, all of which lacked spontaneous activity in control conditions. In support of our prediction, vMNs fired rhythmic trains of action potentials at whisking frequencies (1.5–17 Hz; Figs. 1 and 4) in response to trains of action potentials at whisking frequencies (1.5–17 Hz; Figs. 1 and 4) in response to the firing frequency evoked in individual neurons (Prather et al. 2001). We therefore hypothesized that in vMNs, 5-HT produces firing by activating PICs. Because the firing rate of neurons, including motoneurons, is closely related to the magnitude of inward current reaching the soma (Powers and Binder 2001). Neuromodulators that regulate membrane currents can alter this current-frequency relationship and are thus capable of regulating the cell’s firing frequency (Powers and Binder 2001; Rekling et al. 2000). One important neuromodulator is 5-HT, which, acting through 5-HT$_2$ receptors, potently facilitates slowly or noninactivating voltage-dependent inward currents (Harvey et al. 2006a; Heckman et al. 2005; Perrier and Hounsgaard 2003). These PICs have a significant impact on the firing rates of some motoneurons (Prather et al. 2001). We therefore hypothesized that in vMNs, 5-HT produces firing by activating PICs. Because the beginning of drug application and onset of firing may primarily reflect the time required for the drug to diffuse through the tissue to reach the cell. We computed firing rates from the last third of the drug application period (highlighted in red in Fig. 1B) by constructing histograms (bin size = 0.25 Hz) of instantaneous firing frequencies from this period, which we defined as the steady state (Fig. 1C). We defined the magnitude of responses at steady state as the peak of these histograms and identified the whisking frequency at which this peak occurred. We defined the half-width at half-maximum of each histogram as the SD of the steady-state firing rate. Similar results were found for 15 of 16 vMNs tested, as shown in Fig. 1D. For these cells, the higher agonist concentration evoked a significantly higher firing rate (Student’s t-test, $P < 10^{-3}$). In one cell only, the firing rates at the two concentrations were not significantly different ($P = 1$). These findings support the hypothesis that vMNs generate the whisking frequency in response to a varying degree of drive from serotonergic premotoneurons.

vMNs express PICs

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firing rate of vMNs is determined by the magnitude of 5-HT₂ receptor activation (see preceding text), we further predicted that regulation of vMN firing is achieved through a graded facilitation of these PICs.

The presence of a PIC in a vMN is demonstrated by the data depicted in Fig. 2A. To better isolate the persistent currents, we used protocols with slow voltage ramps that minimize the activation of potentially confounding transient Na⁺ currents (Lee and Heckman 1998). Starting from a holding potential of −60 mV, we ramped the membrane potential to −49 mV and held the cell at this potential for three seconds. We examined the voltage dependence of the currents activated by this protocol by increasing the holding potential in 3-mV increments. At more depolarized membrane potentials, the increase in net outward current was reduced (Fig. 2A, middle). Examination of the leak subtracted currents (top) reveals that this reduction resulted from a slowly or noninactivating inward current that lasted for the duration of the voltage command (3 s). Currents with these kinetics and voltage dependence are defined as PICs (Schwindt and Crill 1982).

To quantify these PICs, we used a slowly increasing (8–16 mV/s) triangular voltage command. Like the ramp-and-hold protocol described in the preceding text, this slow-ramp protocol suppressed activation of fast Na⁺ spikes. In addition, the slow ramp is advantageous in that using a single voltage command, it provides information simultaneously about both the PIC activation voltage and magnitude. A representative recording using this protocol is shown in Fig. 2B. The bottom panel depicts the triangular voltage command, whereas the top panel shows the recorded membrane current (gray) and leak-subtracted (see METHODS) current (black). During the ascending phase of the voltage ramp, the currents were dominated by an outward leak current; however, at −58 mV, an inward current was activated (arrow “1”). As the voltage ramp continued to ascend, outward currents dominated. During the descending phase of the voltage ramp, a second inflection was visible, suggesting that the current had remained active.

