Effect of the Stimulation of Sensory Inputs on the Firing of Neurons of the Trigeminal Main Sensory Nucleus in the Rat

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Bernier AP, Arsenault I, Lund JP, Kolta A. Effect of the stimulation of sensory inputs on the firing of neurons of the trigeminal main sensory nucleus in the rat. J Neurophysiol 103: 915–923, 2010. First published December 2, 2009; doi:10.1152/jn.91109.2008. Mastication can be triggered by repetitive stimulation of the cortex or of sensory inputs, but is patterned by a brain stem central pattern generator (CPG). This CPG may include the dorsal part of the principal trigeminal sensory nucleus (NVsnpr), where neurons burst repetitively when the extracellular concentration of Ca2+ ([Ca2+]e) drops. We examined the effects of repetitive stimulation of sensory afferents of the trigeminal tract on activity of NVsnpr neurons recorded extracellularly in vitro under physiologic [Ca2+]e (1.6 mM). Spontaneously active cells had either a tonic (n = 145) or a bursting (n = 46) firing pattern. Afferent stimulation altered burst duration and/or burst frequency in bursting cells and firing frequency in most tonic cells. In 28% of the latter, the firing pattern switched to rhythmic bursting. This effect could be mimicked by local application of N-methyl-D-aspartate and blocked by APV but not DNQX. Detailed analysis showed that rhythm indices (RIs) of 35 tonic neurons that were negative (nonrhythmic) before stimulation became significantly rhythmic (RI > 0.01) after stimulation. Mean and median bursting frequency of these units were 8.32 ± 0.72 (SE) Hz and 6.25 Hz (range, 2.5–17.5 Hz). In seven instances, two units were recorded simultaneously, and cross-correlation analysis showed that firing of six pairs was rhythmic and synchronized after stimulation. Optimal stimulation parameters for eliciting rhythmic bursting consisted in 500-ms trains of pulses delivered at 40–60 Hz. Together, our results show that repetitive stimulation of sensory afferents in vitro can elicit masticatory-like rhythmic bursting in NVsnpr neurons at physiologic [Ca2+]e.

INTRODUCTION

The basic pattern of mastication is generated by a central pattern generator (CPG) located in the brain stem. This CPG can produce repetitive jaw movements in response to inputs from either higher centers or sensory afferents (Dellow and Lund 1971). Although neither input is essential for the production of the basic pattern of mastication, sustained repetitive stimulation of either type can activate the masticatory CPG even in paralyzed animals (fictive mastication) (Dellow and Lund 1971; Lund and Dellow 1971; Sumi 1969). This property is not unique to mastication, because activation of sensory afferents and descending inputs elicits locomotion (Fleshman et al. 1984; Grillner et al. 1981; Rossignol 2000). However, the cellular mechanisms by which sustained activation of these inputs is converted into a rhythmic output by the CPG are unknown. Our previous work suggested that the dorsal part of the trigeminal main sensory nucleus (NVsnpr) is an important part, if not the core, of the masticatory CPG (Athanassiadis et al. 2005b; Brocard et al. 2006; Kolta et al. 2007; Tsuboi et al. 2003 for a review). NVsnpr receives inputs from the masticatory area of the cortex and from trigeminal sensory afferents, and neurons of its dorsal part project directly to the trigeminal motor nucleus (Kolta et al. 2000; Li et al. 1993; Travers and Norgren 1983; Yoshida et al. 1998). The expression of c-Fos (a neuronal marker of activity) increases in neurons of dorsal NVsnpr after bouts of fictive mastication (Athanassiadis et al. 2005a), and about a third of neurons recorded in these are rhythmically in phase with trigeminal motoneurons during fictive mastication (Tsuboi et al. 2003). These rhythmic neurons receive inputs from sensory receptors that provide important feedback during mastication: intraoral touch receptors, periodontal pressure receptors, and muscle spindles (Tsuboi et al. 2003). In vitro studies have shown that many dorsal NVsnpr neurons are intrinsic bursters, and our most recent work has shown that repetitive bursting in these cells depends on a persistent sodium conductance (I\textsubscript{Na}\textsubscript{P}) that is voltage dependent and modulated by the extracellular concentration of Ca2+ ([Ca2+]e). Under a physiologic [Ca2+]e (1.6 mM), only 18% of neurons could be made to burst by depolarizing current injections; however, reducing [Ca2+]e caused a progressive increase in the percentage of neurons that burst spontaneously (Brocard et al. 2006). Brocard et al. (2006) proposed that sustained activation of inputs to NVsnpr causes a fall in [Ca2+]e that, in turn, activates I\textsubscript{Na}\textsubscript{P} and triggers rhythmic bursting. Decreases of [Ca2+]e caused by sustained or intense neuronal activities have been described in the spinal cord (Murase and Randic 1983; Somjen 1980); cerebellum (Nicholson et al. 1978); cortex (Somjen 1980); and hippocampal slices (Benninger et al. 1980; Rusakov and Fine 2003). The objective of this study was to test whether repetitive stimulation of sensory afferents to NVsnpr at physiologic concentrations of Ca2+ can elicit rhythmic bursting in these neurons.

