The identification of thalamocortical axon terminals in barrels of mouse S1 cortex using immunohistochemistry of anterogradely transported lectin (Phaseolus vulgaris-leucoagglutinin)

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The anterograde transport and immunohistochemical demonstration of the lectin, Phaseolus vulgaris-leucoagglutinin (PHA-L), has been used to label thalamocortical axon terminals in barrels of mouse S1 cortex. The reaction product is visible with both the light and electron microscopes so that the distribution of axons and the types of synapses they form can be determined.

Methods involving lesion-induced degeneration or the anterograde transport of radioactively labeled amino acids are commonly used to identify the source of axon terminals in thin sections. One disadvantage of lesion-induced degeneration is that it is usually unreliable for the quantification of axon terminals because in most systems degeneration proceeds at different rates within different axons. Disadvantages of the electron microscopic (EM) autoradiographic method include lengthy exposure times for the autoradiographs and difficulty in accurately delimiting the effective injection site. Further, the identification of labeled terminals is at times complicated because of the indirect nature of the labeling.

Recently, Gerfen and Sawchenko have described a neuroanatomical method for tracing connections in the central nervous system which is based on the anterograde axonal transport of the kidney bean lectin, Phaseolus vulgaris-leucoagglutinin (PHA-L). They report significant advantages of the PHA-L method over the autoradiographic or degeneration methods for the anterograde labeling of axons at the light microscopic level. For instance, available evidence suggests that only neurons at the injection site that are filled with PHA-L transport demonstrable amounts of tracer, implying that the effective injection site can be defined precisely. Further, that fibers passing through the injection site do not take up the injected PHA-L. Another advantage of the PHA-L method is that results can be obtained in a relatively short time by using immunohistochemistry to demonstrate the lectin. Here we report a successful attempt to use this method to identify axon terminals with the electron microscope.

The lectin (Vector, CA) was injected iontophoretically into the vibrissal area of the left ventrobasal complex (VB) in 7, 3-month-old, male CD1 mice. Injections of 20 min duration were made through a glass pipette having a tip diameter of approximately 12 μm using 5 μA pulsed positive DC current delivered by a Midguard CS-3 high voltage current source (Transkinetics, MA).

A posterior approach through the tectum was used so that the pipette did not pass through neocortex or through other structures afferent or efferent to the vibrissal area of primary somatosensory cortex. Four mice were perfused intracardially 6–9 days later, and 3 mice 3 weeks later. Brains were sectioned at...
40 µm and processed according to a protocol based on that developed by Gerfen and Sawchenko. Sections were soaked for two days in biotinylated anti-PHA containing either 0.1% or 0.06% Triton-X. Following exposure to the biotinylated anti-PHA, the sections were reacted using an avidin-biotin-horseradish peroxidase (HRP) system (ABC kit, Vector, CA). The HRP was visualized using a diaminobenzidine (DAB) reaction intensified by the addition of cobalt chloride. Sections were postfixed in a solution of 1.0% osmium tetroxide and 1.5% potassium ferrocyanide for 0.5 h, stained for 1 h with 1.0% uranyl acetate dissolved in 70% methanol, dehydrated in graded ethanolos and propylene oxide and embedded in Epon/Araldite. Series of thin sections cut through layer IV of the posteromedial barrel subfield region of Sml cortex were mounted on formvar-coated slot grids and examined with a JEOL 100SX electron microscope.

Sections were examined with the light microscope to determine the placement and extent of the injection in VB thalamus. In each instance, the distribution of injected lectin occurred within the region of VB which contains the representation of the large mystacial vibrissae and occupied a volume of approximately 400 × 400 × 700 µm. Somata containing large amounts of reaction product occurred within the central one-third of this volume.

In coronal sections through the vibrissal region of Sml cortex, lectin was especially concentrated within axons in the white matter and in the neuropil immediately above it, in the lower part of layer V, in layer IV and in the lower portion of layer III (Fig. 1). The distribution of lectin transported anterogradely to layers IV and III mimicked the distribution of specific thalamocortical afferents to the vibrissal region of Sml cortex as observed in Golgi and in Fink-Heimer preparations. Thus the lectin appeared as clumps of reaction product, presumably filling barrel hollows, separated by relatively clear areas corresponding to the barrel sides and septa. The several clumps shown in Fig. 1 show varying intensities of labeling. This may be related to the amount of each barrel hollow included within the section, or it may reflect differences resulting from the distance of the corresponding part of the thalamus from the center of the injection site. In the latter instance, in-depth quantitative studies will be needed to determine whether lightly stained barrels contain a full complement of labeled thalamocortical axons, just lightly stained, or whether the number of thalamocortical axons is deficient in these barrels. Only tissue taken from the darkly stained barrels was examined with the electron microscope in the present study.

In some animals, a few, very lightly labeled somata occurred at the layer V/VI border, a region known to contain the cell bodies of corticothalamic projection neurons. We concluded that this small amount of retrograde transport did not complicate the identification in thin sections of anterogradely labeled thalamocortical afferents because no 40 µm thick section contained more than 2 or 3 such cells (none are observed in Fig. 1), and because the label was indeed very light. No label was observed in the axons of these cells, in their collaterals or in other than the proximal portions of their dendrites.

