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Behavioral correlates of the activity of serotonergic and non-serotonergic neurons in caudal raphe nuclei

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Abstract

We investigated the behavioral correlates of the activity of serotonergic and non-serotonergic neurons in the nucleus raphe pallidus (NRP) and nucleus raphe obscurus (NRO) of unanesthetized and unrestrained cats. The animals were implanted with electrodes for recording single unit activity, parietal oscillographic activity, and splenius, digastric and masseter electromyographic activities. They were tested along the waking-sleep cycle, during sensory stimulation and during drinking behavior. The discharge of the serotonergic neurons decreased progressively from quiet waking to slow wave sleep and to fast wave sleep. Ten different patterns of relative discharge across the three states were observed for the non-serotonergic neurons. Several non-serotonergic neurons showed cyclic discharge fluctuations related to respiration during one, two or all three states. While serotonergic neurons were usually unresponsive to the sensory stimuli used, many non-serotonergic neurons responded to these stimuli. Several non-serotonergic neurons showed a phasic relationship with splenius muscle activity during auditory stimulation. One serotonergic neuron showed a tonic relationship with digastric muscle activity during drinking behavior. A few non-serotonergic neurons exhibited a tonic relationship with digastric and/or masseter muscle activity during this behavior. Many non-serotonergic neurons exhibited a phasic relationship with these muscle activities, also during this behavior. These results suggest that the serotonergic neurons in the NRP and NRO constitute a relatively homogeneous population from a functional point of view, while the non-serotonergic neurons form groups with considerable functional specificity. The data support the idea that the NRP and NRO are implicated in the control of somatic motor output.

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- Startle behavior
- Drinking behavior

Introduction

The two most caudal raphe nuclei, the nucleus pallidus (NRP) and nucleus obscurus (NRO), have been implicated in the control of somatic motor and visceral motor-secretory functions. Three lines of evidence support this view. First, the major projections of these nuclei are directed at the somatic motor/premotor and the visceral motor-secretory/premotor-secretory nuclei of the brainstem and spinal cord. Well-known target structures are the trigeminal motor nucleus (1), facial nucleus (2), nucleus ambiguus (3), hypoglossus nucleus (4,5), the ventral horn of the spinal cord (6-8), the medial medul-

lary reticular formation (9), the ventral respiratory group (10), the red nucleus (11), the dorsal motor nucleus of the vagus (12), the intermediolateral cell column of the spinal cord (13-15) and the rostral ventrolateral medulla and surrounding regions (9,16,17). At least part of the axons terminate directly in motoneurons (7,18) or preganglionic autonomic neurons (15).

Second, increased NRP or NRO activity induced by electrical or chemical stimulation facilitates trigeminal motoneurons (18), facilitates or depresses phrenic motoneurons (19-21), facilitates lumbar motoneurons (22,23), facilitates motor and secretory gastric preganglionic neurons in the dorsal motor nucleus of the vagus (12,24), and facilitates or depresses sympathetic preganglionic neurons to the heart and vessels (25, 26).

Third, the NRP and NRO contain neurons that discharge in association with respiration (27-30), mastication and other orofacial behaviors (30,31), locomotion (30), the cardiac cycle (32,33), and blood pressure (14,34).

The putative output control role of NRP and NRO seems to involve in part serotonergic neurons, which are relatively numerous in both nuclei. In the cat, they represent about 50% of all neurons in the NRP and 35% of all neurons in the NRO (35). Correspondingly, target structures present many serotonergic terminals (18,36). Artificial activation of the NRO was shown to increase the release of serotonin in target structures (37) and blockade of serotonin receptors with antagonists considerably reduces the effects of artificial activation of NRP and NRO on target neurons (18-22). Furthermore, the discharge of some serotonergic neurons in the NRP and NRO is associated with mastication and other orofacial behaviors and locomotion (30,31).

The relative role of non-serotonergic neurons in the NRP and NRO is less well understood. It can be inferred almost exclusively from experiments on anesthetized animals or reduced preparations (decerebrated animals). The only study that used unanesthetized and unrestrained animals was the one done by Heym et al. (27). These investigators described three groups of non-serotonergic neurons in the NRP of cats. One included cells that discharged multiple action potentials; behavioral correlates of this activity were not determined. Another was formed by cells that fired with an oscillating rhythm related to respiration. The last one, the largest, contained cells whose discharge appeared to be associated with somatic movements. Nothing else was reported about these neurons.

The present study extends the investigation begun by Heym et al. (27), Veasey et al. (30) and Ribeiro-do-Valle (31). We (31) examined in detail the activity of a few serotonergic neurons and a large number of non-serotonergic neurons in the NRP and NRO in several physiological conditions. Behaving cats were used. The results clearly support the hypothesis of an involvement of serotonergic and especially non-serotonergic neurons in these nuclei in somatic motor output control.

Material and Methods

Subjects

Male and female adult cats weighing 2.4-3.8 kg were used. The animals were housed individually in stainless steel cages in a naturally illuminated and well-ventilated room. Food and water were available *ad libitum*. Housing conditions of the animals and the experimental protocol were approved by the "Animal Research Ethics Committee" of Instituto de Ciências Biomédicas, Universidade de São Paulo.

Testing booth

The subjects were tested individually in a

wooden booth measuring 60 x 60 x 90 cm, with a clear Plexiglas front door. This booth was ventilated through small holes on the posterior part of its top side.

Recording set

Cortical activity (EEG), muscular activities (EMGs) and single neuron activity were recorded. These signals were led by means of low noise cables to a swivel commutator mounted on a counter-weighted device fixed over the top side of the testing booth (in this way the cables could turn around and move up and down freely). The signals were amplified and bandpass filtered (1-35 Hz for the EEG, 30-3000 Hz for the EMGs and 300-3000 Hz for the neuron activity) using a polygraph (Grass Instrument, Co., Quincy, MA, USA). Single neuron activity was continuously monitored on an oscilloscope and also isolated from background noise by means of a window discriminator (Back Electronics, Inc., Germantown, MD, USA). The TTL (transistor-transistor logic, 5-V electric pulse) output of this window discriminator activated an audio monitor. The EEG, the EMGs and the TTL output of the window discriminator were recorded on paper. The EEG, the EMGs, and raw- and TTL-transformed single neuron activity were digitalized (sampling rate of 11 kHz) by means of an A/D VCR Adapter (Medical Systems Corp., Greenvale, NY, USA) and stored on videotape.

Behavior was filmed with a videocamera and the audio monitor output was recorded together with behavior.

Surgical procedure

The subjects were injected with atropine sulfate (200 µg, *sc*) and, 30 min later, with pentobarbital sodium (40 mg/kg, *ip*). A pair of electrodes fashioned from Teflon-coated multi-stranded stainless steel wire was implanted into the right digastric muscle and another into the right masseter muscle to record their EMG. Three stainless steel screws were threaded into the parietal and frontal bones to serve as anchoring points. A stainless steel screw electrode was threaded into the right parietal bone and another one into the left parietal bone to record the EEG. One stainless steel screw electrode was threaded into the temporal bone in contact with the temporal muscle for electrical grounding.

The two guide cannulae of a microdrive assembly (see Ref. 38) were stereotaxically implanted into the brainstem according to a -15° approach, using the following coordinates (for the anterior cannula): anteroposterior = -11.6, lateromedial = 0.0, and dorsoventral = -0.2, of the Snider and Niemer (39) atlas of the cat brain. The microdrive assembly was then fixed to the skull with dental acrylic. A bundle of 7 microwires (Formvar-insulated nichrome wires, diameters of 32 and 64 µm), previously soldered to a 25-pin connector, was lowered through each guide cannula until it protruded 7 mm beyond its tip into brainstem tissue. The microwire bundles were fixed in place by gluing to the upper extremity of the guide cannulae.