METHODS

Brain slice preparation

Sixty-one Sprague-Dawley rats (13–34 days old) were used for this study. Retrograde labeling of trigeminal motoneurons was performed before slice preparation to help position the recording electrode in NVsnpr. This was done by injection of 5–10 μl of Alexa fluor 488–conjugated cholera toxin (subunit B) into the masseter muscle of cryo-anesthetized rat pups. One to 2 wk later, the animals were decapitated, and the brain stem was rapidly removed and immersed in oxygenated ice-cold (4°C) sucrose-
artificial cerebrospinal fluid (ACSF; composition in mM: 252 sucrose, 3 KCl, 1.25 KH₂PO₄, 4 MgSO₄, 0.2 CaCl₂, 26 NaHCO₃, and 25 d-glucose, bubbled with 95% O₂-5% CO₂, pH 7.4). Transverse slices (400 µm thick) containing NVsnpr and the trigeminal tract were prepared using a vibratome (VT1000 S, Leica). Slices were maintained at 28–32°C in an interface chamber saturated with a humidified mixture of 95% O₂-5% CO₂. The chamber was perfused with normal ACSF (composition in mM: 125 NaCl, 3 KCl, 1.25 KH₂PO₄, 2.8 MgSO₄, 1.6 CaCl₂, 26 NaHCO₃, and 25 d-glucose) at a rate of 1 ml/min. The slices were incubated ≥1 h before the recordings. Three concentrations of CaCl₂ were used during recording of 280 neurons: physiological level (1.6 mM, n = 174, Jones and Keep 1988); low calcium (1.2 mM, n = 69); or high calcium (2.4 mM, n = 15). In some experiments (n = 22), we began with Ca²⁺-free ACSF to facilitate the detection of bursting neurons and changed to physiological concentration in 17 cells. Ca²⁺-free solutions were prepared by equimolar substitution of CaCl₂ with MgCl₂ to maintain the concentration of divalent ions constant. Once a bursting unit was detected, the Ca²⁺ concentration was raised. All procedures for dye injections and slice preparation conformed to National Ethics Committee guidelines and were approved by the Institutional Animal Care Committee at the Université de Montréal, Montreal, Canada.

**Electrophysiological recording**

Extracellular recordings were made with conventional borosilicate electrodes (1.0 mm OD, A-M systems; 10–20 µm at the tip) containing 0.5 M filtered NaCl and an A-M systems 1800 amplifier. The trigeminal tract was stimulated extracellularly using bipolar electrodes (~50 µm between poles) made of twisted nichrome wires (25 µm diam) positioned close to the recording electrode in NVsnpr. The intensity (generally <1.5 mA) was set to the minimum required to obtain responses for each cell. Pulse duration was 0.25 ms. Repetitive stimulation was performed at 40 Hz for all cases and assessed at different frequencies (20–100 Hz) in some cases. The train duration varied from 100 ms to 2 s. The data were digitized at 10 kHz and stored using pClamp 9 (Axon Instruments). The cells selected for analysis had a stable firing pattern under resting conditions during ≥10 min of baseline. For each unit, the protocol of stimulation was repeated 10 times for single pulses and 5 times for trains, with a minimum of 1 min between each stimulation protocol.

