Behavioral/Systems/Cognitive

On the Role of the Pontine Brainstem in Vocal Pattern Generation: A Telemetric Single-Unit Recording Study in the Squirrel Monkey

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In a recent study, we localized a discrete area in the ventrolateral pontine brainstem of squirrel monkeys, which seems to play a role in vocal pattern generation of frequency-modulated vocalizations. The present study compares the neuronal activity of this area with that of three motoneuron pools involved in phonation, namely the trigeminal motor nucleus, facial nucleus, and nucleus ambiguus. The experiments were performed in freely moving squirrel monkeys (*Saimiri sciureus*) during spontaneous vocal communication, using a telemetric single-unit recording technique. We found vocalization-related activity in all motoneuron pools recorded. Each of them, however, showed a specific profile of activity properties with respect to call types uttered, syllable structure, and pre-onset time. Different activity profiles were also found for neurons showing purely vocalization-correlated activity, vocalization- and mastication-correlated activity, and vocalization- and respiration-correlated activity. By comparing the activity properties of the proposed vocal pattern generator with the three motoneuron pools, we show that the pontine vocalization area is, in fact, able to control each of the three motoneuron pools during frequency-modulated vocalizations. The present study thus supports the existence of a vocal pattern generator for frequency-modulated call types in the ventrolateral pontine brainstem.

Key words: **reticular formation; phonatory motor nuclei; telemetry; vocalization; vocal-motor control; vocal pattern generator**

Introduction

The ventrolateral pontine brainstem seems to play a crucial role in motor control of mammalian vocalization: it gets input from the periaqueductal gray of the midbrain, an area indispensable for vocalization initiation (Jürgens and Pratt, 1979; Cameron et al., 1995; Odeh and Antal, 2001; Hannig and Jürgens, 2006); it has connections to all cranial motoneuron pools involved in phonation (Thoms and Jürgens, 1987; Li et al., 1993a,b; Hannig and Jürgens, 2006); stimulation of the ventrolateral pontine brainstem yields vocalization (Jürgens and Ploog, 1970; Jürgens and Richter, 1986; De Lanerolle, 1990; Behrend and Schuller, 2000); and blocking of its excitatory neurotransmission eliminates specific frequency-modulated (FM) vocalizations electrically elicitable from the periaqueductal gray (Jürgens, 2000). In a recent study in the squirrel monkey, we showed that a discrete area exists in the ventrolateral pontine brainstem directly dorsal to the superior olivary complex (VOC region), which behaves like a vocal pattern generator for FM vocalizations: its neurons increase their activity exclusively just before and during frequency-modulated vocalizations; none of these neurons changes its activity during non-frequency-modulated (nonFM) vocalizations, mastication, or quiet respiration. In >80% of vocal-motor neurons in VOC, firing was related to call patterns (Hage and Jürgens, 2006). The question of how VOC controls cranial motoneuron pools involved in FM vocalizations was left open in that study.

In the present study, we compared neuronal activities of vocalization-correlated neurons (VM neurons) from VOC with those of cranial motoneuron pools. We wanted to find out whether there are relationships in the neuronal activity between these regions, supporting the specific role of VOC in vocal pattern generation. We, therefore, explored the ventrolateral pontine and medullary brainstem, including the motor trigeminal nucleus (MV), facial nucleus (NVII), and nucleus ambiguus (NA), three important cranial phonatory motoneuron pools, for vocalization-related neuronal activity. We used a telemetric single-unit recording technique that allows simultaneous recording of extracellular single-unit activity and spontaneously uttered vocalizations in freely moving squirrel monkeys (Grohrock et al., 1997; Jürgens and Hage, 2006).

In the following, we will describe highly specific vocalization-correlated neuronal activities for each cranial motoneuron pool, underlining their different roles in vocal production. By comparing the activity properties between VM neurons of VOC and the three cranial motoneuron pools, we can show that VOC, in principle, is able to control the cranial motoneuron pools during the production of trill vocalizations and, therefore, could serve as a vocal pattern generator for frequency-modulated calls.

Materials and Methods

Animals and anesthesia. The study was performed in three male squirrel monkeys (*Saimiri sciureus*), aged 3 years at the beginning of the experi-

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ments. Surgical anesthesia was induced with ketamine (30 mg/kg body weight) and xylazine (6 mg/kg) intramuscularly; a prolonged anesthetic state was maintained by repeated injections of ketamine and xylazine (one-half and one-quarter of the initial dosages).

Platform and electrode implantation. The anesthetized animals were placed in a stereotaxic apparatus. The surgery was done under aseptic conditions. The skin over the frontal and parietal parts of the skull was removed, and a platform was implanted with the aid of four stainless steel screws, anchored in the skull with nuts, and embedded in dental acrylic (Paladur; Kulzer, Wehrheim, Germany). The platform consisted of a $30 \times 30 \times 4$ mm acrylic plate with an embedded grid of 12×12 stainless steel guiding tubes (outer diameter, 0.81 mm; inner diameter, 0.52 mm; center-to-center distance, 0.81 mm orthogonally, 1.13 mm diagonally) covering the stereotaxic coordinates of the brainstem according to the brain atlas of Emmers and Akert (1963). After fixation of the platform, the wound edges were adapted to the head mount, and the skin incisions rostral and caudal to the mount were sutured. Postoperative care consisted of analgesia with buprenorphine (0.003 mg/kg body weight) and antibiotic treatment. After 4 weeks of recovery, the animals were implanted with pairs of 80-μm-diameter quartz-insulated platinum/tungsten microelectrodes (Thomas Recording, Marburg, Germany) under ketamine/xylazine anesthesia (see above). The electrodes were inserted into the brain by the aid of a custom-made ultra-light microdrive fixed on the platform (Jürgens and Hage, 2006). They were running within stainless steel stabilizing tubes, which themselves ran within another pair of guiding tubes (outer diameter, 0.47 mm; inner diameter, 0.25 mm). The guiding tubes served as indifferent electrodes and ended just above the structures to be explored. The distance between the two electrodes of a pair was 1.13 mm. Finally, the electrodes were connected to the transmitters (see below), and a protection cap (Plexiglas) was fixed on the platform.

Telemetry. The neuronal activity picked up by the electrodes was amplified by a MOSFET preamplifier and fed into a custom-made transmitter circuit. The circuit was completely built with surface-mounted technology components and assembled on a single 20×28 mm printed circuit board. Transmitting coils and radiofrequency stages were mounted on one side of the circuit board, whereas the other side received the preamplifier together with the 3 V lithium battery [for details of the electronic circuitry and telemetry setup, see Grohrock et al. (1997)]. One of the two transmitters mounted on the platform had two channels. The first channel was used to record the neuronal activity, and the second transmitted the signal of a piezo-ceramic skull vibration sensor. This sensor picked up vibrations of the skull during vocalization. By comparing the signals coming from the room microphone and the skull vibration sensor, it was possible to distinguish vocalizations of the experimental animal from those of the group mates: vocalizations recorded synchronously with both skull vibration sensor and room microphone were uttered from the experimental animal, and vocalizations recorded only by the room microphone stemmed from the group mates. Carrier frequencies of the transmitters were between 100 and 150 MHz. The whole head stage, including platform, microdrive, amplifiers, transmitters, and protection cap, had a weight of 32 g (Jürgens and Hage, 2006). The recording sessions took place in a cage of 2.4 m height $\times$ 0.8 m length $\times$ 0.8 m width, in which the experimental animal was housed with one or two other animals. Two additional groups of three squirrel monkeys each were held in separate cages in the same room with visual, acoustic, and restricted tactile contact with the experimental animal. The room was lined with foam mats to reduce reflections.

