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Use of peroxidase substrate Vector VIP® for multiple staining in light microscopy

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Abstract

The study of the distribution of a fiber input to a particular brain area and the visualization of the anatomical relationships of that input with both projection- and interneurons, requires a triple-staining that allows the unequivocal distinction of each of the three components in one and the same histological section. In this regard, we investigated the properties of a recently introduced peroxidase chromogen, VIP® (V-VIP; Vector Labs) in combination with two traditional substrates, standard diaminobenzidine (DAB, brown precipitate) and nickel-enhanced DAB (DAB-Ni, black). In rats, the anterograde tracer biotinylated dextran amine (BDA) and the retrograde tracer fluorogold (FG) were injected in the perirhinal cortex and hippocampus, respectively. Transported BDA was detected with an avidin-biotin-peroxidase complex, whereas the transported FG was detected via a PAP method. Tracing with BDA and FG was combined with parvalbumin- or calbindin-immunocytochemistry. We compared various combinations and staining sequences. The best results were obtained with a staining sequence comprising first the BDA stain with DAB-Ni as chromogen, second the FG protocol with the chromogen DAB and finally, parvalbumin- or calbinding-immunocytochemistry using the chromogen V-VIP. The order with which the chromogens were applied appeared to be critical. Partial or even total loss of V-VIP reaction product has been observed after standard dehydration in ethanol. As an alternative, a quick dehydration procedure in toluene yields much better staining. Colour separation is excellent and the sensitivity is high. This procedure may also be used for detection of any other combination of three different labels, taking the usual care to avoid cross-reactivity between antibodies. © 1997 Elsevier Science B.V.

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1. Introduction

Knowledge of the relationships among different fiber systems and projection and/or interneurons in a particular brain region is an important goal in neuromorphology research. As part of our ongoing efforts to elucidate the anatomical organization of the cortico-hippocampal system in the rat, we are interesed in the postsynaptic target structures of efferent projections from the perirhinal cortex to the entorhinal cortex. Our special interest is to elucidate whether perirhinal efferent axons contact principal cells in the superficial layers of the entorhinal cortex, which in turn project to the hippocampus and/or presumed inhibitory entorhinal neurons (for an overall review of the neuronal circuits of interest see Witter et al., 1989; Amaral and Witter, 1995). Accordingly, we needed to develop a method for the simultaneous visualization, in one and the same section, of each of these three components. Until today,

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apart from dual immunofluorescence-based methods (Wessendorf, 1990), a large number of dual-staining methods for detecting two different antigens have been introduced (for a review, see Groenewegen and Wouterlood, 1990; Wouterlood and Groenewegen, 1991), mainly based on a sequential two-colour immunocytochemical paradigm (Hancock, 1986). According to this paradigm, one antigen is detected first with nickel-enhanced diaminobenzidine (DAB-Ni) and then the other using regular diaminobenzidine (DAB) as a chromogen.

Vector Labs has recently introduced several new peroxidase substrates for both light and electron microscopy. These new chromogens include the peroxidase substrate Vector™ VIP® (V-VIP), which results in a light purple reaction product which is perfectly distinguishable from the black or brown reaction product obtained with DAB-Ni or DAB, respectively. Initial results combining V-VIP and DAB-Ni or DAB look very promising for both light and electron microscopy (Zhou and Grofova, 1995).

In the present study we have chosen to visualize the perirhinal fibers with the use of the anterograde tracer biotinylated dextran amine (BDA). Entorhinal principal neurons in layers II and III were labelled via retrograde tracing with fluorogold (FG), and populations of entorhinal interneurons were stained with immunocytochemical procedures, using antiserum against parvalbumin (Pv) or calbindin (Cb). These labels were visualized in one and the same histological section with a triple staining procedure. This sensitive protocol allows to establish whether a projection targets one out of two types of neurons, or both. Moreover, it allows for the analysis of the relationships between the two neuronal populations in a particular brain structure. It is expected that a similar protocol can be used for any neuroanatomical procedure where three distinguishable chromogens are required. A preliminary report was published in an abstract form (Lanciego et al., 1996).

