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Simultaneous Anterograde Labelling of Two Afferent Pathways to the Same Target Area with *Phaseolus vulgaris* Leucoagglutinin and *Phaseolus vulgaris* Leucoagglutinin Conjugated to Biotin or Dinitrophenol

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ABSTRACT

Anterograde transport of *Phaseolus vulgaris* leucoagglutinin (PHA-L) and PHA-L conjugated to either biotin or 2,4-dinitrophenol were used to simultaneously label two populations of axons converging onto the same target area. Using the rat hippocampus as a test system, the septohippocampal and contralateral hilar afferents were labelled with the tracers. Double immunohistochemical procedures and either nickel-enhanced 3,3′-diaminobenzidine (DAB) reaction (blue-black colour) or DAB alone (brown colour) were used to produce contrasting colours for the different tracers in the same section.

Both the biotinylated PHA-L and PHA-L conjugated with dinitrophenol were used successfully as anterogradely transported axonal tracers. They produced extensive axonal labelling in the hippocampal formation. Excellent double labelling could be produced with the simultaneous application of PHA-L and biotinylated PHA-L. Biotinylated PHA-L was visualized by a sequence of avidin-biotinylated peroxidase complex (ABC), biotinylated goat antiavidin and ABC again using nickel-enhanced DAB as chromogen yielding a blue-black reaction endproduct. PHA-L alone was detected by the unlabelled antibody enzyme (PAP) method using goat antibodies to PHA-L, and DAB as chromogen for the peroxidase reaction, resulting in brown axons. The combination of biotinylated PHA-L and DNP-conjugated PHA-L gave similar results, although the sensitivity of detection by the latter procedure was inferior to that obtained with the other tracers.

These protocols permitted visualization of axons of different origin, together with their terminals, either in a blue-black or brown colour, and also allowed the demonstration of overlapping inputs in strata radiatum and lacunosum moleculare of the hippocampus and stratum moleculare and hilus of the dentate gyrus.

KEY WORDS: Anterograde axonal tracing Double immunostaining PHA-L Biotinylated PHA-L DNP-conjugated PHA-L

INTRODUCTION

The kidney bean lectin, *Phaseolus vulgaris* leucoagglutinin (PHA-L) introduced by Gerfen and Sawchenko,[4] has proved to be a powerful neuronal tracer, which has a number of advantages over other techniques. This lectin is transported predominantly, though not exclusively,[2,4] in the anterograde direction along axons from the application site.[5,20,24] This preferential transport of PHA-L in the anterograde direction is in contrast to the transport of wheat germ agglutinin and horseradish peroxidase, which travel not only in the anterograde but also in the retrograde directions.[8,9,10,17] Another advantage of PHA-L is that small and circumscribed application sites can be produced by extracellular, iontophoretic delivery of the lectin. The lectin is still detectable in the axons and nerve terminals even 2 weeks after the application, and it does not seem to cause any significant degeneration in the labelled structures.[5,24] In addition, PHA-L labelling can be combined with other tracing techniques, e.g. the transport of fluorescent dyes,[16] HRP histochemistry,[21] autoradiography,[4] pre-embedding[5,6,22,23] and post-embedding[3] immunocytochemistry. The PHA-L-labelled axons can be visualized in great detail[4,5,23] together with their terminals.

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Table 1. Summary of experiments and combined procedures used for visualizing two populations of afferents converging to the same area of the hippocampus. For visualizing procedures see the flow diagram in Fig. 1. Colour of afferents revealed with the nickel-enhanced DAB reaction for peroxidase was bluish-black, but black is used for short.

Experiment	Application site	Tracer	Visualization procedure (Fig. 1)	Colour of labelled axons	Figure
1	(a) Septum	PHA-L	D	Brown	
	(b) Hippocampus (contralateral)	PHA-L-biotin	C	Black	5A, B, D
2	(a) Septum	PHA-L-biotin	C	Black	
	(b) Hippocampus (contralateral)	PHA-L	D	Brown	5C, E
3	(a) Septum	PHA-L-DNP	B	Brown	
	(b) Hippocampus (contralateral)	PHA-L	A	Black	5G
4	(a) Septum	PHA-L-DNP	B	Brown	
	(b) Hippocampus (contralateral)	PHA-L-biotin	C	Black	5F

In many areas of the central nervous system it is of interest how different axonal systems, originating from separate areas of the brain, are distributed in relation to each other. One of us (R. A. J. McI.) reasoned that the conjugation of PHA-L to small molecules should not affect its transport characteristics, and therefore allow the simultaneous visualization of two separate populations of afferents converging onto the same target area. In this study we describe the use of the anterogradely transported property of PHA-L and PHA-L conjugated to dinitrophenol (DNP) or biotin as tracers. Immunohistochemical methods were then used to detect these attached molecules. The PHA-L conjugated markers behaved like unconjugated PHA-L as neuronal tracers, and axons originating from the septum or from the contralateral hippocampus could be revealed in the same hippocampal sections by two-colour immunostaining.

