RETROGRADE TRANSPORT AND INTRANEURONAL FATE OF EXOGENOUS HORSERADISH PEROXIDASE IN THE NERVOUS SYSTEM—NIGRONEOSTRIATAL SYSTEM AND SCIATIC NERVE OF ADULT RATS—LIGHT AND ELECTRON MICROSCOPIC INVESTIGATION

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Exogenous horseradish peroxidase (HRP, Type VI, Sigma) was applied as a marker for retrograde neuroanatomical tracing in the nigrostriatal system and the sciatic nerve of adult rats. The retrograde transport and intraneuronal fate of HRP was examined with light and electron microscopy.

In the nigrostriatal system, the labeled neurons containing many brown granules in their perikarya were observed in the ipsilateral substantia nigra 3-4 hours after an injection of HRP, confirming the existence of a nigrostriatal pathway. Ultrastructurally, these granules appear as multivesicular and lysosomal bodies in the perinuclear region.

The labeled nerve cells of the spinal ganglia were also observed within 12 hours after the application of HRP in the sciatic nerve. The HRP positive brown granules had accumulated in the perinuclear region and were rapidly degraded by the lysosomal system disappearing 2 weeks after the injection.

In instances of long periods of survival, characteristic lysosomal bodies which included crystals, crystalloid and laminar structures were found.

The use of exogenous horseradish peroxidase (HRP, E.C. 1.11.1.17. MW. 40,000) as a marker for retrograde axonal transport from the nerve terminals to the parent cell bodies was first described by Kristensson and Olsson (10). Subsequently, many investigators have used this new histochemical method in order to identify the origin of the neuronal projections in the central and peripheral nervous systems (5, 6, 10, 14, 16, 17, 18, 19).

Kristensson and Olsson (11, 12, 13) investigated the subcellular localization of HRP using the hypoglossal and sciatic nerves of mice. Using the chick visual system, LaVail and LaVail (15, 16) studied the uptake and retrograde transport of HRP from the retinal nerve terminals of the ganglion cells to the cell bodies using light and electron microscopy, and clarified the ultrastructural localization of HRP within the axon terminals, axonal processes and parent cell bodies. Many fundamental investigations using this method with light and electron microscopy have been attempted in order to study the features of HRP taken into
the nerve terminals and its localization within the parent cell bodies in detail (11, 12, 13, 16, 19, 20).

Presently, studies on ultrastructural changes involving the elimination of HRP are incomplete. Therefore, the present study was designed to examine the retrograde axonal flow of exogenous horseradish peroxidase in the central nervous system using the nigroneostriatal system, and in the peripheral nervous system using the sciatic nerve. We have attempted to follow the intraneuronal fate of HRP transferred to the perikarya with light and electron microscopy.

**MATERIALS AND METHODS**

Fifty adult albino rats of both sexes were used in this study. Horseradish peroxidase (HRP, MW. 40,000, Type VI, Sigma Chemical Co.), dissolved in 0.9% saline, was used as a marker of retrograde neuroanatomical tracing.

Experimental Procedures:
A) **Nigroneostriatal system:**

The animals were anesthetized with Nembutal® and placed in a stereotaxic head holder. The cortex was exposed, and 0.2–2 µl of freshly prepared 50% HRP solution was stereotaxically injected into the neostriatum (left side) using a microsyringe. The enzyme was injected gradually over a period of 10–20 min. Thereafter, the microsyringe was left in place for an additional 10 min. The animals were allowed to survive for 2 h, 3 h, 4 h, 6 h, 24 h, 3 days and 1 week, respectively, after the injection of HRP. Both sides of the substantia nigra and neostriatum were examined.