2. Materials and methods

Female wistar rats ($n = 5$; body weight 200–230 g) were deeply anaesthetized (0.1 ml/100 g body weight) with an intramuscularly injected mixture of 4:3 parts of Ketaset® (1% solution of ketamine, Aesco, Boxtel, The Netherlands) and Rompun® (2% solution of xylazine, Bayer, Leverkusen, Germany) respectively. The rats were then placed in a stereotaxic frame. In a single surgical session, 5% solution of biotinylated dextran amine (BDA; Molecular Probes, Eugene, OR) in 0.01 M phosphate buffer, pH 7.25 was iontophoretically delivered into the perirhinal cortex through a glass micropipette (inner tip diameter 20–30 μm) using a 5 μA positive pulsed direct current (7 s on/off for 5 min). Immediately afterwards, a 2% solution of fluorogold (Fluorochrome, Englewood, CA) in 0.1 M cacodylate buffer, pH 7.3 was iontophoretically injected into the ipsilateral dorsal hippocampus using the same parameters described above for BDA injections. All coordinates were taken from the stereotaxic atlas of Paxinos and Watson (1986).

2.1. Tissue processing

After a survival time of 7 days, the animals were deeply anaesthetized via an intraperitoneal injection of Nembutal (sodium pentobarbital, Ceva, Paris, France, 60 mg/kg body weight) and perfused transcardially with 100 ml of 0.9% saline solution (at body temperature) followed by 1000 ml of a cold fixative containing 4% paraformaldehyde, 0.1% glutaraldehyde and 0.2% saturated picric acid in 0.125 M phosphate buffer, pH 7.4. After the perfusion, the skull was opened and the brain removed and stored in a cryoprotective solution containing 2% dimethylsulphoxide (DSMO) and 20% glycerin in 0.125 M phosphate buffer, pH 7.4 (Rosene et al., 1986). Coronal sections (40 μm thick) were obtained with a freezing microtome and collected in 0.125 M phosphate buffer, pH 7.4.

2.2. Staining

It is well known that subsequent immunostaining procedures with different antibodies and different chromogens may have differential results, depending on the sequence of staining of each particular label. Therefore, various combinations and staining sequences were tested in order to establish the optimal procedure (Fig. 1). The procedure eventually selected since it produced the best results is described below.

The BDA staining procedure was carried out according to Veenman et al. (1992): sections were incubated in ABC solution (ABC kit; standard PK-4000, Vector Labs, Burlingame, CA) for 90 min and then reacted with DAB-Ni (Sigma, St. Louis, MO) for 3–5 min. After rinsing in Tris-buffered saline, incubation was continued in a cocktail of primary antiserum composed of 1:2000 rabbit anti-fluorogold (kindly provided by Dr H.T. Chang, Department of Anatomy and Neurobiology. University of Tennessee, Memphis, TN) and 1:2000 monoclonal mouse anti-parvalbumin (Sigma) or 1:2000 mouse anti-calbindin (Sigma). This incubation took 60 h at 4°C under gentle agitation (Please note that a commercial antibody against fluorogold is now commercially available from Chemicon, Temecula, CA. For more details using this antisera, see Van Bockstaele et al., 1994). Subsequently, sections were incubated with a mixture of swine anti-rabbit IgG (1:50, Dako, Copenhagen, Denmark) and goat anti-mouse IgG (1:50, Dako) for 120 min at room temperature. The

next step consisted of incubation with a peroxidase-antiperoxidase complex raised in rabbit (1:600 rabbit-PAP, Dako) for 90 min, followed by reaction for 20–30 min in DAB solution (Sigma). Finally, sections were incubated in a monoclonal PAP complex raised in mouse (1:100 mouse-PAP, Dako) and stained for 15–30 min using V-VIP (Vector) as a chromogen. Following staining, the sections were mounted on glass slides using a 2% solution of gelatin in 0.05 M Tris/HCl, pH 7.6; quickly dehydrated in toluene, and coverslipped with Entellan® (Merck, Darmstadt, Germany). All the antisera used throughout this procedure were diluted in 0.05 M TBS buffer with 0.5% Triton X-100 (Sigma, St. Louis, MO), pH 8.0.