We defined the PIC activation voltage as the membrane potential where the slope of leak-subtracted current first becomes negative (Fig. 2B, arrow “1”). We defined the PIC magnitude as the peak of the leak-subtracted inward current during the ascending phase of the voltage command (Fig. 2B, arrow “2”). Cells without a net inward leak-subtracted current were considered to not have a PIC. We observed a similar PIC in 26 of 34 vMNs tested, indicating that most vMNs express PICs, even in the absence of agonists. The magnitude of the PIC averaged −120 ± 60 pA (median, −150 pA) and had an activation voltage of −53 ± 6 mV. These PICs persisted in the presence of antagonists of fast glutamatergic and GABAergic receptors (n = 20, see METHODS), indicating that the PICs are an intrinsic property of vMNs.

**PICs are facilitated by 5-HT₂ receptors**

To examine the effects of α-Me-5HT on the PICs, we focused on currents evoked during the ascending phase of the voltage ramps and compared, in each cell, the leak-subtracted activation voltage and current magnitude before and after drug application (1–30 μM). For the 26 vMNs that expressed a PIC in control conditions, the average PIC magnitude in the presence of α-Me-5HT increased from −120 ± 60 to −500 ± 200 pA (median, −500 pA). α-Me-5HT also significantly shifted the PIC activation voltage to a more hyperpolarized value of −57 ± 5 mV (P = 0.029, Kolmogorov-Smirnov test). In the eight vMNs that did not express a PIC in control conditions, the agonist evoked a PIC of −360 ± 140 pA with an average activation threshold of −56 ± 5 mV.

To examine the effects of increases in serotonergic drive on the facilitation of PIC magnitude, we applied multiple agonist concentrations (1–30 μM) to 19 of these cells. A representative example is shown in Fig. 3A. This vMN had a PIC in control conditions (black trace) with a peak magnitude of −19 ± 10 pA. Application of 1 μM agonist (red trace) enhanced the PIC magnitude to −198 ± 20 pA. Increasing the agonist concentration to 5 μM (green trace) further enhanced the PIC magnitude to −396 ± 21 pA. The impact of the agonist on the PIC magnitude for all 19 motoneurons is shown in Fig. 3B, where the peak PIC magnitude is plotted as a function of agonist concentration normalized to the lowest concentration applied to each cell. In every neuron, the increase in PIC magnitude from control to low concentration was significant (P < 10⁻³). Increasing the concentration of the agonist resulted in an additional, significant increase in PIC magnitude in 18 of 19 cells (P < 10⁻⁵). These results indicate that, through the graded facilitation of PICs, 5-HT can modulate the magnitude of PICs.

**Fig. 2.** vMNs express persistent inward currents (PICs). A: ramp-and-hold protocol reveals the existence of a PIC in a representative vMN in control conditions. Note the nearly uniform reduction in the recorded membrane current (Iₘ) at more depolarized holding potentials. Leak subtracted currents reveal that the observed reduction in Iₘ resulted from a voltage-dependent PIC. B: protocol for measuring the PIC magnitude and activation voltage. The membrane currents (gray) generated in response to a triangular voltage command (16 mV/s) are dominated by an outward current. The downward inflections in the recorded membrane current traces during both the ascending and descending phases result from a PIC, seen more clearly in the leak-subtracted current trace (black). The PIC activation voltage was defined as voltage where the slope of the leak subtracted current trace first becomes negative (1). The peak PIC magnitude was measured at the peak of the inward inflection during the ascending phase of the voltage command (2).
of inward currents in vMNs, thereby generating the full range of whisking frequencies.

**PICs are largely composed of sodium currents**

PICs in spinal (Harvey et al. 2006a), hypoglossal (Powers and Binder 2003), and trigeminal (Hsiao et al. 1998) motoneurons consist of Na$^+$ and Ca$^{2+}$ currents. To determine if Na$^+$ currents contribute to PICs in vMNs, we suppressed Na$^+$ conductances with TTX (1 μM), currents that contribute to PICs in vMNs, we suppressed Na$^+$ conductances with TTX (1 μM). A representative example is depicted in Fig. 3C. In response to application of 10 μM α-Me-5HT (green trace), this motoneuron had a PIC magnitude of $-741 \pm 31$ pA. Co-application of 1 μM TTX (red trace) reduced the PIC magnitude to $-210 \pm 6$ pA. Group data for 23 motoneurons are shown in Fig. 3D. After application of TTX, the agonist-facilitated PIC was reduced in magnitude by $79 \pm 16\%$ (median $= 81\%$). In each case, the reduction was significant ($P < 10^{-2}$, Student’s t-test).

