Exp Brain Res 40, 383–392 (1980)

Experimental Brain Research

Double Retrograde Neuronal Labeling Through Divergent Axon Collaterals, Using Two Fluorescent Tracers with the Same Excitation Wavelength Which Label Different Features of the Cell

H.G.J.M. Kuypers, M. Bentivoglio[1], C.E. Catsman-Berrevoets, and A.T. Bharos

Erasmus University Rotterdam, Medical School, Dept. of Anatomy II, Rotterdam, The Netherlands

Summary. Recent studies show that several fluorescent substances are transported retrogradely through axons to their parent cell bodies and label in different colors different features of the cell at the same 360 nm excitation wavelength. Thus, Bisbenzimide (Bb) and "Nuclear Yellow" (NY; Hoechst S 769121) produce green and golden-yellow retrograde labeling of the neuronal nucleus. "True Blue" (TB) and "Fast Blue" (FB) produce blue retrograde labeling of the neuronal cytoplasm. In the present study the possibility of retrograde double labeling of neurons by way of divergent axon collaterals using combinations of Bb or NY with TB or FB has been explored in rat and cat. The findings show that in these animals these tracer combinations are transported retrogradely through two axon collaterals to one and the same cell. Neurons which are retrogradely double-labeled with these tracer combinations display a blue fluorescent cytoplasm and a white or golden-yellow fluorescent nucleus at the same 360 nm excitation wavelength. Therefore, these tracer combinations can be successfully used to demonstrate the existence of divergent axon collaterals in the brain.

Key words: Fluorescent retrograde tracers – Retrograde double labeling – Mammillothalamic connections – Nigral efferents – Cerebellar efferents

Several fluorescent substances, i.e., Evans Blue (EB), DAPI, Primuline (Pr), Propidium Iodide (PI), Bisbenzimide (Bb), "Nuclear Yellow" (NY), "True Blue" (TB), and "Fast Blue" (FB) are transported retrogradely through axons to their parent cell bodies and produce retrograde fluorescent labeling of different features of the cell (Kuypers et al. 1977, 1979; Vanderkooy et al. 1978; Bentivoglio et al. 1979b, 1980a, b). Neurons labeled with some of these retrograde tracers fluoresce in different colors at different excitation wavelengths. Thus, EB labeled neurons fluoresce red at 550 nm excitation wavelength, whereas DAPI-Pr labeled ones fluoresce blue at 360 nm excitation wavelength. These two tracers may be combined in double labeling experiments, such that they are transported retrogradely through two divergent axon collaterals to one and the same cell (Vanderkooy et al. 1978; Bentivoglio et al. 1979b). The double labeling of the neurons can then be demonstrated by illuminating the cell with the two wavelengths, under which circumstances the cell fluoresces red and blue, respectively. It has been demonstrated by means of this technique that in rat individual neurons in the lateral mammillary nucleus distribute divergent axon collaterals to ipsilateral and contralateral anterior thalamus (Fig. 1) (Vanderkooy et al. 1978) and that individual neurons in the juxtapeduncular portion of substantia nigra pars reticulata (SNR) distribute divergent axon collaterals to tectum and ventromedial thalamus (Fig. 2) (Bentivoglio et al. 1979b).

The combination of EB with DAPI-Pr, however, has two disadvantages. First, in scrutinizing the material for double-labeled neurons the areas under study continuously have to be illuminated with two wavelengths. Second, these tracers are not transported very effectively over long distances, especially not in cat. The combination of Bb or NY with TB or FB promised to overcome these disadvantages, because all four tracers fluoresce in different colors at the same 360 nm excitation wavelength. Moreover, Bb and NY label preferentially the nucleus, while TB and FB label preferentially the cytoplasm. Double-labeled neurons were therefore expected to display at

1 Present address: Istituto di Neurologia, Università Cattolica, Roma, Italy

Offprint requests to: Dr. H.G.J.M. Kuypers (address see above)

0014–4819/80/0040/0383/$ 2.00

360 nm excitation wavelength a green (Bb) or golden-yellow (NY) fluorescent nucleus and a blue (TB, FB) fluorescent cytoplasm. Finally, each of the four tracers, except TB, is rather effectively transported over long distances also in cat.

Double labeling by means of the combination of TB or FB with Bb or NY was tested in rat and cat using the mammillo-thalamic system (Fig. 1) and the nigro-tectal and -thalamic system (Fig. 2) as models. The tracer combinations were also tested on the connections from the cerebellum to meso-diencephalon and spinal cord in rat and cat (Fig. 3), because when two tracers are injected in rat thalamus and tectum and in spinal cord, respectively, many neurons in the contralateral deep cerebellar nuclei are single-labeled from the thalamus, some are single-labeled from the spinal cord, and some in fastigial and interpositus nuclei are double-labeled (Bentivoglio and Kuypers, unpubl. observ.).

The findings in these experiments, to be reported below, confirmed the above expectations and showed that especially the combinations of "Nuclear Yellow" (Hoechst S769121, NY) with "True Blue" (TB) and "Fast Blue" (FB) can be successfully used in double labeling experiments. However, in cat FB is used instead of TB, because FB is more effectively transported over long distances in this animal (Bentivoglio et al. 1980b).

Material and Methods

The tracers were dissolved or suspended in water and were injected in various brain areas of Nembutal anesthetized rats and cats by means of a Hamilton microsyringe with a 22 G needle or by means of a glass micropipette. TB and FB were injected in combination with Bb and with NY. However, Bb and NY are more rapidly transported retrogradely than TB and FB. Moreover, Bb and NY soon migrate out of the retrogradely labeled neurons while TB and FB migrate after much longer survival times (Bentivoglio et al. 1980a). Therefore, the two tracers were always injected in two different sessions, i.e., TB or FB first and Bb or NY later. The total survival time (see tables) will be referred to as TB or FB survival time, while the interval between the Bb and NY injections and perfusion of the animals will be referred to as Bb and NY survival time.

