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Combined axonal transport tracing and immunocytochemistry for mapping pathways of peptide-containing nerves in the peripheral nervous system

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Summary. The various combinations of axonal transport tracing and immunocytochemistry used for mapping pathways of peptide-containing nerves, and in particular those of the peripheral nervous system, are reviewed. The advantages and disadvantages of these methods are discussed. The applications and results presented illustrate the future potential value of this approach.
Key words. Anterograde tracing; axonal transport; immunocytochemistry; neuropathways; peptide-containing nerve; retrograde tracing.

Introduction

In the beginning of the 1970s neuroanatomy experienced a methodological revolution with the development of a new technique for tracing neural pathways based on the axonal transport of compounds such as horseradish peroxidase (HRP)[50, 55, 56, 100], radiolabelled amino acids[14, 20, 22] and the fluorescent dye Evans blue (bound to albumin)[49]. New tracers, both ingenious and powerful, are still rapidly being added to the tools of the neuroanatomist[1, 2, 23, 28, 31, 44, 48, 52–

[54, 57, 62, 63, 76, 80, 92, 93, 101]. Tracers, such as the fluorescent dyes[1, 2, 53, 54], are taken up by the axonal processes of neurones and retrogradely transported to the parent cell bodies after being administered into their peripheral terminal fields. Others, for example radioactively labelled amino acids[21] and *Phaseolis vulgaris*-leucoagglutinin (PHA-L)[28] are taken up by neuronal cell bodies and subsequently anterogradely transported to their terminals. Compounds, such as HRP[65], wheat germ agglutinin (WGA) or WGA-HRP[57, 81] and cholera toxin[92, 104], are transported efficiently in both anterograde and retrograde directions simultaneously. Retrograde and anterograde tracing techniques now offer convenient methods for mapping nerve pathways and tracing connections of central neurones, peripheral ganglia and nerves supplying muscles, skin and viscera. However, the use of axonal transport techniques alone does not determine the transmitter content of defined neuronal pathways.

Radioimmunoassay[5, 106] and immunocytochemistry[12, 13, 67, 90] were developed at about the same time as these transport techniques and have been used to investigate neuropeptides; these techniques have yielded extensive valuable information about neuropeptides. More than 40 peptides have been localised in the central and peripheral nervous system[3, 25, 29, 34, 36, 45, 70, 71, 73, 79]. Therefore, in addition to classical neurotransmitters, neuropeptides in growing number are now strong candidates for neurotransmitter status.

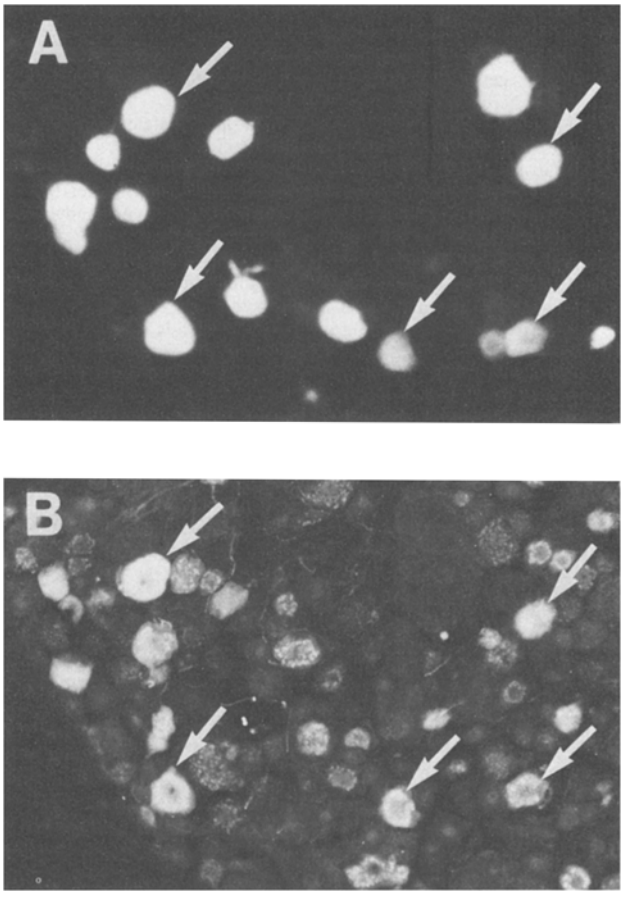
In order to trace neurotransmitter-specific pathways, especially peptide-containing neuropathways, axonal transport combined with immunocytochemistry has been successfully applied[6–11, 32, 33, 35, 38–40, 60, 66, 75, 77, 82–84, 94–96, 102]. In principle, all the combination methods are two-step procedures[37]. First, tracers are non-specifically taken up and transported by neurones regardless of their transmitter content. In the second step neurotransmitters or neurotransmitter candidates in the tracer-labelled neurones are demonstrated by immunocytochemistry. The interpretation of the results is based on the assumption that the neurotransmitter present in the cell body is the same as the one stored and released at its terminals. Combination of axonal transport and immunocytochemistry seems to be the most elegant approach for tracing neurotransmitter-specific pathways. The various combinations used for tracing peptide-containing neurones are reviewed here with emphasis on investigation of the innervation of peripheral organs.