In 13 of these motoneurons, we subsequently applied 50–200 μM cadmium (Cd$^{2+}$) to determine whether the TTX-insensitive component resulted from Ca$^{2+}$ currents. For the motoneuron shown in Fig. 3C, application of 50 μM Cd$^{2+}$ (black trace) completely suppressed the TTX-insensitive component of the PIC. We recorded a similar complete suppression of PICs in 10 of 13 vMNs (Fig. 3D). In the remaining three neurons, Cd$^{2+}$ application significantly ($P < 10^{-5}$), but not completely ($38 \pm 21\%$), further suppressed PIC amplitudes. Thus in vMNs, PICs are predominantly composed of sodium currents, suggesting that a persistent Na$^+$ current ($I_{NaP}$) plays a central role in generating firing in these cells.

**Riluzole modulates PIC magnitudes in vMNs**

Because $I_{NaP}$ accounted for most of the PIC in the vMNs, we focused on these currents in the remaining experiments. At low concentrations (2–5 μM), riluzole selectively antagonizes this current without significantly affecting fast Na$^+$ currents that underlie action potentials (Wu et al. 2005). We took advantage of this specificity to test the hypothesis that $I_{NaP}$ undergoes a central role in generating firing in these cells.

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Riluzole modulates firing in vMNs

The effects of riluzole are illustrated for a representative motoneuron in Fig. 3E. Riluzole (2 μM) significantly (Student’s t-test, P < 10^{-3}) decreased the magnitude of the agonist-facilitated PIC from −396 ± 21 to −143 ± 10 pA. Increasing the concentration of riluzole to 5 μM further suppressed the PIC to −50 ± 7 pA. The subsequent addition of 1 μM TTX completely abolished the PIC in this motoneuron. Group data for nine neurons tested at both riluzole concentrations, and four vMNs tested only at the 5 μM concentration, are shown in Fig. 3F. In all 13 vMNs, riluzole significantly suppressed PIC magnitudes. The amount of suppression with 5 μM riluzole was significantly (P ≤ 10^{-3}, n = 9) greater than the suppression in the presence of 2 μM riluzole. Subsequent addition of TTX suppressed the agonist facilitated PIC by a total of 80 ± 20% (n = 9), eliminating the majority of the PIC. We obtained similar results in the presence of ionotropic glutamatergic and GABAergic antagonists (see METHODS), suggesting that riluzole acted directly on the recorded vMNs. These findings strengthen the premise that INaP is a major component of PICs in vMNs, and that its activity may underlie the generation of the whisking rhythms by vMNs.

Riluzole modulates firing in vMNs

The postulated role of INaP on regulating the firing rate of vMNs is demonstrated in the current-clamp recordings shown in Fig. 4A. In control conditions (top), the motoneuron was silent; however, application of 20 μM α-Me-5HT caused the vMN to fire regularly at ~2 Hz. After the addition of 5 μM riluzole, the firing frequency began to decline and was eventually completely suppressed. Riluzole’s effects were reversible: After washout with artificial cerebrospinal fluid (ACSF) for 22 min, re-application of 20 μM α-Me-5HT evoked firing in the vMN near 2 Hz. Group data for eight vMNs tested at both 2 and 5 μM riluzole and nine vMNs tested only at 5 μM are shown in Fig. 4B. In the eight cells tested at both concentrations, 2 μM riluzole significantly reduced firing frequency by 28 ± 17% (P < 10^{-3}) in the remaining five neurons. Subsequent application of 5 μM riluzole to these five vMNs caused a complete suppression of firing in four cells and an additional significant 59% reduction in the remaining vMN (P < 10^{-3}). In the nine vMNs tested only at 5 μM riluzole, firing was completely suppressed in six cells and significantly reduced by 35 ± 21% in the remaining three (P < 10^{-3}). Washout was successful in seven out of eight cells tested.

