Mapping of afferent and efferent pathways in the central nervous system using transneuronal transport of free horseradish peroxidase in bonnet monkeys

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To plot the position of second order neurons in the lingual proprioceptor path and to map the distribution pattern of sympathetic supply to a given viscus, HRP in Ringer's solution was injected into (i) various sites of the peripheral lingual proprioceptor pathway and (ii) one of the typical intercostal space muscles in bonnet monkeys. After appropriate post-injection survival period, the animals were sacrificed and relevant spinal segments were removed, transverse frozen sections were cut and processed for the demonstration of HRP labeled neurons in the medulla and the spinal cord respectively. From the results obtained it may be concluded that (i) under appropriate care, HRP can be used as a successful transneural marker to mark next order neurons both in the sensory and motor pathways; (ii) the dorsal nucleus of the vagus and the nucleus of the tractus solitarius are the probable sources of second order neurons in the lingual proprioceptor path and (iii) the sympathetic supply to the vasculature of a typical intercostal space is discrete.

BASED on the process of axoplasmic transport, many macromolecular tracer substances are used to explore the structural organization of the nervous system[1–4]. Of these, horseradish peroxidase (HRP) has proven to be one of the successful and promising tracers[5–8]. By virtue of its ability to travel both in the anterograde and retrograde directions in sensory and in motor axons, HRP has been used by many investigators to study the intricate circuitry of the nervous system since its introduction in 1971 (ref. 9). It has been pointed out that in contrast to nucleosides, amino acids and wheat germ agglutinin, free HRP appears to undergo transcellular transport very rarely[5]. Even when injected along with additives like poly-L-ornithine and penetrants like DSMO, HRP did not show marked transcellular transport, though its uptake was enhanced[10]. Long survival period, varying from 24 to 168 h following adminstration of HRP, did not yield appreciable transcellular labelling; possibly because of exocytosis of HRP from the target neuron[5]. This led to the conclusion that free or unconjugated HRP is not a favourable marker to define the pattern of neuronal connections. As a result, investigators started using either conjugated forms of HRP—example HRP–WGA (refs 2, 3, 4, 11) or certain viruses like herpes simplex virus and pseudorabies as the markers[12–14] to define the pattern of neuronal connections.

However, in the course of neural tract tracing studies in the Neurophysiology Laboratory, Christian Medical College and Hospital, Vellore, we found that, if adequate care is taken, unconjugated HRP can be used to mark higher-order neurons with precision by its transcellular transport in the sensory as well as in the motor pathways[8]. This potential of the marker was used in the present study to identify and map the distribution pattern of central neurons in specific sensory and motor paths in bonnet monkeys.

In the bonnet monkey, the peripheral pathway for proprioception from tongue muscle is fairly well established[15]. However, the sources of second-order neurons in this pathway remain obscure. Bowman[16] is of the view that the cuneate nuclei are the sources of second-order neuron. There is no concrete evidence in favour of this view. In light of this, it was decided to locate the central nucleus containing second-order neurons of this particular sensory pathway, by injecting free HRP into a specific site of its peripheral path in bonnet monkeys.

On the basis of extensive work, it has been established that motor innervation to any skeletal muscle is very specific; i.e. distribution patterns for motor system innervating voluntary muscles are defined[17]. However, this has not been established for motor system innervating smooth muscle and glands. This may be due to the fact that unlike the somatomotor system, the autononic motor system is a two-neuron system and access to it for any anatomical study is not easy; since its distribution is confined to deeply-placed structures. Studies so far conducted on this part of the nervous system speak of the

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location of preganglionic neurons and the target areas they supply[17]. However, nothing is said about its distribution pattern, i.e. whether innervation to any given viscous or gland is very specific or diffuse. In the light of this lacuna in the literature, it was decided to map the distribution pattern of preganglionic neurons that innervate the vasculature of a particular segmental region (any one typical intercostal space) in bonnet monkeys, using transneural transport of free HRP.

Materials and method

Adult male bonnet monkeys (*Macaca radiata*) were used for the study. All experiments were performed under aseptic conditions. Monkeys were anaesthetized by intravenous injection of ketamine hydrochloride (2 to 5 mg/kg body weight) along conjunction with diazepam (10 mg in 2 ml solution). Further maintenance doses were given as and when required.

