Properties and Interconnections of Trigeminal Interneurons of the Lateral Pontine Reticular Formation in the Rat

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Received 8 January 2001; accepted in final form 12 June 2001

The basic circuits forming the central pattern generator (CPG) for mastication are located in the brain stem (Dellow and Lund 1971). Several studies have described neuronal populations, within this region, that fire in relation to mastication and that are influenced by the cortex, the basal ganglia, the cerebellum, and sensory pathways (see Lund 1991; Lund et al. 1998; Nakamura and Katakura 1995 for reviews). Despite this knowledge, the circuits and mechanisms responsible for generation of rhythmic jaw movements remain to be identified. This issue is common to many rhythmic movements, and as emphasized by other authors in the field, the first step is to identify the components of the CPG, map their synaptic connections, and examine their intrinsic properties using intracellular methods (Kiehn et al. 1997; Marder and Calabrese 1996).

Recent evidence suggests that the minimal circuitry required to induce rhythmic activity in trigeminal efferent nerves is located in the rostral lateral portion of the brain stem at the pontine level (Tanaka et al. 1999). This region comprises the main sensory trigeminal nucleus (NVSnpr), the rostral division of the subnucleus oralis of the spinal nucleus (NVspo), the trigeminal motor nucleus (NVmt), and the region bordering it dorsally, medially, laterally, and ventrally. The ventral part, referred to here as the parvocellular reticular formation (PCrt), corresponds to the ventral and caudal parts of “Region h” described by Meessen and Olszewski (1949) in the rabbit and the area adjacent to NVspo (sometimes called juxtratrigeminal area). The other three regions are collectively called the peritrigeminal area (PeriV). Dorsal PeriV corresponds to the dorsal part of Regio h and the supratrigeminal nucleus (Lorente De No 1922; Torvik 1956) and will be referred to here as the supratrigeminal area (SupV). The lateral area will be called the intertrigeminal region (IntV) in agreement with Lorente de No’s (1922) description, whereas the medial part will be referred to as medial PeriV. PeriV and PCrt contain the largest proportion of trigeminal last-order interneurons. These neurons receive excitatory inputs from small intra- and peri-orai sensory fields, short-latency inputs from ipsi- and contralateral sensorimotor cortex (Donga and Lund 1991; Inoue et al. 1992; Olsson et al. 1986) and from parts of the medial reticular formation thought to contribute to masticatory rhythmo genesis (Kolta et al. 2000; Lund et al. 1998). Many neurons in PeriV, PCrt, and NVspo fire rhythmically during fictive mastication (Donga and Lund 1991; Donga et al. 1990; Inoue et al. 1992, 1994; Westberg et al. 1998) and have bilateral projections to...
NVmt, making them ideal to coordinate a bilateral structure like the mandible (Donga et al. 1990; Kamogawa et al. 1994; Landgren et al. 1986; Li et al. 1993a; Mizuno et al. 1978; Roks et al. 1986). It is still not known whether rhythmic activity in these populations is the result of network properties, intrinsic neuronal properties, or both. Networks that produce coherent rhythms often involve reciprocally connected GABAergic neurons (Marder and Calabrese 1996; Wang and Buzsaki 1996; Wang and Rinzel 1993), while intrinsically generated self-sustained oscillations described in several areas of the brain depend on ionic currents, such as the low-threshold Ca\(^{2+}\) current (Bal et al. 1995; Bleasel and Pettigrew 1992; Warren et al. 1994) or voltage- and/or Ca\(^{2+}\)-dependent K\(^{+}\) currents (Del Negro and Chandler 1997; Grillner et al. 2000; Kiehn et al. 1997; Puil et al. 1988). Cationic rectifier currents (such as I_{\text{NaP}}) and noninactivating currents, such as the persistent Na\(^{+}\) inward current (I_{\text{NaP}}), may also be involved in some cases (Cattaert and el Manira 1999; Del Negro et al. 1999; Franceschetti et al. 1995; Hsiao et al. 1997). An increasing body of evidence also suggests that rhythm and burst properties emerge from transmitter-induced modulation of conductances or changes in membrane current-voltage (I-V) relationships that will alter the response to other synaptic inputs (see Grillner et al. 2000 and Kiehn et al. 1997 for reviews).

In this study, we have used intracellular recordings in an in vitro brain stem slice preparation to assess the burst-inducing ability of neurons of the lateral tegmentum between the trigeminal and facial motor nuclei and the networks that they form.

**Methods**

**Brain stem slice preparation**

One- to 4-wk-old Sprague-Dawley rats (Charles River, Montreal, Quebec) were anesthetized by methoxyflurane inhalation (Metofane, Janssen Pharmaceutica, Ontario, Canada) and decapitated. The brain stem slice preparation was described in detail before (Kolta 1997). Briefly, 5–10 mL of a solution (in mOsm) 225 sucrose, 5 KCl, 1.25 KH\(_2\)PO\(_4\), 2.0 CaCl\(_2\), 25 NaHCO\(_3\), and 10 d-glucose (Aghajanian and Rasmussen 1989) was injected into the digastric muscles of cryoanesthetized rat pups (0–2 days), while crystals of the carboxyamine dye DiI C18(1,1'-dioctadecyl-3,3',3'-tetra-methylindocarbo-cyanine perchlorate; Molecular Probes, Eugene, OR) were inserted in the musc exterous muscles. Both tracers were allowed to diffuse for 1–3 wk. Slices were visualized at low magnification (5×) under epifluorescence, and the massecerous and dianxin dye injections and slice preparation conformed with national ethics committee guidelines and were approved by an institutional ethics committee.

