Emergence of Intrinsic Bursting in Trigeminal Sensory Neurons Parallels the Acquisition of Mastication in Weanling Rats

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Submitted 5 April 2006; accepted in final form 9 August 2006

Brocard, Frédéric, Dorly Verdier, Isabel Arsenault, James P. Lund, and Arlette Kolta. Emergence of intrinsic bursting in trigeminal sensory neurons parallels the acquisition of mastication in weanling rats. J Neurophysiol 96: 2410–2424, 2006. First published August 16, 2006; doi:10.1152/jn.00352.2006. There is increasing evidence that a subpopulation of neurons in the dorsal principal sensory trigeminal nucleus are not simple sensory relays to the thalamus but may form the core of the central pattern generating circuits responsible for mastication. In this paper, we used whole cell patch recordings in brain stem slices of young rats to show that these neurons have intrinsic bursting abilities that persist in absence of extracellular Ca2+ channel blockers. The proportion of bursting neurons increased dramatically in the second postnatal week, in parallel with profound changes in several electrophysiological properties. This is the period in which masticatory movements appear and mature. Bursting was associated with the development of an afterdepolarization that depend on maturation of a persistent sodium conductance (INaP). An interesting finding was that the occurrence of bursting and the magnitude of INaP were both modulated by the extracellular concentration of Ca2+. Lowering extracellular [Ca2+] increased both INaP and probability of bursting. We suggest that these mechanisms underlie burst generation in mastication and that similar processes may be found in other motor pattern generators.

INTRODUCTION

The basic pattern of masticatory movement is produced by a brain stem central pattern generator (CPG) (Dellow and Lund 1971), the core of which lies between the rostral borders of the Vth and VIIth cranial motor nuclei (Kogo et al. 1996; Nakamura et al. 1999; Tanaka et al. 1999). Several models of the CPG have been proposed (Lund et al. 1998; Nakamura and Katakura 1995; Nozaki et al. 1986), all of which include medullary reticular nuclei and last-order interneurons in the lateral reticular formation and spinal trigeminal nucleus.

The trigeminal principal sensory nucleus (NVsnpr) was long regarded as a relay station to thalamus and other regions of the somatosensory system. However, it also contains neurons that project to the motor nuclei that control feeding, the Vth, VIIth, and XIIth cranial motor nuclei (Kolta et al. 2000; Li et al. 1993; Pinganaud et al. 1999; Travers and Norgren 1983; Yoshida et al. 1998). It was recently shown in rabbits that many neurons in the dorso-anterior region of NVsnpr burst rhythmically during fictive mastication (Tsuboi et al. 2003). The same region also expressed c-Fos-like protein, a functional marker of activity, after repeated episodes of fictive mastication (Athanasiadis et al. 2005a). Furthermore, Sandler et al. (1998) have shown that about one-half of NVsnpr neurons recorded in vitro in slices of the gerbil brain stem have plateau properties that allow them to transform a depolarizing input into bursts. Such behavior is more compatible with a role in rhythm generation than with the faithful transmission of sensory information.

METHODS

Slice preparation

All surgical and experimental procedures conformed to guidelines of the Canadian Institutes of Health Research and were approved by the University Animal Care and Use Committee. Experiments were performed on NVsnpr neurons in slices obtained from 5- to 17-day-old Sprague-Dawley rats. Rats were anesthetized with isoflurane (Abbott Laboratories, Saint-Laurent, Quebec, Canada) inhalation and decapitated. The brain was quickly taken out and placed in cold (4°C) sucrose-based artificial cerebrospinal fluid (ACSF, composition in mM: 225 sucrose, 3 KCl, 1.25 KH2PO4, 4 MgSO4, 0.2 CaCl2, 20 NaHCO3, and 10 D-glucose) bubbled with 95% O2-5% CO2, pH 7.4. In the same medium, transverse slices (300 μm thick) through the NVsnpr were prepared using a Vibratome (VT1000 S, Leica). Slices were incubated at room temperature (21–24°C) in the holding chamber filled with normal ACSF (in mM: 125 NaCl, 3 KCl, 1.25 KH2PO4, 1.3 MgSO4, 2.4 CaCl2, 26 NaHCO3, and 25 D-glucose). The slices were transferred to an immersion slice chamber and perfused with normal ACSF at a rate of ~2 ml/min. The slices were allowed ≥1 h before the experiment was started.

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Electrophysiological recordings

Neurons were visualized using a fixed stage microscope (Eclipse E600FN, Nikon) coupled with a 40× water immersion lens. The image was enhanced with an infrared-sensitive CCD camera and displayed on a video monitor. Whole cell patch-clamp recordings in current-clamp mode were performed from visually identified cells located in the dorsal part of the NVsnpr using an axoclamp 2B amplifier (Axon Instruments, Foster City, CA). Patch electrodes (6–9 MΩ) were pulled from borosilicate glass capillaries (1.5 mm OD, 1.12 mm ID; World Precision Instruments, Sarasota, FL) on a Sutter P-97 puller (Sutter Instruments, Novato, CA) and filled with a K+ glutamate based solution (in mM: 140 K+ glutamate, 10 NaCl, 2 MgCl2, 10 HEPES, 0.5 EGTA, 2 ATP, and 0.4 GTP). All drugs were purchased from Sigma-Aldrich (Oakville, Ontario, Canada), kept as a concentrated stock solution, and diluted in the bath perfusing ACSF to their final concentration using a syringe pump. The following pharmacological agents were used: TTX (1 μM); tetraethylammonium chloride (TEA, 10 mM); riluzole (20 μM); apamin (100 nM); charybdoxin (100 nM); and ZD 7288 (10–20 μM). In the experiments examining the role of calcium in burst generation, the amount of CaCl2 removed from the normal ACSF was replaced by an equivalent amount of MgCl2.