Surgical and injection procedure

(i) *For tracing central connections of tongue proprioceptors*: In a given experiment the carotid triangle of any one side was opened. The hypoglossal nerve, the vagus nerve, and the two roots of ansa cervicalis were exposed. All the central and peripheral connections to the hypoglossal nerve, excepting the connections with the ansa cervicalis and with the first cervical spinal nerve, were severed. The vagus nerve was cut very close to its exit from the skull. The segment of the hypoglossal nerve, distal to the origin of the superior root of the ansa cervicalis, was crushed well with artery forceps to enhance uptake of HRP (Figure 1)[5]. Using a 26 gauge hypodermic needle, about 40 mg of HRP (Sigma VI) dissolved in 0.5 ml of Ringers solution was injected into the crushed segment of the hypoglossal nerve (Figure 1). In all experiments cotton padding moistened with saline was used to cover exposed tissues other than the site of injection to absorb HRP spill if any. By this means, transport of HRP through systemic circulation and possible erratic labelling of nervous elements was excluded. The distance between the site of injection and the medulla oblongata was measured. The wound was sutured and the animal was allowed to recover in the cage.

(ii) *For mapping preganglionic neurons innervating vasculature of a typical intercostal space*: Under anaesthesia, a typical intercostal space on any one side was exposed from sternal end to the angle of the rib. Intercostal muscles of the exposed space were moistened with Ringers solution. Using a 26 gauge hypodermic needle, about 40 mg of HRP in 5 ml of Ringers solution was injected into the intercostal muscles at multiple points covering the entire exposed area. For identification purposes, a stitch was placed in the intercostal muscles of the space immediately cranial to the one where HRP was injected. The distance between the site of injections and the corresponding spinal cord segment was measured. The wound was closed and the animal was allowed to recover in the cage.

After five trial experiments, the optimal time for HRP travel was estimated. Based on this and also based on Mesulam's recommendation[5], the animals were allowed to survive for a period ranging from 24 to 36 h and occasionally 48 h.

Perfusion and removal of tissue

After anaesthetizing the animal with ketamine hydrochloride, about 5000 units of heparin was given

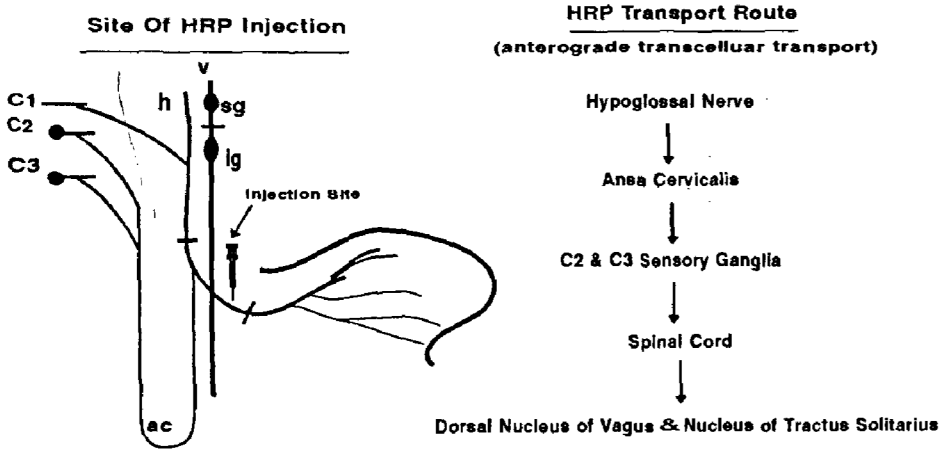

Figure 1. Path of tongue proprioceptor. C, C1, C2: Cervical nerves and ganglia; v: vagus nerve; sg: superior ganglia; ig: inferior ganglia; h: hypoglossal nerve; ac: ansa cervicalis; —: sites of nerve transection.

intramuscularly. The medulla oblongata or the spinal cord (as required) was fixed by intra-aortic perfusion with paraformaldehyde and glutaraldehyde mixture followed by 3M sucrose solution for cryoprotection in phosphate buffer (pH 7.4).

In order to identify the location of the second-order neurons belonging to tongue proprioceptor pathway, the medulla oblongata was removed *in toto* and the non-injected side was marked by a longitudinal cut. In animals where intercostal muscles were injected with HRP, the ventral roots of the relevant spinal segment were identified and traced to the cord. The segment of interest along with one segment cranial and one segment caudal to that was removed. A longitudinal cut was placed on the non-injected side of the entire length of spinal segments which had been removed to identify the injected side in sections. The tissues were stored in 3 M sucrose solution at 4°C overnight.

