HHS Public Access

Author manuscript

Brain Res. Author manuscript; available in PMC 2016 January 27.

Published in final edited form as:

Brain Res. 1984 January 9; 290(2): 219–238.

An anterograde neuroanatomical tracing method that shows the detailed morphology of neurons, their axons and terminals: Immunohistochemical localization of an axonally transported plant lectin, *Phaseolus vulgaris*- leucoagglutinin (PHA-L)

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Abstract

A new neuroanatomical method for tracing connections in the central nervous system based on the anterograde axonal transport of the kidney bean lectin, Phaseolus vulgaris-leucoagglutinin (PHA-L) is described. The method, for which a detailed protocol is presented, offers several advantages over present techniques. First, when the lectin is delivered iontophoretically, PHA-L injection sites as small as 50-200 μm in diameter can be produced, and are clearly demarcated since the neurons within the labeled zone are completely filled. Second, many morphological features of such filled neurons are clearly demonstrated including their cell bodies, axons, dendritic arbors and even dendritic spines. Third, there is some evidence to suggest that only the neurons at the injection site that are filled transport demonstrable amounts of the tracer, raising the possibility that the effective injection site can be defined quite precisely. Fourth, even with the most restricted injections, the morphology of the labeled axons and axon terminals is clearly demonstrated; this includes boutons en passant, fine collateral branches, and various terminal specialization, all of which can be visualized as well as in the best rapid Golgi preparations. Fifth, when introduced iontophoretically, PHA-L appears to be transported preferentially in the anterograde direction; only rarely is it transported retrogradely. Sixth, PHA-L does not appear to be taken up and transported effectively by fibers of passage. Seventh, there is no discernible degradation of the transported PHA-L with survival times of up to 17 days. Finally, since the transported marker can be demonstrated with either peroxidase or fluorescent antibody techniques, it may be used in conjunction with other neuroanatomical methods. For example, double anterograde labeling experiments can be done using the autoradiographic method along with immunoperoxidase localization of PHA-L, and the retrogradely transported fluorescent dyes can be visualized in the same tissue sections as PHA-L localized with immunofluorescence techniques.

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Keywords

Neuroanatomy; axonal tract tracing

The anterograde axonal tracing technique utilizing the plant lectin, *Phaseolus vulgaris*-leucoagglutin (PHA-L) was developed in 1983 (Gerfen and Sawchenko, 1984), and has been used in over 1000 published studies. The technique involves iontophoretic injection of the plant lectin PHA-L into the brain and, following a 1-3 week survival period, the tracer is visualized using standard immunohistochemical methods. Neurons at the injection site are labeled in their entirety including the cell body, dendrites and axonal projections. Labeling of neurons appears to fill neuronal processes displaying fine morphologic details including dendritic spines and axon terminal specializations throughout the axonal projection field.

Axonal tract tracing methods evolved using agents that interact with various mechanisms of neuronal function. In the 1970s, the autoradiographic axonal tract tracing method was developed that utilized ^{3}H-amino acid injections into the brain (Cowan et al., 1972). This technique was based on the incorporation of ^{3}H-amino acids into proteins that are axonally transported from the cell body to axon terminals. As labeled tracer is incorporated into neurons only in the cell body, this technique only labeled axonal projections of neurons at the injection site, with no labeling of fibers of passage. The tracer was visualized by mounting brain sections on glass slides, dipping the slides in photographic emulsion and then developing the slides to produce black silver grains to reveal the neurons at the injection site and their axonal projections. The autoradiographic technique was used in nearly 1500 published studies that established many principles of the connectional organization of the brain.

Another method developed in the 1970s used horseradish peroxidase (HRP) to retrogradely label neurons projecting to injected brain sites (Lavail and Lavail, 1972). Injected HRP taken up by endocytosis into axon terminals and transported retrogradely to the neuronal cell body is detected using the HRP enzymatic activity to convert the chromagen, diaminobenzidine (DAB), to an insoluble brown reaction product. Two advances to this technique expanded its utility. One was the use of tetramethylbenzidine (TMB) as a chromagen, which provided considerable amplification revealing both retrograde and anterograde transport of the tracer (Mesulam, 1978). The second advance was the conjugation of HRP with the plant lectin, wheat germ agglutinin (WGA-HRP) (Staines et al., 1980). WGA binds to sialic acid moieties of glycoproteins on neuronal membranes, which increased the incorporation into neurons and limited the spread of the tracer at the injection site. The use of WGA-HRP combined with the TMB process had advantages over the autoradiographic axon tracing method in being somewhat easier to perform due to the reaction being produced directly in the neurons rather than indirectly in an emulsion coating of the section and greater sensitivity particularly for labeling of sparse axonal projections. Problems with the WGA-HRP/TMB method included uptake by fibers of passage, the difficulty of delineating the exact effective injection site and the fact that the crystalline TMB reaction product distorted the underlying morphology of the neuronal structure.

