Midbrain Periaqueductal Gray and Vocal Patterning in a Teleost Fish

J. Matthew Kittelberger, Bruce R. Land, and Andrew H. Bass

Department of Neurobiology and Behavior, Cornell University, Ithaca, New York

Submitted 19 January 2006; accepted in final form 3 April 2006

Kittelberger, J. Matthew, Bruce R. Land, and Andrew H. Bass. Midbrain periaqueductal gray and vocal patterning in a teleost fish. J Neurophysiol 96: 71–85, 2006. First published April 5, 2006; doi:10.1152/jn.00067.2006. Midbrain structures, including the periaqueductal gray (PAG), are essential nodes in vertebrate motor circuits controlling a broad range of behaviors, from locomotion to complex social behaviors such as vocalization. Few single-unit recording studies, so far all in mammals, have investigated the PAG’s role in the temporal patterning of these behaviors. Midshipman fish use vocalization to signal social intent in territorial and courtship interactions. Evidence has implicated a region of their midbrain, located in a similar position as the mammalian PAG, in call production. Here, extracellular single-unit recordings of PAG neuronal activity were made during forebrain-evoked fictive vocalizations that mimic natural call types and reflect the rhythmic output of a known hindbrain–spinal pattern generator. The activity patterns of vocally active PAG neurons were mostly correlated with features related to fictive call initiation. However, spike trains in a subset of neurons predicted the duration of vocal output. Duration is the primary feature distinguishing call types used in different social contexts and these cells may play a role in directly establishing this temporal dimension of vocalization. Reversible, lidocaine inactivation experiments demonstrated the necessity of the midshipman PAG for fictive vocalization, whereas tract-tracing studies revealed the PAG’s connectivity to vocal motor centers in the fore- and hindbrain comparable to that in mammals. Together, these data support the hypotheses that the midbrain PAG of teleosts plays an essential role in vocalization and is convergent in both its functional and structural organization to the PAG of mammals.

INTRODUCTION

The midbrain periaqueductal gray (PAG) is proposed to play an essential role in the production of vocal communication signals across vertebrates, including humans (Esposito et al. 1999; Jurgens 1994; Kennedy 1975; Seller 1981; Wild 1997). Although inputs from forebrain limbic structures, as demonstrated in mammals, support the PAG’s role in coupling motivational state to vocal behavior (Bandler and Shipley 1994; Behbehani 1995; Holstege 1998; Jurgens 2002; Seward and Seward 2003), few studies show how PAG neuronal activity shapes the temporal parameters of vocal motor output. PAG activity is clearly necessary for call initiation (Behbehani 1995; Jurgens 2002). Single-unit recordings in vocalizing macaques show PAG activity correlated with temporal features (e.g., duration) of vocalizations (Larson 1991; Larson and Kistler 1986), implying a more direct role in vocal patterning than classically assumed (Davis et al. 1996; Jordan 1998; Jurgens 1994, 2002; Swanson 2000). However, similar recordings in squirrel monkeys do not reveal such correlations (Dusterhoft et al. 2000, 2004). Although these findings may represent species differences, the interpretation is currently complicated by an incomplete understanding of the relationship between ensemble muscle activity and the acoustic properties of primate vocalizations. This makes it difficult to establish the PAG’s exact role in vocal patterning. We therefore chose to examine this question in a species with a simple vocal repertoire and a more direct translation from neural activity pattern to muscle action and vocal output.

Midshipman fish (Porichthys notatus) depend on vocal communication for successful courtship and reproduction. Territorial males use sonic swim bladder muscles (Fig. 1A) to produce several call types, differing primarily in duration (Brantley and Bass 1994) (Fig. 1B). The sonic muscles are innervated by a hindbrain–spinal sonic motor nucleus (SMN) that receives input from nearby pacemaker neurons (PN) (Bass and Baker 1990) (Fig. 1C). The rhythmic output of the PN–SMN circuit, referred to as fictive vocalization, directly establishes muscle contraction rate and, in turn, call fundamental frequency (Bass and Baker 1990). A ventral medullary nucleus (VM) links the PN–SMN circuitry to a midbrain region similar in location to the mammalian PAG (Fig. 1C) (Bass et al. 1994; Goodson and Bass 2002). Electrical stimulation of this midbrain region elicits vocalization (Demski and Gerald 1972, 1974; Fine 1979; Goodson and Bass 2002). The simplicity of vocal motor production, specifically a one-to-one translation from the SMN spike train to acoustic properties of the call, make this an ideal system for identifying the role of PAG neurons in vocal initiation and/or patterning.

Here, we show that the activity patterns of a subset of PAG neurons are correlated with both call initiation and duration. In addition, direct manipulation of PAG activity by electrical microstimulation of descending PAG axons resulted in changes in call duration, further supporting a role for the PAG in temporal patterning. The necessity of PAG activity for vocal initiation is shown by PAG-specific, reversible lidocaine blockade. Focal neurobiotin injections confirm that the vocal motor connectivity of the midshipman PAG compares closely to that in mammals. Thus two groups of distantly related vertebrates, teleost fish and mammals, share many functional and structural similarities in the descending control of vocalization.

METHODS

Animals

Midshipman fish (Porichthys notatus) have two male reproductive morphs that differ in vocal and spawning behaviors (Bass 1996). All
experiments were performed on adult type I (territorial) males, which have the most dynamic vocal repertoire (Bass et al. 1999). Fish were collected from tidal pool nesting sites or by offshore trawls in northern California and Washington, shipped to Cornell University, and maintained in artificial seawater (ASW) tanks at about 15°C. All experimental procedures were approved by the Cornell University Institutional Animal Care and Use Committee.

**Surgeries**

Surgical procedures were similar to those described previously (Bass and Baker 1990; Goodson and Bass 2000c). Fish were anesthetized by immersion in 0.025% benzocaine (ethyl p-amino benzoate, Sigma, St. Louis, MO). Local anesthetic [0.2 ml of 0.25% bupivacaine (Abbott Labs, North Chicago, IL) with 0.01 mg/ml epinephrine (International Medication Systems, South El Monte, CA)] was then injected subdermally to the top of the head. The hypothalamus, midbrain, and hindbrain were exposed by dorsal craniotomy. Before transfer to the experimental apparatus, fish were immobilized with an intramuscular injection of pancuronium bromide (about 5 mg/kg, Baxter Healthcare, Deerfield, IL). For all experiments, fish were suspended in a parafilm sling in a Plexiglas tank with head stabilized, and ASW at about 15°C was perfused continuously across the gills. Exposed portions of the brain were kept covered with an inert, electrically conductive fluorocarbon (Fluorinert, 3M, St. Paul, MN). Fish were left to rest for about 1 h before starting electrophysiology experiments to allow all residual benzocaine to wash out of their system.