MATERIALS AND METHODS

Tracers

PHA-L and PHA-L conjugated to biotin were obtained from Vector Laboratories and both were used as a 2.5 per cent solution in 10 mM-phosphate-buffered saline (pH 7.4).

DNP was conjugated to the lectin by dissolving PHA-L (5 mg) in 0.5 ml of 0.3 M-$NaHCO_3$ and adding 50 µl of a 1 per cent 2,4-dinitro-1-fluorobenzene (FDNB) solution in ethanol. The reagents were mixed by rotation in a sealed Eppendorf tube for 1.5 h at room temperature, and then separated on a 10 ml Sephadex G-25 column using 5 mM-phosphate buffer (pH 8.0) containing 10 mM-NaCl as the eluant. The void fractions containing the conjugated PHA-L were pooled, the optical density at 360 nm was determined, and the pooled fractions were freeze dried. The substitution of PHA-L with FDNB was calculated from the optical density at 360 nm and routinely was 10.5 molecules of DNP/molecule of PHA-L. The freeze-dried reagent was reconstituted to a final PHA-L concentration of 2.5 per cent in distilled water, and frozen in aliquots at −20°C.

Iontophoretic injection of lectins and preparation of tissue sections

Experiments were carried out on 16 female Sprague–Dawley albino rats. The skull was opened with a dental drill under deep chloral hydrate anaesthesia (350 mg/kg, i.p.). Glass micropipettes with a tip diameter of 10–20 µm were filled with one of the tracers just before application using a vacuum line. Stereotaxic coordinates, according to the atlas of Paxinos and Watson,[14] were the following: for the septum, AP, +0.8 and 0.2 (from bregma); L, 0.3; V, 6.2, 5.7, 5.2; for the hilus, AP, −2.8 and −3.4 (from bregma); L, 1.9; V, 3.3. The tracers were applied by iontophoresis using a positive constant current of 5 µA. The current was delivered in a cycle of 7 s and 5 s off over a period of 15–20 min. Altogether 32 sites were injected, some of them receiving the PHA-L application at two closely placed locations.

Tracers were applied in four combinations to the medial septum and the contralateral hippocampus as summarized in Table 1. After 7–9 days survival period the animals were given an overdose of chloral hydrate and perfused transcardially, first with Tyrode's solution (oxygenated with a mixture of 95 per cent O_2, 5 per cent CO_2) for 1–2 min, followed by a fixative containing either 2.5 per cent glutaraldehyde, 0.5 per cent paraformaldehyde, and approximately 0.2 per cent picric acid in 0.1 M-phosphate

