Abstract
Using injections of small molecular weight fluorescein dextran amines, combined with activity-dependent uptake of sulforhodamine 101 (SR101), brainstem circuits presumed to be involved in feeding motor output were investigated. As has been shown previously in other studies, projections to the cerebellar nuclei were identified from the cerebellar cortex, the trigeminal motor nucleus, and the vestibular nuclei. Results presented here suggest an additional pathway from the hypoglossal motor nuclei to the cerebellar nucleus as well as an afferent projection from the peripheral hypoglossal nerve to the Purkinje cell layer of the cerebellar cortex. Injections in the cerebellar cortex combined with retrograde labeling of the peripheral hypoglossal nerve demonstrate anatomical convergence at the level of the medial reticular formation. This suggests a possible integrative region for afferent feedback from the hypoglossal nerve and information through the Purkinje cell layer of the cerebellar cortex. The activity-dependent uptake of SR101 additionally suggests a reciprocal, polysynaptic pathway between this same area of the medial reticular formation and the trigeminal motor nuclei. The trigeminal motor neurons innervate the m adductor mandibulae, the primary mouth-closing muscle. The SR101 uptake clearly labeled the ventrolateral hypoglossal nuclei, the medial reticular formation, and the Purkinje cell layer of the cerebellar cortex. Unlike retrograde labeling of the peripheral hypoglossal nerve, stimulating the hypoglossal nerve while SR101 was bath-applied labeled trigeminal motor neurons. This, combined with the dextran labeling, suggests a reciprocal connection between the trigeminal motor nuclei and the cerebellar nuclei, as well as the medulla. Taken together, these data are important for understanding the neurophysiological pathways used to coordinate the proper timing of an extremely rapid, goal-directed movement and may prove useful for elucidating some of the first principles of sensorimotor integration.

Keywords
Hypoglossal nerve · Cerebellar nuclei · Cerebellar cortex · Sulforhodamine 101 · Anuran amphibian

Introduction
Recently much work has focused on identifying the descending premotor pathways involved in the generation of amphibian feeding motor patterns (Dicke et al. 1998; Ewert et al. 1999; Roth et al. 1999). Identifying the neuronal basis of these goal-directed movements is critical to our understanding of motor coordination tasks. In our studies, one major focus has been to identify the feedback mechanisms that occur during the initiation of feeding movements in frogs. Previously published data has shown the presence of a unique sensory pathway in the leopard frog, Rana pipiens, that originates from the tongue epithelium (Harwood and Anderson 2000) and is carried in the hypoglossal nerve (Anderson and Nishikawa 1997). This feedback pathway coordinates the timing of the tongue with the jaws so that the muscular system can properly time these movements during feeding (Anderson and Nishikawa 1993; Nishikawa and Gans 1992). Anatomical data has shown that these sensory neurons from the hypoglossal nerve project to a specific portion of the medulla, the medial reticular formation, and it is here that the integration of sensory and motor commands probably occurs before the muscle commands are sent out to the various skeletal muscles (Anderson and Nishikawa 1997; Weerasuriya 1989). In addition to this hypoglossal-reticular formation feedback pathway, hypoglossal afferents have been shown to project to the cerebellum (Anderson and Nishikawa 1997), a pathway likely involved in coordinating the proper timing of various postural and locomotor movements during feeding.

From an anatomical perspective, the afferent input into the anuran cerebellum has been reasonably well stud-
ied. A large number of fibers project into the cerebellum, including trigeminal fibers (Herrick 1948), trochlear nerve fibers, spinocerebellar fibers, bulbo-cerebellar tract fibers from various nuclei of the medulla, and tectocerebellar fibers originating from the presumptive undifferentiated inferior colliculus (Larsell 1923). In addition, there are cerebellar afferents from the vestibular system (Amat 1982; Hillman 1969; Larsell 1923; Llinas et al. 1971; Mateus 1979; Montgomery 1988), dorsal root fibers (Antal et al. 1980), multiple forms of somatic inputs from the sciatic and radial nerves (probably corresponding to afferent inputs from muscle spindles; Amat et al. 1984), as well as the glossopharyngeal-taste sensory system (Hanamori and Nobusada 1987a, 1987b). Therefore, it is not surprising to identify sensory information from the tongue (and carried in the hypoglossal nerve) that projects into the cerebellum. This evidence supports the hypothesis that the cerebellum may play a role in coordinating the timing of motor output during directed movement in frogs.