B) **Sciatic nerve:**

The sciatic nerve was exposed and crushed with forceps 1.0 cm below the lower margin of the pifiform muscle. Five µl of freshly prepared 50% HRP solution was applied to the left sciatic nerve using a microsyringe, while the contralateral sciatic nerve remained crushed by forceps only. In order to investigate the uptake and intraneuronal fate of HRP transported to the perikarya, the animals were allowed to survive for 2 h, 3 h, 4 h, 6 h, 9 h, 12 h, 24 h, 2 days, 3 days, 1 week, 2 weeks and 3 weeks, respectively, after the administration of HRP. In this series, we examined both sides of the sciatic nerves and both sides of the corresponding dorsal spinal ganglia. The contralateral spinal ganglia (uninjected side) were examined as a control.

Histochemical Procedures:

At the previously designated times, the experimental animals were anesthetized with Nembutal® and perfused through the left cardiac ventricle with heparinized 0.9% saline, followed by perfusion with 1% glutaraldehyde and 1% paraformaldehyde buffered with phosphate at pH 7.4. The brain, sciatic nerve, and the dorsal spinal ganglia of the animal were removed and postfixed in the same fixative over night.

Specimens were transferred into phosphate buffer containing 20% sucrose for 1 day. Sections of 40 µm thickness were then prepared on a freezing microtome.
The sections were incubated in 3,3'-diaminobenzidine and hydrogen peroxide buffered with Tris-HCl buffer at pH 7.6 as described by Graham and Karnovsky (4). The unstained sections and those counterstained with 0.1% cresyl violet-acetic acid were mounted and examined with a light microscope and a differential interference contrast microscope (Nomarski).

For electron microscopic investigation, the sections after the incubation for HRP reaction were postfixed in 1% osmium tetroxide buffered with phosphate at pH 7.4 after being washed with the same buffer. They were then dehydrated by a graded series of acetone and embedded in Epon 812 mixture. Ultrathin sections made by a Porter Blum II ultramicrotome were stained with uranyl acetate and lead acetate, and examined under a JEM 100B electron microscope. Unstained sections were also examined.

RESULTS

A) Nigroneostriatal system:

In the frontal sections, the injection sites in the neostriatum were seen macroscopically as deep brown regions of variable size, depending on the amount of injected HRP. The brown coloration at the injection sites appeared to decrease as survival periods increased.

With light microscopy, the injection sites were seen as stained diffusely brown, except for the fiber bundles of the internal capsule (Fig. 1a, 1b). Following the injection of HRP, almost all neuronal and glial elements in the injected area were stained completely with a deep brown color. Small brown granules were also visible around the cell elements, some of which seemed to be included in processes and the others to be free in the extracellular region (Fig. 1b).

With electron microscopy, various forms of the HRP positive electron dense bodies were observed in the perikarya of the nerve cells in the neostriatum. The cell membrane of these cells was coated with fine granules of HRP (Fig. 1c). The electron dense HRP granules in the cytoplasm were classified into the following 3 types: small granular, multivesicular and large lysosomal types (Fig. 1c).

At 3–4 hours after the injection of HRP, labeled neurons which contained many granules in their perikarya were observed light microscopically in the ipsilateral substantia nigra (Fig. 2). Many dark brown granules were found not only in the perikarya but in the processes as well.

At 6–12 hours following injection, the number of HRP labeled neurons in the ipsilateral substantia nigra reached a maximum while nearly all neurons in the substantia nigra were labeled by HRP. This decreased gradually with the prolongation of survival time.

Ultrastructurally, neurons of the ipsilateral substantia nigra were found to contain great numbers of various sizes of electron dense materials with a HRP positive reaction in their cytoplasm (Fig. 3a). In some cases, the same granules were found in the dendritic processes (Fig. 3b). These electron dense materials were not observed in the nuclei in any cases. When the survival period was short, electron dense long tubular structures containing fine granules could be seen in the axons (Fig. 3c).
Fig. 1a. Frontal section of the neostriatum. The injection sites of HRP are stained with a brown color. CC: Corpus callosum. ×20

Fig. 1b. Highly magnified light micrograph of the injection sites of the neostriatum. The injection sites are stained brown with the exception of the fiber bundles of the internal capsule. The brown granules are visible around the cell elements stained a deep brown color. ×100

Fig. 1c. Electron micrograph of the neuron at the injection sites. The various forms of the HRP positive bodies, i.e., small granular, multivesicular and large lysosomal types are observed in the perikarya and the cell membrane is coated with fine granules of HRP. ×15,000
In the perikarya of the neurons of the contralateral substantia nigra, we were unable to find any HRP positive electron dense bodies with either light or electron microscopy.