The transmitter signals were picked up by two interconnected, orthogonally arranged antennae (length, 2.4 and 1.2 m) within the animals' cage. The antenna signals were preamplified (PA-21; Conrad, Hirschau, Germany) and sent via a coax-cable to three receivers (VR-5000; Yaesu, Cypress, CA). After demodulation, the telemetric signals were sent to a four-channel video recorder (BR-S611E; JVC, Friedberg, Germany) for long-term storage and to a personal computer (Pentium IV, 2 GHz) via an analog/digital interface (Micro 1401 mkII; Cambridge Electronics Design, Cambridge, UK) for data analysis. The video recorder stored an additional signal from a video camera installed in the animal room. This signal was also displayed on a monitor (TC-1470Y; Panasonic, Hamburg, Germany) and served for continuous observation of the experimental animals. Furthermore, the output of a microphone (ME 64 + K6; Sennheiser, Wedemark, Germany) placed in the animal room was sent via a microphone preamplifier (Audio Buddy; M-Audio, Oehringen, Germany), an audio amplifier (NL 120; Digitimer, Hertfordshire, UK), and a high-pass filter (cutoff frequency 300 Hz; NL 125; Digitimer) to the video recorder and via the Cambridge Electronics Design analog/digital interface to the personal computer.

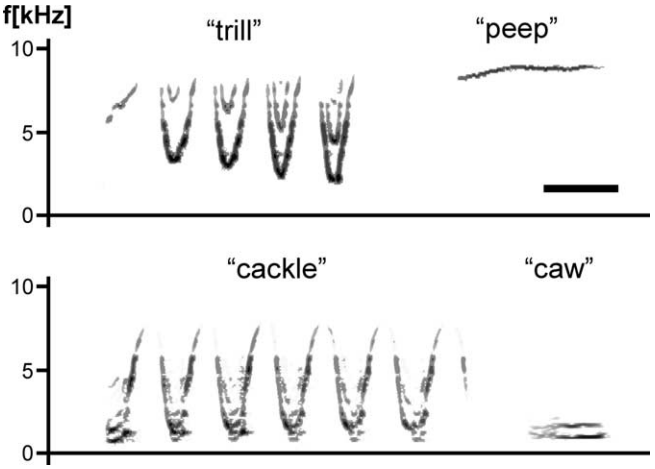

Figure 1. Examples of spontaneously uttered trill, cackle, peep, and caw vocalizations of the squirrel monkey. Sonograms as calculated from vocalizations recorded with the bone vibration sensor are shown. Intensity is represented by gray level. *x*-axis, Time. Calibration bar, 100 ms.

Recording procedure. Recording sessions lasted 10 min and were held twice a day during feeding time, because the occurrence rate of vocalizations was the highest during that time. Before each session, the experimental animal was caught and placed in a monkey chair. There, the electrodes were moved into a new position and batteries were exchanged, if necessary. The distance between successive recording positions was 50 or 100 μm, depending on the recording site. After the electrodes had been advanced to the new positions, the animal was brought to the observation cage and recording started. At the end of an electrode track, the electrodes were removed together with their guiding tubes, and a new pair of electrodes was implanted at a new position.

Neuronal activity was recorded during all call types uttered. Quantitative data analysis was done for two highly frequency-modulated call types with a rhythmical character (trill, cackle), a high-pitched (peep), and a low-pitched nonrhythmic call (caw). Examples of these call types are depicted in Figure 1. To test whether the recorded neurons showed a consistent auditory response, bursts of white noise, covering the complete hearing range of the squirrel monkey (0.3–32 kHz) (Pelleg-Toiba and Wollberg, 1989; Wienicke et al., 2001), were used as acoustic stimuli beside the animal's own vocalization and vocalizations from its group mates. Broadband noise (20 Hz to 200 kHz, 80 dB sound pressure level) was produced by a generator (Sine/Noise Generator Type 1049; Brüel and Kjær, Quickborn, Germany) and shaped by an electronic switch (Uni-Ulm-Elektronik, Ulm, Germany) into 300 ms bursts (including 10 ms rise and fall times) with 700 ms intertone intervals. The output signal of the generator was fed through a power amplifier (TA-F335R; Sony, Köln, Germany) to a dynamic speaker (Mariner 300; Mediacraft, Frankfurt, Germany) located in the animal room. The speaker covered frequencies between 65 Hz and 30 kHz and had a flat ± 3 dB response in a range of 1–20 kHz at the site of the animal's ear. Bursts were presented while the experimental animal was sitting quietly in a distance of 60–120 cm from the speaker. The sound pressure level of the white noise bursts was measured at a distance of 90 cm from the speaker (6.5 mm calibrated microphone 4135 and measuring amplifier 2231; Brüel and Kjær). White noise was used because almost all auditory neurons in the pontine brainstem show responses to this kind of stimuli (Tsuchitani and Boudreau, 1966; Tsuchitani, 1977; Caird and Klinke, 1983; Irvine and Jackson, 1983).

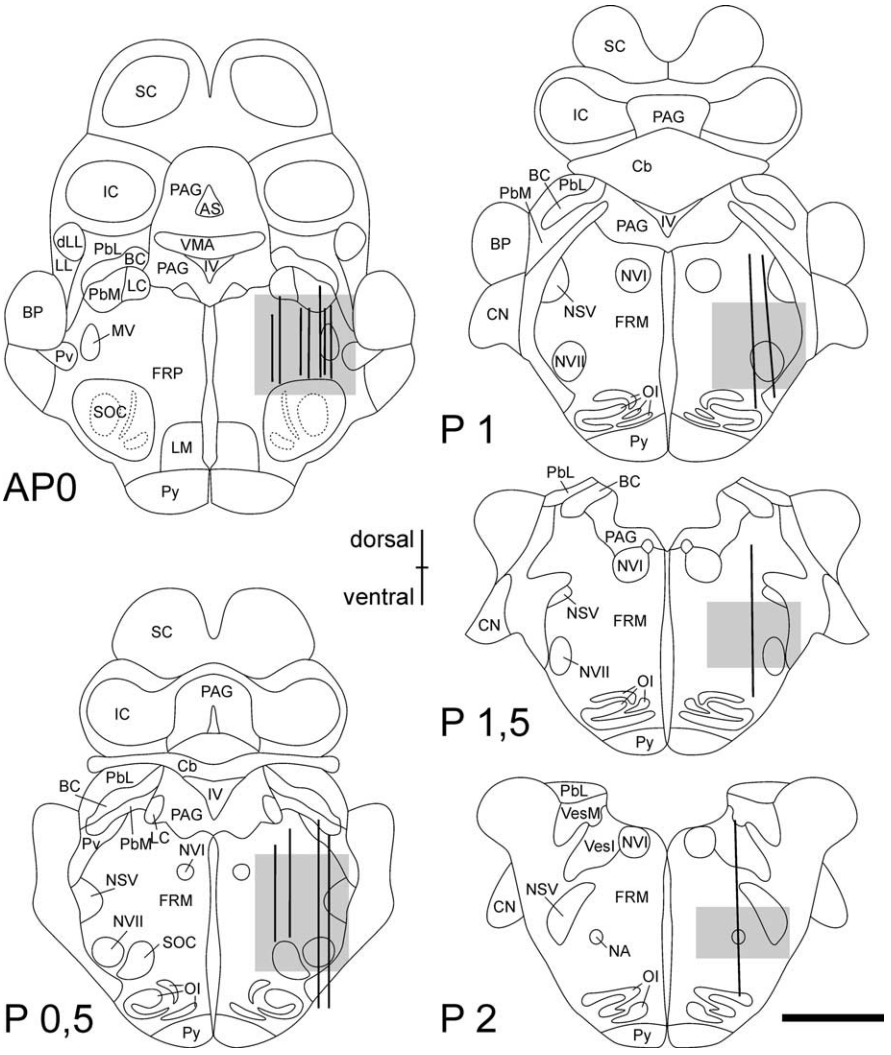

Figure 2. Frontal sections of the squirrel monkey's brainstem at the midbrain-pons transition (stereotaxic coordinates from Emmers and Akert, 1963), with vertical lines indicating the extent of the explored electrode tracks. Electrode tracks originally positioned on the left side were mirrored to the right for better overview. Highlighted squares indicate the ventrolateral brainstem shown enlarged in Figures 3, 6, and 9. AS, aquaeductus sylvii; BC, brachium conjunctivum; BP brachium pontis; Cb, cerebellum; CN, cochlear nucleus; dLL, dorsal nucleus of the lateral lemniscus; FRM, medullary reticular formation; FRP, pontine reticular formation; IC, inferior colliculus; IV, fourth ventricle; LC, locus ceruleus; LL, lateral lemniscus; LM, medial lemniscus; MA, group of neurons showing neuronal activity to vocalization and mastication; MV, motor trigeminal nucleus; NA, nucleus ambiguous; NSV, spinal trigeminal nucleus; NVI, nucleus abducens; NVII, facial nucleus; OI, inferior olive; PAG, periaqueductal gray; PbL, lateral parabrachial nucleus; PbM, medial parabrachial nucleus; Pv, nucleus principalis nervus trigemini; Py, pyramidal tract; SC, superior colliculus; SOC, superior olivary complex; VesI, inferior vestibular nucleus; VesM, medial vestibular nucleus; VOC, group of neurons showing neuronal activity exclusively to vocalization. Scale bar, 3 mm.

