Original Article

Characterization of Retrograde Axonal Transport of Antibodies in Central and Peripheral Neurons1

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Retrograde axonal transport of antibodies against synaptic membrane glycoproteins was studied in the hypoglossal nerve and several CNS pathways of the rat. Injection into the tongue of polyclonal antibodies against synaptic membrane glycoproteins produced immunocytochemically labeled cells in the hypoglossal nucleus 4–5 hr later. Immunoreactive staining increased through 48 hr after injection and then declined. Injections of Fab preparations of the antibody gave labeling patterns indistinguishable from those of the whole antibody. The specificity of this method is shown by control studies in which antibodies against antigens that are not known to be present on the surface of presynaptic membranes were injected and gave no retrograde labeling. Retrograde labeling was also demonstrated in CNS pathways. However, labeling was never as intense as that seen in the hypoglossal nucleus, and some CNS pathways failed to show any retrograde labeling. Furthermore, retrograde labeling after control injections could be demonstrated in some cases. To determine if antibodies were also transported anterogradely, injections were made into the vitreous body of the eye, and the superior colliculus was processed for immunocytochemistry. Unlike wheat-germ agglutinin and several other tracers, antibodies were not found to be anterogradely transported in the optic nerve.

KEY WORDS: Retrograde axonal transport; Synaptic membrane antigens; Synaptic membrane glycoprotein; Antibodies; Retrograde labeling.

Introduction

The axon and axon terminal are dependent on the neuronal cell body for the continued replenishment of macromolecules which are delivered by the process of axonal transport (Grafstein, 1977; Grafstein and Forman, 1980; Lasek and Hoffman, 1976; Schwartz, 1979; Wilson and Stone, 1979). Once delivered, some of these macromolecules, particularly those supplied in the fast component of anterograde transport, are returned to the cell body by retrograde transport. This may serve as a normal degradation process and may possibly involve recycling of specific proteins. There is evidence, however, that retrograde axonal transport may also provide a mechanism for functional communication between the synaptic terminal and the cell body (Bisby, 1982, 1984; Grafstein, 1977; Grafstein and Forman, 1980; Kristensson, 1978, 1984; Schafer et al., 1983). A detailed knowledge of the molecules involved in retrograde transport may give new insights into both the mechanisms of synaptic protein degradation and the nature of this presumed communication.

A number of exogenous substances, including horseradish peroxidase (HRP), lectins, and dyes, have been shown to be retrogradely and, in some cases, anterogradely transported in neurons (LaVail, 1978; Mesulam, 1982). Such substances have been widely applied as tracers for the mapping of neuronal pathways in both the central and peripheral nervous systems. Little is known about the molecular bases of these transport processes, although the mechanisms of uptake and the ultimate fate of the retrogradely transported substances are well documented (LaVail, 1978; Mesulam, 1982). Certain lectins have been reported to be selectively transported in some populations of neurons (Wiley et al., 1983), but lectins recognize rather common sugar residues and are selective only for broad classes of macromolecules (Borges and Sidman, 1982; Margolis et al., 1981; Ruda and Coulter, 1982; Schnyder and Kunzle, 1983; Trojanowski, 1983). Retrograde transport has also been studied by covalently attaching a radioactive probe to neuronal proteins, followed by analysis of radioactivity after transport (Fink and Gainer, 1979; 1980a; 1980b), and by ligation experiments in which macromolecules accumulating distal to an axonal ligature are analyzed (Grafstein and Forman, 1980; Bisby, 1982). Although such approaches have been useful, they are often unsatisfactory because of lack of sensitivity or because they cannot be applied to a wide variety of neuronal pathways. Recently, Dahlstrom et al. (1985) have demonstrated anterograde and retrograde transport of antibodies against synaptic membrane glycoproteins. This work was supported by grants NS 18907, NS 06887, HD-03352, and BNS-7912939 and by the Intramural Research Program, NINCDS.

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transport of synaptic vesicle antigens by localizing immunoreactive material accumulating on both sides of a crush in the sciatic nerve. This approach may be useful in characterizing the axonal transport of known antigens.