B) Sciatic nerve:

a) Sciatic nerve: After the application of HRP directly to the crushed sciatic nerve, HRP diffusely entered the axoplasm through the disrupted perineurium, and the endoneurium was seen as stained brown with light microscopy. This brown coloration of the nerve fibers extended in a proximal direction for 1 cm, but we were unable to find any brown colored axons beyond this in the proximal portion (Fig. 4a).

With electron microscopy, dark granules ascribed to HRP appeared in contact with the basement membrane of Schwann cells (Fig. 4b). Electron dense HRP positive bodies were found in unmyelinated or myelinated fibers. Small portions of these granules formed a multivesicular body-like structure (Fig. 4b). Neurotubules containing HRP were also found in axons of the sciatic nerve.

b) Nerve cells of the spinal ganglia: Small brown colored granules were observed in the cytoplasm of the nerve cells of the spinal ganglia within 12 hours after the application of HRP. The number of brown colored HRP positive granules in the perikarya was at a maximum and almost all nerve cells of the spinal ganglia were labeled by HRP within 2–3 days after application (Fig. 4c). Using differential interference contrast microscopy, the granules were seen to be accumulated in the perinuclear region of the cytoplasm (Fig. 4d). As the survival times were prolonged, the number of HRP positive neurons decreased. Within one week after HRP
application, only a few nerve cells retained HRP positive granules, and 2 weeks later, brown colored HRP positive granules had completely disappeared from the cytoplasm of the nerve cells of the spinal ganglia.
Fig. 4a. Light micrograph of the sciatic nerve fibers of the injected side. The brown coloration of the nerve fibers can be seen. ×190

Fig. 4b. Electron micrograph of the sciatic nerve of the injected side. Electron dense HRP positive bodies can be seen and granules of HRP appear to be in contact with the basement membrane of Schwann cells. M: mitochondria ×45,000

Fig. 4c. Light micrograph of HRP labeled nerve cells in the spinal ganglia. Almost all nerve cells are labeled and small brown colored granules are found in their cytoplasm. ×120

Fig. 4d. Differential interference contrast micrograph of the HRP labeled nerve cells of the spinal ganglia. The granules have accumulated in the perinuclear region of the cytoplasm. N: nucleus. ×500
One to two days after the application of HRP, the brown colored HRP positive granules which had accumulated in the perinuclear region were seen as variably sized electron dense organelles. Therefore, the electron density of these organelles was high (Fig. 5a). When the survival period was prolonged, these organelles became low in density and large in size (Fig. 5b). One to two weeks after HRP application, characteristic lysosomal bodies which contained crystals, crystalloids and laminar structures were seen. These bodies were greater in number in cases of longer survival period animals (Fig. 5c).

DISCUSSION

Following the histochemical fluorescence method of Falck et al. (3), the unilaterally ascending nigro-striatal tract was detected by fluorescence microscopy (1, 8.). In the present experiment, the nigro-neostriatal system in the central nervous system was examined, the existence of which has been clarified by other methods. Application of HRP using microsyringe into the restricted area of the neostriatum is possible, since it has adequate size for the treatment and it also distant from the substantia nigra.