**Extracellular recordings**

**FICTIVE VOCALIZATIONS.** The vocal–motor output of the hindbrain vocal pattern generator—the “fictive vocalization”—was monitored with an extracellular electrode [75-μm diameter Teflon-coated silver wire (A-M Systems, Sequim, WA) with an exposed ball tip, 125–200 μm in diameter] placed on an occipital nerve root that carries the
PAG neurons. Extracellular recordings from PAG neurons were obtained using glass microelectrodes (6010, A-M Systems) pulled to a tip resistance of 10–15 MΩ on a Flaming/Brown micropipette puller (Model P-97, Sutter Instruments, Novato, CA) filled with 2 M NaCl. The electrode tip solution also contained 5% dextran tetramethylrhodamine (10,000 MW, D-1868, Molecular Probes, Eugene, OR) to label each recording site. Preamplified signals were amplified 1,000× total (Model NB-100 amplifier, Biomedical Engineering, Thornwood, NY; and an A-M Systems Model 1700 differential AC amplifier) and band-pass filtered from 0.3 to 10 kHz.

Forebrain stimulation of PAG neurons and fictive vocalizations

Rationale. Previous studies established the medial longitudinal fasciculus (MLF) of the midbrain as a site where low-amplitude stimulation evokes naturalistic vocalizations (Demske and Gerald 1972; Fine and Perini 1994). Thus our preliminary experiments focused on the MLF and showed that stimulation here elicited vocal responses that did not fatigue with repeated stimulation. These experiments facilitated the reliable localization and isolation of single PAG neurons with stimulus-modulated activity concurrent with stimulus-evoked fictive vocalizations. However, our ongoing anatomical studies showed the MLF to be the main descending pathway for PAG axons (see last section of RESULTS). Thus changes in PAG neuron firing evoked by MLF stimulation were likely antidromically mediated and therefore would not resemble the endogenous pattern of vocal-related PAG activity during spontaneous vocalization. Neuroanatomical and brain stimulation studies suggested that the ventral tuberal hypothalamus (vT) was a likely candidate for a source of endogenous PAG vocal activity patterns. Therefore, for the purposes of this paper, we present data from 48 PAG neurons recorded during vT stimulation. The results of the MLF stimulation experiments were used to analyze how descending midbrain output, inclusive of PAG axons, affects features of the vocal response (see below).

Procedures. Surface landmarks and micromanipulator coordinates were used to guide insulated tungsten stimulating electrodes (125-μm diameter, 8° tip angle, 5-MΩ impedance; A-M Systems) to sites in or near either vT or the MLF. Brief trains of stimuli (3–15 pulses, 1-ms pulse duration, 333-Hz repetition rate, 50–75 μA) were delivered using a WPI stimulus isolation unit (Model 850A, World Precision Instruments, Sarasota, FL), with stimulus timing parameters driven by Tucker-Davis System II software and hardware (Alachua, FL). The stimulating electrode was lowered into the brain until a stable reliable vocal output was obtained. The extracellular electrode was then positioned over the PAG and advanced into the brain using a Burleigh microdrive (Model LSS-1000, Burleigh Instruments, Fishers, NY). Once the electrode was roughly at the correct depth, we began searching for units whose activity was clearly modulated by the stimulus. The latency to the first poststimulus vocalization was then confirmed in our own anatomical studies (see RESULTS). Spontaneous vocal-related PAG activity during spontaneous vocalization. Neuroanatomical and therefore would not resemble the endogenous pattern of firing evoked by MLF stimulation were likely antidromically mediated.

Data analysis and statistics of PAG neuronal activity

Both PAG axons, and nerve root signals, digitized at 50 kHz, were acquired using a Tucker-Davis System II data acquisition system with Brainware v6.3 software (Tucker-Davis). Initial post hoc analyses of the extracellular and vocal nerve recordings were performed with Brainware v6.3 software (Tucker-Davis). The shapes of all action potentials were examined, and multiple units, when present, were defined based on differences in various parameters of spike shape (amplitude of each peak, interpulse interval). When all spikes were plotted in parameter space, distinct clusters were readily apparent. Visual inspection of voltage versus time plots of each cluster confirmed that all spikes within a cluster were from the same unit (see, e.g., Fig. 1D). The few spikes that did not conform to cluster boundaries were discarded. Of the 143 total neurons recorded, the majority of all recording sites were single units (112 of 127); of the 15 multunit sites, 14 revealed two clearly distinguishable units each, and one revealed three clear units. The 48 units recorded during vT stimulation represent 42 separate recording sites: 36 sites with only a single unit plus six sites each yielding two clearly distinguishable single units. The spike times for each unit, and for the coincident nerve recording of the vocal response pulses, were exported for further analysis.

Custom Matlab scripts [version 7.0.1 (R14), The MathWorks, Natick, MA] were used to compile, annotate, and analyze the spike records. For each unit confirmed by histology to be in the PAG (see following), we calculated (Table 1): 1) the mean spontaneous firing rate (from trials with no stimulus), 2) the mean number of stimulus-evoked spikes (over the first 800 ms poststimulus, reduced by the number of expected spontaneous spikes), 3) the mode of the interspike interval distribution, 4) the latency to the first poststimulus spike, and 5) the mean lag time between the first poststimulus unit spike and the first pulse of the vocal response. In addition, for each of these fish, we determined various parameters of the vocal response, including (Table 1): 1) the total mean number of pulses (over the first 800 ms poststimulus), 2) the mean number of pulses in the first vocal burst (a burst was defined as a series of pulses, each separated from the next by no more than 50 ms), 3) the latency to the first pulse of the vocal response, 4) the mean interpulse interval, and 5) the mean interburst interval. Poststimulus time histograms (PSTHs) for each unit and vocal response, perievent time histograms (PETHs) for each unit (centered on the time of the first vocal response pulse), and frequency distributions of interspike intervals (for each unit and vocal response) were plotted using Origin version 6.1 software (Origin Lab, Northampton, MA).

Statistical analyses, including tests for trial-by-trial correlations between the unit spike activity and the vocal response, were performed using JMP version 5.0.1a software (SAS Institute, Cary, NC). For each unit, we tested for three possible correlations: 1) between the total number of stimulus-evoked unit spikes and the total number of vocal response pulses, 2) between the net unit spikes and the latency of the vocal response, and 3) between the latency of the unit response and the latency of the vocal response. The total number of vocal response pulses per trial reflects the product of the probability of a vocal response × the duration (number of pulses) of each vocal pulse.
Most PAG neurons fired spontaneously at only a very low rate. Most of the single-unit data distributions were skewed toward the lower end of the range, as indicated by the differences between the medians and means. Note that the average modal interspike interval of PAG neurons poststimulus was much higher (about fourfold) than the interpulse interval of the vocal response. The properties of the fictive vocal response (pulses per burst, duration, interpulse interval, and interburst interval) were very similar to those of natural grunts (see Bass et al. 1999). All data presented here result from constant-duration (12-pulse) stimulation of VP.

