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Protocol

Multiple neuroanatomical tracing in primates

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Abstract

The present report deals with a multiple tract-tracing procedure in non-human primates enabling the simultaneous visualization of retrogradely transported Fluoro-Gold (FG) and cholera toxin B subunit (CTB) in combination with anterogradely transported biotinylated dextran amine (BDA). Two issues have played key roles on the achievement of this reliable procedure: first, the recent development of a commercial antiserum against FG that allows us to convert the original fluorescent signal of this dye in a permanent precipitate via standard peroxidase-anti-peroxidase methods; second, the introduction of the novel peroxidase substrate Vector® VIP (V-VIP), resulting in a purple precipitate. The combination of these neuroanatomical tracers in one and the same histological section opens a possibility for the permanent visualization of the convergence of inputs from a particular brain area onto identified, two different subsets of projection cells of another area. Furthermore, this combination of three tracers emerges as a powerful technical tool for obtaining broad amounts of complementary data regarding the monkey brain connectivity, thus significantly reducing the number of animals needed to complete a particular study.

Themes: Cell biology

Topics: Staining, tracing, and imaging techniques

Keywords: Axonal tracing; Fluoro-Gold; Cholera toxin; BDA; HRP; V-VIP; Peroxidase; Neuroanatomy

1. Type of research

1. Triple labeling studies.
2. Multiple axonal tracing combining anterograde and retrograde tract-tracing methods.
3. Analysis of the convergence of one particular projection onto two different types of projection neurons.

2. Time required

1. Surgery
   - Deep intracranial stereotaxic surgery in large monkeys (*Macaca fascicularis*) is very demanding regarding the time spent on it. Pre-surgical care including anesthesia and placement of the monkey in the stereotaxic frame is about 1 h, immediately followed by a ventriculography in order to find the appropriate horizontal position of the head. This latter procedure takes about 1 h more. The time spent on tracer delivery varies depending on the accuracy of the injections, each one of them monitored under radiological control. Overall, the total surgical procedure takes about 9 h to be completed, plus another 2 or 3 h of post-surgical care until the monkey shows full recovery.
2. Survival time
   - The survival time should be adjusted to the length of the pathway being studied. In our experimental design, satisfactory labeling of long pathways (for example, from the caudate nucleus to the substantia nigra) have been obtained with survival times of two weeks.
3. Perfusion/fixation and sectioning
   - The total time spent on this procedure is approximately 1 h. Careful dissection of the brain takes 4 h.

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Cryoprotection of the tissue lasts about two days. Sectioning of the entire brain for light microscopy is done on a freezing microtome through an entire day of work.

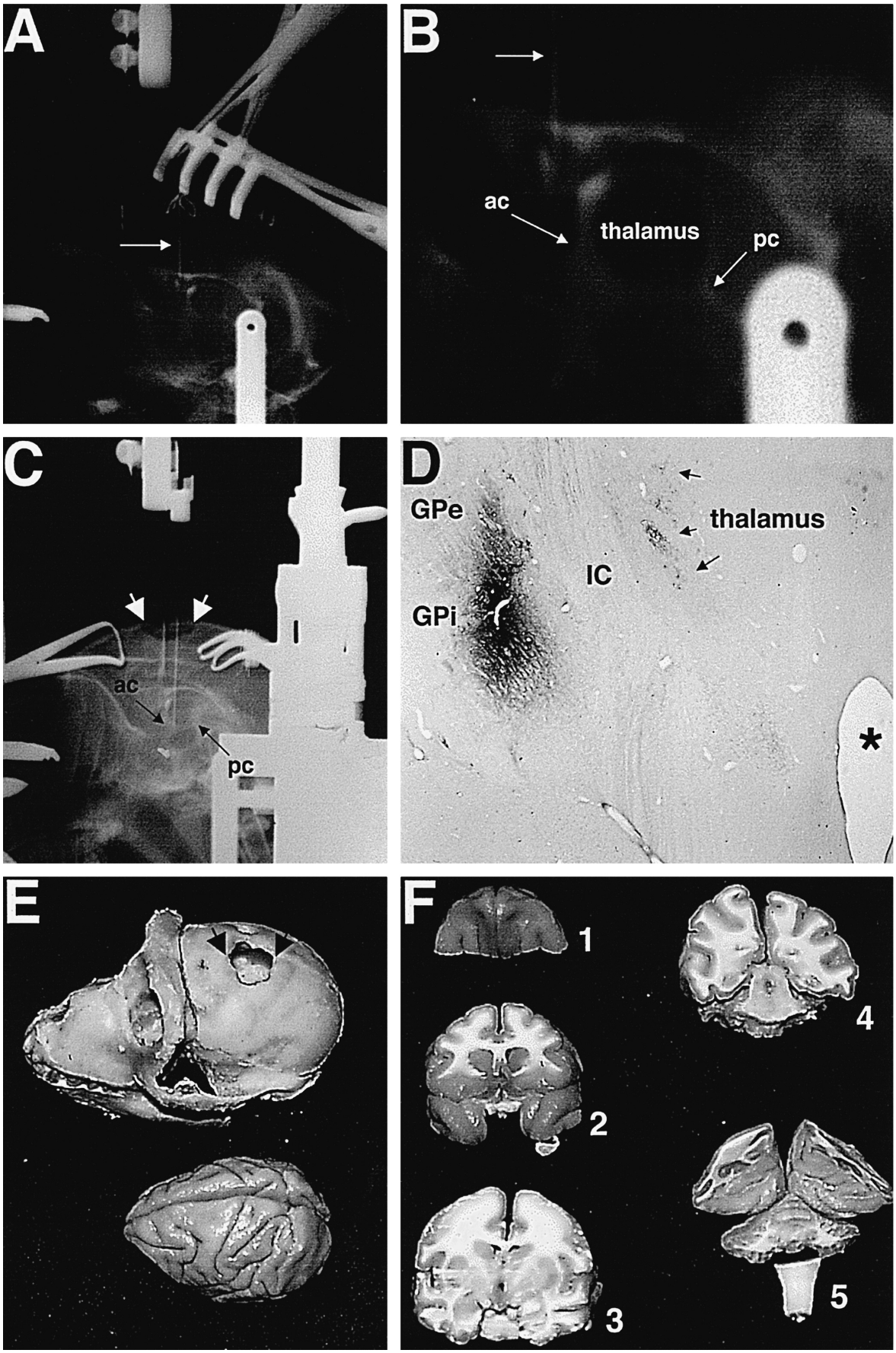
4. Staining
   - The biotinylated dextran amine (BDA) protocol is completed in 4 h, to which 72 h have to be added to complete both the cholera toxin (CTB) and the Fluoro-Gold (FG) protocol. Additional processing of the sections (mounting on glass slides, dehydration and coverslipping) means several more days.
5. Total estimation
   - Overall, this entire technical procedure requires almost one month to be fully completed, from the beginning to the end.