A Teflon-coated multi-stranded stainless steel wire electrode was implanted into the right splenius muscle and another one into the left splenius muscle.

The EEG, EMG and ground electrodes were soldered to the 25-pin connector. This was then secured to the skull with dental acrylic.

The animals were injected *im* with benzathine penicillin (two doses of 300,000 units, 12 h apart) for prophylaxis.

Testing procedure

The animals started to be tested after a recovery period of about 2 weeks. Two to three times a day each subject was placed in the testing booth and all microwire leads were screened for the presence of spikes

that could be clearly distinguished from the background noise. In the absence of such an activity, the microelectrodes were moved about 80 µm ventrally by turning the microdrive assembly screw about 1/4 turn.

When a consistently distinct spike (usually a signal to noise ratio of at least 3) was found, the subject was further tested. Neuron activity, as well as the EEG and the splenius, digastric and masseter EMGs, were recorded during exposure to a series of visual stimuli (100 light flashes, 3 x 10^3 cd/m^2 peak intensity, 1-ms duration, 0.5 Hz), a series of auditory stimuli (100 clicks, 80 dB, 1-ms duration, 0.5 Hz) and a series of visual + auditory stimuli (light flashes plus clicks), in this order, with the animal awake but quiet. These signals were also recorded during spontaneous behaviors like drinking milk, water or fish soup and during quiet waking (QW), slow wave sleep (SWS) and fast wave sleep (FWS). Finally, they were recorded during painful stimulation (strong pressure on the animals' ear exerted by the experimenter's thumb and index finger for 5 s, twice on each side, with an interstimulus interval of 55 s).

The microelectrodes were moved ventrally for a total distance of about 5 mm.

Histological analysis

The animals were deeply anesthetized with pentobarbital sodium. A direct anodal current (0.2 mA for 5 s) was passed through each microelectrode that recorded cells at the exact dorsoventral level where these cells were found. Saline, formalin solution and potassium ferrocyanide-formalin solution were then perfused through the ascending aorta. The encephalon was removed and stored in formalin. After at least one week the encephalon was frozen and the hindbrain was cut transversally into 40-µm thick sections which were stained with neutral red. The outline of the relevant sections and the location of the recorded cells, indicated by a blue color, were drawn on sheets of paper.

Data processing and statistical analyses

Behavioral data recorded on videotape were played back on a TV monitor. At the same time the corresponding electrophysiological data recorded on paper were played back on the polygraph. The beginning of each new behavior and its identity were indicated at the proper position on the paper record.

Paper records were mainly used to select stretches of data of interest, which were transferred from the videotapes to the hard disk of a microcomputer. First they were analogized back by the A/D VCR Adapter and then the EEG and EMGs were digitalized again with an A/D Interface (Cambridge Electronic Design Ltd., Cambridge, England) at a sampling rate of 200 Hz for the EEG and 5000 Hz for each EMG. The TTL-transformed single neuron activity was acquired by the A/D Interface as a digital signal.

The data were further processed and statistically analyzed as follows:

Evaluation of neuron activity dependency on waking-sleep cycle state

a) Number of spikes per 10 s was calculated for 5 min of QW, 5 min of SWS and 5 min of FWS. Firing rates corresponding to the three states were compared using Friedman analysis of variance. The Wilcoxon matched pairs test was used for subsequent pairwise comparisons when appropriate. A significance level of 0.01 was adopted. According to its relative firing rate across the three states, the neuron was classified into one of 27 possible subtypes.

b) The interspike interval coefficient of variation was calculated for 5 min of QW, 5 min of SWS and 5 min of FWS.

Evaluation of neuron responsiveness to sensory stimulation

a) Spikes discharged by the neuron during consecutive 1-ms bins, 500 ms before and 500 ms after the occurrence of the visual, auditory and visual + auditory stimuli were added across 100 trials on a bin basis. A response to the stimulus was considered to have occurred when the total number of spikes on each of three successive time bins, during the first 100 ms after the stimulus, became larger (smaller) than the mean total number of spikes per time bin for the 500 ms before the stimulus ± 3 SD (standard deviation). The first significant time bin was considered the beginning of this response and the first of three consecutive nonsignificant time bins, its end. The latency (in ms), duration (in ms) and magnitude (mean total number of spikes per time bin divided by prestimulus mean total number of spikes per time bin) of this response were evaluated.

b) The total number of spikes discharged by the neuron during the four 5-min periods of painful stimulation was compared to that during the four immediately preceding 5-min periods. An increase greater than 1.5 times or a decrease greater than 0.5 times was considered as a response. The result of the division of the number of counts during stimulation by the number of counts before stimulation represented the magnitude of this response.

Evaluation of the association of neuron activity with respiration

Spike autocorrelation (10-s time window, 10-ms bin width) was performed for 5 min of QW, 5 min of SWS and 5 min of FWS. Bin counts were divided by the total number of spikes considered. For those neurons whose curve exhibited a clearcut wax and waning pattern upon visual inspection (normalized amplitude of oscillation equal to or greater than 0.01), the time interval between the first two peaks was evaluated. An oscillation period of 1 to 4 s was considered to be suggestive of a relationship between the activity of the neuron and respiration. This possibility was reinforced by relating the changes in firing rate of the neuron (as heard through the loudspeaker) to the respiratory movements of the thoracic cage (as observed visually).

Evaluation of the association of neuron activity with splenius muscle activity

a) Stimulus-triggered averaging (-502.5- to 502.5-ms time window, 1.5-ms bin width) of the rectified and integrated (over 1.5 ms) activity of the splenius muscle during auditory stimulation (100 clicks) was performed. A response was considered to have occurred when total muscle activity for each of three consecutive time bins during the first 100.5 ms after the stimulus became larger (smaller) than the mean total muscle activity per time bin for 502.5 ms before the stimulus +3 SD. The first significant time bin was considered to be the beginning of this response and the first of three consecutive nonsignificant time bins, its end. The latency (in ms) and duration (in ms) of this response were evaluated.

b) Spike-triggered averaging (-502.5- to 502.5-ms time window, 1.5-ms bin width) of the rectified and integrated (over 1.5 ms) activity of the splenius muscle during auditory stimulation (100 clicks) was performed. Only spikes occurring during a period of 100.5 ms immediately after the stimulus were considered. A possible response was considered to have occurred when total muscle activity for each of three consecutive time bins during the first 20 ms after the stimulus became larger (smaller) than the mean total muscle activity per time bin for the 10-ms period immediately before the spike +3 SD. The first significant time bin was considered to be the beginning of this possible response and the first of three consecutive nonsignificant time bins, its end. The latency (in ms)

and duration (in ms) of this possible response and the latency of its peak/valley were evaluated. Latency to peak/valley had to be below 20 ms and duration had to be longer than 3 ms for this possible response to be considered a true response (these criteria were established on the basis of observations made by Fetz and Cheney (40)). This analysis could suggest a relatively direct phasic influence of the neuron on splenius muscle motoneurons.