Injections of current pulses (200 ms) during the peak effects of riluzole confirmed that the neurons (n = 15) were still capable of generating trains of fast action potentials. This ensured that the fast sodium channels underlying the action potentials had not been affected by the riluzole. Together, the effects of riluzole on the magnitude of INaP and its impact on the agonist-induced firing in vMNs support the hypothesis that the facilitation of a PIC by 5-HT is necessary and sufficient for vMNs to generate whisking over a range of frequencies.

Activity in putative serotonergic premotoneurons is correlated with whisking frequency

The results of our in vitro experiments support the hypothesis that vMNs generate rhythmic whisking in response to a graded facilitation of a PIC by 5-HT. In the intact animal, serotonergic drive to vMNs originates from a subset of serotonergic neurons in the brain stem that both project to vMNs in the lateral facial nucleus and receive projections from the vibrissa motor cortex (vMCx) (Hattox et al. 2003; Hattox et al. 2002). Thus these serotonergic premotoneurons likely serve as the endogenous source of the 5-HT employed by vMNs to generate whisking rhythms. As the frequency of the motor pattern generated by vMNs varies with the level of serotonergic drive they receive, we predict that the output from these serotonergic premotoneurons should correlate positively with whisking frequencies.

To test this prediction, we evoked rhythmic whisking by applying ICMS to vMCx, as previously described (Cramer and Keller 2006; Haiss and Schwarz 2005), while recording extracellularly from putative serotonergic premotoneurons. Our attempts to unambiguously identify the premotoneurons by evoking antidromic action potentials after stimulation of the facial nucleus were unsuccessful. This is most likely due to the difficulty in activating small-diameter, nonmyelinated fibers characteristic of serotonergic neurons (Azmitia and Gannon...
1983) and the close proximity of the recording and stimulation sites. As the electrophysiological properties (e.g., spontaneous activity and spike shape indices) of serotonergic neurons show considerable variation (Zhang et al. 2006), we concluded that these criteria cannot be used reliably to identify serotonergic neurons. We therefore accepted for analysis, and defined as putative serotonergic premotoneurons, all cells recorded in the rostral lateral (juxtafacial) paragigantocellularis nucleus (LPGi) according to stereotaxic coordinates. This nucleus is composed predominantly of serotonergic neurons (Bowker and Abbott 1990; Darnall et al. 2005) that both receive direct inputs from vMCx and project to vMNs in the lateral facial nucleus (Hattox et al. 2002, 2003).

An example of ICMS-evoked whisking, monitored by recording EMG activity from the whisker pad, is shown in Fig. 5A. As we reported previously, whisking frequency was positively correlated with ICMS intensity ($r = 0.98$, $P = 0.0004$; Fig. 5B), enabling us to control whisking frequency in the anesthetized animal. This allowed us to investigate the relationship between whisking frequency and activity in serotonergic premotoneurons.

Figure 6A shows the response of putative serotonergic premotoneuron to ICMS (50 Hz, 125 μA, 1-s duration) of vMCx. This neuron responded to vMCx stimulation with a latency of $6 \pm 2$ ms (Fig. 6B). Increasing ICMS intensities resulted in an increase in the number of spikes evoked by this neuron (Fig. 6C) without affecting response latency. Group data for 16 putative serotonergic premotoneurons are shown in Fig. 6D, where the premotoneuron spike count and stimulus intensity have been normalized to their threshold values. The activity in these premotoneurons was positively correlated with the ICMS intensity ($r = 0.92$, $P = 10^{-3}$).

