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Distribution of hypoglossal motor neurons innervating the prehensile tongue of the African pig-nosed frog, *Hemisus marmoratum*

Curtis W. Anderson[a,*], Kiisa C. Nishikawa[b], Joyce Keifer[a]

[a]*Department of Anatomy and Structural Biology, University of South Dakota School of Medicine, Vermillion, SD 57069, USA*
[b]*Department of Biological Sciences, Physiology and Functional Morphology Group, Northern Arizona University, Flagstaff, AZ 86011, USA*

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Abstract

Using retrograde neuronal tracers, a study of the distribution of hypoglossal motor neurons innervating the tongue musculature was performed in the African pig-nosed frog, *Hemisus marmoratum*. This species is a radically divergent anuran amphibian with a prehensile tongue that can be aimed in three dimensions relative to the head. The results illustrate a unique rostrocaudal distribution of the ventrolateral hypoglossal nucleus and an unusually large number of motor neurons within this cell group. During the evolution of the long, prehensile tongue of *Hemisus*, the motor neurons innervating the tongue have greatly increased in number and have become more caudally distributed in the brainstem and spinal cord compared to other anurans. These observations have implications for understanding neuronal reconfiguring of motoneurons for novel morphologies requiring new muscle activation patterns. © 1998 Elsevier Science Ireland Ltd.

Keywords: Anuran amphibian; Hypoglossal nerve; Motor nuclei; Tongue

Recent studies of feeding in anurans have revealed a diversity of tongue morphologies and feeding mechanisms [1,5,7,8,10,11,15]. Despite the multiple lineages that have evolved long tongues independently, the morphology and function of the tongue musculature has remained remarkably consistent [11,14]. In this study, we investigated one species of frog, *Hemisus marmoratum*, that exhibits a radically divergent tongue morphology. The aim of this study was to determine whether the motor neurons controlling this unusual tongue have undergone divergence as well.

The African pig-nosed frog, *Hemisus marmoratum*, is a member of the family Hemisotidae and is considered part of the Ranoidea [6]. A recent analysis of the kinematics and mechanisms of prey capture in *H. marmoratum* has revealed an unusual prehensile tongue that can be aimed in three dimensions relative to the head. The mechanism of tongue prehension used by *Hemisus*, hydrostatic elongation, is possessed by members of the families Hemisotidae and Microhylidae [12,15]. In hydrostatic elongation, the tongue protractor muscle (M. genioglossus) has two types of muscle fiber orientation. As in all frogs, there is a set of muscle fibers oriented parallel with the long axis of the tongue. Additionally, in hydrostatic elongators, there is a group of muscle fibers oriented vertically to the long axis of the tongue [12]. Thus, there is a priori evidence to suggest that the hypoglossal motor neurons innervating the tongue of hydrostatic elongators may have been reconfigured during the evolution of this novel morphology.

Hemisus marmoratum ($n = 6$; 2.0–3.25 cm snout vent length) were obtained from a commercial supplier (Glades Herp; Fort Meyers, FL, USA) and maintained in captivity. Hypoglossal motor neurons innervating the tongue protractor muscle, M. genioglossus, were retrogradely labeled using either 3000 MW fluorescein dextran ($n = 4$; Molecular Probes, Eugene, OR, USA), 10 000 MW fluoro-ruby ($n = 2$; Molecular Probes), or neurobiotin ($n = 2$; Vector, Burlingame, CA, USA). The hypoglossal nerve was labeled bilaterally with the fluorescein dextran. Due to the similar transport times of the fluoro-ruby and neurobiotin, one tra-

* Corresponding author. Tel.: +1 605 6776883; fax: +1 605 677 6381.

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cer was placed on each side to compare labeling between the fluoro-ruby and the neurobiotin.

The frogs were anesthetized by immersion in 0.1% tricaine methane sulfonate (MS-222) buffered with sodium bicarbonate for approximately 8–10 min. They were then wrapped in damp paper towels and placed under a surgical microscope. The hypoglossal nerve was exposed by making a small incision through the skin of the lower jaw and the Mm. intermandibularis and geniohyoideus. A few crystals of the tracers were mixed with a drop of distilled water and dimethyl sulfoxide and allowed to harden. Using a fire-polished glass micropipette, a few crystals of the neuronal tracers were applied bilaterally to the cut nerve stumps of the hypoglossal nerve peripherally at the base of the tongue. After approximately 30 s, the cut nerve and the incision were rinsed with distilled water and the incision was closed with veterinary grade cyanoacrylate surgical glue.