Lectin labeled axons and their terminals were recognizable in thin sections because of the electron-dense HRP reaction product they contained (Figs. 2–9). Reaction product was especially prominent in axon terminals where it tended to adhere to synaptic vesicle membrane. The ability to identify labeled axon terminals was directly related to the concentration of Triton-X employed in the immunohistochemical procedures; the higher the concentration of Triton-X, the easier it was to identify labeled terminals. For example, the labeled terminal in Fig. 2 is more easily distinguishable from unlabeled terminals (e.g. Fig. 3) than are the labeled terminals shown in Figs. 4 and 5, the latter having been taken from a preparation in which comparatively little Triton-X was used. An advantage of using lower concentrations of Triton-X is that cell membranes and cytoplasmic structure are far better preserved in these preparations than when higher concentrations of detergent are employed.

Fig. 1. Light micrograph of a 40 µm thick coronal section through mouse barrel cortex showing the distribution of PHA-L transported anterogradely from the injection site in VB thalamus. Reaction product is particularly dense in barrel hollows in layer IV, in layer III above barrel hollows, in the lower parts of layers V and VI and in the white matter. Roman numerals indicate cortical laminae; boundaries between laminae occur approximately half-way between these numerals. × 220.
Fig. 2. Electron micrograph showing two asymmetrical, axospinous synapses made by a thalamocortical axon terminal (TA) labeled by the anterograde transport of PHA-L. The high intensity of labeling results from the use of relatively high concentrations of Triton-X. S, spine. × 44,500.

Fig. 3. Electron micrograph from the same preparation as Fig. 2 showing, for comparison, asymmetrical synapses made by two unlabeled axon terminals (A). S, spine. × 44,500.

Figs. 4 and 5. Electron micrographs showing thalamocortical axon terminals (TA) labeled by PHA-L in a preparation in which relatively low concentrations of Triton-X were used. Mitochondria of TA terminals appear somewhat larger and have less regularly arranged cristae than those in unlabeled processes. A, unlabeled axon terminal; D, dendrite; S, spine. × 44,500.

Fig. 6. Electron micrograph showing an asymmetrical, axodendritic synapse made by a degeneration axon terminal (DA). At the upper left is shown an unlabeled axon terminal (A), and below it a thalamocortical axon terminal which is lightly labeled with transported lectin (TA, arrow). × 38,000.
Figs. 7–9. Electron micrographs of adjacent serial thin sections showing two PHA-L-labeled boutons (TA) which are joined by a pre-terminal axon segment. Each bouton makes asymmetrical synapses with spines (S). At the right of Figs. 8 and 9 is shown a synapse between an unlabeled axon terminal (A) and a spine (S). × 43,000.
Degenerating terminals having no trace of lectin within them were observed in all animals at a frequency of about 0.5% of all terminals in layer IV (Fig. 6). The possibility exists that these are thalamocortical axon terminals which are degenerating in response to mechanical or electrical damage at the injection site, or as a result of necrosis caused by PHA-L at the injection site in thalamus or in the cortex itself. For instance, the lectins ricin, abrin and modeccin are known to cause death of neurons20, and wheat germ agglutinin has been shown to cause inflammation2 or necrosis at the injection site12. As yet, PHA-L has not been observed to cause cell death; on the contrary, the occurrence of transported PHA-L within neuronal somata, dendrites and axons as long as 3 weeks following injection would seem evidence against any necrotic action of PHA-L. The amount of degeneration was, in any event, slight and thus was not considered to significantly affect the identification of thalamocortical axon terminals.

All synapses made by lectin labeled axon terminals possessed a plaque of electron-dense material adherent to the cytoplasmic surface of the postsynaptic membrane and thus were considered to be of the asymmetrical variety4. No thalamocortical axon terminals made symmetrical synapses as has been reported for cat visual cortex6. In comparison with lesion-induced degeneration, an advantage of the anterograde PHA-L method is that it permits the identification of preterminal segments and thus individual boutons can be identified as belonging to a single axon (Figs. 7–9).

Quantitative analyses in which lesion-induced degeneration has been used to identify thalamocortical afferents to mouse Sml barrels have demonstrated that thalamocortical synapses form from 18 to 23% of the asymmetrical synapses present in the neuropil of barrel hollows in CD/l mice10,11,17–19. To examine the potential of the PHA-L method for quantitative studies, we examined serial thin sections through neuropil taken from the barrel hollows of 3 animals. These series were cut only within the superficial 10 µm of the 40 µm thick sections so as to avoid complications which might result from any decrease in the penetration of immunological reagents into the depths of the thick sections. Counts were made of all synapses occurring in 4 samples which ranged in size from 180 to 240 µm³. Results showed that lectin labeled axon terminals formed from 18 to 21% of all asymmetrical synapses occurring within these samples. This finding is interpreted to mean that the anterograde transport of PHA-L is as effective as lesion-induced degeneration for the quantification of synapses made by thalamocortical afferents in mouse barrels. In addition, the similarity between results obtained with these two very different methods implies that labeling of axon terminals by the retrograde transport of PHA-L is not an important factor in this system.

The neuroanatomical tracing method based on the anterograde transport and subsequent immunohistochemical localization of the lectin, Phaseolus vulgaris-leucoagglutinin, represents a significant advance in the ability to identify terminal axonal projections in the mammalian brain. Among the advantages of this method are that axonal transport is predominantly anterograde, the injection is probably not taken up by fibers of passage8, and as shown in this study, the PHA-L method is suitable for the identification and quantification of synapses made by thalamocortical axon terminals. Because the anterograde PHA-L method labels preterminal axon segments it is now possible to determine how many of these synapses are made by single thalamocortical afferents, and further, how many afferents contact individual postsynaptic elements. For these reasons, we conclude that the anterograde PHA-L method can be used to quantify synapses made by thalamocortical or other types of axons in regions of the brain where lesion-induced degeneration has proven inadequate.

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