**Drug applications**

Drugs were either added to the perfusing medium or locally applied with a Picospritzer II ejection system (General Valve). Local applications were made by pressure-ejecting the drug contained in a pipette placed in the vicinity of the recorded cell. D,L-2-amino-5-phosphonovaleric acid (APV; Sigma-Aldrich) was either bath-applied (75 µM) or locally applied (50 µM). N-methyl-D-aspartic acid, (NMDA; 1–2 mM; Sigma-Aldrich) was always locally applied, whereas 6,7-dinitroquinoxaline (DNQX; Sigma-Aldrich) was bath applied (100 µM). In a few experiments, a mixture of APV, DNQX, and picrotoxin (50 µM) were applied together to block major excitatory and inhibitory synaptic potentials.

**Analysis**

Data were analyzed off-line using Spike2 (Cambridge Electronics Division). Spike detection and unit discrimination were performed with the template matching and waveform processing tools, which insured that every action potential in the recording trace is sorted according to its waveform signature. This enabled us to discriminate firing of different cells when the activity of more than one unit was picked up by the recording electrode. All subsequent analyses were conducted on events detected by the software. Burst detection was performed using the script Burst.s2s, in which we arbitrarily defined a burst as a cluster of three or more action potentials occurring at a shorter interspike interval. When such bursts occurred in a regular periodic manner, they were referred to as “rhythmic bursting.” Firing in single spikes or in doublets, even if occurring in a regular periodic manner, was defined as “tonic.” Thus in this study, the term “rhythmic” refers only to rhythmic bursting. To analyze rhythmicity in the firing pattern, we used a method developed by Sugihara et al. (1995) to calculate rhythm indices (RIs) from the autocorrelograms. Autocorrelograms were performed for all neurons that appeared to change from tonic firing to bursting after trigeminal tract stimulation and for 10 neurons that did not appear to burst. Interval histograms were used to set the time bin size of the autocorrelograms (5–10 ms) as described in Lang et al. (1997, 1999). The peaks and valleys in the autocorrelograms were identified if their amplitude exceeded ±2 SD of the baseline, which was calculated using the following formula: baseline level = (total number of spikes)²/(recording time)(autocorrelogram bin width).

Rhythm indices were calculated as follows: rhythm index = Sa / z + 2Sb/z, where a is height difference of the ith peak with baseline + SD and b is the depth difference of ith valley with baseline – SD. Z is defined as the difference between the zero time bin and the baseline level. The height of the zero time bin indicates the total number of spikes in the trace. Following criteria established by Sugihara et al. (1995), firing patterns were considered to be rhythmic if the RI was >0.01. The greater the RI, the tighter and stronger the oscillatory activity. The lower the RI value, the closer the cell firing is to randomness. When the autocorrelogram had no recognizable peaks and valleys, a value of zero was given to the RIs. The rhythm frequency was defined as the reciprocal of the interval between the start of the autocorrelogram and the first peak. Alternatively, fast Fourier transforms (FFTs) were performed to identify the predominant frequency. The RIs and FFTs were carried out with Matlab. All statistical calculations were run in SigmaStat 3.1 (Systat Soft), and three-dimensional plots (3D spline surfaces) were made with Matlab (Mathworks).

**RESULTS**

**Age dependency of firing pattern**

Recordings were obtained from 280 neurons spread throughout the entire dorso-ventral extent of the nucleus. Two main categories of spontaneous firing patterns were identified: tonic and bursting (Fig. 1A). Tonic units fired single spikes or doublets, whereas bursting units fired clusters of spikes separated by silent periods (see METHODS above for definition of tonic, bursting, and rhythmic bursting). Under [Ca²⁺]₀ of 1.6 mM (n = 191) spontaneous bursting was almost nonexistent in animals younger than 15 days old (Fig. 1B). In agreement with our previous findings (Brocard et al. 2006), the proportion of bursting neurons increased with age and reached nearly 40% from 17 days old.