After a survival period of between 16 and 20 h for the fluorescein dextran and 1.5–2.5 days for the neurobiotin and fluoro-ruby, the animals were euthanized by immersion in 0.1% buffered MS-222. The brains were removed, placed in 4% paraformaldehyde overnight, mounted in gelatin and sectioned at 50 μm on a sliding microtome. For the fluorescein dextran cases, the sections were mounted in phosphate buffered saline (PBS), air dried, coverslipped using glycerol and PBS, and photographed. For the neurobiotin cases, every other section was reacted and the other sections were evaluated for fluoro-ruby transport. The sections to be reacted for neurobiotin transport were washed once in 0.1 M PBS and incubated, shaking continuously for 2.5 h, in an avidin-biotin-peroxidase complex with 0.3% Triton X-100 (ABC kit PK 4000; Vector). They were then reacted with the DAB substrate kit (SK 4100) using nickel intensification for 8 min. The sections were rinsed twice in PBS and mounted. The slides were allowed to air dry, and then were dehydrated in alcohol, cleared in xylene and mounted using Accu-Mount (Baxter).

Retrograde labeling from the peripheral hypoglossal nerve revealed two populations of motor neurons, the ventrolateral nucleus (VLN) and the dorsomedial nucleus (DMN) (Fig. 1). These two nuclei exhibit a very distinctive arrangement of the cell groups and have previously been described in detail in other anurans [4,13,16–18]. The neurons of the DMN are located more rostrally in a distinct spherical cell group. The VLN is caudal to the DMN and the cell group is much more elongate. In addition, a small number of labeled neurons could not easily be assigned to either of these motor nuclei because their distribution was along an axis connecting the VLN and the DMN. They were thus labeled as VLN-DMN hypoglossal motoneurons. An intermediate group of neurons located between the VLN and DMN was also identified in the leopard frog, *Rana pipiens* [16].

The DMN of *Hemisus* is distributed rostrocaudally from 416 ± 44 μm rostral to the obex to 266 ± 33 μm caudal to the obex (Fig. 2). This does not appear to differ from that of other anuran amphibian species studied. The number of motoneurons contained within this nucleus (174 ± 19; Fig. 3) is less than that observed in *Rana pipiens* (approx. 302; see Fig. 3 of [17]), or *Bufo japonicus* (approx. 348; see Fig. 14 of [13]), although the much larger sizes of *Rana* or *Bufo* has not been taken into account. The VLN-DM was found to contain 51 ± 3 stained cell bodies. The number of cell bodies in this intermediate group has not been reported in any other anurans.

