EFFECT OF SURVIVAL TIME ON RETROGRADE NEURONAL LABELING WITH HORSERADISH PEROXIDASE (HRP), USING RAT'S FACIAL NUCLEUS AS A MODEL

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SUMMARY

The effect of survival time on horseradish peroxidase (HRP) histochemical procedures has been commented upon in the past but has never itself been the subject of systematic study. We studied the effect of this variable as a potential source of inconsistencies in the final results of tracing neural pathways. The facial nucleus of five male albino rats was studied as a model. To select a suitable survival time and to observe the effect of different survival periods on neuronal labeling in the facial nucleus in general, HRP was applied to the proximal stump of the transacted facial nerve. After a post operative survival time of 24, 36, 48, 72 and 96 hours, the other variables of the procedure remaining constant, the tissues were processed to visualize the transported enzyme. The animal with survival time of 48 hours showed discrete and intensive labeling patterns in the ipsilateral facial nucleus with good identification of somata and their processes. In view of the superior quality labeling in the majority of neurons (4600 in the present study), a 48 hours survival period was selected as a standard for similar studies.

INTRODUCTION

The retrograde transport of horseradish peroxidase has recently become a widely used technique for tracing neuronal connections and determining the cells of origin in various peripheral and central nervous system pathways. A brief comparison with other methods illustrates some of the advantages of this technique. The principal experimental technique available to neuroanatomists for identification of cell bodies of origin of axons projecting to particular region rests on the appearance of certain "retrograde" changes in the cell body of neurons whose axones have been interrupted.[8,9] The HRP method may eliminate the question of complicating fibers of passage. Another handicap of the retrograde cell degeneration method lies in the fact that not all neurons show chromatolysis or die following injury to their axons; presumably the non reacting cell bodies are sustained by intact collateral axone branches.[4,5] This does not appear to be a problem using the retrograde transport method. A further difficulty with the previous method has been the possible involvement of anterograde trans-synaptic changes. Since HRP appears not to be transported in an anterograde direction as judged by light microscopic criteria, this is not a complicating factor.[11]

Despite standardization of experimental conditions, considerable variation can be observed in the extent of labeling by the horseradish peroxidase method.[7] In addition, certain studies also report variable results

TABLE – I

THE EFFECT OF DIFFERENT SURVIVAL PERIODS ON THE NUMBER AND DEGREE OF LABELING IN THE FACIAL MOTOR NUCLEUS

S. No.	Animal	Survival period OBEX	Distance of FMN FMN	Restrocaudal extent of	No. of labeled	Degree of labeling
1.	R-6	24 hours	1.28 mm	1.40 mm	3536	+
2.	R-7	36 hours	1.28 mm	1.36 mm	3312	++
3.	R-8	48 hours	1.28 mm	1.44 mm	4600	+++
4.	R-9	72 hours	1.32 mm	1.72 mm	4195	++
5.	R-10	96 hours	1.28 m	1.24 mm	3285	+

Key: FMN = Facial motor nucleus;

+++ = Heavy labeling of both soma and neurits;

++ = Faint labeling in few of the labeled cells;

+ = Faint labeling in most of the labeled cells.

with respect to the number of cells labeled by the retrograde transport and in the grain density within the cell bodies.[1] Various factors in certain pathways and the rapidity of inactivation of enzyme by cellular mechanisms have been invoked to explain the variations in results obtained with the HRP method.

Another important factor, which may affect the number of labeled cells as well as the intensity of labeling, is the length of the survival period. The rate of retrograde transport varies somewhat between species and fiber systems but is generally of the order of 60-100mm/day.[13] Depending upon the length of the axons under study as well as certain other factors, a survival period of 24 hours to 2-3 days was usually best suited in studies of this kind. Apart from axonal length, transganglionic route added further to the problem of selecting a suitable survival time. The present study was aimed at finding the effect of different survival periods on the extent and degree of neuronal labeling and to conclude a standard survival time.

MATERIALS AND METHODS

In the present experimental study five male 60-120 days old albino rats (Sprague-Dawley strain), weighing between 150-300gms were used.

Surgery and HRP application:

The animals were deeply anaesthetized with ether and intraperitoneal injection of 7% solution of chloral hydrate in a dose of 35mg/g body weight.[18]

The dorsolateral, retroauricular aspect of the upper neck was dissected to expose the facial nerve trunk. The nerve was dissected free of surrounding connective tissue, blood vessels and epineural sheath. The main trunk of the nerve was cut obliquely before its entrance into the parotid gland. The proximal stump was mounted on a dry parafilm "M" sheet to prevent leakage of the enzyme. The axonal fascicles were exposed by everting the epineural sheath of the stump towards the stylomastoid foramen.