Drug application

All drugs were purchased from Sigma-Aldrich (Oakville, Ontario, Canada), kept as a concentrated stock solution, and diluted in the bath perfusing ACSF to their final concentration using a syringe pump. The following pharmacological agents were used: TTX (1 μM); tetraethylammonium chloride (TEA, 10 mM); riluzole (20 μM); apamin (100 nM); charybdoxin (100 nM); and ZD 7288 (10–20 μM). All drugs were purchased from Sigma-Aldrich (Oakville, Ontario, Canada), kept as a concentrated stock solution, and diluted in the bath perfusing ACSF to their final concentration using a syringe pump. The following pharmacological agents were used: TTX (1 μM); tetraethylammonium chloride (TEA, 10 mM); riluzole (20 μM); apamin (100 nM); charybdoxin (100 nM); and ZD 7288 (10–20 μM). In the experiments examining the role of calcium in burst generation, the amount of CaCl2 removed from the normal ACSF was replaced by an equivalent amount of MgCl2.

Data acquisition and analysis

Electrophysiological data were acquired through a Digidata 1322A interface and analyzed with Clampex 9 software (Axon Instruments). Passive membranes properties of cells were measured by injecting small hyperpolarizing currents pulses to avoid the activation of voltage-sensitive currents. Data are presented in the text and in the tables as mean ± SE. The input resistance is measured by the slope of the linear portion of the I-V relationship. The membrane time constant was determined by fitting an exponential function to the rising phase of the voltage trace used for determining the input resistance. In some cells, there was evidence of inward rectification (“sag”) during strong hyperpolarization. The size of the sag was expressed as the ratio of the peak negative voltage to the steady-state membrane potential. The rheobase was defined as the minimum current intensity necessary to fire the cell. Single spike analysis was performed on the first spike elicited near the rheobase. Peak spike amplitude was measured from the threshold potential, and spike duration was defined as the time to fall to half-maximum peak. To study the afterdepolarizations (ADPs) and the afterhyperpolarizations (AHPs), single spikes were evoked by brief intracellular pulses at holding potential. The peak amplitude and duration (to one-half the peak height) of ADPs and AHPs were measured from the same holding potential (~50 mV for the AHP and ~70 mV for the ADP). Firing patterns were studied with 1-s-long depolarizing current pulses of varying amplitudes. The average instantaneous firing frequency during the last 500 ms of the 1-s pulse was defined as the steady-state firing frequency. We used one-way ANOVA for age-dependent comparisons with two posttests: Tukey’s test was used to evaluate the effect of age on other variables. The pharmacological effects of drugs were evaluated using Student’s paired t-test. Values of P < 0.05 were considered significant (GraphPad Prism 4.0, GraphPad Software, San Diego, CA).

RESULTS

Data were collected from 292 NVsnpr neurons from 77 rats aged between P5 and P17 (with P0 defined as the 1st 24 h after birth). Only neurons exhibiting a stable (>15 min) resting membrane potential more negative than ~50 mV were included in the study.

NVsnpr neurons displayed four firing patterns

The firing patterns of neurons recorded in normal ACSF were classified as adaptive (25.6%, n = 53), tonic (59.9%, n = 124), or bursting (14.5%, n = 30). Adaptive neurons generated a train of spikes in response to current injection near rheobase (Fig. 1A, bottom), but further depolarization caused a progressive change in spike form: amplitude fell and duration increased (Fig. 1A, top) until sustained depolarization without waveletst remained. Tonic neurons were characterized by a pattern of single spikes that persisted for the entire duration of the depolarization (Fig. 1B), even with large depolarizing current injections. The spike shape remained nearly constant throughout the firing period. Bursting firing patterns were divided into two subtypes: burst-and-tonic (Fig. 1C1) and repetitive bursting (Fig. 1D1). In all bursting cells, an initial burst was elicited at the onset of the current injection (Fig. 1, C2 and D2) followed by either a regular spike train (burst-and-tonic spiking, n = 23) or recurrent bursts (repetitive bursting, n = 7). Adaptive or tonic cells could not be converted into bursting cells by large changes in the holding potential.

Some membrane properties varied significantly between classes of neurons (Table 1). Adaptive cells had a higher mean input resistance and membrane time constant, and displayed broader spikes followed by more prolonged AHPs. The primary property that distinguished bursting neurons from the other two classes was their higher excitability, shown by their tendency to have a lower rheobase and significantly lower firing threshold. In addition all bursting cells, but only one-half of the tonic and one-third of the adaptive cells displayed an ADP (P < 0.0001, Fisher’s test).

Age-related changes in firing patterns

To determine whether these firing patterns constitute distinct neuronal populations in the adult NVsnpr nucleus or represent successive stages in the development of the firing pattern, we compared the proportions of the four groups of cells in animals divided into three age groups: P5–P8 (n = 68), P9–P12 (n = 68), and P13–P17 (n = 73). These three age groups represent key periods of development in the rat in which the pattern of mastication is absent, emerging or of adult form, respectively (Westneat and Hall 1992).

In the youngest age group (P5–P8), only adaptive and tonic neurons were encountered in the proportion of one third and two thirds, respectively (Fig. 2A). From P9 to P12, when the first bursting cells were detected, tonic cells were dominant (~85% of the total). In the more mature animals (P13–P17), the proportion of bursting cells increased to nearly 40% of the total, whereas adaptive neurons were very rare (1.4%). After P12, the incidence of bursting cells increased rapidly progressing from 6% at P12, through 25% at P13, to 44% at P14 (Fig. 2B), after which it remained nearly constant. Note that all repetitive bursting neurons were observed in the P13–P17 age group.