**Electrophysiology**

Neurons were impaled with sharp microelectrodes (made with borosilicate glass, 1.0 mm OD, A-M systems) filled with a solution containing 2% neurobiotin in 1 M filtered potassium acetate. Recordings were made in the bridge mode using capacitance neutralization with an Axoclamp-2B amplifier (Axon Instruments). To evoke synaptic responses, sites around NVmt were stimulated extracellularly using bipolar nichrome electrodes (25 µm diam) that were placed several hundreds of micrometers from the recording electrode. The intensity (0–3 mA) and duration (0.05–0.2 ms) of pulses were gradually increased to obtain reliable subthreshold synaptic potentials and/or antidromic spikes. Frequency following was used in an attempt to establish monosynaptic connections. We initially used 25-Hz stimulation, on the basis of published criteria (Jahr and Yoshioka 1986), but later adopted 50 Hz, since almost all (but 1) responses, including those occurring at the longest latencies (8.6 ms), could follow 25 Hz. However, even 50 Hz was judged to be insufficient since most responses (30/33 tested) could follow this frequency, even those with latencies as long as 6 ms.

**Drug application**

Drugs were bath-applied at the following concentrations: bicuculline methiodide (BIC, 10 µM; Sigma), strychnine (Strych, 5 µM; Sigma), 6,7-dinitroquinoxaline (DNQX, 10 µM; RBI), and N-methyl-D-aspartate (NMDA) receptor–mediated currents, respectively. In some experiments, glutamate (1 mM) was pressure applied locally by a Picospritzer II ejection system (General Valve) using patch-like pipettes (~5 µm tip diameter).

**Morphological examination**

Impaled cells were filled with neurobiotin by passing depolarizing current (1.0–1.5 nA, 1 Hz, 500 ms duration) for 20–30 min through the recording electrode. Extracellular labeling was obtained by passing anodal current (1 µA or less; 7 s on, 7 s off during 10 min) through an extracellular electrode (8–20 MΩ, 3 µm tip diameter) filled with biocytin (1.5% in 0.5 M NaCl) and positioned near active neurons at least 150 µm below the surface of the slice (Pinault 1994). The slice was kept in the chamber for at least 2 h following injection and then immersed in a fixative solution containing 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB; pH 7.4) overnight at 4°C. The slices were then resectioned to a thickness of 40–60 µm using a freezing sliding microtome. The sections were treated with 0.5% H\(_2\)O\(_2\) for 1 h, washed 3 times (10 min) in PB, incubated overnight in an avidin-biotin complex (ABC kit; Vector Laboratories), and reacted with an avidin-biotin complex. Images were acquired with a color video camera (HV-C20) and Northern Exposure software (Empix Imaging).

**Measurements**

Data were digitized (10 kHz) and analyzed with pClamp 6.0 and 8.0 (Axon Instruments). Cells selected for analysis had a stable resting membrane potential (RMP) more negative than −50 mV and a spike that overshoot zero. Action potential height was measured from RMP, and spike duration was measured from the spike onset to the point at which
which the spike downstroke crossed the spike threshold. The amplitude of the afterhyperpolarization (AHP) was measured from RMP, and its duration was measured from the point at which the spike downstroke crossed the spike threshold to the return to RMP. The steady-state voltage responses to hyperpolarizing current pulses (100 ms duration) were plotted against current amplitude for each cell, and input resistance was determined by the slope of the line fitted to the straight portion of the curve. Reported values were obtained in control conditions. Threshold for firing was defined as the voltage at the last point preceding the inflection in the waveform caused by the fast rising phase of the spike. Synaptic response latencies were measured on individual traces from the beginning of the stimulus artifact to the foot of the rising or falling phase of the postsynaptic potential (PSP). The coefficient of variation of this measure was calculated for all cells in which at least 20 responses were obtained. The amplitude of the PSP was measured from the baseline to the peak of the average of all responses (excluding those in which the cell spiked).

Data are represented as means ± SE. Tests used for statistical analysis are mentioned in the text.

R E S U L T S

The results presented here are based on recordings from 91 cells in 50 animals. The number of neurons recorded in each region and their basic characteristics are described in Table 1.

The cells in this sample had an average RMP of $-57 ± 1$ (SE) mV, an input resistance of $47 ± 5$ MΩ, a firing threshold of $-46 ± 1$ mV, and spikes that reached $56 ± 1$ mV in height that were followed by a small, but long AHP (amplitude: $-7 ± 0.7$ mV; duration $74 ± 11$ ms). None of these parameters differed significantly between groups of neurons recorded at different locations, nor did they vary significantly with age, within the period studied, although a trend was sometimes apparent (linear regression; $r^2$ ranged from 0.005 to 0.07).

Anatomical labeling

A few neurons of each region were successfully filled with neurobiotin ($n = 8$). They were all fusiform or triangular and small in size (Fig. 1A). Their dendrites extended horizontally by 10.2 ± 0.3 mm and generally remained confined within their subdivision. Axonal trajectories could not always be reconstructed with this method.

Small extracellular injections of biocytin were used to label bundles of axons from groups of neurons. Although the exact termination site of these axons could not be determined by this method, trajectories could at least be described. Injections in SupV marked axons that coursed in IntV ventrally toward...
PCrT and laterally toward the NVSnpnpr and others that reached the mesencephalic and motor trigeminal nuclei. Fibers crossing the adjacent medial reticular formation and traveling toward the midline were also seen. Injections in medial PeriV also labeled fibers heading toward the midline, other crossed NVmt and reached IntV. Ventral and dorsal projections toward PCrT and SupV also originated from this area. Fibers marked by injections in PCrT entered NVmt or passed alongside giving off branches in all subdivisions of PeriV (Fig. 1B) and NVSnpnpr. Many commissural fibers were also seen originating from this region and reaching its contralateral counterpart.