Evaluation of the association of neuron activity with masticatory muscle activity

a) Number of spikes per 10 s, digastric muscle rectified and integrated activity (over 10 s) and masseter muscle rectified and integrated activity (over 10 s) were calculated for 60 s of drinking behavior. Neuron activity and the activity of each muscle were correlated using the Spearman R correlation test. A significance level of 0.01 was adopted. This analysis could suggest a tonic influence of the neuron on masticatory muscle activity.

b) Neuron activity was analogized by substituting an excitatory postsynaptic potential-like curve (1-ms rising time and 20-ms decay time) for each pulse representing a spike, for 60 s of drinking behavior. The rectified activity of each masticatory muscle was integrated over 1 ms for the same period of time. Neuron activity and the activity of each masticatory muscle were then cross-correlated (500-ms time window, 1-ms bin width). Latency and amplitude of the cross-correlation peak and the cross-correlation valley in a range of lag times from -250 to 250 ms were calculated (for exactly equal signals, maximum amplitude would be 1.0 or -1.0). Peak/valley latencies between -50 and 50 ms and amplitude equal to or above 0.10/equal to or below -0.10 were considered to be indicative of an association between the activity of the neuron and the activity of the muscle (the 0.10 and -0.10 amplitude values exceeded in every case the maximum/minimum mean coefficient ± 3 SD obtained by cross-correlating 10 times a pseudorandom activity with muscle activity). This analysis could suggest a phasic influence of the neuron on masticatory muscle activity.

The criteria adopted here to consider a change in neuronal activity as a response or as related to another physiological change were strict. It was deliberately decided to reduce the chance of type I error to a minimum, even at the cost of losing true responses or relationships. This means that more cells than were reported could eventually be implicated in each investigated function.

Results

Location of the recorded neurons

A total of 8 serotonergic neurons (identified as such by the following criteria: action potential duration of about 2.0 ms, regular discharge during QW and marked reduction in activity or silence during FWS; see Ref. 27) and 60 non-serotonergic neurons (action potential duration of about 1.5 ms) were recorded in the medulla midline between the rostral and the caudal poles of the inferior olivary complex (lateromedial levels from 0.0 to ± 0.5 and anteroposterior levels from -8.0 to -12.5 of the Snider and Niemer (39) atlas). Four serotonergic neurons were located in the NRP region and the other 4 serotonergic neurons were located in the NRO region. Thirty-three and 21 non-serotonergic neurons were located, respectively, in the NRP region and in the NRO region. The location of the remaining 6 non-serotonergic neurons, whether in the NRP region or in the NRO region, could not be determined.

All sampled neurons probably had a medium to large size soma (20-40 μm in diameter). This is a limitation of the recording technique used (see Ref. 38).

Neuronal activity across the waking-sleep cycle

Six serotonergic neurons and 52 non-serotonergic neurons could be recorded for 5 min of QW, 5 min of SWS and 5 min of FWS. The characteristics of the activity of these serotonergic neurons in each state have been previously described (31) and are reviewed in Table 1 for comparison. The discharge rates of the non-serotonergic neurons in any state and their interspike interval coefficients of variation in any state could be quite different.

Ten (19%) non-serotonergic neurons discharged more during QW than during SWS and FWS and 10 (19%) other neurons discharged less during QW than during the other two states. Just 1 (2%) neuron discharged more during SWS than during QW and FWS; 25 (48%) neurons discharged less during SWS than during the other two states. Thirty (58%) neurons discharged more during FWS than during QW and SWS; only 2 (4%) neurons discharged less during FWS than during the other two states.

Differences between states in the interspike interval coefficients of variation were not analyzed statistically. It should be noted, however, that 6 (12%) non-serotonergic neurons exhibited a maximum value and 27 (52%) neurons a minimum value for this variable, for QW. For SWS, 5 (10%) neurons exhibited a maximum value and 18 (35%) neurons a minimum value for this variable. For FWS, 41 (79%) neurons exhibited a maximum value and 5 (10%) neurons a minimum value for this variable.

The neuron that discharged more during SWS, the 2 neurons that discharged less during FWS and the 5 neurons whose activity varied the least during FWS were all located in the NRP. The other groups of non-serotonergic neurons characterized above had elements in both NRP and NRO.

Non-serotonergic neurons could be grouped into 10 subtypes (from A to J) according to their relative discharge rates across the three states (as defined statistically). The relationships that characterize each subtype are the following: A) QW>SWS, SWS<FWS, QW<FWS; B) QW<SWS, SWS<FWS, QW<FWS; C) QW = SWS, SWS<FWS, QW<FWS; D) QW>SWS, SWS<FWS, QW>FWS; E) QW>SWS, SWS<FWS, QW = FWS; F) QW>SWS, SWS = FWS, QW>FWS; G) QW = SWS, SWS = FWS, QW = FWS; H) QW = SWS, SWS>FWS, QW>FWS; I) QW<SWS, SWS>FWS, QW<FWS; J) QW = SWS, SWS>FWS, QW = FWS. The actual discharge rates and interspike interval coefficients of variation in each state of these subtypes are indicated in Table 1. It is apparent from this table that certain patterns of relative discharge rates across states were much more common than others. For all subtypes of neurons the median for the interspike interval coefficients of variation for FWS was higher than those for QW and SWS. No spatial location selectivity occurred for subtype A to F neurons. Subtype G, H and I neurons were found in the NRP, and subtype J neurons were found in the NRO. The functional characteristics of the non-serotonergic neurons should be

Table 1. Median discharge rate (Hz) and median interspike interval coefficient of variation for quiet waking (QW), slow wave sleep (SWS) and fast wave sleep (FWS) of serotonergic (Ser) and non-serotonergic neurons (A to J).

	Discharge rate			Coefficient of variation		
	QW	SWS	FWS	QW	SWS	FWS
Ser (6)	3.69	2.80	0.09	0.21	0.27	1.98
A (13)	6.38	2.42	12.51	1.25	1.21	2.56
B (9)	3.95	7.65	28.41	1.79	1.67	2.63
C (8)	2.29	1.90	19.13	1.76	1.64	2.64
D (7)	8.76	1.06	4.22	0.89	1.28	2.64
E (5)	13.35	6.99	15.72	0.84	1.26	3.00
F (3)	2.59	2.14	2.15	1.10	1.15	2.65
G (3)	3.09	2.90	5.36	0.63	0.83	1.16
H (2)	9.75	9.51	3.40	0.34	0.42	3.00
I (1)	3.11	10.24	5.26	1.51	1.22	2.09
J (1)	3.00	3.80	2.21	1.13	1.62	4.86

The number of neurons is given in parentheses.

compared with those of the serotonergic neurons.

Neuronal responsiveness to sensory stimulation

The 8 serotonergic neurons and 57 non-serotonergic neurons were tested with visual, auditory and visual + auditory stimuli. Excitatory responses could be identified and evaluated quantitatively using the statistical criterion.

One (13%) serotonergic neuron responded to the auditory stimulus and another (13%) to the visual + auditory stimulus. Table 2 shows the latency, duration and magnitude of these responses. The neuron responsive to the auditory stimulus was located in the NRO and the neuron responsive to the visual + auditory stimulus was located in the NRP.

Only 5 (9%) non-serotonergic neurons responded to the visual stimulus. Responses to the auditory stimulus and to the visual + auditory stimulus, on the other hand, were very common: 39 (68%) neurons responded to the auditory stimulus and 30 (53%) neurons responded to the visual + auditory stimulus. Seven (12%) neurons responded only to the auditory stimulus, 2 (4%) to the visual and the auditory stimuli, 27 (47%) to the auditory and the visual + auditory stimuli, and 3 (5%) to the visual, auditory and visual + auditory stimuli. Table 2 presents the median latency, median duration and median magnitude of these responses. These parameters were similar for the serotonergic and non-serotonergic neurons and were also similar for the 4 groups of non-serotonergic neurons. Responses to the visual, auditory and visual + auditory stimuli were not very different. Four non-serotonergic neurons that were excited by the auditory stimulus or by the auditory and visual + auditory stimuli appeared to present a subsequent inhibitory rebound, which was not quantified (see below). The responses of a neuron that was influenced by the auditory and the visual + auditory stimuli are illustrated in Figure 1. Neurons of each kind were found in both NRP and NRO.

Table 2. Median latency (ms), median duration (ms) and median magnitude (times baseline) of neuronal excitatory response to visual, auditory and visual + auditory stimulation.

