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Research Note

A Note on the Transneuronal Transport of Wheat Germ Agglutinin-conjugated Horseradish Peroxidase in the Avian and Rodent Visual Systems*

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Summary. While using horseradish peroxidase conjugated to the plant lectin wheat germ agglutinin (WGA-HRP) as an anterograde marker to label the developing retinofugal projection in the chick, we have found that a significant amount of the tracer can escape from the axons and axon terminals of retinal ganglion cells and be subsequently taken up and transported both anterogradely and retrogradely by neighboring neurons and/or axon terminals. The release and uptake of the tracer appears to be non-specific, and is particularly striking at embryonic stages; at these stages there is also appreciable uptake of the WGA-HRP by ependymal cells and by radial glial processes. Subsequent experiments in rats have shown that as early as 2 days after an injection of WGA-HRP into an eye there is clear labeling of geniculo-cortical axons in the striate cortex. Since WGA-HRP is being used increasingly in neuroanatomical studies, it is now clear that when interpreting the results of experiments with this marker the possibility of transneuronal labeling must be borne in mind, and especially after relatively long post-labeling survival periods. At the same time the secondary transport of WGA-HRP may, in certain neural systems, provide a useful tool for analyzing second-order connections.

Key words: Wheat germ agglutinin conjugated horseradish peroxidase – Transneuronal transport – Chick visual system – Visual cortex

It is now known that many different substances are released at nerve terminals. In addition to the various neurotransmitters, there is experimental evidence for the release of nucleosides (Schubert and Kreutzberg 1974), several different enzymes (Cubeddu et al. 1974; Greenfield et al. 1980), neurotropic viruses (such as poliomyelitis and herpes simplex, Knotts et al. 1974), certain bacterial toxins (Schwab et al. 1979), some plant lectins (Coulter et al. 1980), and various glycoproteins or their constituent amino acid or sugar moieties (Grafstein 1971). The latter, when radioactively labeled and used as markers of anterograde axonal transport, have been used effectively to demonstrate the secondary projections of the cochlea upon the auditory relay nuclei (Kelly et al. 1978) and of the retina upon the visual cortex (Grafstein 1971; Specht and Grafstein 1973; Dräger 1974; Wiesel et al. 1974).

The fact that substances which are commonly used as axonal tracers can be released from, or leak out of, axon terminals and then be taken up and secondarily transported by the surrounding neurons obviously calls for special care in the interpretation of experimental neuroanatomical studies. Several workers have cited the absence of such transneuronal transport as one of the major advantages of using horseradish peroxidase (HRP) either alone, or conjugated with the plant lectin wheat germ agglutinin (WGA-HRP), as an anterograde marker. However, we have recently found, while using WGA-HRP to study the development of the central connections of the retina in the chick, that a considerable amount of the tracer may be released by the axons and axon terminals of the retinal ganglion cells and that a significant proportion of the released tracer can be taken up and secondarily transported both anterogradely and retrogradely. These findings prompted us to examine whether this phenomenon also occurs in the mammalian visual system, and when it became evident that WGA-HRP injected into the eye can lead to clear labeling of the rat geniculo-striate

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projection as early as 2 days after the injection, it seemed appropriate, given the widespread use of WGA-HRP as a neuroanatomical tracer (Gonatas et al. 1979; Staines et al. 1980), to place our observations on record.

