

# Anterograde axonal tracing with the subunit B of cholera toxin: a highly sensitive immunohistochemical protocol for revealing fine axonal morphology in adult and neonatal brains

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## Abstract

We report an improved immunohistochemical protocol for revealing anterograde axonal transport of the subunit B of cholera toxin (CTB) which stains axons and terminals in great detail, so that single axons can be followed over long distances and their arbors reconstructed in their entirety. Our modifications enhance the quality of staining mainly by increasing the penetration of the primary antibody in the tissue. The protocol can be modified to allow combination in alternate sections with tetramethylbenzidine (TMB) histochemical staining of wheat germ agglutinin conjugated to horseradish peroxidase (WGA-HRP).

Using this protocol, we tested the performance of CTB as an anterograde tracer under two experimental paradigms which render other anterograde tracers less sensitive or unreliable: (1) labeling the entire retinofugal projection to the brain after injections into the vitreal chamber of the eye, and (2) labeling developing projections in the cortex and thalamus of early postnatal mammals. Qualitative comparisons were made with other tracers (*Phaseolus vulgaris* leucoagglutinin, dextran rhodamine, biotinylated dextran, free WGA, or WGA-HRP) that were used to label these same projections. From these observations it is clear that CTB, visualized with our protocol, provides more sensitive anterograde labeling of retinofugal projections as well as of axonal connections in the neonatal forebrain.

**Keywords:** Retinal projection; Visual; Cortex; Thalamus; Development; Tetramethylbenzidine histochemistry

## 1. Introduction

Few known anterograde axonal tracers reveal in detail the fine morphology of axons and their terminals. Among these are the plant lectin *Phaseolus vulgaris* leucoagglutinin (PHA-L) (Gerfen and Sawchenko, 1984), biocytin (King et al., 1989), biotinylated dextrans (Brandt and Apkarian, 1992) and dextran-conjugated rhodamine (Shmued et al., 1990). A limitation common to these tracers, however, is their relative lack of versatility. Factors such as the species or the age of the animal, or the particular neuronal pathway, may render the tracers unsuitable, due to reduced uptake, transport, or detectability, or a

combination of these factors. Several of these problems occur, for example, when tracers are injected in the vitreal chamber of the eye to label the entire retinofugal projection. Similarly, most anterograde axonal tracers show a markedly reduced sensitivity when applied to label central connections in neonatal animals (Payne et al., 1988; Ramirez et al., 1990; Claps and Casagrande, 1991).

In the course of a series of studies on the retinofugal and thalamocortical pathways in adult and neonatal ferrets, we employed a variety of anterograde tracers. Among them, we tried the subunit B of cholera toxin (CTB). Although well known as a retrograde tracer (Luppi et al., 1987, 1990; Ericson and Blomqvist, 1988) CTB had been only occasionally used for anterograde tracing, mainly due to the poor detail of the fiber labeling compared to other tracers (Ericson and Blomqvist, 1988; Bruce and Grofova, 1992). We have introduced modifications to the immunohistochemical method of Mikkelsen (1992) that significantly enhance the quality of the fiber staining. We report that, using this method, CTB yields complete and highly

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Table 1  
Intraocular injections

Tracer	Solution	Eyes adults ( <i>n</i> )	Survival adults (days)	Eyes postnatals ( <i>n</i> )	Survival postnatals (days)
Subunit B of cholera toxin (Low salt; List Biological Labs)	1%, H <sub>2</sub> O	18	3–6	15	1–2
Free WGA (Vector Labs)	10%, saline	2	2		
WGA conjugated with horseradish peroxidase (WGA-HRP; Sigma)	5%, saline	6	2	12	1–2
Biotinylated dextran (10000 MW; Molecular Probes)	20%, saline + 2% DMSO	2	7		
Biotinylated dextran (3000 MW; Molecular Probes)	20%, saline + 2% DMSO	2	4–7		
Dextran rhodamine (10000 MW, Lysine fixable; Molecular Probes)	5%, H <sub>2</sub> O + 2% DMSO	2	6		

detailed anterograde axonal labeling, both after injections in the eye and in the brain of young postnatal animals. In addition, we have developed a protocol that allows one to combine, in alternate sections, CTB immunostaining with tetramethylbenzidine (TMB) histochemical staining of wheat germ agglutinin conjugated to horseradish peroxidase (WGA-HRP) (Mesulam, 1978).

## 2. Materials and methods

### 2.1. Intraocular injections of tracers

To label anterogradely the entire retinofugal projection, we made unilateral or bilateral intraocular injections of one of the tracers indicated in Table 1, in adult or in young postnatal (aged 4–54 days) pigmented ferrets (*Mustela putorius furo*). Animals were purchased from a commercial supplier or bred in our colony. Some of the CTB and WGA-HRP eye injections (18 in adults and 22 in postnatals) were made in animals that had been surgically manipulated at birth to reroute retinal axons to the auditory thalamus (Sur et al., 1988b; Roe et al., 1993).

Animals older than 27 days were anesthetized with ketamine (30 mg/kg) and xylazine (1.5 mg/kg). Between the age of 14 and 27 days only ketamine (40 mg/kg) was administered, while younger animals were anesthetized by deep hypothermia. A 0.5% proparacaine hydrochloride

solution was applied to the conjunctiva. Using a Hamilton microsyringe, we injected 10  $\mu$ l (in adults) or 2–6  $\mu$ l (in postnatals) of a solution of one of the tracers listed in Table 1. After allowing a survival period for axonal transport (Table 1), the animals were administered an overdose of sodium pentobarbital (80 mg/kg) and transcardially perfused with saline for 5 min, followed in most cases by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 for 30 min. The WGA-HRP-injected animals were perfused with 1% paraformaldehyde and 1.25% glutaraldehyde for 30 min, followed by a 5–10% sucrose solution in PB for 30 min. The brains were removed from the skull, post-fixed overnight in the same fixation solution at 4°C, and cryoprotected by soaking in 30% sucrose in PB for 24–48 h at 4°C. The brains injected with WGA-HRP were soaked in 30% sucrose in PB without post-fixation. The tissue was then freeze-sectioned at 40–50  $\mu$ m.

Some of the animals (*n* = 13) received injections of CTB into one eye and of WGA-HRP into the other eye. In animals older than 27 days we injected CTB into one eye as described above and, 2 days later, we injected 10  $\mu$ l of a 4–5% solution of WGA-HRP into the other eye. Two further days of survival were allowed prior to perfusion. In younger animals, 2–6  $\mu$ l of each tracer was administered on the same day, and 1–2 days of survival allowed for transport. The animals were perfused with saline, followed by 2% paraformaldehyde in PB at 4°C for 30 min, and then the excess fixative was removed from the tissue by

Table 2  
Injections in the cortex and thalamus

Tracer	Solution	Adults ( <i>n</i> )	Survival adults (days)	Postnatals ( <i>n</i> )	Survival postnatals (days)
CTB	1–2%, 0.1 M PB (pH 6.0)	3	3–10	6	1–5
WGA-HRP	1–3%, 0.1 M PB or saline	4	2	6	1–2
Biotinylated dextran (3000 MW)	10%, saline	2	10–12	6	1–5
Dextran rhodamine	5%, H <sub>2</sub> O	5	10–14	4	1–4
PHA-L (Vector Labs)	2.5%, 10 mM PBS (pH 8.0)	6	10–14	7	1–5

perfusing with 5–10% sucrose in PB for 30 min, before cryoprotecting and sectioning as above.

## 2.2. Intracerebral injections

We tested the performance of CTB as an anterograde tracer when injected directly into the brain tissue. For this purpose, a series of CTB injections was made in the cerebral cortex or thalamus of adult ferrets and postnatal animals aged 1–21 days. For comparison, we made similar injections of PHA-L, biotinylated dextran, dextran rhodamine and WGA-HRP in some further adult and postnatal cases (Table 2).