3. Materials

The animals used in this study were male monkeys (*Macaca fascicularis*) with a body weight ranging from 2500 to 3200 g. The animals were handled at all times according to the Society for Neuroscience Policy on the Use of Animals in Neuroscience Research. Animal handling was conducted under supervision of the Ethical Committee of the University of Navarra. Animals were supplied by R.C. Hartelust (Tilburg, The Netherlands).

1. Special equipment
   - Siemens Rx tube SR 90/20 FN. Germany.
   - Vertical glass capillary puller Narishige PB-7
   - Stereotaxic frame David Kopf model 1730. David Kopf Instruments, Tujunga, CA.
   - Micromanipulator David Kopf model 1760-61.
   - Hamilton syringe 801-RNE 10 μl model H-0084804. Hamilton, Reno, NV.
   - Perfusion peristaltic pump Watson–Marlow model 501S-170. Falmouth, Cornwall, England.
   - Surgical microscope Zeiss Power Supply 11. Germany.
   - Freezing sliding microtome Microm model HM-400. Germany.
   - Orbital shaker Janke and Kunkel model IKA vibrax Vx1. Germany.
2. Chemicals and reagents
   - Xilonibsa® (10% of a solution of lidocaine). Labs. Inibsa, 08029 Barcelona, Spain.
   - Ketolar® (ketamine, 50 mg/ml). Parke Davis, Warner Lambert, Morris Plains, NJ.
   - Dormicum® (Midazolam 5 mg/ml). F. Hofmann-La Roche, Basel, Switzerland.
   - Finadyne (Flunixin meglumine). Schering-Plough, Madrid, Spain.
   - Ampicilina® (ampiciline 1 mg) ref. 620583. Instituto Llorente, Madrid, Spain.
   - Omnigraph® (330 X, 50 ml). Juste SAQF, Madrid, Spain.
   - Biotinylated dextran amine (BDA; dextran, biotin, 10.000 MW, lysine fixable). Molecular Probes Europe, Leiden, The Netherlands.
   - Fluoro-Gold. Fluorochrome, Englewood, CO.
   - Cholera toxin B subunit ref. 104. List Biological Laboratories, Campbell, CA.
   - ABC kit standard ref. PK-4000. Vector Labs, Burlingame, CA.
   - Rabbit anti-FG ref. AB-153. Chemicon International, Temecula, CA.
   - Swine anti rabbit IgG ref. Z0195. Dakopatts a/s, Glostrup, Denmark.
   - Rabbit-PAP ref. Z0113. Dakopatts.
   - Goat anti-CTB ref. 703. List Biological.
   - Donkey anti-goat IgG ref. 03N09-181. Nordic Immunological Laboratories, Tilburg, The Netherlands.
   - Goat-PAP ref. 03N15-005. Nordic.
   - Albumine fraction V (BSA) ref. 1.12018 Merck, Darmstadt, Germany.
   - 3-3′ diaminobenzidine (DAB) ref. D-5637. Sigma Chemical, St. Louis, MO.
   - Nickel ammonium sulfate ref. 464545. Farmitalia Carlo Erba, Milano, Italy.
   - Vector® VIP (V-VIP) ref. PK-4600. Vector.
   - Trizma 7-9® ref. T-1378. Sigma.
   - Entellan® ref. 1.07961. Merck, Darmstadt, Germany.