**TABLE 1. Description of the firing properties of PAG neurons, both spontaneous and stimulus-evoked, and of the fictive vocal response**

<table>
<thead>
<tr>
<th>Property</th>
<th>Mean</th>
<th>SD</th>
<th>Range</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PAG single-unit data</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spontaneous activity rate, Hz</td>
<td>1.4</td>
<td>2.1</td>
<td>0–8.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Stimulation-evoked spikes, net</td>
<td>3.7</td>
<td>6</td>
<td>0.3–28.8</td>
<td>1.5</td>
</tr>
<tr>
<td>Interspike interval, mode, ms</td>
<td>44</td>
<td>44</td>
<td>7–145</td>
<td>21</td>
</tr>
<tr>
<td>Latency to first spike, ms</td>
<td>43</td>
<td>45</td>
<td>5–191</td>
<td>24</td>
</tr>
<tr>
<td>Lag first spike to vocal response, ms</td>
<td>−166</td>
<td>110</td>
<td>−350–85</td>
<td>−161</td>
</tr>
<tr>
<td><strong>Vocal response data</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Trials with vocal response</td>
<td>62</td>
<td>29</td>
<td>17–98</td>
<td>65</td>
</tr>
<tr>
<td>% Vocal response with &gt;1 burst</td>
<td>18</td>
<td>22</td>
<td>0–62</td>
<td>8</td>
</tr>
<tr>
<td>No. of pulses, first vocal burst</td>
<td>7.1</td>
<td>2</td>
<td>3.7–10.9</td>
<td>7.6</td>
</tr>
<tr>
<td>Duration, first vocal burst, ms</td>
<td>62</td>
<td>19</td>
<td>27–106</td>
<td>61</td>
</tr>
<tr>
<td>Latency to vocal onset, ms</td>
<td>201</td>
<td>76</td>
<td>92–317</td>
<td>216</td>
</tr>
<tr>
<td>Interpulse interval, ms</td>
<td>10.3</td>
<td>0.9</td>
<td>9.0–12.0</td>
<td>10.1</td>
</tr>
<tr>
<td>Interburst interval, ms</td>
<td>349</td>
<td>65</td>
<td>287–498</td>
<td>341</td>
</tr>
</tbody>
</table>

MLF stimulation of vocal output

We wanted to directly test whether altering the ensemble output of PAG neurons influenced specific parameters of the vocal response, including temporal features such as call duration and interpulse interval. To do so, we analyzed the properties of fictive vocal responses elicited by stimulation of the MLF (as described above). All stimulation sites were confirmed histologically to be in the MLF, through which PAG axons descend to connect with the hindbrain vocal circuit. We systematically altered the number of stimulation pulses (stimulus duration) and analyzed four features of the vocal response that might change in correlation with stimulus duration: 1) the duration of the stimulus-evoked vocal burst (number of vocal pulses), 2) the probability of eliciting a vocal response, 3) the latency to the vocal response, and 4) the mean interval between pulses within the vocal response. All four features were quantified at each stimulus duration. Using SAS version 9.1.3 statistical software (SAS Institute), we ran repeated-measures ANOVAs to determine whether there was a significant effect of stimulus duration on each of these four vocal parameters across experiments.

Lidocaine inactivation experiments

In these experiments, we sought to determine whether reversible inactivation of the PAG affected the stimulus-evoked vocal output. Surgeries, VP stimulation, and vocal nerve recording were as described above. Once a stable and reliable vocal response was obtained, we recorded the baseline response for ≥40 min (16–24 stimulus trials every 10 min). About 6–7 min after the final baseline recording, a glass micropipette containing 4% lidocaine hydrochloride (Sigma) and 4% fluorescent dextran conjugate (either fluorescein or tetramethylrhodamine, Molecular Probes) in 0.1 M phosphate-buffered saline (PBS) was stereotactically guided to PAG. Micropipettes were made as described above for extracellular recording electrodes, but the tips were broken back to an inner diameter of about 5–10 μm. Either lidocaine or control (vehicle plus dye only) solution was pressure-
ejected from the pipette using a picospritzer (Biomedical Engineering) set to deliver 1 pulse/s, 10- to 50-ms duration each, at 25–30 psi, for 30 s, for a total injection volume of about 0.5 nl. We continued to record the stimulus-evoked vocal responses for up to 1 h postinjection. PAG injections were bilateral, ipsilateral to the stimulus, or contralateral. In some fish, control injections were targeted to midbrain areas outside the PAG, including the torus and ventral tectum. In some cases post hoc analysis revealed that injections intended for the PAG had in fact missed; these injections were also treated as controls. Vehicle (PBS) only or sham injections to the PAG were used as additional controls. Typically, one experimental and one control injection were made in each fish, with ≥60 min between the two injections, allowing for complete recovery from any effect of the first injection on the vocal response. The locations of the injection sites were confirmed post hoc (see following text).

Post hoc analyses were similar to the extracellular single-unit analysis described above. Vocal response pulses were discriminated using Brainware and pulse times were exported for further analysis. Matlab scripts were used to compile and annotate the data and to calculate the mean number of vocal response pulses over the first 800 ms poststimulus for each time point pre- and postinjection. Data were graphed in Origin and statistical analysis was performed using JMP. Within each experiment, an injection was determined to have had a significant effect on the vocal response if: 1) there was a significant overall effect of time on the mean vocal response over the duration of the experiment and 2) a Tukey–Kramer test indicated that the vocal response at a minimum of at least one time point within the first 20 min postinjection was significantly less than at a minimum of at least two time points during the preinjection baseline. To analyze the data by treatment type, we first normalized the postinjection vocal response data to the mean preinjection baseline response for each experiment and then pooled these normalized data by treatment type. Within each treatment type, we performed a repeated-measures ANOVA across the experimental time points. Because the data were normalized to the preinjection baselines, a significant effect of time indicates a consistent postinjection effect within the group. In such cases we also determined which postinjection time points were different from pre-injection using a Tukey–Kramer test, with a significance level of 0.05.

**Tract-tracing experiments**

To characterize the connectivity, both antero- and retrograde, of neurons in the PAG, we made focal iontophoretic injections of 5% neurobiotin (Vector Labs, Burlingame, CA). Surgeries were as described above, except that only a small craniotomy was made over the midbrain on one side. Fish were transferred to the recording rig and glass micropipettes were stereotactically guided to the PAG. Micropettes were the same as those used as extracellular recording electrodes. Neurobiotin (in 2 M KCl) was iontophoresed for 5–10 min using +3-μA pulsed current (15 s on/15 s off). Only a single injection was made in each fish. After a 10-h survival time to allow transport of the tracer, fish were perfused and brains processed as described below.