	Visual			Auditory			Visual + Auditory		
	Lat.	Dur.	Magn.	Lat.	Dur.	Magn.	Lat.	Dur.	Magn.
Ser (1)	-	-	-	79.0	10.0	5.52	-	-	-
(1)	-	-	-	-	-	-	52.0	7.0	11.83
N-Ser (7)	-	-	-	49.0	4.0	10.87	-	-	-
(2)	33.0	6.5	9.40	54.0	8.5	8.95	-	-	-
(27)	-	-	-	24.0	8.0	8.70	24.0	11.0	9.09
(3)	42.0	5.0	9.47	42.0	11.0	15.08	29.0	11.0	8.00

Eight serotonergic neurons (Ser) and 57 non-serotonergic neurons (N-Ser) were tested. Lat., latency; Dur., duration; Magn., magnitude. The number of neurons is given in parentheses.

Inhibitory responses to visual, auditory and visual + auditory stimulation could not be characterized properly using the statistical criterion. The spontaneous firing rate of most recorded neurons was relatively low and variable. Consequently, the reference value for deciding whether a significant reduction in activity occurred (mean spontaneous firing rate -3 SD) tended to be negative and as such useless. Visual inspection of the individual peri-stimulus time histograms suggested that 1 (2%) non-serotonergic neuron (distinct from those mentioned above) was inhibited by the visual + auditory stimulus and 2 (4%) (also distinct from those mentioned above) were inhibited by the auditory and visual + auditory stimuli.

All serotonergic neurons and 47 non-serotonergic neurons were tested with the painful stimulus. None of the serotonergic neurons responded to this stimulation. Three (6%) non-serotonergic neurons were excited by it and 9 (19%) non-serotonergic neurons were inhibited by it. The median magnitude of the excitatory responses was 1.96 times the baseline value and the median magnitude of the inhibitory responses was 0.38 times

the baseline value. Non-serotonergic neurons of these two kinds were found in both NRP and NRO.

Non-serotonergic neurons exhibiting each kind of sensory responsiveness were distributed without any obvious selectivity among subtypes A to H and J.

Neuronal activity possibly associated with respiration

No cyclic fluctuation in firing rate during QW, SWS or FWS was observed for the 6 serotonergic neurons evaluated.

Of the 52 non-serotonergic neurons examined, 11 (21%) exhibited this pattern of activity during QW and/or SWS and/or FWS. One of them always showed the pattern. The period of oscillation decreased from 2.42 s during QW to 1.99 s during SWS and to 1.74 s during FWS (see Figure 2). One showed the pattern during QW (oscillation period of 3.24 s) and SWS (oscillation period of 2.52 s), but not during FWS. Six showed the pattern only during QW (median oscillation period of 2.70 s), 2, only during SWS (oscillation periods of 2.48 and 3.12 s), and 1, only during FWS (oscillation period of 1.20 s). For 10 of these neurons a discharge phase alternated with a silence phase. The discharge rate during the discharge phase increased continuously, increased and then decreased or stayed about the same. The duration of the discharge phase tended to be longer than that of the silence phase. There was considerable variation both in the pattern during the discharge phase and in the relative duration of the two phases from one cycle to another.

For 6 neurons the cyclic changes in activity were synchronized with the respiratory movements of the thoracic cage. For the remaining neurons the existence of such an association could not be determined either because the respiratory movements could not be clearly seen on the films (3 cases) or because behavior films were not available (2 cases).

Five of the 11 neurons were located in the NRP and the other 6 were located in the NRO.

The 11 neurons were distributed evenly among subtypes A, B, C, D and E.

Neuronal activity associated with splenius muscle activity

The auditory stimulus produced a phasic response of the splenius muscle on 36 of the

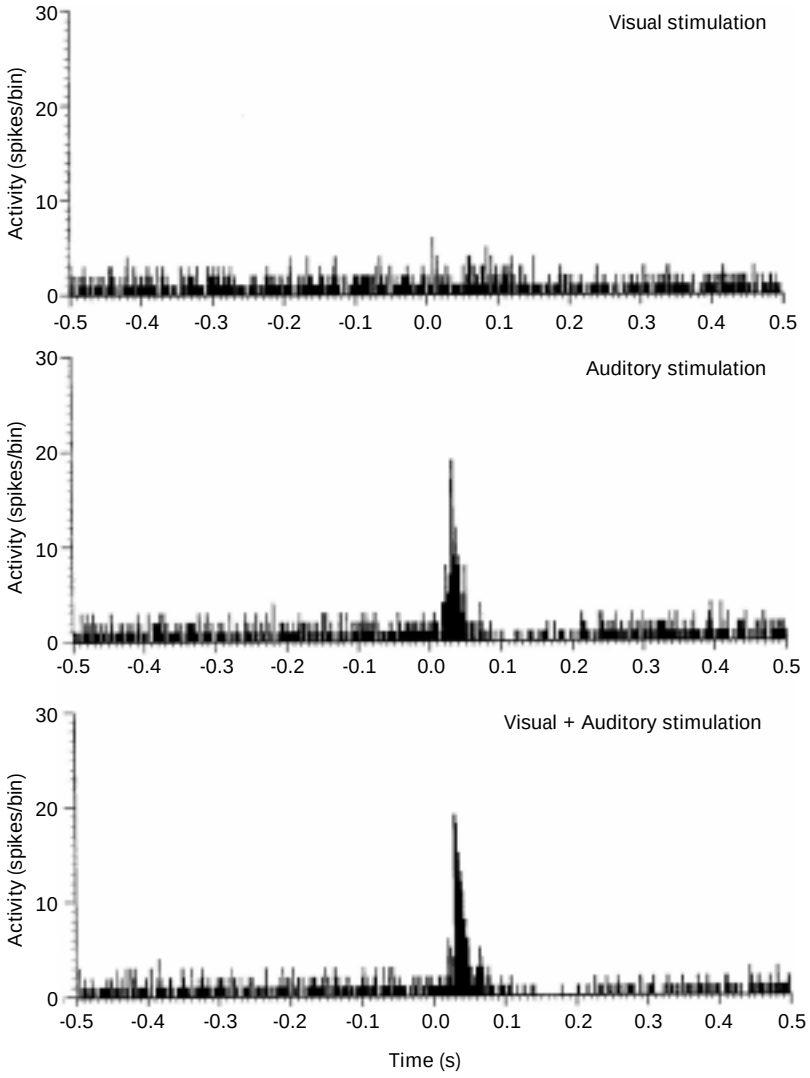

Figure 1. Peri-stimulus time histograms of a non-serotonergic neuron that did not respond to visual (upper panel) stimulation, but responded to auditory stimulation (middle panel) and to visual + auditory stimulation (bottom panel). One hundred stimuli were presented in each case. This neuron was located in the nucleus raphe pallidus. Bin width is 1 ms.

65 occasions it was used to test the neurons (see Figure 3). Only 3 responses were seen for the visual stimulus and 38, for the visual + auditory stimulus. The median latency of the responses was 38 ms and their median duration was 6 ms.

No significant spike-triggered response of the splenius muscle occurred for the 8 serotonergic neurons during the auditory stimulation. A response was obtained for 11 (19%) of the 57 non-serotonergic neurons tested. The median latency of the responses was 11 ms. The median duration of the responses was 6 ms. The median latency of the response valley was 15 ms. Three of the neurons were located in the NRP and 8 in the NRO. Eight neurons were distributed among subtypes A, B, C, D, F, H and J and 3 did not have their subtype determined. The auditory stimulus activated both the neuron and the muscle in 7 of the 11 cases.