2.3. Comparative stains

Dual-staining procedures were done combining BDA histochemistry with FG immunocytochemistry, testing the quality of contrast between the DAB-Ni reaction product (black) and the DAB precipitate (brown), in comparison with contrast obtained by combining the DAB-Ni reaction product (black) and V-VIP precipitate (brown).

A different sequence for triple-staining procedures was also performed by testing the V-VIP chromogen for the BDA stain. In these cases, the parvalbumin- or calbindin-immunocytochemistry was conducted first and visualized with DAB-Ni, then the FG stain was conducted with DAB as chromogen, while finally the BDA staining was done using with V-VIP.

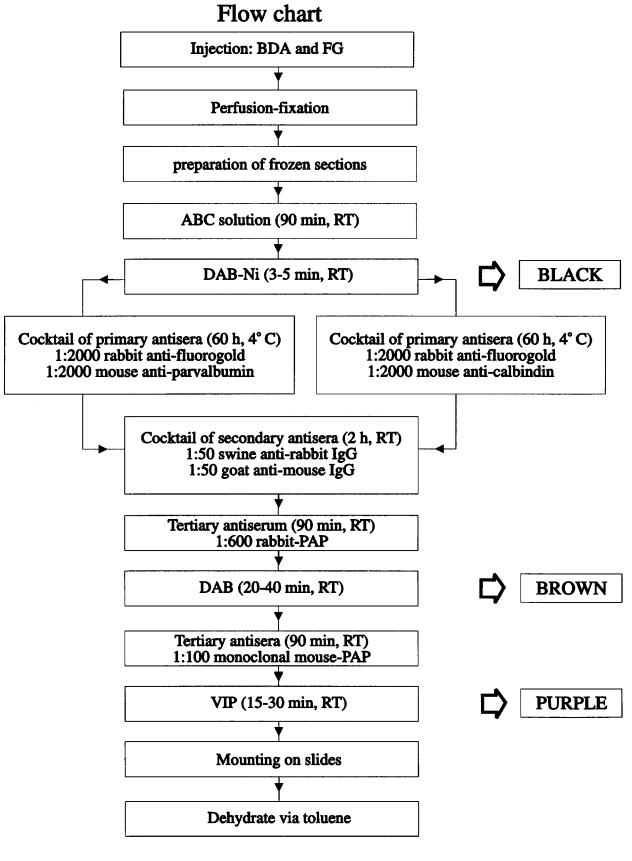

Fig. 1. Flow chart illustrating the various steps, antisera and chromogens used in the experimental protocol described in the present study for simultaneous labelling of (i) BDA-containing perirhinal afferents to the entorhinal cortex, black-labelled with DAB-Ni, (ii) FG-containing layer II-III entorhinal principal neurons projecting to the hippocampus, brown-labelled using DAB, and (iii) entorhinal interneurons containing the calcium binding proteins parvalbumin or calbindin, purple-stained with V-VIP.

2.4. Peroxidase substrates and reactions

The DAB-Ni substrate was prepared by dissolving 0.2 g of nickel ammonium sulphate and 7.5 mg of DAB in 50 ml of 0.05 M Tris/HCl, pH 8.0. Immediately prior to use, 10 μl of 30% H_2O_2 was added. The reaction takes approximately 5–10 min, resulting in a blue-black reaction product. The regular DAB solution was prepared by dissolving 5 mg of DAB in 10 ml of 0.05 M Tris/HCl, pH 7.6. Prior to use, 3.3 μl of 30% H_2O_2 was added. Sections were incubated in this solution for 20–40 min, inspected at intervals in a microscope control in order to stop the reaction just before background stain began to develop. The V-VIP solution was prepared in higher dilutions than those recommended by Vector; instead of three drops of each vial to prepare 5 ml of V-VIP solution, as indicated by the manufacturer, we used one drop of each vial to prepare 3.5 ml of the incubation solution.