Histology. At the end of the experiments, the animals were killed with an overdose of pentobarbital sodium. They underwent perfusion with warm physiological saline, followed by 4% paraformaldehyde. The brains were removed and, after a postfixation time of 1 week in 4% paraformaldehyde followed by 20% sucrose for cryoprotection, were cut on a cryotome at 40 μm in the frontal plane. Every section was stained with cresyl violet. Every second section was counterstained immunohistologically against glial fibrillary acidic protein according to Benevento and McCleary (1992). With this technique, it was possible to identify the electrode positions even several months after removal of the electrodes. Histological evaluation was made using the stereotaxic atlas of Emmers and Akert (1963).

The experiments were approved by the animals ethics committee of the district government Braunschweig, Lower Saxony, Germany. The experiments were performed according to the principles regarding the care and use of animals adopted by the American Physiology Society, the Society for Neuroscience, and the specifications of the German Animal Welfare Law for the prevention of cruelty to animals. Care was taken to minimize the number of animals used and their suffering. All three experimental animals behaved normally to human caretakers and to other animals in the colony.

Data analysis. Data analysis was performed using the software Spike2, version 5 (Cambridge Electronics Design). First, the original recording has been submitted to a spike-sorting procedure (template-based spike-clustering) as reported previously (Hage et al., 2006). To avoid measuring the same unit at two consecutive positions, only the largest spike form recorded at a specific position was used for data analysis. For the identification of vocalization-correlated and auditory units, conventional perievent time histograms (PETHs) and peristimulus time histograms, respectively, were constructed. As a reference for the PETHs, the start of the vocalization was determined by examining the sonogram of the bone vibration sensor (see Fig. 4). In the same way, the end of the vocalization was determined.

Statistical analysis. The Pearson's correlation was used to examine the correlation between call duration and the duration of the corresponding neuronal activity. Because the number of measures per neuron differed, a correction of the correlation coefficients was done by calculating the Fisher's Z prime values.

To test for significant differences in neuronal firing between four brain areas, six variables were tested: activity type, timing of activity, excitation/inhibition, call-pattern correlation, call specificity, and pre-vocal onset time. Statistical analyses were performed for the syllable pre-onset times by a univariate variance analysis (general linear model) comparing general differences between all brain regions tested. When significant differences were found, *post hoc* tests with Bonferroni's were conducted to identify significant differences between single brain areas. A multivariate analysis of all six variables seems not to be adequate because some variables do not show a normal distribution and differ in measurement scale. For nonparametric variables, such as activity type, timing of activity, excitation/inhibition, call-pattern correlation, and call specificity, the χ^2 test was performed to find significant differences between the four brain areas tested, in general. After significant differences were found, the χ^2 or Fisher's exact test, respectively, was conducted *post hoc* to identify brain areas with significant differences in detail.

To test differences between two brain structures, e.g., when comparing NA and pre-ambiguus VM neurons, the χ^2 and Fisher's exact test, respectively, were used for the nonparametric variables; the Mann–Whitney U test was performed for the parametric variables. All tests were made with SPSS 12.0.1 (SPSS, Chicago, IL). Correlations and differences in distributions were considered significant if the probability of error was $<5\%$.

Results

A total of 519 positions in 15 electrode tracks were explored in the ventrolateral brainstem during self-produced vocalizations, feeding behavior, and auditory stimulus presentation in three squirrel

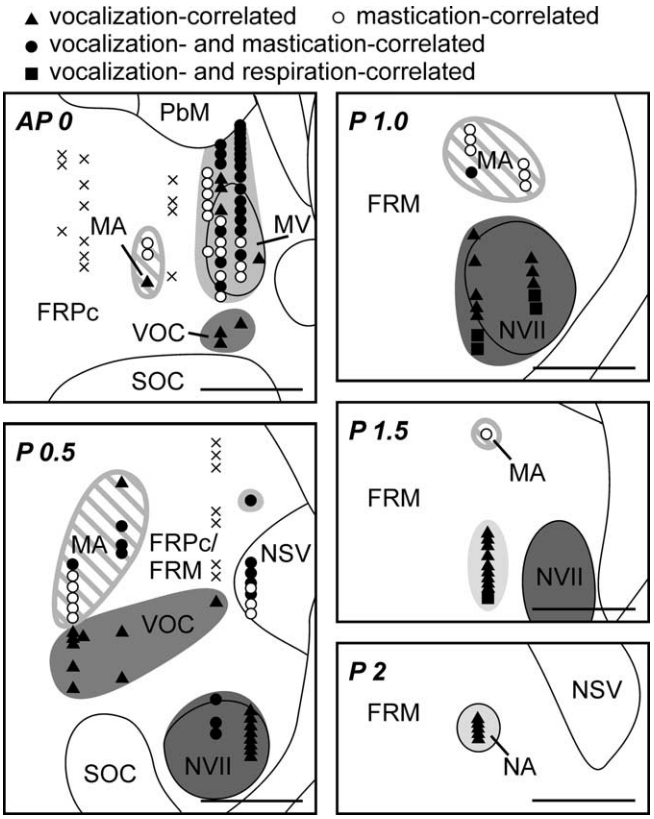

Figure 3. Frontal sections of the squirrel monkey's brainstem, showing the positions of different types of vocalization-correlated neurons (filled symbols), purely mastication-correlated (open circles), and auditory neurons (crosses) in various subdivisions. Purely vocalization-correlated neurons are indicated by filled triangles, vocalization/mastication-correlated neurons by filled circles, and vocalization/respiration-correlated neurons by filled squares. VM neurons were divided into various subdivisions. For additional explanation, see Results. For abbreviations, see Figure 2. Scale bars, 500 μm.

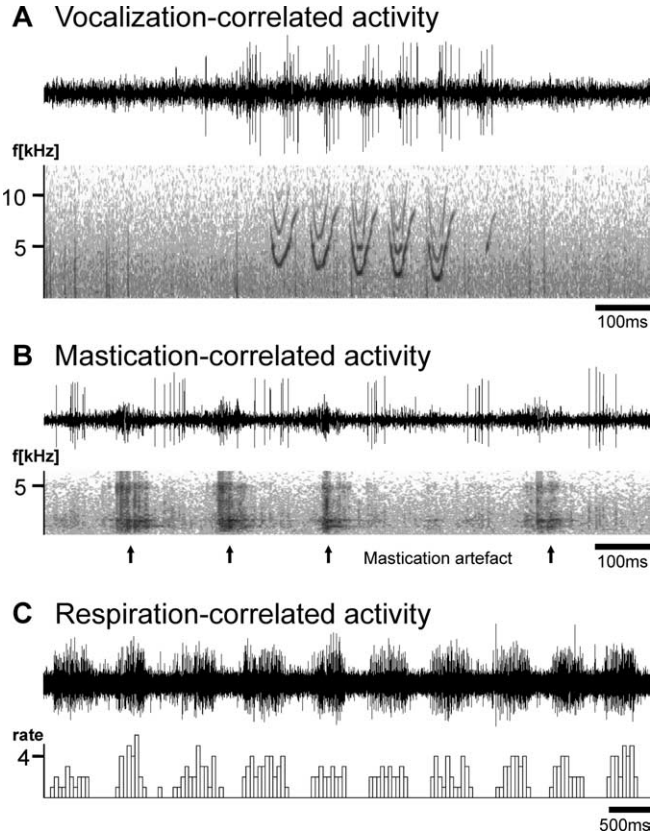

Figure 4. Examples of telemetrically recorded vocalization-correlated (***A***), mastication-correlated (***B***), and respiration-correlated (***C***) activity from the lateral brainstem. Top traces, Extracellular single-unit recordings. Bottom traces, Spectrographic representation of skull vibration signals showing a trill vocalization (***A***) and mastication artifacts (***B***). ***C***, Time–rate diagram of the single unit from the top trace (bin width, 50 ms).

monkeys (*S. sciureus*). The positions of the electrode tracks in the ventrolateral brainstem are shown in Figure 2.

