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## **DISTRIBUTION OF ACCESSORY AND HYPOGLOSSAL NERVES IN THE HINDBRAIN AND SPINAL CORD OF LUNGLESS SALAMANDERS, FAMILY PLETHODONTIDAE**

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Study of the innervation of the musculature related to feeding behavior in plethodontid salamanders by means of the horseradish peroxidase (HRP) technique has demonstrated the existence of a true spinal accessory nerve which innervates neck musculature, enters the brain via the ganglion of IX/X cranial nerves and has its motor neurons within the nucleus of the second spinal nerve. Further, it has been shown that the first spinal nerve, being strictly motor, alone constitutes the ramus hypoglossus and is, therefore, homologous to the hypoglossus of amniotes.

Amphibians are believed to possess only 10 cranial or cerebral nerves. The 11th nerve, or *accessorius*, which innervates branchiomic neck musculature (e.g. m. cucullaris/trapezius), is described both in anurans and urodeles to be part of the system of the 10th nerve (vagus) since it has its motor units in the hindbrain as the posterior part of the IX/X motor nucleus. No *spinal* accessory nerve, typical for amniotes, with motor units in the spinal cord at the level of spinal nerves 2–5, seems to be present [1, 4, 8].

The homologue of the 12th cranial nerve, or *hypoglossus*, which innervates hypobranchial musculature of the tongue and the throat region, is considered to be composed of the ventral roots of the first two spinal nerves in urodeles. An anastomosis exists peripherally between the first and second spinal nerves. Most authors believe that via this anastomosis fibers from the second spinal nerve join those of the first to form the ramus hypoglossus [1, 4, 8, 9]. In anurans the hypoglossus is constituted by the second spinal nerve alone [7].

Most species of the family Plethodontidae or lungless salamanders possess a highly developed feeding mechanism, a protrusible tongue that enables them to catch even fast moving prey [5, 6, 10, 11]. We studied the sensory motor control of the feeding behavior of two American species, *Batrachoseps attenuatus* (n = 20)

and *Bolitoglossa subpalmata* (n = 16), and of the European species *Hydromantes italicus* (n = 6). We systematically stained all nerves involved in the tongue reaction (cranial nerves VII, IX, X, XI and spinal nerves 1 and 2) [12]. Horseradish peroxidase (HRP, Boehringer, grade I) was applied in crystals to the cut stumps of the nerves both peripherally and near their entrance to the hindbrain. After a survival time of 48–72 h the animals were perfused with isotonic saline, followed by a solution of 1% glutaraldehyde + 1% paraformaldehyde + 2.5% sucrose in 0.12 M phosphate buffer (pH 7.4). The brains were rinsed in 0.1 M cacodylate buffer (pH 7.2) for 2 h. They were reacted as whole mounts in a solution of 0.2% diaminobenzidine (Sigma) + 0.01% H<sub>2</sub>O<sub>2</sub> in 0.1 M cacodylate buffer (pH 5.45) for 1 h. They