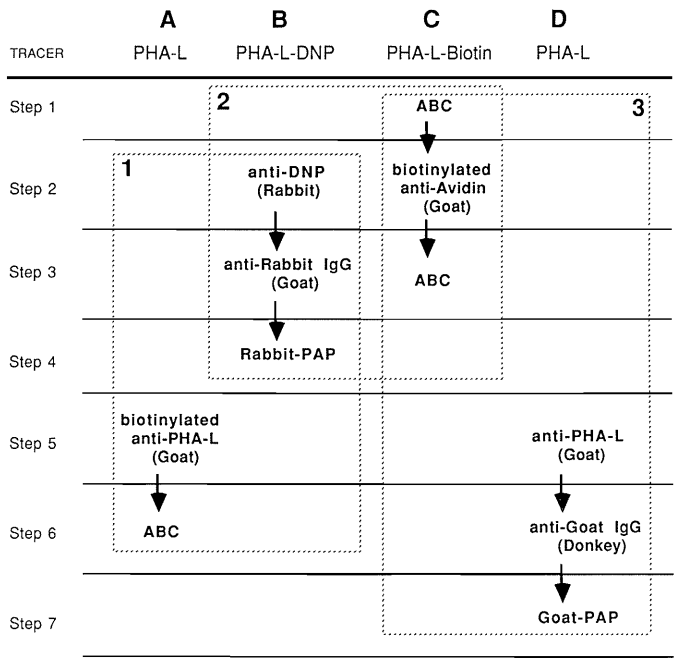


Fig. 1. Flow diagram of antibody sequences used for immunohistochemical detection of tracers alone, or in three different combinations (1–3, dashed line frames). The unconjugated lectin, PHA-L, was reacted in two different ways (columns A and D). The antibody sequence used to reveal dinitrophenol coupled to PHA-L (PHA-L-DNP) is shown in column B, and the incubation protocol for the detection of biotin coupled to the lectin (PHA-L-biotin) is summarized in column C. Steps 1–7 indicate the order in which the antibodies were used for a particular incubation. Reagents, in two columns occupying the same step and horizontal line within frame 2, were mixed and applied together to the sections.

buffer with a pH 7.4, or 0.1 per cent glutaraldehyde, 4 per cent paraformaldehyde and appr. 0.2 per cent picric acid in the same buffer.[18] The brain was removed and fixed by immersion in the same fixative for 1–3 h. Injection sites and the intact (non-injected) hippocampus were dissected and washed in 0.1 M-phosphate buffer followed by 10 per cent and 20 per cent sucrose dissolved in the same buffer until the blocks sank. Blocks were frozen in liquid nitrogen then thawed in 0.1 M-phosphate buffer. Sections of 60 μm thickness were cut on vibratome and washed in several changes of phosphate buffer.