While the WGA-HRP/TMB technique was an extremely useful axonal tracing technique we had the idea that other plant lectins binding to other sugar moieties might be even more efficacious. In addition to WGA, we selected PHA-L, which binds to a complex oligosaccharide containing galactose, N-acetylglucosamine and mannose as well as peanut agglutinin (PNA), which binds glycoproteins including Gal-β(1-3)-GalNAc. We decided to visualize these lectins immunohistochemically. Application of immunohistochemical techniques to the nervous system had been developing in parallel with the first generation axonal transport methods, and provided considerably higher resolution of morphologic details of neuronal processes than did TMB-based or autoradiographic methods. Additionally, we decided to use an iontophoretic injection method to produce restricted injections. These two innovations proved critical for the PHA-L technique.

Comparison of WGA, PNA and PHA-L injected iontophoretically into the striatum revealed very distinct results. Injected WGA produced a diffuse injection site with retrograde labeling of neurons in the cerebral cortex and substangia nigra, whereas injected PNA labeled only axonal fiber fascicles in the striatum with no evidence of axonal transport. On the other hand, at the PHA-L striatal injection site there were approximately 30 medium spiny neurons in an area about 500 μm in diameter that were labeled so as to ostensibly fill the cell bodies and all the dendritic processes, revealing fine morphologic details, such as dendritic spines. Axons of these neurons were also completely labeled which could be traced into terminal areas in the globus pallidus and substantia nigra pars reticulata where terminal varicosities were clearly evident. There was limited retrograde labeling of neurons in the substantia nigra pars compacta. These results indicated that different lectins label different components of neurons, depending on the sugar moieties to which they bind. The utility of PHA-L as an anterograde tracer was evident in that it provided complete labeling of neurons at the injection site as well as their axonal projections with what at the time was unprecedented clarity. Injections into multiple other brain areas provided similar labeling. Moreover, we adduced no evidence of uptake by fibers of passage following iontophoretic administration of PHA-L. A major advantage of the PHA-L labeling was that the neurons at the injection site could be clearly identified such that all labeled axonal projections could be explicitly determined as having arisen from those neurons.

A distinguishing feature of PHA-L is that it provides visualization of the fine morphologic detail of the labeled neurons. This results from PHA-L being incorporated into the cytoplasm of the neuron contrasted with WGA being incorporated through endocytosis into neuronal vesicles. The most likely mechanism is due to the parameters of the iontophoretic injection technique, which are similar to those used for electroporation providing for transient opening of the membrane to allow access of PHAL directly to the cytoplasm. Pressure injections of PHA-L do not provide the same discrete filling of neurons or their axonal projections. In addition to providing superior labeling of the fine morphologic details of neurons and their processes obtained with iontophoretic injections the labeling is stable for extended survival periods, which allowed for labeling of very long axonal processes. This stability is presumably a result of PHA-L not being incorporated into the neuronal vesicular compartments, whereas tracers incorporated by endocytosis are transported in the endoplasmic reticular compartment, which result in their degradation.

During the 1980s antibodies were developed for numerous neuronal proteins and peptides, which enabled immunohistochemical studies to map the organization of neurochemical systems in the brain (Sawchenko and Swanson, 1981). Combining PHA-L with these techniques allowed the neurochemical phenotype of axonal projections to be determined as well as the ability to map the distribution of axon projections in relationship to neurochemical markers that defined the organization of brain regions. For example, this approach established principles of the organization of the basal ganglia (Gerfen 1984, 1989). The striatum, which is the major input structure of the basal ganglia is composed of patch and matrix compartments identified with immunohistochemical markers such as calbindin in the matrix and mu-opiate receptors and various peptides in the patch compartment. Combining PHA-L labeling of corticostriatal projections and striatal projections demonstrated that different layers in the cortex differentially target the patch and matrix striatal compartments, which in turn provide differential inputs to dopamine and GABAergic neurons in the substantia nigra. These studies identified functional channels originating the cerebral cortex that pass through the striatal patch and matrix compartments to differentially affect the dopamine and GABAergic output of the substantia nigra (Gerfen, 1992).