3. Results

3.1. Double-staining procedures

Two different dual-staining sequences were used to evaluate the usefulness of the V-VIP substrate as a substitute for DAB. One series of sections from each rat was used for BDA staining with DAB-Ni and FG with DAB, while in the other series BDA was stained with DAB-Ni and FG with V-VIP. In all cases, the V-VIP reaction product showed a distribution identical to that obtained when DAB was used. The appearance of the FG injection sites was similar, regardless of the chromogen employed. The same was observed in the case of the somata that were retrogradely-labelled with FG. All the cell bodies showed the same granular label contents, and the overall distribution of labelled cells in the entorhinal cortex was similar, irrespective of the chromogens used (Figs. 2A,B).

The contrast achieved between the reaction products obtained in both staining sequences (black contrasted to brown or black contrasted to purple, respectively) was found to be excellent for both procedures and no

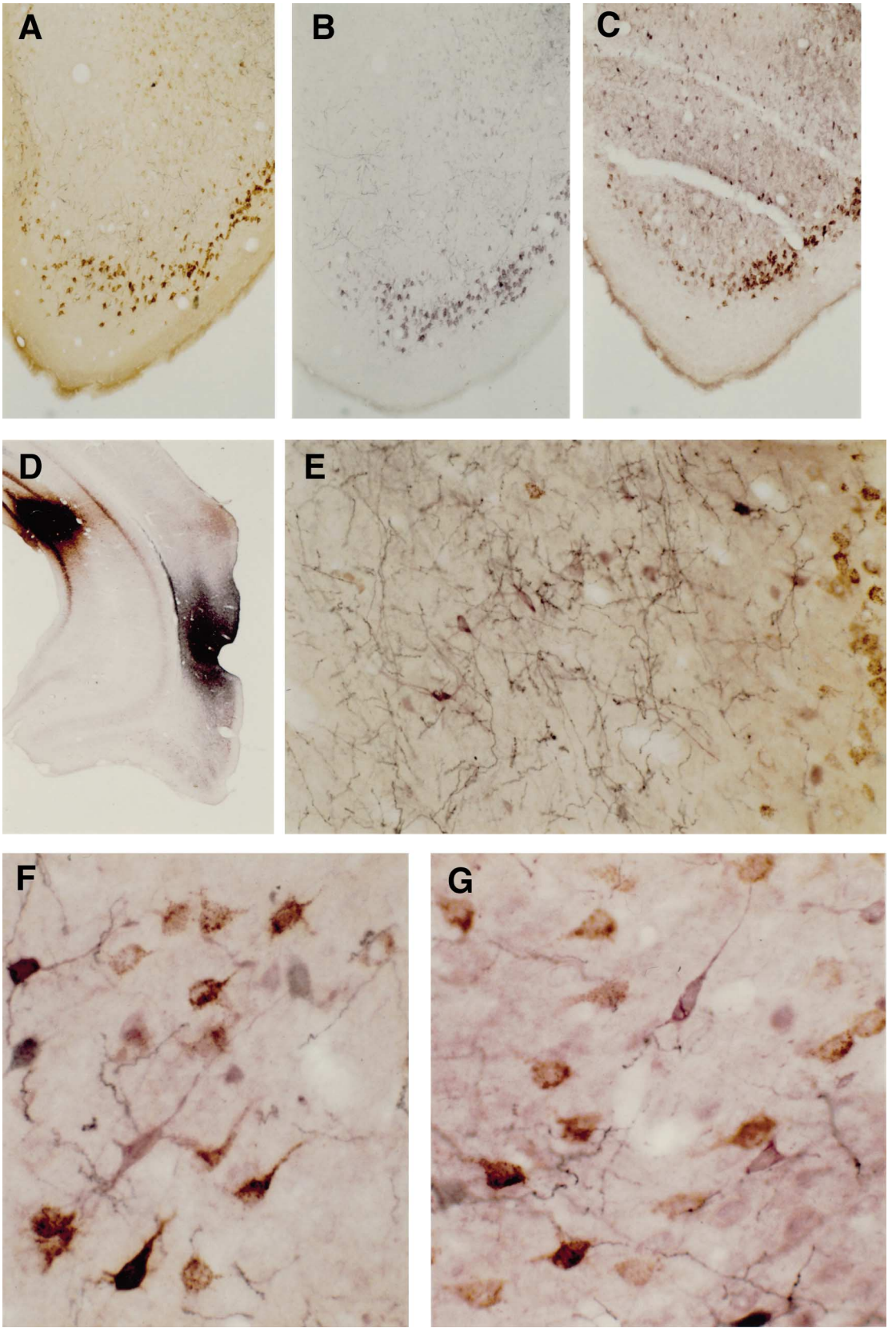

Fig. 2.

decrease in the staining intensity of either label due to their simultaneous visualization has been observed (Figs. 2A,B). A greater background staining was observed when V-VIP was used as the second chromogen when this reagent was prepared exactly according to the manufacture's instructions. The use of higher dilutions of this reagent almost completely abolished this background staining. Another phenomenon that may hinder the interpretation of the results is the phenomenon of so-called 'colour mixing', that is, a shift in colour of the reaction product of the first chromogen due to incubation in the second chromogen. The black colour resulting from developing BDA with DAB-Ni was perfectly conserved when DAB was used to stain the second label (FG). A certain degree of colour mixing appeared when V-VIP was used to visualize FG. In such cases, the BDA injection sites changed colour rapidly, losing the black precipitate corresponding to DAB-Ni and acquiring a purple colour, corresponding to the colour of the V-VIP substrate used to complete the FG protocol. However, this change in colour was only noticeable in the BDA injection sites, i.e. in the zones where DAB-Ni precipitate was greater. In contrast, the original black colour was conserved in all the BDA-labelled fibers and axon terminals. This problem can be circumvented with higher dilutions of the V-VIP chromogen, such that the BDA injection sites maintain their original colour (Fig. 2D).