As part of a companion study to understanding the physiological role of the cerebellum during the generation of feeding movements in frogs, we investigated in more detail the anatomical connections between motor neurons innervating the tongue musculature and projecting to the medulla and cerebellum. Using retrograde dextran amine labeling as well as the activity-dependent dye sulforhodamine 101 (sulforhodamine 101), we describe evidence for an afferent projection from the peripheral hypoglossal afferents, not to the granular layer as previously reported, but rather to the Purkinje cell layer of cerebellar cortex in the leopard frog, Rana pipiens. Labeling the peripheral hypoglossal nerve combined with an injection into the cerebellar cortex double-labeled the same population of neurons in the medial reticular formation. Data are additionally presented illustrating a projection from the bilateral hypoglossal motor nuclei to the cerebellar nuclei.

Using bath-applied SR101 to a semi-intact brainstem preparation, the peripheral hypoglossal nerve was stimulated. This resulted in labeling of the ventrolateral hypoglossal nuclei, the raphe nuclei, the spinal trigeminal and trigeminal motor nuclei, the medial reticular formation, and the cerebellar cortex. These data suggest a reciprocal loop between the trigeminal motor nucleus and medial reticular formation. These anatomical pathways will be investigated physiologically to identify the exact role of the described pathways, but provide further evidence of the neuronal basis of feeding behavior in the leopard frog, Rana pipiens.

### Methods and Materials

*Rana pipiens* (n=17; 6.5–7.5 cm snout vent length) were obtained from a commercial supplier (Sullivan, Tenn., USA) and maintained in captivity. Neuronal labeling was done initially in an intact animal (peripheral hypoglossal nerve labeling), followed by an in vitro injection into either the cerebellar cortex or cerebellar nuclei. In five cases, a semi-intact cranial preparation was used in conjunction with bath-applied SR101 to investigate activity-dependent dye uptake following hypoglossal nerve stimulation.

To label the afferent projections traveling in the peripheral hypoglossal nerve, as well as the hypoglossal motor neurons, the frogs were anesthetized by immersion in 0.01% tricaine methane sulfonate (MS-222, 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 then transected distal to the branch that innervates the geniohyoid muscle to ensure that the afferent fibers labeled were only those that originated from the tongue and the retrogradely labeled motor neurons were those providing innervation to the geniohyoid muscle of the tongue. Either 3,000 MW fluorescein dextran amine (FDA) or 3,000 MW tetramethylrhodamine dextran amine (TMR; Molecular Probes, Eugene, Ore., USA) was used as the retrograde neuronal tracers. A few milligrams of the neuronal tracer was dissolved in a distilled water-DMSO solution and allowed to dry. The dried crystals are applied to the nerve stump using a fire-sealed, pulled glass micropipette and allowed to soak into the cut nerve for approximately 30 s. The cut nerve was then rinsed with distilled water and closed with veterinary grade cyanoacrylate surgical glue.

Initial transport time for the labeling of the hypoglossal nerve ranged from 4–6 h. Following this, the frogs were deeply anesthetized (0.01% MS-222), decapitated, and the upper spinal cord, brainstem, and cerebellum were exposed. Ten nanoliters of either 0.2% or 0.5% 3,000 MW FDA or TMR was pressure-injected unilaterally into either the cerebellar nuclei or the cerebellar cortex using a nanoliter injector (Fig. 1; WPI nanoliter injector with Micro 1 microsyringe pump; WPI Instruments, Sarasota, Fl., USA). In three cases, the hypoglossal nuclei (and thus motor neurons) was directly injected unilaterally, and double-labeled with a cerebellar cortex injection. To accomplish this, 10 nl of 0.5% TMR was pressure-injected into the ventrolateral hypoglossal nuclei using the same protocol. This was immediately followed by injecting the cerebellar cortex with FDA.

Following the injection, the preparations were flushed with cold Ringer's and then left in fresh, cold Ringer's for between 6 and 12 h, periodically changing the Ringer's solution. The brains were postfixied in cold 3% paraformaldehyde overnight. The brains were removed, mounted in gelatin, and sectioned at 50 µm on a sliding microtome with a freezing platform. The sections were mounted in phosphate-buffered saline and air-dried, dehydrated through an ethanol series, cleared in Hemo-de (Fisher Sci-