Other applications have included a delineation of the organization and neurochemical specificity of afferent projections providing for reflex control of multiple visceromotor (autonomic and neuroendocrine) neuron populations housed in the paraventricular nucleus of the hypothalamus (e.g., Cunningham and Sawchenko, 1988; Cunningham et al., 1990). This work predicted a critical role for catecholamine-containing (adrenergic and noradrenergic) projections in this regard, many aspects of which have subsequently been confirmed experimentally. More recent extensions of this work have sought to define the organizational features that provide for modulatory (inhibitory) influences of the limbic forebrain on stress-related neuroendocrine responses. In these studies PHA-L was combined with immunohistochemical and/or retrograde and/or other anterograde tracing methods to define a discrete relay in the bed nucleus of the stria terminalis that serves as a point of convergence through which limbic inhibitory influcnces on the stress axis are effected (Radley et al., 2009; Radley and Sawchenko, 2011).

The PHA-L technique has been used in over 1000 published studies that established many of the principles of the connectional organization of brain systems in relationship to the neurochemical structure of the brain. Most recently the method was used in a comprehensive mapping of the neural networks of the cerebral cortex (Zingg et al., 2014), which indicates that the technique has contributed to studies for over 30 years.

Over the past 10 years the development of molecular genetic tools for axonal tracing have supplanted the PHA-L method and other conventional axonal tracing techniques (Luo et al., 2008). The use of adeno-associated viral (AAV) vectors that contain constructs to express GFP or other fluorescent proteins provide selective anterograde axonal labeling comparable to the morphologic detail produced with PHA-L (Oh et al., 2014). The development of transgenic mouse lines with Cre-recombinase expression directed to specific neuronal cell types (Gerfen et al., 2013) combined with AAV vectors with Cre-dependent GFP expression constructs enable specific labeling of axonal projections specific neuronal subtypes. Notably, the Allen Institute for Brain Science (AIBS) has used these techniques to provide a

comprehensive mapping of the axonal projections of over 1000 mouse brain sites registered to a common reference atlas (Oh et al., 2014). The AIBS mouse connectivity database provides a powerful resource for quantitative analysis of the organization of brain circuitry. Another powerful approach is the use of genetically modified rabies virus with Cre-dependent expression constructs that provide the ability to trans-synaptically label selective inputs to specific neuron subtypes (Luo et al., 2008). These approaches are revealing ever greater specificity of the connections within neural circuits. For example, the PHA-L method demonstrated that distinct cortical subtypes project differentially to the macroscopic patch/matrix compartments in the striatum, while these new techniques demonstrate an even finer-grained specificity of inputs from different cortical subtypes to striatal direct and indirect pathway neurons intermingled with one another. In addition, the development of optogenetic techniques employing AAV constructs to target specific neuron subtypes in transgenic Cre lines allow for studies to determine the behavioral function of specific neuronal circuits (Zhang et al., 2010). For example these techniques have been used to determine the functional role of subtypes of layer 5 cortical neurons in preparatory motor activity (Li et al., 2015). The autoradiographic, PHA-L and other axonal tracing methods established many of the basic principles of the connectional organization of brain systems. The newer molecular genetic techniques not only reveal additional selective structure in neuroanatomical circuits, but also provide the ability to determine how these circuits function to produce behavior.