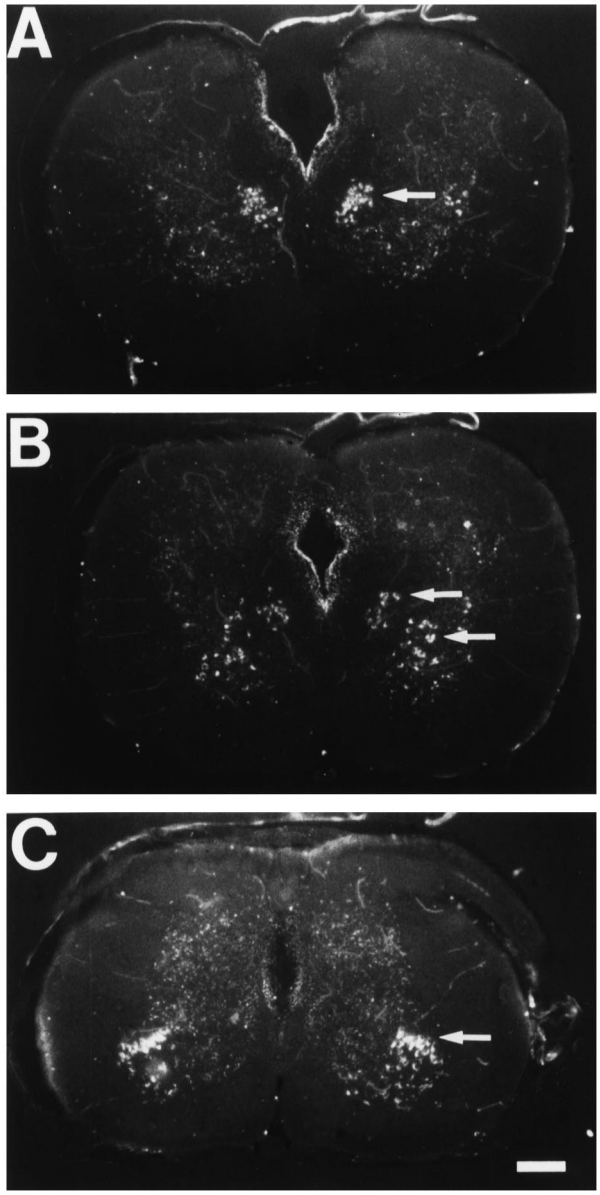

Fig. 1. Cross sections through the brainstem of *Hemisus marmoratum* following retrograde labeling of the lingual branch of the hypoglossal nerve. (A) Section just caudal to the obex showing the dorsomedial hypoglossal nucleus (DMN; arrow). (B) Section rostral to the obex showing stained motoneurons that were located intermediate to the hypoglossal nuclei and were thus considered the DMN-VLN (arrows). (C) Section through the third spinal nerve showing the stained cell bodies of the ventrolateral hypoglossal nucleus (VLN; arrow). Scale bar, 200 μM.

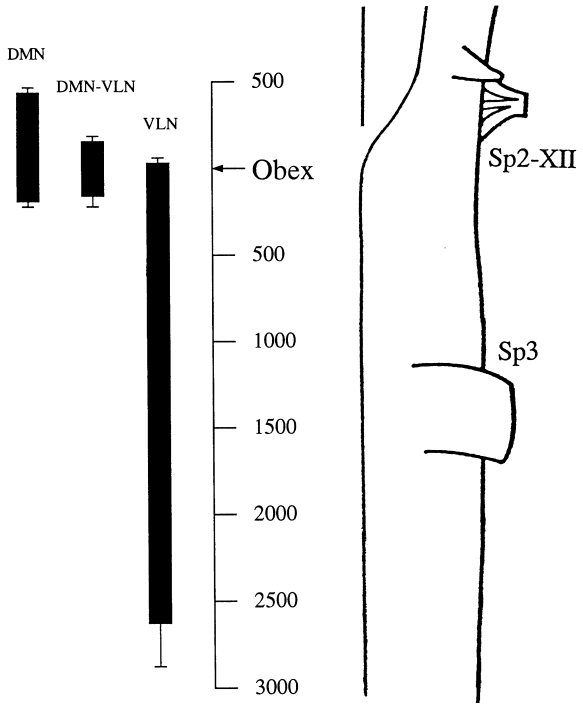

Fig. 2. Schematic drawing of the right half of the lower brainstem of *Hemisus marmoratum.* On the left, the bars illustrate the rostrocaudal distribution of hypoglossal motor neurons (in μm, $\pm$ SEM) above and below the level of the obex. Sp2-XII, second spinal and hypoglossal nerve complex; Sp3, third spinal nerve.

Relative to other frogs, the VLN has a much larger rostrocaudal distribution ranging from 17 ± 17 μm rostral to the obex to 2667 ± 248 μm caudal to the obex (Fig. 2).

Additionally, the number of motoneurons contained within the VLN (778 ± 102) far exceeds the number observed in *Rana pipiens* using retrograde HRP labeling (approx. 91) [17], or *Bufo japonicus* using cobaltic lysine (approx. 116) [13].