For neurons exhibiting a train of single spikes (i.e., tonic and burst and tonic spiking cells), the frequency-current relationship (F-I) was studied (Fig. 2C1). Examples of F-I relationships for neurons at P7, P12, and P17 are shown in Fig. 2C2 and were best fitted with sigmoidal functions. In the initial portion of the curve, the mean firing frequency increased...
rapidly with the current intensity. Linear regression analyses were performed on the first part of the curves ($r > 0.95$). There was no difference in slope between P5–P8 and P9–P12 (85 ± 11 vs. 103 ± 9 Hz/nA; $P > 0.05$, 1-way ANOVA), but a significant increase was observed between P9–P12 and P13–P16 (145 ± 16 Hz/nA at P13–P16; $P < 0.05$). As current was increased, slopes flattened, and mean firing frequency eventually reached a maximum that was least for the P7 neuron. Indeed, the maximum frequency in the P5–P8 group was significantly lower than that of the P9–P12 group (18 ± 1 vs.

**FIG. 1.** Typical firing patterns recorded in trigeminal principal sensory nucleus (NVsnpr) neurons. Responses to depolarizing current pulses were obtained from resting membrane potential. A: adaptive neurons showed regular firing when depolarized just above threshold, but spikes were damped with larger current injections. B: in tonic neurons, firing is sustained throughout pulse. C: in burst-and- tonic neurons, an initial 2-spike burst is followed by tonic firing. D: repetitive-bursting pattern is characterized by recurrent bursts of action potentials. Shaded areas of recordings in C1 and D1 are enlarged in C2 and D2, respectively. Bottom traces: injected currents, superimposed.

### TABLE 1. Passive and active membrane properties of NVsnpr neurons classified according to their firing patterns

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Adaptive</th>
<th>Tonic</th>
<th>Burst and Tonic</th>
<th>Repetitive Bursting</th>
</tr>
</thead>
<tbody>
<tr>
<td>$N$</td>
<td>53</td>
<td>(53)</td>
<td>124</td>
<td>(23)</td>
</tr>
<tr>
<td>$V_{m}$ mV</td>
<td>-57 ± 1</td>
<td>-56 ± 0.4</td>
<td>-54 ± 1</td>
<td>-58 ± 1</td>
</tr>
<tr>
<td>$R_{m}$ MΩ</td>
<td>521 ± 32 vs T, vsBT, RB</td>
<td>347 ± 17</td>
<td>360 ± 32</td>
<td>219 ± 57</td>
</tr>
<tr>
<td>$\tau$, ms</td>
<td>53.6 ± 2.5 vs T, BT, RB</td>
<td>(121)</td>
<td>27.8 ± 3.1</td>
<td>18.1 ± 4.8</td>
</tr>
<tr>
<td>AP amp, mV</td>
<td>63 ± 2</td>
<td>62 ± 1</td>
<td>62 ± 3</td>
<td>67 ± 8</td>
</tr>
<tr>
<td>AP dur, ms</td>
<td>2.9 ± 0.2 vs T, BT, vsRB</td>
<td>1.6 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>AP threshold, mV</td>
<td>-37 ± 1</td>
<td>-39 ± 1</td>
<td>-43 ± 1 vsA, T</td>
<td>-48 ± 2 vsA, T</td>
</tr>
<tr>
<td>Rheobase, nA</td>
<td>0.042 ± 0.008</td>
<td>0.072 ± 0.009</td>
<td>0.021 ± 0.005</td>
<td>0.015 ± 0.004</td>
</tr>
<tr>
<td>ADP, %</td>
<td>34.9</td>
<td>47.5</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>AHP amp, mV</td>
<td>-5.9 ± 0.6</td>
<td>-7.5 ± 0.3</td>
<td>-6.5 ± 0.9</td>
<td>-5.4 ± 1.3</td>
</tr>
<tr>
<td>AHP dur, ms</td>
<td>167 ± 92 vs T, BT</td>
<td>103 ± 4</td>
<td>82 ± 10</td>
<td>119 ± 25</td>
</tr>
</tbody>
</table>

†$P < 0.01$, ‡$P < 0.001$ (1-way ANOVA). $N =$ number of cells. Number of observations are in parentheses. NVsnpr, trigeminal principal sensory nucleus; A, adaptive; T, tonic; BT, burst and tonic; RB, repetitive bursting; ADP, afterdepolarization, AHP, afterhyperpolarization, AP, action potential.
32 ± 4 Hz; \( P < 0.01 \), 1-way ANOVA), and there was a nonsignificant trend toward a further increase at P13–P16 (44 ± 5 Hz).

For neurons exhibiting repetitive bursting \((n = 7)\), the bursting frequency increased linearly with current intensity (Fig. 2, D1 and D2), and the \( F-I \) relationship was fitted with a linear regression function (Fig. 2D3; mean slope: 113 ± 33 Hz/nA, mean \( r = 0.987 \)). The mean burst frequency ranged from 3 ± 1 Hz with near-threshold depolarizations to 9 ± 2 Hz with larger currents. The firing pattern switched from recurrent bursting to burst and tonic spiking with further depolarization (see Fig. 8C).

The following experiments were performed to determine which cellular mechanisms underlie the emergence of bursting.

**Age-related changes in electrophysiological properties**

An analysis of the voltage responses to hyperpolarizing currents (Fig. 3A) revealed that input resistance and membrane time constant were significantly reduced from P5 to P17 by \(~40\%\) (Fig. 3B1; \( P < 0.001 \), 1-way ANOVA) and \(~70\%\) (Fig. 3B2; \( P < 0.0001 \), 1-way ANOVA), respectively. The largest reduction of the membrane time constant occurred during the second postnatal week. In contrast, the resting membrane potential did not change with age (Fig. 3B3; \( P = 0.213 \), 1-way ANOVA). In \(~90\%\) of the cells, application of large hyperpolarizing current pulse resulted in the appearance of an inward rectifying response (see arrowheads in Fig. 3A). However, the magnitude of this rectification increased twofold over the age range studied (Fig. 3B4; \( P < 0.0001 \), 1-way ANOVA).