**Histology**

At the end of all experiments, fish were deeply anesthetized (0.025% benzocaine) and perfused with ice-cold, teleost Ringer solution with 10 units/ml of heparin (Elkins-Sinn, Cherry Hill, NJ), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were removed, postfixed overnight, and transferred to 0.1 M PB (pH 7.2) for storage. Brains were equilibrated in 30% sucrose for 24 h and then sectioned transversely at 50 μm on a freezing microtome. For brains with fluorescent dye injections (fluorescein- or rhodamine-dextran, as in the recording and lidocaine inactivation experiments), sections were collected in 0.1 M PB, mounted immediately on gelatin-subbed slides, dried overnight, and coverslipped using a fluorescent mounting medium (Vectorshield, Vector Labs).

Neurobiotin injections, as used in the tract-tracing experiments, were visualized using a standard avidin–biotin–peroxidase protocol. Briefly, sections were collected in PBS, permeabilized for 30 min in PBS with 0.04% Triton-X100 (Sigma), incubated for 3 h at room temperature in avidin–biotin–peroxidase in PBS (Vectorstain ABC Elite, Vector Labs), rinsed twice in PB, reacted with 0.05% diaminobenzidine (Sigma) and 0.0072% H2O2 in PB, rinsed twice more in PB, and mounted on gelatin-subbed slides. Alternate sections were mounted on separate slides, dried overnight, cleared in xylene, and coverslipped. Later, one set of sections was stained with 0.5% cresyl violet (Sigma) to delineate the borders of the different cell clusters. Sections were examined and photomicrographs taken on a Nikon Eclipse E-800 microscope.

**RESULTS**

**Extracellular recording of vocal-related activity in PAG**

Extracellular recordings were made from a total of 143 isolated units in the midbrains of 53 type I male midshipman fish, while stimulating different sites to elicit vocal output (see METHODS). Of these, a total of 48 neurons were recorded from 23 fish while vocal responses were being elicited by microstimulation of 6V, a known vocal structure providing descending afferent input to the PAG (see METHODS and the last section of RESULTS). Twenty-four of these 48 neurons, in 12 fish, were confirmed histologically post hoc to be in the PAG (e.g., Fig. 6C), a compact cell layer along the periventricular surface of the midbrain (Fig. 6, A, B, and K). Recording sites were often (21/24) localized to the PAG axons just ventral to the main PAG cell body layer (e.g., Fig. 6C), but all 24 of these sites had clearly back-filled neurons within the PAG proper. Of the 24 neurons outside the PAG whose activity was also modulated by 6V stimulation, 11 were localized to the ventral tectum, nine were in the midbrain tegmentum, and four were rostral of the PAG, in the dorsal thalamus; there were no back-filled neurons in the PAG in any of these recording sites. The mean and median values, including range and SDs, for spontaneous and stimulus-evoked firing properties of PAG neurons are summarized in Table 1. All cells fired spontaneous action potentials at either a low rate (≤8 Hz; 17 cells) or not at all (seven cells). Ventral tuberal stimulation induced an excitatory response in all PAG neurons recorded. From cell to cell, this response ranged from a single, short-latency spike (nine cells), to either a discrete burst of spikes or a broad, longer-lasting increase in firing that decayed back to the spontaneous baseline over several hundred milliseconds. In 18 of the 24 cells, the mean latency to the first poststimulus spike was <50 ms.

Stimulus-evoked unit spiking typically began well before the vocal response, and some units continued to fire during the fictive vocalization (Fig. 2, A1 and B1). This relationship is best visualized by comparing the PSTHs of vocal and unit activity (e.g., Fig. 2, A2 vs. A3, B2 vs. B3). In 23 of the 24 PAG units, the peak of the unit PSTH preceded the peak of the vocal PSTH, across all trials. In addition, we computed the mean lag time as the difference between the times of the first unit spike and of the first vocal pulse, using only trials where both occurred. The mean lag time for the population of PAG units (165.6 ± 110 ms) was significantly <0 (P < 0.0001, ANOVA). All but two PAG units had mean lag times <0, that is, unit leading vocal. However, there was a great deal of variation, both from unit to unit, and from trial to trial within each unit, in the lag time magnitude (see Table 1).
When examining the trial-to-trial raster plots of spike versus vocal activity (e.g., Fig. 2, A1 and B1), it was clear that much of the trial-to-trial variance in either the latency or duration of the vocal response, or in whether a vocal response even occurred, could not be explained by variance in either the number or latency of spiking of any individual PAG neuron. Indeed, vT stimulation elicited excitatory responses in PAG neurons in nearly all trials, regardless of whether such stimulation elicited a subsequent vocal response. Therefore several statistical analyses were used to determine whether the stimulus-evoked firing of PAG neurons was significantly related to the stimulus-evoked vocal response. Neuronal activity was considered to be vocal related if either 1) spike number (as in Fig. 2, A4 and B4) or latency was significantly different in trials with vocal responses than in trials in which the vocal response failed, with stimulus parameters held constant; or 2) there was a significant correlation, across trials, between the number of net unit spikes evoked by the stimulus and either the number of
vocal response pulses (e.g., Fig. 2, A5 and B5) or the latency of the vocal response (e.g., Fig. 2B6; see METHODS for details of statistical tests). By these criteria, 13 of the 24 PAG neurons, including both neurons illustrated in Fig. 2, showed a significant relation between their spike activity and the vocal response. There was no clear spatial specificity within the PAG between neurons classified as vocal and those classified as nonvocal using these criteria. Furthermore, none of the basic firing properties of the neurons listed in Table 1 (e.g., spontaneous firing rate) was statistically different between these two populations (Wilcoxon tests).

In nine of the 13 “vocal” neurons, the net number of stimulus-evoked spikes (i.e., total spike count minus spontaneous activity) was significantly correlated with the total number of vocal response pulses (Table 2, Fig. 2, A5 and B5), suggesting that spike number is correlated with either the duration (i.e., number of vocal pulses) of each vocal burst or with the number of vocal bursts. Alternatively, these data could reflect either increased or decreased neuronal firing when a vocal response occurred, with no correlation to the duration of the response itself. This latter possibility would support the hypothesis that PAG neuronal activity contributes to initiation of the vocal response, but has no clear role in the patterning of response duration. To directly test whether spike number in any of the vocal neurons predicted the duration of the subsequent vocal burst, we reran the correlation analyses excluding all trials when the vocal response failed, considering only the duration of the first vocal burst on each trial and considering spikes occurring only before the end of this first vocal burst. In five of the nine neurons that initially showed significant correlations between net spike number and total vocal pulses, significant correlations (three positive, two negative) persisted between net spike number and vocal burst duration (Fig. 2A6).

