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The functional anatomy and evolution of hypoglossal afferents in the leopard frog, *Rana pipiens*

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Abstract

Previously, we suggested that afferents are present in the hypoglossal nerve of the leopard frog, *Rana pipiens*. The basis for this was behavioral data obtained after transection of the hypoglossal nerve. These afferents coordinate the timing of tongue protraction with mouth opening during feeding. The goal of the present study was to define anatomically these hypoglossal afferents in *Rana pipiens*. Retrograde tracing was performed using horseradish peroxidase, fluorescent dextran amines and neurobiotin. Data show that the cell bodies of hypoglossal afferents are located in the dorsal root ganglion of the third spinal nerve and enter the brainstem through its dorsal root. The afferents ascend in the dorsomedial funiculus and move laterally after they pass the obex. They project in the granular layer of the cerebellum and the medial reticular formation. The cervical afferents that travel in this pathway are known to carry proprioceptive and cutaneous sensory information. We hypothesize that the presence of afferents in the hypoglossal nerve is a derived characteristic of anurans, which has resulted from the re-routing of afferent fibers from the third spinal nerve into the hypoglossal nerve. The appearance of hypoglossal afferents coincides with the morphological acquisition of a highly protrusible tongue. © 1997 Elsevier Science B.V.

Keywords: Hypoglossal nerve; Afferent; Tongue; Anuran; Sensory feedback

1. Introduction

During evolutionary changes in morphology, neuronal pathways may be reconfigured to control the new structures, and novel sensory mechanisms may be required to coordinate their movements. Among anuran amphibians, the acquisition of a long, protrusible tongue represents such a phylogenetic event. The primitive condition for frogs is a tongue that protrudes only slightly beyond the tips of the mandibles [20]. A highly protrusible tongue has evolved several times independently [22]. Therefore, an interesting system has emerged to investigate evolutionary changes in the neural control of the highly protrusible tongues. In this study, we address the phylogenetic origins and anatomical projections of sensory neurons that coordinate tongue movements in the leopard frog, *Rana pipiens*.

The tongues of frogs, in all but a few cases, are attached anteriorly, and are protracted and retracted by the contraction of the extrinsic muscles, mm. genioglossus and hyoglossus. The m. genioglossus rotates the tongue over the mandibular symphysis and out of the mouth [11,12,22] and the m. hyoglossus pulls the tongue back into the mouth. The protractor and retractor muscles of the tongue are controlled by motoneurons of the hypoglossal nerve.

The hypoglossal (CN XII) motoneurons of vertebrates have been well documented [4,6,17,25,31]. Rami of the hypoglossal nerve innervate the neck musculature and the lateral muscles of the otic capsule. Ultimately, the terminal branches (ramus hypoglossus) innervate the hypobranchial muscles, including the hyoglossal and genioglossal muscles [5,13]. In extant amphibians, the brainstem has become compressed and, with the exception of a spino-occipital nerve present in a few urodele amphibians and caecilians which is composed in part by the hypoglossal nerve, the hypoglossal is formed from the combined branches of the first and second spinal nerves [3,6].

Urodele amphibians represent the primitive condition for tetrapods; the hypoglossal nerve is a purely motor

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nerve and no afferent fibers are associated with it [27]. However, hypoglossal afferents have evolved several times independently among vertebrates. Because the hypoglossal nerve does not have a dorsal root ganglion, several investigators have demonstrated alternative pathways by which anastomoses from other nerves may contribute afferent fibers to the ramus hypoglossus (for review, see [17]). In primates, the hypoglossal afferents are re-directed cervical spinal neurons as well as neurons from the nodose ganglion of the vagus nerve and are used in modulation of tongue movements during vocalization [7]. These neurons enter the tongue through the hypoglossal nerve. In the dog and cat, Zimney et al. [36] found a small number of afferent fibers in the proximal vagus nerve that show degeneration following transection of the hypoglossal nerve. Among finches, Wild [35] has shown that Herbst corpuscles and terminal cell receptors of the tongue papillae are innervated by the lingual branch of the hypoglossal nerve. Their cell bodies are located in the jugular ganglion and then ascend within the trigeminal tract.