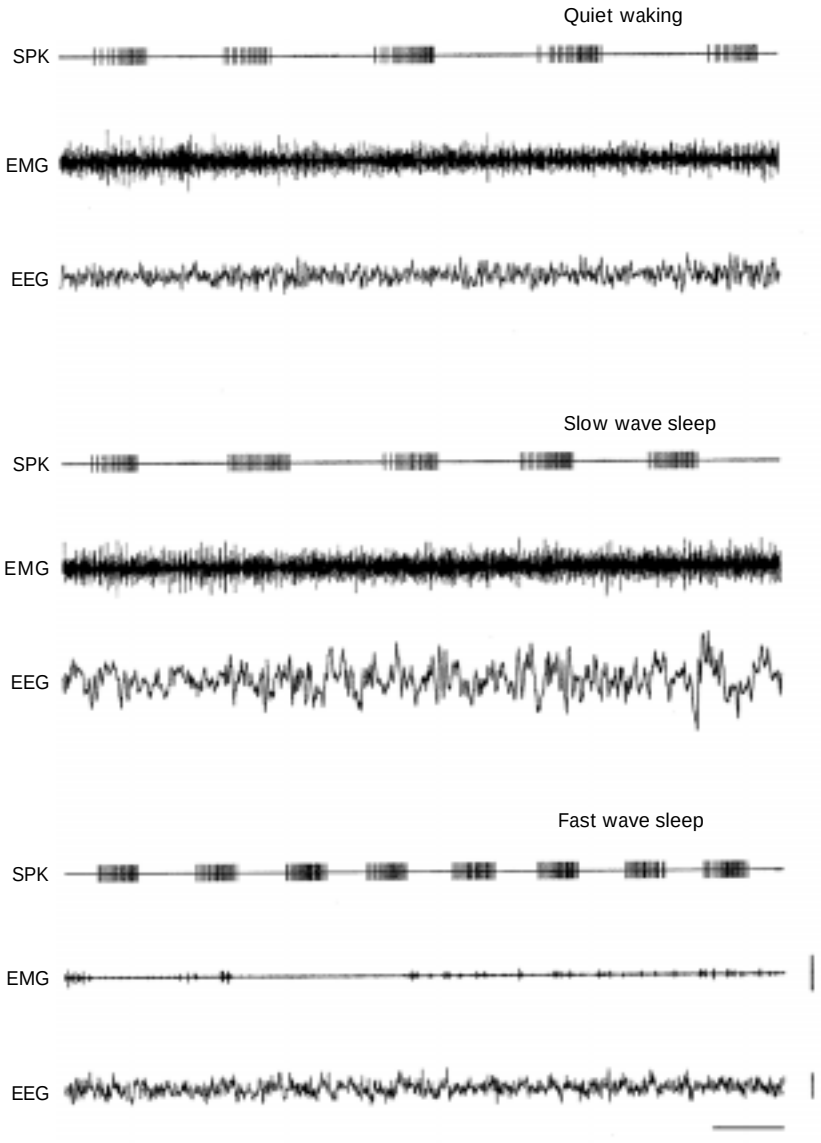

Figure 2. Cyclically changing activity during 10 s of quiet waking, slow wave sleep and fast wave sleep of a non-serotonergic neuron in the nucleus raphe pallidus. Bursts of activity occurred in synchrony with the inspiratory movements of the thoracic cage of the animal. SPK, neuronal activity; EMG, splenius electromyographic activity; EEG, parietal electrocorticographic activity. Vertical bars at bottom right represent 100 µV. Horizontal bar at bottom right represents 1 s.

Neuronal activity associated with masticatory muscle activity

Six serotonergic neurons and 43 non-serotonergic neurons were recorded during 60 s of drinking behavior. A tonic relationship between the activity of one of these serotonergic neurons and digastric muscle activity was found, as reported previously (31). This relationship was also observed for 5 (12%) non-serotonergic neurons. Activity was correlated only with digastric muscle activity in 2 of them ($r = +0.94$, $P = 0.005$; $r = -0.94$, $P = 0.005$), only with masseter muscle activity in 2 ($r = +0.93$, $P = 0.008$; $r = -0.94$, $P = 0.005$) and with both muscle activity in 1 ($r = +0.94$, $P = 0.005$, for the digastric muscle; $r = -1.00$, $P<0.001$, for the masseter muscle). The first 4 neurons were located in the NRP and the last one in the NRO. The first two neurons belonged to subtype A, the third and fourth to subtypes A and D, and the fifth to subtype A.

A phasic relationship was observed between the activity of 1 serotonergic neuron and the activity of the digastric muscle. The change in neuron activity preceded the change in muscle activity. The maximum cross-correlation coefficient and its lag time for this neuron are shown in Table 3. This neuron was located in the NRP.

A phasic relationship was also found between the activity of 21 (49%) non-seroto-

nergic neurons and digastric and/or masseter muscle activity. For a group of 11 neurons, the change in muscle activity preceded the change in neuron activity. The activity of 7 neurons was related to digastric muscle activity, the activity of 3 neurons was related to masseter muscle activity, and the activity of the remaining neuron was related to digastric muscle and masseter muscle activity. The lag time of the maximum cross-correlation coefficient was equal to or less than 10 ms for 2 cases involving the digastric muscle and 1 case involving the masseter muscle. For another group of 9 neurons, the change in neuron activity preceded the change in muscle activity (Figure 4). The activity of 3 neurons was related to digastric muscle activity, the activity of 5 neurons was related to masseter muscle activity, and the activity of the remaining neuron was related to digastric muscle and masseter muscle activity. The lag time of the maximum cross-correlation coefficient was less than 10 ms for 1 case involving the digastric muscle and 1 case involving the masseter muscle. For a last group represented by only one neuron, the change in masseter muscle activity preceded the change in neuron activity, which in turn preceded the change in digastric muscle activity. The activity of this neuron was related to the activity of these muscles. The median of the maximum cross-correlation coefficients for these groups of neurons and their respective median time lags are shown in Table 3. Six neurons in the first group were located in the NRP, 4 in the NRO, and 1 could be in either nucleus. Four neurons in the second group were located in the NRP, 3 in the NRO, and 2 could be in either nucleus. The neuron in the third group was located in the NRP. Three neurons in the first group belonged to subtype A, 3 to subtype B, 1 to subtype D and 1 to subtype E; the remaining 3 neurons did not have their subtype determined. Two neurons in the second group belonged to subtype A, 2 to subtype C and 2 to subtype G; the remaining 3 neurons belonged to subtypes D, E and F. The neuron in the third group belonged to subtype E.

There was some overlap between the population of neurons whose activity was related to respiration, the population of neurons whose activity was related to splenius muscle activity and the population of neurons whose activity was related to masticatory muscle activity. Of 21 neurons evalu-