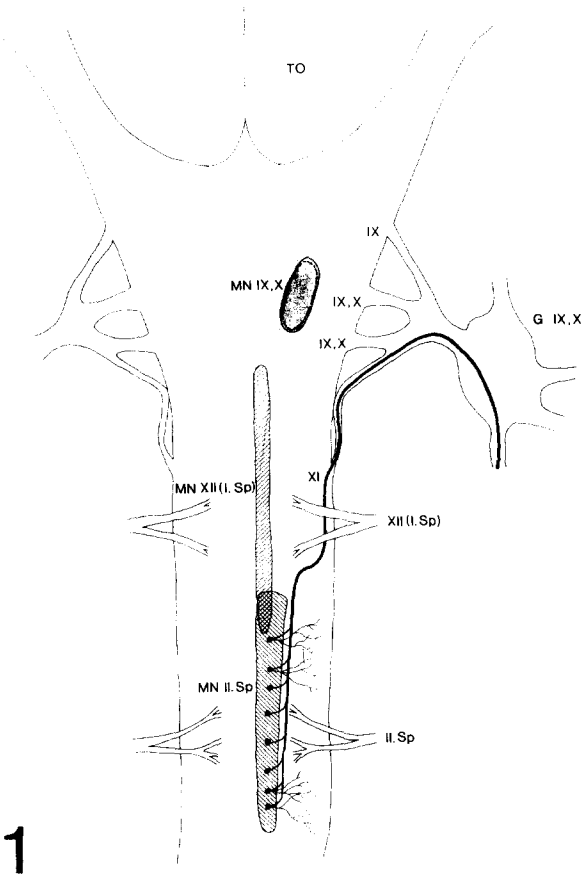


Fig. 1. Schematic drawing of the arrangement of cranial nerves IX, X, XI and XII/1st spinal and 2nd spinal nerves in the hindbrain and rostral spinal cord of the plethodontid salamander *Batrachoseps attenuatus*. Dorsal view. Notice the newly discovered spinal accessory nerve (XI) with its motor nucleus contacting the motor nucleus of the 2nd spinal nerve (MN II.Sp.). The elongate nucleus of the hypoglossal nerve (MN XII (I.Sp.)) occupies most of the motor column between the nuclei of the IX/X cranial and 2nd spinal nerves.

were cleared in cedarwood oil or methyl salicylate, photographed as whole mounts, embedded in Epon and cut in coronal sections of  $30\ \mu\text{m}$ . For further control of the

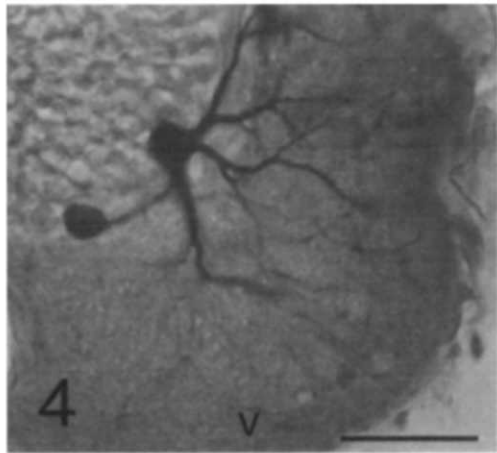
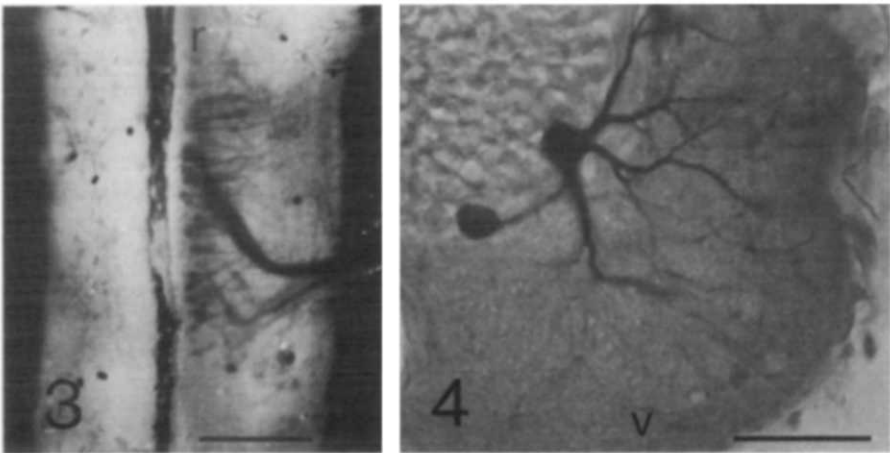
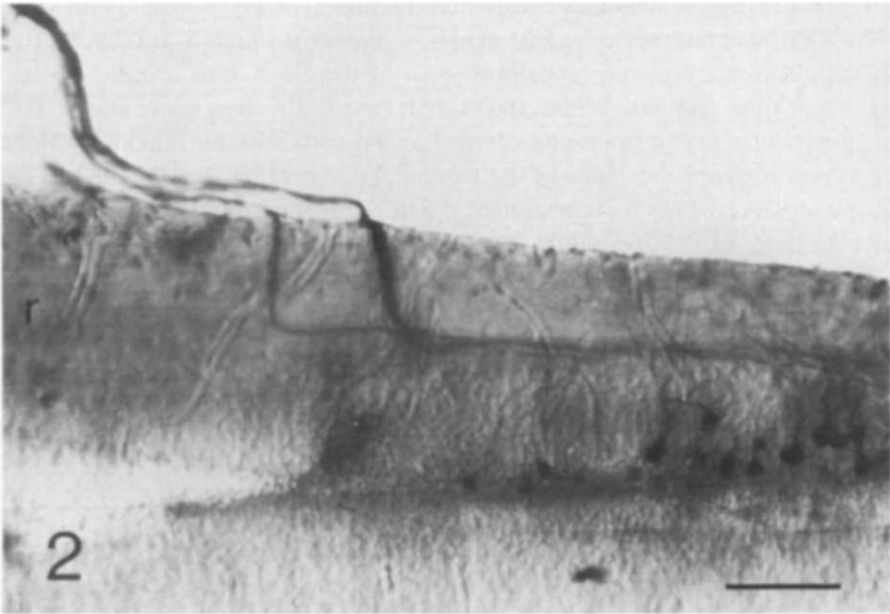