3.2. Triple-staining procedures

Two series of sections from each rat were used for triple staining of three labels, combining anterograde tracing with BDA and FG retrograde tracing with parvalbumin immunocytochemistry in one series (Fig. 2F), and with calbindin immunocytochemistry in the other (Fig. 2G). In most cases, the same staining sequence was followed, comprising first BDA histochemistry with DAB-Ni substrate, second a FG protocol with DAB substrate and then a parvalbumin or calbindin stain with V-VIP substrate. One series of sections of each rat was processed through a different sequence (parvalbumin or calbindin immunocytochemistry with DAB-Ni, FG with DAB and BDA with V-VIP), although this option was soon abandoned due to the high background stain always obtained when DAB-Ni is applied to parvalbumin or calbindin procedures, reducing the usefulness of the triple staining method.

In all the series used for triple staining, the distribution and characteristics of the BDA-labelled fibers and FG-labelled neurons were the same as that obtained in the dual stained series, which were used as controls (Figs. 2A–C). The distribution and morphological features of the parvalbumin- or calbindin-containing neurons (stained with V-VIP substrate) were similar to that previously reported (Wouterlood et al., 1995). Colour separation as well as specificity were excellent (Figs. 2F,G).

The degree of background stain obtained in triple procedures was always greater than that observed in dual staining procedures, although careful microscopic monitoring of the progress of staining (especially recommendable because the third label is always detected with V-VIP chromogen) enable us to obtain a minimum background in the present procedure (Figs. 2C,E–G). Quite the opposite was found when DAB-Ni solution was used to disclose parvalbumin- or calbindin-containing neurons, in the sense that the reaction progresses so rapidly (30–60 s) that it was later almost impossible to distinguish FG-labelled neurons (DAB chromogen) and BDA-labelled fibers (V-VIP chromogen).