Fig. 1. Composite plate showing several steps related to primate surgery and dissection of the brain. (A) shows the first ventriculography, done as an assessment of the appropriate, horizontal position of the head. Once the needle is placed on the lateral ventricle of the right brain hemisphere (arrow), a volume of 0.3 ml of radiographic contrast was injected to fully delineate the ventricular system. (B) Higher magnification view taken from (A) in order to better appreciate the ventricular system, the position of the needle, as well as the anatomical localization of both the anterior and posterior commissures. It can be easily noticed that both commissures are not located within the same horizontal line, and hence being necessary to correct the position of the head. (C) Ventriculography showing the position of the needle within the medial part of the left globus pallidus (the second needle, just caudal to the one of the ventriculography). Once the plate has been carefully checked, BDA is injected through this needle. (D) Low-power magnification microphotograph showing the BDA injection site in the medial part of the globus pallidus. This injection site corresponds to the ventriculography of the former picture. Small arrows indicate three small patches of BDA-labeled fibers within the thalamus. An asterisk is placed in the third ventricle. Abbreviations: GPe: external globus pallidus; GPi: internal globus pallidus; IC: internal capsule. (E) Dissection of the brain. Please note the craniotomy that can be seen in the dorsal part of the skull, which corresponds to the one shown in (C) (see arrows). The picture is taken to compare the relative sizes of the brain and the skull. (F) Once the brain is removed from the skull, a total number of five blocks were cut from rostral to caudal (blocks 1 to 5), at approximate intervals of 15 mm. Several basal ganglia nuclei can easily be appreciated in blocks 2 and 3.


A
B
ac
thalamus
pc
C
ac
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D
GPe
thalamus
IC
GPi
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1
2
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4
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- Dimethylsulphoxide (DMSO) f.a. ref. 131954. Panreac, Barcelona, Spain.

4. Detailed procedure

4.1. Surgery

4.1.1. Anesthetic solution and post-surgical care

Preliminary anesthesia was induced by intramuscular injection of a solution of Ketolar® (10 mg/kg). Surgical anesthesia was achieved with an 1:1 mixture containing Ketolar® (10 mg/ml) and Dormicum® (5 mg/kg), resulting in deep anesthesia for 2 to 3 h. Two additional doses of this anesthetic solution were given throughout the surgical procedure. Local anesthesia was induced with Xilonibsa® immediately prior to the surgery. Once the surgery is completed and prior to the monkey recovery, analgesia was achieved by an intramuscular injection of 1 ml of Finadyne® (5 mg/kg). This injection was repeated 24 and 48 h post-surgery. A similar schedule was also performed for the application of antibiotics to prevent infections. The daily intramuscular injection of Ampicilina® (0.5 ml/day) was administered through an entire week. During the survival time, animals were kept under constant control in single cages and fed with food and water ad libitum.

4.1.2. Ventriculography

Attempts to find the real brain size and the most appropriate horizontal position of the head are crucial to ensure the right target for tracer delivery. Accordingly, a ventriculography was performed in the opposite brain hemisphere. Each ventriculography consisted of the delivery of 0.2 ml of Rx contrast (Omnigraph® 300) into the frontal horn of the lateral ventricle to obtain a complete filling of the ventricular system. The coordinates for the lateral ventricle were taken 1 mm lateral from the sagittal sinus. Once the Rx plate was developed, both the anterior (ac) and posterior (pc) commissures were localized. The ac–pc was measured and used as a reference for positioning the head parallel to the horizontal plane. The coordinates for the different target areas were calculated according the length of the ac–pc line. Additional help regarding stereotaxic coordinates was taken from the atlas of Szabo and Cowan [26]. These procedures are illustrated in Fig. 1A,B.

4.1.3. Tracer delivery

All tracers were pressure-injected with a Hamilton syringe (0.2 μl each). FG was injected in the putamen as a 2% solution in 0.1 M cacodylate buffer, pH 7.3. CTB was delivered in the ipsilateral caudate nucleus as a 2% solution in 0.1 M phosphate buffer (PB), pH 6.0. Finally, BDA was injected in the medial part of the ipsilateral globus pallidus as a 10% solution in 0.01 M PB, pH 7.25.

Once each syringe was placed in the calculated target area and before the tracer delivery, an additional ventriculography was made to assess the accurate localization of the needle. When the syringe was not placed into the right target, it was necessary to correct the position of the syringe, resulting in more additional ventriculographies, until reaching the almost complete security of the appropriate placement of each individual injection (Fig. 1C).