In adult ferrets, anesthesia was induced with ketamine (30 mg/kg) and xylazine (1.5 mg/kg). Anesthesia was subsequently maintained with 1–2% isoflurane in 1:1 mixture of nitrous oxide and oxygen. Young animals were anesthetized as described above. A small craniotomy and durotomy were performed over the area of interest, and the tracers (with the exception of dextran rhodamine) delivered iontophoretically through glass micropipettes (5–20  $\mu\text{m}$  inside tip diameter) by applying positive current in 7 s on/off cycles. For CTB injections we used 2–3  $\mu\text{A}$  current for 2–10 min, while 7–8  $\mu\text{A}$  of current for 10–20 min were used to deliver biotinylated dextran, PHA-L and WGA-HRP. Dextran rhodamine (0.5–2  $\mu\text{l}$ ) was pressure injected using a Picospritzer (General Valve) and glass micropipettes of 20–30  $\mu\text{m}$  inside tip diameter. After appropriate postinjection survival (Table 2), the brains were fixed and sectioned as described above.

## 2.3. Staining procedures

To visualize anterogradely transported CTB, PHA-L, or free WGA, we used peroxidase-based immunohistochemistry. WGA-HRP was revealed by TMB histochemistry (Mesulam, 1978) and biotinylated dextran either using the high-affinity binding of an avidin–alkaline phosphatase conjugate (AP), or that of the avidin–biotin–HRP complex (ABC; Elite<sup>®</sup>, Vector). Dextran rhodamine was directly visualized under epifluorescence microscopy.

### 2.3.1. CTB immunohistochemical protocol

The following steps were carried out on free-floating sections, under constant agitation at room temperature (RT) unless otherwise specified:

1. Rinsing 3  $\times$  5 min in 0.1 M phosphate-buffered saline (PBS) pH 7.4.
2. Incubation in 0.3%  $\text{H}_2\text{O}_2$  in PBS for 20 min.
3. Rinsing 3  $\times$  5 min in PBS, and then in 0.1 M glycine (1  $\times$  30 min), followed again by rinsing in PBS (3  $\times$  5 min).
4. Incubation in 4–5% normal rabbit serum (NRS; Vector Labs), 2.5% bovine serum albumin (BSA; Boehringer Mannheim) and 0.3–0.5% Triton X-100 in PBS overnight at 4°C.

5. Rinsing 2  $\times$  5 min in PBS.
6. Incubation in goat anti-CTB (List Biological Labs, Campbell, CA) diluted 1:4000 in a PBS solution containing 2% NRS, 2.5% BSA, 2% Triton X-100, for either 2 days at RT or 4 days at 4°C.
7. Rinsing for 1 h in 3–4 changes of PBS, followed by 1  $\times$  10 min. in 2% NRS + 2.5% BSA.
8. Incubation in biotinylated rabbit anti-goat IgG (Vector Labs) diluted 1:200 in 2% NRS, 2.5% BSA and 1% Triton X-100 in PBS, for 1 h.
9. Repeat step 7.
10. Incubation in ABC diluted 1:100 in PBS, for 1 h.
11. Rinsing 4  $\times$  15 min in PBS and then 2  $\times$  5 min in 0.05 M Tris buffer (TB) pH 7.4.
12. Incubation in 0.5%  $\text{CoCl}_2$  in TB for 10 min.
13. 1  $\times$  1 min rinse in TB followed by 2  $\times$  5 min rinse in PBS.
14. Soaking in 0.025% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) in PBS. After 5 min, 0.004%  $\text{H}_2\text{O}_2$  was added to the solution, and 2–3 min were allowed for development.
15. Rinsing 5  $\times$  1 min in PBS, to stop the reaction.

As a non-carcinogenic alternative to DAB, Vector VIP<sup>®</sup> (Vector Labs), which produces an intense purple stain, can be used. Right after step 10, sections were washed for 1 h in 3–4 changes of PBS and then incubated in VIP for 3–5 min. The reaction was then stopped by rinsing 2  $\times$  5 min in distilled water.

After the immunostaining was completed sections were immediately mounted onto gelatin–chromalum-coated slides, air dried, dehydrated in graded alcohols, defatted in HistoClear (National Diagnostic) for 20 min, and coverslipped with DePeX. We used bright- and dark-field optics at 32–800  $\times$  for analyzing the labeling.

For brevity, the protocol described above is the one which yielded the best staining. In the course of this study, however, we systematically modified various parameters such as: (1) time of incubation (1–5 days) in primary antibody (at RT vs. 4°C) and in Triton X-100; (2) concentration of Triton X-100 (0–2%); (3) thickness of the tissue sections (40–50  $\mu\text{m}$ ); and (4) use of metal-enhanced vs. plain DAB staining, or Vector VIP<sup>®</sup> staining.

### 2.3.2. CTB immunohistochemistry and HRP histochemistry combined in adjacent tissue sections

For this procedure, we perfused the brains according to the light fixation protocol described above. We collected serial 40- $\mu\text{m}$ -thick freeze-sectioned tissue slices in PB. WGA-HRP was revealed in one series of sections according to the TMB method of Mesulam (1978). The adjacent series of sections was post-fixed in 2–4% paraformaldehyde for 2–24 h. Peroxidase activity was bleached away by incubation for 20 min in a solution containing 90% methanol and 0.3%  $\text{H}_2\text{O}_2$  in distilled water, and CTB immunohistochemistry was carried out as described above, starting from step 3.

### 2.3.3. PHA-L immunohistochemistry

PHA-L immunostaining was carried out using a slightly modified version of the protocol of Gerfen and Sawchenko (1984). Sections were first incubated overnight in a blocking solution containing 2% NRS, 2.5% BSA and 0.5–0.7% Triton X-100 in 0.02 M potassium phosphate-buffered saline (KPBS) pH 7.4, and then transferred for 48 h to a 1:3000 dilution of primary goat anti-PHA-L (Vector Labs) in KPBS containing 2% NRS and 2.5% BSA at 4°C. After a 3 × 10 min rinse in KPBS, the tissue was incubated in a 1:200 dilution of biotinylated secondary antibody (rabbit anti-goat IgG; Vector Labs) in the same buffer with 0.5% Triton X-100, for 1 h at RT. The final steps of the immunostaining were performed as for CTB (see above), starting from step 10.

### 2.3.4. Free WGA immunohistochemistry

We incubated the tissue sections at 4°C in one of the following dilutions of primary goat anti-WGA IgG (Vector Labs): 1:1000, 1:2000, 1:3000 and 1:5000 in PB containing 2% NRS, 2.5% BSA and 1% or 2% Triton X-100. After 1–4 days of incubation at 4°C, we rinsed the sections in PBS (5 × 5 min), and incubated them in a 1:200 dilution of biotinylated rabbit anti-goat IgG for 1 h at RT. We performed the remaining steps as for CTB (see above), starting from step 10.

### 2.3.5. Staining of biotinylated dextrans

For detection of biotinylated dextrans by means of alkaline phosphatase binding, we followed a modified version of the protocol of Brandt and Apkarian (1992). We pre-incubated the sections for 20 min in 90% methanol and 0.3% H<sub>2</sub>O<sub>2</sub> in distilled water. After rinsing in Tris buffer, pH 8.0 (5 × 5 min), the tissue was incubated overnight at RT in a solution containing 0.1% Extravidin<sup>®</sup>-AP (Sigma), 0.1% sodium azide, and 1% Triton X-100 in Tris buffer, pH 8.0. We then rinsed (6 × 5 min) in Tris buffer, pH 9.5, and developed the staining in a solution containing 0.08% 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma), 5% *N,N*-dimethyl-formamide (Sigma), 0.0125% Levamisole (Sigma), and 0.008% Nitro Blue Tetrazolium (NTB; Sigma) in Tris buffer, pH 9.5. After 2–3 h, we stopped the developing by rinsing in PB (5 × 5 min). For dehydration, exposure to alcohols was short (20 s in 70%, 90% and 100% alcohol solutions, respectively). Defatting and coverslipping were as for CTB.

For visualization of biotinylated dextrans by means of ABC binding, we first incubated the sections overnight in a blocking solution containing 2% NRS and 0.7% Triton X-100 in 0.02 M PB overnight. After rinsing in PBS (5 × 5 min), the tissue was transferred to a 1:100 solution of ABC Elite<sup>®</sup> for 2 h at RT. Subsequently, we proceeded as for CTB (see above) from step 11 on.