In the remaining four neurons, this second test revealed no significant relation between spike count and vocal duration, implying that the initial correlation between spike count and total vocal pulses in these four neurons was attributed to an underlying relationship between spike count and the binary presence or absence of a vocal response or between spike count and the number of vocal bursts. Indeed, in seven of the 13 vocal neurons, including these four, there was a significant relationship between the net stimulus-evoked spike number and the simple presence or absence of a vocal response (Fig. 2, A4 and B4), evidence that further supports a role in initiation (but not excluding a role in patterning). Only a minority of the 13 vocal neurons revealed significant correlations either between vocal neuron activity and vocal latency (three cells, Fig. 2B6) or between mean unit latency and the presence of a vocal response (four cells). Finally, in none of the 24 confirmed PAG neurons was there any significant relationship between the firing frequency of the unit spike train and the discharge frequency of the vocal response (i.e., rate of fictive sound pulses; see Table 1) or between the latency of the first unit spike and the latency of the vocal response. Histograms of interpulse interval (ISI) distributions for each neuron never showed a clear peak at or near 10 ms, the characteristic interpulse interval of both stimulus-evoked fictive vocalizations (see Vocal response data in Table 1) and natural calls (Fig. 1B). In summary, approximately half of all histologically

![FIG. 2.](http://jn.physiology.org/)

**TABLE 2.** Heterogeneity of correlations between unit activity and vocal response

<table>
<thead>
<tr>
<th>Net Unit Spikes vs.</th>
<th>Vocal response pulses, to end of 1st vocal burst (vocal failure trials excluded)</th>
<th>Vocal response latency</th>
<th>Trials With vs. Trials Without Vocal Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total vocal response pulses (vocal failure trials included)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>“Vocal” (n = 13)</td>
<td>8 + 3 + 1 +</td>
<td></td>
<td>5 + 2 +</td>
</tr>
<tr>
<td>“Nonvocal” (n = 11)</td>
<td>0 0 0</td>
<td></td>
<td>0 0</td>
</tr>
</tbody>
</table>

Vocal neurons were those with at least one of the indicated statistically significant correlations; most exhibited more than one significant correlation. For each type of correlation, the number of units with a significant positive (+) or negative (−) correlation is indicated. The last two columns indicate the number of units with a significant positive (or less) correlation on trials with vocal response than on trials without a vocal response.
confirmed PAG neurons recorded were significantly correlated with the initiation and/or duration of the vocal output.

There were no obvious patterns across neurons in the combination of these correlations that might indicate the presence of distinct subpopulations of vocal PAG neurons. The different categories of correlations described in the preceding paragraph were not mutually exclusive, as the spike activity of some neurons predicted multiple parameters of the vocal response, such as the presence of a vocal response and vocal burst duration (the neuron in Fig. 2A), or the presence of a vocal response and the latency of the response (the neuron in Fig. 2B) (see also Table 2). On the other hand, overlap between these categories of correlations did not appear to be obligatory. For example, cells with a significant positive correlation between the number of net stimulus-evoked unit spikes and the number of vocal response pulses were neither more nor less likely than other cells to have a significant correlation between the number of unit spikes and the vocal latency. The one possible exception is one of the two cells that showed a significant negative correlation between the number of unit spikes and vocal burst duration. This cell was also one of only two cells with significantly fewer spikes on trials with a vocal response than on trials without. Thus there may be a small subset of PAG neurons with net inhibitory effects on vocal initiation and/or duration.

Another approach to examining the relationship between spike trains and vocal output was to replot each unit’s PSTH as a perievent time histogram (PETH) relative to the onset of the vocal response on each trial. By comparing the PSTH and PETH for each unit, it is possible to quantify how tightly each unit’s spiking is distributed relative to the stimulus versus to the onset of the vocal response (Fig. 3). We quantified this difference by measuring the width of the peak of each distribution at half of the maximum height (see METHODS) and comparing the widths of the two distributions for each unit. Across all PAG neurons the PETH width was, on average, 119 ± 29 ms (SE) greater than the PSTH width, indicating that spiking was more tightly time locked to the stimulus than to the vocal response. However, when cells were subdivided into “vocal” and “nonvocal” categories, based on correlations with the vocal response as described above, the vocal cells had a significantly smaller difference between the PETH and PSTH

![Fig. 3. Spike timing in vocal PAG neurons. Spike timing in vocal PAG neurons was more tightly distributed relative to the onset of vocalization than in nonvocal PAG neurons. PSTHs are shown in the top row and perievent time histograms (PETHs, relative to the time of vocal onset) are shown in the bottom row. Shown here are PSTHs and PETHs of spikes in a neuron classified as vocal (left column) because its net spike activity was significantly negatively correlated with vocal latency (P < 0.03, ANOVA) and in another neuron classified as nonvocal (right column) because it had no significant correlations between its spike train and the vocal response. In the vocal neuron, the spike timing was just as tightly distributed relative to the onset of the vocal response as it was to the stimulus itself, as determined by comparing the width, at half-maximal height, of the peaks of the PSTH and PETH spike distributions (see METHODS). In contrast, in the nonvocal neuron, spikes were much more closely time locked to the stimulus than to the vocal response. This effect was consistent across the populations of PAG neurons. That is, the difference between the PSTH and PETH peak width was significantly smaller in vocal neurons than in nonvocal neurons (by Wilcoxon test; inset, top left).](http://jn.physiology.org/)

J. M. KITTELBERGER, B. R. LAND, AND A. H. BASS

J Neurophysiol • VOL 96 • JULY 2006 • www.jn.org
distribution widths (Fig. 3, top left inset). In seven of the 13 cells classified as vocal (and only one of the nonvocal cells), the PETH width was within ±50 ms of the PSTH width. Thus in these cells, spiking was nearly as well time locked to the vocal response as to the stimulus itself. Although the difference between the PETH and PSTH half-height widths was significantly less in vocal cells than in nonvocal, the absolute values of these widths for either PETH or PSTH distributions were no different between the two groups (P > 0.2 and P > 0.9, respectively, Wilcoxon test). This analysis validates the separation of PAG neurons into vocal and nonvocal subgroups and shows that the spike trains of vocal neurons were not only significantly correlated with the vocal response, but were also more tightly distributed relative to the onset of the fictive vocalization.