4.2. Survival time

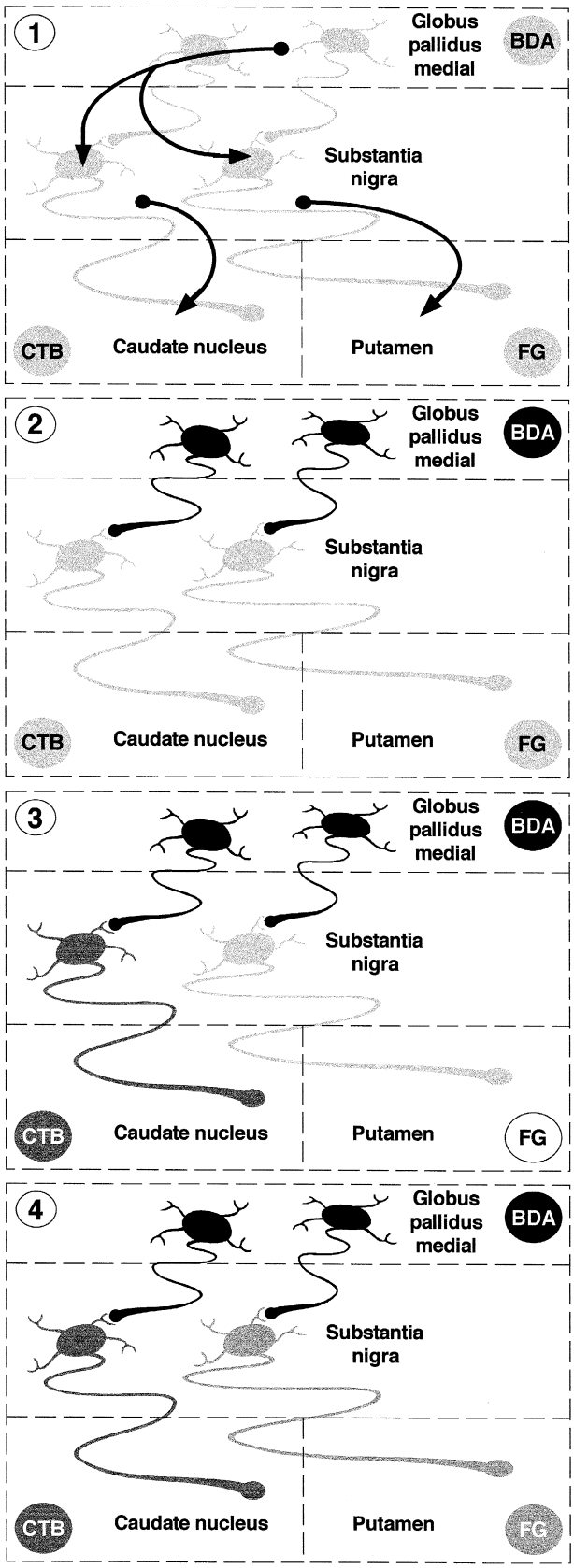
Survival time has to be adapted to the length of the pathway being studied. There is little information available on the detectability of BDA as a function of the survival time. BDA has a wide range of survival time [5,12–14,18,19,31,34]. The BDA's longest reported survival time is seven weeks for squirrel monkeys [5]. In our case, two weeks were enough to obtain a nice staining of the pallidonigral pathway. Similar statements can be used when considering FG and CTB survival times. FG has a wider spectrum of survival time, ranging from two days to one year [1,16,17,22–24]. Tract-tracing with CTB [6,11,15,20,25,29,32] varies as a function of survival time. Short survival times (24–48 h) resulted in intense anterograde labeling [2,28], while longer survival times display a tendency to enhance the reliability of CTB for retrograde axonal tracing. The survival time used in the present procedure resulted in intense retrograde labeling with CTB, as well as a slight amount of CTB anterogradely labeled fibers.

4.3. Perfusion / fixation / sectioning

The animals were re-anesthetized with an overdose of 10% chloral hydrate in distilled water and perfused transcardially. The perfusates consisted on a Ringer saline rinsing solution (at body temperature), immediately followed by 3000 ml of cold fixative containing 4% paraformaldehyde, 0.1% glutaraldehyde and 0.2% saturated picric acid in 0.125 M PB, pH 7.4. Subsequently, the perfusion was continued by using 1000 ml of a cryoprotective solution containing 1% DMSO and 10% glycerin in 0.125 M PB, pH 7.4. After the perfusion, the skull was opened and the brain removed (Fig. 1E,F). Tissue blocks (1.5 cm thick) were stored for 48 h in a cryoprotective solution [21] containing 2% DMSO and 20% glycerin in 0.1 M PB pH 7.4. Finally, frozen coronal sections (40 μm thick) were obtained and collected in 0.125 M PB 0.125 M pH 7.4.

4.4. Histology

Step 1: Wash the sections three times (10 min each) in 0.05 M TBS-Tx, pH 8.0. [6.06 g Trizma® (Sigma) + 8.85



g NaCl + 975 ml H_2O + 25 ml 20% Triton X-100 (Sigma). Then add a few drops of 1 N HCl to reach the adequate pH].