When depolarized to threshold, NVsnpr neurons produced a single spike (Fig. 4A1). Mean threshold became more negative with age, going from \(-36\) to \(-43\) mV between P5 and P17 (\( P < 0.01 \), linear regression \( r = -0.73 \)). The spike amplitude was unchanged (Fig. 4A2; \( P = 0.665 \), 1-way ANOVA) but its duration was shortened by \(~75\%\) (Fig. 4A3; \( P < 0.0001 \), 1-way ANOVA), with the largest reduction occurring during the first 11 postnatal days.
Spikes were followed by a prolonged AHP that also changed with age (Fig. 4B1). Its amplitude increased by 55% (Fig. 4B2; \( P < 0.001 \), 1-way ANOVA) between P5 and P17, whereas duration decreased by 65% (Fig. 4B3; \( P < 0.001 \), 1-way ANOVA). The decrease in duration mainly occurred between P9 and P14 (\( P < 0.01 \); 1-way ANOVA, Tukey post hoc).

An ADP (Fig. 4C1, arrowheads) was observed in a subpopulation of NVsnpr neurons that grew in size during postnatal development. Approximately 30% of neurons exhibited an ADP at P6–P7, but this increased to 70% at P12–P13 and to 87% at P16–P17 (Fig. 4C3). During the same period, ADP amplitude decreased significantly by about 35% (see linear regression in Fig. 4C2; \( P < 0.05 \), \( r = -0.63 \)).

ADP and sag are known to play an important role in activation and maintenance of regenerative bursting in other neurons (Azouz et al. 1996; Pape 1996). Past studies suggest that voltage-dependent Ca\(^{2+}\) currents contribute to the ADP (Kobayashi et al. 1997; Wong and Prince 1981) and that the mixed cation conductance \( I_h \) underlies the sag (Pape 1996). We sought to determine if the development of these two specific membrane conductances is related to the emergence of burst firing.

**Ionic basis of recurrent bursts**

We first examined the effect of decreasing the external Ca\(^{2+}\) concentration (\([\text{Ca}^{2+}]_E\)) on the firing properties of 19 NVsnpr neurons recorded from P12 to P16. Although spike amplitude and duration did not change, the amplitude of the ADP was significantly increased (Fig. 5A1; Table 2; \( P < 0.0001 \), paired \( t \)-test), whereas AHP amplitude was significantly reduced (Fig. 5A2; Table 2; \( P < 0.0001 \), paired \( t \)-test) in Ca\(^{2+}\)-free medium. Occasionally, enhancement of the ADP reached threshold and triggered some spikes (Fig. 5A1, right). These reversible changes occurred without significant alteration in input resistance and resting membrane potential (Table 2). However, the most obvious effect of removing Ca\(^{2+}\) was that 80% of tonic and burst and tonic spiking cells recorded in normal ACSF were converted to repetitive bursting in Ca\(^{2+}\)-free ACSF (Fig. 5B).
In standard solution, the age of the rat had a profound effect on the probability of observing repetitive bursting. In the next step of the experiment, we asked if the bursting activity observed under Ca\(^{2+}\)/H11001-free ACSF is developmentally regulated. In 26 neurons examined before P9, only 11.5% showed repetitive bursting (Fig. 5C). After P9, the incidence of repetitive bursting increased substantially from 58% at P10, through 73% at P12 and P14, to 85% at P16 (Fig. 5C).

Persistence of bursting activity in Ca\(^{2+}\)/H11001-free ACSF suggests that 1) entry of Ca\(^{2+}\) is not essential for the generation of repetitive bursts and 2) that the ability to generate bursts results from intrinsic voltage-dependent properties of the membrane because synaptic transmission is abolished. Furthermore, the increase in the incidence of bursting under Ca\(^{2+}\)-free ACSF is likely to be linked to the reciprocal changes in ADP and AHP amplitudes. A reduction of the...
AHP would allow a larger ADP to occur, which could lead to bursting.

**Ca²⁺-activated K⁺ current**

As in most neurons, Ca²⁺-activated K⁺ current probably underlie the AHP in NVsnpr neurons and it is not surprising that elimination of Ca²⁺ reduces AHP amplitude. The reduction of the AHP amplitude in Ca²⁺-free saline could constitute a critical factor in the emergence of bursting activities. To test this hypothesis, the effect of specific $I_{KCa}$ blockers on burst generation were studied in older rats (P14–P17). In standard solution, apamin (100 nM), a selective blocker of the small conductance Ca²⁺-activated K⁺ (SK) channels, depressed the amplitude of the AHPs (from 3.2 ± 0.4 to 2.1 ± 0.7 mV, $n = 4$) but did not generate recurrent bursts in response to steady...
depolarizing current (Fig. 6A, right). The latter finding did not result from the incapacity of the cells to fire in burst because they generated bursting activity in Ca\(^{2+}\)-free saline (Fig. 6A, left). Frequency and duration of burst during Ca\(^{2+}\)-free were unchanged by bath application of apamin. On the other hand, when charybdotoxin (100 nM), a selective blocker of the big conductance Ca\(^{2+}\)-activated K\(^+\) (BK) channels, was bath-applied in standard solution, not only AHP amplitude decreased (from 5.3 ± 1.9 to 1.1 ± 1.1 mV, n = 3), as with apamin, but when depolarized to threshold, NVsnpr neurons produced doublets of spikes rather than single spikes as in control (n = 3; Fig. 6B2), probably caused by enhancement of the ADP. Nevertheless, these cells did not show robust recurrent bursting as observed in calcium-free ACSF (Fig. 6B, left), but they only discharged a doublet or a triplet of spikes at the onset of long depolarizing pulses in two cases while recurrent doublets under steady-state depolarization was only observed in one case. As for apamin, frequency and duration of burst during Ca\(^{2+}\)-free were unchanged by bath application of charybdotoxin. Similarly, bath application of TEA (10 mM), a K\(^+\) channels blocker, did not prevent bursting in Ca\(^{2+}\)-free saline, but it did reduce burst frequency and greatly increase their duration (Fig. 7A; n = 5). Together, these results suggest that TEA-sensitive K\(^+\) currents shape the bursting activity, likely by controlling the repolarization.

