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Brief Review

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Neuronal tracing

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ABSTRACT

The tracing of neural pathways continues to be an important concern for neuroanatomy. During the second half of the nineteenth century, at a time when the foundations of neuroanatomy were being established, tracing pathways often required laborious dissections by hand. Then, neuroanatomy experienced a methodological revolution in the beginning of the 1970s with the development of powerful techniques based on the axonal transport of tracers. Axonal tracing of neuronal pathways using anterograde and retrograde transport is available. Axonal transport can be combined with immunohistochemistry for the neurochemical characterization of specific neuronal pathways. After tracing, tissue is collected following appropriate survival time for the tracer to be transported. Immunohistochemical methods, virus immunolabeling or autoradiography at the axon terminals are used for expressing the tracers. Retrograde axonal transport is used by certain viruses (e.g., herpes and pseudorabies virus) to spread from one neuron to the next in a chain of neurons (transneuronal transfer). It is also the method whereby toxins (e.g., tetanus) are transported from the periphery into the central nervous system. This has led to the idea that viral tracing may be an important way as a possible gene delivery method for the gene therapy in nervous system diseases.

Key words: neuronal tracing, axonal transport, anterograde, retrograde

Introduction

Histologically, the nervous system contains different types of nerve cells (neurons) and supporting cells (glial cells) which are essential for neuron function. Neurons are composed of a cell body (soma, perikaryon), dendrites and an axon. Axons transmit impulses to other neurons or effector cells, namely, cells of muscle and glands. In addition to impulse conduction, an important function of the axon is axonal transport of the materials between the soma and the axon terminals. In anterograde transport, the direction is to axon terminal; in retrograde transport, the direction is from the axon terminal to the cell body [1].

Axonal tracing of neuronal pathways using anterograde and retrograde transport is available. Neuronal tracing studies are powerful because they allow for a precise identification of neuronal pathways and functions. Since the 1970s, much has been learned about the nature and functioning of the neuron through study of the mechanism of axonal retrograde transport with the use of the enzyme horseradish peroxidase (HRP) [2].

In a healthy nerve cell, a large fraction of all metabolic effort is expended in managing the intracellular movement of molecules and organelles. This intracellular informational and metabolic challenge is managed through a phenomenon called axonal transport (axoplasmic flow). Axoplasmic flow precedes interference with the transmission of electrical impulses or actual vascular compromise of the nerve [3]. Axonal transport continues in crushed nerves with accumulation of transported substances both proximal and distal to the site of injury [4] and so could be used to mark the location of the compression. Axonal transport is disordered in diabetic neuropathy and amyotrophic lateral sclerosis, there is at least reason to hope that clinical imaging of transport could aid in diagnosis and management. Axonal transport also provides a powerful highway for transporting useful pharmaceuticals, nucleic acids, and other physiologically active compounds from easily accessible sites in the periphery to important targets behind the blood/nerve and blood/brain barrier. During the 1990's it began to be apparent that magnetic resonance imaging could be capable of evaluating the physiology and pathology of axonal transport by the means of a novel class of intraneural contrast agents [5].

Physiology of Axonal Transport

Axoplasmic flow is not a single constant rate of movement of the axoplasm, but rather can be divided into several different kinetic components and can proceed in both directions. Axons will transport a variety of substances, several of which are relatively well studied . Anterograde transport is used in the translocation of membranous organelles (e.g., mitochondria) and vesicles as well as of macromolecules, such as actin, myosin, and clatrin, and some of the enzymes necessary for neurotransmitter synthesis at the axon terminals mediated by kinesin-family proteins. Items returned to the cell body from the axon in retrograde transport include protein building blocks of neuroflaments, subunits of microtubules, soluble enzymes and materials taken up by endocytosis (e.g., viruses and toxins) mediated by cytoplasmic dyneins. Additionally, small molecules and proteins destined for degradation are transported to endolysosomes of the soma. The earliest view into these was provided by studies of amino acids which are incorporated into proteins in the cell body and then transported anterograde. However, there are a variety

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of cell surface receptors that first bind ligands and then are endocytosed and transported anterograde or retrograde. When these endogenous ligands are conjugated to exogenous substances, the ligands act to facilitate transport of the exogenous substance via an unique and specific intraneural pathway [6].