Single neurons were isolated at 322 positions. Neurons were located in the MV, NVII, NA, spinal trigeminal nucleus (NSV), and the adjacent reticular formation. Of these, 88 neurons showed vocalization-correlated activity (VM neurons), 31 were active during mastication only, and 24 neurons responded to external auditory stimuli (Fig. 3). The anatomical position and physiological response of the auditory neurons are in agreement with previous studies (Irvine and Jackson, 1983). Auditory neurons were not interspersed with purely mastication-correlated or VM neurons in any of the electrode tracks (Fig. 3). Because this type of neuron is not the subject of this article, it will not be considered further. The remaining 159 neurons recorded were spontaneously active but showed no activity during vocalization, mastication, or quiet respiration and could not be driven by acoustical stimuli. These neurons were rarely interspersed in the clusters of VM neurons (<6%) and were mainly located in the marginal parts of the electrode tracks. This type of neuron is also not the subject of this article and will not be discussed here.

Vocal-motor activity in the ventrolateral brainstem

Quantitative analysis of VM neurons was conducted mainly with neuronal activities to trill vocalizations because trills were uttered at all but three positions at which VM neurons could be isolated (median number of trill calls per position, 19.2 ± 12.1; $n = 85$ positions). Quantitative analysis for caw, cackle, and peep vocalizations, because of their rarer occurrence, could be performed only at 48, 34, and 10 positions, respectively. The median number of caw calls per position was 10.5 ± 8.3, that of cackle calls 8.7 ± 4.1 per position, and that of peep calls 4.6 ± 2.1 per position. The VM neurons recorded were analyzed with respect to their neuronal firing properties (activity type, time of activity, excitation/inhibition, call-pattern correlation, call-type specificity, and pre-onset time).

Activity types

Most of the VM neurons were exclusively active during vocalization, that is, they did not change their activity during mastication, swallowing, or quiet respiration (48 of 88). Some neurons showed additional activity during mastication (35 of 88), and only a few (5 of 88) showed activity changes in the rhythm of quiet respiration (~1 Hz) (Häusler, 2000). None of the VM neurons reacted to auditory stimuli. Examples for neural activities correlated to vocalization, mastication, and quiet respiration are shown in Figure 4. The spatial distributions of different types of VM neurons are shown in Figure 3.

Time of activity

Almost 90% of the VM neurons showed a vocalization-correlated activity before and during vocalization (78 of 88). Only 11 neurons showed a different timing, such as activity only before (2 of 88), only during (3 of 88), only after (1 of 88), or before, during, and after vocalization (4 of 88). Examples of the most common activity types are shown in Figures 4*A*, 5, 7*A*, and 8.

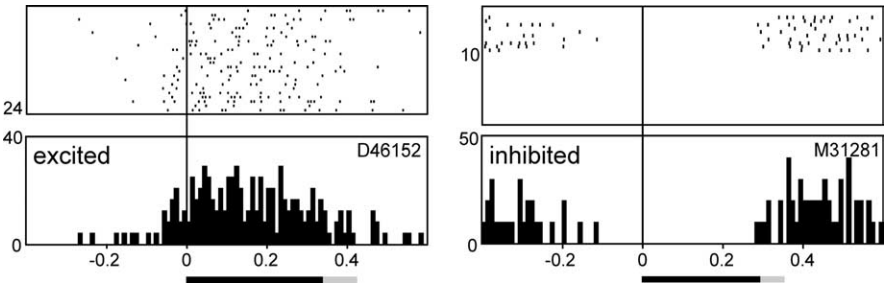

Figure 5. Trill-correlated activity of two VM neurons. Left, VM neuron showing a vocalization-correlated increase in neuronal activity (excited). Right, Neuron with a vocalization-correlated decrease in its activity (inhibited). Each panel shows as raster displays (top) rows of dots, each representing series of action potentials during self-produced vocalizations, and the summation of this activity in perievent time histograms (bottom). Black bars below the trill-related activity indicate the onset and mean duration of vocalizations; the gray bars indicate SD.

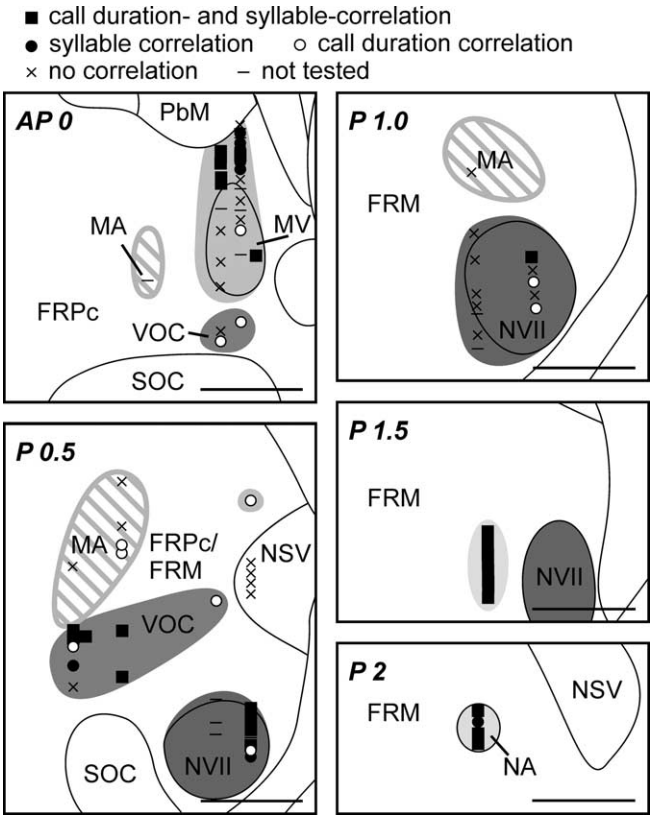

Figure 6. Frontal sections of the squirrel monkey's brainstem showing the positions of VM neurons classified according to their activity patterns during trill vocalizations into various subdivisions: call length- and syllable-correlated activity (filled squares), syllable-correlated activity only (filled circles), call length-correlated activity only (open circles), neither call-duration- nor syllable-correlated activity (crosses), and specific correlations could not be tested (bars). For abbreviations, see Figure 2. Scale bar, 500 μm.

Excitation/inhibition

Most VM neurons showed an increase in neuronal activity during vocalization (77 of 88); 11 neurons showed a decrease in their activity. Examples of both activity types are depicted in Figure 5.

Call-pattern correlation

Some VM neurons showed vocalization-correlated activity that reflected specific acoustic features of trill vocalization: in 40 of 78 VM neurons, activity changed in the rhythm of the syllable structure of the trill vocalizations (syllable correlation) (Fig. 6, filled symbols). Syllable-correlated activity was determined by examining the PETHs for syllable-synchronous neuronal activity changes (Fig. 7*A*). Syllable-synchronized PETHs were used for the measurement of syllable pre-onset times (Fig. 7*A*, *B*′). The median syllable pre-onset time was 32.8 ± 8.7 ms ($n = 40$). In 43 of 76 VM neurons, a significant correlation between the duration of vocalization and the duration of the corresponding neuronal activity was found (call-duration correlation) (Fig. 6, filled squares, open circles). Call-duration-correlated neurons had p values of <0.05 (30 of 43 had p values of <0.001; Pearson's correlation) and R values between 0.43 and 0.99 (median p value of 0.95, after correction with the Fisher's Z prime value). Thirty-two neurons showed both syllable- and duration-correlated activity (Fig. 6, filled squares). Examples of neurons showing duration correlation are shown in Figure 7*B*. For 15 neurons, syllable and/or duration correlation could not been tested because of small sample size (nine), low spontaneous activity (four), or the absence of trill vocalizations during recording time (three).

