In Vitro Investigation of Synaptic Relations Between Interneurons Surrounding the Trigeminal Motor Nucleus and Masseteric Motoneurons

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Kolta, Arlette. In vitro investigation of synaptic relations between interneurons surrounding the trigeminal motor nucleus and masseteric motoneurons. *J. Neurophysiol.* 78: 1720–1725, 1997. Because of their many inputs and bilateral projections, interneurons surrounding the trigeminal motor nucleus (MotV) are thought to be very important in control of jaw movements and reflexes. However, their interactions with the trigeminal motoneurons are almost unknown. In the present study an in vitro slice preparation was used to investigate this relationship in rat. The zone bordering MotV has been subdivided into four regions: the supra-, juxta-, and intertrigeminal areas (SupV, JuxtV, and IntV, respectively) and the parvocellular reticular formation ventral and caudal to MotV. Stimulation of all areas evoked short-latency excitatory postsynaptic potentials (EPSPs) in masseteric motoneurons. Frequently the EPSPs masked inhibitory postsynaptic potentials (IPSPs) or were followed by long-lasting inhibitory potentials. Only responses obtained from stimulation of JuxtV and IntV seemed devoid of inhibitory components. The EPSPs were mediated through kainate/α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors, whereas the IPSPs appear to be due to γ-aminobutyric acid and glycine. EPSPs and IPSPs were also recorded in SupV premotor interneurons after stimulation of IntV and MotV, respectively, thus suggesting that reciprocal connections exist between premotor areas and also between premotor interneurons of SupV and inhibitory interneurons located within MotV. It is concluded that the preparation used here will doubtless prove useful for further investigation of the circuitry involved in the bilateral coordination of the jaw.

INTRODUCTION

In the masticatory system, last-order interneurons located in the area surrounding the trigeminal motor nucleus (MotV), the peritrigeminal area (PeriV), are suspected to play a key role in the brain stem circuitry generating jaw movements and reflexes. First, these neurons are excited by inputs from the oral cavity and muscle and from contralateral sensorimotor cortex (Olsson et al. 1986). Second, many project to ispsilateral MotV and some project to the contralateral nucleus, suggesting that they coordinate a bilateral structure such as the mandible (Appenteng et al. 1990; Donga and Lund 1991; Rokx et al. 1986). Third, neurons in this area are phasically active during fictive mastication (Donga and Lund 1991; Inoue et al. 1992; Moriyama 1987). However, little is known about their actions at their targets and detailed investigation of this in vivo is difficult because of the proximity of these neurons to MotV.

A better description of the microcircuitry within PeriV and with MotV is needed if we are to understand how jaw movements are controlled. Thus a brain stem slice was used to characterize connections between these interneurons and motoneurons. To define the boundaries of PeriV and record from identified neurons, a method was developed to label masseteric motoneurons and spindle primary afferents. The latter have cell bodies in the mesencephalic trigeminal nucleus (MesV). In addition to showing that connections of neurons from different divisions of PeriV are preserved in the slice, the present study reveals the existence of reciprocal connections between the neurons and with a new population of interneurons within MotV.

METHODS

Crystals of a carbocyanine dye [1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, DiI; $DiIC_{18}$ (3); Molecular Probes] were injected into the masseters of hypothermia-anesthetized rat pups (2–5 days old) and allowed to diffuse for 7–16 days. On the experimental day, 9- to 21-day-old rats were anesthetized with methoxyflurane (Metofane) and quickly decapitated, and their brain stems were put into ice-cold sucrose artificial cerebrospinal fluid (composition, in mM: 225 sucrose, 5 KCl, 1.25 KH_2PO_4, 4 $MgSO_4$, 0.2 $CaCl_2$, 20 $NaHCO_3$, and 10 D-glucose, pH 7.4) (Aghajanian and Rasmussen 1989). The block was embedded in agar and placed on its side. The rostral end was cut at 55° from the long axis of the brain stem (see Fig. 1*B*) and the block was glued to a vibratome stage with the collicules facing down and the obex facing up. Transverse slices (400 μm) cut parallel to the basis of the block (diagonal dashed line in Fig. 1*B*) were used in all experiments ($n = 12$) except for one experiment in which parasagittal slices were used. In some experiments the injections of DiI crystals were made directly into MotV (instead of the masseters) after the slice was fixed in a 4% paraformaldehyde solution and allowed to diffuse for 3–4 wk.