## 3. Results

First, we describe our results after CTB injections in the vitreal chamber of the eye, either alone or combined with injections of WGA-HRP in the other eye for analysis of binocular relationships. Subsequently, we give an account of our observations after injections of other anterograde tracers in the vitreal chamber. Finally, we report our findings after injections of tracers into the brain tissue of adult and postnatal animals.

### 3.1. Labeling of retinofugal projections after CTB eye injections

Following injections of CTB in the eye, both in adult and neonatal ferrets, our immunohistochemical protocol revealed heavy anterograde axonal transport to all retinal targets (Fig. 1). High-contrast fiber staining was present in major targets, such as the eye-specific layers of the lateral geniculate nucleus (LGN; Fig. 1A–C), the medial interlaminar nucleus (MIN; Fig. 1A) the superior colliculus, and the pretectal nuclei (PT; Fig. 1E). Sharp labeling was also present in the suprachiasmatic nucleus of the hypothalamus (SCN; Fig. 1D), and the nuclei of the accessory optic system. Likewise, in animals which had developed ectopic retinal projections as a result of surgical manipulations at birth (cf., Sur et al., 1988b), such projections were also clearly labeled (Fig. 1F,G). Retinofugal axons were stained not only in the terminal fields, but also along their path through myelinated tracts such as the main (Fig. 1A,B) or accessory optic tracts. Labeled fibers, particularly the thinnest ones, were sharply refringent under dark-field illumination, a feature that increased their detectability, even at relatively low magnification (Fig. 1E; see also Fig. 2D). In cases with high background or heavy staining, the fibers were less refringent in darkfield.

The most remarkable feature of the staining was the continuity of the fiber filling (Figs. 1C,F,G and 2D). Moreover, bouton-like enlargements appeared specifically in areas known to receive retinal terminations, while axons that simply traverse an area known not to be a retinal target typically showed a smooth appearance (Fig. 1C,F,G). The latter observation suggests that the technique reveals true terminal specializations rather than artifactual swellings. We observed slight differences in the quality of staining between the various terminal regions. For example, in areas such as the contralateral LGN (Fig. 1A) where the density of retinal terminations is very high, the amount of reaction product was such that single fibers were impossible to follow, whereas in less densely innervated areas, the morphology of axon arbors and their terminal specializations was visible in striking detail (Fig. 1F,G).

Labeling was optimal at all survival times allowed after the injection (2–7 days), both in adults and young animals. The parameters that mostly influenced the quality of the

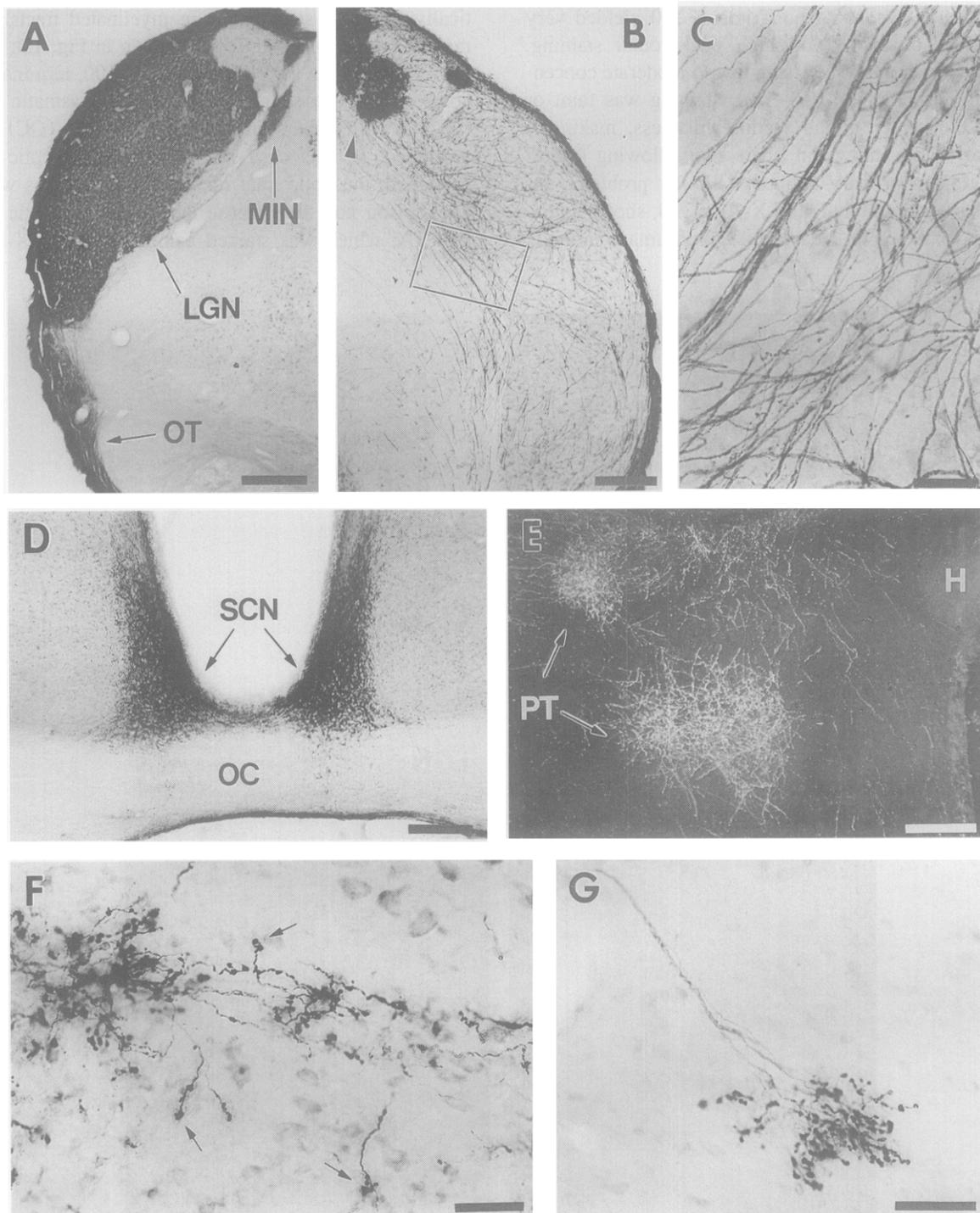


Fig. 1. Retinofugal projections labeled by intravitreal injections of CTB in ferrets. A: Coronal view of the lateral geniculate thalamic nucleus (LGN) contralateral to the injected eye in an adult ferret. Midline is to the right and dorsal is to the top of the image. Notice the heavy labeling in the eye-specific layers of LGN and the medial interlaminar nucleus (MIN), as well as in the myelinated fibers of the optic tract (OT). B: Labeling in the LGN ipsilateral to the injected eye. The labeling mirrors that of A. Fibers can be seen passing through layer A of the LGN on their way towards layer A1 (arrowhead). Medial is to the left, dorsal is up. C: High-power detail of the passing fibers shown in B (inset). Note the continuous, smooth appearance of the axons. D: Terminal retinal arborizations stained in the suprachiasmatic nucleus (SCN) of the hypothalamus, above the optic chiasm (OC). E: Anterograde labeling in the pretectal nuclei (PT) of a 21-day-old ferret pup viewed under dark-field illumination. H, habenula. F: High-power view of stained ectopic retinal axon arbors in the medial geniculate nucleus of an adult ferret. These projections are induced by means of surgical manipulations at birth (see text). Various terminal specializations (arrows), boutons en passant, and thick and fine collateral branches can be clearly discerned. G: A stained ectopic retinal axon arbor in another subdivision of the auditory thalamus. Scale bars = 500  $\mu\text{m}$  (A), 300  $\mu\text{m}$  (B), 200  $\mu\text{m}$  (D,E), 50  $\mu\text{m}$  (C,F,G).