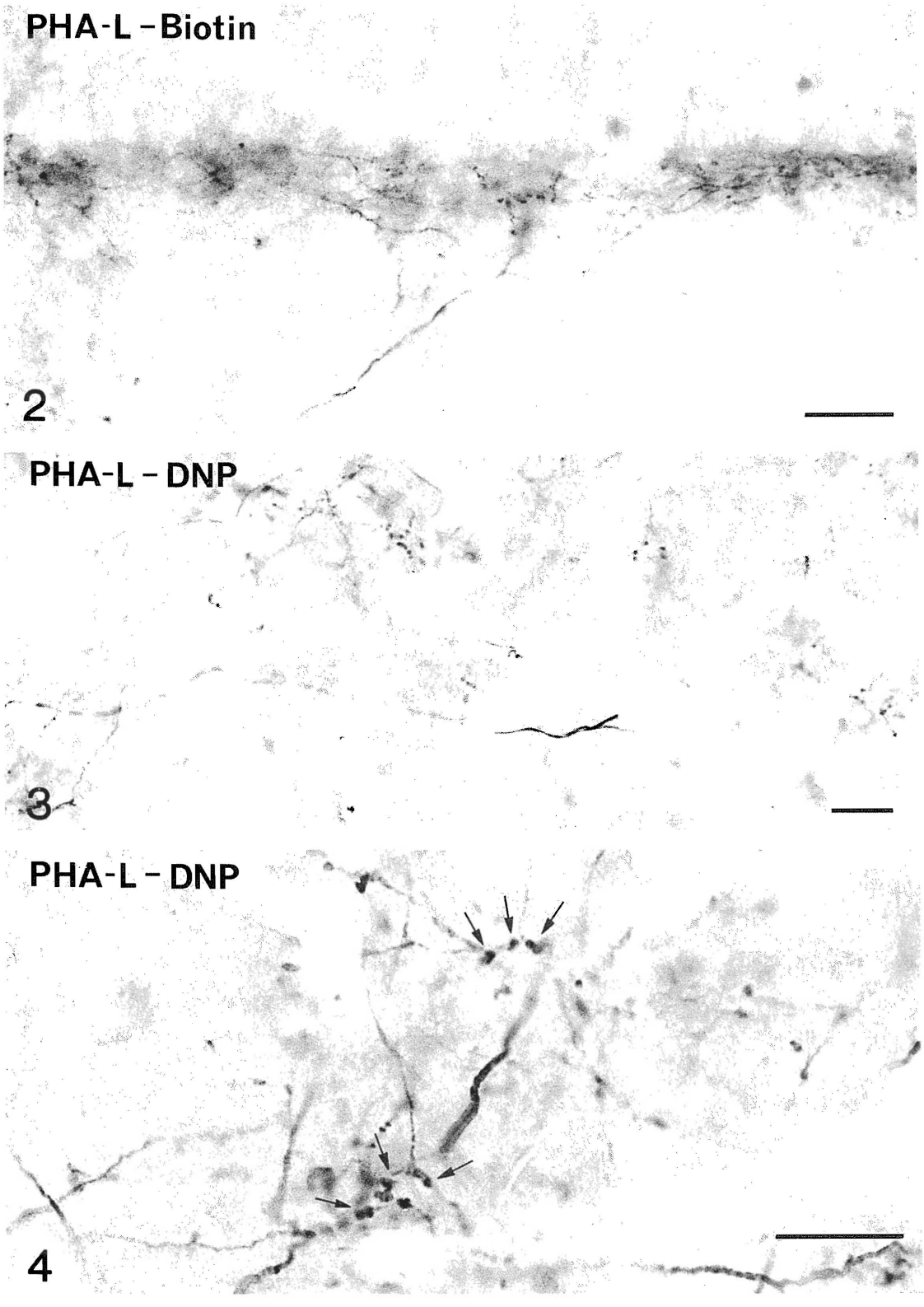
Localization of PHA-L, DNP and biotin

Two immunostaining protocols were used to localize PHA-L (Fig. 1). The first involved incubating sections with biotinylated goat anti-PHA-L (Vector Labs, diluted 1:200) for 2 days at 4°C, before transfer to a solution of avidin-biotinylated peroxidase complex (ABC, Vector Labs, 90 μl reagent A and 90 μl reagent B mixed in 10 ml buffer) for 4 h at room temperature. In the second protocol after incubation in the goat anti-PHA-L antibody (Vector Labs, diluted 1:1000), the sections were transferred to donkey anti-goat IgG (ICN, diluted 1:40) for 10–12 h at room temperature, before an overnight treatment with peroxidase goat-antiperoxidase (PAP, Miles, diluted 1:100) at 4°C.

To reveal DNP conjugated to PHA-L, sections were treated with anti-DNP antibodies. Antibodies from four sources were tried (Sigma, Sera Lab, ICN), but they all produced high non-specific background staining. Finally a fifth antiserum from ICN (rabbit anti-DNP, code 61–006–1) was chosen, which at a dilution of 1:10 000 produced the lowest background. The serum was applied for 2 days at 4°C, followed by goat anti-rabbit IgG (ICN, diluted 1:40) for 10–12 h at room temperature, and an overnight incubation at 4°C in rabbit-PAP (Dako, diluted 1:100).

Biotinylated-PHA-L was visualized by reacting sections for 4 h at room temperature with ABC, prepared as above, followed by an incubation in biotinylated goat anti-avidin (Vector Labs, diluted 1:1000) for 2 days at 4°C, and the reaction completed by a second ABC treatment.

Prior to the antibody treatments sections were kept in 20 per cent normal serum for 40–50 min. All incubations were performed under continuous gentle agitation, and all of the antibodies were diluted in 50 mM-TBS (pH 7.4) to which 0.5 per cent Triton-X 100 and 1 per cent normal serum were added. Except for the second immunostaining sequence against PHA-L in all of the incubations normal goat serum (ICN) was added to the buffer. When PHA-L was reacted with the goat anti-PHA-L/donkey anti-goat IgG/goat-PAP sequence, normal donkey serum (ICN) was used in the antibody solutions. Between incubations in the antibody solutions sections were rinsed three times for 30 min in the same buffer. Peroxidase enzyme



Figs 2–4.

Fig. 2. Commissural axons, bearing nerve terminals and labelled with biotinylated PHA-L that was injected into the hilus of the contralateral hippocampus. Axons terminate in a well-delineated lamina of the inner stratum moleculare of the dentate gyrus. Bar: 20 μm.

Figs 3 and 4. Axons terminating in the stratum radiatum (Fig. 3), or stratum oriens (Fig. 4) of the CA3 region, and labelled by anterogradely transported PHA-L-DNP that was injected into the medial septal nucleus. Some of the axons with large varicose boutons form baskets (arrows) around cell bodies. Sections were reacted with antibodies to DNP. Bar: 20 μm.

reaction was carried out by reacting the sections with 0.05 per cent diaminobenzidine (DAB, Sigma) and 0.01 per cent H_2O_2 in 50 mM-Tris buffer (pH 7.4). Immunostained sections were mounted on chrome alum-gelatin coated slides, air dried, dehydrated in ethanol, cleared in xylene and mounted under coverslips with XAM neutral medium.

Two-colour immunoreactions

Depending on the tracer combinations one of three different protocols were used and these are summarized in Fig. 1.