**[Ca²⁺]₀ dependency of firing pattern**

The [Ca²⁺]₀ had a marked effect on the firing pattern (see Fig. 4 for firing of the same neuron under different [Ca²⁺]₀). Figure 1C shows the distribution of rhythmic and tonically firing neurons recorded under different [Ca²⁺]₀. More than 90% of cells had a spontaneous bursting pattern in Ca²⁺-free ACSF, whereas >90% fired tonically when [Ca²⁺]₀ was 2.4 mM. From these, 17 neurons were recorded first in Ca²⁺-free...
ACSf, and it was confirmed that they could burst spontaneously in this condition. Twelve of these (71%) became tonic after increasing \([Ca^{2+}]_o\) to 1.6 mM.

**Effect of stimulation of the trigeminal tract on cells firing pattern**

All stimulations were carried out with \([Ca^{2+}]_o = 1.6\) mM and on neurons from rats \(\geq 13\) days old. Single shocks delivered to the trigeminal tract had little sustained effect on the spontaneous firing of NVsnpr neurons, whereas repetitive stimulation was effective in 77% of cases. In our sample, 124 neurons fired tonically before stimulation, 46 were bursting cells, and 21 were silent. These 21 silent cells were detected only after stimulation, which produced tonic firing that lasted 5.3 \(\pm\) 1.3 (SE) s (Fig. 2A). Of the 124 tonically firing units, 52 (42%) increased their firing frequency (from 6.2 \(\pm\) 0.8 to 28.5 \(\pm\) 3.2 Hz) for a period of 4.4 \(\pm\) 0.6 s after stimulation (Fig. 2B). Inhibition of firing lasted in average 2 \(\pm\) 0.5 s and was observed in 17 cells (14%; Fig. 2C). Twenty cells (16%) did not respond to stimulation. Finally, in 35 cells (28%), stimulation caused tonic firing to become rhythmic bursting with an average bursting frequency of 8.3 \(\pm\) 0.7 Hz for \(\sim\) 2.2 \(\pm\) 0.5 s (Fig. 2D). The effects on burst firing units \((n = 46)\) were also both excitatory and inhibitory. In eight cells (17%), stimulation caused an increase of burst frequency from 5.7 \(\pm\) 1.8 to 21.5 \(\pm\) 5.1 Hz that lasted 1.5 \(\pm\) 0.56 s (Fig. 2E). In 10 cases (22%), stimulation increased burst duration from 37 \(\pm\) 8.2 to 180.4 \(\pm\) 43.6 ms for \(\sim\) 3.7 \(\pm\) 1.5 s (Fig. 2F). Bursting was suppressed in 4 neurons (9%; Fig. 2G) for 1.7 \(\pm\) 0.36 and bursting was unchanged in 24 cases (52%).

**Distribution of neurons and effects of repetitive stimulation**

Different recording and stimulation positions were assessed. Figure 3 shows the distribution of the effects obtained according to their recording locations. Effects were more readily observed when cells were recorded from the dorsal half of the nucleus, and the stimulation electrode was located close to the recording electrode. Stimulation of the dorsal portion of the tract produced an effect in 87% of cases, whereas stimulation of the ventral portion of the tract had an effect in only 13% of cases. Excitatory effects (on tonic firing and bursting neurons) were mostly seen in neurons of the dorsal half; whereas inhibitory effects were most often observed in the middle part of the nucleus. Conversions from tonic to burst firing were exclusively observed in the dorsal part of the nucleus. Cases where stimulation had no effect were spread throughout the nucleus.

**Analysis of conversion from tonic to burst firing**

Analyses were carried out on the 35 units that were initially identified by visual inspection of data. The neuron shown in Fig. 4 was first identified as a bursting cell in \([Ca^{2+}]_e\) (Fig. 4A, 3rd trace). The two top traces show action potentials...
(Events) and bursts detected by the acquisition software (Spike 2). Although bursting is somewhat irregular under this condition, an RI of 0.9 was calculated from the autocorrelogram (middle), which shows distinct peaks and a dominant frequency of 6.5 Hz in the FFT analysis (bottom). Raising [Ca$_{2+}$]$_{e}$ to 1.6 mM caused the cell to fire tonically (Fig. 4B). The autocorrelogram was flat and the RI dropped to −0.25. However, repetitive stimulation of the trigeminal tract (40 Hz for 1 s) switched the firing pattern to rhythmic bursting (Fig. 4C). Bursting was even more regular than in 0 Ca$_{2+}$. The RI increased to 3.0, but the dominant frequency remained at 6.5 Hz.