Sectioning and processing

Fifty micrometer thick transverse frozen sections were cut. While the sections of medulla oblongata were not serial, those of the spinal cord were serial. The sections were processed histologically using tertramethyl benzidine as chromogen according to the method described by Mesulam[5]. The sections were mounted on glass slides pretreated with chrome alum, dried and counterstained with neutral red solution. All sections were analysed under light microscope, and the results were documented.

Results

In experiments which were conducted for tracing the tongue proprioceptor pathway, labelled neurons were found in the dorsal nucleus of vagus and in the nucleus of the tractus solitarius, the post-injection time period being fixed to about 36 h. In all the cases the labelled neurons were found located bilaterally. Since the sections were not serial, the distribution pattern was not pictured. However, it was noticed that ipsilateral nuclei showed more number of labelled neurons. Labelled neurons in the dorsal nucleus of vagus exceeded those found in the nucleus of the tractus solitarius. None of the other sensory nuclei in the medulla concerned with proprioceptive sensation showed marked neuronal somata or nerve processes.

In experiments done for labelling preganglionic neurons innervating vasculature of a specific intercostal muscle, it was found that both ipsilateral and contralateral side ventral horns showed labelled neurons in Rexed lamina IX. In addition, the preganglionic sympathetic neurons of the ipsilateral lateral horn showed HRP marked cells, the post-injection time interval being between 32 and 40 h. Nerve processes of these two areas were also labelled. However, cells of the dorsal column did not take up the label. Labelled neurons – both in the ventral as well as in the lateral horns – were confined solely to the spinal segment concerned (i.e. the segment that innervates the intercostal muscles and vessels of the injected intercostal space).

Discussion

The present study attempted to answer two questions: one pertaining to the proprioceptor sensory pathway in the cranial region, and the other pertaining to the autonomic motor distribution to a specific organ (blood vessel specific to a specific region) by using the method of transneuronal transport of free HRP.

As shown in Figure 1, HRP injected into the hypoglossal nerve reached the vagal nucleus and the nucleus of the solitary tract anterogradely: (i) by transganglionic transport at the cervical dorsal root ganglia of C2 and C3, and (ii) by transcellular transport at the junction of the nerve fibres originating from the spinal cord to neurons of the vagal and the solitary tract nuclei/at the junction of fibres originating from the cells of the C2 and C3 ganglia with these two cranial nuclei. Since the spinal segments of C2 and C3 were not processed for demonstration of labelled neural elements in this set of experiments, it is not known whether crossing of the neural barrier by the marker occurred in the spinal cord itself. The authenticity of the conclusion drawn in this study was based on results obtained by conducting experiments in twelve adult monkeys under the same experimental conditions.

It is therefore suggested that in the bonnet monkey neurons of dorsal nucleus of the vagus and the nucleus of the tractus solitarius are involved in processing sensory input from tongue muscles. This is contrary to the earlier view that the rostral projections of lingual proprioceptors is through neurons of the cuneate nuclei[8-20]. However, it has some relevance with the findings of Wozniak and Young[21]. According to these authors, afferents present in the hypoglossal nerve are relayed to neurons of the hypoglossal nuclear complex, and to the tractus solitarius nucleus. Further, it is stated that the dorsal nucleus of vagus is a mixed nucleus containing two types of neurons; one type innervating smooth muscle and the other type is possibly concerned with visceral sensation[17]. The nucleus of the tractus solitarius represents the centre for gustatory and general visceral sensation[17]. On these bases it may be reasonable to argue that the dorsal vagal nucleus and the nucleus of the tractus solitarius are probable sites for central projection of hyoglossal afferents.

Figure 2 shows the route taken by HRP to label the neurons of spinal cord when injected into intercostal