The physiology of axonal transport has been a very active area of research, with a literature that parallels and in some matters, overlaps the work on tracer methods carried out by neuroanatomists and histochemists and has been reviewed extensively [7]. Interest in transport includes studies on the mechanisms by which various substances enter the neuron, the rates at which various substances travel, and in the various skeletal and motile proteins involved.

ENDOCYTOSIS

The ability of neurons to engulf large intact proteins was a surprise finding that grew out of early electron microscope work, and it was immediately apparent that this process involved the formation of membrane bound vesicles [8]. In general, cellular endocytosis of receptor-ligand complexes involves a first stage event of creation of "endosomes". The endosomes have a highly acidic pH which tends to dissociate the ligand [9]. In this sequence, the receptor is then recycled back to the cell membrane for reuse while the ligand is passed along to a lysosome for degradation. It is known that this sequence can be altered for e.g. transferrin when carrying colloidal gold. In this situation, the receptor and ligand remain complexed and are passed onto the lysosome as a unit [10]. This sort of unit, packaged in the lysosome, would then undergo fast retrograde transport in the neuron such as is known for Wheat Germ Agglutinin-Horseradish Peroxidase (WGA-HRP). Retrograde transport of exogenous molecules apparently involves three distinct mechanisms of intake into the cell. Fluid phase endocytosis and adsorptive endocytosis as observed with HRP and WGA-HRP respectively both result in these substances being introduced into lysosomes [11]. The third mechanism, transcytosis [12] is slower than normal retrograde transport, takes place in a limited compartment immediately beneath the axolemma, and differs from other mechanisms in that the receptor and ligand are not subject to lysosomal digestion during transport.

RATES OF TRANSPORT

It has been clear for many years that the rate of transport of a given substance is independent of electrical activity within an axon. It was also appreciated early on that different classes of substances moved at different rates [13]. Anterograde axonal transport has been shown to have a major fast and a slow component. The slow component is divided into "slow component a" and "slow component b" at rates of approximately 0.1 and 6 mm/day respectively. These slow components apparently reflect gradual structural repair and replacement of the subunits of the cytoskeleton and are not involved in the fast components important for tracer studies. The fast component of transport demonstrates distinct maximal rates for anterograde (200-400mm/day) and retrograde (100-200 mm/day) transport (Table 1). The maximal rates of transport apply to small membrane vesicles. Further, there are a variety of "waves" or distinct

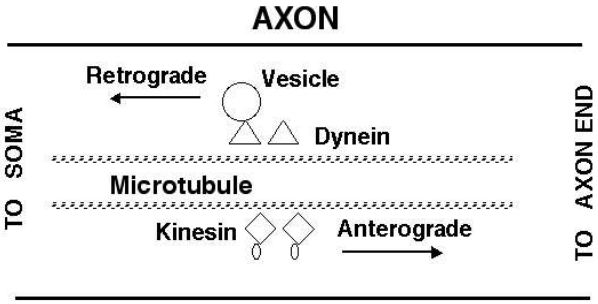

Figure 1 | Molecular mechanism of axonal transport.

sets of slower transport rates exhibited in characteristic fashion by various molecules. Rates of progression and spreading of wave fronts have been assessed using pulse labeled 35S-protein in rat sciatic nerve subsequently excised, and "desheathed" for measurement with a linear array of proportional ß-counters [14].