The slices were transferred to an interface-type chamber perfused with normal artificial cerebrospinal fluid (composition, in mM: 125 NaCl, 5 KCl, 1.25 KH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 26 $NaHCO_3$, and 25 D-glucose, pH 7.4), maintained at 30–32°C, and exposed to a humidified mixture of 95% O_2-5% CO_2. MotV and MesV were visualized with an epifluorescence microscope (Nikon). Conventional sharp microelectrodes filled with 3 M potassium acetate (70–120 MΩ) were lowered in the densely labeled area of MotV or in the zone dorsal to it and ventral to MesV (300–400 μm away from the labeled cells) corresponding to the supratrigeminal area (SupV). Intracellular records were obtained

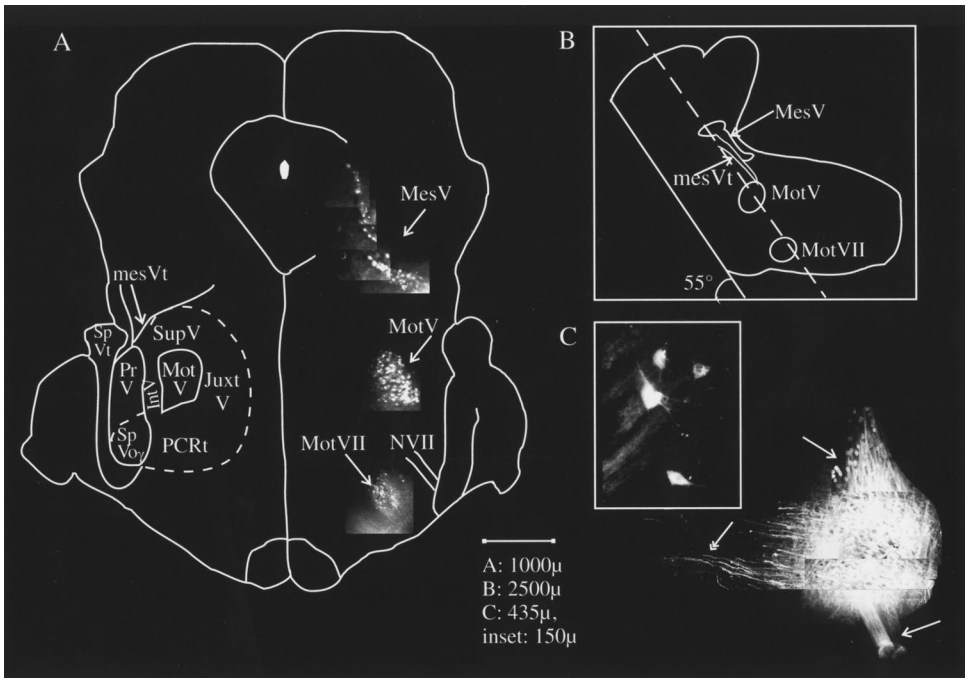

FIG. 1. *A*: crystals of $DiIC_{18}(3)$ (1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate, DiI) injected into masseteric muscle label primary afferent and motoneuron somata in mesencephalic trigeminal nucleus (MesV) and trigeminal motor nucleus (MotV), respectively. Facial nucleus is also labeled because injections were made through skin. *A, right*: micrographs of labeled neurons superposed to drawing of transverse section. *A, left*: well-defined anatomic structures found at same level. Dashed line: peritrigeminal area (PeriV) and parvocellular reticular formation ventral and caudal to MotV (PCRt). IntV, intertrigeminal area; JuxtV, juxtatrigeminal area; mesVt, mesencephalic tract of trigeminal nerve; MotVII, facial motor nucleus; NVII, facial nerve; PrV, trigeminal principal sensory nucleus; SpVoγ, spinal trigeminal nucleus oralis γ; SpVt, spinal trigeminal tract; SupV, supratrigeminal area. *B*: schematic drawing (location of nuclei and scale according to rat atlas of Paxinos and Watson 1982) of sagittal section of brain stem illustrating cutting plane. Slices were obtained at approximate level of dashed line. *C*: deposits of DiI crystals placed directly into MotV label motor root anterogradely and round somata of MesV retrogradely (single-headed arrows). Commissural fibers (double-headed arrow) and neurons in contralateral MotV (*inset*) are also marked by this procedure.

with the use of the bridge mode on an Axoclamp 2B amplifier (Axon Instruments). All but two of the neurons studied had resting membrane potentials (RMPs) negative to −50 mV and discharged overshooting action potentials in response to depolarizing current pulses (100–500 ms). Two interneurons from SupV having RMPs positive to −50 mV (−42 and −38 mV) were included in the analysis because of their particular response to MotV stimulation (see RESULTS and DISCUSSION). Input resistance was determined from the plateau portion of transmembrane responses to hyperpolarizing