HRP (Sigma type-VI, Sigma chemical C0. St. Louis, USA) in the form of 25%

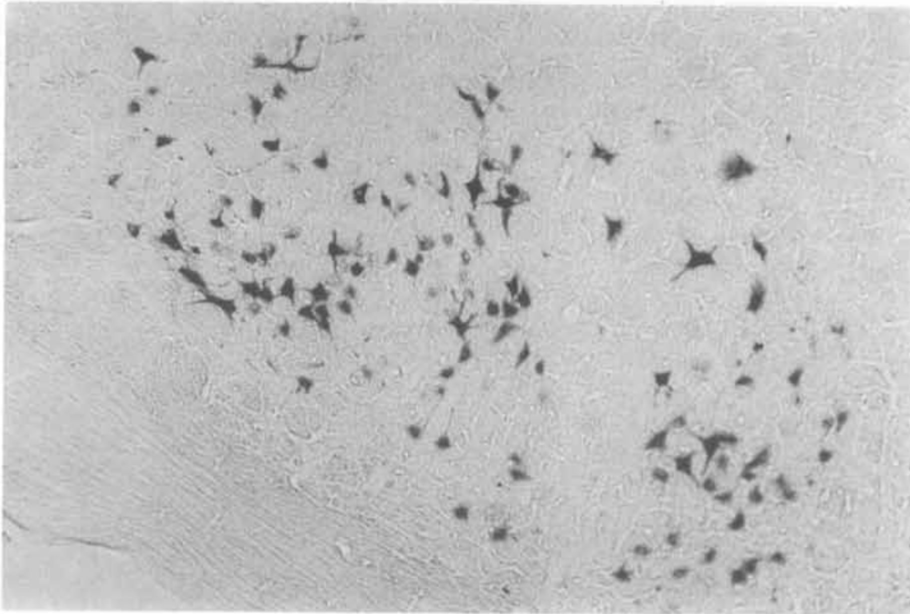

Fig. 1. Photomicrograph of 40-micron thick transverse section through the middle level of facial nucleus showing sparse retrograde labeling following application of HRP to the central cut end of the facial nerve trunk and a survival period of 24 hours. The section was counterstained with neutral red X 500.

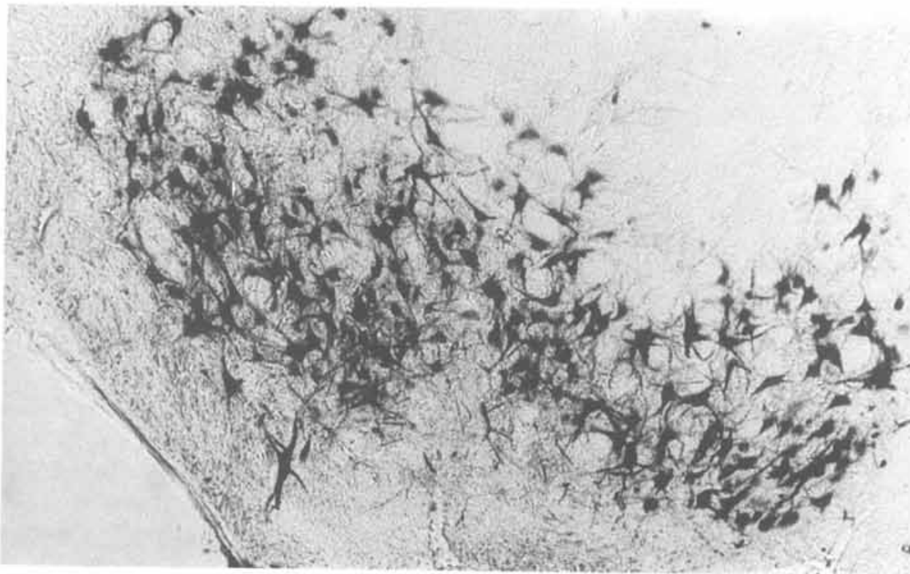

Fig. 2. Photomicrograph of 40-micron thick transverse section through the middle level of facial nucleus after expsure to HRP of the proximal cut end of the facial nerve and a survival time of 48 hours. It is clear that this survival time which we recommend in this report demonstrates many more labeled neurons. The section was counterstained with neutral red X 500.

solution in 0.9% physiological saline was applied to the proximal cut end of the nerve at frequent intervals for 1-1/2- 2 hours. During exposure to HRP, the nerve stump was kept moist and gently pinched to avoid axonal blockage.[3] After the application of HRP, the nerve stump and operative field were thoroughly washed with saline. The wound was closed in layers and the animals allowed to recover form anaesthesia.