CYTOSKELETAL MECHANISMS

There are three major cytoskeletal elements in the axon: 1) microtubules made up of alpha and ß-tubulin monomers, 2) microfilaments composed of actin monomers, and 3) neuroflaments which are a type of intermediate filament composed of three distinct subunits. A variety of studies involving intact and skinned axons with specific antibodies and various specific poisons and depolymerizing agents have proven that it is the microtubules which are the most directly involved in axonal transport. The various cytoskeletal elements are effectively stationary in the axon. Anterograde transport involves the packaging of various proteins into the membranes of transport vesicles. In anterograde transport, these vesicles and similarly sized mitochondria are pulled along the microtubules by movable bridges of "kinesin" at a characteristic rate. The various slower rates of transport in anterograde motion appear to reflect molecules that either bind poorly with kinesin or that pass in and out of the vesicles during transport. Retrograde transport moves along the same microtubules but the driving protein is "dynein" which operates in a direction opposite to kinesin [15] (Fig. 1).

All of this movement is ATP and calcium dependent. The metabolism involved is local, i.e., mitochondria bound to the axolemma as well as mitochondria being transported on the microtubules use glucose and oxygen absorbed through the cell membrane along the axon to generate ATP locally.

When a nerve is ligated, transport continues for many hours both proximal and distal to the ligation. Transported vesicles, proteins, and mitochondria pile up at the ligation site. Some signal which turns around and heads back to the cell body then causes anterograde fast transport to halt [16]. This situation continues until a critical concentration of cytoskeletal subunits accumulates via slow transport and at that time, axonal growth begins.

Tracing Methods

New tracing and labelling methods have revolutionised neurobiology in the last 30 years. Some of the methods which are shortly presented in Table 2 include; anterograde and retrograde labelling. Neuroanatomical tracing methods

Table 1 | A summary for components of axonal (axoplasmic) transport.

Axonal Transport	Velocity (mm/day)	Transporting Substances
Anterograde Transport		
Fast Transport	200-400	Synaptic vesicle, enzymes, neurotransmitters
Mitochondrial Transport	50-100	Mitochondria
Slow Transport		
Slow Components a (SCa)	0.1 - 1.0	Tubulin, neurofilament protein
Slow Component b (SCb)	2 - 6	Actin, clathrine, calmodulins, spectrin, cytoplasmic enzymes
Retrograde Transport	100-200	Prelysosomal vesicles, recycled proteins, HRP*, WGA**, neurotrophic viruses

*: Horseradish Peroxidase (HRP), **: Wheat Germ Agglutinin (WGA).

Table 2 | Some methods and materials used for neuronal tracing.

Methods	Materials
Anterograde Tracing Methods	
Marchi Method	OsO_4 after tract of nerve lesion
Nauta Method - Fink Heimer Method	Reduced Silver Method after tract lesion
Autoradiography with Radiolabelled Amino Acid	Tritiated Glycine
Anterograde Tracer	Phageolus Vulgaris Leucoagglutinin (PHA-L)
Retrograde Tracing Methods	
Nissl Reaction	Methylene blue, Toluidine blue, Thionin, Cresyl violet
Retrograde Tracer	Horseradish Peroxidase (HRP), Wheat Germ Agglutinin (WGA)
Fluorescence Tracer	Lucifer Yellow, Fast Blue, Nuclear Yellow
Viruses and Toxoids	Herpes Simplex Virus, Adeno Virus, Pseudo rabies, Cholera toxin-B chain (CTB), Tetanus toxin-fragment C (TTC)

can be combined with a variety of other techniques for the aim of study.

Tracer Application

The tracer is injected into (1) a peripheral organ (e.g. the stomach) for retrograde transport, using fine gauge needles or micropipettes and a dissecting microscope to guide injections, or (2) a ganglion (e.g. the nodose ganglion) or (3) selected areas of the brain, for anterograde transport, with the aid of a stereotaxic apparatus to guide the exact site of injection. Three different techniques are used to introduce tracer into the tissue: pressure injection, iontophoretic injection and the insertion of dye crystals [6].