**TABLE 2. Effects of external Ca\(^{2+}\) removal on intrinsic membrane properties of NVsnpr neurons**

<table>
<thead>
<tr>
<th></th>
<th>Standard ACSF</th>
<th>Ca(^{2+})-Free ACSF</th>
</tr>
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<tbody>
<tr>
<td>(V_{m}), mV</td>
<td>−60 ± 1</td>
<td>−60 ± 1</td>
</tr>
<tr>
<td>(R_{m}), MΩ</td>
<td>279 ± 36</td>
<td>281 ± 42</td>
</tr>
<tr>
<td>AP amp, mV</td>
<td>61 ± 3</td>
<td>63 ± 4</td>
</tr>
<tr>
<td>AP dur, ms</td>
<td>1.1 ± 0.1</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>ADP amp, mV</td>
<td>13.6 ± 1.3</td>
<td>21.7 ± 1.1*</td>
</tr>
<tr>
<td>AHP amp, mV</td>
<td>−8.5 ± 1</td>
<td>−19.9 ± 0.5*</td>
</tr>
<tr>
<td>Percent of repetitive</td>
<td>0%</td>
<td>80%</td>
</tr>
<tr>
<td>bursting cells</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.001. Paired r-tests (n = 15). NVsnpr, trigeminal principal sensory nucleus; ACSF, artificial cerebrospinal fluid; AP, action potential; ADP, afterdepolarization; AHP, afterhyperpolarization.

FIG. 6. Independence of burst-firing on Ca\(^{2+}\)-activated K\(^+\) currents. A: bursting elicited by a 0.03-nA depolarizing current pulse in absence of external Ca\(^{2+}\) was converted to tonic firing in standard ACSF and not restored by addition of apamin (100 nM). B: bursting elicited by 0.02-nA depolarizing current in absence of external Ca\(^{2+}\) was converted to tonic firing in standard ACSF. Addition of charybdotoxin (100 nM) to the bath, enhanced the ADP (B2) and caused the cell to fire doublets of action potentials but was not sufficient to induce plateau potentials and bursts similar to those observed in Ca\(^{2+}\)-free conditions (B1). Shaded areas of recordings in B are enlarged in B1 and B2.
Ih current

The evidence that the depolarizing sag increases with age led us to assess the role that Ih might play in the emergence of bursting activity. ZD 7288 (10–20 μM), applied at a concentration that blocks the sag in older animals (P14–P17; Fig. 7B, bottom left), did not prevent bursting in Ca2+-free ACSF; instead, it caused an increase of 158 ± 64% in burst frequency (Fig. 7B, bottom right; n = 4). This increase in frequency was associated with a decrease in amplitude of hyperpolarization that follow bursts. Shaded areas of recordings in C are enlarged in insets.

Persistent sodium currents

We then examined the role of sodium influx in burst generation, first by using TTX (1 μM), a specific sodium channel blocker, in Ca2+-free conditions. A brief bath application of TTX (5–10 min) reversibly inhibited the bursting activity of the four neurons tested without abolishing spikes (Fig. 8A). However, continued superfusion of TTX led to a disappearance of spikes (data not shown). These results suggested involvement of a persistent sodium current (INaP) in burst generation. This hypothesis was supported by the observation that riluzole (20 μM), a drug that interferes with INaP, disrupted the generation of repetitive bursts (Fig. 8B, n = 3) without blocking spikes. This effect did not reverse even after 1–2 h of washout. These results confirm a critical role for INaP in burst generation.

Age-related changes in INaP-dependent bursting activity

To define the contribution of INaP in the development of bursting, we compared the effects of injected current on neurons from animals of different ages in absence of [Ca2+]E and in presence of TEA. In two of seven neurons tested in young animals (P5–P7), a brief threshold depolarizing pulse evoked a compound response consisting of a fast spike followed by a train of spikes superimposed on a plateau potential that outlasted the current pulse (Fig. 8C, P6). Addition of TTX to the medium abolished the plateau potential and the spike train before the initial spike was affected (Fig. 8C, insets), indicating

FIG. 7. Independence of burst-firing on tetraethylammonium chloride (TEA)-sensitive K+ currents and Ih. A: bursting elicited by a 0.06-nA depolarizing current pulse in Ca2+-free ACSF before, 30 min after adding TEA (10 mM), and 30 min after removing the drug. B: ZD 7288 (10 μM) blocked the depolarizing sag (arrowhead) induced by a hyperpolarizing current pulse of –0.4 nA in Ca2+-free ACSF and increased the frequency of bursting. Increase in frequency was accompanied by a decrease in amplitude of hyperpolarization that follow bursts. Shaded areas of recordings in C are enlarged in insets.
again that the plateau depolarization is \( I_{\text{NaP}} \) dependent. In the five other cells recorded, the fast spike was not followed by the plateau potential (data not shown). The incidence of \( I_{\text{NaP}} \)-dependent plateau potentials increased with age and was observed in 80 and 100% of neurons recorded at P9–P12 (Fig. 8C; P11, \( n = 5 \)) and P14–P17 (Fig. 8C; P16, \( n = 5 \)), respectively. The mean duration of the plateau potential was 0.3 ± 0.2 s at P5–P7, was unchanged at P9–P12 (0.4 ± 0.1 s), but tended to be longer at P14–P17 (2.5 ± 0.8 s; \( P = 0.07 \), 1-way ANOVA). In contrast, peak amplitude tended to increase from P6 to P9–P12 (26.6 ± 3.4 and 38.1 ± 4.9 mV, respectively) but remained stable thereafter (41.7 ± 1.7 mV at P14–P17; \( P = 0.07 \), 1-way ANOVA). As a consequence of the increase in peak amplitude, spikes were totally inactivated during plateau potentials. Interestingly, cells that did not generate bursts in Ca\(^{2+}\)-free ACSF did not display \( I_{\text{NaP}} \)-dependent plateau potentials (data not shown).