In some anurans, the hypoglossal nerve has been shown to contain sensory neurons that are used in the coordination of tongue movements during feeding [2–4,11]. Additionally, in two species of frogs that have evolved a long tongue independently, the sensory fibers were found to have originated from anastomoses from different sources. In the leopard frog, *Rana pipiens*, the hypoglossal afferents were found to be re-directed cervical spinal neurons from the third spinal nerve [4]. Toads of the genus *Bufo* were shown to contain hypoglossal afferents that represented sensory fibers of the glossopharyngeal nerve re-directed into the peripheral hypoglossal nerve [11]. In both of these examples, the afferents within the hypoglossal nerve are used to coordinate the rapid tongue and jaw movements [2,9]. Although *Hemisus marmoratum* is a neobatrachian anuran similar to *Bufo* and *Rana*, no afferents were observed in any of the cases in the present study. Relative to other anurans with long tongues, the tongue of *Hemisus* is protracted extraordinarily slowly [15]. This may explain the lack of afferents within the hypoglossal nerve, as jaw/tongue coordination may not be as crucial for successful prey capture.

In addition to the slow speed of tongue protraction, *Hemisus* exhibits the largest degree of precision and fine motor control of the tongue observed in any anuran. For example, it can control the trajectory of the tongue in three dimensions relative to the head [15]. The mechanism of tongue protraction in *Hemisus*, hydrostatic elongation, utilizes a tongue morphology in which the genioglossus muscle has fibers arranged longitudinally to the long axis of the tongue, as found in other frogs. However, there is an additional group of genioglossus muscle fibers that are arranged vertically in the tongue. It has been shown in other frogs that the

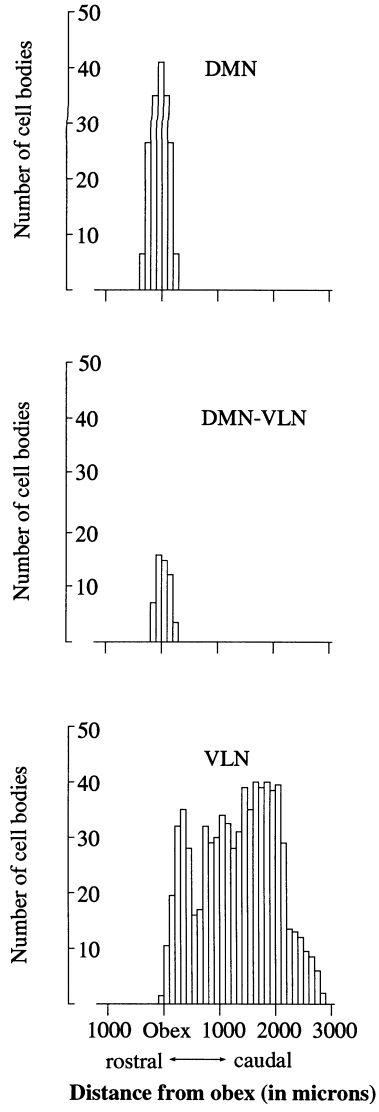

Fig. 3. Histograms showing the mean number of stained hypoglossal motor neurons distributed on a rostrocaudal axis along the brainstem. The DMN is the dorsomedial nucleus ($n = 174$), the DMN-VLN represents an intermediate group of neurons between the two nuclei ($n = 51$), and the VLN is the ventrolateral nucleus ($n = 778$). Abscissa indicates the distance (in μm) from the obex. Ordinate indicates the number of stained hypoglossal cell bodies. Bin width, 100 μm.

motor neurons innervating the M. genioglossus are located more caudally in the brainstem than are the neurons innervating the tongue retractor (M. hyoglossus) [16,19]. Therefore, in *Hemisus*, the large increase in the caudally distributed VLN motor neurons most likely arises from an increase in the innervation of the M. genioglossus and may play a role in the fine control of the hydrostatic tongue.

We conclude that in the African pig-nosed frog, *Hemisus marmoratum*, the hypoglossal nerve complex that innervates this radically divergent tongue morphology has conserved a distribution of hypoglossal motor neurons relatively similar to that of other anurans, however, the number of motor neurons has been drastically altered. The dorsomedial cell group is indistinguishable in the number of cell bodies and the rostrocaudal distribution in the brainstem from other anurans studied. However, the ventrolateral cell group has been extended to a much larger caudal distribution and contains a significantly larger number of motor neurons. This suggests that during the evolution of a hydrostatic tongue in the African pig-nosed frog, *Hemisus marmoratum*, the ventrolateral hypoglossal motoneuron pool was reconfigured for this novel muscle morphology.

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