(A) Double labeling with TB and Bb was tested (1) in the mammillo-thalamic system (Fig. 1) of nine rats and two cats in which TB was injected in one anterior thalamus and Bb in the other; (2) in the nigro-tectal and -thalamic system (Fig. 2) of eight rats in which TB was injected in tectum and Bb in ipsilateral ventral thalamus (Table 1). *(B) Double labeling with TB and NY* was tested (1) in the mammillo-thalamic system of two rats; (2) in the nigro-tectal and -thalamic system of two rats in which TB was injected in tectum and NY in ipsilateral ventral thalamus, and (3) in the cerebello-thalamic and -spinal system (Fig. 3) of two rats in which TB was injected in thalamus and tectum and NY ipsilaterally in C2 spinal segments (Table 2). *(C) Double labeling with FB and Bb or NY* was tested (1) in the mammillo-thalamic system of two rats; (2) in the nigro-tectal and -thalamic system of two rats in which FB was injected in tectum and Bb or NY in ipsilateral ventral thalamus, and (3) in the cerebello-thalamic and -spinal system of two cats, in which FB was injected in thalamus and tectum and NY ipsilaterally in C2 spinal segment (Table 3).

The survival times as well as the concentrations and the quantities of the injected tracers are shown in Tables 1–3 (see Results).

All animals were killed under deep Nembutal anesthesia by transcardial perfusion with 0.9% saline followed by 10% formalin. In the cats of group C3 cacodylate buffered formalin was used (pH 7.2). The brains were generally soaked in 30% cacodylate buffered sucrose (pH 7.2) for 12–48 h, with the injection sites being kept separate from the other tissue slices (Bentivoglio et al. 1980b). However, in the more recent experiments in cats (group C3) soaking tissue slices in 30% sucrose was discontinued and the formalin perfusion was followed by a perfusion with cacodylate buffered (pH 7.2) 8% to 10% sucrose, after which the brains were immediately transferred to the microtome. All brains were cut transversely in frozen sections (20–35 μm thick). The sections were collected in distilled water and in most of the cases were then immediately mounted and air dried at room temperature, but not coverslipped. Drying of the sections at 60° C was discontinued, because it produced some tissue autofluorescence. In some cases the sections, before mounting, were kept in distilled water at 4° C up to 8 h. However, in the more recent experiments in cats (group C3) to avoid in vitro migration of Bb and NY (Bentivoglio et al. 1980a), each section to be studied, after being cut, was immediately mounted from distilled water and air dried.

The material was viewed with a Leitz Ploemopack fluorescence microscope equipped with filter systems A, D, and N2 providing excitation light of 360 nm, 390 nm, and 550 nm wavelength, respectively. The immersion oil was applied directly to the dry sections. Some sections were kept without oil for microphotography since the oil eventually seems to affect the brightness of the fluorescence. The sections were studied as soon as possible after mounting since after 2–3 weeks they begin to show a glistery tissue autofluorescence which ultimately may obscure the fluorescence of the retrogradely labeled neurons. However, the appearance of the glistering autofluorescence will be delayed by storing the material at 4° C.

Results

Prior to reporting the double labeling obtained with the different tracer combinations, the individual characteristics of each of the four tracers will be summarized.

Bisbenzimide (Bb) and "Nuclear Yellow" (NY) give a brilliantly fluorescent retrograde labeling of neurons (Kuypers et al. 1979; Bentivoglio et al. 1980b) and both are transported very effectively over long distances in rat and cat (Kuypers et al. 1979; Bentivoglio et al. 1980b). At 360 nm excitation wavelength Bb and NY labeled neurons show a green (Bb) or golden-yellow (NY) fluorescent nucleus including a brilliantly fluorescent ring around the nucleolus. After prolonging the survival time Bb labeled neurons also display a granular yellow fluorescence of the cytoplasm. However, NY labeled ones show only faint fluorescence of the cytoplasm, especially after perfusion with buffered (pH 7.2)

formalin and sucrose solutions (Bentivoglio et al. 1980b). Bb and NY also produce fluorescence of glial nuclei around retrogradely labeled neurons and along axons through which they are transported (Kuypers et al. 1979; Bentivoglio et al. 1980b). However, glial nuclei lack the fluorescent ring around the nucleolus which is characteristically present in neuronal nuclei. The fluorescence of the glial nuclei results from migration of the tracers, e.g., out of the retrogradely labeled neurons into surrounding cells which, because of the Bb and NY affinity for nucleotides, produces labeling of the nuclei. This migration should be minimized because it may produce fluorescence of neuronal nuclei which are not retrogradely labeled.

Migration of Bb and NY occurs gradually: in vivo during the survival time and in vitro during the storage of the sections in water which has been noticed mainly in cat material (Bentivoglio et al. 1980a). The in vivo migration can be prevented by using 1% Bb or 1% NY and restricting the survival time. Labeled neurons then show only a fluorescent nucleus and either lack surrounding fluorescent glial nuclei, or are surrounded by a few dull fluorescent glial nuclei, which shows that only minimal migration of the tracer has occurred. As a consequence under these circumstances neuronal nuclei, which are much more brilliant than the surrounding dull fluorescent glial nuclei, can with confidence be regarded to be genuinely retrogradely labeled. In this respect NY is a more attractive tracer than Bb, because the NY retrograde labeling of neuronal nuclei and the subsequent fluorescence of surrounding glial nuclei takes approximately twice as long as with Bb, which facilitates the titration of the survival time (Bentivoglio et al. 1980a). Moreover, the fluorescent neuronal labeling obtained with 1% NY after short survival times is more brilliant than that obtained with 1% Bb (Bentivoglio et al. 1980a). In vitro migration, which has mainly been observed in cat material (Bentivoglio et al. 1980a) may be prevented by rapid processing of the material (see Material and Methods).