In all 35 neurons that converted from tonic firing to rhythmic bursting, the calculated RIs were negative (nonrhythmic) before repetitive stimulation of the tract (Fig. 5A) and positive (rhythmic) after stimulation (Fig. 5B; mean: 1.9 ± 0.17). The RI analysis was first conducted on cells that seemed to fire rhythmic bursts on visual inspection of the raw records. To validate the method, we conducted the same analysis on 10 randomly chosen cells that had been considered to be either tonically excited (n = 5) or tonically inhibited (n = 5) by stimulation of the tract. In all 10 cases, the RIs were negative before and after stimulation.

Poststimulus bursting frequencies calculated from the autocorrelograms and from FFTs gave similar results (mean of 8.75 ± 0.78 Hz calculated on autocorrelograms and 8.32 ± 0.72 Hz using FFT). The median was 6.25 Hz (range, 2.5–17.5 Hz) using both methods. Figure 5C shows the distribution of the frequencies calculated with FFTs. These ranged from 1 to 20 Hz. Among the 35 neurons that began to fire rhythmically after stimulation, two units could be clearly detected in the raw data in seven cases. In such cases, cross-correlograms were also computed. Figure 6 shows an example where two units were recorded: one with a large action potential that fired tonically and one with a smaller action potential that fired in bursts. There was no clear temporal relationship between firing of both units before stimulation as shown in the flat cross-correlogram (Fig. 6A, bottom). However, stimulation of the tract caused firing of the large unit to become rhythmic and bursts were synchronized in both units at 8.3 Hz (Fig. 6B). In this case, the latency between firing in the two units was 10 ± 1.5 ms as shown in the cross-correlogram (Fig. 6B, bottom, arrowhead). Synchronization between two units was also observed in the other five cases, and the average latency between units calculated in the cross-correlograms was 9.17 ± 1.54 ms.

**Optimal parameters of stimulation for the initiation of rhythmic activity**

Stimulation parameters were varied in frequency (20–100 Hz) and duration (100–2,000 ms) to identify the optimal conditions for burst generation in nine cases of conversion from tonic to bursting (Fig. 5D). The z-axis in the figure represents the number of neurons that burst for each combination of frequency (x) and duration (y). A rhythmic bursting case is represented only once in each different combination of parameters so the z-axis represents the number of cells that burst after tract stimulation in this combination of parameters.
The peak in the figure was found for the combination 40–60 Hz in the frequency domain and 500 ms in duration. That was the combination used for most cases.

Role of ionotropic glutamate receptors in bursting

Repetitive stimulation of the descending tract presumably causes a massive release of glutamate from the primary afferent terminals. To test whether glutamate is responsible for the bursting induced by stimulation we assessed the effects of ionotropic glutamate receptor agonists and antagonists. In 22 tonically firing neurons, NMDA pressure ejected from a pipette near the recorded cell was as efficient as tract stimulation to elicit bursting. Figure 7A shows tonic firing of a cell in control conditions ([Ca\(^{2+}\)]\text{mem} = 1.6 \text{ mM}). About 8 s after NMDA application, the cell began to burst (5 Hz; Fig. 7B), but firing returned to a tonic pattern again with the washout of NMDA (Fig. 7C). NMDA was tested in 22 of the 191 cases recorded in physiological [Ca\(^{2+}\)]\text{mem}. In one half of these, an increase of tonic firing was observed, but rhythmic bursting was induced in seven cases (33%). No effects were seen in two cases. In 2 of 22 cases (6%), rhythmic bursting could be obtained with NMDA application and also with stimulation of the trigeminal tract.