Direct manipulation of PAG output alters fictive vocal initiation and duration

If PAG activity does contribute to both initiation of the vocal response and establishing vocal duration, one prediction is that increasing or decreasing the duration of PAG ensemble spiking should cause changes in both the probability and the duration of the resultant vocal output. Our preliminary recording experiments involved stimulation in the MLF (see METHODS), which is the sole pathway by which PAG axons connect to the hindbrain vocal circuit (see following text). Thus MLF stimulation presumably elicits vocal responses by excitation of the descending PAG axons, and changes in the duration of such stimulation will produce longer or shorter trains of action potentials in these descending inputs to the hindbrain. We examined the relationship between stimulus duration and vocal response properties in 11 fish in which the stimulation site was histologically confirmed post hoc to be in the MLF and in which vocal responses had been recorded at multiple stimulus durations. Repeated-measures ANOVAs revealed a significant positive relationship across experiments between stimulus duration and both the duration of each vocal burst (Fig. 4A) and the probability of a vocal response (Fig. 4B). In contrast, stimulus duration did not significantly affect either the latency of the vocal response (Fig. 4C) or the interval between vocal pulses within the response (Fig. 4D). Thus not only is PAG neuronal activity correlated with the initiation and duration of the vocal output, but directly altering the output of these neurons also causes changes in these same vocal parameters.

Reversible inactivation of the PAG blocks vocal output

To directly test whether PAG activity was necessary for the stimulus-evoked vocalizations to occur, focal injections of the reversible sodium channel blocker lidocaine were made into the PAG and surrounding midbrain structures after recording baseline vocal responses to vT stimulation. After injection, we continued recording to monitor any effects of the injection and recovery from these effects. Fluorescent dyes included with the lidocaine allowed us to examine post hoc the location of each injection and to estimate the relative size and spread away from the injection site.

Representative results of two lidocaine inactivation experiments are shown in Fig. 5, A–D. The vocal response was completely suppressed immediately after all lidocaine injections to the PAG ipsilateral to the site of vT stimulation (n = 6) and nine of ten lidocaine injections to the PAG bilaterally. In contrast, none of the control injections, including sham (n = 3) and vehicle (n = 4) injections to ipsilateral PAG and lidocaine injections to either the lateral midbrain tectum (n = 3) or the underlying torus semicircularis (n = 2), resulted in the complete and immediate blockade of the vocal response (see summary in Fig. 5E).

Lidocaine effects were reversible: vocal responsiveness returned within 60 min after all ipsi- or bilateral PAG injections. Qualitatively, the effects of the various control treatments varied. Lidocaine injections outside of the PAG (including tectal and toral injections) reduced the number of vocal response pulses evoked, but this effect was always delayed by ≥10 min postinjection and, in two of these five cases, the vocal response was not completely blocked at any postinjection time.
point. The delayed effect of these injections was consistent with the diffusion of lidocaine from the injection site to the PAG. Indeed, in all but one of these injections, we confirmed the spread of fluorescent dye from the injection site to structures surrounding the ventricle, including the PAG (see example in Fig. 5B'). In two of four cases, vehicle injections to the PAG caused a decrease of >70%, but not a complete suppression, in the vocal response immediately after injection; in both cases the response returned to baseline levels within 10 min. Although this shows an apparent influence of the vehicle alone, the results, we emphasize that these results sharply contrast with the lidocaine injections in the ipsilateral PAG that show an immediate and complete cessation of vocal activity (see above and statistical tests below). Sham injections to the PAG never resulted in any detectable change in the vocal response (see METHODS for how we defined an effect of treatments within each experiment).

The effects of lidocaine injections to only the PAG contralateral to the stimulation were inconsistent. Of the four injections, three caused an immediate decrease in the vocal response, with two of these completely blocking the vocal response. The latter two injection sites both showed clear diffusion of dye across the midline to the ipsilateral PAG, but the immediacy of the effects nonetheless suggests the involvement of the contralateral PAG in vocalizations evoked by unilateral vT stimulation. That the results of these experiments were inconsistent may imply relatively weak contralateral connectivity (see anatomical experiments below for confirmation of bilateral PAG to PAG connectivity). Nevertheless, only lidocaine injections directly in the ipsilateral PAG blocked the vocal response consistently, immediately, and completely, demonstrating that neuronal activity in the PAG, but not in the surrounding midbrain structures, is necessary for stimulation-evoked vocal responses.

These results were confirmed by statistical analyses of the different treatment types. Repeated-measures ANOVAs confirmed that there was a significant overall effect across postinjection time of both bilateral and ipsilateral lidocaine injections to the PAG (P < 0.0001, both groups). Toral and tectal lidocaine injections also had significant effects on the vocal response (P < 0.0001 and P < 0.001, respectively), but the timing of these effects was different. Tukey–Kramer tests indicated that lidocaine injections to the PAG, both ipsi- and bilateral, caused a significant decrease in the vocal response, relative to baseline, at the 0, 10, and 20 min postinjection time points. In contrast, lidocaine injections to the torus and tectum caused decreases at the 10, 20, and 30, but not 0, min time points. This confirms that the effects of lidocaine injections outside the PAG were indeed delayed relative to injections that directly hit the PAG, supporting the hypothesis that activity only in the PAG, and not in neighboring midbrain structures, is essential for vocal initiation. By this analysis, there were no significant overall effects of vehicle or sham injections to the PAG or of lidocaine injections to the contralateral PAG (P > 0.6, P > 0.35, and P > 0.06, respectively).

![Fig. 5. Lidocaine inactivation of the PAG reversibly blocked fictive vocalization. A–D: representative experiments from 2 fish. Summed vocal response to vT stimulation was recorded at 10-min intervals. Photomicrographs of coronal brain sections showing the spread of fluorescent dye from the 4 injection sites shown in the top row for experiments A–D are shown immediately below in A’–D’. Immediately after focal lidocaine injections to the PAG either bilaterally (as in injection A, top left), or ipsilateral to the stimulation site (injection D, top right), the vocal response was completely suppressed; the effect was reversible, as the vocal response returned within an average of 42 ± 7 min (SE). Control injections of lidocaine were made to other midbrain structures, including the tectum (injection B) and the torus semicircularis. In contrast to the PAG injections, which immediately and completely blocked the vocal response; these control site injections had either no detectable effects on the vocal response or reduced the response, but only either partially or with a delay of ≥10 min (as per injection B). Injections to the contralateral PAG sometimes had no detectable effect on the vocal response (as per injection C), but in other instances did block the vocal response immediately (see text for details). Note the spread of dye into the ventricle from the tectal injection B’. Dye spreads medially toward the PAG. Note also that the contralateral PAG injection (C’), which had no effect on the vocal response, is well matched in both size and position with the ipsilateral PAG injection (D’), which immediately and completely blocked the vocal response. E: summary of all experimental injections showing a significant difference across experimental groups in whether the vocal response was immediately and completely blocked.
Anatomical connectivity of the PAG