Step 2: Incubation in ABC solution (90 min, room temperature). To prepare 1 ml of the ABC solution, add 8 μl of solution A (avidin DH) to 1 ml of 0.05 M TBS-Tx, pH 8.0 and shake briefly. Then add 8 μl of solution B (biotinylated horseradish peroxidase H) and shake for several seconds. Please note than once the solution is prepared, it is necessary to wait 30 min before use.

Step 3: Wash the sections twice (10 min each) in 0.05 M TBS-Tx pH 8.0.

Step 4: Wash the sections twice (10 min each) in 0.05 M Tris/HCl pH 8.0. 6.06 g Trizma® + 1000 ml H_2O. Then add a few drops of 1 N HCl to reach the final pH.

Step 5: Histochemical reaction. BDA stain with a DAB–Ni solution (5 to 10 min, room temperature). 200 mg nickel ammonium sulfate + 50 ml 0.05 M Tris/HCl pH 8.0. When dissolved, add 7.5 mg of DAB and store in the dark. Immediately before use, add 10 μl 30% H_2O_2.

Step 6: Wash the sections three times (10 min each) in 0.05 M Tris/HCl pH 8.0.

Step 7: Wash the sections three times (10 min each) in 0.05 M TBS-Tx, pH 8.0.

Step 8: Incubation in a cocktail solution of primary antisera (60 h, 4°C). Goat anti-CTB (List Biological) and rabbit anti-FG (Chemicon) are used at a dilution of 1:2000 in TBS-Tx, pH 8.0 containing 2% BSA.

Step 9: Wash the sections three times (10 min each) in 0.05 M TBS-Tx, pH 8.0.

Step 10: Incubation in a cocktail solution of secondary antisera (120 min, room temperature). Donkey anti-goat (Nordic) and swine anti-rabbit (Dako) are used at a dilution of 1:50 in TBS-Tx, pH 8.0.

Step 11: Wash the sections three times (10 min each) in 0.05 M TBS-Tx, pH 8.0.

Step 12: Incubation in a peroxidase complex raised in goat (goat-PAP; 90 min, room temperature). Goat-PAP (Nordic) is used at a dilution of 1:600 in TBS-Tx, pH 8.0.

Step 13: Wash the sections twice (10 min each) in 0.05 M TBS-Tx, pH 8.0.

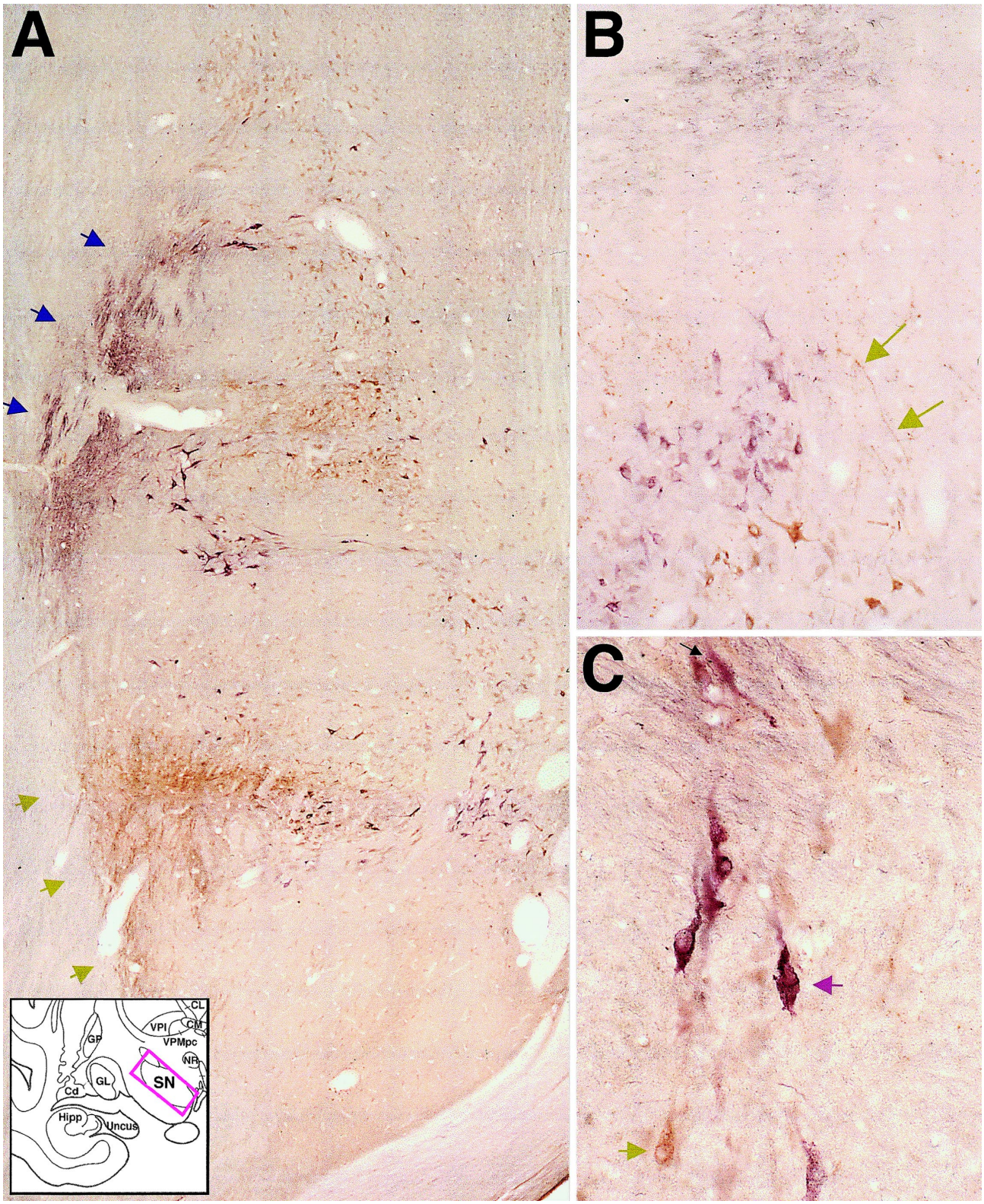
Step 14: Wash the sections once (10 min) in 0.05 M Tris/HCl, pH 8.0.