At the injection sites of the neostriatum three different forms of HRP, i.e., fine granular, multivesicular and large lysosomal types in the cytoplasm were found. It was assumed that these were the structures of different stages of the elimination of HRP. In the neurons of the ipsilateral substantia nigra, small brown granules were found in not only the perikarya but in the processes as well. Using electron microscopy, HRP positive multivesicular and lysosomal bodies were observed in the perikarya, and large vesicles containing HRP were also observed in the dendrites. Based on these observations, the existence of the nigro-neostriatal pathway was confirmed using the HRP retrograde transport method. It seems that exogenous horseradish peroxidase was taken up in a fine granular form and transported by the neuronal tubules to the parent cell bodies, ultimately degraded by lysosomes. Neurotubules containing HRP positive material in the axons were observed. These findings reveal that the neurotubules play an important role in retrograde transport of HRP (2).

Furthermore, the intraneuronal fate of exogenous horseradish peroxidase in this system was investigated. The labeled cells were not found in the restricted area, but were distributed in a large area of the substantia nigra. Therefore, the sciatic nerve was chosen as a suitable material for the following reasons: 1) It is relatively easy to apply HRP solution to the sciatic nerve in a restricted area when the perineurium, the barrier to diffusion of macromolecular substances such as

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**Fig. 5a.** Electron micrograph of the HRP labeled nerve cells of the spinal ganglia 1 day after the application of HRP. The brown colored HRP granules are seen as variably sized electron dense organelles. N: nucleus. × 18,000

**Fig. 5b.** Electron micrograph of the HRP labeled nerve cells of the spinal ganglia 4 days after the application of HRP. The organelles containing HRP decreased in density and increased in size. × 23,000

**Fig. 5c.** Electron micrograph of the nerve cells of the spinal ganglia 2 weeks after the application of HRP. The characteristic lysosomal bodies contain crystals, crystalloid and laminar structures. × 30,000
HRP (9, 13), is broken down. In the present study the nerve fibers were crushed with forceps, thus aiding HRP dispersion to the nerve fibers (13). 2) Parent cells are compactly distributed in the corresponding spinal ganglia and are at a distance from the injection sites.

HRP positive bodies in the corresponding nerve cells of the spinal ganglia were found electron microscopically within 12 hours after HRP application. The accumulation of brown colored materials with an HRP positive reaction was observed within 24 hours after HRP application by light and electron microscopy, and the number of HRP positive granules in the ganglion cells reached a maximum 2 days after HRP application. From these observations the speed of the retrograde axonal flow of exogenous HRP was calculated to be 0.2–0.5 cm/hour in the sciatic nerve system of the rat.

HRP positive brown granules were detected in the cytoplasm of the nerve cells of the spinal ganglia within 12 hours after HRP application, which were accumulated in the perinuclear regions. No granules were found in the nucleus. Kristensson and Olsson (13) reported the same localization of HRP granules and emphasized that this phenomenon was important in information transport to the nucleus. These granules disappeared completely from the perikarya within 2 weeks after HRP application, as seen with light microscopy. Kristensson and Olsson (12) stated that HRP disappeared from the perikarya of the hypoglossal neuron within 6–11 days after an HRP injection to the tongue and present results agree well with these.

Ultrastructurally, the brown colored HRP positive granules were seen as variably sized HRP-containing granular and lysosomal bodies, and localization of HRP in this system agrees with that in other investigations (13, 20).

Two weeks after HRP application, the HRP-containing lysosomal bodies increased in size, while density decreased. The characteristic lysosomal bodies which contained crystals, crystalloids, or laminar structures were found. These characteristic structures increased in number in animals with long survival periods. On the other hand, these materials were never found in the control ganglion cells. Exogenous transported horseradish peroxidase is rapidly degraded by the lysosomal system in the parent cells, and many lysosomal enzymes are capable of breaking down many types of macromolecules such as HRP (7). Characteristic lysosomal bodies like these may represent final stage form of lysosomal degradation in the elimination of exogenous HRP in the nerve cells of the spinal ganglia. In the sciatic nerve, the appearance of crystals, crystalloids, and laminar structures in the lysosomes may be due to the break down of the endoneurium and perineurium of the axons by forceps, used when exogenous HRP was injected into the sciatic nerve.

REFERENCES