pulses (100 ms). Threshold was determined by injecting incrementing depolarizing pulses (steps of 0.2 nA) and was defined as the first membrane potential at which spikes were triggered. In all but four cases spikes were evoked synaptically or occurred spontaneously. In these cases the amplitude was measured from the preceding baseline. In the four remaining cases, spikes were elicited with depolarizing pulses and their amplitude was measured from the potential corresponding to threshold.

Synaptic responses were evoked by electrical stimulation with the use of bipolar nichrome electrodes (25 μm diam, insulated except at the tip, stimulus duration 0.05–0.2 ms). The latency was estimated from the beginning of the stimulus artifact. To map the areas projecting to MotV, the stimulating electrodes were first moved throughout PeriV and the adjacent reticular formation both ipsi- and contralaterally. At each location, stimulus intensity was increased gradually from 0 to 10 mA. Stimulation of areas outside PeriV, the parvocellular reticular formation ventral and caudal to MotV (PCRt), MesV, and contralateral MotV failed to induce any response in masseteric motoneurons. The electrodes were left at positions where stimulation yielded a postsynaptic potential and stimulus intensity was adjusted as to obtain a stable and reliable response that was just subthreshold for firing (usually <300 μA). Labeled axons and visible tracts were avoided when placing the stimulating electrodes. To ensure that the observed responses did not result from direct activation of the recorded cell consequent to current spread, at the end of the recording session the stimulating electrode was moved within MotV or SupV very close to the recording electrode and the same stimulus was delivered. Although this was not done for all cells, waveforms resembling the synaptic responses reported here were never observed. All drugs were obtained from RBI. Data were digitized at 5–10 kHz and stored on disk with the use of pClamp 6 system (Axon Instruments, Foster City, CA). Results are given as means ± SE.

RESULTS

The preparation is shown in Fig. 1*A* with the labeled neurons and their corresponding nuclei at *right* and the interneuron-containing area investigated, delimited by a dashed line, at *left*. Lorente de No (1922) subdivided PeriV into SupV, the juxtatrigeminal area (JuxtV), and the intertrigeminal area (IntV). Injections of DiI crystals directly into MotV revealed some of MotV's inputs and outputs that are preserved in the slice. Arrows in Fig. 1*C* indicate the motor root, retrogradely labeled MesV somata, and commissural

TABLE 1. *Responses of masseteric motoneurons to stimulation of PCRt and subdivision of PeriV*