In the leopard frog, *Rana pipiens* (Family Ranidae), Stuesse et al. [32] showed that the distal portion of the hypoglossal nerve contains afferents, although neither the root in which the fibers entered the brainstem, nor their projections were described.

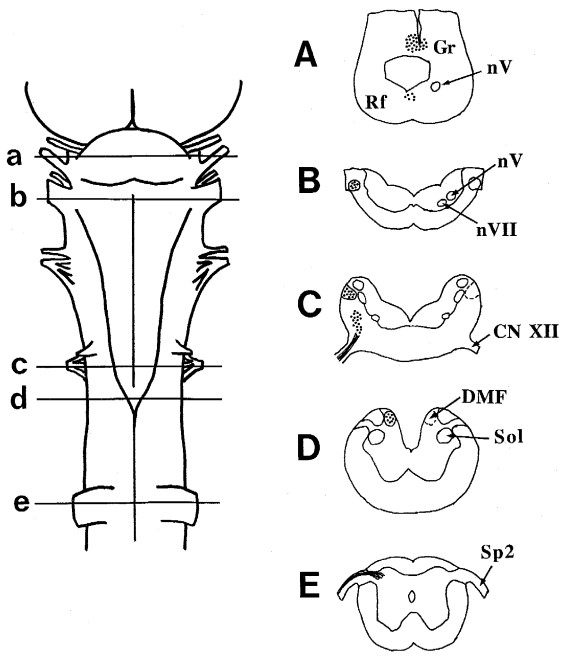

Fig. 1. This schematic summarizes the results of the neurobiotin and fluorescent tracings. The dyes were placed on the cut stump of the hypoglossal nerve in the tongue. The afferent fibers enter the brainstem through the second most anterior dorsal root, the third spinal nerve (E). They travel in the dorsomedial funiculus (D) and move laterally as they ascend past the obex (C–B). The solitary tract is included for reference. There does not appear to be a direct synaptic connection with either the nV or nVII nuclei. The fibers split near the entrance to the trigeminal root, with many fibers projecting to the granular layer of the cerebellum (A). A small number of fibers also project bilaterally to the reticular formation (A). Sp2, second spinal nerve; Sol, solitary tract; DMF, dorsomedial funiculus; CN XII, hypoglossal ventral root; nVII, facial nerve nuclei; nV, trigeminal nerve nuclei; Rf, reticular formation; Gr, granular layer of the cerebellum.