In our initial experiments, we prepared the V-VIP substrate according to the guidelines provided by the supplier. This resulted in an extremely high background staining in all our experiments and thus was unacceptable. Subsequently, higher degrees of V-VIP dilutions were tested. The optimal results for the presently described triple staining procedure were obtained in agreement with what has been mentioned in the Section 2. All the reagents included in the V-VIP substrate kit (four different vials) were dissolved in 0.05 M Tris/HCl, pH 7.6 and the washing steps before and after the incubation were carried out in this same buffer.

Fig. 2. Multiple staining procedures using V-VIP as a substrate. (A–C) illustrate a comparison between adjacent sections that have been processed in a different way. (A) Dual staining method combining BDA (black-stained using DAB-Ni) and FG (visualized in brown with DAB). Scale bar = 135 μm. (B) Shows a similar procedure, but in this case V-VIP substrate has been used as an alternative to DAB for the immunocytochemical detection of FG-labelled neurons. Note that the spatial distribution of the FG-labelled neurons is the same than those showed in Fig. 1, irrespective of the chromogen used to detect FG-labelled cells. Scale bar = 135 μm. (C) Illustrates a triple staining procedure with BDA-labelled fibers labelled in black (DAB-Ni substrate), FG-labelled neurons in brown (DAB) and populations of parvalbumin-containing neurons in purple (V-VIP stain). Note the slight higher background stain in (C) as compared with (A and B). Scale bar = 150 μm. (D) Ultralow-power magnification microphotograph showing the injection sites corresponding to BDA (perirhinal cortex, black colour), and FG (dorsal hippocampal formation, brown colour). Scale bar = 1000 μm. (E) Panoramic view of the lateral entorhinal area, in which the three labels overlap with a high density. A BDA retrogradely-labelled cell can also be appreciate at the right apical corner of the photomicrograph. Scale bar = 80 μm. (F) High-power magnification photomicrograph of a triple staining procedure combining anterograde tracing with BDA (black fibers; DAB-Ni chromogen), retrograde tracing with FG (brown-labelled cells, DAB chromogen), and parvalbumin-containing neurons (purple-labelled neurons, V-VIP chromogen). Scale bar = 45 μm. (G) High-power microphotograph showing a three-colour paradigm, combining BDA histochemistry (DAB-Ni chromogen, black), FG immunocytochemistry (DAB, brown), and immunocytochemistry using antibodies against calbindin (V-VIP, purple). Background stain in (G) is higher as compared as the one obtained in (F) due to a lower specificity of the antibody used to detect calbindin-containing neurons. Both pictures (F and G) were taken from the same animal, corresponding to different series. Scale bar = 45μm.

Finally, two issues seem to influence the results of staining with V-VIP. First, according to Vector's guidelines, V-VIP chromogen needs to be the last one to be applied, in order to maintain the brilliant purple colour. Second, we observed that the V-VIP substrate is very sensitive to strong dehydration procedures using ethanol, resulting in partial or sometimes even total loss of the V-VIP reaction product. As an alternative, toluene has been tested to dehydrate the sections. In these cases, fading of V-VIP staining was never observed.

4. Discussion

The sequential triple staining procedure described here, combining the peroxidase substrates DAB-Ni, DAB and V-VIP, resulted in a sensitive three-colour paradigm that allows unequivocal detection of three different labels. The two tracers used in the present study have been widely used and various application procedures as well as uptake and transport characteristics have already been reported in detail (Schmued and Heimer, 1990; Brandt and Apkarian, 1992; Veenman et al., 1992; Rajakumar et al., 1993; Reiner et al., 1993; Warr et al., 1993; Lanciego and Wouterlood, 1994; Wouterlood and Jorritsma-Byham, 1994).