staining were, in order, the concentration of Triton X-100 used, the time of incubation in primary antibody and Triton X-100, and the thickness of the tissue sections. Tissue series assayed with no Triton X-100 yielded very poor labeling of axons and high background staining, precluding further analysis. Using low to moderate concentrations of Triton X-100 (0.3–1%), staining was faint or absent at the center of the section thickness, making it difficult to follow individual fibers even allowing 4 days of incubation in primary antibody. Similar problems appeared when, using high Triton X-100 (2%), short incubation times (for example, 2 days at 4°C) in primary antibody

were allowed, as well as when, the other parameters being optimal, tissue sections were thicker than 40  $\mu\text{m}$ . In addition, experiments using low Triton (< 2%) characteristically failed to stain axons in myelinated tracts. A dramatic example of this effect is shown in Fig. 1D. Using a low concentration (0.3%) of Triton X-100, terminal retinal arborizations are stained in the suprachiasmatic nucleus (SCN), while fibers in the optic chiasm (OC) remain unstained. In this case not only was the optic chiasm unstained, the optic tract remained unstained as well. For comparison note the intense labeling in the optic tract in Fig. 1A, which was stained using 2% Triton X-100. As

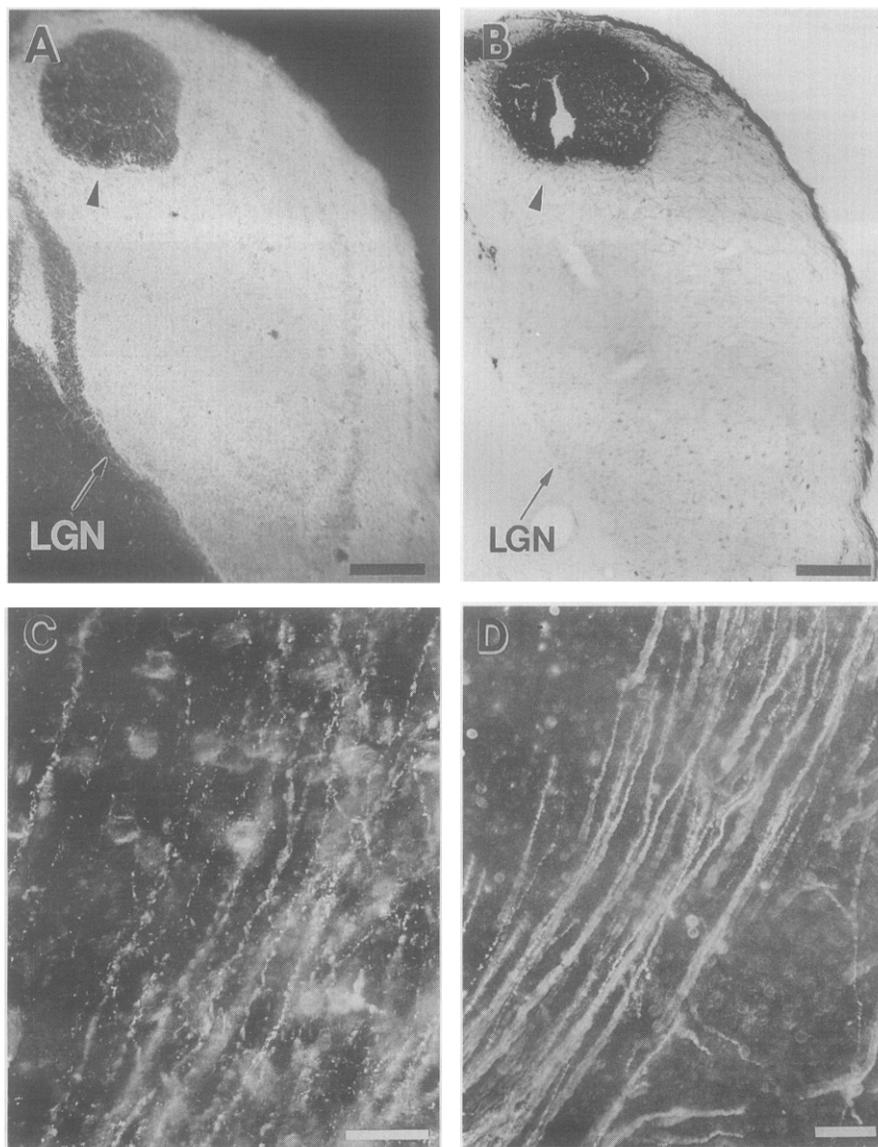


Fig. 2. Combined CTB immunohistochemistry and TMB histochemical staining of WGA-HRP in adjacent sections. A: Labeling in the LGN contralateral to the eye injected with WGA-HRP, viewed under dark-field illumination. Only lamina A of the LGN is stained. Arrowhead points to the unstained lamina A1. Dorsal is up and medial to the left. B: Bright-field micrograph of the section adjacent to A, showing the immunostaining in lamina A1 (arrowhead) of the LGN ipsilateral to the eye injected with CTB. Notice that, due to the methanol and  $\text{H}_2\text{O}_2$  treatment, the WGA-HRP staining in lamina A has completely been bleached away in this section. C: Detail of the WGA-HRP labeling in the contralateral LGN at high power. Note the discontinuity and grainy appearance of the TMB reaction product. D: High-power view of the CTB labeling in lamina A of the ipsilateral LGN. Scale bars = 300  $\mu\text{m}$  (A,B), 50  $\mu\text{m}$  (C,D).

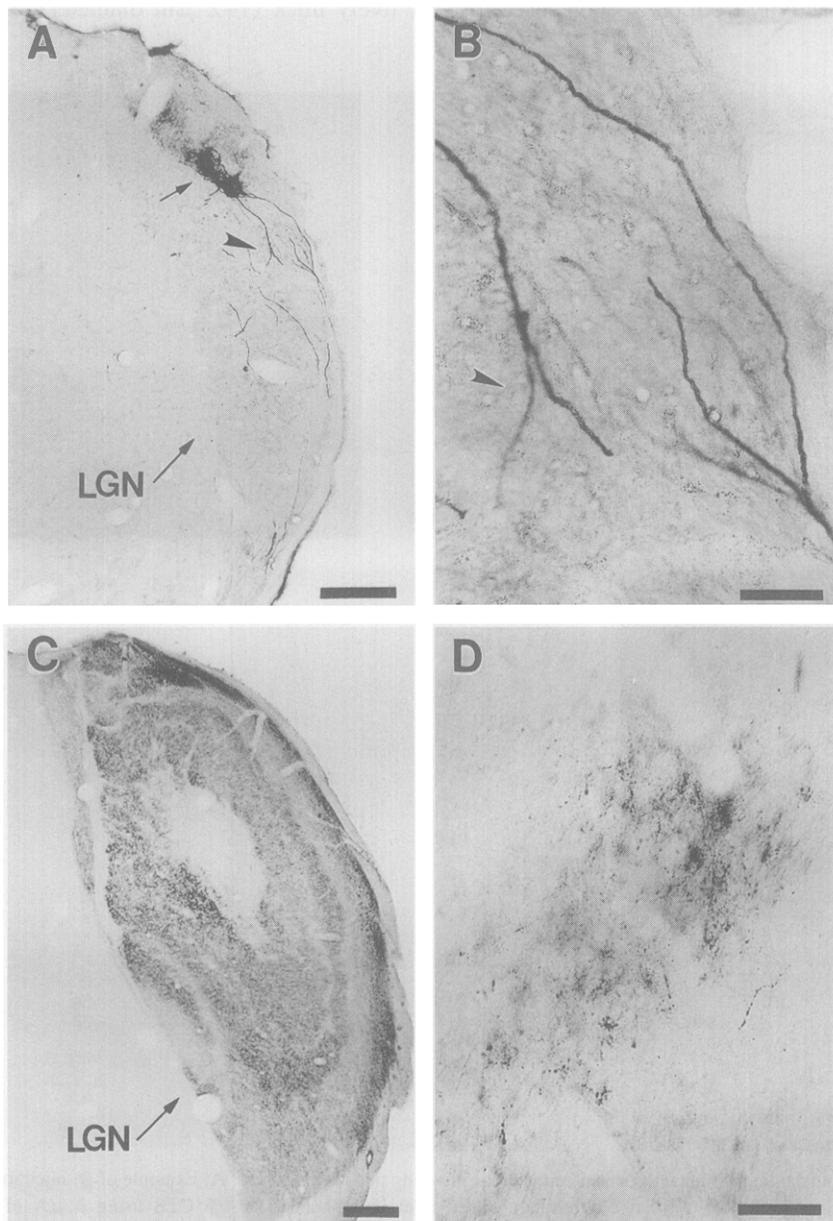
indicated in Section 2, the best results were obtained using 40  $\mu\text{m}$  sections, high (2%) Triton X-100 concentrations, and long incubation times (2 days at RT or 4 days at 4°C) in primary antibody. In addition the concentration of anti-CTB antibody (1:4000) was higher than that recommended in previous protocols.

