Ultrastructure of Spinal Relay of Hypoglossal Afferents to the Parabrachial Nucleus

By

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Summary: Wheat germ agglutinin-conjugated horseradish peroxidase (WGA-HRP) injection into the hypoglossal nerve mainly resulted in retrograde labeling in the superior ganglia of the glossopharyngeal and vagal nerves ipsilaterally. Anterogradely labeled fibers were found in lamina I of the ipsilateral upper cervical spinal cord with a few distribution to laminae IV-V and VII-VIII. WGA-HRP injection into the PBN revealed intensive labeling of lamina I neurons of the upper cervical spinal cord ipsilaterally. These light microscopic observations appear to indicate the hypoglossal sensory inputs to the PBN through the spinal cord. In order to investigate the synaptic nature of this spinal relay, electron microscopic observations were carried out on lamina I of the first and second cervical spinal cord after cutting the hypoglossal nerve and WGA-HRP injection into the PBN in the same animal. The spinoparabrachial projection neurons were demonstrated to show a low cytoplasmic/nuclear ratio and have an oval or deeply indented nucleus with a centrally located nucleolus. Furthermore, dark and light type degenerating fibers were observed to make synaptic contacts with HRP-labeled somata and dendritic profiles.

Recent anatomical data have made possible a better understanding of the neural organization with respect to the motor and autonomic components in the hypoglossal nerve^{10,32,33}. Concerning sensory components, previous studies mainly focused on the location of ganglion cells^{4,31,37}. Subsequent studies revealed the central distribution of sensory fibers in the hypoglossal nerve utilizing transganglionic transport of HRP^{20,28}. In these works, lamina I of the upper cervical spinal cord was demonstrated to be one of the major target of the afferents. However, it is likely that there are significant lacunae about the secondary pathway of the sensory information. In particular, ascending projections from lamina I remain to be ascertained.

Lamina I, as well as marginal layer of the trigeminal spinal caudal subnucleus, contains c-fos immunoreactive neurons which are considered to play an important role in modulatory mechanisms related to nociception^{9,11,17,34}. On the other hand, lamina I neurons are reported to respond exclusively to noxious stimuli and send axons to the parabrachial nucleus (PBN)¹³. Taken together, these anatomical

and physiological findings seem to suggest that sensory information in the hypoglossal nerve is conveyed to the PBN through lamina I of the cervical spinal cord. Therefore, the present study was done to elucidate the synaptic morphology of the spinal relay on lamina I which connects between the primary afferent fibers in the hypoglossal nerve and the PBN in the cat.

Materials and Methods

Experiments were performed on nineteen cats weighing 0.7–4.6 kg and anesthetized with intraperitoneal injections of pentobarbital soda (40 mg/kg). The anesthesia was maintained through small doses of pentobarbital administered intraperitoneally.

Light microscopic experiments

One side of the neck was opened by a longitudinal incision and the hypoglossal nerve was exposed widely around the common carotid artery. After cutting the superior radix of the ansa cervicalis.

2–5 μ l of 0.2% WGA-HRP (Toyobo) dissolved in sterile 0.9% saline were injected into the hypoglossal nerve using a 10 μ l Hamilton microsyringe (11 cats). In the other experiment, 0.02–0.03 μ l of 5% WGA-HRP were injected stereotactically into the lateral portion of the PBN using a glass micropipette (tip diameter: 50–60 μ m) attached to a 1- μ l Hamilton microsyringe (3 cats). After a survival period of 2–3 days, the animals were deeply anesthetized again and sacrificed by perfusion through the ascending aorta with 0.1 M phosphate buffer (pH 7.4) followed by a fixative containing 8% formalin in 0.1 M phosphate buffer. The brain and spinal cord were removed from the skull immediately together with the trigeminal and geniculate ganglia, superior and inferior ganglia of the glossopharyngeal and vagal nerves, and C1–C3 spinal ganglia on the side ipsilateral to the injection. The lower brain stem through the hypoglossal nucleus, spinal cord and each ganglion were cut transversely at 60 μ m-thickness on the freezing microtome. All sections were processed for the demonstration of HRP reaction product according to the tetramethyl benzidine (TMB) protocol of Mesulum¹⁸⁾ and were counterstained with neutral red.

Electron microscopic experiments

The hypoglossal nerve was cut at the portion distal to the superior radix of the ansa cervicalis, followed 1–2 weeks later by injection of 0.02–0.03 μ l of 5% WGA-HRP into the lateral portion of the PBN on the side ipsilateral to dissection (3 cats). After a survival period of 2–3 days, the animals were sacrificed by perfusion with 0.1 M phosphate buffer followed by a fixative of 1% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). In order to confirm the injections of WGA-HRP into the PBN and hypoglossal nerve, 60 μ m-thick frozen sections through the lower brain stem containing the PBN and hypoglossal nerve were cut transversely and reacted with TMB. 200 μ m-thick transverse sections through the C1 and C2 spinal cord were made. These sections were immersed in 2% ammonium molybdate, dissolved in a 5% solution of 0.2 M acetate buffer (pH 3.3) for 30 min, to stabilize the HRP reaction products according to the method reported previously²⁷⁾. Then, the sections were postfixed in buffered 1% osmium tetroxide for 2 hours, block-stained with saturated uranyl acetate for 1 hour, dehydrated in a graded acetone series and embedded in Epon. Lamina I of the C1 and C2 spinal cord was identified on 1 μ m-thick sections stained with toluidine blue. Ultrathin sections through lamina I were observed without further staining in a JEOL 200 CX electron microscope.