Fig. 2. Scheme of the striatonigral connectivity and the use of the present multiple tracing technique to visualize it. (1) Panel illustrating the connectivity itself as shown by the involved neurons, as well as the related nuclei, in which each tracer was delivered: BDA (anterograde tracer) was injected in the medial part of the globus pallidus, CTB (retrograde tracer) in the caudate nucleus, and FG (retrograde tracer) in the putamen. (2) The first step comprised the detection of transported BDA using an ABC solution, and then stained in blue/black with DAB–Ni as a chromogen. (3) the second step consisted of the detection of the transported CTB via a PAP procedure, brown-stained with DAB. (4) Finally, another PAP procedure was conducted for the detection of the FG-containing neurons, and the reaction was completed with the peroxidase substrate V-VIP (purple precipitate).

Step 15: Wash the sections twice (10 min each) in 0.05 M Tris/HCl, pH 7.6.

Step 16: Immunoreaction. CTB stain with a DAB solution (30–40 min, room temperature). 5 mg DAB + 10 ml 0.05 M Tris/HCl, pH 7.6. Store in the dark and filter. Immediately before use, add 3.3 μl 30% H_2O_2.

Step 17: Wash the sections twice (10 min each) in 0.05 M Tris/HCl, pH 7.6.



Step 18: Wash the sections once (10 min) in 0.05 M Tris/HCl, pH 8.0.

Step 19: Wash the sections twice (10 min each) in 0.05 M TBS-Tx, pH 8.0.

Step 20: Incubation in a peroxidase–antiperoxidase complex raised in rabbit (rabbit-PAP; 90 min, room temperature). Rabbit-PAP (Dako) is used at a dilution of 1:600 in TBS-Tx, pH 8.0.

Step 21: Wash the sections twice (10 min each) in 0.05 M TBS-Tx, pH 8.0.

Step 22: Wash the sections once (10 min) in 0.05 M Tris/HCl, pH 8.0.

Step 23: Wash the sections twice (10 min each) in 0.05 M Tris/HCl, pH 7.6.

Step 24: Immunoreaction. Transported FG is stained with a V-VIP solution [35]. (5–10 min, room temperature). V-VIP peroxidase substrate is presented as a kit containing four vials. In order to prepare 3.5 ml of the working solution, one drop of each vial has to be used: 1 drop vial A + 1 drop vial B + 1 drop vial C + 1 drop peroxidase reagent in 3.5 ml 0.05 M Tris/HCl pH 7.6.

Step 25: Wash the sections three times (10 min each) in 0.05 M Tris/HCl, pH 7.6.

Step 26: Mounting. 2 g gelatin (Merck) + 1000 ml warm (60°C) 0.05 M Tris/HCl, pH 7.6.

Step 27: Coverslipping. Once the sections were dried, dehydration and clearing is achieved by using two rinsing steps (4 min each) in toluene. Finally, sections were coverslipped with Entellan® (Merck).

4.5. Safety requirements

(a) Animal handling: All the monkeys used in the present study have been tested by the supplier for several distinct kinds of infectious diseases, including TB, HSV-B, SIV, salmonella, shigella and philovirus. Nevertheless, animals were kept in single cages for a minimum of 40 days upon receipt and then tested again for TB, prior to undergoing surgery. Throughout surgery, we strongly recommended the use of double pair of gloves and mask, plus the standard safety precautions when dealing with experimental animals.