The above results suggest that the emergence of repetitive bursting depends on a concomitant increase in amplitude and duration of the \( I_{\text{NaP}} \)-dependent depolarization.
Characteristics of bursting activity

[Ca\textsuperscript{2+}]_E DEPENDENCY FOR GENERATION OF BURSTING. An intriguing question is the relationship between \(I_{NaP}\) and the [Ca\textsuperscript{2+}]_E. The proportion of repetitively bursting cells was small (3.4%, \(n = 7/207\)) in normal ACSF, but lowering [Ca\textsuperscript{2+}]_E increased their incidence. To determine the [Ca\textsuperscript{2+}]_E threshold below which most of NVsnpr neurons fire in bursts, the effect of a gradual reduction of [Ca\textsuperscript{2+}]_E on firing patterns was studied (Fig. 9A) in six tonic neurons from animals older than P14. Lowering [Ca\textsuperscript{2+}]_E from 2.4 to 1.2 mM did not change the tonic firing pattern, but a further reduction to 0.6 mM induced bursting in five of the six cells. Subsequent exposure to Ca\textsuperscript{2+}-free ACSF, increased the amplitude and duration of the plateau potential, but reduced burst frequency (Fig. 9A, right). Lowering [Ca\textsuperscript{2+}]_E also caused a concentration-dependent increase in the amplitude of the ADP (Fig. 5A) and in the duration of the \(I_{NaP}\)-dependent plateau potential elicited by a brief depolarizing stimulus (Fig. 9B). Thus the increase in duration of the \(I_{NaP}\)-dependent plateau observed in low-[Ca\textsuperscript{2+}]_E seems to be responsible for the switch from tonic firing to bursting.

Voltage dependency of burst generation

We assessed the voltage dependency of bursting in Ca\textsuperscript{2+}-free ACSF by sustained injection of currents in six neurons from animals older than P14 (Fig. 9C). Bursting was observed...
at membrane potentials between $-59 \pm 2$ and $-41 \pm 3$ mV. Below $-60$ mV, most cells were silent; above $-41$ mV, most were tonically active. The duration and area of the $I_{NaP}$-dependent plateau potential tested in absence of $[Ca^{2+}]_E$ and in presence of TEA ($n=5$) were also voltage dependent (Fig. 9, $D_1$ and $D_2$). The area of the plateau potential was considerably reduced in a linear manner with hyperpolarized membrane potential (Fig. 9, $D_1$ and $D_2$, circles). This effect was a direct function of the plateau duration that proportionally decreased with holding membrane potential (Fig. 9$D_2$, triangles). No relationship was observed with the plateau amplitude (Fig. 9$D_2$, squares).

Together, these results strongly suggest that the bursts are $I_{NaP}$ dependent and are modulated by $[Ca^{2+}]_E$ and by the transmembrane potential.

**Discussion**

Our study shows that the membrane properties of dorsal NVsnpr cells undergo important changes in the first 3 postnatal wk. These include decreases in input resistance, membrane time constant, action potential and AHP durations, and increases in $sag$ and ADP amplitudes. During the same period, there are major changes in firing properties, in particular, the emergence of an ability to intrinsically generate rhythmic bursts in low-$[Ca^{2+}]_E$. This property seems to be $I_{NaP}$ dependent and modulated by $[Ca^{2+}]_E$. Intrinsic bursting is rare or absent before P12, which is approximately the age at which the first masticatory movements appear (Westneat and Hall 1992), but it becomes common later in development.

Development of membrane properties

It is likely that the decrease of input resistance and membrane time constant of NVsnpr neurons after birth is caused by a gradual increase in their cell size (Jacquin et al. 1996; Miller and al-Ghoul 1993), accompanied by an increase in density of ionic channels (Cameron et al. 2000). Unlike genioglossal motoneurons (Nunez-Abades et al. 2000), the decrease in input resistance does not result from an increase in synaptic inputs because it is still observed under conditions that prevent synaptic transmission (in Ca2+-free medium).

Surprisingly, there was a decrease of rheobase, which shows that NVsnpr neurons become more excitable with age, even though input resistance and membrane time constant decrease. The negative shift in action potential threshold probably reflects a decrease in activation threshold of Na+ channels (Gao and Ziskind-Conhaim 1998). This and the increase of the $I_{NaP}$ during development could contribute to the age-related decrease of the rheobase. Accompanying these changes in excitability, action potential duration shortened as depolarization and repolarization both become faster. This is probably caused by increases in the density of Na+ and K+ channels during development that have been found elsewhere in the rat nervous system (Gao and Ziskind-Conhaim 1998; Martin-Carballo and Greer 2000; Nerbonne and Gurney 1989).