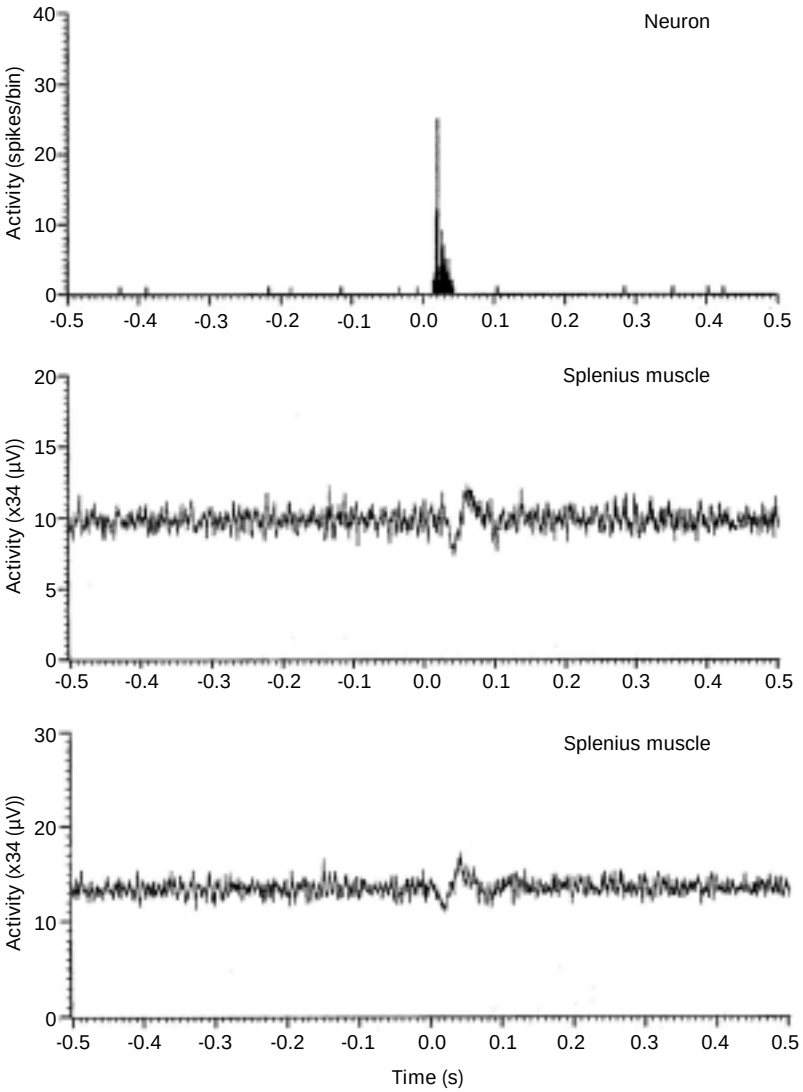

Figure 3. Peri-stimulus time histogram of the responses of a non-serotonergic neuron to the auditory stimulus (1-ms bin width) (upper panel). Corresponding auditory stimulus-triggered average of splenius electromyographic activity (1.5-ms bin width) (middle panel). Spike-triggered average of this same electromyographic activity (1.5-ms bin width) (bottom panel). One hundred auditory stimuli were presented. The neuron was located in the nucleus raphe obscurus.

ated for all three relationships, 6 had activity related to respiration and to masticatory muscle activity, 2 had activity related to splenius muscle activity and to masticatory muscle activity and 1 had activity related to respiration, to splenius muscle activity and to masticatory muscle activity.

Discussion

The present study examined the behavior of medium to large size neurons in the NRP and NRO in several physiological conditions. These may represent 80 to 90% of all neurons present in these nuclei (41). A few serotonergic neurons and many non-serotonergic neurons were characterized. There was not much difference between the behavior of the distinct serotonergic neurons. Non-serotonergic neurons, on the other hand, tended to behave very differently from one another.

Table 3. Median of the maximum neuron-muscle cross-correlation coefficients and median of their respective lag times (in ms).

	Digastric muscle				Masseter muscle			
	Direct		Inverse		Direct		Inverse	
	Lag	CC	Lag	CC	Lag	CC	Lag	CC
Ser (1)	-	-	27	-0.12	-	-	-	-
G1 (2)	-30	0.13	-	-	-	-	-	-
(5)	-	-	-25	-0.12	-	-	-	-
(1)	-	-	-	-	-24	0.11	-	-
(2)	-	-	-	-	-	-	-15	-0.13
(1)	-34	0.17	-	-	-	-	-20	-0.17
G2 (2)	38	0.22	-	-	-	-	-	-
(1)	-	-	4	-0.19	-	-	-	-
(4)	-	-	-	-	-34	0.13	-	-
(1)	-	-	-	-	-	-	46	-0.12
(1)	16	0.17	-	-	-	-	29	-0.18
G3 (1)	-	-	26	-0.11	-	-	-39	-0.11

Only significant values occurring between lag times of -50 ms and 50 ms were considered. Positive and negative coefficients indicate direct and inverse cross-correlations, respectively. Negative lag times indicate that a change in muscle activity preceded a change in neuron activity; positive lag times indicate that a change in neuron activity preceded a change in muscle activity. Ser., serotonergic neurons. G1, G2 and G3, groups of non-serotonergic neurons. Lag, lag time; CC, cross-correlation coefficient. The number of neurons is given in parentheses.

The firing rates of the serotonergic neurons changed in a characteristic way along the waking-sleep cycle. As previously reported by us (31) and by others (27,30), these neurons showed a small decrease in their discharge rate from QW to SWS and a large decrease from SWS to FWS. The non-serotonergic neurons exhibited ten different patterns of relative discharge rates across the three states (theoretically, 27 patterns would be possible). Five of these patterns were much more common than the others. These were, in decreasing order of occurrence: 1) a decrease in discharge rate from QW to SWS but an increase above the QW level from SWS to FWS, 2) an increase in discharge rate from QW to SWS and a much larger increase from SWS to FWS, 3) the same discharge rate from QW to SWS but a marked increase from SWS to FWS, 4) a decrease in discharge rate from QW to SWS but an increase below QW level from SWS to FWS, and 5) a decrease in discharge rate from QW to SWS but an increase to QW level from SWS to FWS. The first of these patterns was also observed by Heym et al. (27) for their non-serotonergic "motor-related" neurons in the NRP. It is interesting that the larger discharge during FWS as compared to SWS, characteristic of the five most common neuronal subtypes here, is also a pattern exhibited by about two-thirds of the medium to large size neurons in the medial medullary reticular formation, lateral to the raphe nuclei (42). This pattern is observed equally in the NRP and NRO as well as in the medial medullary reticular formation (and other medullary and pontine regions) during the FWS-like state induced by microinjection of cholinergic agonists into the rostral pontine tegmentum (43). This suggests that this pattern might have a common origin for all of these regions.

The variability of the activity of the serotonergic neurons during QW and SWS was very low (the regularity of the activity of these neurons during QW has been previ-

ously pointed out by Ribeiro-do-Valle (31)). It increased several times during FWS. Similar findings were reported by Heym et al. (27). Non-serotonergic neurons tended to exhibit a higher discharge variability than the serotonergic neurons for any particular state.

The patterns of activity observed across the waking-sleep cycle for the serotonergic and the non-serotonergic neurons in the NRP and NRO are compatible with the putative role of somatic motor output control of these nuclei. The net serotonergic influence on motoneurons and pre-motoneurons seems to be mainly facilitatory (44-48). The decreasing activity of the serotonergic neurons from QW to SWS to FWS might then have contributed to the general reduction in striated muscle activity that normally occurs across these states. Their regular discharge during QW and, to a certain extent, SWS would have been important for maintaining the basal activity level of striated muscles during these states (see Ref. 49). So far the nature of the non-serotonergic influence on motoneurons and pre-motoneurons does not seem to have been determined. The diversity of activity patterns found for the non-serotonergic neurons suggests that their action on any target is not uniform. Changes across states in the activity of at least part of these neurons may have been related to the postures adopted in each state. A role of NRP and NRO in posture maintenance is suggested by the strong otolithic organ input that they receive (50). Some neurons, that discharged much more during some periods of FWS than others, would have contributed to generating the muscle twitches that characterize the active phases of this state. Similar explanations were proposed by Siegel et al. (42) for the discharge of medial medullary reticular formation neurons in QW, SWS and FWS. One should also consider the possibility that some neurons in the NRP and NRO may have contributed to generating the muscle atonia of FWS, as proposed by Yamuy et al. (43) and others for neurons in the medial medullary reticular formation.