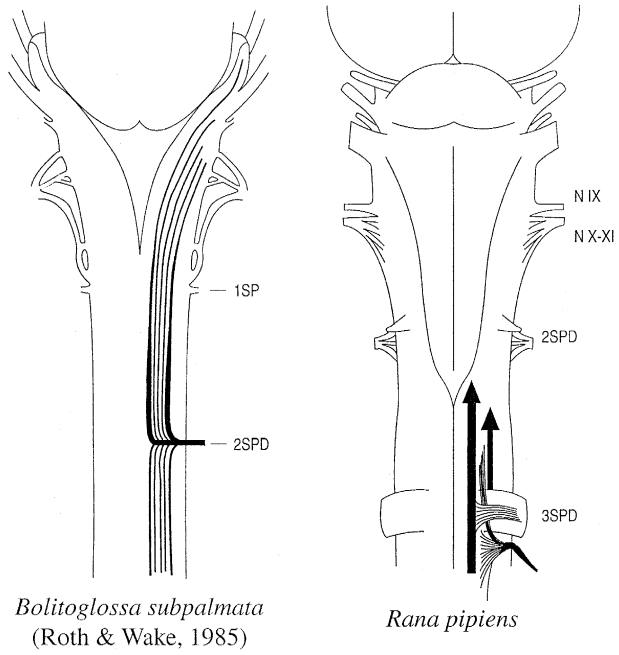

Fig. 2. These two drawings illustrate the ascending and descending pathways of the spinal nerves after staining of the entire second spinal nerve in the salamander *Bolitoglossa subpalmata* (from Roth and Wake [27]) and after staining the peripheral hypoglossal nerve with HRP in the leopard frog, *Rana pipiens*. In *Rana*, the projections could not be traced in the whole mount beyond the point of the arrows. In both species, the pathways ascend in two distinctly separate fasicles. This suggests that the stained afferents in the hypoglossal nerve of *Rana* are re-routed cervical spinal afferents.

Previous studies of feeding motor patterns in *Rana pipiens* have shown that hypoglossal afferents coordinate the timing of tongue movements [1]. When feeding on small sizes of prey, the hypoglossal nerve must be intact for the mouth to open during feeding. When the hypoglossal nerve is transected, the frogs lunge forward and attempt to capture the prey, but the mouth does not open during the feeding cycle. Hypoglossal afferents have also been shown to gate incoming visual information and influence the choice of motor programs used during prey capture [2]. Therefore, sensory information traveling within the hypoglossal nerve presumably carries information about movements of the tongue and is used to coordinate tongue protraction and mouth opening [2].

In more basal lineages of frogs, the hypoglossal nerve appears to be purely motor. Transection of the hypoglossal nerve does not prevent these frogs from opening their mouths during feeding [8,23]. Therefore, we hypothesize that the presence of afferents in the hypoglossal nerve represents a derived condition among anurans. The appearance of these afferents coincides with the morphological acquisition of a highly protrusible tongue. In addition, a phylogenetic survey of hypoglossal afferents among anu-

rans suggests that these afferents have evolved multiple times independently in the ranids and the bufonids [24].

In the present study, we trace hypoglossal afferent fibers from the tongue into the brainstem and illustrate the ascending projections to the cerebellum and reticular formation. We discuss the evolution of afferents from the third spinal nerve which have become re-routed to anastomose with the hypoglossal nerve. Their function is to control the timing of tongue and jaw movements in the frog, *Rana pipiens*.

2. Materials and methods

Adult *Rana pipiens* ($n = 20$), approximately 6.5–7.5 cm snout–vent length, were obtained from commercial suppliers and maintained in glass aquaria at approximately 15°C. To identify the sensory component of the hypoglossal nerve and where it enters the brainstem, three tracers were used: (1) horseradish radish peroxidase ($n = 9$; HRP; Sigma); (2) a 3000 molecular weight fluorescein dextran amine [10] ($n = 2$ whole mount; $n = 5$ sectioned preparations; Molecular Probes Inc., Eugene, OR); and (3) neurobiotin ($n = 4$; Vector Laboratories Inc., Burlingame, CA). All frogs were subjected to only one of the staining protocols. The frogs were anesthetized by immersion in 0.1% tricaine methane sulfonate buffered with sodium bicarbonate for approximately 10 min. They were then wrapped in damp paper towels and placed under a dissecting microscope for surgery. The hypoglossal nerve was exposed by making a small incision through the skin of the lower jaw and the mm. intermandibularis and geniohyoideus. The hypoglossal nerve was transected distal to the branch that innervates the geniohyoideus muscle. This ensures that the fibers stained are only those that enter the tongue to innervate sense organs of the m. genioglossus, and are therefore the same fibers that were previously identified in lesion studies [1].