Both hyperpolarizing (AHP) and depolarizing (ADP) afterpotentials were seen, and the proportion of cells showing an ADP increased with age. Although the ADP is Ca2+- dependent in a variety of vertebrate neurons, it seems to be independent of Ca2+ influx in NVsnpr neurons because removal of extracellular Ca2+ did not block the ADP; instead, it increased its amplitude. Our results suggest that $I_{NaP}$ is necessary for the expression of the ADP in these cells because it is abolished by TTX at doses that do not affect the action potential. These results are congruent with what has been found in adult CA1 pyramidal neurons (Azouz et al. 1996; Su et al. 2001). Despite the increase in frequency of ADP, its amplitude decreases after birth, which may be a reflection of increases of K+ currents. This assumption is supported by the recent finding that, in CA1 pyramidal cells, selective blockade of a noninactivating K+ current (M current) increases the amplitude of the ADP (Yue and Yarai 2004). The ADP is dominated by Ca2+-dependent K+ conductances and is abolished by removal of Ca2+ from the medium. We did not study the mechanisms underlying the age-related decrease in AHP duration, but the shorter membrane time constant, associated with larger inward rectification current observed in older animals, may produce a faster recovery from the AHP. The developmental increase in the inward rectification current likely reflects an increase in the density of $I_h$ channels, as has been shown in hypoglossal motoneurons (Bayliss et al. 1994) and mesencephalic trigeminal neurons (Tanaka et al. 2003).

In general, the age-related changes in membrane properties observed in dorsal NVsnpr neurons are similar to those of the “barrelette” region of NVsnpr (Lo and Erzurumlu 2001), the spinal trigeminal nucleus (Guido et al. 1998), and other brain stem regions of the rat (Bao et al. 1995; Berger et al. 1996; Nguyen et al. 2004; Nunez-Abades et al. 1993; Tanaka et al. 2003; Tsuzuki et al. 1995), suggesting that the maturation of many brain stem neurons follows a common time-course.

Development of firing patterns

We classified NVsnpr neurons into three types, based on the firing pattern during depolarization: adaptive, tonic, and bursting. These patterns were strongly linked to some membrane properties. Bursting cells had a lower input resistance than adaptive cells, suggesting that they had a greater membrane area. This assumption is in agreement with previous morphological studies that reported that bursting cells in the neocortex have larger somata than adaptive- or regular-spiking cells (Chagnac-Amitai et al. 1990; Faulkner and Brown 1999; Kasper et al. 1994a; Larkman and Mason 1990; Schubert et al. 1997; Tseng and Prince 1993; Yang et al. 1996). Bursting neurons also exhibit a prominent ADP and have a lower action potential threshold. These characteristics probably reflect differences in sodium channels properties, densities, and/or location between bursting and nonbursting cells. The greater excitability of bursting cells may result from a prominent $I_{NaP}$. Because this current is activated below the action potential threshold (Crill 1996), it will boost depolarization toward the spike threshold. Previous work has shown that firing patterns usually change during postnatal development. Immature cells tend to adapt rapidly, and the ability to fire repetitively usually appears during the first 2 postnatal wk. This change probably results from an increase in the voltage-gated K+ channels that are responsible for repolarization. We found that duration of the action potentials increased with each firing in adaptive neurons during sustained depolarization, which is consistent with the suggestion that a reduction in the inactivation time of Na+ channels is also required for repetitive firing.
firing (Wang et al. 1997). The age-related decrease in AHP duration may also facilitate sustained firing and probably correlates with the steeper F-I slopes of mature neurons. However, the most significant developmental change in firing patterns is the emergence of bursting ability. As in the neocortex (Franceschetti et al. 1993, 1998; Kasper et al. 1994b; Kriegstein et al. 1987) and the ventrobasal thalamus (Perez Velazquez and Carlen 1996), burst firing did not appear until the end of the second postnatal week.

Ionic mechanisms underlying the emergence of bursting cells

Bursting was maintained and even enhanced under conditions that reduce synaptic transmission, suggesting that it is an intrinsic property of NVsnpr neurons. It was first shown by Sandler et al. (1998) that NVsnpr neurons have the intrinsic ability to burst. The evidence suggests that NVsnpr bursts are \( I_{\text{NaP}} \)-dependent, as they are in the pre-Bötzinger complex (Butera et al. 1999; Del Negro et al. 2002), cultured spinal cord (Darbon et al. 2004), hippocampus (Jinno et al. 2003; Su et al. 2001), subthalamic nucleus (Beurrier et al. 2000), neocortex (Brumberg et al. 2000; Guatteo et al. 1996; Nishimura et al. 2001), and trigeminal mesencephalic nucleus (Wu et al. 2005). Riluzole, which suppresses \( I_{\text{NaP}} \) predominately (Urbani and Belluzzi 2000; Wu et al. 2005), and TTX inhibited bursts without changing the spike. The ability to burst developed in parallel with a TTX-sensitive plateau potential, and both plateau potentials and bursts were only seen between the activation and steady-state inactivation voltages typical of \( I_{\text{NaP}} \) (Crill 1996). Several of our observations indicate that the ADP is also \( I_{\text{NaP}} \)-dependent and must play an important role in the generation of burst firing. It is prominent in bursting neurons, and its amplitude increases under conditions that facilitate bursting.