**Postsynaptic responses**

The electrophysiological results are summarized in Table 2. In 70 of 72 neurons tested (97%), stimulation of an adjacent area in PeriV or PCrT elicited either an excitatory postsynaptic potential (EPSP; n = 47; Fig. 2A) or an inhibitory postsynaptic potential (IPSP; n = 16; Fig. 2B) or both (n = 7). The mixed responses usually consisted of a rapid EPSP followed by an IPSP (Fig. 2C).

**EXCITATORY RESPONSES.** In all recording regions, fast-rising, slowly decaying potentials were evoked at RMP following stimulation of other divisions of PeriV and PCrT. Typically, the amplitude, but not the latency of the EPSP increased with stimulus intensity up to a certain level after which further increases caused spiking. In all cases tested (n = 28), hyperpolarization of the membrane 50 ms prior to stimulation increased the amplitude of the EPSP, while small depolarizations caused it to trigger a spike at a subthreshold potential. Of the 47 EPSPs recorded, 32 did not involve spiking and were assessed for synaptic facilitation or depression; only changes of amplitude >10% were considered. The percentage of responses showing facilitation with paired pulse stimulation (40-ms interval) varied slightly but not significantly (1-way anova, P = 0.1) with the stimulus location and ranged from 16; Fig. 2A) or both (n = 7). The mixed responses usually consisted of a rapid EPSP followed by an IPSP (Fig. 2C).

**INHIBITORY RESPONSES.** Even in the absence of blockade of glutamatergic transmission, short-latency IPSPs were obtained in 22% of cases, mainly on stimulation of medial PeriV, but also sometimes with stimulation of SupV and PCrT. IPSPs induced by repetitive stimulation of medial PeriV (40-ms interpulse interval) showed depression (−35 ± 6% of their amplitude) in 6/12 cases and facilitation (29%) in 2 of the remainder cases. No consistent effects were observed with the IPSPs elicited by stimulation of SupV and PCrT. As would be expected for chloride mediated potentials, all tested IPSPs (n = 10) increased in amplitude when the membrane was depolarized and reversed polarity around −56 ± 9 mV to become positive in the hyperpolarized range. This was also the case for the three IPSPs unveiled by DNQX.

**Pharmacology.** Bath application of the AMPA receptor antagonist, DNQX, reversibly abolished the EPSP in 12 of 13 responses tested and reduced the amplitude of the EPSP in the remaining cell, which was lost before a full effect could be achieved. Figure 3A shows an example where an EPSP obtained at the RMP (−55 mV) is abolished after addition of DNQX and recovered after its wash out. In 3 of the above 13 cases, blockade of the EPSP by DNQX unmasked an IPSP. In the case illustrated in Fig. 3B, the uncovered IPSP occurred at the same latency as the EPSP. Its blockade bystrychnine revealed a second long-lasting excitatory component that appeared at a longer latency (4.3–5 ms). This component had slow kinetics reminiscent of NMDA receptor–mediated responses and was effectively blocked by APV, as was the case of three other long-lasting depolarizing responses.

**TABLE 2.** Connections between interneurons tested by electrical stimulation of neighboring areas

<table>
<thead>
<tr>
<th>Stimulation Site</th>
<th>Recording Site</th>
<th>SupV</th>
<th>Medial PeriV</th>
<th>IntV</th>
<th>PCrT</th>
</tr>
</thead>
<tbody>
<tr>
<td>SupV</td>
<td>EPSP</td>
<td>29 (5/17)</td>
<td>100 (2/2)</td>
<td>80 (4/5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ISPP</td>
<td>65 (11/17)</td>
<td>20 (1/5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>No response</td>
<td>6 (1/17)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medial PeriV</td>
<td>EPSP</td>
<td>73 (8/11)</td>
<td>100 (4/4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ISPP</td>
<td>18 (2/11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mix</td>
<td>9 (1/11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IntV</td>
<td>EPSP</td>
<td>80 (4/5)</td>
<td>33 (1/3)</td>
<td>75 (6/8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ISPP</td>
<td>33 (1/3)</td>
<td>13 (1/8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mix</td>
<td>20 (1/5)</td>
<td>13 (1/8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>No response</td>
<td>33 (1/3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCrT</td>
<td>EPSP</td>
<td>100 (4/4)</td>
<td>67 (4/6)</td>
<td>100 (5/5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mix</td>
<td>33 (2/6)</td>
<td>100 (2/2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Data in each category represent the percentage (%), and number of cells in which these responses were observed are in parentheses. EPSP, excitatory postsynaptic potential; IPSP, inhibitory postsynaptic potential; for other abbreviations, see Table 1. |
Chloride conductance changes are usually mediated by GABA\textsubscript{A} or glycinergic receptors. Antagonists of both types of receptors were tested on nine responses. Bicuculline blocked two of five IPSPs tested and diminished the amplitude of a third one, while strychnine blocked two IPSPs totally and two partially. In two of these cells, blockade of the IPSP revealed a small-amplitude EPSP that was presumably AMPA mediated since it was abolished by further addition of DNQX in at least one of the two cells.