	EPSPs				Biphasic Responses				Unmasked IPSPs			Spikes	
Stimulation Site	*n*	Latency, ms	Amplitude, mV	DTc, ms	*n*	Latency, ms	EPSP amplitude, mV	IPSP amplitude, mV	*n*	Latency, ms	Amplitude, mV	*n*	Latency, ms
PCRt	4	1.8 ± 0.1	8.3 ± 2.2	16.2 ± 1.1					2	1.8	4.5		
JuxtV	9	2 ± 0.1	7.5 ± 1.2	19.2 ± 1.1									
SupV	6	2.5 ± 0.3	6.4 ± 1.7	13.1 ± 1.2	2	1.8	1.6	4.4	1	1.8	3	2	1
IntV	4	1.7 ± 0.3	5.2 ± 0.7	12.7 ± 1.6	1	3.1	3	1.9				5	0.8 ± 0.1

Values in *columns 3–5* and *14* are means ± SE. *n*, number of cells in which this response was observed. Unmasked inhibitory postsynaptic potentials (IPSPs) are inhibitory potentials observed only in presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) or 6,7-dinitroquinoxaline-2,3-dione (DNQX). PCRt, parvocellular reticular formation ventral and caudal to trigeminal motor nucleus; PeriV, peritrigeminal area; EPSP, excitatory postsynaptic potential; DTc, decay time constant; JuxtV, juxtatrigeminal area; SupV, supratrigeminal area; IntV, intertrigeminal area.

fibers. Neurons located in contralateral MotV (*inset*) were also labeled by this procedure.

Recordings from motoneurons

Twenty-four neurons were recorded from the labeled pool in MotV and, on this basis, considered as masseteric motoneurons. These had an RMP of −62 ± 1.2 (SE) mV and an input resistance of 58 ± 8 MΩ. Threshold for firing was from −10 to −64 mV (−36 ± 4 mV) and amplitude of spikes averaged 52 ± 2 mV. Synaptic responses elicited by electrical stimulation of different areas of PeriV and PCRt were obtained in 19 cases (Table 1). Several motoneurons received convergent inputs from two ($n = 6$), three ($n = 2$), or four ($n = 1$) interneuron-containing areas.

Excitatory postsynaptic potentials (EPSPs) or spikes were obtained with stimulation of all divisions of PeriV and PCRt (Table 1, Fig. 2, *A* and *C, left;* Fig. 3, *A* and *B*). Most occurred at short latency and all cases tested ($n = 10$) followed 20-Hz stimulation, suggesting a monosynaptic pathway (Jahr and Yoshioka 1986). Very short-latency spikes evoked by stimulation of IntV (Table 1) that did not arise from an EPSP probably resulted from an antidromic activation of the motor root. In one case this was confirmed by the insensitivity of the spike to antagonists that suppressed synaptic responses in all other tests (Fig. 3*B2*). The excitatory nature of the positive postsynaptic potentials was suggested by the fact that they became a full-blown spike at depolarized potentials and did not reverse polarity at hyperpolarized potentials (Fig. 2*B, left*). Their suppression by addition of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) or 6,7-nitroquinoxaline-2,3-dione [DNQX; 20 μM; kainate/α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor blocker] to the medium (Figs. 2, *A* and *C, right*, and 3*A2*) confirmed this observation in three cases. Abolition of EPSPs evoked by stimulation of PCRt with CNQX unmasked short-latency inhibitory postsynaptic potentials (IPSPs) that were bicuculline insensitive ($n = 2$; Fig. 2*A, right*). These reversed polarity at −64 and −82 mV (Fig. 2*B, right*). Responses evoked by JuxtV had no apparent inhibitory component, leading to a slower decay of the EPSPs (Table 1). CNQX abolished the EPSP but did not unmask other potentials, confirming the lack of IPSPs ($n = 1$; Fig. 2*C, right*).

Biphasic responses (EPSP/IPSP) (Fig. 3*A1*) were also observed following stimulation of SupV ($n = 2$). The IPSPs reversed polarity near the chloride equilibrium potential (−73 and −70 mV, respectively). In one case, addition of DNQX and bicuculline abolished the EPSP and diminished the duration and amplitude of the IPSP (Fig. 3, *A1* and *A2*). The remaining IPSP was eliminated by strychnine (Fig.