Call-type specificity

During the recording of 55 VM neurons, both frequency-modulated call types and non-frequency-modulated calls were uttered by the experimental animals. Thirty-one of these neurons were exclusively active during FM calls (trill, cackle), three neurons were exclusively active during nonFM calls (caw), and 21 were active during both FM and nonFM calls. Examples for call-specific neurons are depicted in Figure 8. The spatial distribution of call-specific neurons is shown in Figure 9.

Anatomical clusters of VM neurons

Using anatomical position, physiological data, and statistical criteria, VM and purely mastication-correlated neurons can be grouped into six clusters (Figs. 3, 6, 9; Table 1).

"NA" neurons

In the region of NA, some of the VM neurons were located in NA itself (Fig. 3, P 2), whereas others were located somewhat rostral to it, that is, medial to the caudal pole of NVII (Fig. 3, P 1.5, light gray). The latter group of neurons did not show any significant differences to those neurons recorded in NA in all parameters tested (for statistical details, see Table 2). Only syllable pre-onset times showed a strong tendency ($p = 0.053$) to be longer for the rostral group than for the NA neurons (see Table 2), suggesting that the rostral group represents pre-motoneurons of NA, an interpretation that is in harmony with Yajima et al. (1982). In the following, we will call the combined group of pre-ambiguus VM neurons and NA neurons proper "NA" neurons.

"NVII" neurons

As can be seen from Fig. 3 (P 0.5, P 1.0, dark gray), in addition to those neurons located within NVII itself, some VM neurons were found immediately adjacent to NVII. These neurons showed reaction characteristics that were also found within NVII. Accordingly, they will be classified in the following as "NVII" neurons.

"MV" neurons

Another group of neurons with rather uniform reaction characteristics was found in MV, dorsally bordering supratrigeminal nucleus and caudally bordering parvocellular reticular formation (Fig. 3, AP 0, P 0.5, medium gray). Statistical analysis revealed for

all but one parameter no statistical differences between MV neurons and those in the supratrigeminal nucleus and bordering reticular formation. Only the number of neurons with syllable-correlated activity was significantly higher in the supratrigeminal nucleus than in MV itself (for statistical details, see Table 2). Because the supratrigeminal nucleus and parvocellular reticular formation bordering MV are known to contain pre-motoneurons of MV (Mizuno et al., 1981, 1983; Li et al., 1995), this might explain the similarities in reaction characteristics found in the present study between MV and bordering reticular formation. In the following, we will call these VM neurons "MV" neurons.

NSV neurons

In NSV, another group of neurons ($n = 7$) could be found, all of which showed mastication-correlated activity. Approximately half of these neurons were also activated by vocalization (Fig. 3). Because of their anatomical position, the NSV neurons form a cluster clearly distinguishable from the other groups of VM neurons. NSV represents a sensory relay nucleus, receiving somatosensory information from the oral region (Altschuler et al., 1989).

VOC and MA neurons

In the pontomedullary reticular formation, another 19 VM neurons were found that could be divided into two groups with respect to their anatomical position. The first group of VM neurons ($n = 12$) was located directly dorsal to the superior olivary complex, containing exclusively purely vocalization-correlated neurons (VOC) (Fig. 3, AP 0, P 0.5). The second group was located dorsal to the VOC neurons and contained almost exclusively purely mastication-correlated and vocalization/mastication-correlated neurons (MA) (Fig. 3, AP 0–P 1.0). Besides the distinct anatomical position of MA and VOC neurons, significant differences were found in the distribution of several neuronal characteristics between these two groups supporting their division (for statistical details, see Table 2). MA neurons will not be discussed further in this study.

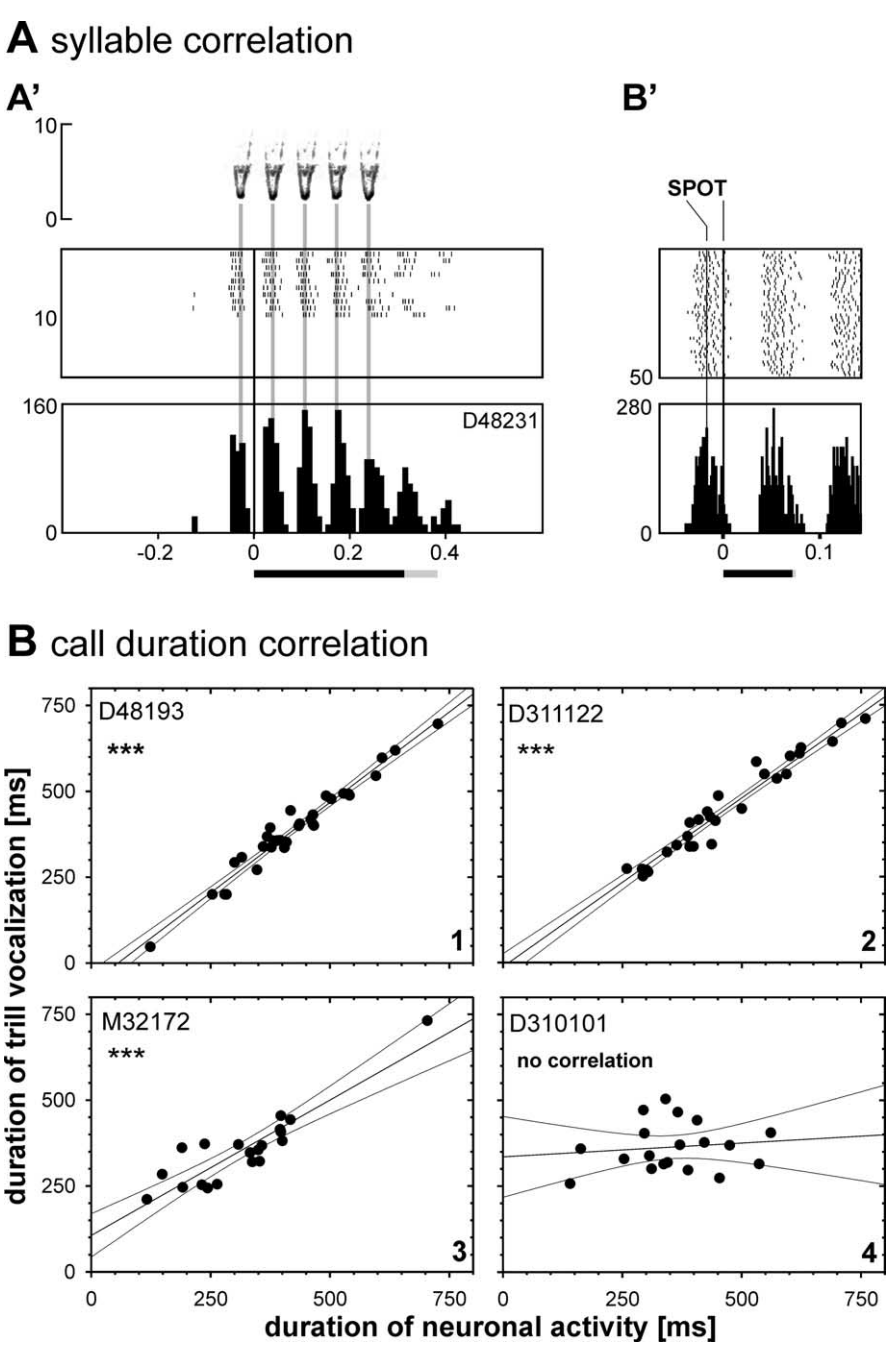

Figure 7. Examples for call-pattern correlation. ***A***, Syllable correlation. ***A′***, VM neuron showing a syllable-correlated activity to trill vocalizations. Gray vertical lines relate the maxima of neuronal activity to the corresponding syllables of a representative trill call. For additional explanation, see legend to Figure 4. ***B′***, Syllable-synchronized PETH of the same VM neuron as in ***A′***. The time between the onset of a syllable and the maximum of the corresponding neuronal activity is defined as syllable pre-onset time (SPOT). The black bar below the histogram indicates onset and mean duration of syllables; the gray bar indicates SD. ***B***, Examples of VM neurons showing an activity correlated significantly with the duration of trill vocalizations (1–3; Pearson's correlation, $^{***}p < 0.001$) and a neuron showing no correlation with call duration (4). Different durations are mainly attributable to different numbers of syllables in a call.