**Organization of pathways**

Some of the recorded PSPs occurred at a very short latency and probably resulted from activation of a direct pathway, but the majority of responses occurred at longer latencies and likely involved oligosynaptic pathways. The individual latencies of the responses ranged from 1.2 to 8.6 ms (see Table 3 for averages). High-frequency stimulation (up to 50 Hz) was judged to be insufficient to determine the synaptic order of recorded PSPs. The coefficient of variation of response latency was used instead, on the assumption that a greater number of synapses in a pathway would presumably cause a greater variability in transmission. However, as shown in Fig. 4A, this measure was not significantly correlated to the latency ($P > 0.05$, Pearson correlation) and could not be used to differentiate between mono- and polysynaptic responses that could follow 50 Hz. The average values of this measure did not vary significantly with the recording nor the stimulating locations (see Table 3).

Arbitrary criteria were fixed on the basis of the distribution histograms of evoked response latencies, shown in Fig. 4B. This figure clearly shows that most events occurred at latencies that are too long to be considered monosynaptic. However, a curve fitted to these histograms reveals several almost equidistant peaks that could be used as indices of synaptic order. The first peak occurred at 1.5 ms (if ignoring one response that occurred at 1.2 ms) and the second at 1.9 ms. On the basis of these peaks, latencies below 1.7 and 2.1 ms were considered as monosynaptic and disynaptic, respectively. Longer latencies were considered as oligosynaptic.

**EFFERENTS.** Stimulation of SupV and PCRt evoked responses in all cells tested ($n = 20$ and 19, respectively). The input from these areas to the other subdivisions was primarily, but not exclusively excitatory (EPSPs, 18 and 15; mixed responses, 2 and 1, respectively). The latencies of IPSPs elicited by stimulation of PCRt were significantly longer than those of EPSPs ($5.5$ vs. $3.2$ ms; $t$-test, $P = 0.035$). In control conditions, activation of IntV elicited only excitatory responses (7/7), but IPSPs were also observed ($n = 2$) after abolition of EPSPs by DNQX. Stimulation of medial PeriV failed to evoke a response in 2 of the 26 cases tested. IPSPs represented one-half of the responses (12/26) obtained with this stimulation site, while EPSPs ($n = 10$) or mixed responses ($n = 2$) composed the rest. The latencies of the IPSPs evoked by stimulation of this region were shorter than those of the EPSPs ($3.1$ vs. $4$ ms; $t$-test, $P = 0.045$).

**AFFERENTS.** Although all areas received extensive excitatory inputs from most adjacent divisions, inhibitory inputs were

<table>
<thead>
<tr>
<th>Recording Site</th>
<th>Stimulation Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>IntV</td>
<td>Medial PeriV</td>
</tr>
<tr>
<td>Latency, ms</td>
<td>3.53</td>
</tr>
<tr>
<td>CV</td>
<td>0.16</td>
</tr>
<tr>
<td>$n$</td>
<td>1</td>
</tr>
<tr>
<td>Medial PeriV</td>
<td>Latency, ms</td>
</tr>
<tr>
<td>CV</td>
<td>0.18</td>
</tr>
<tr>
<td>$n$</td>
<td>9</td>
</tr>
<tr>
<td>SupV</td>
<td>Latency, ms</td>
</tr>
<tr>
<td>CV</td>
<td>0.31</td>
</tr>
<tr>
<td>$n$</td>
<td>2</td>
</tr>
<tr>
<td>PCRt</td>
<td>Latency, ms</td>
</tr>
<tr>
<td>CV</td>
<td>0.26</td>
</tr>
<tr>
<td>$n$</td>
<td>2</td>
</tr>
</tbody>
</table>

Numbers with the same superscript letter differ significantly from each other. $n$ below 4 were excluded from the analysis. CV, coefficient of variation; for other abbreviations, see Table 1.
mostly recorded in SupV. Twelve of the 16 recorded IPSPs (75%) were obtained in this area. The four remaining IPSPs were recorded in medial PeriV and IntV, while none were obtained in recordings made in PCRt. SupV neurons received inputs from the three other divisions. Stimulation of medial PeriV elicited mainly inhibitory responses (11/17; 3 of which falling in the mono- and 2 in the disynaptic range). The EPSPs were all polysynaptic (>2.6 ms). Inputs from IntV were monosynaptic but rare, while those from PCRt all had a polysynaptic latency (>2.3 ms). Connections were found in two of three cases between IntV and medial PeriV. The other responses evoked in these two areas were obtained following stimulation of SupV and PCRt. Of these, six (4 EPSPs, 2 IPSPs) fell in the monosynaptic range and four (3 EPSPs and 1 IPSP) in the disynaptic range.

PCRt also received inputs from all areas of PeriV, but none of the recorded responses was a pure IPSP. Inhibitory potentials were obtained only in conjunction with EPSPs and were elicited by stimulation of medial PeriV and ventral levels of PCRt. Only 2 (1 from IntV and 1 from medial PeriV) of the 18 PSPs recorded in this area were considered to be monosynaptic.

In two cases, glutamate injections were made in lieu of the electrical stimulation and in both cases mimicked its effects except for the duration of the response, which was longer. This confirms that, at least for these two cases, the observed PSPs were mediated by local neurons and not by passing by fibers.

**Inputs from a new group of interneurons located in the trigeminal motor nucleus**

Neurons recorded in PeriV and PCRt were routinely tested for antidromic activation from NVmt. Antidromic potentials were obtained in 11/63 cases (e.g., Fig. 5 in SupV). They occurred at a short and constant latency (0.6 ± 0.06 ms), could follow high stimulation frequencies above 100 Hz, and had no underlying EPSPs. The absence of underlying PSPs was confirmed by the fact that they did not change in amplitude with imposed changes of membrane potentials; instead they disappeared when the membrane was too hyperpolarized and reappeared at potential near resting and above.