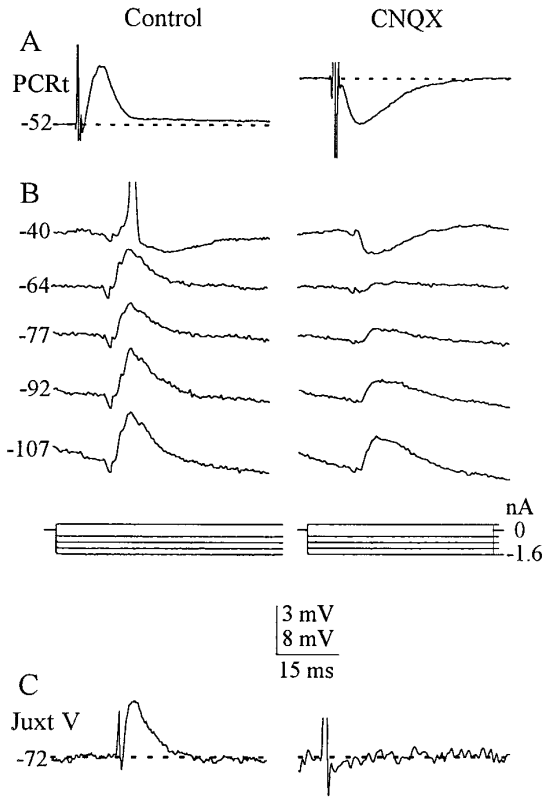

FIG. 2. Intracellular recordings from identified masseteric motoneurons. *A*: under control conditions, stimulation of PCRt evokes excitatory potential (*left*). Abolition of excitatory postsynaptic potential (EPSP) with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM) uncovers inhibitory postsynaptic potential (IPSP, *right*) triggered by same stimulation. *B*: hyperpolarizing cell to different levels increased size but did not reverse polarity of response (*left*), whereas at depolarized potentials spike was evoked (*1st trace*, truncated). In contrast, IPSP uncovered by CNQX (*right*) reversed polarity around −64 mV. *B, bottom traces*: current injected into cell. *C*: EPSP obtained in different motoneuron in response to stimulation of JuxtV before (*left*) and after (*right*) perfusion with CNQX (20 μM). Vertical calibration: 3 mV (*A* and *C*); 8 mV (*B*).