4.1. Colour mixing

A phenomenon that should be taken into account is the so-called 'colour mixing'. This emerges as a slight instability in the colour of the reaction product resulting from the chromogen used first, when proceeding with the incubation in the second chromogen. When colour mixing occurs, there is a tendency for the colour of the first chromogen to take the colour associated with the second chromogen. This especially holds true in zones with high concentrations of the first reaction product (injection sites and peri-injection-labelled neurons). Colour mixing can also occur to the first two chromogens when a third is added. Colour mixing is observed quite often when labels requires long incubations with the chromogens DAB or V-VIP, in order to be detected.

4.2. Advantages

The sequential protocol described here, combines good sensitivity for the simultaneous detection of three labels with excellent contrast quality between three sufficiently separated colours in the visible light spectrum, to be able to obtain an unequivocal distinction (black, brown, purple), even in the brain areas in which the labelled structures overlap with a high density (Fig. 2E). Previous attempts to find a third chromogen for combination with DAB-Ni and DAB Smith and Bolam, 1991, 1992; Dolleman-Van der Weel et al., 1995 did not result in a perfect colour segregation. Smith and Bolam, 1991, 1992, have reported a method combining TMB (blue), DAB-Ni (black) and DAB (brown). This method suffered from a poor segregation of the colours black versus blue. Dolleman-Van der Weel et al., 1995 have recently introduced a sequential protocol combining the chromogens DAB-Ni (black), DAB (brown) and 1-naphthol/Azur B (blue-green). The use of 1-naphthol/Azur B as a chromogen has been originally reported by Mauro et al., 1985 as an alternative to DAB. With this method it may be difficult to differentiate among the three different chromogens in zones where all of them concur massively.

A second, minor advantage of the protocol described here, is that is unnecessary to store the sections under particular conditions to prevent fading of the V-VIP reaction product (for example, 1-naphthol/Azur B requires storage at -20°C). According to our experience, no fading of the V-VIP substrate has been noticed after a 1 year storage of the section under standard conditions (boxes, vertical storage containers, etc.), at room temperature.

4.3. Disadvantages

V-VIP substrate is very sensitive to ethanol. Dehydration procedures in ethanol for longer that 15 min may result in a total loss of the V-VIP reaction product. Partial fading of the substrate was also been noticed with shorter dehydrations in ethanol. This drawback was successfully solved when toluene was used for dehydration.

The level of background staining obtained with this procedure can be considered very low for a triple staining procedure. The distinction between different stained structures was always very clear, such that the use of high-power magnification lens is required only when one wishes to appreciate the finest fibers containing calbindin or parvalbumin. Such fibers show only little contrast against the slight background staining due to their light purple colour.

4.4. Final remarks

To obtain a maximum level of quality in this procedure, the following guidelines should be strictly observed: (a) one should use higher dilutions of the V-VIP substrate than those recommended by the supplier, to prevent unacceptable degrees of background stain and to avoid the appearance of colour mixing phenomena; (b) toluene should be used for the dehydration of sections, in order to avoid fading of the V-VIP reaction product; (c) prolonged incubations in the different chromogen solutions should be avoided, since these

may result in elevated background staining and colour mixing artifacts; and (d) in relationship with the former remark, it is necessary to carefully monitor each of the three incubations with the different chromogens in order to stop the reaction in time and thus avoid unwanted background staining.

Please note that Vector Labs does not provide information concerning the chemical composition of V-VIP substrate. Although V-VIP is considered to be non-carcinogenic (Angelucci et al., 1996), little is known about possible toxicity, and therefore we recommend that the same care be taken in handling the V-VIP substrate as when handling to DAB.

We conclude that the use of the recently introduced peroxidase substrate Vector™ VIP, in combination with the traditional substrates DAB-Ni and DAB, provides a sensitive and reliable method for simultaneous staining of any source of three different labels in one and the same section, taking the usual care to avoid cross-reactivity and choosing the most suitable chromogen for each label.

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