However, as summarized in Table 4 and shown in Fig. 5, PSPs were elicited in nearly all neurons (62/63) from PeriV and PCRt on stimulation of NVmt. EPSPs (as in PCRt and IntV in Fig. 5), IPSPs (Fig. 5, medial PeriV), and mixed responses were elicited at latencies slightly shorter than those described above for PeriV (3.1 ± 0.2 vs. 3.7 ± 0.2 ms, Mann-Whitney Rank sum test; \( P = 0.038 \)). The distribution histogram of the latencies of PSPs elicited by stimulation of NVmt also showed equidistant peaks, separated by 0.3-ms intervals in this case, for responses with latencies <2 ms (Fig. 4B). The first of these peaks occurred after nearly 1 ms. On average, the IPSPs, which accounted for 27% (17/63) of the responses, tended to occur at a longer latency than the EPSPs (3.3 ± 0.33 ms vs. 3.0 ± 0.19 ms, respectively), but this difference was not significant (t-test). All responses followed 50-Hz stimulation. The EPSPs showed similar voltage sensitivity to those obtained from stimulation of PeriV and PCRt and were blocked by DNQX in 9 of the 10 cases tested. In the remaining case, DNQX only reduced the amplitude of the EPSP and APV abolished the remaining component. In three cases, complete blockade of the EPSP by DNQX revealed an IPSP that was abolished by addition of bicuculline in one case and strychnine in another case. Four other IPSPs tested with these drugs showed resistance to either one of the drugs before being blocked by the second. In two of these four cells, blockade of the IPSP revealed an EPSP that was abolished by further addition of DNQX in one case and strychnine in another case. Four other IPSPs tested with these drugs showed resistance to either one of the drugs before being blocked by the second. In two of these four cells, blockade of the IPSP revealed an EPSP that was abolished by further addition of DNQX in one case and strychnine in another case. 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These results suggest the existence of a population of interneurons within NVmt and were further supported by the observation of two morphologically distinct types of neurons within NVmt under infrared microscopy. One type, presumably motoneurons, consisted of multipolar neurons with large somatic compartments. The second type of neurons were much smaller and fusiform. A few of the latter were filled with biocytin to examine their somata and projections. In all cases examined (n = 6), the somatic compartment varied between 11 and 19 \( \mu m \) (mean: 14 \( \mu m \)) on its longest axis, whereas randomly sampled adjacent motoneurons (retrogradely labeled by the fluorescent dye or Nissl-stained) had a significantly larger diameter (36 \( \mu m \), n = 11; t-test, P < 0.001). Figure 6 shows an example of such a small neuron (Fig. 6A) that was located within the masseteric pool as indicated by the fluorescent labeling of the adjacent motoneurons (Fig. 6B). The dendrites of this neuron extended outside the border of NVmt into SupV, while its axon bended medially toward the midline (Fig. 6C).

Extracellular injections of biocytin in NVmt labeled fibers in all areas surrounding the nucleus (Fig. 6D) in addition to...
commissural fibers that could be tracked to the region of the contralateral NVmt near the motor root.

**Intrinsic properties**

**MEMBRANE RESPONSES TO DEPOLARIZING CURRENT PULSES.** The majority of neurons (85%) fired regularly with suprathreshold depolarizing current pulses. Their firing frequency increased with depolarizations, but their firing pattern remained tonic. Nearly one-half of these neurons (45%), named type 1, continued firing regularly even at very depolarized potentials and showed no signs of adaptation (Fig. 7A), while the others primarily of type 2 (40%), stopped firing or displayed membrane oscillations at membrane potentials above −30 mV (Fig. 7B). A minority (15%), named type 3, fired only 1–3 spikes at the beginning of the depolarizing pulse at all membrane potentials (Fig. 7C1). These proportions were roughly maintained throughout the age period examined and in two of the recording regions SupV and IntV. PCRt had no type 3 neurons, and the majority of its cells (75%) were of type 1, while the majority of medial PeriV neurons were of type 2 (63%), and the remainder were split between type 1 (26%) and type 3 (11%). There were no significant differences in the firing threshold and the RMP of neurons belonging to the three types, but the input resistance of type 3 neurons (34 MΩ) was significantly lower than that of types 1 (46 MΩ) and 2 (58 MΩ).

A few type 1 neurons (15%) showed outward rectification with depolarizing current, while more type 2 and 3 neurons did (44 and 77%, respectively; Fig. 7C2). A minority of cells of all types showed an inward rectification with very large depolarizing pulses (5, 2, and 15% of type 1, 2, and 3 neurons, respectively).

**MEMBRANE RESPONSES TO HYPERPOLARIZING CURRENT PULSES.** A few type 1 neurons (13%) fired action potentials at the offset of a hyperpolarizing pulse, while more type 2 and 3 neurons did (44 and 77%, respectively; Fig. 7C2). A minority of cells of all types showed an inward rectification with very large depolarizing pulses (5, 2, and 15% of type 1, 2, and 3 neurons, respectively).

A few type 1 neurons (13%) fired action potentials at the offset of a hyperpolarizing pulse, while a large proportion of type 2 and 3 cells (67 and 45%, respectively) did and, in a few cases (n = 4), the number of spikes was proportional to the amount of injected current (Fig. 7D). These spikes were sometimes riding on top of a Ca$^{2+}$ spike. Most neurons (88%) displayed a linear I−V relationship over a long range of hyperpolarized potentials.
potentials (Fig. 7C2). The remaining 12% that showed an inward rectification were of types 2 and 3.