Fig. 2. Whole mount of the brainstem and rostral spinal cord of the tropical salamander *Bolitoglossa subpalmata*. The fibers of the spinal accessory nerve, cut and stained with HRP near the m. cucullaris, enter the brain as the last root of the IX-X root complex with two rootlets. At the right side retrogradely filled motor neurons can be seen. r, rostral. Scale =  $150\ \mu\text{m}$ .

Fig. 3. Whole mount of the brainstem and rostral spinal cord of *Batrachoseps attenuatus* showing the ventral root and motor nucleus of the 1st spinal/hypoglossal nerve. r, rostral. Scale =  $200\ \mu\text{m}$ .

Fig. 4. Higher magnification photograph of an individual motor neuron of the nucleus of the accessory nerve showing large arborization. v, ventral. Scale =  $100\ \mu\text{m}$ .

innervation pattern coronal sections stained by the Palmgren method were used.

The HRP experiments revealed the existence of a nerve that innervates the m. cucullaris and nearby branchiomic neck musculature. The nerve extends forward to the IX/X (glossopharyngeus/vagus) ganglion, passes through it and enters the brain at the obex region as the caudalmost root of the IX/X root complex, often showing two or three rootlets. Within the brain it runs to the deep white matter and proceeds posteriorly in the brain and cervical spinal cord without making contact with the motor nuclei of the vagus or the 1st spinal nerve. It reaches the nucleus of the 2nd spinal nerve where its retrogradely filled motor neurons are found, intermingled with those of the 2nd spinal.

The characteristics of this nerve are consistent with those of a true *spinal* accessory or XI cranial nerve, previously known only in amniotes: it innervates branchiomic neck musculature, enters the brain via the IX/X root complex and has its motor nucleus in the spinal cord.

The existence of a spinal accessory nerve described here might be restricted to plethodontid salamanders. This nerve was not found in the salamandrid species *S. salamandra* and *Notophthalmus viridescens* (Roth and Wake, unpublished). This could explain why it was not described previously in urodeles [2, 3].

The ramus hypoglossus was cut peripherally in the lower jaw region and near its entrance into the brain. The ventral root of the 1st spinal nerve was stained, and retrogradely filled neurons were found in a narrow, elongate region of the hindbrain and cervical spinal cord, extending from the level of the second vagus root to the midpoint between the ventral roots of the 1st and the 2nd spinal nerves. No dorsal root and sensory fibers were observed. Additionally, there was no staining of the dorsal and ventral roots of the second spinal nerve.

After cutting the anastomosis between the 1st and the 2nd spinal nerves, only cells within the motor nucleus of the second spinal nerve were found and no staining of the distal part of the ramus hypoglossus. We interpret these results as indicating that the ramus hypoglossus is formed by the 1st spinal nerve alone. Fibers from the 2nd spinal nerve which join the ramus hypoglossus through the anastomosis leave almost immediately to innervate the m. rectus cervicis profundus, the retractor of the tongue.

We conclude that the 1st spinal nerve of plethodontid salamanders is the homologue of cranial nerve XII, the hypoglossal, of amniotes. It is not a typical spinal nerve, as has long been recognized, for it has only a ventral root in adults, is strictly motor, and innervates only hypobranchial musculature. Its motor nucleus extends far forward into the hindbrain region. The 2nd spinal is the first of the series to have dorsal and ventral roots, as well as a dorsal ganglion.

Since both nerves described here are deeply involved in the guidance of the feeding behavior (XI by control of neck musculature and, therefore, head movement, 1st spinal/hypoglossus with respect to retraction of the tongue), it may well be that the formation or reorganization of these nerves is an adaptive phenomenon related to feeding mechanisms in plethodontids.

Our findings show that there is less distinction between urodeles and amniotes than formerly believed as far as nerve pattern is concerned. Furthermore, the traditional spatial distinction between hindbrain and spinal cord is unsatisfactory, artificial and not in accordance with functional and phylogenetic considerations.

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