High-contrast staining of the fibers was obtained either by using the  $\text{CoCl}_2$ -enhanced DAB method or the Vector VIP<sup>®</sup> reaction, as opposed to the paler staining obtained with DAB alone. The Vector VIP method yielded a darker staining of densely innervated areas than the  $\text{CoCl}_2$ -DAB.

Retrograde labeling was consistently observed in motoneurons of the oculomotor nuclei, reflecting CTB spillage into extrinsic ocular muscles at the injection site in the eyeball.

### 3.2. Combined labeling of retinofugal projections with CTB and WGA-HRP

The quality of CTB labeling in animals perfused using our fixation protocol for combined WGA-HRP/CTB staining was similar to that in animals perfused for CTB



**Fig. 3.** Staining of retinofugal pathways with biotinylated dextran and free WGA. **A:** Retinal projection to the LGN stained by an injection of biotinylated dextran 3000 MW into the ipsilateral eye. Note the heavy staining of a small subset of fibers crossing lamina A and arborizing in lamina A1 (arrow). Dorsal is up, medial is to the left. Arrowhead points to the retinal axons shown at higher power in B. **B:** High-power view of the same retinogeniculate axons shown in A (arrowhead) **C:** Terminal labeling in LGN obtained after injecting both eyes with free WGA. As a result, labeling is present in all laminae of the nucleus. Dorsal is up, medial is to the left. **D:** Detail of the WGA labeling in the pretectum at high magnification. Note the pale and blurred appearance of the reaction product. Scale bars = 300  $\mu\text{m}$  (A,C), 50  $\mu\text{m}$  (B,D).

immunostaining alone (compare Figs. 1B with 2B and 1C with 2D). Importantly, the TMB staining of WGA-HRP (Fig. 2A,C) was as intense as that observed routinely in ferrets (cf., Roe et al., 1993) using the standard fixation of Mesulam's (1978) method.

We found that while glutaraldehyde is not essential for good TMB histochemistry, limiting exposure to paraformaldehyde is crucial: failure to remove the excess fixative from the brain tissue led to faint or no WGA-HRP staining, even when low concentrations (1%) of paraformaldehyde had been used for the fixation.

Pretreatment with  $H_2O_2$  in methanol completely bleached away the HRP activity from the sections used for

CTB staining, thus allowing an unambiguous identification of the projections arising from each eye (Fig. 2B). In the sections used for revealing CTB, the contrast of the immunostaining was markedly improved by post-fixation in 4% paraformaldehyde after sectioning.

### 3.3. Anterograde labeling of retinofugal fibers using tracers other than CTB

Injections of biotinylated dextrans yielded a striking pattern of labeling: only a small group of retinal axons were heavily stained (Fig. 3A,B). These axons were relatively thick (1–2  $\mu m$  diameter), and present only in the

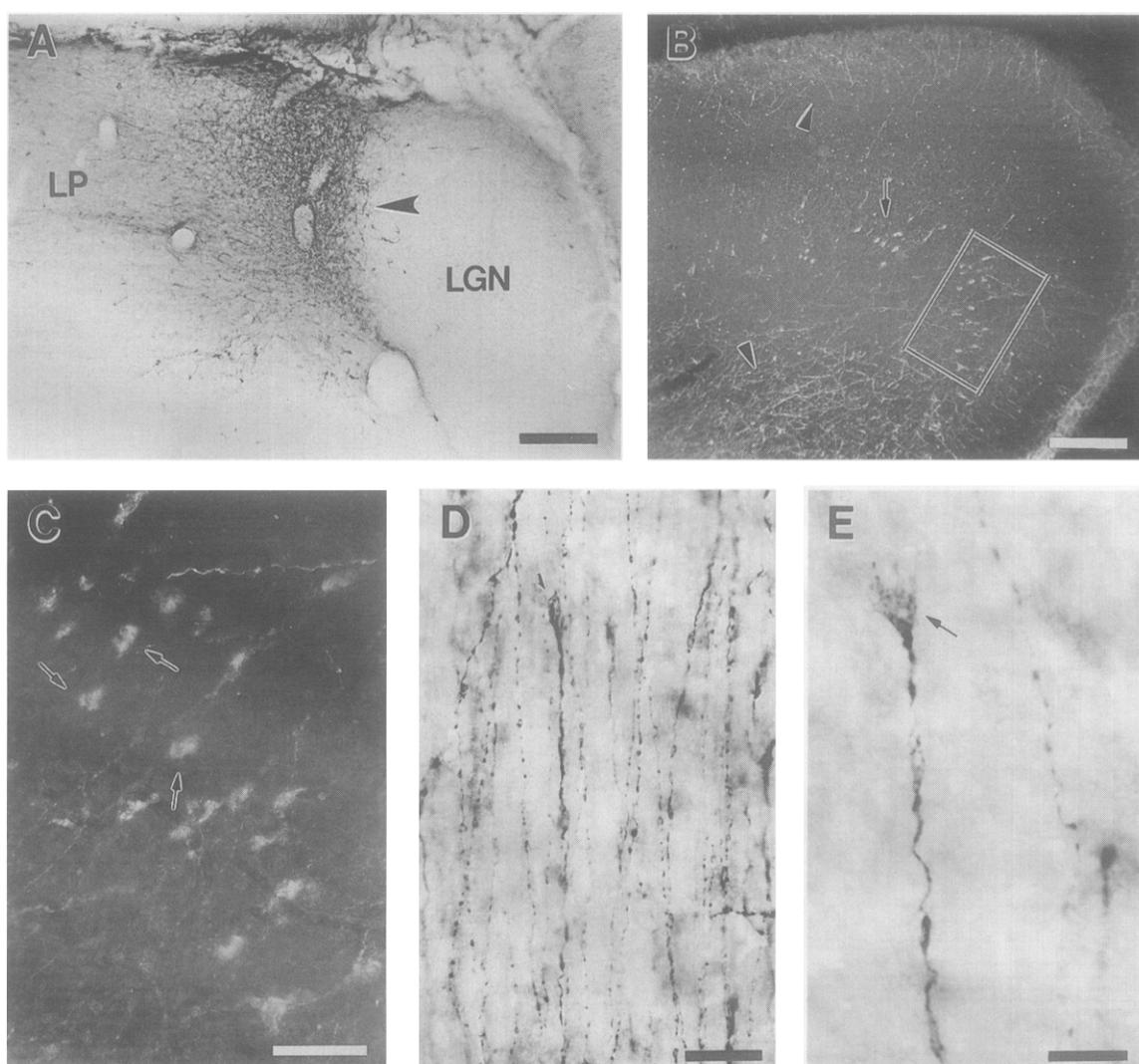


Fig. 4. CTB labeling of corticothalamic and thalamocortical connections in young postnatal animals. A: Example of an injection site in the pulvinar and the medial interlaminar nucleus (arrowhead) of a 9-day-old ferret, obtained after iontophoresis of 1% CTB using 3  $\mu A$  of current for 10 min, and a micropipette with tip diameter of 20  $\mu m$ . LP, lateral posterior thalamic nucleus. Dorsal is up, medial to the left. Bright-field illumination. B: Anterogradely labeled axon arbors (arrowheads) and retrogradely labeled cell bodies (arrow) in the occipital cortex, obtained after the thalamic injection shown in A. The labeling in the inset is shown at higher magnification in C. Dark-field illumination. Dorsal is up medial is to the right. C: Higher-power detail of the retrogradely labeled cortical cells shown in B (inset). Note that only the perikaryon is stained (arrows), while the axon and distal dendrites are not visible. Dark-field illumination. D: Bundle of developing corticofugal axons in the intermediate zone, labeled after an injection in the cortical plate of a 1-day-old animal. Note the growth cones at the tip of some axons (arrow). E: High-power view of a growth cone. Note the delicate detail of the stained terminal specializations (arrow). Scale bars = 200  $\mu m$  (A,B); 50  $\mu m$  (C); 20  $\mu m$  (D) and 10  $\mu m$  (E).