"True Blue" (TB) is almost insoluble in water and is injected as a milky 2% suspension. It gives a deep blue fluorescent retrograde labeling of the cytoplasm of the neuronal cell body and of a major portion of the dendrites as well as of the nucleolus at 360 nm excitation wavelength, but gives little nuclear fluorescence (Bentivoglio et al. 1979a). TB also gives a blue fluorescence of axons through which it is transported retrogradely. Prolonging the TB survival time increases the fluorescence of the cell, but in rats also produces many silver fluorescent granules in the cytoplasm (see later). After very long survival times

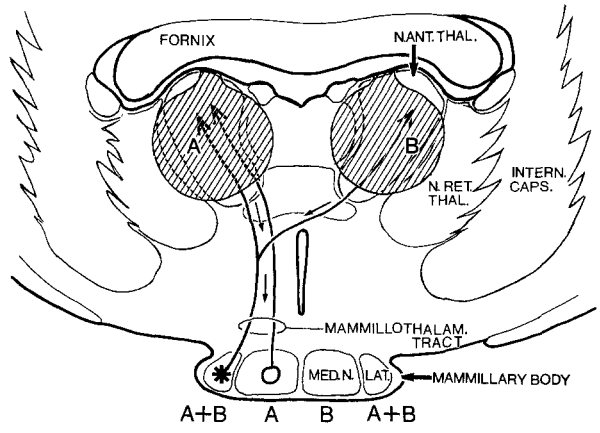

Fig. 1. Diagram of the distribution of mammillo-thalamic fibers and collaterals and the retrograde transport of two tracers (A and B) from anterior thalamus through these fibers to neurons in medial and lateral mammillary nuclei

TB labeled neurons become surrounded by a halo of blue fluorescent neuropil with some fluorescent glial nuclei. TB is effectively transported retrogradely over long distances in rat (Bentivoglio et al. 1979a), but in cat even transport over short distances requires very long survival times (Bentivoglio et al. 1980b).

"Fast Blue" (FB) (Bentivoglio et al. 1980b) is better soluble in water than TB. It produces a relatively lighter blue fluorescence of the cytoplasm of cell body and dendrites, with fine silver fluorescent granules at 360 nm excitation wavelength, but gives little fluorescence of the nucleus. However, occasionally single FB labeled neurons may show a light blue fluorescent nucleus. FB is transported more rapidly and effectively over long distances than TB in both rat and cat and gives a blue labeling with silver fluorescent granules of axons through which it is transported both retrogradely and anterogradely. Prolonging the survival time increases the FB fluorescence of the cell and also produces large orange fluorescent granules in the cytoplasm. After long survival times the neurons become surrounded by a halo of blue fluorescent neuropil with some blue fluorescent glial nuclei (Bentivoglio et al. 1980b).

In the following experiments the double labeling with combinations of the above tracers has been tested.

A. Double Retrograde Labeling with TB and Bb Mammilo-thalamic System

Injections of 10% or 1% Bb in anterior thalamus on one side and 5% TB in the other side (Fig. 1) in the nine rats and two cats of group A1 (Table 1) followed

Table 1. Group A: Double labeling with TB and Bb

A	Animals	% Tracer	µl	Injection site	% Tracer	µl	Injection site	Survival time	Survival time
1.	6 rats	5% TB	0.5	Left Ant. Thalam.	10% Bb	0.1	Right Ant. Thalam.	TB: 3–22 days	Bb: 2–3 days
	3 rats	5% TB	0.5	Left Ant. Thalam.	1% Bb	0.1	Right Ant. Thalam.	TB: 2–3 days	Bb: 1½, 3, 6 h
	2 cats	5% TB	12	Left Ant. Thalam.	10% Bb	0.6	Right Ant. Thalam.	TB: 13, 22 days	Bb: 3 days
2.	5 rats	5% TB	0.5	Tectum	10% Bb	0.2	Ipsil. Vent. Thalam.	TB: 7–21 days	Bb: 3 days
	3 rats	5% TB	0.5	Tectum	1% Bb	0.4	Ipsil. Vent. Thalam.	TB: 4 days	Bb: 6, 12, 15 h

by different survival times gave essentially the same results as obtained with EB and DAPI-Pr (Vanderkooy et al. 1978). Thus, in the mammillary bodies when viewed with filter mirror system A (360 nm excitation wavelength) the medial mammillary nucleus on either side showed only single TB labeled and Bb labeled neurons, respectively, the two populations of which were sharply delineated at the midline. However, in the lateral mammillary nucleus on either side many double labeled neurons were present. They could be easily recognized because they displayed a blue TB fluorescent cytoplasm with a white to yellow fluorescent Bb labeled nucleus.