Comparison of neuronal activity in VOC and cranial motoneuron pools

As described before, VM neurons are found in four motor regions. These are MV ($n = 27$), NVII ($n = 23$), NA ($n = 15$), and VOC ($n = 12$). In the following, these regions were compared with respect to their neuronal characteristics described above.

Activity types

VM neurons showed significant differences in their activity types between the different brain structures (χ^2 test, $p < 0.001$; $n = 77$). Furthermore, *post hoc* χ^2 tests revealed that MV showed significant differences to all other brain structures tested (Fig. 10*A*). This was mainly attributable to the fact that a high number of vocalization/mastication-correlated neurons was found in MV. Such neurons were totally absent in NA and VOC and were rare in NVII. Additionally, MV was the only of the four areas in which purely mastication-correlated neurons were found (see Table 1). Furthermore NVII showed significant differ-

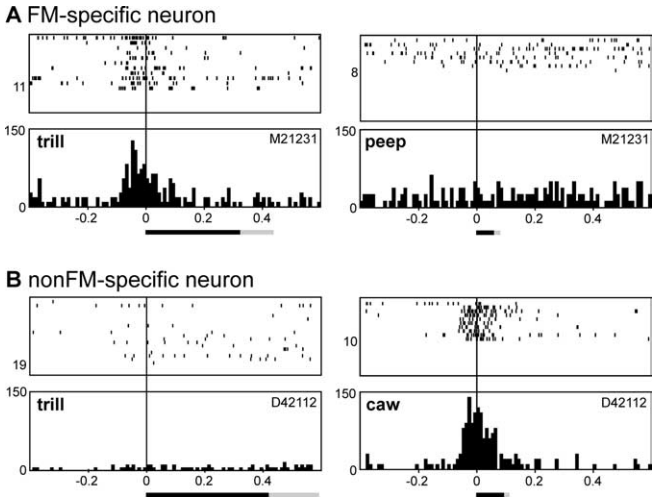

Figure 8. Perievent time histograms of call-specific VM neurons. ***A***, FM-specific neuron showing vocalization-correlated activity to trill calls but not to peeps. ***B***, nonFM-specific neuron with no activity change during trill calls but an increase of activity during caws. For additional explanation, see legend to Figure 4.

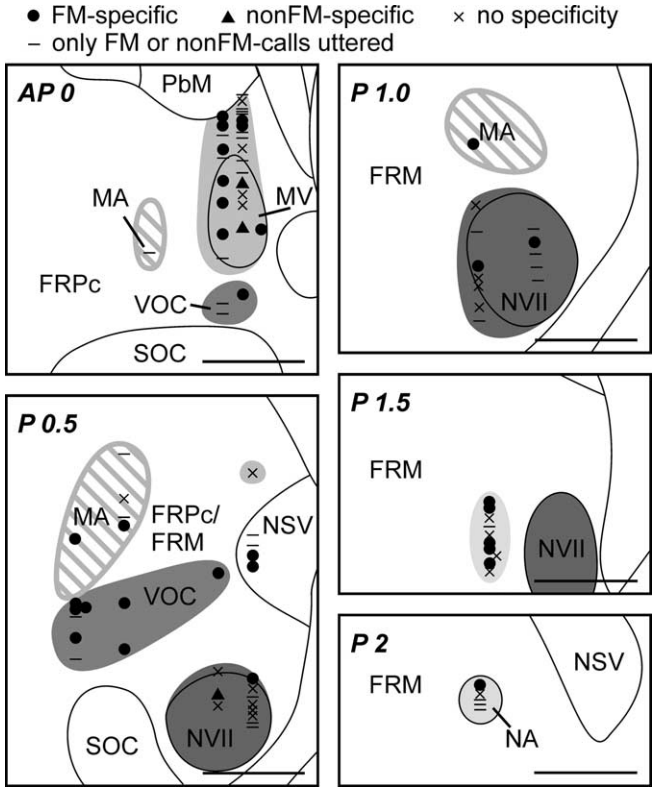

Figure 9. Frontal sections of the squirrel monkey's brainstem showing the distribution of VM neurons according to their call-type specificity in various subdivisions: FM-specific neurons (filled circles), nonFM-specific neurons (filled triangles), no call-type specificity (crosses), and only FM or nonFM calls, respectively, could be tested (bars). For abbreviations, see Figure 2. Scale bar, 500 μm.

ences to VOC, which were mainly attributable to the distribution of vocalization/mastication-correlated and vocalization/respiration-correlated neurons, which were lacking in VOC (*post hoc* χ^2 test) (Fig. 10*A*).

Time of activity

No significant differences were found between the four structures with respect to their time of vocalization-correlated neuronal activity (χ^2 test, $p = 0.85$; $n = 77$). This was attributable to the fact that almost all neurons recorded in the four brain areas showed a vocalization-correlated activity "before and during" vocalization. Only four neurons showed another type of timing, all of which were recorded in cranial motoneuron pools (MV, $n = 2$; NVII, $n = 1$; NA, $n = 1$), that is, none of them was found in VOC.

Excitation/inhibition

All VM neurons in NA and VOC and most neurons in MV and NVII showed a vocalization-correlated increase in neuronal activity (Fig. 10*B*). VM neurons showing a vocalization-correlated decrease in neuronal activity were found in MV ($n = 5$) and NVII ($n = 3$) only. No significant differences were found between the four structures with respect to excitation and inhibition (χ^2 test, $p = 0.16$; $n = 77$).

Call-pattern correlation

A total of 66 of 77 VM neurons could be tested for call-pattern-correlated activity (neurons showing syllable- and/or call-duration-correlated activity). Statistical analysis revealed significant differences in the distribution of call-pattern-correlated neurons in the four brain structures (χ^2 test, $p < 0.05$; $n = 66$) (Fig. 11*A*). *Post hoc* tests showed that these differences are attributable to statistical differences between NA, in which all VM neurons showed call-pattern-correlated activity, and MV and NVII, respectively, in which at least one of three neurons showed no correlation to syllable structure and call duration (Fisher's exact test). The significant differences also held if call-duration and syllable correlation were considered separately. VM neurons of VOC showed an intermediate distribution between MV and NVII on the one hand and NA on the other and revealed therefore no significant differences (*post hoc* Fisher's exact test) (Fig. 11*A*).

Call-type specificity

During the recording of 49 of 77 VM neurons in MV, NVII, NA, and VOC, the experimental animals were uttering FM as well as nonFM call types (Fig. 1). Also for the distribution of call-type-specific VM neurons, significant differences were found between the four brain structures (χ^2 test, $p < 0.05$; $n = 66$) (Fig. 11*B*). Here, *post hoc* tests revealed that these differences were attributable to the fact that all of the neurons located in VOC and being tested with FM as well as nonFM calls ($n = 8$) were exclusively active during FM calls (Fisher's exact and χ^2 tests) (Fig. 11*B*). The remaining four neurons of VOC, which could be tested only with FM calls, were all active during this call type. In contrast, approximately one-third of VM neurons in MV, almost 50% of neurons in NA, and even approximately two-thirds of neurons in NVII were active during both FM and nonFM calls (Fig. 11*B*). VM neurons showing preference to nonFM calls were very rare ($n = 3$) and were recorded in MV and NVII only.

Pre-onset time

A total of 40 of 77 VM neurons in MV, NVII, NA, and VOC showed syllable-correlated activity and thus had a specific syllable pre-onset time. A univariate variance analysis (general linear model) showed that there were significant differences for syllable pre-onset times between the four groups of VM neurons ($p < 0.001$; $n = 40$). All cranial motoneuron pools showed significant differences in their syllable pre-onset times among each other (*post hoc* test with Bonferroni's) (Fig. 12). The median syllable pre-onset time was longest in NVII (43.2 ± 3.5 ms), followed by MV (34.5 ± 4.0 ms) and NA (24.4 ± 4.8 ms). VM neurons in VOC had syllable pre-onset times being widely spread (between

26 and 47 ms; median, 37.9 ± 9.6 ms). A significant difference could be found only between VOC and NA (*post hoc* Bonferroni's) (Fig. 12). The distribution of syllable pre-onset times in the four brain areas is shown in Figure 12. Neuronal activity to caw calls was recorded in MV and NVII. As for syllable pre-onset times, caw pre-onset times of NVII neurons (51.5 ± 15.1 ms; $n = 7$) were significantly longer than those of MV neurons (20.7 ± 7.3 ms; $n = 10$), indicating that these motoneuron pools play a similar role in both call types with respect to vocalization-related activity onset (Mann–Whitney U test, $p < 0.001$; $n = 17$).