In our experiments WGA-HRP (Sigma) was injected into the vitreal chamber of chick embryos of different ages, and into the eyes of post-hatched chicks and young adult rats. The WGA-HRP was made up as a 1–2% solution in 0.1 M phosphate buffered saline (pH 7.4). Depending on their ages, between 3 and 10 µl of the WGA-HRP solution was injected into the eyes of chick embryos[1] using a 10 µl Hamilton syringe; for the post-hatched chicks and rats 10–20 µl of the WGA-HRP solution was injected intraocularly using a 50 µl Hamilton syringe. After survival times of between 5 and 40 h (for the embryos) and 2–5 days for the posthatched chicks and adult rats, the animals were perfused sequentially with normal saline, a mixture of 1.0% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), and 10% sucrose in 0.1 M phosphate buffer (pH 7.4). The brains were removed and stored overnight at 4° C in 10% sucrose in phosphate buffer, and then serially sectioned on a freezing microtome. Two, one in two series of sections were mounted onto chrom-alum coated slides, and processed for peroxidase histochemistry following the TMB method of Mesulam (1978). For each brain one series of sections was counterstained with Richardson's stain. In most cases the histochemical procedure used resulted in minimal background labeling and for this reason we believe that the deposition of the TMB chromogen can be attributed with confidence to the presence of transported WGA-HRP.

The most striking evidence we have observed for the transneuronal transport of WGA-HRP is in the visual system of embryonic chicks. Here, after relatively short post-injection survival times (no more than 8–14 h depending on the age of the embryo) we have evidence for the transneuronal spread of the marker to neurons and glial cells along the course of the optic tract and within the various regions of termination of the axons of the retinal ganglion cells, and also for its anterograde and retrograde transport by the neurons in these regions or by other cells whose axons terminate in the same regions. After shorter survival times (6 h or less) the peroxidase labeling is limited to the regions which are in receipt of the primary visual projections as previously defined in axonal degeneration and autoradiographic studies (Cowan et al. 1961; Repérant 1973; Crossland et al. 1975). The great majority of the retinal fibers cross in the optic chiasm and can be followed to a number of diencephalic and mesencephalic nuclei on the contralateral side, and especially to the optic tectum. Here the fibers are distributed over the surface of the tectal lobe in the stratum opticum (SO), and labeled axons can be seen to leave the SO at intervals to turn inwards into the outer layers of the stratum griseum et fibrosum superficiale (SGFS), where they terminate in laminae a through f as defined by Cowan et al. (1961). At these short post-injection times there is no clear evidence for the spread of the WGA-HRP to the cells in any of the primary visual relay nuclei nor in the optic tectum; indeed the only other structure labeled is the isthmo-optic nucleus (ION) which is the site of origin of centrifugal fibers to the retina. The ION shows heavy retrograde labeling of essentially all its cells, and in addition several hundred ectopic ION neurons are labeled in the area surrounding the nucleus (O'Leary and Cowan 1982).

1 Although we have examined embryos at each stage from the fifth day of incubation until just before hatching (on day 21) for the present purposes we shall confine ourselves to cases injected after the 12th day of incubation by which time the retinal projection is well established, at least in the sense that optic nerve fibers have reached virtually all parts of the optic tectum and all of the primary visual relay nuclei

Fig. 1. A A low-power, dark-field photomicrograph through the caudal end of the optic tectum on the side contralateral to a retinal injection of WGA-HRP in a 13-day old embryo (24 h survival). Note the heavy labeling of the outer layers of the optic tectum. The *black arrowhead* marks the deepest level to which optic nerve fibers normally extend (lamina f of the SGFS). There is appreciable labeling in the subjacent layers including the deep cellular layer (the SGC) and in the ependymal lining (E) of the tectal ventricle. Note also the heavy retrograde transneuronal labeling of the nucleus isthmi pars parvocellularis (IPC). This may be compared with the direct retrograde labeling of the cells of the isthmo-optic nucleus (ION). The appearance at this embryonic stage should be contrasted with that seen in an animal that was injected intraocularly with WGA-HRP on the 6th day after hatching and allowed to survive for 48 h (**D**). In this case there is much less spread of HRP into the deeper layers of the tectum, but the cells in the IPC still show heavy retrograde transneuronal labeling. Note also the dense direct terminal labeling in the ectomammillary nucleus (EM). The micrographs in **B** and **E** are from sections adjoining those in **A** and **D**, respectively, to show, at higher magnification, the spread of the label within the optic tectum. Note the dense terminal labeling in laminae b, d and f of the SGFS, and the more diffuse spread of label deep to this. **C** is an even higher magnification view of the area outlined in **B** to show the labeling of individual cells in the SGC (*open arrow*) and the linear distribution of the reaction product in what we take to be the radial processes of ependymel cells (*parallel arrows*). **F** is a higher power micrograph of the retrograde transneuronal labeling of the cells in the region of the IPC marked by the rectangle in **D**. Calibration bar in **A** and **D**: 500 µm, in **B** and **E**: 100 µm, and in **C** and **F**: 50 µm