Some differences occurred between the various cases (Table 1). Thus, *in respect to the Bb labeling,* in the six rats and the two cats with injections of 10% Bb and 2–3 days Bb survival time, the single Bb labeled neurons showed labeling of both nucleus and cytoplasm. Moreover, the single Bb labeled neurons as well as the double-labeled ones were surrounded by brilliantly fluorescent glial nuclei. However, in the three rats with injections of 1% Bb followed by 1½ h, 3 h, and 6 h Bb survival time, the single Bb labeled neurons showed only fluorescence of the nucleus. After 1½ h survival the nuclei were only lightly fluorescent and no fluorescent glial nuclei occurred. After 3 h and 6 h survival time they were brilliantly fluorescent and were surrounded by a few dull fluorescent glial nuclei. The same was true for the double-labeled neurons. Yet, even after 1½ h Bb survival time when no Bb labeled glial nuclei were present, the double-labeled neurons showed already an unequivocal Bb labeled nucleus in a blue fluorescent cytoplasm. This indicates that the double labeling did not result from migration of the tracers, but represented genuine double labeling by way of divergent axon collaterals (Vanderkooy et al. 1978).

In respect to the TB labeling, in the rats with 2–3 days TB survival and 10% Bb injections followed by 2–3 days Bb survival time (Table 1) the blue TB labeling of the cytoplasm of the double labeled neurons was sometimes in part masked with some yellow Bb labeling. This masking of the TB cytoplasmic labeling was less pronounced when the TB

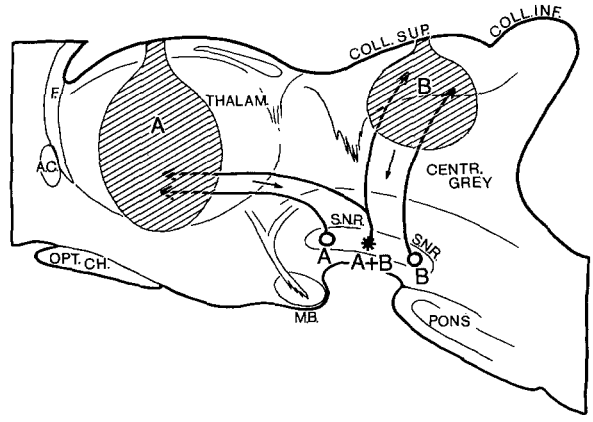

Fig. 2. Diagram of the distribution of fibers and collaterals from substantia nigra pars reticulata (SNR) to ventral thalamus and superior colliculus and the retrograde transport of two tracers (A and B) from thalamus and tectum through these nigral axons to SNR neurons

survival time was prolonged to 4–6 days which increases the intensity of the blue TB cytoplasmic fluorescence. However, in the rats the longer TB survival times (10–22 days) resulted in an accumulation of silver fluorescent granules in the cytoplasm, which tended to mask the blue TB labeling. However, this did not occur in the two cats, even not after 22 days TB survival time. The admixture of the yellow Bb fluorescence of the cytoplasm in double labeled neurons was also less pronounced in the three rats with 1% Bb injections and short Bb survival times (1½, 3, and 6 h), in which cases also single Bb labeled neurons showed little Bb cytoplasmic labeling. However, in this respect 1% NY (see group B) was better than 1% Bb, since NY also gives little cytoplasmic labeling, and after short survival times gives a more brilliant fluorescence of the nucleus (Bentivoglio et al. 1980a).

Nigro-tectal and -thalamic System. In the eight rats of group A2 (Table 1) with TB injections in tectum and 1% or 10% Bb injections in ipsilateral ventral thalamus (Fig. 2) followed by different survival times, several nigral neurons were double labeled. Similar to the findings in the earlier experiments

Table 2. Group B: Double labeling with TB and NY

B	Animals	% Tracer	µl	Injection site	% Tracer	µl	Injection site	Survival time	Survival time
1.	2 rats	2% TB	0.6	Left Ant. Thalam.	1% NY	0.5	Right Ant. Thalam.	TB: 5 days	NY: 5, 9 h
2.	1 rat	2% TB	0.6	Tectum	1% NY	0.6	Ipsil. Vent. Thalam.	TB: 8 days	NY: 12 h
3.	2 rats	2% TB	0.8	Thalam. + Tectum	1% NY	1.0	Ipsil. Spin. C2	TB: 6 days	NY: 17 h

(Bentivoglio et al. 1979b) they were present mainly in its juxtapeduncular portion of the SNR. These neurons contained a green to white fluorescent Bb labeled nucleus and a blue, TB labeled cytoplasm.

In the five rats with 10% Bb injections and 3 days Bb survival time, many brilliantly fluorescent glial nuclei were present around the single Bb and double-labeled neurons, and the TB blue fluorescent cytoplasm of the double-labeled neurons showed some admixture of yellow Bb labeling. However, in the two rats with 1% Bb injections followed by survival times of 6 h and 12 h this admixture of yellow Bb fluorescence was almost absent and virtually no fuorescent glial nuclei or only some dull fluorescent ones were present around the Bb labeled neurons. Yet, even after 6 h Bb survival time the double labeled SNR neurons showed an unequivocal Bb labeling of the nucleus. In the rats with 21 days TB survival time many silver fluorescent granules were present in the cytoplasm, which were absent after 4 days. The findings in this group of cases indicate that TB can be successfully combined with Bb in double labeling experiments provided 1% Bb and relatively short Bb survival times are used and the TB survival time is kept within limits.

B. Double Retrograde Labeling of Neurons with Nuclear Yellow (NY; Hoechst S769121) and True Blue (TB)

In these experiments the use of 1% NY instead of 1% Bb was explored because in several respects NY is a more attractive double labeling tracer than Bb.