LGN. The remainder of the corresponding LGN laminae showed a very faint tint, in which no organized structures were distinguishable. There was no labeling in other retinal targets.

Immunostaining of WGA labeling after injections in the vitreal chamber revealed only the terminal areas of retinofugal axons (Fig. 3C,D) in all retinal targets. However, the fiber staining had a grainy and discontinuous appearance (Fig. 3D), similar to that of WGA-HRP revealed by TMB histochemistry (Fig. 2C). This made it impossible to follow single fibers at branch or cross points, or to distinguish terminal specializations with confidence.

Finally, dextran rhodamine yielded dimly stained fibers in retinal targets such as the LGN and superior colliculus (not illustrated). We did not detect labeling in other retinal targets. An additional shortcoming of this technique was that the rhodamine fluorescence became photobleached under the fluorescence epi-illumination at the high-power magnification required for single axon arbor analysis.

### 3.4. Intracerebral injections

Both in adult and in young postnatal animals, CTB injections in the thalamus or cerebral cortex consistently produced anterograde labeling of the corresponding neural pathways (Fig. 4). The quality of axon staining was comparable to that seen after eye injections. In the adult cases, however, the labeling often showed a blurred appearance in those areas which contained a very high density of labeled terminals. This was particularly apparent after large injections, which labeled massive numbers of fibers.

In postnatal animals, the quality of anterograde labeling was remarkable even after only 1 day of survival postinjection, although the best results were seen allowing 2–4 days of survival. For example, after 1 day of survival in newborn animals that received cortical injections, fibers were already labeled in structures situated as far away as the pyramidal tract. Moreover, CTB not only labeled established connections, but also developing, growth cone-tipped axons (Fig. 4D,E).

Because of retrograde axonal transport in the highly bi-directional pathways between the cerebral cortex and thalamus, CTB injections labeled, along with the anterogradely stained fibers, numerous neuronal bodies (Fig. 4B). However, this retrograde labeling, particularly in the cortex, was conspicuously different from the anterograde: only perikarya and main dendrites were stained, while axons or distal dendrites were always spared. Fig. 4B,C show as an example, neocortical cells. It is unlikely that this reflected a failure of the staining technique, because such retrogradely labeled perikarya usually lay amidst well labeled axon arbors (Fig. 4B).

Using the parameters specified in Section 2, CTB injection sites remained small (200–400  $\mu\text{m}$  diameter) even in the youngest postnatal animals (Fig. 4A). The core of the injection site, which presumably was the region from

which the effective axonal transport originated, appeared as a small area covered by an amorphous grayish DAB-cobalt precipitate. Around this core, there was a dense halo of heavily stained neuronal processes (Fig. 4A). The Vector VIP method yielded a much darker staining of the core of the injection than the  $\text{CoCl}_2$ -DAB (not shown). With the small tip diameters employed, leakage of the CTB along the micropipette track was negligible, even though retention currents were not applied during positioning of the micropipette.

As expected from previous reports, injections of biotinylated dextrans and PHA-L in the thalamus and cortex of adult ferrets were highly effective in labeling axon arbors and terminal specializations. However, in animals younger than 2 weeks, biotinylated dextrans and PHA-L performed worse than CTB. Biotinylated Dextrans labeled only a small numbers of fibers in a faint, discontinuous manner even after 4–5 days of survival. Anterograde labeling with PHA-L was capricious, being absent in many cases and faint in others. In general, well-stained fibers were only seen in the vicinity of the injection site even after relatively long (4 days) survival times. On the other hand, it is to be noted that in newborns, both biotinylated dextrans and PHA-L performed efficiently as *retrograde* tracers.

As in the case of the eye injections, WGA-HRP deposits in the brain of adult ferrets produced heavy (although coarse) anterograde labeling of fibers and terminals. In young postnatal animals, however, WGA-HRP yielded only faint and grainy anterograde labeling as reported by previous authors (Payne et al., 1988; Ramirez et al., 1990). Moreover, in newborns, injection sites were relatively large even after small iontophoretic deposits.

Dextran rhodamine produced detailed labeling of axon arbors after injections in the cortex of adult ferrets. However, we found this tracer completely unsuitable for use in neonatal animals. Apparently, the tracer diffused freely in the tissue, labeling glial and endothelial cells, but no neurons, extensively in the brain. Indeed, the fluorescence was ubiquitous and diluted to the point that even the injection site was barely recognizable.

## 4. Discussion

Using a modified immunohistochemical protocol, we have shown here that CTB stains with Golgi-like resolution axons along their entire length, including myelinated tracts and terminals. CTB performed better than the other tracers assayed (biotinylated dextrans, free WGA, WGA-HRP, dextran rhodamine and PHA-L) after injections into the vitreal chamber of the eye aimed at labeling the entire retinofugal projection, as well as after iontophoretic deposits in the brain tissue of neonatal ferrets aimed at labeling specific pathways between thalamus and cortex. When injected in the adult ferret brain, CTB was as

sensitive an anterograde tracer as PHA-L or biotinylated dextrans, although was inadequate for analyzing dense terminal axonal arborizations.

#### 4.1. CTB as an anterograde tracer

Since its introduction as an axonal tracer, the subunit B of *Vibrio cholerae* toxin has mostly been applied for retrograde labeling of neurons, in the peripheral (Flink and Westman, 1986; Horikawa and Powell, 1986; Hirakawa et al., 1992; Llewellyn-Smith et al., 1992) and in the central (Luppi et al., 1987; Berendse and Groenewegen, 1990; Berendse et al., 1992; Hay-Schmidt and Mikkelsen, 1992; Shinonaga et al., 1992; Gritti et al., 1994) nervous system. Although also reported to undergo anterograde axonal transport (Luppi et al., 1987, 1990; Ericson and Blomqvist, 1988; Bruce and Grofova, 1992), anterograde labeling with CTB was judged inferior to PHA-L and biotinylated dextran (Ericson and Blomqvist, 1988; Bruce and Grofova, 1992). Indeed, our own initial experiments using previously published immunohistochemical protocols (Luppi et al., 1990; Mikkelsen, 1992) did not yield a complete staining of axons. Rather, we observed mainly terminal labeling consisting of swellings interconnected by thin fibers, and occasional, discontinuously labeled axons, similar to those shown in Fig. 4 of Mikkelsen (1992).

The present results demonstrate that, revealed properly, anterograde transport of CTB stains axons along their entire length up to the terminal specializations, including their path along myelinated tracts. The modifications that we have introduced may have improved the quality of staining chiefly by increasing the penetration of the primary antibody in the tissue, since we: (1) used higher concentrations of, and longer exposure to, the tensoactive agent Triton X-100; (2) allowed longer incubation in primary antibody; (3) used thin tissue sections, and (4) used higher concentrations of primary antibody than previous studies (Ericson and Blomqvist, 1988; Berendse et al., 1990; Luppi et al., 1990; Bruce and Grofova, 1992; Mikkelsen, 1992). Although concentrations of Triton X-100 of 0.3–0.5% are commonly used for most immunohistochemical reactions, it has been previously shown that some immunohistochemical stains of visual structures require concentrations of Triton X-100 as high as those employed in the present study (Sur et al., 1988a).