(b) Chromogens: Two peroxidase substrates used in the present study (DAB and DAB–Ni) are suspected carcinogens and need to be handled with care inside a fume hood. V-VIP chromogen is presented by Vector Labs as a non-carcinogenic chromogen [2], but due to the little information provided by the supplier on the chemical composition of this substrate, we recommended the use of the same standard safety considerations for DAB handling.

(c) Solvents: Cacodylate buffer can be considered as a poisonous solution and has to be handled with care.

5. Results

Animals received separate injections of BDA, CTB and FG in different nuclei of the basal ganglia (Fig. 2). The pallidonigral pathway is labeled after a BDA deposit in the medial part of the globus pallidus. A subpopulation of neurons of the substantia nigra, which project to the caudate nucleus, were identified by means of retrograde transport of CTB, while another subpopulation of nigral cells projecting to the putamen were retrogradely labeled after a FG injection in the putamen. A moderate amount of CTB-anterogradely labeled fibers can also be seen in the substantia nigra. Some CTB- and FG-labeled neurons can also be appreciated in the contralateral substantia nigra.

The sensitive protocol reported here resulted in intense labeling of three different structures in the substantia nigra, simultaneously showing up anterogradely transported BDA (blue–black fibers), and retrogradely transported CTB and FG (brown and purple cells, respectively) (Fig. 3A). Higher magnification can be used to disclose the fine morphological details of the anterogradely labeled fibers and the retrogradely labeled neurons within the areas in which the labeled structures show a certain degree of overlapping (Fig. 3B,C).

Non-specific background staining has been kept to minimum levels, specially when considering that we were dealing with triple staining procedures. In this regard, the present combination of three tracers resulted in a slight degree of background staining, similar to the one obtained in other multiple combinations of tracers previously reported [13,14].

6. Discussion

The focus of this report is to extend the use of multiple tract-tracing protocols in the primate brain by showing that three widely used neuroanatomical tracers can be simulta-

Fig. 3. Triple-stained section. (A) Low-power magnification photocomposition showing a coronal section of the primate brain through the substantia nigra. The injected tracers were simultaneously detected in one and the same section according to a three-color paradigm. BDA fibers were black-stained using DAB–Ni, CTB-containing neurons and fibers were stained in brown with DAB and somatopetally transported FG was stained in purple using V-VIP. Blue arrows shows a large patch of BDA-containing fibers, while green arrows indicate anterogradely transported CTB. One can easily notice several distinct patterns of distribution (overlapping and/or segregation) for the types of projections being studied here. (B and C) Pictures taken at higher magnification in order to better see the morphological details that can be observed by using this procedure. The arrows in (B) show a CTB-labeled fiber. (C) Illustrates two well-defined populations of neurons. The purple arrow is showing a FG-positive cell, while the green arrow is pointing at a CTB-containing neuron. Close appositions can also be seen between the BDA-labeled fibers and the FG-positive neurons (small black arrow). Scale bar is 1 mm for (A), 400 μm for (B), and 200 μm for (C).

neously and permanently detected in one and the same section, according to a three-color paradigm [13,14]. The use of monkeys as experimental subjects is technically very demanding, time-consuming and quite expensive. Moreover, there is quite some social controversy in using non-human primates in research. Accordingly, the possibility of simultaneous detection of three tracers resulted in broad amounts of information about the way in which three different kinds of projections interact each other, thus allowing to reduce the number of animals needed to complete a particular study.

6.1. Troubleshooting

6.1.1. The use of Fluoro-Gold in axonal tracing with monkeys

There is some discrepancy regarding the use of FG for tract-tracing studies in monkeys. It has been pointed out that FG may produce variable results when this tracer is used in non-human primates [22], and the reasons for this variability remains not understood. Only very little work has been done using somatopetally transported FG in monkeys [8,9,27]. According to our experience, the best results using FG have been obtained by dissolving the tracer in 0.1 M cacodylate buffer, pH 7.3, as previously reported elsewhere [24]. Dilutions of FG in other solvents rather than cacodylate buffer, such as distilled or deionized water, phosphate buffers or acetate buffers resulted in an non-homogeneous solution that often precipitates, and this may seriously compromise axonal uptake (Please note that FG is a complex mixture of several chemical compounds. For more details regarding FG composition and axonal uptake, see Wessendorf [33]).

6.1.2. Survival time

As previously stated, survival time has to be adapted to the length of the pathways being study. When considering protocols aimed at the simultaneous detection of three axonal tracers in one and the same section, it is mandatory to choose three tracers that can afford a similar survival time. In this regard, the three markers used in the present combination (BDA, CTB and FG) allows a survival time large enough [5,10,11,15,16,23] to be able to adjust the survival time convenient for almost any kind of experimental design in monkeys.