Mammillo-thalamic System and Nigro-tectal and -thalamic System. In the two rats with 1% NY and 2% TB injections in the anterior thalami (group B1, Table 2) and in thalamus and tectum (group B2, Table 2), respectively (Figs. 1, 2) the same findings were obtained as in the corresponding experiments of groups A1 and A2 with 1% Bb injections. However, the double-labled neurons in the lateral mammillary nuclei and the juxtapeduncular SNR showed a golden-yellow NY fluorescent nucleus, a blue TB fluorescent cytoplasm, and very little yellow fluorescence in the cytoplasm. After the thalamic injections followed by 9 h NY survival time (group B1, Table 2) the single NY labeled mammillary neurons as well as the double labeled ones were surrounded by brilliantly fluorescent glial nuclei, but after 5 h only a few dull NY fluorescent glial nuclei occurred (Fig. 4). Yet, the NY labeled nuclei of both the single and the double labeled neurons were more brilliantly fluorescent than the Bb labeled nuclei in the comparable cases of groups A1 and A2. However, in the rat with 2% TB injections in tectum and 1% NY in ipsilateral ventral thalamus followed by 12 h NY survival time some brilliantly fluorescent glial nuclei were present around NY labeled SNR neurons. Yet, in the more caudally located deep cerebellar nuclei, which also contained many retrogradely labeled neurons, only some dull fluorescent glial nuclei were present.

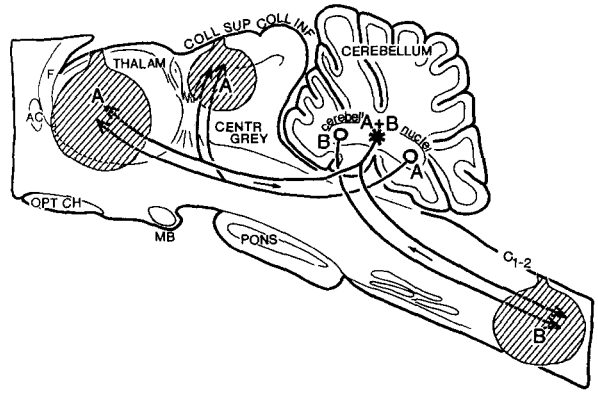

Fig. 3. Diagram of the distribution of fibers and collaterals from deep cerebellar nuclei to tectum and thalamus and to the spinal cord and the retrograde transport of two tracers (A and B) from these areas through the efferent cerebellar fibers to deep cerebellar neurons

Cerebello-thalamic and -spinal System. In the two rats with 2% TB injections in thalamus and tectum and with 1% NY injection ipsilaterally in C2–C3 spinal segments (Fig. 3) followed by 6 days TB survival time and 17 h NY survival time (group B3, Table 2) the intermediate portions of the contralateral fastigial and interpositus cerebellar nuclei contained some double-labeled neurons as observed also with other tracers (Bentivoglio and Kuypers, unpubl. observ.).

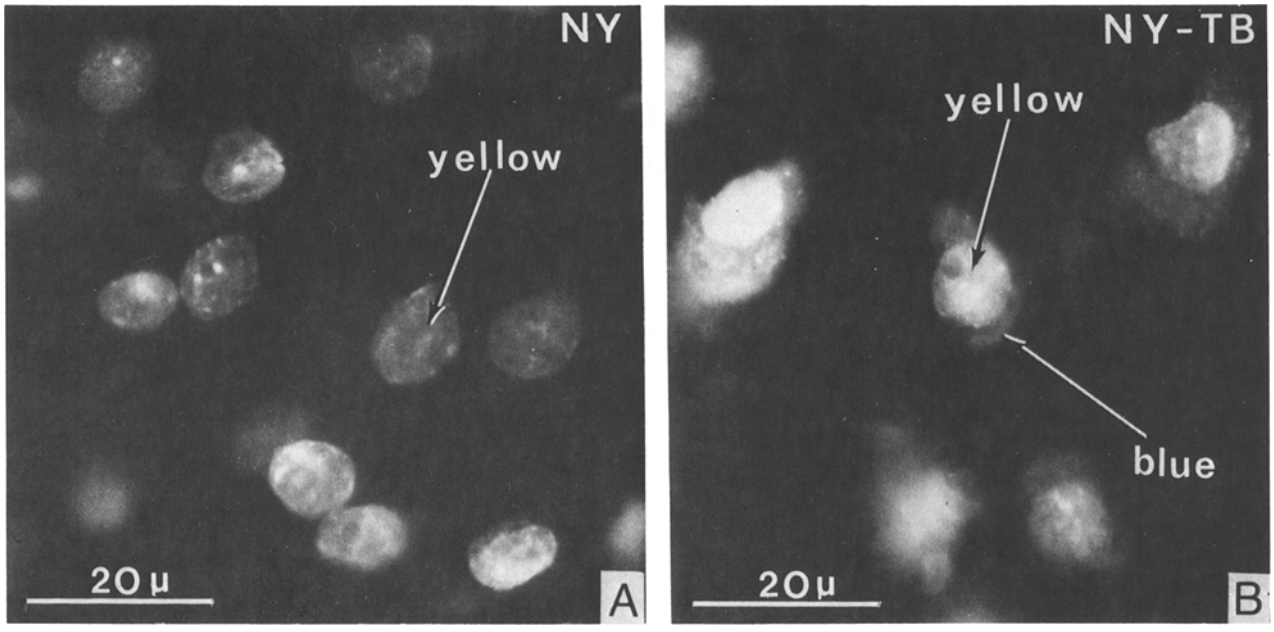

Fig. 4A, B. Photomicrographs of NY and TB labeled neurons in rat mammillary body taken with filter-mirror system A (360 nm excitation wavelength). NY was injected in anterior thalamus on one side and TB in anterior thalamus on the other (group B1, 5 h NY survival time). **A** NY labeled neurons in medial mammillary nucleus on the side of the NY thalamic injection. Note brilliant (golden-yellow) fluorescent nuclei and absence of fluorescence in cytoplasm. **B** NY-TB double-labeled neurons in lateral mammillary nucleus. Note brilliantly (yellow) fluorescent NY labeled nucleus and brilliantly (blue) fluorescent TB labeled cytoplasm. Note presence of only a few dull fluorescent glial nuclei