Combination of fluorescent retrograde tracing and immunofluorescence

Many fluorescent substances for tracing neuronal connections have been described by Kuypers and his colleagues[1, 2, 53, 54]. For details of general properties of fluorescent tracers see Steward[91]. When combining fluorescent retrograde tracing with immunofluorescence, it was noticed that many of the fluorescent dyes tend to diffuse out of the cells during the immunofluorescence procedure. Only a few dyes, e.g. True Blue, Fast Blue, Primuline and propidium iodide, resist diffusion during immunohistochemistry and are reliable for the visualisation concomitantly with the positive immunofluorescence staining[37, 91].

Using retrograde tracing with True Blue fluorescence combined with the indirect immunofluorescence technique for visualising enkephalin-like immunoreactivity, Hökfelt et al.[38] demonstrated immunoreactive neurones in the medulla oblongata projecting to the spinal cord. A simple method for simultaneous localisation of an antigen and retrogradely transported fluorescent dye (True Blue) within single neurones was described in more detail by Sawchenko and Swanson[75]. The method is based on 1) the efficiency of retrograde neuronal labelling with the fluorescent marker True Blue; 2) the near quantitative persistence of retrogradely transported True Blue localisation after subsequent processing of the tissue for immunohistochemistry; 3) the possibility to distinguish clearly between True Blue and immunocytochemically stained cells by simply using appropriate excitation wave-lengths for each. This method has been widely used to trace peptide-containing nerves in both the central nervous system and peripheral organs[30, 32, 38, 40, 42, 75, 82–84, 94–96]. In brief, the procedure is as follows: 1) True Blue (2–5% w/v aqueous suspension) is injected with a Hamilton microsyringe into the terminal fields to be investigated. Multiple injection sites are suggested in peripheral organs. 2) After optimal survival time (see below) the injected animals are anaesthetised and transcardially perfused with ice-cold 4% paraformaldehyde. 3) Relevant tissues are dissected out, placed in the same fixative for 1 h or overnight and washed in 0.1 M PBS (pH 7.4) containing 15% sucrose for at least 24 h. 4) Frozen sections are cut and processed for indirect immunofluorescence using fluorescein-labelled second antibodies. 5) Sections are examined by fluorescence microscopy using 340–360 nm excitation for True Blue and 450–490 nm for fluorescein. Co-localisation of True Blue and fluorescein may be observed by switching the filter systems without altering the plane of focus (fig.).

True Blue and fluorescein thus provide sensitive, stable and clearly distinguishable labels for retrograde tracing combined with immunofluorescence in properly fixed tissue[75]. If fluorescent retrograde tracing is combined with immunostaining of thin adjacent sections with different antisera or



Section from a rat T_9 dorsal root ganglion showing True Blue fluorescence after injection of the dye into the stomach (A). The same field viewed for fluorescein fluorescence showing cells immunostained for CGRP (B). Arrows indicate True Blue retrogradely labelled neuronal cell bodies which display CGRP immunoreactivity. × 157.

with elution of the first antiserum after photography and restaining with a second antiserum[99], it can be used for tracing neurones with multiple putative transmitters[59],
When interpreting the results of combined retrograde tracing and immunocytochemistry, some facts require careful consideration.

1. The degree of specificity of retrograde labelling
False-positive labelling may result from leakage of tracers from the injection sites, the dye being transported to other regions by the vascular system, or spreading to contaminate adjacent organs. It is therefore important that care is taken to avoid any leakage of dye during microinjection. The organs to be investigated are isolated with parafilm during the course of injections. After each injection the needle is left in place for at least 1 min, the organs are then rinsed with saline and thoroughly swabbed and the injection sites inspected. Recently a barrier formed from a plastic wound spray (pyroxylin solution, New Skin) was suggested for application to the surface of injected viscera[24].
Fluorescent dye is avidly taken up not only by nerve terminals, but also by damaged and undamaged fibres-of-passage[75]. Although there are obvious problems associated with interpreting the origins of terminal fields, this feature can be used to advantage in some instances, for example, when labelling a peripheral nerve or a fibre tract in the central nervous system[97]. Recently, the fluorescent retrograde tracer SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid) has been introduced as a cytoplasmic label, which seems to be taken up primarily from axon terminals[41,78].