Unlike that of HRP, the transport of lectins is dependent on the initial receptor-mediated binding of the lectin to the neuronal membrane. This binding mechanism provides an approach for labeling specific macromolecules destined for retrograde transport. Although lectins recognize common classes of sugars, more specific ligands such as cholera toxin, antibodies against dopamine-β-hydroxylase, and nerve growth factor are transported similarly (Dumas et al., 1979; Fillenz et al., 1976; Jacobowitz et al., 1975; Wan et al., 1982). We have reported that antibodies to synaptic membrane components are also retrogradely transported in the rat hypoglossal and facial neurons (Wenthold et al., 1984). More recently, others have reported similar findings studying different neuronal pathways (Fabian et al., 1984; Ritchie et al., 1984). These findings suggest that antibodies can be used for studying retrograde transport; with specific antibodies the fates of individual synaptic proteins could be monitored. In the present study we have extended our initial observation to a characterization of transport in the hypoglossal nerve and in selected CNS pathways.

Materials and Methods

Antibody Preparation. Antibodies were raised in rabbits, guinea pigs, and mice against the whole synaptic membrane fraction of rat brain or the concanavalin A (ConA)-,positive or the wheat-germ agglutinin (WGA)-positive components of the synaptic membrane fraction. Synaptic membranes were isolated using whole brain without brainstem, following the method of Jones and Matus (1974). Synaptic membranes were solubilized in 1% sodium deoxycholate and lectin-affinity chromatography was done in 0.1% sodium deoxycholate. Immunoglobulins were isolated from rabbit serum by ammonium sulfate precipitation and DEAE cellulose chromatography (Hum and Chantler, 1977). Fabs were prepared by papain digestion of the purified IgG fractions and purified by CM cellulose chromatography. Fractions obtained were characterized electrophoretically (Figure 1).

Antibody Injection. Sixty-day-old Sprague-Dawley rats were used in all studies. Animals were anesthetized with ether and injected in the tongue with antiserum in order to study transport in the hypoglossal nerve. Large injections consisted of 25 μl distributed among six different sites, and small injections consisted of 1.5 μl given at a single site. Volumes of serum less than 1.5 μl were diluted to 1.5 μl with phosphate buffered saline before injection. Injections into the vitreous body of the eye were done with a Hamilton syringe, with a maximum of 5 μl injected. Rats were anesthetized with ketamine HCl (70 mg/kg, IP) and sodium thiamylal (25 mg/kg, Sc) for injections in the CNS. The target site was exposed and injections were made using a pressure delivery system and a calibrated micropipet. Usually four injections, with a total volume of 1 μl, were distributed within a 1 mm radius. In many cases the antiserum or antibody preparation was lyophilized and resuspended in a smaller volume to concentrate the injection solution.

Immunocytochemistry. At chosen times after injection of antibody, rats were anesthetized with urethane and perfused with 50-100 ml of phosphate buffered saline (PBS) at room temperature, followed by 300 ml of cold 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.4. The brainstem was removed and post-fixed in the same fixative for 1 hr at 4°C. It was then transferred to PBS containing 10% sucrose and incubated overnight at 4°C. Forty-micron cryostat sections were cut at ~20°C and placed in cold PBS. They were then incubated for 30 min at 37°C in PBS containing 5% normal goat serum and 0.1% Triton X-100. Sections were incubated for 1 hr at 37°C with the IgG fraction of goat anti-rabbit immunoglobulin (Cappel, Cochranville, PA) diluted 1:200 in PBS containing 5% normal goat serum and 0.1% Triton X-100. After three rinses of 15 min each in PBS, sections were incubated at room temperature with rabbit peroxidase-antiperoxidase (1:400, Cappel) in PBS containing 5% normal goat serum and 0.1% Triton X-100. After washing, peroxidase was developed with DAB.


**Gel Electrophoresis and Immunoblotting.** Electrophoresis was done using the method of Laemmli (1970) and immunoblotting was done using the method of Towbin et al. (1979). Incubations with primary antibody were carried out at dilutions ranging from 1:200 to 1:1000. Affinity-purified goat anti-rabbit immunoglobulin conjugated to HRP (BioRad Laboratories, Richmond, CA) was used at 1:1000 dilution to localize rabbit antibodies. For localization of guinea pig or mouse antibodies, HRP-conjugated antibodies were obtained from Cappel and used at a 1:500 dilution.