A few crystals of either HRP or the fluorescent dextran amine were mixed with a drop of distilled water and dimethyl sulfoxide (DMSO) and allowed to harden. They were then placed on the cut nerve stump and allowed to soak into the nerve fiber (approximately 2 min for the HRP and 20–30 s for the dextran amine). A similar procedure was followed with the neurobiotin. A few milligrams of neurobiotin were dissolved in the same distilled

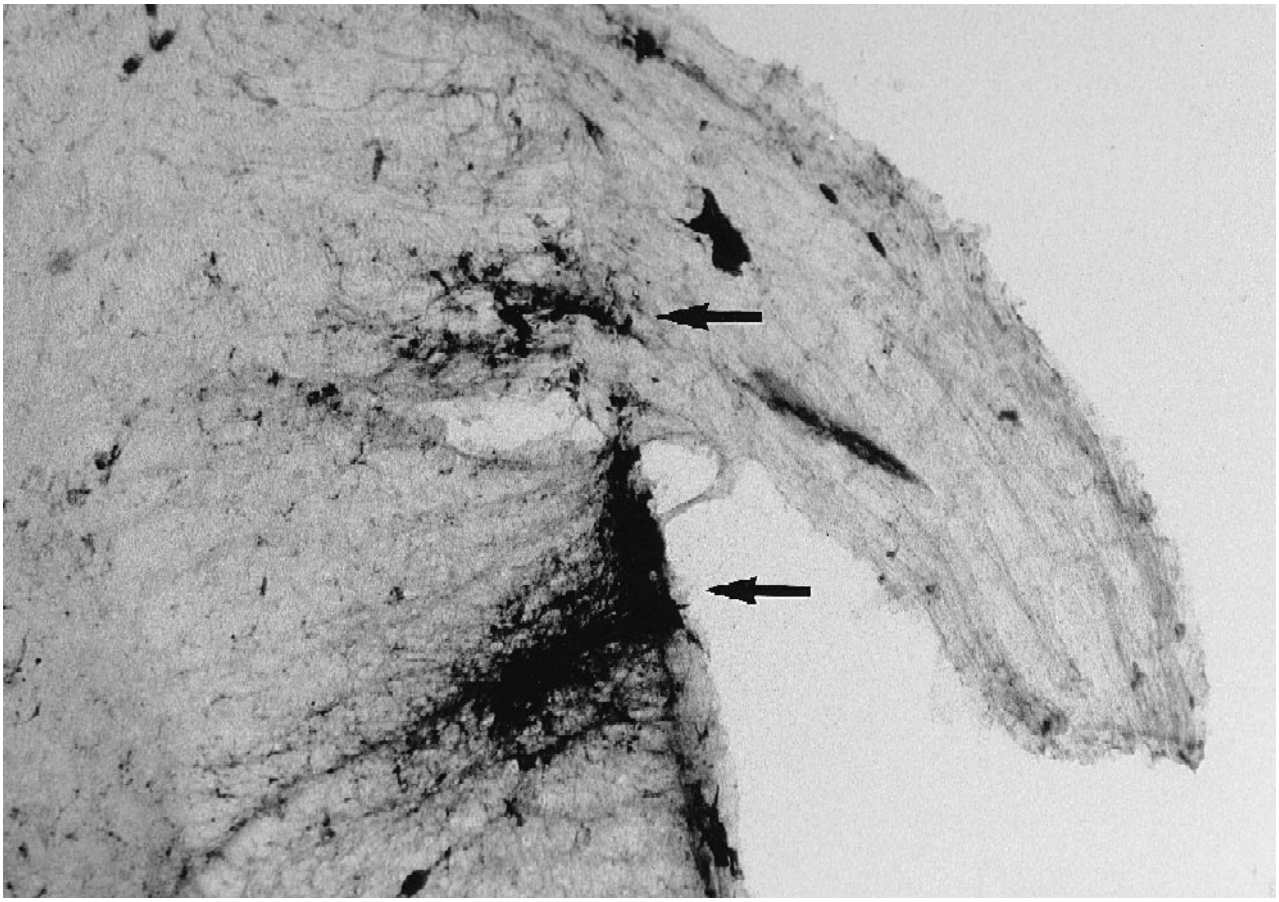

Fig. 3. Photomicrograph showing the dorsal root of the third spinal nerve in *Rana pipiens* following staining of the peripheral hypoglossal nerve with neurobiotin. The arrows indicate two distinct fiber tracts that enter through the main dorsal root as well as slightly caudal to the root in a small nerve rootlet.

water/DMSO solution and allowed to dry. The dried crystals were applied to the nerve stump and allowed to soak into the cut nerve for approximately 30 s. The incision was rinsed with distilled water and closed with a veterinary grade cyanoacrylate surgical glue.