1. PHA-L and DNP-PHA-L visualization combined

First the immunoreaction for DNP was developed in order to mask by peroxidase reaction product the epitopes recognized by anti-PHA-L antibodies on the PHA-L-DNP molecules. This made the second incubation for visualizing PHA-L alone specific for the unconjugated PHA-L. Detection of DNP was carried out as described above (steps 2–4 in column B of Fig. 1); the antibodies were visualized by a DAB reaction, producing brown end-product. After several rinses in 50 mM-Tris buffer (pH 7.4), then in TBS (pH 7.4), the incubation was continued with biotinylated goat anti-PHA-L followed by ABC (steps 5–6 in column A of Fig. 1) in order to reveal the unconjugated PHA-L. The second immunoreaction was completed with a nickel-enhanced DAB chromogen reaction.[7] Briefly, sections were reacted with 0.015 per cent DAB, 0.6 per cent nickel-ammonium sulphate and 0.006 per cent H_2O_2 in 50 mM-Tris buffer (pH 8.0) for 2–5 min under continuous gentle agitation. Axons and cells visualized by nickel-enhanced DAB appear deep blue-black.

2. PHA-L-DNP and biotinylated PHA-L visualization combined

First sections were incubated with ABC (step 1 in column C of Fig. 1) then transferred into a solution containing both rabbit anti-DNP and biotinylated goat anti-avidin antibodies (step 2 in columns B and C of Fig. 1). The anti-DNP serum recognizes DNP conjugated to PHA-L, while biotinylated goat anti-avidin antibodies react with the avidin molecule of the ABC. Sections were then treated with a mixture of goat anti-rabbit IgG, reacting with rabbit anti-DNP IgG, and also with ABC again, thereby increasing the peroxidase content of the complex bound to biotinylated PHA-L (step 3 in columns B and C of Fig. 1). Next a nickel-enhanced DAB reaction was carried out to visualize biotinylated PHA-L-labelled axons. This was followed by an incubation with rabbit-PAP (step 4 in column B of Fig. 1), and finished with a peroxidase reaction using DAB as chromogen producing brown colour.

3. Biotinylated PHA-L and PHA-L visualization combined

First biotin was detected (steps 1–3 in column C of Fig. 1), followed by a nickel-intensified DAB reaction producing blue-black labelled axons. PHA-L was detected subsequently by treating sections with goat anti-PHA-L antiserum, donkey anti-goat IgG, and finally with goat-PAP (steps 5–7 in column D of Fig. 1). This second immunoreaction was developed with DAB alone producing brown-coloured labelled axons.

RESULTS

Application sites

The three tracers, unconjugated PHA-L, PHA-L conjugated to either DNP or biotin were delivered iontophoretically into the medial septum of different animals, and PHA-L or biotinylated PHA-L was injected into the hilar region of the hippocampus. As detected by antibodies to PHA-L, all three tracers produced labelled cells at the application sites in an area of 100–300 μm in diameter. The appearance of these cells and the injection sites were similar to those reported in other studies.[5,20,24] Individual labelled cells could be seen occasionally scattered more widely than the core of the application site. The hilar applications were centered on the hilus proper, but in every case a substantial number of CA3 pyramidal cells and granule cells were labelled as well.

Distribution of anterogradely labelled axons

Following medial septal injections of the tracers the distribution of labelled axons and terminals was similar to that described in other studies.[12,13,15] Axons were most densely arranged in the hilus of the dentate gyrus and in the CA3 region of the hippocampus, preferentially in stratum oriens. The two axon populations of septal origin described by Nyakas *et al.*[13] and Freund and Antal[3] could also be distinguished (Figs 3 and 4). Smaller varicosities with occasional drumstick-like terminals were seen on thinner axons (Fig. 3), whereas the larger axons frequently formed basket-like contacts around cell bodies and proximal dendrites (Fig. 3).

Following injections of biotinylated PHA-L or PHA-L into the hilus of the dentate gyrus, labelled fibres were found in all regions and layers of the contralateral hippocampal formation. The distribution of labelled axons and terminals was similar to that described in earlier studies.[5,15] In the hippocampus, in both the CA1 and CA3 regions, the majority of the fibres were concentrated in the stratum lacunosum-moleculare and outer part of the stratum radiatum. The hilus of the dentate gyrus contained only a small number of fibres but the proximal one third of the molecular layer was occupied by a dense layer of labelled axons (Fig. 2).

Detectability of different tracers

Neighbouring sections, cut either from the site of the application of conjugated PHA-L, or from the hippocampus were reacted with combinations of antibodies to either PHA-L and biotin, or to PHA-L and DNP. The comparison of these neighbouring sections showed that all three tracers can be delivered with approximately the same efficiency. However, at the same application site of the conjugated PHA-L, cells and/or axons were labelled more intensely and detected in larger number with antibodies to PHA-L itself than with the immunoprocedures to the small conjugated molecules.