The findings that ICMS intensity is positively correlated with whisking frequency (Fig. 5) (Cramer and Keller 2006) and that ICMS intensity is correlated also with spike counts of putative serotonergic premotoneurons support the conjecture that spiking output of these premotoneurons is causally related to whisking frequencies. We were able to test this directly for four premotoneurons. This small sample size reflects the difficulty in obtaining stable recordings under the relatively light anesthesia necessary for ICMS-evoked whisking (Cramer and Keller 2006; Haiss and Schwarz 2005). In these neurons, we recorded vibrissal EMG simultaneously with the neuronal recordings. The relationship between ICMS evoked activity in a representative putative serotonergic premotoneuron and the simultaneously recorded EMG is shown in Fig. 7A. Increasing ICMS intensity increased both the number of spikes generated by the premotoneuron and the whisking frequency, revealing a positive correlation between these latter parameters ($r = 0.94$, $P = 0.006$). Group data for all premotoneurons/EMG pairs are shown in Fig. 7B where whisking frequency and premotoneuron activity were normalized to their threshold values. Whisking frequency was positively correlated with the level of activity in the putative premotoneuron ($r = 0.89$, $P = 10^{-7}$). These findings support the hypothesis that LPGi premotoneurons provide the tonic serotonergic drive employed by vMNs to generate the whisking rhythm.

**DISCUSSION**

Using the rodent vibrissa motor system, we provided evidence for a novel mammalian mechanism of rhythm generation that underlies movements. Like CPGs, this system does not rely on cortical inputs (Gao et al. 2003; Lovick 1972; Semba and Komisaruk 1984) or sensory feedback (Welker 1964) to generate rhythmic output. However, whereas mammalian CPGs are typically composed of networked interneurons that generate the motor pattern and subsequently drive motoneurons, in the vibrissa system, our results suggest that rhythmic movements are generated by the motoneurons themselves in response to tonic input from serotonergic premotoneurons. The involvement of vMNs in the rhythmogenesis, the necessity of 5-HT for generating movements, and the sufficiency of 5-HT to generate the motor pattern are unique to this mammalian model of motor control.

In support of the postulate that 5-HT is necessary for rhythmic whisking are our findings that 5-HT receptor antagonists suppress both voluntary whisking (Hattox et al. 2003) as well as ICMS-evoked whisking (Cramer and Keller 2006). The finding that the activity of putative 5-HT premotoneurons co-varies with whisking frequency (Fig. 7) is also in agreement with this postulate. Available evidence suggests that 5-HT interactions with vMNs are also sufficient for generating rhythmic whisking: 5-HT (or its receptor agonists) applied at different concentrations evoke a range of rhythmic firing rates in vMNs (Hattox et al. 2003) (Fig. 1), an effect that is suppressed by 5-HT receptor antagonists (Hattox et al. 2003). These pharmacological manipulations are resistant to suppression of glutamatergic and GABAergic receptors, suggesting that these serotonergic effects are specific to vMNs. This effect appears unique to vMNs as an earlier investigation into the effects of 5-HT on facial motoneurons, which did not restrict sampling to any particular sub-nucleus, did not observe 5-HT-evoked rhythmic firing (McCall and Aghajanian 1979). Similarly,
5-HT does not evoke rhythmic firing in guinea pig trigeminal motoneurons (Hsiao et al. 1997). Thus whereas in other motor systems the actions of 5-HT on motoneurons is described as modulatory, capable of shaping an on-going rhythm but not responsible for the rhythm generation itself (Heckman et al. 2004), in the vibrissa motor system, 5-HT is both necessary and sufficient for generating the whisking motor pattern and thus capable of generating rhythmic whisking.

Our results indicate that 5-HT acts through a graded facilitation of a PIC within vMNs. PICs are voltage-dependent membrane currents that resist inactivation (Heckman et al. 2005; Schwindt and Crill 1980). In many motoneurons, including vMNs (Figs. 2 and 3) these currents are facilitated by 5-HT, frequently through the activation of 5-HT$_2$ receptors (Harvey et al. 2006a; Heckman et al. 2005; Perrier and Hounsgaard 2003). Although these currents are considered to modulate ongoing firing in other motoneurons, in vMNs their presence is associated with the generation of rhythmic firing. We found that concentrations of a 5-HT$_2$ receptor agonist that produced a graded facilitation in PIC magnitude also generated a progressive increase in firing rates in vMNs, suggesting a causal relationship between the two phenomena. The action of riluzole on the agonist-induced firing rates supports this causal relationship. Riluzole, which selectively antagonizes persistent sodium currents at low concentrations (2–5 μM) (Wu et al. 2005) caused a similarly graded suppression in both PIC magnitude and firing rate of vMNs. Together, these data support the hypothesis that vMNs generate whisking rhythms in response to serotonergic drive.