Transport times were 6–10 h for the dextran amine, 2.5–3 days for the neurobiotin and 8–10 days for the HRP. Following transport, the frogs were killed by immersion in 0.1% buffered tricaine methane sulfonate for approximately 30–45 min. Under this deep plane of anesthesia, the frogs were perfused transcardially with Ringers solution followed by 4% paraformaldehyde in 0.1% phosphate buffer.

The HRP-stained brains were removed and placed in fixative. Following postfixing, the brains were rinsed in 0.1 M cacodylate buffer for 2 h. They were reacted whole in 0.2% diaminobenzidine (Sigma) and 0.1% H_2O_2 for 20 min. They were then dehydrated in an alcohol series, cleared in methyl salicylate, and photographed.

For the dextran amines and neurobiotin, the brainstem was dissected out and placed in 4% PFA fixative overnight before sectioning. The brainstems were mounted in 3% agar, immersed in cold 0.05% phosphate buffer, and sectioned into 50 or 60 μm sections on a vibratome. For the fluorescence, the sections were mounted using Vectashield™ mounting medium and photographed immediately.

For neurobiotin, the sections were washed once in 0.1 M phosphate buffered saline (PBS) and incubated, shaking continuously for 2.5 h, in a solution from the Vector Laboratories ABC staining kit (PK 4000). They were then washed twice for 5 min each in PBS and once in Tris buffer (0.1 M, pH 7.2) for 15 min. The sections were then reacted with the Vector Laboratories DAB Substrate Kit (SK4100) using nickel intensification for 10 min. The sections were rinsed once in PBS and mounted. The slides were allowed to air dry, and then were dehydrated and cleared using Hemo-De™ (Fisher).

3. Results

Following application of neuronal tracers to the hypoglossal nerve in the tongue, stained fibers were observed in the dorsal root of the third spinal nerve (Fig. 1). Stained cell bodies were also identified in the dorsal root ganglion of the ipsilateral third spinal nerve (unpublished observations). The fibers ascend through the dorsomedial funiculus and course laterally after passing the obex. They terminate primarily in the ipsilateral granular layer of the cerebellum, with a few stained fibers bilaterally in the medial reticular formation.

The HRP whole mount shows a small rootlet just caudal to the main branch of the dorsal root of the third spinal nerve which is stained intensely (Fig. 2). A few stained fibers also appear in the main branch of the nerve. The neurobiotin illustrates a similar projection pattern. A section through the third spinal root (Fig. 3) clearly shows the