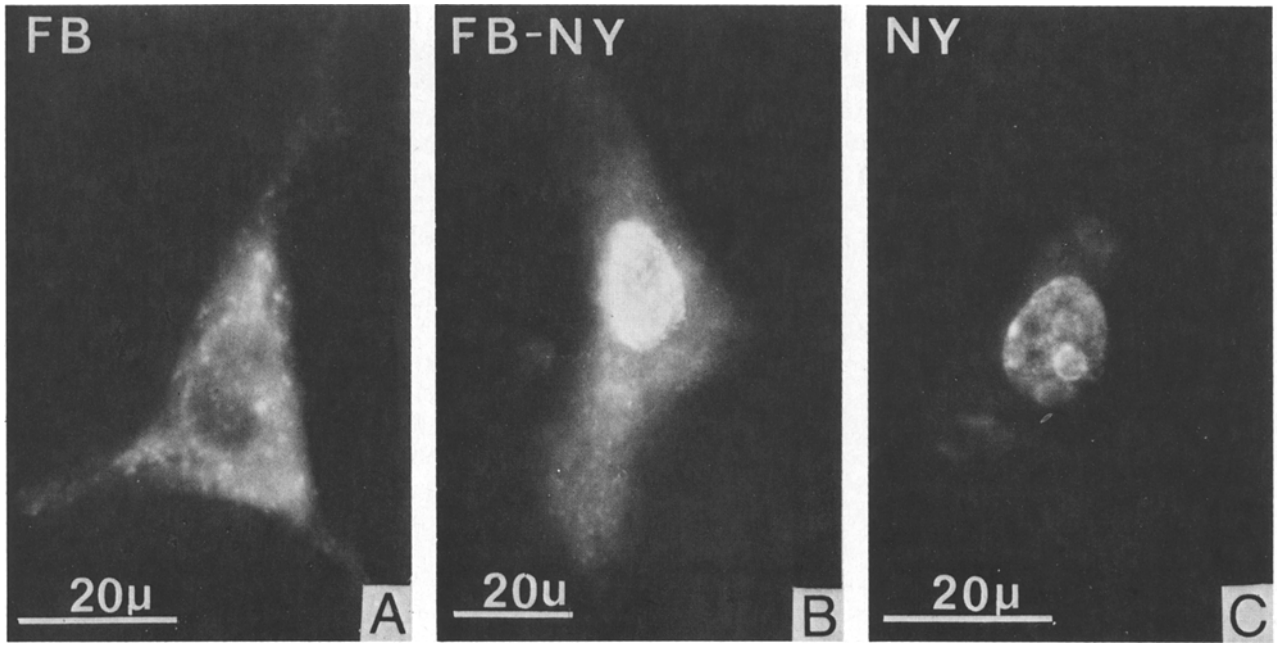

Fig. 5A–C. Photomicrographs of FB and NY labeled neurons taken with filter-mirror system A (360 nm excitation wavelength). **A** Example of FB labeled neuron in rat reticular formation after FB injections in spinal cord (6 days survival). Note brilliantly (blue) fluorescent cytoplasm with silver fluorescent granules and little fluorescence in nucleus. **C** NY labeled neuron in rat deep cerebellar nuclei after NY injections in thalamus (group B2). Note brilliantly golden-yellow fluorescent nucleus with brilliantly fluorescent ring around nucleolus and very dull fluorescent cytoplasm. Note also the dull fluoresence of two adjoining glial nuclei. **B** FB-NY double-labeled neuron in cat cerebellar nuclei after FB injection in thalamus and NY injection in spinal cord (group C3). Note (blue) fluorescence of cytoplasm with silver fluorescent granules and brilliant (yellow) fluorescent nucleus

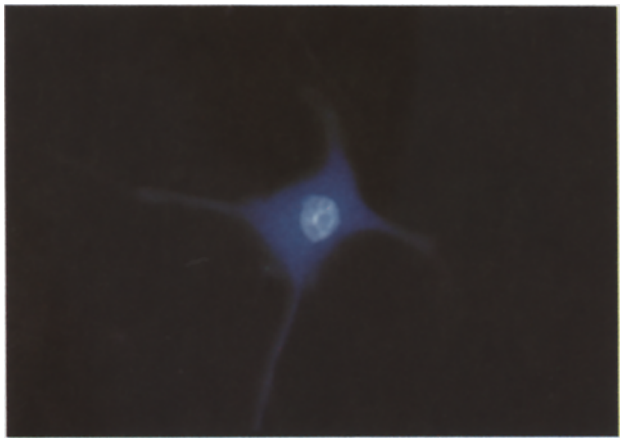

Fig. 6. Photomicrograph of NY-TB double-labeled neuron in rat medullary reticular formation, taken with filter-mirror system A (360 nm excitation wavelength) after TB injection in thalamus and tectum and NY injection at C1–C2 (group B3). Note brilliantly NY fluorescence of nucleus including a brilliantly fluorescent ring around nucleolus and deep TB blue fluorescence of cytoplasm

Some such neurons were also present in the medullary reticular formation (Fig. 6). As in the groups B1 and B2 experiments the blue TB labeled cytoplasm of the double labeled neurons showed virtually no admixture of yellow fluorescence and only few dull fluorescent glial nuclei were present.

These findings indicate that the combination of 2% TB with 1% NY can be very successfully used in double labeling experiments and that this combination is more attractive than the combination of TB with Bb because NY gives a more brilliant nuclear labeling. Moreover, NY gives little cytoplasmic labeling which largely avoids the masking of the TB labeling as sometimes occurs with Bb.