**Results**

Immunoblot analyses of antibodies raised against whole synaptic membrane and the ConA-positive and WGA-positive fractions of the synaptic membranes show that each antibody preparation recognizes a complex pattern of antigens (Figure 2). After injection into the tongue, all showed similar rates of retrograde transport and distribution of label in the hypoglossal nucleus. Antibodies produced in rabbits against the ConA-positive fraction of the synaptic membrane were chosen for more detailed characterization because they gave the most intense immunocytochemical labeling.

**Retrograde Transport in the Hypoglossal Nerve**

Immunocytochemical labeling in the hypoglossal nucleus was studied as a function of survival time after large injections (25 µl) of antisera. Prominent labeling of hypoglossal cell bodies first occurred after survival times of 5 hr (Figure 3a), although light and scattered labeling was detected with survival times as short as 4 hr. After dissecting it free of surrounding tissue, the hypoglossal nerve in the rat is estimated to be 3 cm long; therefore, antibodies are transported at rates of up to 180 mm/day. This compares favorably with rates of retrograde transport determined by other means (Grafstein, 1977; Kristensson, 1978; Grafstein and Forman, 1980; Bisby, 1982). Our value does not account for the time required for the antibody to reach the synaptic area and be taken into the synaptic terminal, as well as for the time for detectable levels of antibody to accumulate in the cell body.

Discrete antibody labeling in the cell body and progressive increases in DAB reaction products were evident, with survival times approaching 48 hr (Figures 3d and 4d). Thereafter, the reaction product was progressively less punctate and less intense until, after a 1-week survival time, the neuropil appeared rather uniformly labeled (Figure 3e). Subsequent increases in the intensity of reaction products, which would be suggestive of the arrival of more slowly transported populations of antibodies, were not seen even with still longer survival times. Rather, labeling continues to decrease 2 and 4 weeks after injection. Punctate reaction product can also be seen in fibers (Figure 4c), presumably representing antibodies that are being retrogradely transported. This observation suggests that the antibodies are transported with an associated organelle which restricts their intra-axonal distribution.

Small injections (1.5 and 0.15 µl) of antibody produced patterns of immunocytochemical labeling in hypoglossal neurons which were qualitatively similar to those of large injection cases. However, antibody labeling from small injections was more confined and punctate (Figures 4a and b) than labeling from large injections at similar survival times. This observation suggests that the diffuse appearance of reaction product at 24 and 48 hr after large injections may simply be the result of a heavy accumulation of labeled antibody in neurons, rather than a diminution of punctate reaction products.

A number of antisera were studied as controls, including normal serum from rabbit, guinea pig, and mouse, rabbit anti-bovine serum albumin, rabbit anti-cytoplasmic aspartate aminotransferase, rabbit anti-neuron-specific enolase, and IgG fraction of rabbit anti-guinea pig immunoglobulins. None of these antisera produced detectable labeling in the hypoglossal nucleus either 24 hr or 2 weeks after injection of 25 µl (Figure 3f).

**Retrograde Transport of Fab Fragments**

Although it was shown that the antibodies are retrogradely transported in the hypoglossal nerve, a question remains if this reflects normal retrograde transport of synaptic molecules or if it was caused by abnormal crosslinking and internalization of the synap-
Figure 3. Labeling of neurons in the hypoglossal nucleus after injection of 25 \( \mu \)l of antiserum into the tongue. Figure shows labeling at 5 hr (A), 6 hr (B), 12 hr (C), 24 hr (D), and 1 week (E) after injection. No labeled cells are seen 24 hr after injection of normal rabbit serum (F). Bar = 50 \( \mu \)m.

Figure 4. Labeling of cell bodies and fibers of hypoglossal neurons after injection of antiserum into the tongue. (A) Labeled neurons 48 hr after injection of 1.5 \( \mu \)l of antiserum. (B) Labeled neurons 24 hr after injection of 0.15 \( \mu \)l of antiserum (diluted to 1.5 \( \mu \)l with PBS). (C) Retrogradely transported antibodies in fibers leading to the hypoglossal nucleus 12 hr after injection of 25 \( \mu \)l of antiserum. (D) Higher magnification of hypoglossal neurons 6 hr after injection of 25 \( \mu \)l of antiserum. Bar = 20 \( \mu \)m.
The present study demonstrates that antibodies to synaptic membrane proteins with polyclonal antibodies. To address this, monovalent Fab fragments produced from the antibodies were injected. The appearance of Fab fragment immunoreactivity in hypoglossal cell bodies followed the same survival time relationships and punctate distributions as described for whole serum injections (Figure 5).