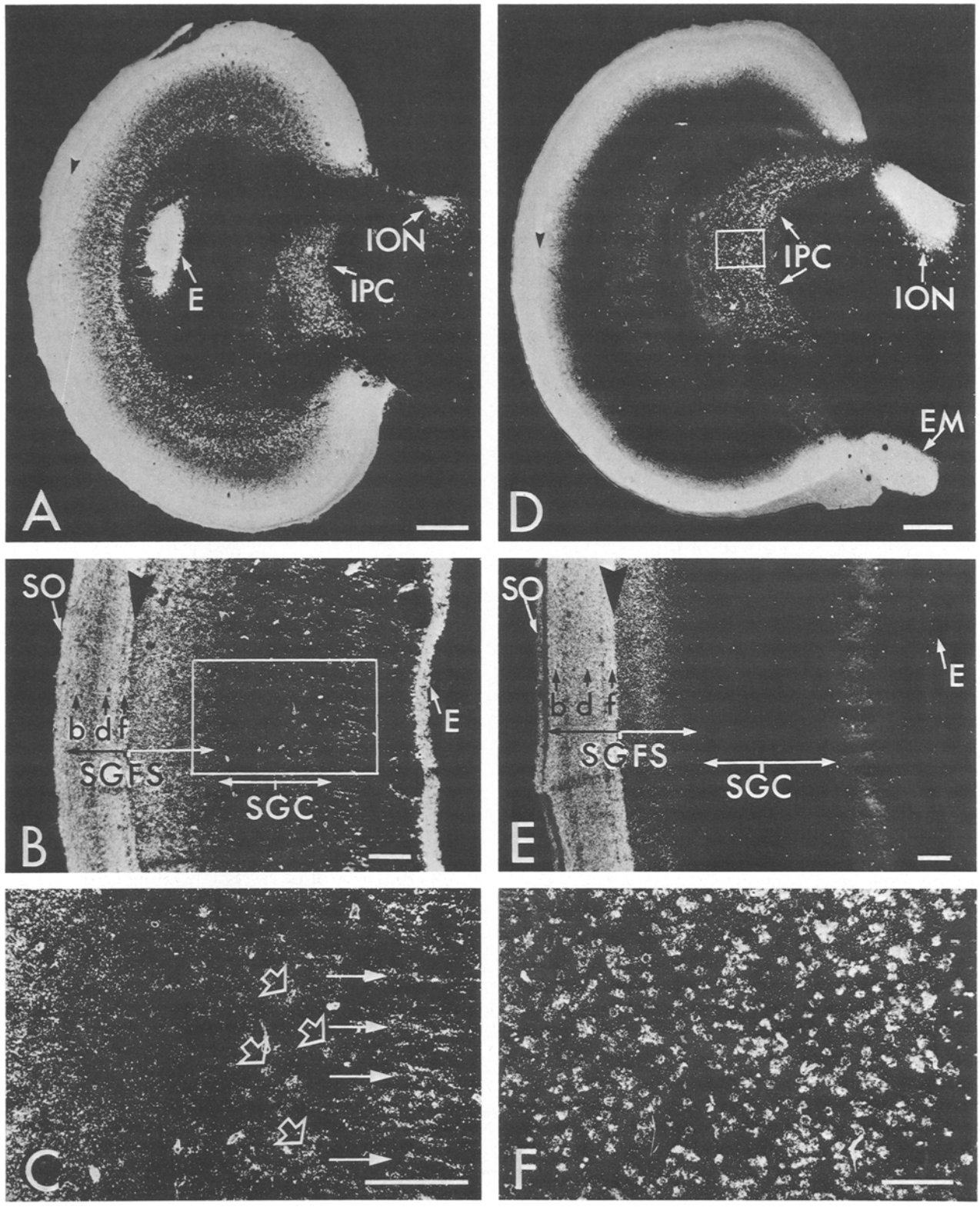

Fig. 1A–F

In animals which survived for 8 h or longer after injections of WGA-HRP the same structures are all heavily labeled, but there is in addition clear evidence for the spread of the marker from the optic tract and from the terminals of the retinal ganglion cells. In all the younger embryos we have examined, a significant amount of the peroxidase reaction product has been seen in the area adjoining the optic tract. In the older embryos this type of "periaxonal" labeling is less evident, but some is still seen at quite late stages in embryonic development. However, the most striking evidence for the transcellular spread of the transported label is seen in the optic tectum. Rather than being confined to the SO and the subjacent laminae of the SGFS, the label can be seen to extend deeply throughout the SGFS, and to have heavily labeled the deeper cellular layers of the tectum including the stratum griseum centrale (SGC) which is one of the principal sources of tectal efferents. Not only are the tectal neurons that lie within and deep to the zone of termination of the retinal fibers labeled, but the marker appears to have been also taken up by the ependymal cells lining the tectal ventricle and by the radial glial processes that extend superficially from the ependymal zone across the tectal wall (see Fig. 1A–C). Perhaps even more surprising is the finding of intense retrograde transneuronal labeling of the neurons in the nucleus isthmi pars parvocellularis (IPC) and, on occasion, in certain of the pretectal nuclei. These nuclei are known to project to those layers of the SGFS that receive retinal fibers (Hunt and Künzle 1976; Hunt et al. 1977; Brecha 1978). The labeling in the IPC is especially striking, and unlike the rather diffuse labeling seen in the deeper layers of the tectum (which is clearly due in part to the escape of the marker from the optic nerve fibers and in part to the transneuronal uptake of the label by tectal neurons) that in the IPC seems to be largely due to the uptake of the released WGA-HRP by the axon terminals of the isthmo-tectal cells and its subsequent retrograde transport to the IPC. There is no