Two main types of neurophysiological data support the hypothesis that vT provides descending afferent input to the PAG. First, the latency of vocal responses elicited by stimulation of vT is much longer than responses evoked by MLF stimulation (roughly 200 vs. 15–30 ms). Second, PAG inactivation blocks vocal responses evoked by vT stimulation. To more clearly define the connectivity of the PAG, both antero- and retrograde, we made focal, unilateral injections of neurobiotin targeted to the vocal PAG regions in four type I male and in one female midshipman (e.g., Fig. 6, A, B, and K). Our two goals were 1) to determine whether there were direct connections from our stimulation site in the anterior hypothalamus (vT) to the PAG and from the PAG to the known vocal motor structures in the hindbrain and 2) to be able to compare the connectivity of the presumptive teleost PAG with that of the mammalian PAG. Photomicrographs of labels resulting from two such injections are shown in Fig. 6, A, B, and K. We found strong retrograde labeling of cell bodies in vT (Fig. 6, D and E), as well as in other vocal active portions of the hypothalamus (the anterior tuberal nucleus) and preoptic area (the anterior and posterior parvocellular preoptic nuclei; not shown, but see Goodson and Bass 2002). In the hindbrain, we found labeled fibers with swellings indicative of presynaptic boutons in VM (Fig. 6, F, G, L, and M) and in an area ventral and medial of VM (Fig. 6, F and H) where commissural axons from VM neurons cross the midline to the contralateral VM (Bass et al. 1994). The projection from the PAG to VM was highly specific because terminals were not found in surrounding structures. In no case did we find fibers or terminals in the PN or SMN, the two other components of the hindbrain vocal motor circuit (Fig. 1C). Labeled axons terminating in VM traveled caudally from the PAG within the MLF. Other labeled axons crossed the midline at the level of the PAG (Fig. 6I) and small numbers of retrogradely labeled neurons and anterogradely labeled terminals were observed in the contralateral PAG (Fig. 6J). This reciprocal connectivity provides an anatomical basis for the immediate effects on the vocal response observed after some contralateral PAG lidocaine injections (see previous section of RESULTS). In sum, these experiments confirm a descending pathway from the anterior hypothalamus (and, more specifically, from vT) to the PAG and then to VM, but neither to the motoneurons innervating the sonic muscles nor to the premotor pacemaker neurons (see Fig. 1C for overview).

Discussion

The data presented here demonstrate for the first time in a teleost fish: 1) the activity patterns of single midbrain PAG neurons that predict the initiation and duration of fictive vocalizations, 2) the necessity of the PAG for vocal production, and 3) the specific anatomical pathway by which descending vocal motor information from the forebrain flows through the PAG to the hindbrain–spinal vocal circuit that determines the fundamental frequency of calls (Bass and Baker 1990).

Single neurons in the midshipman PAG, excited by forebrain hypothalamic (vT) stimulation, showed neuronal responses preceding the evoked fictive vocal response, with significant correlations between spike number and the presence, duration, and/or latency of fictive calls. Such “vocal” neurons were more tightly timed with regard to the vocal onset than were other “nonvocal” PAG neurons. However, the frequency of neuronal firing did not correlate with the discharge frequency of the fictive call. This is consistent with other studies showing that this trait, which establishes the fundamental frequency of natural calls, is primarily, if not solely, determined by the hindbrain–spinal vocal circuitry (Bass and Baker 1990). Stimulation of the MLF, the sole descending pathway for PAG axons connecting to the hindbrain vocal circuitry, affected the probability and duration of vocal bursts, but not their latency or discharge frequency. These data strongly support the hypothesis that the correlations between individual PAG neuron spike activity and vocal output are relevant for vocal initiation and duration patterning, but not for establishing the fine temporal structure (in this case, fundamental frequency) of a vocalization. Consistent with these findings, reversible lidocaine inactivation experiments of the PAG showed that its activity is necessary for the production of vocal responses that are elicited by forebrain stimulation.

Comparisons with mammals

These findings also provide critical empirical support for proposals that this portion of the teleost brain is similar both structurally and functionally to the mammalian, and more generally tetrapod, PAG (Goodson and Bass 2002). Neurons in the vocal portion of the mammalian PAG do not project directly to the motoneuron pools involved in vocalization, but rather to the nucleus retroambiguus (NRA), a primary premotor structure in the caudal medulla (Holstege 1989; Jurgens 2002; Vanderhorst et al. 2000). The NRA then connects to the various motoneuron pools innervating the vocal musculature (f. g., muscles of the larynx, pharynx, tongue, and jaw; see Jurgens 2002). Similarly, we find that the teleost PAG projects to VM, which in turn connects to the PN–SMN circuit that includes sonic motoneurons. As in mammals, we find no evidence of direct connections from the PAG to vocal motoneurons. The mammalian PAG receives dense inputs from a variety of structures in the limbic system, including hypothalamic and preoptic nuclei (Djuardin and Jurgens 2005; Jurgens 2002), inputs that are also present in midshipman (see RESULTS and Goodson and Bass 2002).

An important distinction between our results and those in mammals is that the mammalian PAG appears to be topographically organized. The vocal region itself occupies a discrete portion of the PAG (Holstege 1989; Larson and Kistler 1986; Vanderhorst et al. 2000). Furthermore, there is evidence of topographic mapping of call types across the vocal area of the PAG (Djuardin and Jurgens 2005; Zhang et al. 1994). In contrast, we found no obvious spatial clustering of vocal neurons within the teleost PAG.

Functionally, either lesions or pharmacological inactivation of the mammalian PAG blocks both spontaneous vocalizations
and those elicited by stimulation of PAG afferents (Jurgens 1994; Siebert and Jurgens 2003), just as lidocaine inactivation of the teleost PAG prevented vocal responses to vT stimulation. Single-unit PAG recordings in bats (Suga and Yajima 1988), squirrel monkeys (Dusterhoft et al. 2000, 2004), and macaques (Larson 1991; Larson and Kistler 1986) demonstrate that neuronal activity correlates with vocal output, as we describe here. Generally, PAG neurons in midshipman show similar patterns of vocal-related activity to those described in these other systems. Specifically, both here (see above) and in
mammals: 1) only a small subset of PAG neurons has vocal-related activity; 2) vocal neurons usually begin firing before vocal onset; and 3) vocal responses are heterogeneous, sometimes ceasing to fire before vocal onset and sometimes continuing to fire during vocalization. Furthermore, different neurons are correlated with different aspects of the vocal output. 4) From trial to trial, there is significant variability in the lag time between the onset of neuronal spiking and the vocal onset. Thus the variability in vocal-related spike activity we found in neurons in the midshipman PAG is, in fact, typical of that described previously in the mammalian PAG (Larson 1991; Larson and Kistler 1986). One minor difference between our results and those in mammals is that we found a small subset (2/24) of “vocal” PAG neurons with an apparent net inhibitory relationship to the vocal response. Such neurons have not been described in the mammalian PAG (Dusterhoft et al. 2000, 2004; Larson 1991; Larson and Kistler 1986; Suga and Yajima 1988). Despite the presence of these inhibitory neurons, the summed effect of PAG activity is still excitatory vis-à-vis vocalization, as demonstrated by the fact that PAG inactivation completely blocks vocalization and that MLF stimulation excites vocal responses. Taken together, the structural and functional similarities between the mammalian and teleost PAG suggest that our findings regarding the role of PAG neurons in the initiation and temporal patterning of vocalization are likely to be generally applicable across sonic vertebrates.