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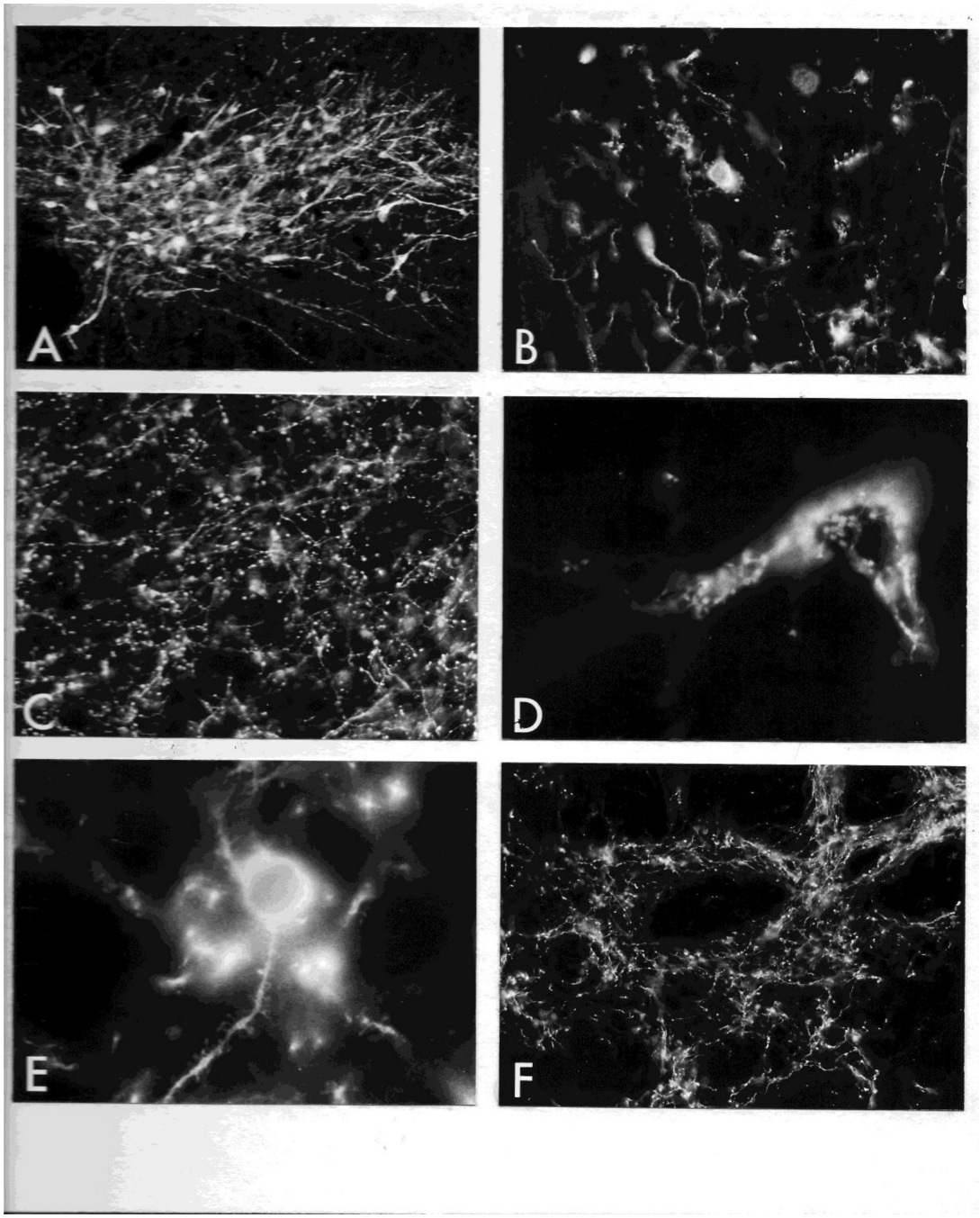

Figure 1.

Examples of types of labeling obtained with PHA-L as localized by an indirect immunofluorescence technique. PHA-L-lableed neurons at an in jection site in the lateral hypothalamus (A) show the typical morphology of labeled neurons, including their dendritic arbors. Projections of these labeled neurons in the lateral septal area (B, low magnification, and D, high magnification) and in the supramammilary nucleus (C, low magnification). Labeled hypothalamic afferents in the lateral septal area target the cell bodies and proximal dendrites of neurons (B) that at higher magnificaction display large boutons (D). On the

other hand, afferents in the supramammilary nucleus ramify extensively and are distributed to more distal dendrites (C). (E) A high-power photomicrograph of one neuron labeled at an injection site in the striatum shows the detailed morphology of dendrites and their spines obtained with PHA-L labeling. (F) PHA-L labeled projections of striatal neurons in the globus pallidus demonstrate the fine details axonal and terminal morphology.