clear evidence for anterograde labeling of the efferent fibers from the tectum to the isthmic nuclei (in the tecto-isthmal tract) but some of the terminals of this tract within the IPC appear to be labeled at these survival times. There is also evidence for the anterograde transneuronal transport of the marker to several secondary visual centers such as the nucleus semilunaris and the lateral pontine nucleus. In embryos that were injected just before hatching, and in chicks injected at different times during the first week after hatching, there is somewhat less, but still substantial, transneuronal spread of label both anterogradely within the tectum and retrogradely from the tectum to the IPC. At these stages there is little evidence that the tracer has escaped from the fibers in the optic tract, but within the optic tectum the WGA-HRP has spread from the zone of termination of the retinal ganglion cells to the deeper laminae of the SGFS (Fig. 1D, E). There is no evidence in these animals of labeling deep to the SGFS (the SGC for example appears to be quite free of label) nor does the marker appear to have been taken up by radial glial processes in the tectum or by the ependymal cells lining the tectal ventricle. However, the retrograde labeling of the neurons in the IPC is as striking as it is at earlier stages (see Fig. 1D and F).

In summary our chick material provides clear evidence for the escape of WGA-HRP along the length of the optic tract in young embryos, and for its release from the terminals of the retinal ganglion cells at every age we have examined (including a small number of adult hens). Some of the material released from the optic nerve fibers is taken up in young embryos by the processes of the radial glial cells and accumulates within their cell bodies in the ependymal lining of the tectal ventricle. This does not seem to occur in older animals but in these, as at earlier stages, there is clear evidence for the uptake and anterograde transport of the marker by neurons in the optic tectum and also for its uptake and retrograde transport by the axons of cells which project into the outer layers of the optic tectum in which the retinal fibers terminate.