FIRING PROPERTIES. Spontaneously active neurons (average firing rate of 14 Hz) were encountered in all regions, but their proportion varied from one area to the other (see Table 1), with PCrt and medial PeriV having the largest proportions (76 and 58%, respectively) and Sup V and IntV having the lowest (15 and 25%, respectively). None of type 3 neurons were spontaneously active, while nearly one-half of types 1 and 2 neurons were (41 and 50%, respectively). These neurons fired tonically at their resting potential at frequencies ranging from 3 to 32 Hz (Fig. 7E, left part of the trace). Bursting was observed in rare cases (n = 5) mostly in SupV (n = 3) following hyperpolarization. In the case shown in Fig. 7E, a 5-mV hyperpolarization of the cell transforms the tonic firing pattern into phasic bursts riding on top of small plateau depolarizations. The instantaneous firing frequency during those bursts is greater than that observed prior to hyperpolarization.

DISCUSSION

Important functions in the control of oral motor behavior have been ascribed to trigeminal interneurons located in the lateral reticular formation at the level of NVmt. However, there has been no systematic effort to characterize, intracellularly, the basic properties and pattern of interconnectivity of these neurons. Therefore this was the primary aim of the present investigation. Most neurons exhibited similar properties and pattern of connectivity, and our data do not provide evidence to divide PCrt and PeriV in distinct subgroups. This view is supported by earlier reports in which SupV, IntV, and PCrt were assumed to be a continuous interneuron system intercalated between the sensory trigeminal system and the cranial motor nuclei (Kuypers 1958; Mizuno 1970; Torvik 1956) and by the absence of cytoarchitectural differences between PeriV and PCrt (Lorente De No 1922; Meessen and Olszewski 1949; Mizuno 1970). Similarities between PeriV and PCrt extend beyond their cytoarchitecture. Both areas receive convergent cortical and trigeminal peripheral inputs (Olsson and Westberg 1989) and cannot be clearly separated on the basis of their peripheral and central inputs (Sessle 1977a). They both contain most last-order neurons projecting to NVmt bilaterally and many rhythmically active neurons during fictive mastication.

In the present study, neurons recorded in these two areas had a fairly low RMP (−57 mV) and high-input resistance (46 MΩ) characteristic of small-sized neurons. These values were obtained from young animals that were still developing but that were almost all in the same developmental stage regarding oral function (preweaning). Although there was a perceived trend for some parameters to vary with age, none of these trends reached statistical significance within the age period examined.
Interconnections

Interconnections between the different subdivisions of PeriV and PCRt as revealed by electrical stimulation and biocytin injections appear to be complex. Figure 8 summarizes the ensemble of the results concerning connectivity between PeriV, PCRt, and NVmt and emphasizes the intricacy of the network they form.

It is clear from the figure that inputs to any of these neurons are likely to be distributed throughout the network. Most responses obtained fell in the di- to polysynaptic range, but many monosynaptic connections were also found between most areas. Monosynaptic latencies reported in the literature range from <1 ms (Agmon and Connors 1992) up to 6.7 ms (Launey et al. 1999), depending on the type of neurons involved and the distance between the stimulating and recording locations. The cutoff limit of 1.7 ms chosen here is compatible with values of many studies using similar preparation of myelinated neurons (Gil and Amitai 1996; Grimwood et al. 1992; Nakanishi et al. 1991). Values between 2 and 2.5 ms are debatable as they are often considered as either monosynaptic or disynaptic latencies and are not easily classified. In our data set, the interval between the first and the second peak of the distribution histograms is 0.4 ms and the second and third peak is 0.45 ms. These values correspond to the synaptic delay of 0.43 ms reported by Appenteng et al. (1989). The conduction time would have to be negligible to consider these as reflecting activation of second and third synapses. This might be the case given the very short distance between recorded and stimulated areas, assuming that cells involved are myelinated. However, following this reasoning, responses occurring at ~4 ms would involve eight synapses. It is unlikely that such a high number of synapses could follow 50-Hz stimulation. Another alternative might be that different pathways are activated with a delay of approximately 0.4 ms in between and where crossing of every additional synapse requires 0.8 ms. This value is compatible with the sum of conduction time (0.35 ms) and synaptic delay (0.43 ms) calculated by Appenteng et al. (1989) for conduction and transmission between PCRt neurons and trigeminal motoneurons in similar conditions and for similar, if not shorter, distances. Thus in the first pathway, monosynaptic responses would occur at 1.5 ms, then disynaptic responses at 2.3, and trisynaptic at 3.1, and so on, while the second would start at 1.1, 1.9, 2.7, 3.5, . . . , etc. The fact that the fastest responses elicited by stimulation of NVmt tend to occur at a slightly shorter latency than those elicited by PeriV and PCRt lends support to this hypothesis and suggests that many areas of PeriV and PCRt may be connected through interneurons in NVmt. In any case, independently of the number of synapses involved, transmission in these pathways is reliable and robust since responses rarely failed and latency variability was similar for responses with short and long latencies.

At least some of the monosynaptic connections reported here are supported by anatomical evidence. For instance, projections from SupV to IntV and PCRt and from PCRt to SupV have already been described (Kolta et al. 2000; Mizuno 1970; Rokx et al. 1986; Shammah-Lagnado et al. 1992).

