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ELUCIDATION OF AXONAL TRANSPORT BY RADIOAUTOGRAPHY

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Nerve cells afford cell biologist a unique opportunity to analyze intracellular movements of molecules and to detect intercellular exchanges with glia. The machinery required for biosynthesis of most macromolecules is confined to the perinuclear cytoplasm. In contrast, the axon forms an elongated cytoplasmic process devoid of ribosomes and Golgi apparatus. Hence the axon depends almost entirely on the nerve cell body which supplies macromolecules required to maintain axonal functions. The uncommon distance separating the perikaryal sites of biosynthesis from the axonal sites of utilization can reach several mm or cm in usual laboratory animals. Therefore the axonal length allows us to decipher the subcellular pathway followed by migrating macromolecules with a resolution in space and time never achieved in other cell types.

The idea that some material moves along the axon was long ago put forth from observation of wallerian degeneration after a nerve transection. This concept was clearly explicated by Weiss and Hiscoe (1948) on the basis of experiments performed on constricted nerves. However it was necessary to prove that the axonal migration of substances took place in intact nerves and did not result from a local injury. The answer was given by the use of tritium to label molecules and of high resolution radioautography to locate them within the axon.

#### Radioautography and axonal transport of protein

After injection of  $^3\text{H}$ -leucine to rats, Droz and Leblond (1962, 1963) have shown that radioautographs made a few minutes later displayed a strong reaction over the nerve cell bodies of various neurons but none over the axons. Several hours later, the radioautographic reaction was found to extend to the proximal regions of the axons. After several days, the label was progressing to more distal regions of the axons. On the basis of these radioautographic results, it was concluded that proteins, once synthesized in the nerve cell body, enter the initial segment of

the axon and move along its length at rates of 1 to 3 mm per day. Electron microscope radioautographic studies revealed that, in the course of the slow axonal flow of radioactive proteins, the label was associated with areas occupied by axoplasm, elements of the cytoskeleton and, to a lesser extent, mitochondria (Droz, 1965). Biochemical analyses confirmed that mainly soluble proteins are conveyed by slow axonal transport ~~(Lasek and Hoffman, 1976)~~; recently subunits of neurofilaments have been identified in the slowly moving phase of axoplasmic proteins (Lasek and Hoffman, 1976).

Faster rates of axonal transport were independently discovered by Lasek (1967) and Grafstein (1967). Since most of the label was recovered in particulate fractions rather than supernatant, membrane constituents were assumed to be preferentially delivered to nerve endings by fast axonal transport. This hypothesis was supported by radioautographic studies of Hendrickson (1972) and Cuénod *et al.* (1972). A decisive proof was provided by Bennett *et al.* (1973) who used  $^3\text{H}$ -fucose or  $^3\text{H}$ -glucosamine to visualize by radioautography the specific labeling of the axolemmal membrane along the axon and of the presynaptic plasma membrane and synaptic vesicles in nerve endings.

The use of radioautography to investigate the axonal transport of phospholipids.

Biochemical studies have shown that phospholipids are transported along axonal tracks in peripheral and central nervous system (Miani, 1963; Grafstein *et al.*, 1975). Owing to special features characterizing the axonal transport of phospholipids, it was felt that a radioautographic investigation could help to elucidate the kinetics of renewal of these macromolecules.

The radioautographic localization of labeled phospholipids in nervous tissue raises however two major points: first, the choice of a precursor and second, the *in situ* preservation of radioactive lipid compounds.

Since glycerol forms the backbone of glycerophospholipids, its incorporation reflects a net synthesis of these compounds. 2-<sup>3</sup>H-glycerol offers the advantage to be virtually not recycled nor reincorporated ( Benjamins and McKhann, 1973). Hence, the movement of phospholipid labeled with this precursor can be safely studied by radioautography. When <sup>3</sup>H-choline is used as precursor, this base is also incorporated into phospholipids in the course of their synthesis, but once incorporated into choline-phosphoglyceride <sup>3</sup>H-choline can be freed and reincorporated into other lipid molecule. In addition, labeled choline can be exchanged with another base borne on phospholipid (Brunetti et al. 1979).

Nervous tissue fixed in glutaraldehyde containing Ca<sup>++</sup>, postfixed in OsO<sub>4</sub> and dehydrated through acetone at low temperature were directly embedded in epon without propylene oxide. This procedure adapted from Gould and Dawson (1976) was found to preserve more than 96% of labeled lipids. Analysis of the label distribution in electron microscope radioautographs of thin frozen sections and of epon-embedded ganglion cells indicates that labeled phospholipids are not displaced in the course of the histological processing, but retained in situ (Boyenval and Droz, 1976).