Prompted by these findings we have examined the brains of a series of rats in which WGA-HRP was injected into the vitreous of one eye. In every case that had been allowed to survive for more than 2 days after the injection the entire retinofugal projection was labeled, including the projections to the suprachiasmatic nucleus of the hypothalamus, to the ventral and dorsal lateral geniculate nuclei, to the accessory nuclei of the optic tract, to the pretectal complex and to the superior colliculus. Since the pattern of distribution of the label is comparable in virtually every respect to the known distribution of retinal fibers as described in axonal degeneration and anterograde labeling studies (see for example, Hayhow et al. 1960; Hendrickson et al. 1972; Scalia 1972) it need not be considered further. But what is of particular interest is that in every brain there is also clear evidence of transneuronal labeling from the dorsal lateral geniculate nucleus to the primary visual cortex (see Fig. 2A). Within the striate cortex there is a distinct band of label in layer VI and a much heavier zone of labeling in layer IV (Fig. 2B) which suggests that most of the transneuronally transported material is within, or close to, the terminals of the geniculo-cortical fibers. In this respect the pattern of

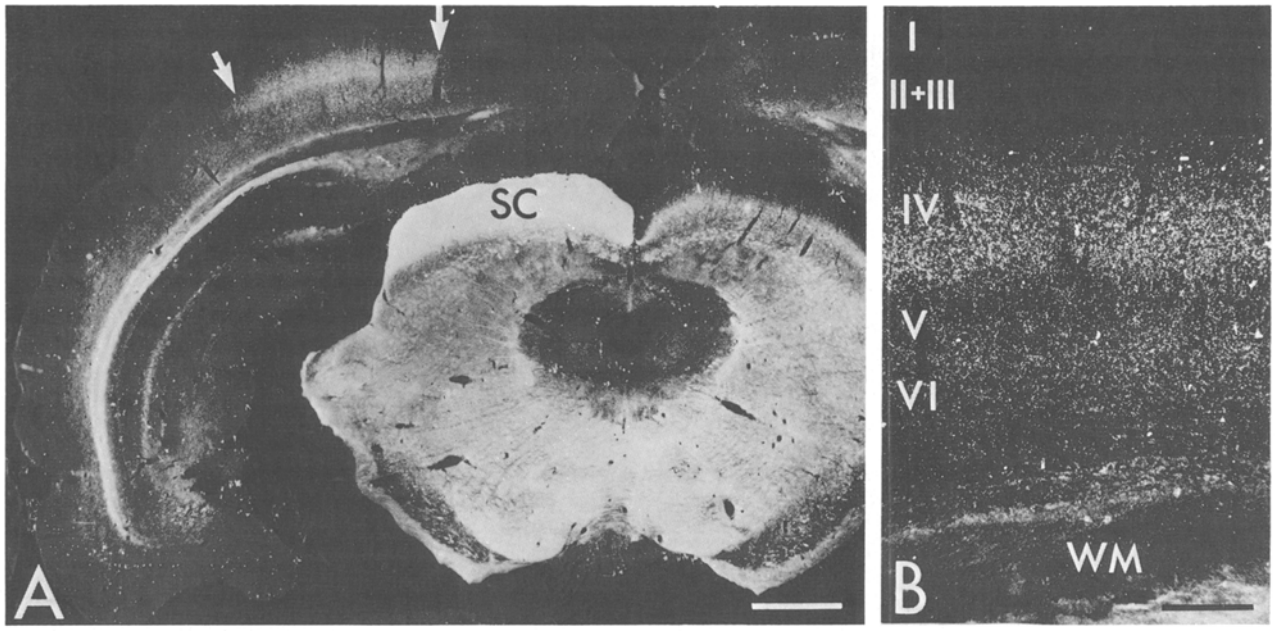

Fig. 2. A A low-power dark-field photomicrograph of a section through the midbrain of a young adult rat in which WGA-HRP was injected into the right eye 5 days earlier. Note that in addition to the dense labeling in the superior colliculus (SC), there is clear evidence of anterograde transneuronal labeling in the visual cortex (between the *white arrows*). **B** A high-power photomicrograph of the striate cortex in this case to show the distribution of the anterogradely transported label in layers IV and VI. Note that the underlying white matter is relatively free of label as are the cortical fields that overlie the superior colliculus (**A**). Calibration bar in **A:** 500 μm and in **B:** 100 μm

transneuronal labeling in the cortex is similar to that seen after injections of ^{3}H-amino acids or glycoprotein precursors into the eye (Specht and Grafstein 1973; Dräger 1974). However, in one important respect the two procedures seem quite different. Invariably after the administration of ^{3}H-amino acids or ^{3}H-fucose there appears to be a considerable amount of diffusion of the label from the lateral geniculate and superior colliculus to such overlying "non-visual" structures as the entorhinal cortex, the subicular complex and hippocampus (Specht and Grafstein 1973; Dräger 1974). In this respect our experiments with WGA-HRP seem quite different: as Fig. 2A shows there is no indication that the marker has spread from the superior colliculus to the overlying cortical areas, and it is evident from its distribution that most of the labeling found within the striate cortex reaches it by way of the geniculo-cortical projection.