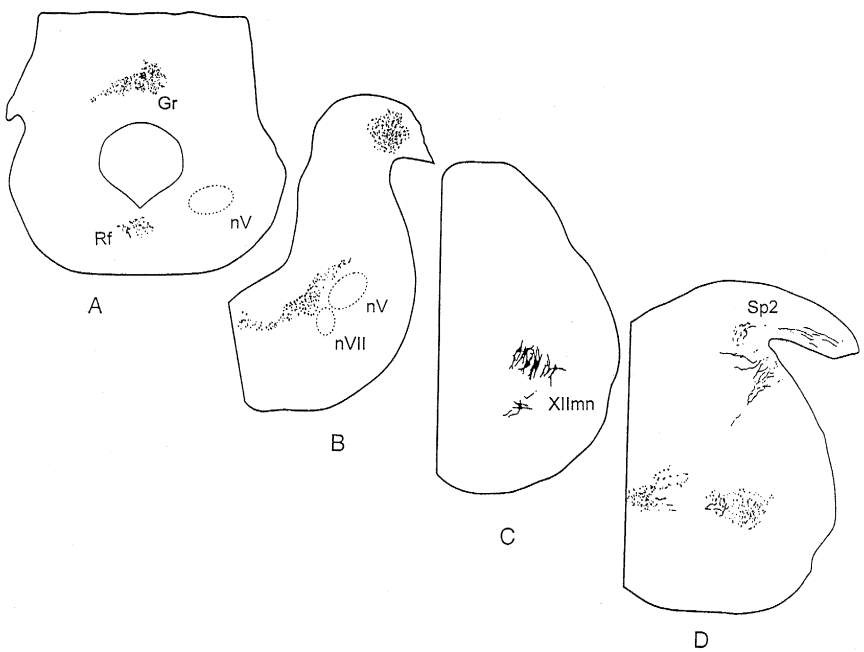

Fig. 4. Camera lucida drawings of brainstem cross sections after application of neurobiotin to the hypoglossal nerve innervating the tongue. A: section through the cerebellum and medulla showing staining in the granular layer of the cerebellum and the medial reticular formation. B: section through the medulla at the level of nV. Staining is evident in the lateral dorsal column and the reticular formation. C: section just rostral to CN XII/Sp2. Hypoglossal motor neurons are clearly stained. D: section through Sp3 showing the dendrites of hypoglossal motor neurons and the hypoglossal afferents entering the dorsal root of Sp3. Sp2, second spinal nerve; XIImn, motor neurons of the hypoglossal nerve; nVII, facial nerve nuclei; nV, trigeminal nerve nuclei; Rf, reticular formation; Gr, granular layer of the cerebellum.

stained fibers entering the brainstem at the posterior-most edge of the third spinal nerve, and through a small caudal rootlet entering the gray matter slightly caudal and ventral to the main fibers of the third spinal nerve. They descend very little, although a few thin descending fibers were stained immediately posterior to the main incoming fiber bundle. At this level, motor neurons of the hypoglossal nucleus are present. As they approach the obex, the hypoglossal afferents ascend in the submarginal zone, and move laterally as they pass the obex and the fourth ventricle (Fig. 4). As the fibers course near the entrance to the trigeminal root, some fibers migrate dorsally and project to the granular layer of the ipsilateral cerebellar cortex. Stained fibers were also found ventral and medial to the fourth ventricle in the medial reticular formation. Most of the stained axons were ipsilateral to the stain application. However, there is a bilateral projection that corresponds with the median and medial reticular formation [19].

4. Discussion

4.1. Anatomy

All three staining methods illustrate that sensory fibers are present peripherally in the hypoglossal nerve and carry sensory information from the tongue. These fibers enter the brainstem at the level of the dorsal root of the third spinal nerve. The whole mount HRP brains clearly illustrate that the fibers ascend in two fascicles. The second spinal nerve of the salamander *Bolitoglossa subpalmata* shows a nearly identical pattern (Fig. 2) [27]. Joseph and Whitlock [16] also found that the dorsal root of the third spinal nerve in *Rana pipiens* divides into two fascicles as the fibers ascend in the spinal cord. The majority of stained neurons projected into the dorsal column, and a smaller projection passed into the dorsolateral fasciculus.

Previous work has described afferents in the hypoglossal nerves of frogs, although there has been some confusion concerning their central projections. Stuesse et al. [32] identified a number of afferent fibers ''descending the spinal cord in the tract of Lissauer'' when HRP was applied to the cut nerve stump of the hypoglossal nerve in *Rana pipiens*. Because all of the sections they observed were made rostral to the third spinal root, these probably represent the same fibers as the ones we have described, and hence are ascending from their entrance into the spinal cord at the third spinal root.

In frogs, it has been shown that the bundle of fibers in the dorsomedial funiculus contains afferents that transmit propriospinal and cutaneous sensory information [19]. Therefore, it is logical to assume that the receptors of the hypoglossal afferents traveling in this tract are either muscle spindles or cutaneous sensory receptors. Careful dissections have failed to reveal the presence of spindle or spindle-like receptors, and the fibers did not appear to be coming from the muscle, but instead terminated in the tongue epithelium. Therefore, the receptors are most probably cutaneous mechanoreceptors.