To assess whether NMDA receptors also contributed to the bursting induced by afferent input, we tested the ability of APV to block rhythmic bursting caused by stimulation of the tract in 15 neurons in physiological [Ca\(^{2+}\)]\text{mem}. In nine of these cases (60%), APV completely blocked the effect of stimulation. Figure 7D shows an example of a tonic firing neuron that bursts rhythmically after repetitive stimulation of the trigeminal tract. Local application of APV (Fig. 7E) prevented the change in firing pattern, which recovered after washout (Fig. 7F). In contrast, DNQX, an AMPA/kainate receptor antagonist, had no effect on stimulation-induced bursting (n = 4; Fig. 8).

**DISCUSSION**

The goal of this study was to assess the effects of repetitive stimulation of peripheral inputs on the firing pattern of NVsnpr neurons in vitro under physiological [Ca\(^{2+}\)]\text{mem} and mostly to see whether it is capable of eliciting rhythmic bursting within the frequency range of mastication.

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**FIG. 4.** Analysis of burst firing units. The 3rd trace from the top shows the raw records, the traces above show spikes (Events) and bursts (Bursts) detected by the software. The middle part of each panel represents the autocorrelogram (bin size is 10 ms), whereas the bottom graphs show the fast Fourier transforms (FFTs). A: Ca\(^{2+}\)-free ACSF. B: ACSF [Ca\(^{2+}\)] = 1.6 mM. C: Ca\(^{2+}\) 1.6 mM ACSF changes the firing pattern to rhythmic bursting. Some rhythmicity can be detected in A, as indicated by a positive rhythm index (RI), but no peaks clearly dominate the FFT. Stimulation increases the RI and a clear peak appears at 6.5 Hz.

**FIG. 5.** Analysis of the rhythmic bursting and of the optimal stimulation parameters. In all 35 cases that changed firing pattern after stimulation, the RIs were negative before stimulation (A) and above the threshold for significance (0.01) after stimulation (B). The mean RI after stimulation was 1.90 ± 0.17, with a median of 2 and range of 0.56–3.9. C: frequency of the bursts elicited by repetitive stimulation calculated from the FFT analysis. D: distribution of the number of neurons in which bursting was induced by different combinations of frequencies and duration of the stimulation train. All combinations were tested in nine cases. The arrow indicates the combination (40–60 Hz, 500 ms) that caused a maximal number of cells to burst (6/9).
make monosynaptic glutamatergic connections with neurons in NVsnpr (Bae et al. 2000; Clements and Beitz 1991; Waite et al. 2000), so we were not surprised that the majority of the short-latency and tonic effects observed were excitatory. The few inhibitory responses are probably caused by activation of inhibitory neurons that have been found within NVsnpr and surrounding areas (Li et al. 1996; Turman and Chandler 1994).

We have already shown that stimulation of adjacent trigeminal and reticular areas elicit GABAA and glycine-sensitive inhibitory postsynaptic potentials in NVsnpr (Athanassiadis et al. 2005b; Bourque and Kolta 2001).

Effects on bursting

Neurons that fired spontaneously in 1.6 mM [Ca\(^{2+}\)]_e were found throughout the nucleus, but only those in the dorsal three fifths were affected by the stimulation (Fig. 3). About one third fired in bursts, and stimulation increased burst frequency or duration of ~40% of these. Two thirds fired tonically, and in ~30% of these, firing became rhythmic after stimulation. Many of these transformed neurons were found to be in the most dorsal portion of the nucleus. Calculations of RIs conducted in all of these 35 cases confirmed that their firing pattern was not rhythmic before stimulation and became rhythmic after stimulation. This method has been developed by Sugihara et al. (1995) to detect periodicity in firing patterns using autocorrelation. Indeed, cells that did not appear to have regularity in their firing on visual inspection also had negative RIs (indicating that they were not rhythmic). Conversion from tonic firing to rhythmical bursting during stimulation is probably caused by the release of glutamate and involves activation of NMDA receptors because it was blocked by APV and not affected by DNQX. NMDA-induced bursting has previously been described in several preparations and systems. In most of these cases, the NMDA-induced bursting relied on a plateau potential that involved Ca\(^{2+}\) entry into the cell (Bertrand and Cazalets 2002; Büschges et al. 2000; Gabriel et al. 2008; Hsiao et al. 2002; Zhu et al. 2004).