2. Survival time
Proper postinjection survival time is important for optimal neuronal labelling. Survival times must be varied to suit the different tracers used and the system being studied, but they may be divided into three groups, short (less than 48 h), intermediate (2–5 days) and long survival times (5 days to several weeks)[37]. Among the fluorescent tracers which are good for combining with immunocytochemistry, Fast Blue and propidium iodide belong to the group with intermediate survival times, whereas True Blue and Primuline have longer survival times. Although True Blue can be used over a relatively wide range of survival times, it is advisable to determine empirically an optimal time for each system under study.

3. Visualisation and evaluation of immunostaining
Although experiments have proved that True Blue labelling does not affect the immunostaining, it is possible that some apparently non-immunoreactive cell bodies may contain very low concentrations of neuropeptides which are immunocytochemically undetectable. Immunocytochemical visualisation of some peptides requires prior treatment of the animals with colchicine to inhibit axonal transport and cause accumulation of peptides in the cell bodies[15,16,19]. Since colchicine treatment also arrests transport of the retrogradely transported fluorescent dye, these procedures have to be carried out 2–3 days after tracer injection or 24–48 h before sacrifice.
A significant limitation of immunocytochemistry in the study of regulatory peptides as for all other substances is the fact that only material stored in the cell at the moment of death can be visualised. The results do not reflect the biosynthetic activity of the cell. More recently, in situ hybridisation has been employed to demonstrate various types of mRNA in cells, including those coding for peptides in neurones[4,26,61,68,86,87,105]. Hybridisation histochemistry has also been combined with retrograde axonal transport of fluorescent dyes to identify the projections of transmitter-specific neurone populations[74,77].

Combined horseradish peroxidase (HRP), conjugated HRP or other protein tracing with immunocytochemistry

Ljungdahl and his coworkers[60] established a combination of HRP retrograde tracing and immunofluorescence staining of neural pathways. After the optimal postinjection survival time, animals are perfused with fixative and tissues processed. Since the peroxidase-diaminobenzidine reaction product at least partly obscures the immunofluorescence, indirect immunofluorescence is carried out first. The sections are examined under a fluorescence microscope and the stained cells photographed. The coverslips are then removed and the section treated with diaminobenzidine for visualisation of peroxidase. The fluorescence and light micrographs of identified region are then compared. When sections are examined by fluorescence microscopy, it is important to keep the exposure time as short as possible, because the ultraviolet light seems to inactivate the peroxidase.
Successful combination of HRP or WGA-HRP retrograde tracing with peroxidase anti-peroxidase (PAP) immunocytochemistry techniques has been reported by several groups[7–9,57,72,89,103]. Both steps involve a development procedure

Anatomically defined peripheral peptide-containing neuropathways

Injection site	Peptide	Localisation of cells containing both tracer and peptides	
Digestive system			
Stomach	Substance P	DRG[82,96]	
	CGRP	DRG[95,96]	NG[95,96]
	NPY	CG[59,95,96]	
Pancreas	Substance P	DRG[82]	
	CGRP	DRG[96]	NG[96]
	NPY	CG[96]	
Colon	Substance P	DRG[96]	
	CGRP	DRG[96]	
	NPY	IMG[96]	
Parotid gland	Substance P	OG[83]	TG[83]
Tongue	CGRP	TG[98]	
Urinary system			
Kidney	Substance P	DRG[51]	
	CGRP	DRG[94]	
Ureter	CGRP	DRG[94]	
Bladder	Substance P	DRG[84]	
	CGRP	DRG[94,107]	
	VIP	PG[30]	DRG[30]
	NPY	PG*	
Urethra	CGRP	DRG[94]	
Female genitalia			
Uterus	CGRP	DRG[42]	
	NPY	PG*	
	VIP	PG[32]	
Vagina	CGRP	DRG[42]	
Preputial gland	CGRP	DRG[42]	
Peripheral ganglia			
IMG	Substance P	DRG (Thoracolumbar)[17]	
	Enkephalin	IML[18]	
Peripheral nerve			
Pelvic nerve	CGRP	DRG[30]	
	VIP	DRG[30,47]	PG[30]
	Substance P	DRG[30,47]	
	Enkephalin	DRG[30,47]	
Pudendal nerve	CGRP	DRG[43]	Motoneurones[43]
	Substance P	DRG[43,47]	
	Enkephalin	DRG[47]	
	VIP	DRG[47]	
Sciatic	CGRP	DRG[43]	Motoneurones[43]
	Substance P	DRG[43]	