The increased penetration did not lead to a higher background staining. We attribute this to (1) pretreating the tissue with  $H_2O_2$  to inactivate endogenous peroxidases, (2) post-fixation of the tissue, and (3) blocking of unspecific binding with NRS and BSA.  $CoCl_2$  or Vector VIP<sup>®</sup> intensification added to the enhanced contrast of the staining.

Previous studies have shown that in the peripheral nervous system, CTB is selectively uptaken by a specific class of nerve fibers (Robertson and Arvidsson, 1985; Robertson and Grant, 1985). In the present study, follow-

ing eye injections, we observed labeling in all known retinal targets of both thin (0.3–0.5  $\mu m$ ) and thick (2–3  $\mu m$ ) axons, suggesting that such a selectivity of staining does not occur in the CNS, at least not in the retinofugal pathways. Similarly, in newborn animals, CTB stained developing retinofugal connections in all known targets up to their growth cones.

Luppi et al. (1990), analyzing the connections of the amygdala, noted that when injected in the brain, retrogradely transported CTB is detected in cell bodies and proximal dendrites but not in axons or fiber bundles. The present results, using a more sensitive staining method, confirm that CTB is undetectable in retrogradely transporting axons. This remarkable difference between anterograde and retrograde staining may prove to be advantageous for analyzing highly reciprocal neural connections, such as those between the cerebral cortex and thalamus. As a shortcoming, it is to be noted that when examined at high-power magnification, CTB fiber labeling often showed a blurred appearance in the most densely labeled areas. For brain injections, this problem can be sidestepped by making smaller CTB injections, that label a more restricted number of fibers. In the retinofugal pathway, CTB is best suited for labeling the retinal projections as a whole, or for detailed analysis of small fiber contingents such as the accessory optic system, the retino-pretectal and retinohypothalamic projections, or the ectopic thalamic projections induced by experimental manipulations (Sur et al., 1988b; Roe et al., 1993).

#### 4.2. Combination of CTB and WGA-HRP staining

We have shown here that CTB immunohistochemistry can be combined in alternate sections with Mesulam's (1978) TMB histochemical staining of HRP. Such combination does not reduce the sensitivity achieved when using either technique alone. This double anterograde staining may be very useful for the analysis of converging axonal projections, such as the retinofugal projections arising from each eye.

The key steps for achieving a successful combination were (1) the perfusion protocol, and (2) the pretreatment of the sections to be used for CTB staining. Regarding the perfusion, first we removed glutaraldehyde from the perfusate because it can interfere with CTB immunostaining (Ericson and Blomqvist, 1988). Second, the long fixation and post-fixation in 4% paraformaldehyde, which is usually employed for immunohistochemistry, but which sharply decreases the HRP activity, was replaced with a limited exposure to paraformaldehyde: 2% solution for 30 min, then removing the excess by perfusing with a sucrose solution. The light fixation did not affect CTB immunostaining, provided that the sections were post-fixed after cutting (see Section 2).

The pretreatment of sections intended for CTB staining was aimed at inactivating any HRP activity from the

tissue. This was necessary because we were using a HRP-based immunostain for revealing CTB. The sections were pretreated by a prolonged exposure to a solution of  $H_2O_2$  in methanol. Bleaching of peroxidase activity was so complete that even those regions most heavily stained by WGA-HRP were free of labeling in the adjacent sections processed for CTB.

#### 4.3. Labeling of retinofugal projections by intravitreal injections

The pattern of labeling of the retinofugal projections after intraocular injections of the various tracers assayed in this study suggests that a critical limiting factor for anterograde axonal tracing with substances delivered into the vitreal chamber may have been the ability of the retinal ganglion cells to uptake the tracers.

Among the tracers assayed in this study, only CTB, free WGA and WGA-HRP were uptaken and transported by a majority of retinal ganglion cells. As discussed above, CTB proved to be the most sensitive, since it labeled all known retinal projections up to the thinnest terminal branches in complete detail, indicating that it had been uptaken in significant amounts by virtually all retinal ganglion cells. WGA and WGA-HRP showed fewer projections to some retinal targets such as those to the suprachiasmatic nucleus of the hypothalamus (Johnson et al., 1988; Mikkelsen and Servi re, 1992) or some of the ectopic projections induced by surgical manipulation at birth (Sur et al., 1988b; Roe et al., 1993). Moreover, they consistently produced a grainy, discontinuous labeling, unsuited for detailed analysis of axonal arbors and terminals. It is interesting to note that fibers immunostained for WGA showed the same discontinuous appearance as fibers histochemically stained for WGA-HRP. This suggests that the incomplete fiber labeling is not an artifact of the TMB procedure, but rather reflects a real lack of continuity in the impregnation of the fibers by WGA.

Dextran rhodamine labeling of the retinofugal projection was limited to targets which are massively innervated by the retina, such as the LGN and the superior colliculus. In addition, the lability of the dye under epi-illumination at high magnification rendered it unsuitable for detailed axon analysis.

When injected in the eye, biotinylated dextrans labeled in detail only a small subset of axons. Results were similar using the 10000 and 3000 MW compounds. Such axons were usually thick (2–3  $\mu m$ ), and present only in the LGN, suggesting that they were part of a specific subclass of retinal ganglion cells. The remainder retinal projections were not stained, or only faintly impregnated.

#### 4.4. Anterograde axonal tracing in young postnatal animals

It is known that most anterogradely transported tracers are less sensitive in newborn mammals than in adults

(Payne et al., 1988; Ramirez et al., 1990; Claps and Casagrande, 1991). This limitation becomes particularly an issue in species such as ferrets, hamsters, or marsupials, which are born very immature. Because of this limitation, techniques based on physical diffusion of dyes (Godement et al., 1987), instead of on axonal transport, have become the standard method for fiber labeling in developing animals. Physical diffusion techniques, however, require very long times for labeling long pathways, become markedly less sensitive at progressively older postnatal ages (Ghosh and Shatz, 1992), and importantly, may lead to false-positive staining because of intercellular diffusion (Godement et al., 1987; Clasc a et al., 1995).

In very young brains, most water-soluble tracers, even when iontophoresed in minute amounts, tend to spread widely, creating big injection sites which are inadequate for selective analysis of pathways. This may relate to the fact that interstitial space in early brain tissue is wide (Rakic, 1972), as well as to the absence or immaturity (Bayer and Altman, 1991) of high-affinity binding glial cells (Stendler and Cooper, 1986). Among the tracers assayed in this study, dextran rhodamine epitomized this problem: following small iontophoretic deposits in the thalamus, the dye diffused so widely in the tissue that the injection site became unrecognizable, while labeling in endothelial and glial cells was present over most of both hemispheres of the brain. Similarly, although to a lesser degree, WGA-HRP showed a tendency to create big injection sites even after minute deposits, as noted in previous studies (Payne et al., 1988; Ramirez et al., 1990).

A conceivable explanation for the poor performance of anterograde axonal tracers in early postnatal ages could be that the molecular systems for binding and/or uptake of the tracers (Rhoades et al., 1986; Stendler and Cooper, 1986) are not fully functional yet. However, the fact that all the injected tracers (except dextran rhodamine, see above) yielded good retrograde labeling even at the earliest ages examined argues against this possibility. On the other hand, given the heterogeneous nature of the various tracers, the fact that one of them (CTB) yielded good anterograde labeling from the earliest ages examined, while the rest performed poorly until older ages (cf., Claps and Casagrande, 1991), suggests that, more likely, the cellular systems used for the anterograde transport of some particular molecular species (Trojanowski and Schmidt, 1984) are less efficient in neonatal animals. Whatever the case, it is clear from our study that, among the known neuronal tracing methods based on anterograde axonal transport, CTB is decidedly the tracer of choice for use in early postnatal brains.