Both conjugated forms of the PHA-L produced extensive anterograde axonal labelling. As with PHA-L itself, no retrogradely labelled cell bodies could be found in the hippocampal formation in our experiments.

In the hippocampus, where anterogradely labelled axons were detected, staining for unconjugated PHA-L alone always resulted in more extensive immunostaining than for either of the two conjugates. Biotinylated PHA-L, when detected by the biotin moiety, always produced a significantly stronger signal than DNP-PHA-L, when detected by the DNP moiety. Generally the detection of DNP conjugated PHA-L gave a higher non-specific background staining than the detection of biotinylated-PHA-L. The detection of biotinylated PHA-L also produced high background staining when the fixative containing low glutaraldehyde was used. However this problem could be circumvented by using 2.5 per cent glutaraldehyde when fixing the brain.

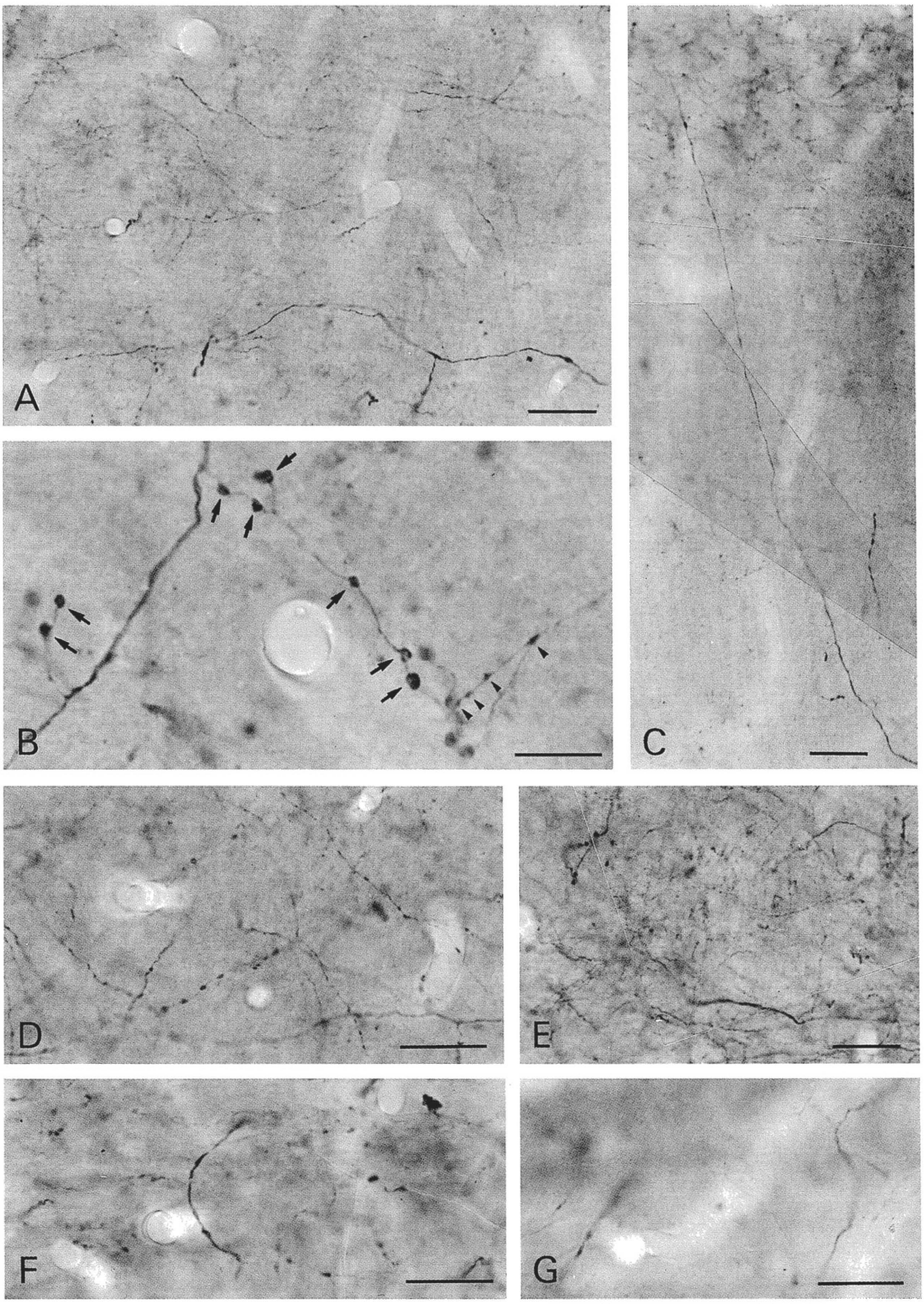
Combined labelling of two axon populations