Neurochemical identity of neurons

It is widely believed on the basis of indirect electrophysiological evidence that SupV neurons are inhibitory and mediate the disynaptic inhibition of jaw closing motoneurons from a variety of peripheral inputs (Goldberg and Nakamura 1968; Kamogawa et al. 1988; Kidokoro et al. 1968; Nakamura et al. 1973). Our results suggest that they are mostly excitatory since 80% of responses induced by stimulation of this area were EPSPs. However, IPSPs and mixed responses were also obtained, suggesting that SupV contains a mixed population of excitatory and inhibitory neurons. Stimulation of medial PeriV and PCRt also evoked, in different proportions, a mix of excitatory and inhibitory potentials sensitive to glutamatergic, GABAergic, and glycineric receptors antagonists. It could be argued that only excitatory neurons are found in these areas and that they activate inhibitory neurons responsible for the IPSPs. However, at least for responses elicited by stimulation of SupV and medial PeriV, the EPSPs occurred at a longer latency than the IPSPs, and, in several cases, their blockade by DNQX unveiled IPSPs at the same latency. This was not the case for responses elicited by stimulation of PCRt where IPSPs occurred at a significantly longer latency than EPSPs and where blockade of the EPSPs (n = 6) never uncovered an IPSP. Grimwood et al. (1992) also reported that PCRt-induced IPSPs recorded in motoneurons occurred at a longer latency than the EPSPs. These authors raised the possibility that inhibitory interneurons have a slower axonal conduction velocity. This is unlikely because IPSPs elicited by stimulation of other areas had shorter latencies than EPSPs.

Anatomical evidence supports the existence of mixed populations of interneurons in both PeriV and PCRt. For instance, both pleomorphic and round synaptic vesicles are found in the terminals of SupV neurons (Mizuno et al. 1978, 1983), while immunocytochemical studies in the rabbit and guinea pig describe glutamatergic, GABAergic, and glycineric immunore-
active cells in PeriV and PCRt (Kolta et al. 2000; Turman and Chandler 1994a,b). In the rat, glutamate immunoreactivity of trigeminal premotoneurons have not been assessed, but glycinergic and GABAergic premotoneurons were identified in PeriV and PCRt (Ginestal and Matute 1993; Li et al. 1996; Rampon et al. 1996). In this study, bicusculine- and strychnine-sensitive IPSPs were observed on stimulation of PCRt and IntV, although anatomical investigations in the rat had identified only glycinergic premotoneurons in IntV (Li et al. 1996). Both bicusculine-sensitive and -insensitive IPSPs were induced by stimulation of medial PeriV, while only strychnine-sensitive IPSPs followed stimulation of SupV. These data may either represent the differential distribution of glycinergic and GABAergic interneurons, or, more likely, the distribution of postsynaptic GABAergic and glycinergic receptors on the recorded cells. The latter possibility is based on the observation that GABA and glycine are often co-localized, particularly in last-order interneurons, and synaptic boutons immunoreactive to both GABA and glycine have been described on dendrites of masseter motoneurons (Bae et al. 1999; Shupliakov et al. 1993; Taal and Holstege 1994).

Thus, all areas recorded from had neurons carrying AMPA/ kainate receptors. Evidence for the presence of NMDA receptors were also found in recordings from IntV, SupV, and PCRt neurons. These results corroborate the observation that SupV and IntV neurons express both the NR1 and NR2A/B subunits of NMDA receptors (Turman et al. 1999). At least some neurons of SupV, PCRt, and medial PeriV had GABA receptors, while some neurons of IntV and SupV expressed glycergic receptors.

The fact that PCRt seems to receive primarily excitatory inputs from ipsilateral PeriV and NVmt interneurons is noteworthy because these neurons have been shown to receive phasic glycinergic inputs and phase unrelated GABAergic inputs during fictive mastication (Inoue et al. 1994). PCRt is thought to play an important role in coordinating oro-facial movements because some of its neurons send bifurcating axons to trigeminal and facial or hypoglossal motor nuclei (Li et al. 1993b). Our results imply that the inhibition observed in these neurons during mastication must originate either from the contralateral side or from other CPGs.

**Inputs from interneurons in NVmt**

Some previously reported electrophysiological observations hinted to the existence of interneurons in NVmt. These were often considered to be of artifactual origin (Sessle 1977b; Shigenaga et al. 1988). However, anatomical reports also support this assertion. For instance, commissural neurons linking the two motor trigeminal nuclei have been described in the rat (Kolta 1997; Ter Horst et al. 1990), while glycine and glutamic acid decarboxylase–immunoreactive and GABA- and gluta- mate-immunoreactive premotoneurons have been identified within NVmt in the rat and rabbit, respectively (Kolta et al. 2000; Li et al. 1996). Some of these immunoreactive neurons in the rabbit were also labeled by injections of a retrograde tracer in the dorsal and lateral zones of PeriV (Kolta et al. 2000).

Our results add up to these data and provide strong evidence for the existence of a mixed population of excitatory and inhibitory interneurons within NVmt that project, at least, to the surrounding premotoneurons. Commisural fibers leaving NVmt and reaching the area of the contralateral nucleus further confirm the above-mentioned reports. Stimulation of NVmt evoked short-latency DNQX-sensitive EPSPs and/or IPSPs in all areas recorded from. The IPSPs were mediated by either GABA or glycinergic receptors and presumably involved a chloride conductance. Many of these PSPs occurred within a monosynaptic latency, followed 50-Hz stimulation, and were mimicked by glutamate injection nearby the stimulation location. Petropoulos et al. (1999) obtained similar findings in the lamprey. The observation of small fusiform neurons adjacent to large motoneurons in NVmt further support the localization of interneurons in this nucleus. These small neurons could be gamma motoneurons. However, preliminary evidence suggest that, at least for some of them, this is unlikely since they were not retrogradely labeled by the tracer injected in the muscle, nor did they fire an antidromic spike on stimulation of the motor root (unpublished observations).