6.1.3. Application of antibodies to Fluoro-Gold

The somatopetally transported FG shows a relative resistance to fading, a common problem to all fluoroprobes. In order to avoid fading problems, photoconversion of the dye in an stable diaminobenzidine (DAB) reaction product can be obtained either via photo-oxidation under UV light [3,4] or by means of the application of antibodies against FG [7,30]. Photoconverted FG resulted in a permanent DAB reaction product that facilitates the ultrastructural examination of the structures displaying tracer. Photoconversion under UV light is partially limited by the fact that only one marker can be photoconverted in a single section, while the application of antiserum against FG allows several combinations of this tracer either with the detection of neuroactive substances [7,30], or with other protocols commonly used for tract-tracing [13,14]. As far as we know, two reliable antiserum against FG are currently commercially available (Chemicon ref. No. AB-153 and Biogenesis ref. No. 4512-4018).

6.1.4. Special remarks regarding triple staining protocols

There are several issues that play key roles when trying to obtain fine technical results using the three color paradigm described in the present report.

(a) The main problem one may encounter when dealing with the simultaneous detection of three markers is the possible appearance of high degrees of non-specific background staining, which may hinder the results. The best way to solve this kind of problem is to undergo a careful control under the microscope of the progression of the different reactions corresponding to each particular chromogen. This procedure may enable to halt the reaction just in time, before background staining begins to show up. Otherwise, the existence of a slight degree of background stain during the first incubation may enhance the non-specific stain obtained in the second reaction, and both will therefore have a kind of multiplying effect on the degree of background stain one may find in the third final reaction; thus sometimes almost impossible to clearly visualize the three final colors corresponding to each particular chromogen. Additionally, prolonged incubations in the different chromogens solutions resulted in the appearance of color mixing phenomena (i.e., the tendency of a particular chromogen for taking the color corresponding to another peroxidase substrate). This particular phenomenon is observed quite often in the areas displaying large amounts of the reaction product (for example, the injection sites).

(b) The strongest chromogen used in this procedure obviously is the nickel-enhanced DAB substrate (DAB–Ni). This substrate has been chosen to detect the BDA-labeled fibers, according to a standard histochemistry by using an ABC solution. In other words, there are no antibodies involved in the BDA detection, so the degree of non-specific background staining is kept to a minimum during the first incubation. Accordingly, one would expect higher amounts of background staining during the second and the third incubations (both are standard PAP procedures). In this regard, the use of 2% BSA as an agent for binding non-specific tissue antibodies resulted in an evident improvement of the final staining quality. Separate controls showed that the rabbit and the goat antisera did not cross-react.

(c) The use of higher dilutions of the V-VIP substrate (as compared to the original guidelines provided by the supplier), resulted in a more handily substrate, so the way in which the reaction progress is much slower, thus facili-

tating to halt the reaction just in time, and hence avoiding background staining, as well as the appearance of color mixing phenomena. The resulting final purple precipitate is unstable under standard ethanol treatments. Therefore, toluene can be used as an alternative to ethanol for dehydration procedures.

6.2. Alternative protocols

This same multitracer study has been successfully used to investigate basal ganglia circuits in rats. Moreover, the three-color paradigm described in the present report allows several other possibilities, such as the simultaneous detection of anterogradely transported BDA and PHA-L in combination with retrogradely transported FG [14], or the visualization of two tracers (BDA and FG) plus the immunocytochemical detection of the calcium binding proteins parvalbumin or calbindin [13]. The use of the peroxidase substrate V-VIP can be extended to the electron microscopy, resulting in granular electron dense deposit. This characteristic of the V-VIP substrate is very helpful in the case of double pre-embedding protocols, first detecting a particular antigen with DAB substrate, and then another marker by using V-VIP substrate [35].

Another reliable option for the simultaneous detection of three tracers can be achieved by using the fluorescence microscope. FG is visible after UV-excitation; BDA can be detected by red fluorescent carbocyanine 3 (Cy3)-streptavidin and CTB might be visualized by indirect immunofluorescence based on goat anti-CTB and green fluorescent Cy2-donkey anti-goat.

Finally, there are other chromogens currently available, such as alkaline phosphatase, BDHC, TMB, etc., which might be explored in order to design several other combinations of multiple tract-tracing procedures.

7. Quick procedure

(A) Tracer delivery, survival time, perfusion, cryoprotection and sectioning.

(B) Incubation in the ABC solution (90 min, room temperature).

(C) DAB–Ni reaction (5–10 min, room temperature).

(D) Incubation in a cocktail of primary antiserum containing 1:2000 goat anti-CTB + 1:2000 rabbit anti-FG (60 h, 4°C).

(E) Incubation in a cocktail of secondary antiserum containing 1:50 donkey anti-goat + 1:50 swine anti-rabbit (120 min, room temperature).

(F) Incubation in the tertiary antiserum 1:600 goat-PAP (90 min, room temperature).

(G) DAB reaction (20–40 min, room temperature).

(H) Incubation in the tertiary antiserum 1:600 rabbit-PAP (90 min, room temperature).

(I) V-VIP reaction (5–10 min, room temperature).

(J) Mount sections on gelatin, dehydrate via toluene and coverslip with Entellan®.

8. Essential literature references

Original papers: Refs. [5,7,11,13,14,18,19,22–25,30,31,35].

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