C. Double Labeling of Neurons with FB in Combination with NY and Bb

The effectiveness of FB in combination with Bb and NY has also been explored using the mammillo-thalamic (Fig. 1), the nigro-tectal and -thalamic (Fig. 2) and the cerebello-thalamic and -spinal connections (Fig. 3) as models (group C, Table 3). In the two rats with 3% FB and 1% Bb injections in anterior thalamus on either side (group C1, Table 3) and in tectum and ipsilateral ventral thalamus (group C2, Table 3), respectively, the same results were obtained in the mammillary nuclei and SNR as with 5% TB and 1% Bb injections (groups A1, A2, Table 1) and many double-labeled neurons with a blue (FB) cytoplasm and a green to white (Bb) nucleus occurred. However, the cytoplasm of the single FB labeled neurons as well as of the double-labeled ones characteristically showed a lighter blue fluorescence than with TB and in addition contained some silver fluorescent granules.

In view of the advantages of NY over Bb, in two rats (groups C1, C2) FB was combined with NY. In the rat with the anterior thalamic injections on both sides, 24 h FB survival and 5 h NY survival time was used. However, in the rat with the tectal and thalamic injections the blue FB cytoplasmic labeling was strengthened by using 8 days FB survival time combined with 12 hours NY survival. In both cases the double labeled neurons showed a brilliant NY labeled golden-yellow fluorescent nucleus and a FB labeled blue fluorescent cytoplasm with some fine silver fluorescent granules and some large orange fluorescent ones. Only a few dull NY fluorescent glial nuclei were present around the retrogradely labeled neurons indicating that only minimial NY migration had occurred.

The combination of NY and FB was also used in cats, in which FB is more effectively transported over long distances than TB (Bentivoglio et al. 1980b). In two cats (group C3, Table 3) 3% FB was injected in thalamus and tectum and 1% NY was injected ipsilaterally in upper cervical cord, followed by 7 days FB survival and 45 h NY survival time. Single FB labeled neurons and single NY labeled ones were present contralaterally in the deep cerebellar nuclei. Occasionally, single FB labeled neurons showed a light blue fluorescent nucleus. In addition, several

Table 3. Group C: Double labeling with FB and Bb or NY

C	Animals	% Tracer	µl	Injection site	% Tracer	µl	Injection site	Survival time	Survival time
1.	1 rat	3% FB	0.6	Left Ant. Thalam.	1% Bb	0.4	Right Ant. Thalam.	FB: 24 h	Bb: 5 h
	1 rat	3% FB	0.6	Left Ant. Thalam.	1% NY	0.5	Right Ant. Thalam.	FB: 24 h	NY: 5 h
2.	1 rat	3% FB	0.6	Tectum	1% Bb	0.5	Ipsil. Vent. Thalam.	FB: 48 h	Bb: 10 h
	1 rat	3% FB	0.6	Tectum	1% NY	0.6	Ipsil. Vent. Thalam.	FB: 8 days	NY: 12 h
3.	2 cats	3% FB	12	Thalam. + Tectum	1% NY	12	Ipsil. Spin. C2	FB: 7 days	NY: 45 h

very strikingly double-labeled neurons occurred in both the fastigial and the interpositus nuclei (Fig. 5) as well as in the medullary reticular formation. Moreover, in these cases, in which the material was processed very rapidly (see Material and Methods) either no NY fluorescent glial nuclei were present around the single NY labeled neurons, and the NY-FB double-labeled ones or only a few dull fluorescent glial nuclei occurred. This indicates that no appreciable NY migration had taken place and that the brilliantly NY labeled neurons including the double-labeled ones were genuinely retrogradely labeled. Finally, in these cases the blue fluorescent labeling of nerve fibers through which FB was transported retrogradely was especially striking. Thus, fibers of superior cerebellar peduncle and medial lemniscus contralateral to the injections site were brilliantly blue fluorescent with silver fluorescent granules and could be traced to the contralateral deep cerebellar nuclei and the dorsal column nuclei, respectively.

The findings in this group indicate that Bb, but especially NY, may also be successfully combined with FB in double labeling experiments. This combination has the advantage that in cats FB is more effectively transported over long distances than TB.

Discussion

Fluorescent retrograde tracers may be used to demonstrate the topographical relationship of neighboring groups of neurons projecting to different areas (Kuzuhara et al. 1980; Vanderkooy 1979). They may also be used in combination with histofluorescent (Björklund and Skagerberg 1979a, b; Hökfelt et al. 1979; Vanderkooy and Wise 1980) and immunohistofluorescent (Hökfelt et al. 1979; Hökfelt et al. in press) techniques to demonstrate the distribution of the fibers of certain neurons which contain specific transmitters. However, fluorescent retrograde tracers may also be used in combination to determine whether individual neurons give rise to divergent axon collaterals (Catsman-Berrevoets et al. 1980; Vanderkooy and Hattori 1979; Hattori and Vanderkooy 1979; Rosina et al. 1979; Bentivoglio and Kuypers 1979; Vanderkooy et al. 1978; Vanderkooy and Kuypers 1979). For this same purpose other methods have also been developed (Stewart et al. 1977; Olsson and Kristensson 1978; Steiger and Büttner-Ennever 1978; Hayes and Rustioni 1979). In the technique based on retrograde transport of HRP and ^{3}H-apo-HRP through two different collaterals to one and the same cell, the substances can be demonstrated by means of the combined HRP histochemistry-autoradiography technique (Hayes and Rustioni 1979).