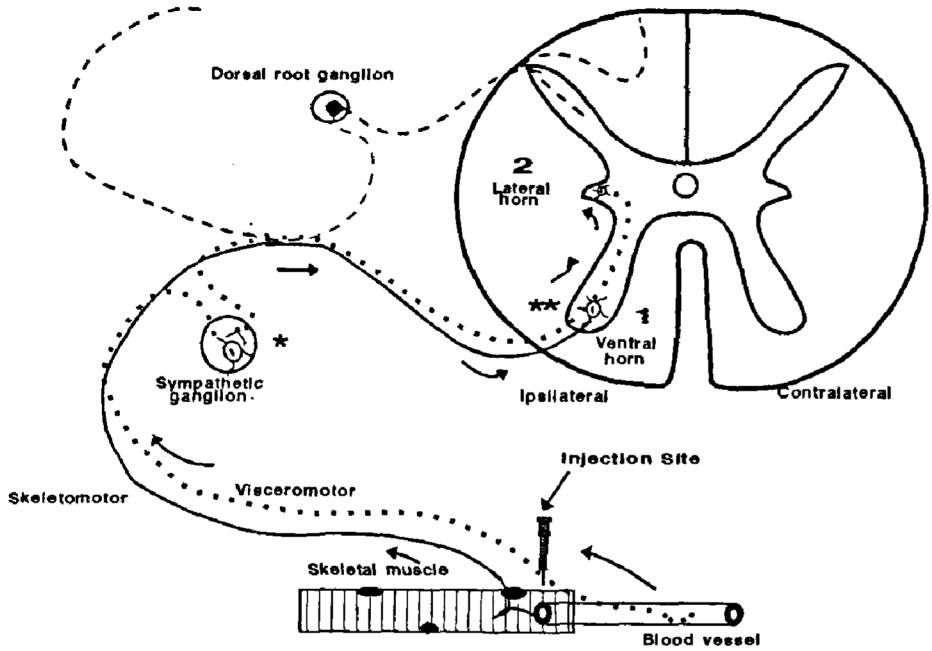

Figure 2. Pattern of visceromotor. →: retrograde transport of HRP; *,**: sites of transneural transport of HRP; 1,2: sites of location of HRP labelled neurons.

muscles of a typical intercostal space. The marker was picked up by the motor terminals present in the skeletal muscles and in the smooth muscles of the blood vessels and transported retrogradely to the concerned neural somata: (i) in the ventral horns (motoneurons), and (ii) in the paravertebral sympathetic ganglion/ganglia. Presence of labelled cells in the lateral horn indicates that after reaching the sympathetic chain the marker had crossed the cellular barrier retrogradely within the ganglion and was transported again in the retrograde direction to reach the lateral horn neurons. So far tracing second-order cells (or higher-order cells) along the autonomic pathways has been done using pseudorabies virus or herpes simplex virus; the virus has been injected into the cortex of the kidney and second-order cells in the lateral horn of the thoracic spinal cord have been recovered[12–14]. Dehal *et al.*[12], Schramm *et al.*[13] and Joshi *et al.*[14] have also reported contralateral marking of sympathetic preganglionic neurons unlike ipsilateral marking of neurons in the present study.

The cranio caudal extent of the lateral horn containing labelled neurons was mapped. When compared with the spread of labelled ventral horn neurons, it was found that labelling of the neurons in the lateral horn was confined to the segment concerned and did not extend into neighbouring segments. This suggests that sympathetic innervation to the vasculature of the given intercostal space is segmental. In other words, its distribution is discrete in this case.

Transneural transport of free HRP in the anterograde and in the retrograde directions to label somata of higher-order neurons is so far not reported in the literature. In this way the present study is the first of this kind.

From the above results regarding sensory pathway and motor distribution pattern, the following conclusions may be drawn: (i) HRP, in its free state can be used for marking higher-order neurons both in motor and in sensory system by transcellular transport, provided travelling time is standardized. (ii) There is no obvious time difference in marking second-order neurons by anterograde or by retrograde routes, though it is said that the marker travels at a faster rate in the anterograde direction than in the retrograde direction[5].

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MEETINGS/SYMPOSIA/SEMINARS

31st Indian Pharmacological Society Conference (IPS-1998)

Date: 18–20 December 1998
Place: Lucknow

The meeting will cover a wide spectrum of topics related to basic aspects of drug action, receptors, ion channels, new drug development, drug toxicity and side effects, pharmacokinetics, clinical pharmacology, and bioinformatics.

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National Seminar on Bayesian Analysis: Theory and Application (NSBA-TA)

Date: 16–18 January 1999
Place: Amravati

The seminar intends to provide a thorough discussion on the theory and applications of the Bayesian Statistics. The delegation at the seminar will cover the use of Bayesian ideas in various fields of statistics like statistical inference, prediction, classification, design of experiment, time series, quality control, survival analysis, software reliability, etc.

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DAE Solid State Physics Symposium

Date: 27–31 December 1998
Place: Kurukshetra
Subject categories included in the symposium: Phase transitions; Complex systems; Novel materials; Experimental techniques and instrumentation; Liquids, glasses and amorphous systems; Relaxation studies; Surface science; Phonons; Electronic structure; Superconductivity; Magnetism; Transport properties; Semiconductor physics.

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