Results

Light microscopic experiments

WGA-HRP injection into the hypoglossal nerve resulted in retrograde labeling through the whole of the hypoglossal nucleus (Fig. 1). With respect to labeling in the ganglia, a lot of neurons in the superior ganglia of the glossopharyngeal and vagal nerves were labeled with HRP ipsilaterally (Figs. 3 and 4). A few labeled neurons occurred in the inferior ganglion of the vagal nerve and spinal ganglion of the C1 segment. Fewer labeled neurons were found sporadically in the mandibular branch of the trigeminal ganglion, inferior ganglion of the glossopharyngeal nerve and spinal ganglia of the C2 and C3 segments. There was no labeling in the ophthalmic and maxillary branches of the trigeminal ganglion and geniculate ganglion. Central projections of the hypoglossal nerve were demonstrated utilizing trans-ganglionic transport of HRP. HRP-labeled glossopharyngeal and vagal afferent fibers entered into the brain stem from the lateral surface at the level from the facial nucleus to the inferior olivary nucleus ipsilaterally. These fibers were observed to distribute to the marginal layer of the spinal trigeminal caudal subnucleus and the solitary regions lateral and medial to the solitary tract across the dorsal portion of the spinal trigeminal tract. Other target areas of the fibers were found in the lateral region of the medial cuneate nucleus and commissural nucleus. From these glossopharyngeal and vagal fibers distributing to various areas in the brain stem, labeled fiber bundles were observed to descend through the dorsal portion of the spinal trigeminal tract. Labeled fibers could be traced as far as the C3 segment with a gradual decrease of labeling. It was of particular interest that in the cervical spinal cord labeling was exclusively confined to lamina I with a few distribution to laminae IV–V and VII–VIII ipsilaterally (Fig. 5).

In order to identify the cells of origin of the spinoparabrachial pathway, WGA-HRP was injected into the PBN. The site of injection was confined to the lateral portion of the PBN (Fig. 2). This injection resulted in retrograde labeling in lamina I with the ipsilateral predominance. Labeled neurons were characterized by the morphology of spindle or triangular shape and long dendrites extending mediolaterally (Fig. 6). This labeling was most conspicuous at the C1 segment. A few labeled neurons were also observed to be present bilaterally in the spinal trigeminal caudal subnucleus, solitary nucleus, medullary reticular formation and laminae IV–V and VII in the upper cervical spinal cord.

Electron microscopic experiments

After transection of the hypoglossal nerve, WGA-HRP was injected into the PBN in the same animal. The tracer enzyme was largely confined to the lateral portion of the PBN although a slight diffusion occurred into the ventral and medial portions of the PBN. 1 μ m-thick sections were prepared prior to electron microscopic observation. The region of lamina I, containing primary afferent fibers in the hypoglossal nerve and spinoparabrachial projection neurons, was identified under the light microscope. In the neuropil of lamina I, ultrathin sections revealed the typical morphology of transganglionic degenerating afferent fibers and retrograde labeling of somata and dendritic profiles. HRP-labeled neurons showed a low cytoplasmic/nuclear ratio and had an oval or deeply indented nucleus with a centrally located nucleolus. The relatively developed cytoplasm contained many electron-dense HRP reaction products (Figs. 7 and 8). These products were best visualized when lead citrate staining was omitted. Degenerating axon terminals were identified as the dark type which was characterized by irregular shape, dark matrix and swollen mitochondria (Figs. 9–13). The majority of these terminals had a long axis ranging from 0.8 to 2.1 μ m and frequently contained both small round and large dense cored vesicles (Figs. 9 and 12). A few degenerating terminals were classified as the light type which was mainly characterized by accumulation of small round vesicles and light matrix (Fig. 10). These degenerating terminals of the dark and light types made synaptic contacts with HRP-labeled somata (Figs. 9 and 10) and dendritic profiles of 2.5–4.1 μ m in diameter (Figs. 11 and 12). Fewer terminals were observed to synapse upon spine-like protrusions less than 1 μ m (Fig. 14).