Voltage-activated K\(^+\) conductances seem to be important for burst termination because burst duration increased substantially under TEA. Big \( \text{Ca}^{2+} \)-activated K\(^+\) conductances may counteract depolarization and indirectly play a role in limiting burst duration because their blockade enhanced the ADP and promoted firing of a second or third spike but was insufficient to induce repetitive bursting in presence of \( \text{Ca}^{2+} \). No obvious role could be attributed to small \( \text{Ca}^{2+} \)-activated K\(^+\) conductances. Other factors, such as the slow inactivation of \( I_{\text{NaP}} \) (Del Negro et al. 2002; Ellerkmann et al. 2001; Fleidervish et al. 1996) and the hyperpolarizing action of the \( \text{Na}^+/\text{K}^+ \) electrogenic pump (Ballerini et al. 1997; Darbon et al. 2003) may also contribute to burst termination. Neither was burst generation dependent on \( \text{Ca}^{2+} \) currents at all ages examined. This contrasts with the findings of Chen et al. (2005), who reported that bursting in developing CA1 pyramidal neurons is transitionally dependent on calcium currents. With the use of focal application of calcium and sodium channels antagonists, they found that, in animals younger than 25 days, bursting rely on activation of somatic persistent voltage-gated \( \text{Na}^+ \) channels and activation of T/R- and L-type voltage gated \( \text{Ca}^{2+} \) channels on the distal dendrites.

Furthermore, repetitive bursting activity was rare at \( [\text{Ca}^{2+}]_E > 1.2 \text{ mM} \), and this probably explains the small proportion of repetitive bursting in our standard ACSF (2.4 mM). However, a \( [\text{Ca}^{2+}]_E \) of 2.4 mM may be abnormally high. Under resting conditions, the \( [\text{Ca}^{2+}]_E \) in rat cerebrospinal fluid decreases from \( \sim 1.6 \text{ mM} \) in the fetus to 1.2 mM in the adult (Jones and Keep 1988). Moreover, under both physiological and pathological conditions, neuronal activity decreases \( [\text{Ca}^{2+}]_E \) (Amzica et al. 2002; Nicholson et al. 1978; Somjen 1980). There is even evidence that activation of a single synapse can cause a transient depletion of \( \text{Ca}^{2+} \) at that synapse (Rusakov and Fine 2003).

Functional implications

To induce the first cycle of mastication by stimulation of the cortical masticatory area, a train of shocks of moderate frequency (10–100 Hz) and a minimum duration of several hundred milliseconds is usually required in anesthetized or awake animals before mastication starts (Dellow and Lund 1971; Lund and Lamarre 1974). Similarly, sustained activity in sensory afferents is necessary to trigger mastication. This is surprising given the fact that the cortical and sensory afferents project massively and at monosynaptic or very short latency to dorsal NVsnpr (Tsuboi et al. 2003) and to the surrounding areas (Westberg et al. 1998) with which it is strongly interconnected (Athanassiadi et al. 2005b). We propose that repetitive stimulation of these inputs causes massive neuronal activation that is responsible for a drop in \( [\text{Ca}^{2+}]_E \), that in turn enhances \( I_{\text{NaP}} \) and initiates bursting. Such a relationship has been found in hippocampal pyramidal cells (Yue et al. 2005). The pathway through which \( [\text{Ca}^{2+}]_E \) modulates \( I_{\text{NaP}} \) is not known, but it may involve \( [\text{Ca}^{2+}]_E \)-sensitive second-messenger cascades because \( I_{\text{NaP}} \) can be modulated by a protein kinase C-dependent mechanism (Carr et al. 2002; Franceschetti et al. 2000; Yue et al. 2005).

The transition from suckling to chewing occurs gradually in the course of the second postnatal week in rats. The first masticatory movements appear around P12, and the adult masticatory pattern is established by P18–21 (Westneat and Hall 1992). In this study, we show that there is a close correspondence between the emergence of bursting in NVsnpr neurons and the development of \( I_{\text{NaP}} \) during the period in which masticatory movements emerge. This adds to recent evidence that suggests that NVsnpr plays a determinant role in the generation of the masticatory pattern. First, neurons of this nucleus project to the facial (Pinganaud et al. 1999; Travers and Norgren 1983), trigeminal (Kolta et al. 2000; Li et al. 1993; Yoshida et al. 1998), and hypoglossal (Pinganaud et al. 1999; Travers and Norgren 1983) motor nuclei, which participate in several coordinate orofacial behaviors. In addition, experiments carried in anesthetized and paralyzed rabbits in which the masticatory pattern was generated by stimulation of the motor cortex show that neurons of the dorsal area of NVsnpr increase expression of c-fos (Athanassiadi et al. 2005a). Extracellular recordings in a similar preparation have shown that a third of the population in this area are rhythmically active in phase with the masticatory cycle (Tsuboi et al. 2003).

NVsnpr is traditionally viewed as a sensory relay to the thalamus. Although there is increasing interest in the roles that bursting may play in sensory encoding (Krahe and Gabbiani 2004; Sandler et al. 1998), it has also been implicated in sensori-motor transformations in simple systems (Viana Di Prisco et al. 2005). We have proposed that NVsnpr neurons may form the core of the masticatory CPG (Tsuboi et al. 2003), because they have both intrinsic burst-generating properties and direct connections to other parts of the CPG and to the
three motor nuclei (facial, trigeminal, and hypoglossal) controlling orofacial behaviors (Kolta et al. 2000; Li et al. 1993; Pinganau et al. 1999; Travers and Norgren 1983; Yoshida et al. 1998). They are capable of generating bursts within the frequency range of natural mastication and the rate of bursting is a direct reflection of the level of tonic depolarization and \([Ca^{2+}]_E\). These neurons also have inputs from muscle spindle, periodontal, and other intraoral mechanoreceptors (Tsuboi et al. 2003), which provide the feedback that is necessary for rapid adaptation of burst parameters and of the motor pattern.

**Acknowledgments**

We thank L. Gronding and D. Veilleux for technical assistance. Present address of F. Brocard: Laboratory Plasticité et Physio-Pathologie de la Motricité, UMR 6196 CNRS, Université de la Méditerranée, 31 Chemin Joseph Aiguier, F-13402 Marseille cedex 20, France.

**Grants**

This work was supported by the Canadian Institutes of Health Research.

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