Although in a number of other structures anterograde transneuronal transport may have occurred (e.g., to the pretectal area) it is difficult in most of these regions to rule out the direct diffusion of the marker from the terminals of the retinal ganglion cells. But in a number of other regions, such as the zona incerta, part of the pontine nuclear group, the parabigeminal nucleus, and the oculomotor complex (each of which is known to receive an input from one or another of the visual relay nuclei, Hayhow et al. 1960; Ribak and Peters 1975; Perry 1980) there is clear evidence of labeling which can only be attributed to anterograde transneuronal transport comparable to that seen in the striate cortex. We have not seen convincing evidence of retrograde transneuronal transport in the rat visual system after the survival periods we have examined. There are labeled neurons in the pretectal area and in the superior colliculus, but one cannot be sure that this is not due to direct uptake of the released marker by the relevant neurons rather than its retrograde transport; certainly there is no indication of retrograde labeling of the cells in layers V and VI of the striate cortex which project to the superior colliculus and dorsal lateral geniculate nucleus, respectively. It is also of interest that most of the geniculo-cortical radiation is unlabeled; it is only within the zones of termination of the geniculo-cortical fibers in area 17, and to a lesser extent in area 18, that significant labeling is seen.

There have been previous reports that both WGA and HRP can be transported transneuronally (Coulter et al. 1980; Nässel 1981; Wilczynski and Zakon 1982) but until recently it has generally been assumed that this is not true of WGA-HRP conjugates. While our paper was in preparation, a brief report by Itaya and Van Hoesen (1982) appeared

describing the anterograde transneuronal spread of WGA-HRP after intraocular injections of WGA-HRP in adult monkeys and rats. Their findings, taken in conjunction with our own, clearly indicate that in a number of neural systems substantial transneuronal labeling of WGA-HRP can occur in both the anterograde and retrograde directions, and that in interpreting the results of experiments in which this tracer is used, it is important to bear this possibility in mind. This is especially true in tracing developing neural pathways and whenever long post-labeling survival periods are used. At the same time the occurrence of transneuronal labeling with WGA-HRP provides an alternative to the use of isotopically labeled amino acids or sugars for deliberately labeling secondary projections; the findings that it occurs after relatively short survival periods and after the injection of small amounts of the tracer, even in mature animals, and since there is little or no diffusion of the tracer away from the sites of transneuronal transfer, suggest that this approach may offer several advantages.

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