Discussion

With respect to the location of the sensory cells of afferent fibers in the hypoglossal nerve, previous studies led to varied conclusions. Morphologically, the sensory cells were demonstrated to locate along the course of the hypoglossal nerve^{22,23,31,37}. In the other reports these cells were described to be present in the spinal ganglia of the cervical spinal cord^{4,14,35}, inferior^{30,37} and superior ganglion^{4,21} of the vagal nerve. Further anatomical studies have revealed the additional labeling of sensory cells in the trigeminal ganglion and superior ganglion of the glossopharyngeal nerve^{20,28}. On the other hand, physiologically, the existence of the afferent fibers was also proposed by recording of afferent impulses in the isolated filaments of the hypoglossal nerve^{2,6}, stimulation of the hypoglossal nerve evoked vascular reac-

tions^{8,30} and reflex activation of the facial^{16,29,36} and laryngeal muscles^{25,36}. This variety of anatomical and physiological findings apparently implies that there are various pathways of the afferent fibers in the hypoglossal nerve. However, considering the degree of labeling in ganglia, the glossopharyngeal and vagal nerves appear to give rise to major pathway of the afferents.

Central distribution of the afferent fibers in the hypoglossal nerve was elucidated exactly utilizing transganglionic transport of HRP^{20,28}. In these reports it is of particular interest that the fibers distribute exclusively to lamina I of the upper cervical spinal cord, because lamina I is considered to be the significant site for receiving nociceptive informations^{5,11,15}. In fact, expression of c-fos, enkephalin and substance P is found in lamina I dorsal horn and marginal layer of spinal trigeminal caudal subnucleus following stimulation of cutaneous sensory afferents^{9,11,17,26,34}. In the present study, ultrastructure of lamina I frequently revealed terminals which contained large dense cored vesicles. These vesicles in terminals are considered to be associated with the presence of biogenic amines and peptides as the neurotransmitter³ and play an important role in transmission of pain²⁶.

The present light and electron microscopic observations revealed the morphological characteristics of spinoparabrachial projection neurons. These neurons in lamina I, which are spindle or triangular in shape and have 2–3 long dendrites extending mediolaterally, are quite similar to those sending axons to the thalamus and hypothalamus^{12,13}. It is of interest that the pontine termination of these neurons is exclusively located to the lateral portion of the PBN which participates in regulation of cardiovascular^{7,19}, respiratory²⁴ and grooming activity¹. In the recent study, greater than 80% of neurons in lamina I projecting to the thalamus were demonstrated to have axon collaterals terminating in the PBN¹². Furthermore, physiological data led to conclusions that neurons in lamina I projecting to the lateral portion of the PBN respond exclusively to noxious stimulation¹³. The present study provided an anatomical substrate for spinal relay of afferents of nociceptive information in the hypoglossal nerve to the midbrain including the PBN.

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Abbreviations

BC	brachium conjunctivum	IXn	glossopharyngeal nerve
DAT	degenerating axon terminal	Xn	vagal nerve
HRP	horseradish peroxidase	I-IV	laminae I-IV of spinal cord
NUCL	nucleus		

Explanation of Figures

Plate I

Figs. 1 and 2. Photomicrographs of retrogradely labeled neurons in the hypoglossal nucleus after WGA-HRP injection into the hypoglossal nerve (Fig. 1) and the site of WGA-HRP injection into the PBN (Fig. 2). Note that the tracer enzyme is exclusively confined to the lateral portion of the PBN (asterisk). Calibration bars in Fig. 1 = 200 μ m, Fig. 2 = 0.5 mm.

Plate II

Plate II

Figs. 3 and 4. Photomicrographs of retrogradely labeled neurons in the superior ganglia of the glossopharyngeal (Fig. 3) and vagal nerve (Fig. 4). Calibration bars in Figs. 3 and 4 = 200 μ m.

Plate III

Figs. 5–7. Photomicrographs of HRP-labeled fibers (Fig. 5) and spinoparabrachial projection neurons (Fig. 6) which were exclusively confined to lamina I of the C1 spinal cord. Note that retrogradely labeled neurons were spindle or triangular in shape and had two or three dendrites extending mediolaterally (Fig. 7). Spinoparabrachial projection neurons in Fig. 6 (framed area) are magnified in Fig. 7. Calibration bars in Figs 5 and 6 = 300 μ m, in Fig. 7 = 100 μ m.

Plate IV

Figs. 8 and 9. Electron micrographs of spinoparabrachial projection neurons in lamina I of the C1 spinal cord. Note that these neurons contained electron-dense HRP reaction products in cytoplasm and had deeply indented (Fig. 8) or round nucleus (Fig. 9) with centrally located nucleolus. Calibration bars in Figs. 8 and 9 = 3 μ m.

Plate V

Figs. 10 and 11. Electron micrographs of degenerating axon terminals making synaptic contacts with HRP-labeled neurons (Figs. 10 and 11). Note that a few terminals were classified as the light type which was characterized by accumulation of small vesicles in light matrix (asterisk). Arrows and arrowhead indicate HRP reaction products in cytoplasm and large dense cored vesicle in degenerating axon terminal, respectively. Calibration bars in Figs. 10 and 11 = 0.5 μ m.

Plate VI

Figs. 12–14. Electron micrographs of degenerating axon terminals making synaptic contacts with HRP-labeled dendritic profiles (Figs. 12 and 13) and spine-like protrusion (Fig. 14). Arrows and arrowheads indicate HRP reaction products and large dense cored vesicles, respectively. Calibration bars in Fig. 12 = 1 μ m, Figs. 13 and 14 = 0.5 μ m.