Temporal patterning of vocalization

A role for the PAG in the direct patterning of the temporal and motor structure of vocalization remains unresolved. In macaques, the activity of single PAG neurons is correlated both with the activation of single or functionally related groups of vocal muscles and with various acoustic properties of vocalizations, including duration and fundamental frequency (Larson 1991; Larson and Kistler 1986). These data have been interpreted to imply a role for the PAG in vocal patterning. However, other data imply that the PAG initiates each call type, but that detailed patterning of the temporal and acoustic features of calls occurs in premotor and motor circuitry of the hindbrain (Jurgens 1994). Consistent with this, the activity of single PAG neurons in squirrel monkeys correlates with call duration, with concurrent effects on the probability of eliciting a vocal response, but not on discharge frequency. MLF stimulation likely excites descending axons from mid- and forebrain sources other than the PAG. Thus, taken alone, these experiments demonstrate only that call duration can be influ-

FIG. 6. Vocal motor connectivity of the PAG. Neurobiotin injections into the lateral (A, B, D–J) and medial (K–M) PAG in type I male midshipman fish. A: low-power view of midbrain cytoarchitecture showing position of the periventricular region designated as the PAG. Illustrated here is an injection site in the lateral PAG showing axons extending ventrolaterally through the tegmentum. B: higher-magnification view of the same injection site, before Nissl stain. Injection was restricted to the PAG, without extending laterally into the torus semicircularis (TS). Examples of labeled PAG neuronal somata are denoted with arrowheads. C: photomicrograph showing the fluorescent dye injected at a representative physiological recording site in the bundle of axon fibers just ventral to the PAG. Backfilled somata in the PAG are indicated with arrowheads. Recording sites (this one is from the neuron whose activity is illustrated in Fig. 2) are highly comparable to the neurobiotin injection sites, implying that both physiological and anatomical data reflect the same population of PAG neurons. D: dense cluster of backfilled neurons in the ventral tuberal hypothalamus (vT) resulting from the injection site shown in A. vT is the stimulation site used to elicit vocal responses in all recording and lidocaine inactivation experiments in this study. E: higher-magnification view of the section shown in D, before Nissl stain. F: Nissl-stained section in the caudal medulla of the same brain, at the level of the ventral medullary nucleus (VM), a component of the hindbrain vocal pattern generator (Fig. 1C; see Bass et al. 1994 for detailed mapping). G: high-magnification view of the indicated portion of VM in F, showing axon fibers with distinct swellings indicative of presynaptic boutons (examples denoted with arrowheads). H: high-magnification view of the indicated area in F that is ventral and medial of VM, another area where axon fibers with presumptive presynaptic boutons (arrowheads) were found. This relatively cell-free area is where a dense commissural bundle of axons connects VM–VM bilaterally (Bass et al. 1994). I: labeled fibers from the same injection site crossing the midline (from left to right) at the level of the PAG. Some of these fibers terminated in the medial contralateral tegmentum; others extended to the contralateral PAG. J: high-magnification view of backfilled neurons and fibers with presumptive presynaptic boutons in the PAG contralateral to the injection site in A. K: neurobiotin injection in another fish into the PAG medial and rostral to the injection in A. L: Nissl-stained section at the level of VM. M: high-magnification view of the indicated area at the medial edge of VM in L. Labeled axons, after exiting the MLF, enter VM from the right; presumptive boutons (arrowheads) are found only within the boundaries of VM. Inset: dense cluster of boutons at the medial edge of VM in an adjacent section. All sections coronal; scale bars as indicated. All sections, except J, are oriented as in A. Other abbreviations: Te, midbrain tectum; Teg, midbrain tegmentum.
enced by activity manipulation above the level of the hindbrain. However, several pieces of evidence suggest the most parsimonious interpretation is that the effects of MLF stimulation on vocalization are attributable to effects specifically on descending PAG axons. First, injections of biotin tracers into the hindbrain region of VM that innervates the PN–SMN circuit (Fig. 1) mainly label PAG neurons (Goodson and Bass 2002). The tract-tracing experiments reported here confirm the specificity of this projection. Consistent with this circuitry, the current study also demonstrates that PAG activity is predictive of a vocal response, and lidocaine experiments show that activity in the PAG, but not in midbrain structures surrounding the PAG, is necessary for forebrain-evoked fictive vocalizations. Previously published reports also support a role for the midshipman PAG in patterning vocal duration. First, neuropeptide injections near the PAG induce dose-dependent changes in fictive call duration but not in discharge frequency (Goodson and Bass 2000b). Furthermore, certain effects of androgenic steroids on vocal duration have been localized to the midbrain (Remage-Healey and Bass 2004); a particularly high density of androgen receptors in the PAG (Forlano et al. 2005) makes it a likely locus for these effects.

The PAG is a critical site in the initiation of a variety of “emotional” behaviors that involve vocal communication signals, from defense and escape to courtship and reproduction (Behbehani 1995; Holstege 1998). Similarly, the multiple midbrain regions that constitute the mesencephalic locomotor center initiate locomotion by projections to reticulospinal neurons driving spinal motoneuron pools (Grillner 2003; Jordan 1998). In all of these contexts, midbrain neuronal activity has been thought to play only a minor role in directly patterning the motor output. Here we show that PAG neuronal activity correlates with discrete aspects of vocal initiation. Furthermore, we demonstrate that PAG activity correlates with the duration of the vocal output, but clearly not with other fine temporal features, such as fundamental frequency. In general, the results of this study provide a compelling example of how distantly related groups of vertebrates have adopted similar mechanisms to solve common problems in vocal–acoustic communication, in this case the patterning of brain stem vocal motor output. To the extent that the specifics of these findings prove to be generally applicable across motor systems and groups of vertebrates, initiation and patterning functions may not be localized in distinct, separate nodes of the descending motor pathway, but rather may be distributed properties of the motor circuit considered in its entirety.

ACKNOWLEDGMENTS

We thank A. Melin and M. Marchaterre for technical assistance and B. Arthur, J. Fetcho, B. Johnson, and R. Harris-Warrick for helpful comments on the manuscript. Professor James Booth, Dept. of Biological Statistics and Computational Biology, provided helpful advice on statistical methods.

GRANTS

These studies were supported by National Science Foundation Grants IBN 9987341 and 0516748 to A. H. Bass and National Institute of Deafness and Other Communication Disorders Grants ROI DC-00092 to A. H. Bass and F32 DC-06156 to J. M. Kittelberger.

REFERENCES