Table 1. Anatomical distribution of recorded neurons

	Vocalization-correlated neurons			
Σ ($n = 119$)	Purely	+ Mastication	+ Respiration	Mastication-correlated neurons
MV ($n = 41$)	4 (9.8%)	23 (56.1%)		14 (34.1%)
NVII ($n = 23$)	16 (69.6%)	3 (13.0%)	4 (17.4%)	
NA ($n = 15$)	14 (93.3%)		1 (6.7%)	
NSV ($n = 7$)		4 (57.2%)		3 (42.8%)
MA ($n = 21$)	2 (9.5%)	5 (23.8%)		14 (66.7%)
VOC ($n = 12$)	12 (100.0%)			

Table 2. Overview on the statistical analyses made for the verification of the grouping of NA with the pre-ambiguus neurons (X) and MV with the adjacent VM neurons (Y), as well as for the separation of VOC from MA

	p value			
VM neuron parameters	NA versus X	MV versus Y	VOC versus MA	Statistical test
Activity types	*a*	0.16	**0.002****	Fisher's exact
Time of activity	*a*	0.21	**0.009****	Fisher's exact
Excitation/inhibition	*a*	0.13	**0.04***	Fisher's exact
Call-pattern correlation	*a*	**0.04***	**0.03***	χ^2
Call-type specificity	*a*	*a*	0.3	Fisher's exact
Syllable pre-onset time	0.053	0.31	*b*	*U* test

[a]Could not be tested because all neurons showed the same characteristic.

[b]Could not be tested because no syllable-correlated neurons were recorded in MA.

Significant differences are highlighted in bold; *$p < 0.05$, **$p < 0.01$.

Discussion

The present study compared the neuronal activities in three cranial motor nuclei involved in phonation (MV, NVII, and NA) with that of an area in the parvocellular reticular formation (VOC) proposed recently to act as a vocal pattern generator for frequency-modulated vocalizations (Hage and Jürgens, 2006). The main findings are (1) that there are highly significant differences in the vocalization-correlated neuronal activities in MV, NVII, and NA according to their different roles in vocal production and (2) that the activity of area VOC is compatible with that of a vocal motor-coordinating center controlling directly MV, NVII, and NA.

Activity properties of MV neurons

The MV is known to control, among others, all jaw-closing and the majority of jaw-opening muscles (Matsuda et al., 1978; Mizuno et al., 1981). Because vocalizations often are accompanied by jaw movements, it is not surprising that vocalization-correlated activity has been found in MV in the present study. The fact that 85% of the VM neurons changed their activity not only during vocalization, but also during mastication, makes clear that these neurons are not vocalization specific but are "multipurpose" neurons, serving several functions. Despite their multipurpose nature, not all neurons showing a vocalization-correlated activity changed their activity during all call types. More than two-thirds of the VM neurons changed activity during either FM or nonFM calls but not during both. This call specificity probably is attributable to the fact that MV contains the motoneurons of quite a number of different jaw muscles that are involved in FM and nonFM calls in differential way (Kirzinger and Jürgens, 1994). A differentiation within MV is not only found with respect to call type but also with respect to activity type. Whereas some neurons increased their activity during vocalization, others decreased it, and still others changed their activity in the rhythm of frequency modulation (syllable-correlated neurons). These observations can be explained by the fact that MV contains motoneurons of antagonistic muscles. Jaw opening is associated with an activation of the jaw-opener motoneurons and inhibition of the jaw-closer motoneurons and vice versa (Nakamura et al., 1982, 1999). Accordingly, jaw opening during vocalization is to be expected to be accompanied by excitation in some MV neurons and inhibition in others. Those neurons showing alternating increases and decreases of activity in the rhythm of frequency modulation during one and the same call might be

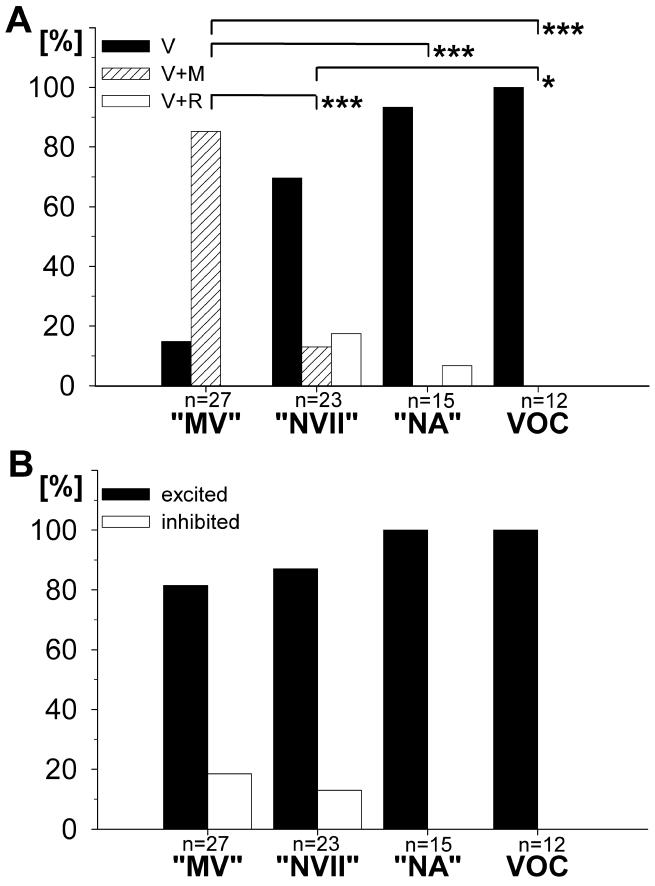

Figure 10. ***A***, Percentages of vocalization-correlated (V), vocalization/mastication-correlated (V + M), and vocalization/respiration-correlated (V + R) VM neurons in the areas MV, NVII, NA, and VOC. Statistically significant differences between the groups are indicated by asterisks (χ^2 test, *$p < 0.05$, ***$p < 0.001$). ***B***, Percentages of neurons showing excitatory or inhibitory vocalization-correlated activity changes in MV, NVII, NA, and VOC.

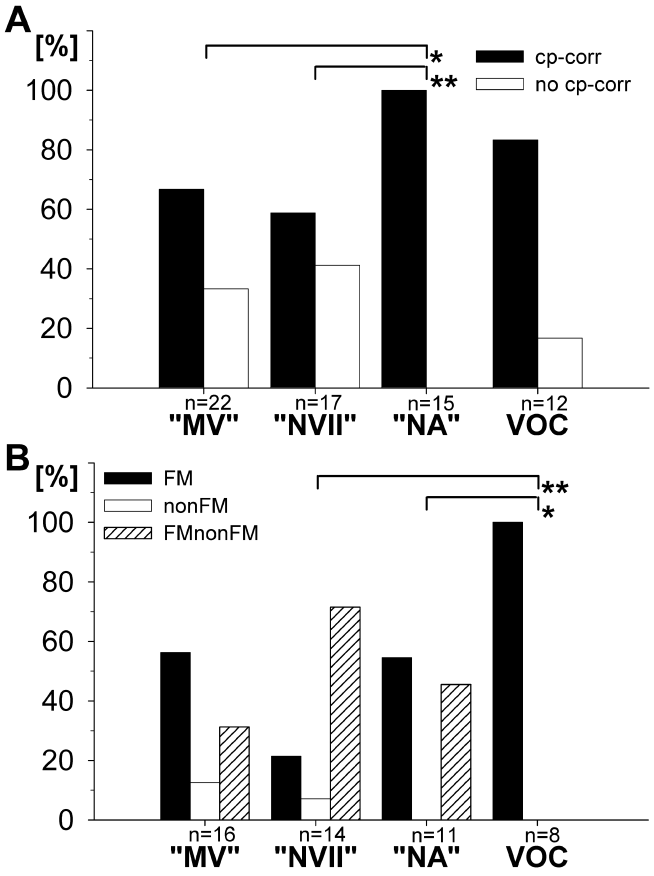

Figure 11. ***A***, Percentages of VM neurons with (cp-corr) and without (no cp-corr) call-pattern-correlated activity in MV, NVII, NA, and VOC. ***B***, Percentages of FM-specific neurons (FM), nonFM-specific neurons (nonFM), and neurons without call specificity (FMnonFM). Statistically significant differences between the groups are indicated by asterisks (Fisher's exact test/χ^2 test, $^*p < 0.05$, $^{**}p < 0.01$).