Survival period:

In order to investigate the difference in the retrograde axoplasmic transport of the tracer, the animals were allowed survival period of 24,36,48,72 and 96 hours respectively. After the survival period, the animals were deeply reanaesthetized with ether and perfused through the left cardiac ventricle with physiologic saline followed by a fixative composed of 1.25% glutaraldehyde and 1% paraformaldehyde in 0.2M phosphate buffer (pH 7.4). The brain stem was then removed and stored over night in 30% sucrose phosphate buffer solution. Transverse serial frozen sections of the brain stem and associated ganglia were made at 40-um thickness and treated for the detection of HRP activity by the tetramethyl benzidine method.[15] The incubating procedure consisted of a 20-minutes prereaction soak in medium without substrate and a subsequent 20-min immersion the same solution to which 0.5ml 0.3% hydrogen peroxide/100ml medium was added as the substrate. The incubation was at 19-23°C; the medium had the same composition as Mesulam described.[16] After the histochemical reaction, the sections were mounted on glass slides and counterstained with 1-% neutral red.

Morphometeric analysis:

The sections were examined under a light microscope at a magnification of x4 for labeled neurons. The sections with no labeling were excluded from the study. The positive slides were then examined under higher power objective lenses such as x80 and x320, for details of neuronal morphology.

The counting of neurons was done by the "Profile count" method.[6] The facial nucleus was visualized and focussed initially by scanning through the section. Only those neurons were counted which were either homogeneously filled with reaction product or showed a centra halo; the representative of nucleus.

RESULTS

After HRP application, the five selected animals were allowed to survive for 24,36,48,72 and 96 hours. The extent of labeled nuclear column, number and quality of labeled cells both in the facial motor nucleus and ganglia showed significant difference. The maximum number of labeled motor neurons (4600) was obtained after 48 hours survival period, which was significantly higher than the rest of the counts. Similarly a greater number of labeled sensory and postganglionic sympathetic neurons were obtained after 48 hours survival of the animal.

Majority of the facial motoneurons displayed intensive labeling after 48 hours survival period so much so that in most cases their nucleoli as well as nuclei were obscured by the accumulated HRP-TMB reaction product. Some cells were so well filled with the marker granules that fine cytological details such as dendritic processes and collaterals were also visible. This type of high degree of filling also occurred in ganglionic neurons. The count of labeled neurons obtained after survival periods other than 48 hours though acceptable was comparatively poor in intensity of labeling. The same was true in case of sensory and postganglionic sympathetic neurons. The labeled nuclear column length obtained after 72 hours survival time was few sections longer than that obtained after 48 hours, but it was not significant enough to affect the final selection of standard survival period.

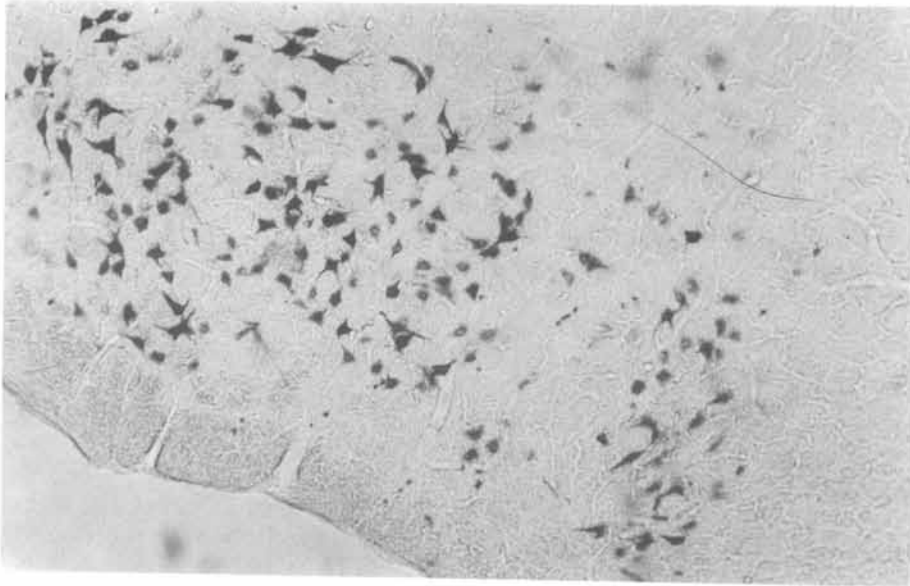

Fig. 3. Photomicrograph of transverse section through the mid-level of facial nucleus showing retrograde labeling of neurons; the animal had a survival time of 72 hours. 40-micron section X 500. Section was counterstained with 1% neutral red.