The activity of several of the recorded non-serotonergic neurons (but not of serotonergic neurons) was shown to be related to the respiratory movements of the animal. Neurons with the same behavior were described by Lindsey et al. (28) and Hosogai et al. (29) in the NRP and NRO of anesthetized or decerebrated cats, and by Heym et al. (27)

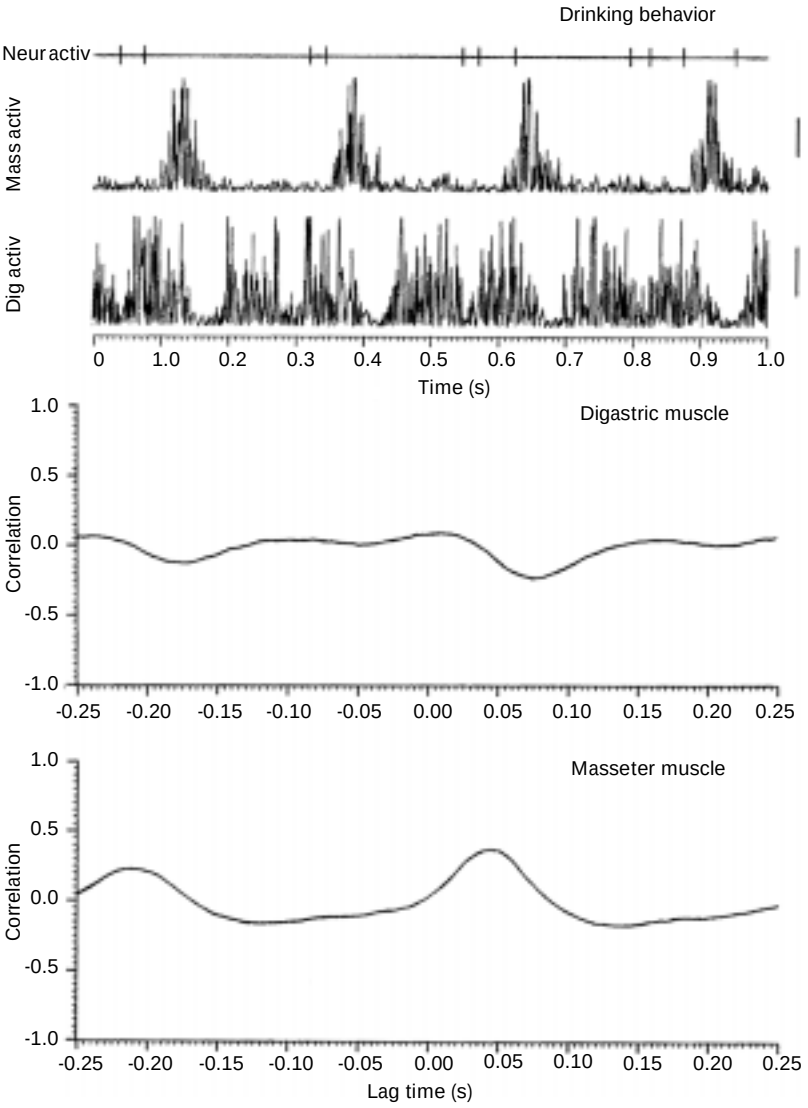

Figure 4. Activity of a non-serotonergic neuron located in the nucleus raphe obscurus and activity of the digastric and masseter muscles during 1 s of drinking behavior. Neur activ, neuronal activity; Mass activ, masseter electromyographic activity; Dig activ, digastric electromyographic activity. Vertical bars on the right represent 100 µV (upper panel). Cross-correlation between neuronal activity and digastric (middle panel) and masseter (bottom panel) muscle activities (1-ms bin width in each case).

in the NRP of behaving cats. Perhaps the most interesting finding here is that the relation to respiration of neuronal activity tends to depend on state. It can occur in one state and apparently disappear completely in another state. The activity of the respiration-related non-serotonergic neurons in the NRP and NRO would be determined in part by the rostral ventral respiratory group in the medulla and also by their companion raphe neurons (see Ref. 28). Those neurons would feedback the rostral ventral respiratory group and send signals directly to phrenic motoneurons, modulating the oscillatory activity of these targets. As the number of neurons showing oscillatory activity in the NRP and NRO decreases from QW to SWS to FWS and the neurons with this discharge pattern differ somewhat from one state to another, one can conclude that the modulatory influence of these nuclei on respiratory motor structures also changes across states. This would help to explain the ventilatory changes that occur along the waking-sleep cycle.

Responsiveness to sensory stimulation was very different for the serotonergic and the non-serotonergic neurons recorded here. Just one serotonergic neuron responded to the auditory stimulus and another one to the visual + auditory stimulus. Heym et al. (27) reported that of 11 serotonergic neurons tested in the NRP, 4 responded to repeated light flashes and 7 to repeated clicks (these authors did not use a visual + auditory stimulus). The intensity of their light flashes might be comparable to the one used here; however, the clicks were louder. The other stimulatory parameters were practically identical in the two studies. A major difference between the study of Heym et al. (27) and the present one is the much less stringent criterion for a response adopted in the former. This would have inflated the number of cells considered to be responsive. It is possible that some real responses were missed here (in fact the neuron that responded to the visual + auditory stimulus seemed, upon visual inspection, to have responded to the auditory stimulus too; the criterion, however, was not satisfied). It is very likely, on the other hand, that several false responses were unduly considered in that study (the peri-stimulus time histograms shown to demonstrate the occurrence of responses to visual and to auditory stimulation are hardly convincing).

In the present study, a few non-serotonergic neurons responded to visual stimulation. Responses to auditory stimulation and to visual + auditory stimulation were much more frequent. About two-thirds of the cells responded to the auditory stimulus and about half of the cells to the visual + auditory stimulus. In all three cases, excitation was the rule. All cells influenced by the visual stimulus were also influenced by the auditory stimulus. The fact that part of these cells did not respond to the visual + auditory stimulus and that several cells that responded to the auditory stimulus responded less (a decrease in magnitude of 30% or more) or did not respond to the visual + auditory stimulus indicates that the visual input could interact negatively with the auditory input. Less frequently, these inputs could interact positively as indicated by the fact that some cells that responded to the auditory stimulus did respond more (an increase in magnitude of 30% or more) to the visual + auditory stimulus. Similar visual and auditory responsive neurons were described by Meredith and Stein (51) in the stratum intermedium and stratum profundum of the superior colliculus. As observed here, positive and negative interactions between visual and auditory influences did occur. These cells were considered to be efferent. Their axons might contact cells in the caudal raphe nuclei (see Ref. 52). If this were the case, they could very well be involved in the determination of the behavior of the neurons recorded in the present study.

Also Yen and Blum (34) and Blair and Evans (53) observed responses of NRP and

NRO neurons to visual and auditory stimulation. Unfortunately the experimental procedures employed by these authors do not allow any straightforward comparison between their results and those obtained here. They did not determine the neurochemical nature of the recorded cells, their auditory stimulus was a hand clap, the visual stimulation rate was higher and the auditory stimulation rate lower than in the present study, and their animals were anesthetized. Blair and Evans (53) did not distinguish neurons localized in the NRP and NRO from neurons localized in the nucleus raphe magnus. What seems to be relevant in both investigations is the evidence that many visual and auditory responsive neurons project into the ventral quadrants of the spinal cord. The authors implicated these neurons in the control of somatic motoneurons.