Stimulus-evoked rhythmical firing in NVsnpr has been reported previously in vivo by Tsuboi et al. (2003). They recorded extracellular neuronal activity during repetitive stimulation of the masticatory area of the cerebral cortex of decerebrate and paralyzed rabbits and found a group of neurons that fired bursts in phase with fictive mastication. This group was located in the rostral and dorsal one third of the nucleus, a region that projects to the trigeminal motor nucleus (Kolta et al. 2000; Li et al. 1993). Tsuboi et al. (2003) showed that these
rhythmic masticatory neurons receive sensory inputs from muscle spindle, periodontal, and mucosal afferents.

Three in vitro studies described intrinsic rhythmic fluctuations of membrane potential and associated bursting in NVsnpr of gerbils (Sandler et al. 1998) and rats (Athanassiadis et al. 2005b; Brocard et al. 2006). Brocard et al. (2006) showed that bursting depended on a persistent sodium current ($I_{NaP}$). Recently, persistent sodium currents have also been described in spinal cord neurons linked to pattern generation in locomotion (Tazerart et al. 2007; Zhong et al. 2007).

Link to mastication and whisking

Brocard et al. (2006) found that both $I_{NaP}$ and bursting were potentiated by reducing $[Ca^{2+}]_e$. They showed that the proportion of NVsnpr neurons that burst spontaneously in low $[Ca^{2+}]_e$ increased dramatically in the second postnatal week, in parallel with maturation of $I_{NaP}$ and with the appearance of mastication in rat pups (Westneat and Hall 1992). Our results are consistent with this because bursting whether spontaneous or evoked by stimulation was very low in animals younger than 15 days.

Furthermore, stimulation was carried out with $[Ca^{2+}]_e$ at 1.6 mM, which is at the high end of the physiological range. Resting $[Ca^{2+}]_e$ in rat cerebrospinal fluid ranges from a high of $\sim 1.6$ mM in the fetus to 1.2 mM in adults (Jones and Keep 1988). Our finding that bursting activity can be modified or evoked in vitro by synaptic inputs from trigeminal sensory afferents provides further support to the hypothesis that NVsnpr rhythmic activity may be an essential component of the masticatory central pattern generator.

Westneat and Hall (1992) showed that the frequency of EMG bursts in the jaw muscles of freely behaving rats ranged from 5 to 11 Hz, with a mean of 8.5 Hz, which corresponds very well to the burst frequency of the great majority of our neurons.

The burst frequency of six of our neurons was $> 12$ Hz, suggesting that these could correspond to the “nonmasticatory” rhythmic neurons of Tsuboi et al. (2003). These were found ventral to the masticatory neurons and fired bursts at two to three times the masticatory rate that were not phase-linked to the masticatory motor pattern. They had receptive fields on the lips and face, suggesting that they could be part of a CPG for whisking, which has a frequency range of 5–15 Hz in rats (Berg and Kleinfeld 2003). NVsnpr, particularly the ventral part, projects to facial motoneurons in NVII (Li et al. 1997; Pinganaud et al. 1999).

The masticatory CPG can be activated by tonic inputs from the motor cortex and from the oral cavity (Dellow and Lund 1971; Lund and Dellow 1971; Sumi 1969). Many primary afferent terminals in NVsnpr are glutamatergic (Bae et al. 2000; Clements and Beitz 1991; Waite et al. 2000). Corticofugal axons also reach NVsnpr directly (Gobel et al. 1971; Kuypers 1958), and many of their terminals are likely to release glutamate, as they do in the spinal cord (Valtsohanoff et al. 1993). However, the brief trains of high-frequency stimulation of the cortical masticatory area that produce intense corticobulbar volleys and strong short-latency muscle twitches in jaw or limb muscles never trigger mastication. Instead, mastication is produced by trains of intermediate frequency (20–100 Hz) (Huang et al. 1989; Lund and Dellow 1971). The optimum stimulation frequency found to be most efficient in this study (40–60 Hz) was in the middle of this range. Interestingly, at steady state, intraoral mechanoreceptors and periodontal receptors, which project heavily to dorsal NVsnpr neurons, fire between 50 and 60 Hz during mastication (Appenteng et al. 1982; Trulsson 2007; Trulsson and Johansson 2002), which fits very well with our findings. In vivo, trains can last $\approx 10$ s and still evoke mastication, but in our preparation, the optimal train duration was $\sim 500$ ms. This may be because of the fact that the sensory fibers have been severed from their cell bodies and therefore prolonged stimulation may deplete transmitter stores.