CG, coeliac ganglion; DRG, dorsal root ganglia; IMG, inferior mesenteric ganglion; IML, intermediolateral column; NG, nodose ganglia; OG, otic ganglion; PG, pelvic ganglion; TG, trigeminal ganglion; *unpublished observations.

based on the presence of HRP, but the retrogradely transported HRP appears as punctate granules, whereas the HRP demonstrating immunoreactivity appears as a diffuse homogeneous stain in the cytoplasm[8,103]. Using cobalt chloride to intensify the diamidinobenzidine product of the first peroxidase reaction, it is possible to increase the colour difference between the retrogradely transported HRP (black) and the antibody-coupled HRP (brown)[6].

However, a disadvantage of these methods is that the granular black reaction product can sometimes obscure a lightly immunostained cell. In contrast, if little HRP has been taken up in the neurones, the reaction product may not be detectable. The most important shortcoming is due to the fact that the appearance of most peptide positive staining does not produce the same homogeneous brown as the conventional transmitters, serotonin and choline acetyltransferase, for which the method was developed[8,103]. Instead, the appearance is granular. This gives rise to much more difficulty in distinguishing retrogradely labelled HRP and the enzyme marking of the immunoreaction. Lechan et al.[57] also pointed out that cells containing the WGA-PAP complex exhibited diminished immunostaining. This was interpreted as being due to steric hindrance to antibody penetration by the WGA accumulation. Furthermore, the fixatives suitable for immunocytochemistry tend to inhibit HRP activity, which is used as an index of the tracer[88]. Thus, the sensitivity of the retrograde HRP technique used in this way is less than that in the usual HRP technique. The tetramethyl benzidine (TMB) method is more sensitive than diaminobenzidine (DAB) chromogen[64] and it would be ideal for such combination studies. However, the low pH required to maintain stability of the chromogen and the reaction product is not always compatible with immunocytochemistry. Peschanski and Ralston[69] adapted the chromogen benzidine dihydrochloride (BDHC) for light and electron microscopic tracing studies. They found that excellent sensitivity could be obtained even when the pH was raised above 6.0. At the light microscopical level, the benzidine dihydrochloride reaction product is bluish-green and crystalline. Therefore, using the benzidine dihydrochloride reaction it may be possible to combine HRP/or wheat germ agglutinin HRP retrograde tracing with PAP immunocytochemistry.

More recently, Shiosaka et al.[85] established a sensitive double-labelling technique of retrograde biotinized tracer (biotin-WGA) and immunocytochemistry, which overcame the above disadvantages. Wheat-germ agglutinin conjugated with biotin is used as a tracer. In principle, biotin-WGA labelled cells are visualised with a streptavidin-Texas red conjugate and the antigen localised in neurones is visualised with immunofluorescence. In this method, the detection systems for immunoreactivity and for the tracer (the streptavidin-biotin reaction) are different. There is no interference between the two reactions and the two labels fluoresce with different colours, red and green. Colloidal gold can also be used combined with a tracer. When PAP and the diamidinobenzidine reaction are used for immunocytochemistry and colloidal gold particles for detection of the tracer, it is possible to identify double labelled cells, dendrites and axons under the electron microscope. This method may also be used in the peripheral nervous system.

Combined anterograde tracing and immunocytochemistry

A sensitive anterograde tracing method using the lectin, *Phaseolus vulgaris*-leucoagglutinin (PHA-L) as a tracer has been reported and was recently successfully combined with immunocytochemistry to trace chemically specified circuits in the central nervous system by Gerfen and Sawchenko[27,28].

PHA-L is obtained from the red kidney bean. When it is delivered into the central nervous system by iontophoresis, the lectin provides by far the most complete labelling of neurones at the site of injection and is almost exclusively transported in the anterograde direction. Both the transported lectin and the neurotransmitter or related enzyme are visualized in the axons and terminals by a double immunostaining method, which provides a powerful way of tracing antigen-specific projections in the central nervous system. Coexistence of transported PHA-L and neuroactive substance could be demonstrated, for instance, by sequential immunostaining of the same tissue section, after elution of the first reaction product, or by simultaneous double immunofluorescence using primary antisera raised in different species, and localised by non-cross-reacting second antibodies labelled with different fluorochromes. For further details of the procedures see Gerfen and Sawchenko[27,28].

Major disadvantages of the method are: firstly, pressure injections or direct application of the tracer do not always result in effective anterograde labelling; secondly, it may have only limited use in developing systems, since in early postnatal rats the tracer is invariably taken up by astroglia as well as by neurones. We also do not know yet whether this combination method is able to trace the chemical-specific pathways in the peripheral organs.