ICMS-evoked whisking displayed a relatively long onset latency (Fig. 7). This delay may reflect the kinetics of the transduction cascades initiated after activation of metabotropic 5-HT$_2$ receptors on vMNs. In addition, 5-HT axon terminals in the facial nucleus, as in other brain regions, are thought not to form classical chemical synapses. Rather activation of 5-HT receptors is thought to occur through the slow diffusion of 5-HT and the activation of extrasynaptic receptors (De-Miguel and Trueta 2005). We have recently reported that increasing ICMS intensity significantly decreases whisking onset latency, most likely by increasing the output from 5-HT premotoneurons (Cramer and Keller 2006), consistent with the postulate that extrasynaptic transmission contributes to the delayed whisking. It is pertinent that the long latencies between cortical activation and EMG onset are consistent with our previous studies in behaving rats (Friedman et al. 2006).

The mechanisms responsible for terminating whisking remain to be determined. Inhibitory inputs are effective in rapidly terminating PICs (Heckman et al. 2005), suggesting that inhibitory inputs to vMNs may be involved. Indeed, we have previously found that both the onset and offset of a whisking epoch is preceded by a brief increase in activity in vMCx (Friedman et al. 2006), suggesting vMCx sends commands to both start and stop a whisking epoch.

Exploratory whisking occurs at frequencies between 5 and 15 Hz (Berg and Kleinfeld 2003), a range that is encompassed by the agonist induced firing rates (Figs. 1 and 4). The correspondence between vMN firing rates in vitro and whisking frequencies in vivo suggests that during voluntary whisking, vMNs fire a single action potential per whisk. Previously we demonstrated that some vMNs do indeed fire in a one-to-one manner during ICMS-evoked whisking (Cramer and Keller 2006). Some vMNs, however, fire bursts of action potentials per whisk. The failure to evoke a similar bursting firing pattern in vMNs in vitro may result from the reduced nature of this preparation. In particular, the partial truncation of the dendritic tree, where the channels that carry PICs are thought to reside (Heckman et al. 2003), might impact the ability of the vMNs to burst in vitro. In addition, nonserotonergic inputs present in the
During rhythmic whisking evoked by ICMS of vMCx, the endogenous source of 5-HT used by vMNs can be activated and regulated by vMCx. In support of this, we found that the activity of putative serotonergic premotoneurons is positively correlated with the whisking frequency (Fig. 7). We have shown previously that this stimulation-evoked whisking is suppressed by 5-HT receptor antagonists (Cramer and Keller 2006), further supporting the hypothesis that voluntary control of the whisking rhythm is achieved by actions of vMCx on 5-HT premotoneurons.

Additional lines of evidence support the role of vMCx in regulating whisking through a 5-HT-dependent mechanism. The activity of vMCx neurons does not co-vary with whisking frequency, suggesting that vMCx does not directly drive vMNs (Carvell et al. 1996). Increased vMCx activity does, however, precede both the onset of whisking and changes in whisking kinematics (Friedman et al. 2006). These observations are consistent with vMCx modulating the activity of a subcortical structure, such as serotoninergic premotoneurons, to initiate and modulate whisking. A role for the motor cortex as a coordinator of movement patterns is supported by studies in primates, demonstrating that the motor cortex may control higher-order movement parameters, such as ethologically relevant motor behaviors, rather than activation of individual muscles or movements (Graziano 2006).