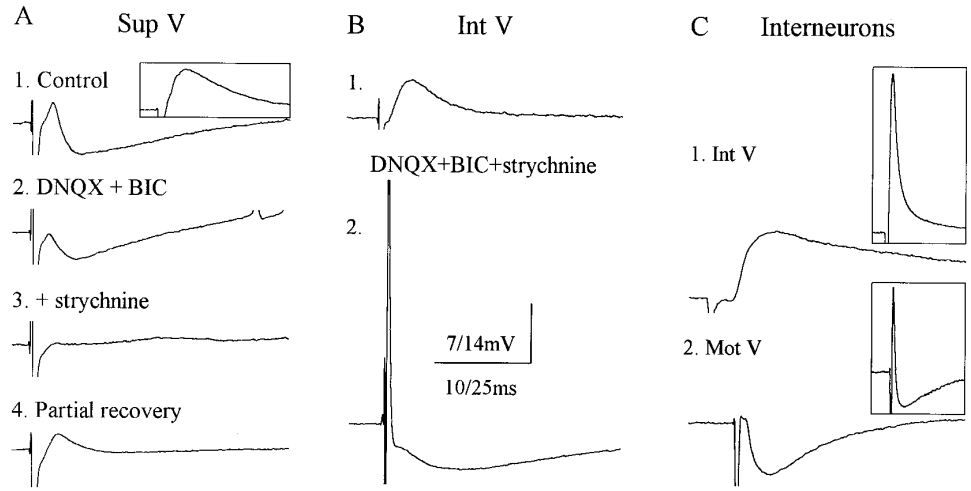

FIG. 3. *A* and *B*: intracellular recording from masseteric motoneurons. *A1*: stimulation of SupV evokes EPSP followed by long-lasting IPSP. In other motoneurons, stimulation of SupV evoked monophasic EPSP (*inset*). *A2*: addition of 6,7-nitroquinoxaline-2,3-dione (DNQX) and bicuculline (BIC; 20 μM each) abolished EPSP and reduced IPSP. Remaining IPSP was eliminated (*A3*) by strychnine (5 μM). *A4*: partial recovery after 30 min of washout, before loosing cell. *B*: in same neuron, stimulation of IntV triggered antidromic spike that was insensitive to DNQX, bicuculline, and strychnine (*B2*). In other neurons stimulation of same site sometimes elicited EPSP (*B1*). *C*: intracellular recordings obtained from 2 interneurons located in SupV. Both neurons fired antidromically after stimulation of MotV (*insets*). *C1*: 1 neuron showed EPSP when IntV was stimulated. *C2*: the other neuron was inhibited by stimulation of MotV at lower intensity than that required to trigger antidromic spike. Calibration: larger numbers apply to *insets* in *A* and *C*.

3*A3*). A similar biphasic response was observed with stimulation of IntV, but after 3.1 ms, indicating involvement of at least two synapses.

Finally, in one particular case, a spike (77 mV) was elicited at very short latency (0.6 ms) by stimulation of the contralateral MotV, which suggests direct antidromic activation.

Recordings from interneurons in SupV

Six neurons were recorded from SupV; four of these were considered premotor because they fired an antidromic spike on stimulation of MotV (Fig. 3, *C1* and *C2*). Four (including 3 MotV projecting neurons) were excited by stimulation of IntV (Fig. 3*C1, inset*) at a short latency (2.5 ± 0.3 ms). In two neurons that had depolarized potentials at rest (−38 and −42 mV, respectively), stimulation of MotV also evoked IPSPs (5.4 and 6.6 mV) at a monosynaptic latency (1.2 and 2.0 ms); one of these also projected to MotV (Fig. 3*C2*).

DISCUSSION

Interneurons located in PeriV presumably play a central role in controlling jaw movements. This assumption is based on indirect evidence, because synaptic relations between these premotor neurons and trigeminal motoneurons have been directly investigated only for interneurons of PCRt (Curtis and Appenteng 1993; Grimwood et al. 1992). The aim of this study was to document these relations with the use of an in vitro model. PCRt was also examined because it contains a large proportion of neurons projecting to MotV (Kolta et al. 1995; Turman and Chandler 1994a,b). The results reported here suggest that both excitatory and inhibitory interneurons are located in this area, because its stimulation evoked EPSPs and IPSPs (unmasked) at the same latencies in motoneurons. This is in agreement with the results of Curtis and Appenteng (1993) and Grimwood et al. (1992) who found, with the use of spike-triggered averaging, that both EPSPs and IPSPs recorded in trigeminal motoneurons shortly followed (0–1.7 ms) spikes recorded from single units in this area. The differences between the latencies reported here and those from the group of Appenteng may reflect recruitment of small-diameter axons having slower conduction by electrical stimulation.

Little is known about the output of IntV and JuxtV; our results indicate that only excitatory interneurons were activated by stimuli delivered in these two areas. The long-latency biphasic response obtained with stimulation of IntV in one case probably involved a multisynaptic pathway. SupV has long been thought to inhibit jaw closing motoneurons (Kidokoro et al. 1968). The present study shows that inhibitory responses in masseteric motoneurons were sometimes caused by stimulation of SupV. However, when present, IPSPs were either masked by or followed EPSPs. Like masseteric motoneurons, neurons in SupV are activated by spindle afferents (Miyazaki and Luschei 1987). It is therefore not surprising that SupV comprises interneurons that are excitatory to masseteric motoneurons. Stimuli delivered to SupV most probably activated both excitatory and inhibitory interneurons, as they did in PCRt. This is in agreement with reports that interneurons containing glutamate, γ-aminobutyric acid (GABA), and glycine are intermingled in PeriV and PCRt of guinea pig and rabbit (Kolta et al. 1995; Turman and Chandler 1994a,b).