Applications and recent discoveries

Retrograde tracing combined with immunocytochemistry has been extensively used to study the projections of peptide-containing neurones in both the central and peripheral nervous system. It is not possible to present all the discoveries here, instead, the principal findings on peripheral peptide-containing neuropathways are reviewed briefly (table).

Sensory substance P and calcitonin gene-related peptide (CGRP) of visceral structures in the rat have been identified and quantified[51,82,84,94,95]. After injections of True Blue into both stomach and pancreas, labelled afferent neurones were visualised in spinal ganglia (mainly at levels T_8–T_{11}) and nodose ganglia. Substance P immunoreactivity was found in 35–50% of gastric spinal afferent and in about 15% of pancreatic spinal afferent neurones[82,96]. Nearly all gastric and pancreatic spinal afferent neurones (about 95%) contain CGRP immunoreactivity[95,96]. Although the nodose ganglia harbour substance P- and CGRP-immunoreactive cell bodies, no True Blue and substance P co-localisation was seen and only a few True Blue-labelled cells contained CGRP immunoreactivity.

The substance P innervation of the rat parotid was also investigated by Sharkey and Templeton[83]. Direct evidence for a dual origin of substance P from the otic and trigeminal ganglia was provided.

Using retrograde tracing and immunocytochemistry it was demonstrated that two segmental groups of dorsal root ganglia at T_{12}-L_2 and L_6-S_1 innervating the bladder contain substance P (10–16%)[84]. Similarly, Kuo et al.[51] demonstrated substance P in renal afferent perikarya. Recently, CGRP immunoreactivity has been shown in a major proportion of afferent neurones supplying the urinary system of the rat[94,107]. Following injection of True Blue into the kidney hilum, ureter or bladder, more than 90% of the True Blue-labelled cells in dorsal root ganglia T_{10}-T_3 contained CGRP-immunoreactive material. Similarly, some 90% of the labelled cells in levels L_6 and S_1 resulting from injections in the ureter and urethra were CGRP-immunoreactive. However, after injection into the wall of the bladder base and dome only 60% of the retrogradely labelled cell bodies in L_6 and S_1 dorsal root ganglia displayed CGRP immunostaining.

It has also been reported that CGRP-immunoreactive nerves in the genitalia of the female rat originate from dorsal root ganglia T_{11}-L_3 and L_6-S_1[42].

A vasoactive intestinal polypeptide (VIP)-containing path-

way linking pelvic viscera and the sacral spinal cord has been established by retrograde tracing combined with immunocytochemistry[30,46]. Pelvic visceral afferents with cell bodies in the dorsal root ganglia are a significant source of VIP-containing fibres in the dorsal horn of the sacral spinal cord.

The projection of the postganglionic sympathetic neurones was studied with special reference to the pylorus using a combination of retrograde axonal tracing and indirect immunofluorescence technique[58]. After injection of True Blue into the pyloric sphincter, labelled neurones were found in the coeliac ganglion which also contained NPY- or somatostatin-like immunoreactivity. In elution-restaining experiments, it was established that the majority of these cells were also immunoreactive for tyrosine hydroxylase. Our observations have also proved that extrinsic neuropeptide tyrosine (NPY)-immunoreactive nerves supplying the stomach, pancreas and colon represent postganglionic sympathetic neurones[96].

The origins of the substance P and enkephalin fibres in the inferior mesenteric ganglion have also been characterised by the use of retrograde tracing in combination with indirect immunohistochemistry. Substance P-containing primary sensory neurones[17] and enkephalin-containing sympathetic preganglionic neurones[18] have both been found to project to the inferior mesenteric ganglion.

Retrograde tracing combined with immunocytochemistry has also been used to analyse the peptide composition of peripheral nerves. For example, among the population of afferent neurones projecting in the pelvic nerve L-enkephalin immunoreactivity was present in 60%, substance P in 30%, and VIP in 15%. Among the population of afferent neurones projecting in the pudendal nerve, L-enkephalin was present in 50%, substance P in 30%, VIP in 3%[47].

Concluding remarks

The combinations of retrograde or anterograde tracing techniques and immunocytochemistry, which have been developed in recent years, have proven their value in defining neuropathways on the basis of their transmitters. However, each of the individual techniques mentioned in this review article has its advantages and drawbacks. The more recently established methods have yet to be applied to the investigation of peripheral organs. Wider use of these methods should provide more data and give guidelines as to which of the newly developed techniques should be chosen for the study of peptide-containing neuropathways.

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