During voluntary whisking, vibrissae often move in unison (Carvell and Simons 1990; Gao et al. 2001). Although the synchrony between vibrissae in the same whisker pad (Sachdev et al. 2002) and bilaterally (Towal and Hartmann 2006) does not occur during all behaviors, the prevalence of such synchrony suggests the presence of underlying coordinating mechanisms. Because the facial nucleus is thought not to contain interneurons (Courville 1966) and because facial motoneurons do not have axon collaterals (Fanardjian et al. 1983), unilateral synchrony may be achieved through electrical coupling. Electrical coupling through gap junctions enhances synchronous activity in neurons (Connors and Long 2004) and is important for generating coordinated output from motoneuron pools (Kiehn and Tresch 2002; Tresch and Kiehn 2000). The presence of gap junction proteins in the facial nucleus (Rohlmann et al. 1993) further suggests that the unilateral synchronous movements of vibrissae result from the coordinated discharge of electrically coupled vMNs. Such a mechanism is supported by findings in the developing mouse hindbrain, where 5-HT generates widespread synchronous activity that is abolished by gap junction blockers (Hunt et al. 2006). Alternatively, synchronous whisking, both unilateral and bilateral, may be achieved through the action of premotoneurons. These may include premotoneurons in LPGi as these project bilaterally to vMNs (Hattox et al. 2002, 2003) or premotoneurons in one of the numerous nuclei that project to vMNs (Hattox et al. 2002).

Many of the nuclei that project to vMNs contain nonsertonegic neurons the role of which in the regulation of whisking remains to be established (Hattox et al. 2002). Within these nuclei may reside a more classically composed whisking CPG that delivers rhythmic inputs to the motoneurons. vMNs also receive direct, albeit sparse, inputs from the vMCx (Grinevich et al. 2005), and although vMCx is not necessary for whisking (Gao et al. 2003; Lovick 1972; Semba and Komisaruk 1984), it has been proposed that vMCx is capable of generating the whisking rhythm itself on a cycle-by-cycle basis (Berg and Kleinfeld 2003). Additionally, in some pathological states vMCx activity is phased-locked to individual whiskers (Castro-Alamancos 2006). In light of these observations, it is unlikely that the circuits described in the preceding text suggest that the endogenous source of 5-HT used by vMNs can be activated and regulated by vMCx. In support of this, we found that during rhythmic whisking evoked by ICMS of vMCx, the activity of putative serotonergic premotoneurons was positively correlated with the whisking frequency (Fig. 7). We have shown previously that this stimulation-evoked whisking is suppressed by 5-HT receptor antagonists (Cramer and Keller 2006), further supporting the hypothesis that voluntary control of the whisking rhythm is achieved by actions of vMCx on 5-HT premotoneurons.

Intact animal but missing in vitro may be essential for bursting in vMNs.

vMNs in the lateral facial nucleus are densely innervated by serotonergic neurons, many of which receive direct projections from the vMCx (Hattox et al. 2002, 2003). The highest density of serotoninergic inputs to vMNs appears to originate from the rostral (juxtafacial) LPGi (Hattox et al. 2002), and stimulation of these neurons produces vibrissa movements (Hattox et al. 2003). These observations suggest that serotoninergic LPGi neurons are strategically placed to act as the source of 5-HT used by vMNs to generate whisking. Indeed our results indicate that the activity of neurons within this nucleus is positively correlated with whisking frequency. However, because LPGi contains a subset of non5-HT neurons (Bellintani-Guardia et al. 1996), we cannot conclusively determine the identity of the neurons we recorded from and therefore referred to them as putative serotoninergic premotoneurons.

The circuits described in the preceding text suggest that the endogenous source of 5-HT used by vMNs can be activated and regulated by vMCx. In support of this, we found that during rhythmic whisking evoked by ICMS of vMCx, the

![Image](http://jn.physiology.org/content/jn/97/3/2156/F7.large.jpg)
that any one mechanism operates in isolation to generate the full range of vibrissal movements. Nevertheless, our findings support the hypothesis that vMNs require only serotonergic inputs to generate the whisking motor pattern. The involvement of vMNs in rhythmogenesis and the necessary and sufficient role of 5-HT in generating the whisking motor pattern establish this network as a novel mechanism for the generation of movements in mammals.

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