**Retrograde Transport of Antibodies in the CNS**

To determine if antibodies to synaptic membrane components are also retrogradely transported in the CNS, antibodies were injected into the inferior colliculus. The inferior colliculi and brainstem were processed for immunocytochemistry after a 24-hr survival time. Although antisera was used in some cases, most experiments were done with the isolated IgG fraction, which enabled a higher concentration of antibody molecules to be injected.

After injection of antibody into the inferior colliculus, immunoreactive neurons are seen in the superior olivary complex, cochlear nuclei, and inferior colliculi (Figure 6). The injection site is filled with peroxidase reaction product. In the surrounding zone many labeled fibers can be discerned, but only a few cell bodies can be distinguished. Injection of control antibodies into the inferior colliculus was also found to produce labeling of cells in the superior olivary complex. However, control injections were always found to produce less labeling than injections of antibodies against synaptic membrane glycoproteins. For example, in Figure 6a, 60 μg of protein from the IgG fraction of rabbit anti-synaptic membrane glycoproteins gave heavy labeling of cells, whereas 160 μg of the IgG fraction of rabbit anti-guinea pig immunoglobulins gave only light labeling. However, in the hypoglossal nerve, injections of up to 800 μg of the IgG fraction of rabbit anti-guinea pig immunoglobulins gave no retrograde labeling.

**Anterograde Transport of Antibodies**

Many exogenous substances, including lectins and HRP, are transported in the anterograde as well as retrograde direction in neurons. To determine if antibodies were transported anterogradely, injections were made into the vitreous body of the eye and the superior colliculus was processed for immunocytochemistry 24 hr after injection. Antisera, purified IgG, and Fab fragments did not give labeling in the superior colliculus.

**Discussion**

The present study demonstrates that antibodies to synaptic mem-

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Figure 5. Labeling of neurons in the hypoglossal nucleus after injection of Fabs prepared from rabbit antibodies against the ConA fraction of rat synaptic membranes. 25 μl was injected at six sites on the tongue. Survival times are 5 hr (A), 6 hr (B), 24 hr (C), and 48 hr (D). Bar = 50 μm.
brane components are retrogradely transported in both peripheral and central neurons. In the hypoglossal nerve a rate of transport of greater than 180 mm/day is obtained. This figure is a minimum, since it does not take into account the time required for the antibody to reach and bind the synaptic antigen as well as the time required for a detectable level of antibody to accumulate in the cell body. Our estimate lies in the range of retrograde transport rates obtained using other tracers and ligation studies (Grafstein and Forman, 1980; Kristensson, 1978). After antibody first appears in the hypoglossal nucleus, it continues to increase through 48 hr after injection. This may reflect several events. Since synaptic molecules are known to turn over at many rates, injection of antibody could be viewed as a pulse label of exposed synaptic antigens. Those which turn over rapidly would be rapidly lost from the synaptic membrane, along with the attached antibodies, and many would be removed by retrograde transport. Antibodies to slowly turning-over proteins would be lost more slowly and could be responsible for the late-arriving antibodies if they are also retrogradely transported. However, the injection of antibody probably does not represent a pulse label, but free antibodies may remain for some time after injection and could bind to newly exposed synaptic proteins. This situation could also account for the late-arriving antibodies. Finally, there may be several discrete waves of retrograde transport during the first 48 hr, reflecting different transport rates of individual synaptic molecules. After 48 hr a decrease in immunoreactive labeling is seen in the hypoglossal nucleus, which continues through 4 weeks after injection. Therefore, a slower rate of retrograde transport does not appear to be present; however, this cannot be ruled out since a minor slow component may not be detected. A slow component of retrograde transport has been reported using a covalent labeling technique (Fink and Gainer, 1979; 1980a; 1980b). These may represent soluble or cytoskeletal proteins which would be inaccessible to the injected antibodies.