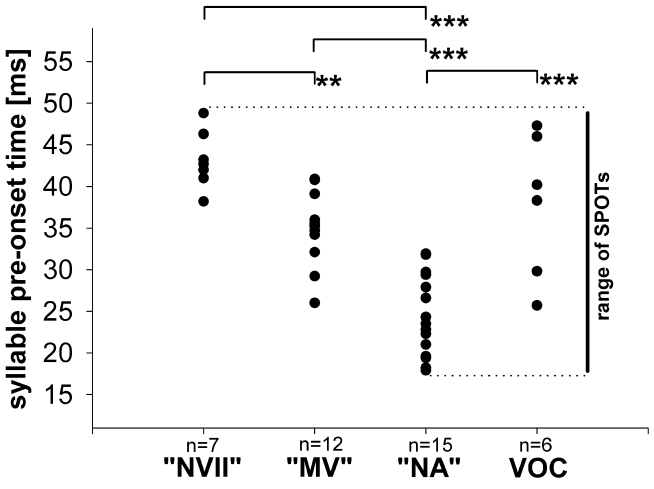

Figure 12. Distribution of syllable pre-onset times (SPOT) in NVII, MV, NA, and VOC. Each single dot stands for a syllable pre-onset time of one syllable-correlated VM neuron. Statistically significant differences between the groups are indicated by asterisks (*post hoc* Bonferroni's, $^{**}p < 0.01$, $^{***}p < 0.001$).

explained by the fact that jaw movements during vocalization serve to modulate the resonance frequencies of the vocal tract and that changes of fundamental frequency are associated with changes in vocal tract resonance frequencies.

Besides jaw-moving muscles, MV neurons, as well as NVII neurons, innervate middle ear muscles, which are also active during vocalization to protect the inner ear from damage caused by loud vocalizations [MV, musculus tensor tympani (Gannon and Eden, 1987); NVII, musculus stapedius (Thompson et al., 1985)]. In the present study, a possible role of the VM neurons of MV and NVII in the control of middle ear muscles could be excluded, because these muscles and therefore their motoneurons are also active during loud external acoustical stimuli (Suga and Jen, 1975); such audiovocal neurons, however, were not found in the present study.

Activity properties of NVII neurons

Vocalization-correlated activity was also found in the facial nucleus. In contrast to MV, NVII neurons only rarely were activated by mastication, in addition. This is in harmony with the innervation field of NVII, which comprises mainly the facial muscles and only to a very small degree jaw-moving muscles (Welt and Abbs, 1990; Horta-Junior et al., 2004). NVII, in the present study, contained a relatively large number of neurons (69.6%) that were vocalization specific in the sense that they did not change their activity during mastication, quiet respiration, or acoustic stimulation. Facial movements, like jaw movements, are a means to change the resonance frequencies of the vocal tract (Leanderson et al., 1971). The high number of VM neurons in NVII underlines the importance of articulation, that is, resonance frequency modulation, in monkey call production. In the squirrel monkey, a very common articulatory gesture is retracting of mouth corners during high-pitched vocalization (Marriott and Salzen, 1978). This gesture is controlled by NVII (Horta-Junior et al., 2004). Approximately two-thirds of VM neurons of NVII did not show any call specificity. This means that most of the motoneurons innervated muscles active during both FM and nonFM calls. A few of the neurons within NVII and its border were found to fire in the rhythm of quiet respiration. Such neurons were also reported by Zheng et al. (1998). The relative number of such neurons was similar in both studies (17% in the present study vs 11% in the Zheng et al. study).

Activity properties of NA neurons

The NA contains all motoneurons of the intrinsic laryngeal muscles (Hinrichsen and Ryan, 1981; Davis and Nail, 1984; Bieger and Hopkins, 1987). Accordingly, its activity is closely related to vocalization (Schuller and Suga, 1976; Yajima et al., 1982; West and Larson, 1993; Chiao et al., 1994). NA shows a partial somatotopic organization, with the cricothyroid motoneurons located rostral to the motoneurons of the remaining laryngeal muscles (Yoshida et al., 1985). In the present study, only the most rostral part of NA was explored, containing the cricothyroid motoneurons. The reason for this was that the focus of this study was on the neuronal activity underlying frequency-modulated calls, such as trills and cackles. The cricothyroid muscle has been shown in a previous study to be responsible for pitch control in the squirrel monkey (Jürgens et al., 1978). The cricothyroid muscle, however, remains inactive during the production of low-pitched nonFM calls, such as caw. This explains why we did not find vocalization-correlated activity during the production of caw calls but found activity during trill and cackle calls. Here, a tight relationship was found between NA activity and call structure of trill vocalization. All but one NA neuron active during vocalization showed a correlation between duration of trill calls and duration of neuronal activity; furthermore, all NA neurons showed activity changes synchronous to fundamental frequency changes (i.e., syllable

correlation). These findings suggest that the recorded VM neurons in NA were cricothyroid motoneurons. In contrast to MV and NVII, none of the NA neurons was activated by mastication.

Comparison of pre-onset times in the cranial motoneuron pools

The various cranial motoneuron pools tested differed significantly in their syllable pre-onset times of trill vocalizations and in pre-onset times of caw vocalizations (for MV and NVII). The longest latencies were found in NVII, the shortest in NA, with MV in between. This means that, in squirrel monkey vocalization, articulation starts before laryngeal (cricothyroid) activation and that, within articulation, facial muscles are activated before jaw muscles.

Comparison of cranial motoneuron pools with VOC

The area VOC in the parvocellular reticular formation of the caudal ventrolateral pons has been proposed in a recent study to act as a motor-coordination center for frequency-modulated calls in the squirrel monkey (Hage and Jürgens, 2006). The present study shows that, whereas the pre-onset times of MV, NVII, and NA differ highly significantly between each other, the pre-onset times of VOC neurons overlap almost completely with the pre-onset times of cranial motoneuron pools as a whole. The shortest VOC pre-onset times, however, are clearly above the shortest cranial motoneuron pools pre-onset times (Fig. 12). These findings support the notion that VOC is an area capable of coordinating the activity of MV, NVII, and NA during vocalization. Additional support comes from the fact that some activity characteristics being essential for the production of frequency-modulated calls showed significantly different distributions in between the cranial motoneuron pools but not when compared with VOC. In detail, whereas significant differences were found between NVII and MV on the one hand, in which at least one-third of VM neurons did not show any call-pattern correlation, and NA on the other hand, in which all VM neurons showed call-pattern correlation, no significant differences were found between VOC and the cranial motoneuron pools as a whole. This is solely possible because of an intermediate distribution of VOC in relation to NVII and MV on the one hand and NA on the other. All VM neurons in VOC were exclusively active during FM vocalizations; none of them changed activity during nonFM vocalizations, mastication, or quiet respiration (Hage and Jürgens, 2006). From previous studies, it is known that VOC gets a strong input from the periaqueductal gray (Jürgens and Pratt, 1979; Hannig and Jürgens, 2006) on the one hand and is connected directly with MV, NVII, and NA on the other hand (Thoms and Jürgens, 1987; Hannig and Jürgens, 2006). These anatomical findings, together with the electrophysiological findings of the present study, suggest that VOC exerts a coordinating, direct control on the various phonatory motoneuron pools during frequency-modulated but not during non-frequency-modulated vocalization. The latter might be coordinated in the reticular formation caudal to VOC because this region also has been shown to be connected with the various phonatory cranial motoneuron pools (Thoms and Jürgens, 1987; Cunningham and Sawchenko, 2000).

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