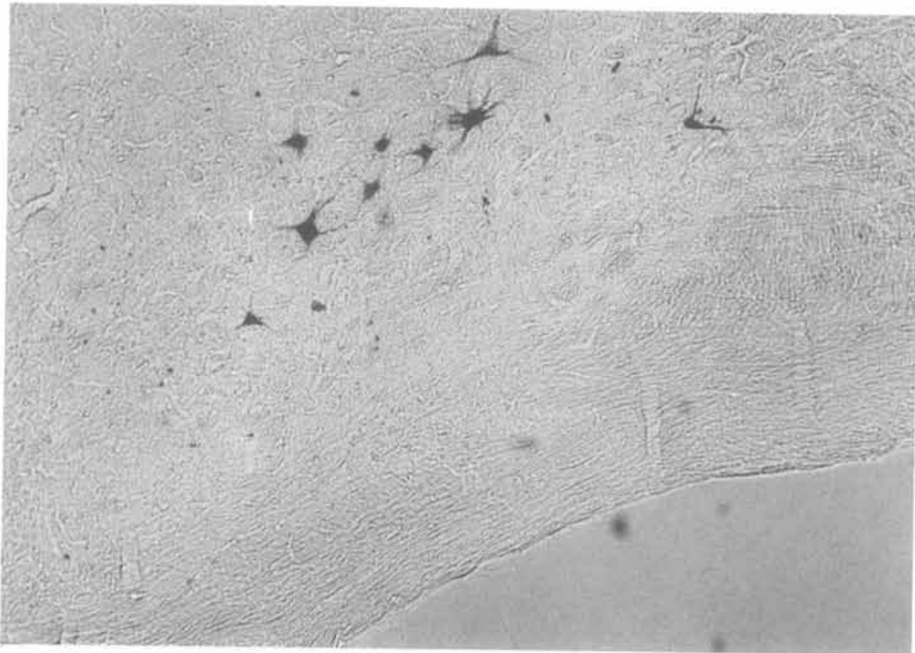

Fig. 4. Photomicrograph (X 500) of 40-micron thick transverse section through the middle of the facial nucleus showing few retrogradely labeled cells after HRP application to the central cut end of the facial nerve trunk and a survival period of 96 hours. Section was counterstained with 1% neutral red.

The above finding provides enough evidence to select 48 hours as a standard survival time for HRP studies on facial and other nerves of similar length.

DISCUSSION

Research methods based on degeneration[8,9] have been replaced by much more sensitive techniques that reveal both cells of origin and sites of termination of axons. The results of the extensive use of methods based on axoplasmic transport have currently necessitated a substantial revision of earlier accounts of neuronal connections in the central nervous system. These recent techniques take the advantage of the uptake and transport of proteins and other substances. A histochemically detectable protein or a suitable fluorescent dye is injected into the region concerned. The foreign molecules are imbibed and retrogradely transported to the corresponding perikarya. The protein tracer is localized by histochemical means, thus revealing the neuronal cell bodies that innervated the site of injection.[13] The first protein to be used extensively as a tracer in this way was the enzyme peroxidase, extracted from the root of horseradish plant. With the development of increased sensitivity in methods for the histochemical detection[15] of peroxidase, it has become possible to study the anterograde as well as the retrograde transport of tracer protein.[16] The method therefore provides, for a smaller investment of time and effort, results comparable to those obtained by autoradiographic method.

In experiments based on the direct histochemical detection of HRP, the distribution of reaction product at the time of microscopic examination provides the only information for detecting the site of HRP administration, the distribution of resultant transport, and consequently, the pattern of neural connectivity. The accuracy in determining neural pathways is therefore influenced by a number of factors related to the enzymatic activity of HRP.[12]

Survival time is one of the most important factors, which determine the outcome in HRP experiments. It depends not only on the axonal length but also whether or not a transganglionic route is involved. Survival periods in the range of 24-72 hours are more likely to produce better results in the central nervous system.[5] However, special considerations become necessary when longer peripheral pathways are under study. Selection of 48 hours survival period correlates well with the observation of Kim and Strick.[12] Studying the effects of different survival periods on neuronal labeling in the lateral geniculate nucleus and visual cortex, these authors found more labeled neurons in animal sacrificed after 48 hours postoperatively than 72 hours. The poor quality of labeling in the facial nucleus after 24 hours survival might be due the fact that the label might not have effectively accumulated in the neuronal soma which is in contrast to the observation of Mesulam and Brushart[14] who found maximum labeling 24 hours following HRP application to the severed sciatic nerve of rat at mid-thigh. The difference might be attributed to the difference in the diameter and number of motor fibers in the two nerves. In addition, the zig zag route followed by the facial axons may also account for the slow transport of the enzyme. The less intense facial neuron labeling after 72 hours survival of the animal may be due to lysosomal degradation of the HRP-TMB reaction product which is in complete accord with the observation of Mesulam[16] and La Vail and La Vail.[13]