The results reported here support the hypothesis that the medial reticular formation is part of the motor pattern generator for prey capture in frogs [33]. A motor pattern generator for feeding in frogs should receive input from all of the sensory modalities that are known to modulate feeding behavior in frogs. The optic tectum is the primary visual input for prey capture in amphibians [7,9,14,28], and it projects to the medullary reticular formation, as has been demonstrated anatomically [15,34] and electrophysiologically [18,28,29]. In addition, Schurg-Pfeiffer [30] recorded from several classes of medullary neurons in this area which contribute to snapping during anuran prey capture, suggesting that the medial reticular formation contains the premotor circuitry for feeding in frogs. We report here that hypoglossal afferents project to the medial reticular formation and this area may be where the interaction between vision and hypoglossal afferents arises.

4.2. Functional consequences

It has been shown that sensory information carried by the hypoglossal nerve modulates the phase of activity in the jaw muscles [1,21]. When the hypoglossal nerve is transected, *Rana pipiens* does not open its mouth when feeding on small prey items and the mouth does not open at all in *Bufo marinus*. Electromyographic studies demonstrate that the mandibular depressors and levators (the mouth opening and closing muscles) normally reach maximum activation asynchronously, with the mandibular depressors reaching peak activation 90–120 ms before the levators [1,21]. Following hypoglossal transection, these two muscles fire nearly simultaneously. The sensory neurons in the hypoglossal nerve control the timing of the opening of the mouth, and we have also shown them to be involved in motor program choice [2].

Previous studies [1,21] suggest that there is an inhibition of trigeminal motoneurons during prey capture allowing the levators mandibulae to contract in the proper temporal sequence. This inhibition is generated through hypoglossal afferent feedback. Therefore, hypoglossal afferents must converge at some point with trigeminal motoneurons. The nV nuclei appear more lateral than would be required for a direct synaptic connection (Fig. 4) [19,26]. However, this possibility cannot be excluded on the basis of the data presented here. In addition, there is most likely an interaction with nVII motoneurons (that innervate the mandibular levators). The nVII motor nuclei appear more caudal than is indicated by the hypoglossal afferent projections [19,26]. Based on these data, the most likely scenario would be a convergence of nV, nVII and nXII projections onto the reticular formation. Direct hypoglossal projections and nV and nVII projections into the reticular formation must be pursued further to definitively rule out these

alternative possibilities. The current findings establish the anatomical basis for the origins of these hypoglossal afferents that are coordinating behavioral modification of the frog feeding motor program.

4.3. Evolutionary considerations

During the evolution of a highly protrusible tongue, sensory neurons were re-routed to coordinate the timing of tongue protraction with the timing of mouth opening. As there are no afferent fibers intrinsic to the hypoglossal nerve, alternative pathways for afferent neurons to enter the tongue must have developed. These fibers come from vagal and cervical afferent neurons in mammals, and trigeminal afferents in finches.

In *Rana pipiens*, it appears that sensory projections from the hypoglossal nerve are cervical spinal neurons that have been re-directed into the hypoglossal nerve. Sensory neurons from the dorsal root of the third spinal nerve have anastomosed with the hypoglossal nerve to coordinate tongue and jaw movements. It has been hypothesized that a highly protrusible tongue has evolved independently multiple times among frogs [23]. Thus, it will prove interesting to compare these results in *Rana pipiens* to other anurans with highly protrusible tongues. Preliminary studies have shown that in the marine toad, *Bufo marinus*, hypoglossal afferents enter the brainstem at the level of the glossopharyngeal nerve and have their cell bodies within the Gasserian ganglion [24]. Thus, hypoglossal afferents have evolved independently even in two anuran species.

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