PRESSURE INJECTIONS

Injections require the tracer to be administered in liquid form. The dissolved dye or other tracer is usually pressed through glass micropipettes (capillaries drawn in a standard electrode puller for electrophysiology) or Hamilton syringe. This can be done either with gas pressure, or by means of an hydraulic system. The latter is often more practical, because it permits to accurately control the injected volume.

IONTOPHORETIC (MICROIONTOPHORESIS) INJECTIONS

This technique requires an iontophoresis unit. Current is applied through the electrode that contains the tracer solution at concentrations of about 1 - 5%. Tracer molecules carry an electrical charge and are thus driven by force of the electrical field. With appropriate electrode tip diameters, this technique is suited for both intracellular and extracellular applications. In addition, the application site may be precisely located prior to iontophoresis by conventional electrophysiological techniques with the same electrode.

INSERTION OF DYE CRYSTALS

Insertions of crystalline dyes are feasible when the tissue is openly accessible. As the labeling efficiency is very high and can be done under microscopic control, this is the method of choice for focal applications of carbocyanine dyes like DiI, DiAsp etc.

Dedection of Tracers

When the enzyme injected into the axon terminal, it can be detected later by histochemical techniques that mark its pathway to the cell body. In studying anterograde axonal transport, researchers inject radiolabeled amino acids into the cell body and then later determine the radioactivity at the axon terminals using autoradiography. Tissue is collected following appropriate survival time for the tracer to be transported (this varies according to the tracer used and the distance of the neuronal circuitry under investigation) and analyzed using fluorescent light (for dyes), or immunohistochemistry with specific antibodies for the tracers. Appropriate controls are performed to assess the specificity of the transport, including time course and interruption of selected neuronal pathways to determine whether the tracer is specifically transported. For the chemical characterization of identified neuronal circuits, sections of the appropriate region are processed for immunohistochemistry. Attention is paid to maintaining the tracer into the labeled neurons (e.g. reducing the use of detergents when utilizing liposoluble tracers such as DiI) when immunohistochemical procedures are applied. The use of neurotrophic viruses such as Herpes Simplex, and rabies virus as tracers, in some cases with cDNA probes used to locate the endpoint of transport [17]. The availability of confocal microscopy increases the power of axonal transport studies, providing a better resolution than conventional microscopy to identify the relationship between nerve terminals visualized with a tracer and distinct neuroanatomical structures.

Viral Transneuronal Tracing

Neurotropic alpha-herpesviruses are used for transneuronal tracing, i.e., for investigating the structural organization of multisynaptic pathways or circuits of several neurons. The utility of this technique is based on the ability of the virus to invade and replicate in neurons following central and peripheral inoculation and then infect synaptically connected nerve cells. Injection of the virus, as pseudorabies virus, into a peripheral organ or the brain is followed by the uptake of the virus by nerve endings located in the injected area. The virus then travels along the axonal flow to the first-order neurons innervating the organ. After reproductive infection within the nerve cell, the virus passes through the synapse and infects the second-order neuron The infection proceeds to further synaptically linked neurons. The infected neurons eventually degenerate and are phagocyted by macrophages [18]. The kinetic of

virus infection implies that the analysis of virus-infected neurons with a well-defined time course is critically important to the interpretation of the data [19]. Jansen et al. [20] have reported that there is a narrow time window in which specific transneuronal labeling occurs. The viral infected neurons can be visualized by immunohistochemistry. Thus, it may be concluded that the viral transneuronal tracing technique is a useful tool to get information about the organization of multisynaptic neuronal pathways.

Recently, the success of human gene therapy relies on efficient delivery and appropriate expression of therapeutic genes which will cure or slow the progression of a disease. Herpesviruses are large DNA viruses which possess a number of advantages as gene delivery vectors [21]. Combination of gene therapy and viral tracing methods are getting very important way to deliver gene vector into the specific area.

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