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## References

- Bayer, S.A. and Altman, J. (1991) *Neocortical Development*, Raven Press, New York.
- Berendse, H.W. and Groenewegen, H.J. (1990) Organization of the thalamostriatal projections in the rat, with special emphasis on the ventral striatum, *J. Comp. Neurol.*, 299: 187–228.
- Berendse, H.W., Groenewegen, H.J. and Lohman, H.M. (1992) Compartmental distribution of ventral striatal neurons projecting to the mesencephalon in the rat, *J. Neurosci.*, 12: 2079–2103.
- Brandt, H.M. and Apkarian, A.V. (1992) Biotin-dextran: a sensitive anterograde tracer for neuroanatomic studies in rat and monkey, *J. Neurosci. Methods*, 45: 35–40.
- Bruce, K. and Grofova, I. (1992) Notes on a light and electron microscopic double-labeling method combining anterograde tracing with *Phaseolus vulgaris* leucoagglutinin and retrograde tracing with cholera toxin subunit B, *J. Neurosci. Methods*, 45: 23–33.
- Claps, A. and Casagrande, V.A. (1991) The distribution and morphology of corticogeniculate axons in ferrets, *Brain Res.*, 530: 126–129.
- Clascá, F., Angelucci, A. and Sur, M. (1995) Layer-specific programs of development in neocortical projection neurons, *Proc. Natl. Acad. Sci. USA* 92, 11145–11149.
- Ericson, H. and Blomqvist, A. (1988) Tracing of neuronal connections with cholera toxin subunit B: light and electron microscopic immunohistochemistry using monoclonal antibodies, *J. Neurosci. Methods*, 24: 225–235.
- Flink, R. and Westman, J. (1986) Different neuron populations in the feline lateral cervical nucleus: a light and electron microscopic study with the retrograde axonal transport technique, *J. Comp. Neurol.*, 250: 265–281.
- Gerfen, C.R. and Sawchenko, P.E. (1984) An anterograde neuroanatomical tracing method that shows the detailed morphology of neurons, their axons and terminals: immunohistochemical localization of an axonally transported plant lectin, *Phaseolus vulgaris* leucoagglutinin (PHA-L), *Brain Res.*, 290: 219–238.
- Ghosh, A. and Shatz, C.J. (1992) Pathfinding and target selection by developing geniculocortical axons, *J. Neurosci.*, 12: 39–55.
- Godement, P., Vanselow, J., Thanos, S. and Bonhoeffer, F. (1987) A study in developing visual systems with a new system of staining neurons and their processes in fixed tissue, *Development*, 101: 697–713.
- Gritti, I., Mainville, L. and Jones, B.E. (1994) Projections of GABAergic and cholinergic basal forebrain and GABAergic preoptic-anterior hypothalamic neurons to the posterior lateral hypothalamus of the rat, *J. Comp. Neurol.*, 339: 251–268.
- Hay-Schmidt, A. and Mikkelsen, J.D. (1992) Demonstration of a neuronal projection from the entopeduncular nucleus to the substantia nigra of the rat, *Brain Res.*, 576: 343–347.
- Hirakawa, M., McCabe, J.T. and Kawata, M. (1992) Time-related changes in the labeling pattern of motor and sensory neurons innervating the gastrocnemius muscle, as revealed by the retrograde transport of the cholera toxin B subunit, *Cell Tiss. Res.*, 267: 419–427.
- Horikawa, K. and Powell, E.W. (1986) Comparison of techniques for retrograde labeling of the rat's facial nucleus, *J. Neurosci. Methods*, 17: 287–296.
- Johnson, R.F., Morin, L.P. and Moore, R.Y. (1988) Retinohypothalamic projections in the hamster and rat demonstrated using cholera toxin, *Brain Res.*, 462: 301–312.
- King, M.A., Louis, P.M., Hunter, B.E. and Walker, D.W. (1989) Biotin: a versatile anterograde neuroanatomical tract-tracing alternative, *Brain Res.*, 497: 361–367.
- Llewellyn-Smith, I.J., Phend, K.D., Minson, J.B., Pilowski, P.M. and Chalmers, J.P. (1992) Glutamate-immunoreactive synapses on retrogradely-labeled sympathetic preganglionic neurons in rat thoracic spinal cord, *Brain Res.*, 581: 67–80.
- Luppi, P.H., Sakai, K., Salvetti, D., Fort, P. and Jouvet, M. (1987) Peptidergic hypothalamic afferents to the cat nucleus raphe pallidus as revealed by a double immunostaining technique using unconjugated cholera toxin as a retrograde tracer, *Brain Res.*, 402: 339–345.
- Luppi, P.H., Fort, P. and Jouvet, M. (1990) Iontophoretic application of unconjugated cholera toxin B subunit (CTb) combined with immunohistochemistry of neurochemical substances: a method for transmitter identification of retrogradely labeled neurons, *Brain Res.*, 534: 209–224.
- Mesulam, M.M. (1978) Tetramethyl benzidine for horseradish peroxidase neurohistochemistry: a non-carcinogenic blue reaction product with superior sensitivity for visualizing neural afferents and efferents, *J. Histochem. Cytochem.*, 26: 106–117.
- Mikkelsen, J.D. (1992) Visualization of efferent retinal projections by immunohistochemical identification of cholera toxin subunit B, *Brain Res. Bull.*, 28: 619–623.
- Mikkelsen, J.D. and Servi re, J. (1992) Demonstration of a direct projection from the retina to the hypothalamic supraoptic nucleus of the hamster, *Neurosci. Lett.*, 139: 149–152.
- Payne, B.R., Pearson, H.E. and Comwell, P. (1988) Neocortical connections in fetal cats, *Neurosci. Res.*, 5: 513–543.
- Rakic, P. (1972) Mode of cell migration to the superficial layers of fetal monkey neocortex, *J. Comp. Neurol.*, 145: 61–84.
- Ramirez, J.J., Jhaveri, S., Jahn, J.H. and Schneider, G.E. (1990) Maturation of projections from occipital cortex to the ventrolateral geniculate and superior colliculus of postnatal hamsters, *Dev. Brain Res.*, 55: 1–9.
- Rhoades, C.H., Stieber, A. and Gonatas, N.K. (1986) A quantitative electron microscopic study of the intracellular localization of wheat germ agglutinin in retinal neurons, *J. Comp. Neurol.*, 254: 287–296.
- Robertson, B. and Arvidsson, J. (1985) Transganglionic transport of wheat germ agglutinin-HRP and cholera toxin-HRP in rat trigeminal sensory neurons, *Brain Res.*, 348: 44–51.
- Robertson, B. and Grant, G. (1985) A comparison between wheat germ agglutinin and cholera toxin-horseradish peroxidase as anterogradely transported markers in the central branches of primary sensory neurons in the rat with some observations in the cat, *Neuroscience*, 14: 895–905.
- Roe, A.W., Garraghty, P.E., Esguerra, M. and Sur, M. (1993) Experimentally induced visual projections to the auditory thalamus in ferrets: evidence for a W cell pathway, *J. Comp. Neurol.*, 334: 263–280.
- Shinonaga, Y., Takada, M. and Mizuno, N. (1992) Direct projections from the central amygdaloid nucleus to the globus pallidus and substantia nigra in the cat, *Neuroscience*, 51: 691–703.
- Shmued, L., Kyriakidis, K. and Heimer, L. (1990) In vivo anterograde and retrograde axonal transport of the fluorescent rhodamine-dextranamine, fluoro-ruby, within the CNS, *Brain Res.*, 526: 127–134.
- Stendler, D.A. and Cooper, G.F. (1986) Wheat germ agglutinin binding sites in the adult mouse cerebellum: light and electron microscopy studies, *J. Comp. Neurol.*, 249: 170–185.
- Sur, M., Frost, D.O. and Hockfield, S. (1988a) Expression of a surface-associated antigen on Y-cells in the cat lateral geniculate nucleus is regulated by visual experience, *J. Neurosci.*, 8: 874–882.
- Sur, M., Garraghty, P.E. and Roe, A.W. (1988b) Experimentally induced visual projections into the auditory thalamus and cortex, *Science*, 242: 1437–1441.
- Trojanowski, J.Q. and Schmidt, M.L. (1984) Studies with HRP and HRP conjugates of wheat germ agglutinin, cholera toxin and the subunit B of cholera toxin, *Brain Res.*, 311: 366–369.