All EPSPs tested seem to mainly involve kainate/AMPA receptors, because they were abolished by CNQX or DNQX and because depolarizing the cell before synaptic stimulation did not unveil a different component. However, implication of NMDA receptors has not been specifically addressed in

this study and so a contribution of these receptors cannot be ruled out, because their presence in trigeminal motoneurons has been described (Kim and Chandler 1995). The short-latency bicuculline-insensitive IPSPs obtained after stimulation of PCRt and SupV probably involved glycinergic interneurons. This is supported by the results of Castillo et al. (1991), who reported that stimulation of PCRt induced short-latency strychnine-sensitive IPSPs in both jaw closing and jaw opening motoneurons. In at least one case, $GABA_A$ receptors partially mediated an IPSP elicited by stimulation of SupV. This IPSP could have resulted from activation of intercalated interneurons or from the same set of interneurons in SupV releasing glycine. Cotransmission of GABA and glycine has been described in several structures of mammalian nervous system (Chen and Hillman 1993; Lahjouji et al. 1996; Moore et al. 1996). In particular, colocalization of GABA and glycine in axon terminals in motoneuronal cell groups seems to be a fairly common feature in the spinal cord (Shupliakov et al. 1993; Taal and Holstege 1994; Todd et al. 1996).

The results reported here were all obtained with electrical stimulation of premotor areas. This approach, although convenient, raises concerns about which elements are being activated by the stimulation. A number of observations suggests that in the present study stimulation was confined to the vicinity of the stimulating electrode. *1*) Stimulation of areas JuxtV and IntV never elicited responses with inhibitory components at short latencies, as would be expected had the current spread to neighboring areas that all contained inhibitory interneurons. *2*) In all cases tested, responses followed 20-Hz stimulation, suggesting that they were monosynaptic. *3*) IPSPs recorded in presence of CNQX or DNQX, and thus probably monosynaptic, appeared at the same latencies as the EPSPs. The possibility that axons of passage originating from more distant areas might have been stimulated persists. However, mapping over a wide area of stimulation sites (see METHODS) never revealed loci outside of PeriV, PCRt, MesV, and contralateral MotV that could elicit a response in masseteric motoneurons.

Recordings obtained from SupV interneurons indicate that they receive excitatory inputs from IntV. In two cells that had lower RMPs, due either to damage or to their small size, IPSPs were evoked by stimulation of MotV. In these cases the depolarized potential was probably instrumental in revealing the IPSP. These inhibitory potentials, coupled with the fact that we have recently found a population of small GABA-immunoreactive neurons in the same area in the rabbit (A. Kolta and K. G. Westberg, unpublished observations) show that a set of inhibitory GABAergic interneurons, located within MotV, probably project to SupV.

It was recently reported that stimulation of MotV sometimes evokes small IPSPs in contralateral jaw closing motoneurons (Jüch et al. 1993). Taken together, these observations suggest that the commissural fibers and neurons seen in contralateral MotV after injection of DiI in MotV, as well as the neuron recorded in MotV that was antidromically activated from contralateral MotV, belong to the same set of interneurons. These may then play a role analogous to that of Renshaw cells or Ia inhibitory interneurons. They are in an ideal position to coordinate the lateral movements of the jaw during which certain compartments within jaw closing muscles are antagonists. If reciprocally connected, they could also contribute to rhythm generation during mastication.

Together, these results demonstrate the usefulness of this preparation, which preserves much of the circuitry necessary for the bilateral control of the jaw.

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