The multiple retrograde fluorescent tracer technique and the HRP and ^{3}H-apo-HRP double labeling technique produce approximately the same results (Berkley et al. in prep.). However, they each have their own peculiarities. For example, the use of HRP in combination with ^{3}H-apo-HRP has the disadvantage first that the lack of enzymatic activity of the ^{3}H-apo-HRP has to be demonstrated and second that the combined histochemistry-autoradiography required in this technique is a laborious procedure. The fluorescent retrograde tracer technique in itself is much simpler. However, it has the disadvantage that the tracers which were used originally, i.e., EB and DAPI-Pr are not effectively transported over long distances, especially not in cat. Moreover, the study of the neurons double-labeled with these tracers is somewhat laborious because the material continuously has to be illuminated with the two wavelengths. The present findings indicate that these drawbacks may largely be overcome by combining Bb but especially NY with TB and FB; (a) because Bb and NY and to some extent FB are transported rather effectively over long distances even in cats and (b) because after short survival times 1% Bb but especially 1% NY, in frozen section material (Björklund and Skagerberg 1979a) mainly labels the neuronal nucleus which fluoresces green (Bb) or golden-yellow (NY) at 360 nm excitation wavelength while TB and FB mainly label the cytoplasm which fluoresces blue at this same 360 nm excitation wavelength. As a consequence, neurons double-labeled with NY combined with TB or FB when viewed at the single 360 nm excitation wavelength can be immediately recognized since they display a golden-yellow (NY) fluorescent nucleus and a deep blue (TB) or lighter blue (FB) fluorescent cytoplasm. In rats both the NY-TB and NY-FB combinations have given very beautiful results. However, in cat the NY-FB combination is superior because FB is more effectively transported over long distances in this animal than TB. The fact that in the present study these tracer combinations when applied to the mammillothalamic system and the nigro-tectal and -thalamic system in rat gave the same results as obtained with EB and DAPI-Pr (Vanderkooy et al. 1978; Bentivoglio et al. 1979b) indicates that the tracer combinations of the present study are at least as effective as the combination of EB and DAPI-Pr.

It should be stressed, however, that the NY survival time has to be much shorter than the TB of FB survival times and should be titrated for each pathway, because during long survival times NY, as Bb, migrates from retrogradely labeled neurons to

adjoining cells (Bentivoglio et al. 1980a). This may produce false NY neuronal labeling as observed in rats in which callosal neurons in sensorimotor cortex were labeled blue retrogradely by TB injections in the other hemisphere and the corticospinal neurons were labeled green or golden-yellow by injections in spinal cord. After 17 h Bb survival time and 28 h NY survival time only few dull fluorescent glial nuclei were present and all neurons were single labeled (Catsman-Berrevoets et al. 1980). This indicates that corticospinal neurons in rats do not provide callosal collaterals, in agreement with electrophysiological observations (Catsman-Berrevoets et al. 1980). However, when using 10% Bb and 10% NY and 6–10 days Bb and NY survival times the Bb and NY labeled corticospinal neurons were surrounded by many brilliant fluorescent glial nuclei (unpubl. observ.), signaling a considerable Bb and NY migration (Bentivoglio et al. 1980a) and the vast majority of the blue fluorescent callosal neurons contained a green to white (Bb) or golden-yellow (NY) labeled nucleus. This double labeling after long survival times, was regarded as false double labeling in agreement with the electrophysiological findings and the findings obtained with other tracers (Catsman-Berrevoets et al. 1980). However, in the present experiments unequivocal retrograde double labeling occurred already after short survival times when none or only a few dull fluorescent glial nuclei were present, which indicates that only minimal migration of the tracers had occurred. The double labeling in these cases can therefore with confidence be regarded to represent genuine double labeling by way of divergent axon collaterals.

NY is a more attractive tracer than Bb, first because NY proceeds more slowly to the cell body and from there to the surrounding cells which makes it easier to titrate the survival time and second because the golden-yellow nuclear fluorescence obtained with 1% NY after short survival time is more brilliant than the green nuclear fluorescence obtained with 1% Bb after such survival times (Bentivoglio et al. 1980a). Finally, NY has also the advantage that it produces less cytoplasmic labeling than Bb and therefore gives virtually no masking of the blue TB or FB labeling of the cytoplasm.

Obviously it would be advantageous if a fluorescent retrograde tracer would be available which as Bb and NY would preferentially label the nucleus and would combine a rapid transport with a lack of migration from the retrogradely labeled neurons. In that case the survival time of both tracers should be kept the same and would not have to be adjusted. Further research may produce such a tracer. However, at present the combination of NY with TB or FB are the most favorable ones for double labeling experiments demonstrating the existence of divergent axon collaterals because the single- and double-labeled neurons can easily be distinguished at the same 360 nm excitation wavelength even under relatively low magnification. A number of fiber systems have already been studied with this technique (Bentivoglio and Kuypers 1979; Catsman-Berrevoets and Kuypers 1979; Rosina et al. 1979), which has provided several new data on the fiber connections in the brain to be reported separately.

Acknowledgements. The authors thank Mr. E. Dalm for his assistance with the surgery, Mr. H. Klink for his histological assistance, Mr. W. van den Oudenalder and Miss P. van Alphen for their photographical assistance, Dr. R. Lemon for reading the manuscript and Miss G. van Gelder for typing it. This study was supported in part by Grant 13–46–15 of FUNGO/ZWO (Dutch Organization for Fundamental Research in Medicine). The authors also thank the Remmert Adriaan Laan Fonds for helping to defray part of the cost of the color print. The authors thank Prof. Dr. O. Dann for providing TB and FB and Dr. H. Loewe for providing NY (Hoechst S 769121).

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Received February 22, 1980