For the simultaneous visualization of two axonal populations originating from the septum or the contralateral hippocampus, three combinations of tracers were used, as summarized in Table 1. Each yielded satisfactory results, but the third (biotinylated-PHA-L combined with PHA-L) always gave better results than the others. Developing the biotinylated-PHA-L with nickel-enhanced DAB yielded a deep blue-black colour which was unaffected by the second reaction to visualize the PHA-L with DAB alone, giving a brown colour (Fig. 5A–E). Even after the second immunoreaction, the background staining of the sections was very low. The blue-black or brown axons could clearly be distinguished from the background staining. Both blue-black and brown fibres were distributed according to their origin in the hippocampus (Fig. 5A,B,C,D,E) and the dentate gyrus (Fig. 5C), where in many cases they had overlapping arborizations.

In the combined procedure described above antibodies produced in goat were used in the first sequence, and anti-goat IgG produced in donkey was used in the second step of the second sequence (Fig. 1). It might be expected that the anti-goat antibodies would recognize the goat biotinylated anti-avidin antibodies leading to colour mixing because of restaining the blue-black axons to a brown colour, the colour of the second reaction product. However, in practice this never happened, probably because the reaction end-product from the first reaction completely masked the goat anti-avidin antibodies. It should be noted, however, that at the site of tracer application the labelled neurons visualized in blue-black always turned brown in the second peroxidase enzyme reaction irrespective of tracer or reagent combinations. The reason for this different behaviour of the application site and the anterogradely labelled axons is not known, but could be related to the different quantity of tracer present at these sites.

In the second two-colour immunostaining procedure fibres of contralateral hippocampal origin, labelled with biotinylated PHA-L, were visualized with Ni-DAB revealing the axons in a blue-black colour. The other, simultaneously performed immunoreaction for DNP was completed with DAB as chromogen, and demonstrated fibres of septal origin in brown colour (Fig. 1, Fig. 5F). Using the antibodies to DNP and biotinylated PHA-L together did not impair the immunostaining, but as it was pointed out earlier the extensive axonal labelling produced by immunoreaction for PHA-L alone was never obtained with immunoreactions for either biotin or DNP. As a result, with this combination of tracers, and with the particular application sites that we obtained, axons from both sources were found together only in the dentate gyrus (Fig. 5F).

Fig. 5. Two-colour immunostaining for the simultaneous visualization of unconjugated PHA-L, and PHA-L conjugated to biotin (A–E) or dinitrophenol (F,G). DNP-PHA-L is visualized in brown colour in F and G. Biotinylated PHA-L is visualized in blackish colour, while PHA-L alone is visualized in brown in all figures but G, where it is shown in black. (A and B). Axons of septal (brown) and contralateral hippocampal (black) origin are in close vicinity in the stratum radiatum of the CA1 region. Both septal (arrows in B) and commissural axons (arrowheads) bear boutons, but the septal axons have larger varicosities. (C). A long septal fibre (black) running towards a fine network of commissural (brown) fibres in the molecular layer of the dentate gyrus. (D). Thin septal (brown) and contralateral hippocampal (black) axons are mixed in the CA1 region. Note that even the thinnest fibres and their varicosities are labelled. (E). Commissural fibres (brown) form a dense network in stratum lacunosum-moleculare of the CA1 region. Septal axons (black) are sparsely arranged in this network. (F). Septal axons (brown) labelled by DNP-PHA-L and a commissural axon (black) labelled by biotinylated PHA-L in the hilus of the dentate gyrus. (G). Septal fibres visualized by DNP-PHA-L (brown), and a PHA-L-labelled commissural axon (black) in the stratum radiatum in the CA3 region. The labelling is weak and the background is considerably higher than with the previously described tracer combinations. Bars, A and C–G, 20 μm; B, 10 μm.


A
B
C
D
E
F
G

The first immunostaining combination (Fig. 1) was far less successful than the second or the third. In order to avoid the possible cross-reactions, DNP had to be visualized first. This reaction always produced a considerable non-specific background staining, especially when the immunocomplex was visualized with nickel-enhanced DAB reaction. If DAB was used in the background staining was significantly lower, but even then the non-specific binding of the anti-DNP antibodies to the tissue was so strong, that it reduced the immunodetection of PHA-L in the second step (Fig. 5G).