Synchronization and phase-coupling

Synchronization of neuronal firing and phase-coupling are fundamental features of CPGs (Grillner 2006). We were able to examine these in six pairs of spontaneously firing neurons that fired in bursts after stimulation. In all cases, the bursts were of the same frequency after stimulation, and they were phase-coupled. There was also a tendency for spikes of one to occur $\sim 9$ ms after the other. The relatively long latency between the spikes of the two units argues against direct coupling through gap junctions and even direct excitatory projections from one neuron to the other. However, NVsnpr is strongly linked to
interneurons in adjacent structures, the periglomerular area surrounding the trigeminal motor nucleus (NVmot), NVmot itself, and nucleus pontis caudalis, and these are similarly interconnected by polysynaptic pathways that, however, follow relatively high frequencies (e.g., 50–70 Hz) (Athanassiadis 2005b; Bourque and Kolta 2001; McDavid et al. 2006). We presume that these circuits participate in the pattern generating process, and indeed, many neurons in these nuclei fire rhythmically during fictive mastication. However, very few have intrinsic bursting properties, and neurons firing in the two phases (jaw opening, jaw closing) are intermingled (Westberg et al. 1998). This is unlike NVsnpr, in which most of the neurons that fire during closing occupy the most dorsal and anterior part of the nucleus, whereas opener neurons are clustered more posteriorly and ventrally (Tsuboi et al. 2003). Our finding of burst phase coupling between adjacent neurons is consistent with this and suggests the existence of strong local interconnections.

Functional implications

Rhythmic bursting in NVsnpr neurons is initiated by activation of \( I_{NaP} \) and can only occur when the membrane potential is between \(-59\) and \(-41\) mV, which corresponds to activation range of this persistent sodium current (Azouz et al. 1996; Brocard et al. 2006; Darbon et al. 2004; Del Negro et al. 2002; Su et al. 2001; Wu et al. 2001). We proposed that sustained activity of depolarizing afferent inputs to the nucleus not only brings the level of depolarization into the \( I_{NaP} \) range but also causes the Ca\(^{2+}\) depletion that is necessary to activate it (Brocard et al. 2006; Kolta et al. 2007). Sustained neural activity does lead to large drops in \([\text{Ca}^{2+}]_e\) in other systems (Benninger et al. 1980; Rusakov and Fine 2003; Somjen 1980). Within the \( I_{NaP} \) range, the frequency of bursting depends on the level of depolarization, providing a mechanism by which the frequency of masticatory movements can be adjusted by interactions with other cell groups of the CPG and by sensory feedback to the changing mechanical properties of the food as it is broken down by the teeth (Lund and Kolta 2006; Peyron et al. 1997; Thexton et al. 1980).

Conclusion

Neurons of dorsal NVsnpr have several properties that suggest a major role in masticatory pattern generation. They fire rhythmically during fictive mastication and project to trigeminal, facial, and hypoglossal motor nuclei and to other premotor regions. In addition, they have an intrinsic capacity to burst that depends in part on a persistent sodium current that is modulated by the extracellular concentration of Ca\(^{2+}\). This study provides evidence that repetitive stimulation of afferent inputs to the nucleus can trigger rhythmic bursting in NVsnpr through an NMDA-dependent mechanism and supports our hypothesis that the level of afferent fibers activity can induce and modify rhythmic bursting by a combination of direct depolarization, local reduction of the extracellular calcium concentration, and through CPG network interactions.

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