After injection of antibodies into the inferior colliculus, labeled neurons were consistently seen in the superior olivary complex and cochlear nucleus. However, several observations suggest that the present technique is not as effective in the CNS as in the hypoglossal system. For example, neurons were never as intensely labeled as hypoglossal nucleus cells, even when larger amounts of antibody were injected. Some CNS neurons may not take up or transport antibody molecules as readily as hypoglossal neurons.

Figure 6. Retrograde labeling of neurons in the CNS. After injection of antiserum into the inferior colliculus, labeled cells are seen in the superior olivary complex (A), the cochlear nucleus (C), and the contralateral colliculus (D). Injection of large amounts of control antibodies also produces labeling in the superior olivary complex (B). Bar = 100 μm for A, B, D; 50 μm for C.
Alternatively, little antibody may be available for binding to synaptic molecules. Since antibodies were made to fractions of CNS synaptic membranes, much antibody may be bound to neurons, terminals, and glial cells at the injection site, and only a small fraction may be associated with the pathway of interest. A heavy reaction product is always seen at the injection site.

Another limitation of CNS injections was that control antisera often produced retrogradely labeled cells. The labeling obtained with control sera was light compared to that obtained with antibodies against synaptic components. Nevertheless, control labeling was never seen in the hypoglossal or facial nerves. This non-specific labeling is probably related to the damage caused by CNS injections, which could give the antibodies access to the interior of the axon, rather than through binding to membrane receptors. It cannot be ruled out, however, that some synaptic molecules have a weak affinity for the antibodies, which could also lead to light retrograde labeling.

Finally, CNS injection of antibody may not label all central neurons. In the course of these studies, other central pathways were tested. For example, injections into the superior colliculus did produce labeled retinal ganglion cells. However, injection into the cerebral cortex failed to produce labeling in the contralateral cortex, and injections into the cerebellum failed to label cells in the inferior olive. In these cases, insufficient antibody may have been transported to allow detection, but the injection protocols were the same as those for the inferior colliculus study. Alternatively, accessibility of particular antigens may be more restricted at certain synapses, or antigens recognized by antibodies used in this study may be absent, or present at low concentrations, on these neocortical and cerebellar neurons.

Injections into the vitreous body of the eye also did not give labeling in the superior colliculus, suggesting that the antibodies are not anterogradely transported. Since synaptic proteins are synthesized in the cell body and transported to the synaptic terminal, this probably results from the lack of accessibility of the antibody to the antigen. Again, antibodies may have been transported but in insufficient amounts to allow immunocytochemical detection.

The most straightforward interpretation consistent with our findings is that injected antibodies bind to antigens on the surface of the synaptic terminal and remain with the antigen through endocytosis and retrograde transport. In the axon and cell body, the reaction product appears associated with vesicular organelles, consistent with the proposed fate of retrogradely transported substances (Kristensson, 1978). Therefore, we propose that antibodies can be used, at least in peripheral neurons, to study the toponography, turnover, retrograde transport, and degradation of synaptic membrane proteins. However, several questions remain. Although it appears that the antigen must be associated with the synaptic membrane, and must probably be exposed on the external surface, for retrograde transport to occur, it is not known if the antibody remains attached to the antigen after endocytosis and during retrograde transport. A protein that is degraded at the synaptic terminal may release its antibodies, which may then be carried back to the cell body unattached to an antigen.

The effect of antibody binding to synaptic antigens is also unknown. Antibodies can alter protein conformation and they have been shown to increase degradation. For example, in cultured muscle cells the degradation rate of the cholinergic receptor is more than doubled when antibody is bound to it (Heinemann et al., 1977). Monovalent Fabs, however, did not change the degradation rate of the cholinergic receptor. In our studies monovalent Fabs produced retrograde transport indistinguishable from that obtained with intact antibodies.

The antibodies used in these studies were raised against complex mixtures of antigens obtained from the synaptic membrane fraction. We have assumed that most antibodies are against synaptic proteins, but antibodies against lipids, glycolipids, and several classes of small molecules may also be present. These substances are also known to be axonally transported (Grafstein and Forman, 1980). However, it is unlikely that antibodies against small molecules are present, since the antigen preparations were extensively dialyzed before injection to produce antibodies. Many of the uncertainties that remain can be addressed by using antibodies specific for individual synaptic proteins. This approach may also address many questions concerning retrograde axonal transport, including which proteins are transported, rates of transport, and conditions that may change retrograde transport.

Literature Cited


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