DISCUSSION

Properties and detectability of conjugated PHA-L

Both the biotinylated PHA-L and DNP-PHA-L proved to be good axonal tracers, which preserved the neuronal tracing characteristics of the native lectin. Contrary to a recent report that PHA-L is transported not only anterogradely but also retrogradely[11] we never observed retrogradely labelled cells in this particular system. The conjugates of PHA-L behaved in the same way as the unconjugated lectin in that they were transported exclusively in the anterograde direction.

The appearance of axons and their terminals labelled with either biotinylated PHA-L or DNP-PHA-L was the same as of fibres labelled with native PHA-L. There was no sign of degeneration of labelled fibres at the light microscopic level, even the thinnest axons and varicosities along them appeared to be intact. However, the detection of the conjugated forms of the lectin, especially DNP-PHA-L, never gave as extensive axonal labelling as that produced with PHA-L alone. These differences are probably due to the lower sensitivity of the immunoreactions for the conjugated molecules. The conjugates probably have a lower epitopic density than the native lectin, resulting in a weaker amplification of the signal due to the reduced binding of the detecting antibody. If this was the case then this problem could be overcome by using higher substitution ratios for the moieties conjugated to PHA-L.

The other problem we have identified is the persistent non-specific background staining obtained in the reaction for DNP using commercial antisera. Whilst this is a less serious problem when visualizing DNP-PHA-L alone, it significantly reduced the sensitivity of detection of PHA-L in the two-colour experiments. This background was present whichever of the five commerical reagents to DNP, including two monoclonal antibodies, was used. The binding of anti-DNP antibodies to the sections either reflects some non-specific anti-brain element in the antisera, or more likely a genuine cross-reactive component in the fixed brain. This problem too could be overcome by producing a more specific antiserum, possibly to the DNP-PHA-L itself.

Combined labelling of two populations of axons

The main goal of this study was to establish a method for the simultaneous labelling of two axonal populations of different origin terminating in the same target area. The properties of PHA-L, namely its excellent cell filling, and predominantly anterograde transport[3–5,19–24] made it the tracer of choice. We reasoned that by coupling PHA-L to small, immunocytochemically detectable molecules (biotin and DNP) the properties of the lectin which make it so useful as a neuronal tracer might be preserved, and enable the different forms to be detected separately following their transport. Therefore we prepared DNP-PHA-L and also experimented with biotinylated PHA-L. The latter has been available for some time, but to our knowledge has not been used previously in neuronal tracing studies. DNP is a widely used protein hapten, to which a wide variety of commercial antibodies are available.

Three different combinations of the tracers were used. As discussed above the combined use of DNP-PHA-L with PHA-L requires further improvements, but the other two tracer combinations can now be used routinely. Excellent double labelling was obtained when PHA-L and biotinylated PHA-L were used together. The first immunoreaction for biotin did not impair the subsequent detection of the unconjugated PHA-L, and the deposition of reaction product from this first reaction successfully masked the antibody binding sites on the biotinylated PHA-L, so that it was not detected by the anti-PHA-L antibodies used in the second immunoreaction. This combination of antibodies produced no detectable background staining even after the second chromogen reaction. This combination is the method of choice at present.

The specification for the biotinylated PHA-L stated that at least 99% of the lectin was in the conjugated form. In theory the small amount of unconjugated lectin should not affect the results as long as the reaction end-product of the first reaction completely masks the lectin before the second reaction. Even the conjugated lectin would be recognized by the anti-PHA-L antibodies if not masked.

The combination of biotinylated PHA-L with DNP-PHA-L also gave acceptable results with no apparent cross-reaction between the DNP- and biotin-tagged tracers. Despite the problem discussed above for the anti-DNP sera, the background staining in this combination was never so high as to cause difficulty in the evaluation of the results.

The use of double immunostaining for PHA-L and conjugates of PHA-L described here should greatly extend the utility of this lectin in neuronal tracing and it should be noted that the methodology described here is also suitable for extension to other neural tracers such as wheat germ agglutinin. The ability to detect the different conjugates of the lectin, which preserve all of the properties of the native

lectin means that it is now possible to study at the light microscopic level the relative distribution of the terminals of two different afferent pathways which converge on the same brain area. This makes it possible, as demonstrated in the present study, to study the convergence, overlapping or segregated distribution of axons and their terminals at the levels of columns, patches, laminae, or even on a single cell.

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