Though apparently randomly selected, most of the previous experiments on sympathetic,[10] sensory[17] and motor components[2] as well as somatotopic organization of the facial nerve[18] have used 48 hours as survival period which stands in good accord with the same parameter of our study.

Studying musculotopic organization of the facial nucleus, Welt and Abbs[19] initially perfused two monkeys after 24 hours, which produced discouraging results. A 48 hours survival, which produced improved labeling pattern, was used in subsequent animals.

In conclusion, we have standardized the survival period i.e. 48 hours in neuron marker studies performed on nerves having axonal length and diameter similar to that of the seventh cranial nerve.

REFERENCES

1. Adams JC. Technical considerations on the use of horseradish peroxidase as a neuronal marker. Neurosci. 1977; 2: 141.
2. Baisden RH, Woodsuff ML, Whittington DL, Baker DC, Benson AE. Cells of origin of the branches of the facial nerve.: a retrograde HRP study in the rabbit. Am J Anat. 1987; 178: 175.
3. Baulac M, Meininger V. Postnatal development and cell death in sciatic motor nucleus of the mouse. Exp Brain Res. 1983; 50: 107.
4. Brodal A. Modification of Gudden-method for study of cerebral localization. Arch Neurol Psych. 1940; 43: 46.
5. Bordal A. Neurological anatomy in relation to clinical medicine 3rd edition, oxford University Press, New York, 1981; 4: 448.
6. Coggeshall RE, Lekan HA. Methods for determining number of cells and synapses: A case for more uniform standards of Review. J Comp Neurol. 1996; 364: 6.
7. Courville J, Saint CYR JA. Modification of the horseradish peroxidase method avoiding fixation. Brain Res. 1978; 142: 551.
8. Cragg BG. What is the signal for chromatolysis? Brain Res 1970; 23: 1.
9. Fry FJ, Cowan WM. A study of retrograde cell degeneration in the lateral mammillary nucleus of the cat, with special reference to the role of axonal branching in the preservation of the cell. J Comp Neurol. 1972; 144: 1.
10. Johansson K, Arivdsson J, Thomander L. Sympathetic nerve fibers in the peripheral sensory and motor nerves in the face of rat. J Auto Nerve Syst. 1988; 23: 83.
11. Jones EG. Possible determinants of the degree of retrograde neuronal labeling with horseradish peroxidase. Brain Res. 1975; 85: 249.
12. Kim CC, Strick Pl. Critical factors involved in the demonstration of horseradish peroxidase retrograde transport. Brain Res. 1976; 103; 356.
13. La Vail JH, La Vail MM. The retrograde axonal transport of horseradish peroxidase in the chick visual system: A light and electron microscopic study. J Comp Neurol. 1974; 157: 303.
14. Mesulam MM, Brushart TM. Transganglionic and anterograde transport of HRP across dorsal root ganglia: A TMB method for tracing central sensory connections of muscle and peripheral nerves. Neurosci. 1979; 4: 1107.
15. Mesulam MM. Tetra methyl benzidine for HRP neurohistochemistry: A non-carcinogenic blue reaction product with superior sensitivity for visualizing neural afferents and efferents. J Histochem Cytochem. 1978; 26: 106.
16. Mesulam MM. Tracing neural connections with horseradish peroxidase. In: Method in neuroscience J Wiley and Sons, Chichester 1982; 3.
17. Semba K, Sood V, Shu NY, Negele RG, Egger MD. Examination of geniculate ganglion cells contributing sensory fibers to the rat facial "motor" nerve. Brain Res. 1984; 308: 354.
18. Tsai TC, Wu CH, Wen CY, Shieh JY. Studies of the motoneurons following the injection of horseradish peroxidase into the peripheral branches of the facial nerve in rats. Acta Anat. 1993; 148: 42.
19. Welt C, Abbs JH. Musculotopic organization of the facial motor nucleus in Macaca Fascicularis: a morphometric and retrograde tracing study with choleratoxin B-HRP. J Comp Neurol 1990; 291: 621.