**General basic properties**

Three types of neurons were identified on the basis of their level of firing adaptation with long-lasting depolarizing current pulses. The distribution of each type across all areas recorded from were fairly uniform except for PCRt, which contained only types 1 and 2 neurons. Minkels et al. (1995) distinguished two groups of SupV neurons (T-I and T-II) on the basis of the intensity of stimulation of inferior alveolar nerve required to drive them and on the pattern of action potentials fired following supramaximal stimulation of the nerve. T-I fired a single spike or a small irregular burst of action potentials, whereas T-II fired a series of action potentials, frequently in two separate waves, and required a higher intensity of stimulation. All T-II and some T-I neurons had a low-amplitude (1.5–20 mV), fast rising, and slow repolarizing (~30-ms decay) action potentials. None of our neurons had similar action potentials. This may be due in part to the difference in RMP between our sample and theirs (~57 mV here vs. ~30 mV). Their T-I neurons may correspond to our type 3 neurons, which fired only one to three spikes even with high levels of depolarizations.

In the rabbit and guinea pig, many PeriV and PCRt neurons are rhythmically active during fictive mastication (Donga and Lund 1991; Donga et al. 1990; Inoue et al. 1992, 1994; Westberg et al. 1998). It is, however, unclear whether these neurons play an active part in rhythmogenesis or simply relay the rhythm generated elsewhere to trigeminal motoneurons. Using the en bloc brain stem preparation of newborn rats, Tanaka et al. (1999) have recently implied that the minimal circuitry required to generate rhythmic activity in trigeminal efferent nerves lies in the reticular formation between the rostral borders of NVmt and the facial nucleus and is limited to 200–400 μm medial to NVmt. This conclusion is debatable because the rhythmic activity in their study was recorded from the motor nerves and was induced by a pharmacological mixture that produces rhythmal membrane potential fluctuations in motoneurons deprived of synaptic input (Kim and Chandler 1995). We found little evidence to support an active role for these neurons in rhythm generation since only 5% exhibited recurrent bursting or oscillating activity under all conditions tested. In contrast, a larger proportion of neurons (31%) from...
NVSmpr recorded under the same conditions tend to fire in burst either spontaneously or following synaptic activation (unpublished observations).

Functional significance

One aim of the present investigation was to identify possible mechanisms by which neurons of the lateral pontine reticular formation may generate rhythmic activity. The results obtained suggest that, although neurons of this area are extensively interconnected, activation of these local circuits does not generate bursting or recurrent firing. Nor did cells of PeriV and PCRt present intrinsic bursting properties. Membrane potential–dependent oscillations and negative slope regions in the steady-state I–V curves characteristic of intrinsically bursting neurons (Kiehn et al. 1996) were never observed. Sharp electrodes, as those used in this study, are not ideal to detect these properties (Kiehn et al. 1996). Although this may explain why such characteristics were not observed even in bursting neurons, it did not interfere with the ability to detect spontaneous or induced bursting in other neurons (unshown results from NVSmpr). Thus our results suggest that the rhythmic firing observed in PeriV and PCRt neurons in vivo during fictive mastication is synaptically mediated rather than intrinsically generated.

Nozaki et al. (1993) showed that PCRt neurons that are only active in the jaw closing phase of the fictive masticatory cycle elicit EPSPs in jaw closing motoneurons, while those active during jaw opening inhibit these motoneurons and excite digastic motoneurons. These results suggest that either both inhibitory and excitatory PCRt neurons are phasically activated during jaw opening or that only excitatory neurons contacting other inhibitory neurons are activated to mediate the effects of these neurons on antagonistic motoneurons. Our results would support the latter possibility because IPSPs induced by stimulation of PCRt tended to occur later than EPSPs.

Inoue et al. (1994) confirmed the findings of Nozaki et al. (1993) and further showed that each type of neuron receive phasic excitatory inputs in their respective phase, but only for those active during jaw closing does this drive alternate with phasic glycine-mediated inhibitory inputs. Thus PCRt neurons are driven by phasic excitatory and inhibitory inputs and are not required to be intrinsically bursting to display a rhythmic activity. This is probably the case of PeriV neurons since they seem to share PCRt-neuron characteristics. A similar situation is reported for spinal interneurons, the majority of which (63%) show rhythmic membrane potential oscillation in phase with activity in ventral roots (Kiehn et al. 1996). This rhythmic activity is synaptically induced for the vast majority (88%) of these neurons. Only 8% of the total population displayed intrinsic plateau or bursting properties. This proportion is comparable with the one obtained here (5%), even if whole cell recording electrodes were used. Kiehn et al. (1996) suggested that rather than playing a role in rhythm generation, these cells may serve to shape and time the diffusion of inputs in the network.

In conclusion, our study does not support the view that the premotor interneurons of the lateral reticular formation surrounding NVmt have the ability to generate rhythmic discharge in trigeminal motor nerves. However, other neurons of the lateral reticular formation at this level may contribute more importantly to the process of rythmogenesis. These may include the putative interneurons located in NVmt. These interneurons are in a position to exert a strong control on motoneuron excitability bilaterally. Moreover, they may contribute to coordination of oro-facial movements if they correspond to the NVmt neurons that project to the hypoglossal nucleus described by Manaker et al. (1992). These neurons, through their monosynaptic contacts in PeriV and PCRt, can access a network where inputs are rapidly distributed. The reliability of transmission in this network ensures that rhythmic inputs arriving at one subgroup of interneurons, such as those observed in PCRt, will reach all other fractions.

The authors sincerely thank Drs. James Lund and Richard Warren for commenting on earlier versions of the manuscript. We are also grateful to L. Grondin for technical assistance.

This work was supported by Canadian Medical Research Council Grant MT-14392 and Canadian Natural Sciences and Engineering Research Council Grant OGP0172682.

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J Neurophysiol • VOL 86 • NOVEMBER 2001 • www.jn.org


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