2-<sup>3</sup>H-glycerol or <sup>3</sup>H-choline were injected into the cerebral aqueduct of chickens and incorporated into phospholipids by nerve cell bodies of the preganglionic neurons of the ciliary ganglion. With time, labeled phospholipids were transported by axonal flow to the ciliary ganglion located 10 mm farther. At intervals of time ranging from 3 hours to 21 days, the ciliary ganglia were removed: one was used for biochemical analysis, the other was processed for radioautography.

At early time intervals after the intracerebral injection of 2-<sup>3</sup>H-glycerol or <sup>3</sup>H-choline, light microscope radioautographs showed a moderate reaction over the preganglionic axons and an intense accumulation of silver grains over their nerve endings.

Quantitative analysis of electron microscope radioautographs by direct scoring (Badler, 1979) pointed to the smooth endoplasmic reticulum of axons and nerve endings as the site of the highest concentration of label at 6 hours; hence this fact confirms that the smooth endoplasmic reticulum would act as a preferential vehicle for the rapid movement of membrane constituents along the axon (Droz et al., 1975). In the course of their axonal transport, labeled phospholipids are destined to the axolemma; thus it appears that proteins, glycoproteins and phospholipids of the axolemma are continuously renewed by components transported with the smooth endoplasmic reticulum of the axon (Bennett et al., 1973; Droz et al., 1975; Markov et al., 1976). Once arrived at nerve endings, labeled phospholipids were rapidly redistributed to synaptic vesicles and presynaptic plasma membranes. After 3 days, heavily labeled mitochondria were occasionally found in axons and nerve endings; they probably correspond to mitochondria which have incorporated labeled phospholipids in the brain nerve cell bodies and have migrated to the ganglion along the axons. Thus phospholipids as well as other membrane constituents are continuously produced in the perikaryon and conveyed along the axon by fast axonal flow.

Radioautographic visualization of axon-glia transfer of myelin components

When radioactive proteins or glycoproteins are rapidly transported along the axons, silver grains are found to accumulate over the axolemma; only stray silver grains are found over the encompassing myelin sheath or the Schwann cell cytoplasm. Such an inconsistent labeling cannot therefore result from a significant transfer of labeled molecules from axon to glia (Bennett et al., 1973). The situation is completely different when labeled phospholipids are axonally transported. After an intracerebral injection of 2-<sup>3</sup>H- glycerol, numerous silver grains are present over the myelin sheath. The analysis of the radioactivity concentration in

various zones of the myelin sheath pointed to the Schmidt-Lanterman clefts and to the inner myelin layers as the most labeled structures. In the absence of 2-<sup>3</sup>H-glycerol reutilization, this result would correspond to a direct transfer of phospholipids as a whole from axon to myelin.

After the intracerebral injection of <sup>3</sup>H-choline, a similar pattern of label distribution is observed at early time intervals; later, the density of silver grains increased in the outer Schwann cell cytoplasm then in the outer myelin layers. This late redistribution of the label in Schwann cells and outer myelin leaflets coincides with the presence of free radioactive choline and derivatives in the ciliary ganglia. Thus, in addition to a direct transfer of labeled phospholipids to inner myelin zones, labeled choline released from the axons is taken up by surrounding Schwann cells and reincorporated into myelin phospholipids (Droz et al., 1978). Such a reutilization of bases incorporated into myelin phospholipids has been recently reported after an intraocular injection of <sup>14</sup>C-serine (Haley and Ledeen, 1979).

According to our radioautographic data, two distinct mechanisms would take place to account for the appearance of label in the myelin sheath: first, a direct transfer of phospholipids to the inner myelin leaflets; second, an uptake of axonal choline by Schwann cells and its reincorporation into myelin phospholipids (Droz et al., 1978). Such an axon-glial transfer of large and small molecules could contribute in part to the trophic control exerted by the axon upon its myelin sheath.

### Conclusion

Radioautography permits to distinguish various pathways within the axons: the axoplasm which includes soluble enzymes and constituents of the cytoskeleton ~~for the~~ <sup>moving with</sup> axoplasmic flow; the mitochondria which are conveyed as organelles; the smooth endoplasmic reticulum which ensures the fast axonal transport of membrane constituents delivered to axolemma, synaptic vesicles, presynaptic membranes or mitochondria. Furthermore radioautography makes it possible to visualize intercellular exchanges of molecules between axon and glia.

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