We examined here the possibility that neurons in the NRP and NRO influence splenius muscle motoneurons directly or at most through one intermediary neuron. As demonstrated, this muscle very often reacts to strong auditory stimulation (presumably as a component of the startle behavior caused by the stimulation) that activates many non-serotonergic neurons in these nuclei. Some non-serotonergic neurons located in the NRO were shown to cause a short latency (<20 ms) response in the muscle, suggesting that they might control its motoneurons in a relatively direct way. Other non-serotonergic neurons may influence the splenius muscle more indirectly. Compatible with this idea is the observation that the overall median latency of neuronal responses to the auditory stimulus was approximately 12 ms shorter than that for the muscle response to this stimulus. Results obtained with the visual stimulus contrast to those obtained with the auditory stimulus. The visual stimulus was usually ineffective for activating the neurons and was usually ineffective too for activating the muscle. The present findings reinforce the hypothesis proposed by Blair and Evans (53) of an involvement of the caudal raphe nuclei in the motor expression of the startle behavior. They would share this role with the medullary reticular formation (see Ref. 54).

Painful stimulation did not affect the activity of the recorded serotonergic neurons. Several non-serotonergic neurons were inhibited and a few were excited by this stimulation. The discharge rate of two-thirds of these neurons during QW was lower than the median discharge rate of all non-serotonergic neurons tested. There seem to be no directly comparable data in the literature. Yen and Blum (34) and Blair and Evans (53) described neurons that were influenced - usually excited - by painful stimuli. As mentioned above, however, there are some problems with the experiments of these authors. Dantas et al. (55) found neurons in the NRO that were excited (63% of their sample) or inhibited (15% of their sample) and neurons in the NRP that were excited (60% of their sample) by noxious stimulation. They noticed that responsive neurons had lower firing rates than nonresponsive neurons. The neurochemical characteristics of all these neurons were not identified. In addition, these neurons were tested under the influence of anesthesia. It is curious that results similar to those reported here were obtained by Auerbach et al. (56) for the nucleus raphe magnus. Their serotonergic neurons were activated by painful stimuli as they also were by any other arousing stimuli (in the present case no response was observed for the serotonergic neurons presumably because they would be already discharging at their maximum rate even before the ear was pinched due to the procedure of holding the subject by the scruff during testing), while non-serotonergic neurons were specifically activated by painful stimuli. Yen and Blum (34) and Blair and Evans (53) considered their skin pinching responsive neurons to participate in the modulation of sensory input, more specifically the noxious one, producing analgesia. Such a

role for the responsive neurons here cannot be completely excluded. One should consider, however, the alternative that these neurons were mainly involved in the control of the somatic (and/or visceral) responses to the noxious stimulus. The immobility of the subjects during the period of stimulation may have been partially related to the decreased discharge that most of them presented.

One of the most interesting findings of the present study was the demonstration of a possibly significant involvement of NRP and NRO neurons in the control of jaw movements. A gross tonic relation between the activities of the digastric and the masseter muscles and the activity of 6 of the 7 serotonergic neurons recorded in the NRP and NRO, and a fine tonic relation between the activity of the digastric muscle and the activity of one of the serotonergic neurons recorded in the NRP were described previously by Ribeiro-do-Valle (31). Further testing here for a phasic relationship between the activity of these muscles and the activity of these neurons demonstrated just one positive result. Veasey et al. (30) observed only a tonic relationship between orofacial movements during feeding and the activity of serotonergic neurons in the NRP. These findings suggest that the serotonergic influence of NRP and NRO would be mostly responsible for long-lasting changes in masticatory muscle activity. This would sustain an increased firing rate of the masticatory motoneurons (and pre-motoneurons) and an increased number of these neurons active, and, consequently, an augmented contraction force of the masticatory muscles during feeding.

The possibility of a gross tonic relationship between masticatory muscle activity and the activity of the recorded non-serotonergic neurons was not evaluated here. A fine tonic relationship was observed between digastric muscle activity and/or masseter muscle activity and the activity of some of these neurons during drinking behavior. This relationship could be positive or negative for any of the muscles.

We also observed a phasic relationship between digastric and/or masseter muscle activity and the activity of several non-serotonergic neurons. Maximal cross-correlation between one neuron and the digastric muscle and another neuron and the masseter muscle occurred at very short lag times (4 and 5 ms, respectively). These two neurons possibly afferented directly motoneurons innervating these muscles. This hypothesis is supported by anatomic evidence indicating the existence of direct projections from NRP and NRO to the trigeminal motor nucleus (1). Results obtained by Takatori et al. (57) suggested that NRP and NRO exerted mainly a direct inhibitory influence on masseter motoneurons. Nagase et al. (18) observed just a facilitatory influence of non-serotonergic (and serotonergic) neurons in NRP and NRO directly afferenting jaw-opener (mylohyoid) and jaw-closer (masseter) motoneurons. The cross-correlation involving the digastric muscle here was negative and that involving the masseter muscle was positive. All of these findings suggest the existence of both a positive and negative direct influence of NRP and NRO neurons on jaw-opener and jaw-closer motoneurons.

The other 7 non-serotonergic neurons that showed a maximum cross-correlation with the digastric and/or the masseter muscle at lag times longer than 10 ms (but lower than 50 ms) may indirectly influence trigeminal motoneurons. In the medial medullary reticular formation there are many neurons that act directly and indirectly upon the trigeminal motoneurons. Part of these neurons were shown to excite digastric motoneurons and inhibit masseter motoneurons, and others to excite masseter motoneurons (58-60). As this region receives projections from both the NRP and NRO (9), one wonders whether these neurons may be mediating at least part of the putative long latency influence of NRP and NRO upon trigeminal motoneurons.

The activity of a group of non-serotonergic neurons here may have been influenced by the activity of the masticatory muscles. This group of neurons was completely separated from the one mentioned before that may have influenced the activity of these muscles. The functional role of this group of neurons is not clear at this time. The activity of a third group of only one neuron may have been influenced by the activity of one masticatory muscle and its activity may have influenced the activity of the other masticatory muscle. This behavior, so rare, is what one would expect for most masticatory muscle-related neurons.

This seems to be the first evidence supporting an involvement of NRP and NRO non-serotonergic neurons in the control of masticatory muscle activity in physiological conditions. It complements the evidence presented earlier of a similar role for NRP and NRO serotonergic neurons (31). These observations help to explain the increased activity observed by Veasey et al. (30) in NRP and NRO neurons during feeding.

Veasey et al. (30) reported some overlap between the population of serotonergic neurons related to respiration and that related to feeding. Similarly, several non-serotonergic neurons recorded in the present study were related to more than one activity. These findings are in accordance with anatomical observations that individual NRP or NRO neurons can project to multiple motor nuclei (4). All of this suggests a potential strongly coupled modulation by NRP and NRO of the activity of their effectors. Without doubt this would be interesting for the organism in many physiological situations. During drinking behavior in cats, for example, it would favor the necessary integration of respiratory and jaw-tongue-pharynx movements.

It is noteworthy that neurons recorded in the NRP and neurons recorded in the NRO behaved very similarly under all tested conditions. This implies that these nuclei share at least part of their functions.

The main results of the present study were interpreted within the framework of the hypothesis that NRP and NRO significantly contribute to somatic motor control. While observed changes in neuronal activity across states and responsiveness to sensory stimulation are not unambiguous, the relationship of neuronal activity with respiratory movements and especially neuronal activity relationship with splenius muscle activity during auditory stimulation and with masticatory muscle activity during drinking behavior strongly indicate such a role. The neuronal activity changes across states and in response to sensory stimuli could also be related to visceral (e.g., cardiovascular) activity. Appropriate experiments should be done in the future to examine this possibility.

The NRP and NRO may influence and cooperate with the nearby medial medullary reticular formation (gigantocellular reticular nucleus and ventral gigantocellular reticular nucleus). This region presents connections very similar to those of the NRP and NRO and is afferented by them (see Refs. 3,9) and, as seen, its neurons exhibit many properties in common with neurons in these nuclei. Further research is needed in an attempt to clarify to what extent the contribution of the NRP and NRO to somatic motor control (and to visceral motor and secretory control) differs from that of the medial medullary reticular formation.

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