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Retrograde fluorescent neuronal tracing combined with acetylcholinesterase histochemistry

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The retrograde fluorescent tracing technique was combined with the di-isopropylfluorophosphate (DFP) histochemical procedure for acetylcholinesterase (AChE). Three fluorescent tracers (True blue, Fast blue and Evans blue) were injected into the rat striatum. After the appropriate survival time and after the administration of DFP, AChE reaction products could be observed in the fluorescent retrogradely labeled substantia nigra neurons. The fluorescent retrograde labeling and the AChE brown reaction products were observed in the same cell bodies by simply turning on and off the bright-field illumination while observing with fluorescence. The sensitivity of the method appeared to be related to the length of the survival time after the tracer injection as well as after the DFP administration. This combined method allows to study the efferent connections of AChE-containing neurons in the central nervous system.

Introduction

The enzyme acetylcholinesterase (AChE) has been demonstrated in the central nervous system of many mammalian species, where it can be visualized histochemically in the somata and processes of AChE-containing neurons and in the neuropil surrounding them (Koelle, 1963; Potter, 1970; Silver, 1974). The topography of AChE-containing neurons is known since Koelle's histochemical study (1954). However, only after the introduction of the di-isopropylfluorophosphate (DFP) technique, which suppresses the neuropil staining (Butcher et al., 1975), individual AChE-containing neurons could be studied in detail (see the review by Lehman and Fibiger, 1979).

In order to study the efferent connections of AChE-containing neurons, the retrograde tracer horseradish peroxidase (HRP; Kristensson and Olsson, 1971) was combined with AChE histochemistry (Hardy et al., 1976; Mesulam, 1976; Mesulam and Van Hoesen, 1976). However, the HRP-AChE combined methods require much histochemical manipulation; moreover, due to the resemblance of the HRP and AChE reaction products, it may be sometimes difficult to differentiate them.

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In order to overcome these problems, the present work was aimed at evaluating whether the fluorescent retrograde tracers, which have been recently introduced in the neuroanatomical research (Kuypers et al., 1977; Bentivoglio et al., 1979, 1980), can be combined with the DFP technique for AChE histochemistry. The nigrostriatal projections were used as a test system in the present study, since the substantia nigra pars compacta (SNc) contains a high number of AChE-positive neurons (Butcher and Marchand, 1978).

Materials and Methods

Three fluorescent retrograde tracers were used; Evans blue (EB), which diffusely labels the neuronal cell body and fluoresces red (Kuypers et al., 1977); True blue (TB) and Fast blue (FB), which fluoresce blue. TB mainly labels the nucleolus, cytoplasm and proximal dendrites (Bentivoglio et al., 1979); FB produces a granular labeling of the neuronal cytoplasm and proximal dendrites (Bentivoglio et al., 1980). All the tracers were injected in the caudate-putamen (CPu) in 11 rats by means of a stereotaxic apparatus (David Kopf Instruments), according to the atlas by Pellegrino and Cushman (1967). Before surgery the animals were deeply anaesthetized with sodium pentobarbital (40 mg/kg, i.p.). In 4 rats 0.2 μl of a 2% TB aqueous suspension were injected, followed by a survival of 3, 5, 10 or 14 days respectively; 4 rats were injected with FB (0.2 μl of a 2% aqueous suspension) and survived 2, 4, 7 or 10 days respectively; finally, 3 animals were injected with 0.2 μl of a 10% EB aqueous solution, followed by 2, 4 and 7 days survival respectively. Before the sacrifice all animals were poisoned with DFP (Serva Feinbiochemica; Butcher et al., 1975). An arachid oil 0.15% DFP solution was injected intramuscularly in the dose of 1 mg/kg of body weight, followed by different survival times, i.e. 4, 6 and 8 h. All animals were perfused intracardially with 0.9% saline followed by 10% neutralized formalin. The brains were postfixed for 1 or 2 h in the same formalin solution, then soaked at room temperature in 30% buffered sucrose (pH 7.2) for a period ranging from 4 to 5 h. The injection sites and midbrains were cut on a freezing microtome into 30 μm thick coronal sections. Alternate sections of the injection sites and midbrains were mounted on slides from distilled water; the other series of sections of the midbrains were processed for AChE histochemistry according to the procedure described by Butcher et al. (1975). An incubation time of 3.5–4 h was adopted during this procedure, which was carried out at room temperature on free-floating sections that were gently shaked every 30 min. The sections stained for AChE were then carefully rinsed in distilled water, from which they were mounted on slides and air dried. They were not coverslipped, and were studied with a Leitz Ploemopak epifluorescence microscope, equipped with 3 Leitz filter-mirror system combinations, i.e. system A (340–380 nm light excitation wavelength), system D (355–425 nm light excitation wavelength), system N2 (540–560 nm light excitation wavelength). Systems A and D were used in the study of the TB and FB fluorescence, while system N2 was used in the study of the EB fluorescence.

The sections were stored at 5°C. However, a few days after tissue processing the

sections showed some degree of background autofluorescence. In this respect, the impression was gained that the sections processed for AChE histochemistry deteriorated much quicker than those only containing fluorescent retrograde labeling, which are stored in our laboratory for several months at 5°C without any consistent diminution of the fluorescence or increase of background autofluorescence.

Results

In all cases the SNc contained several fluorescent retrogradely labeled neurons. When, during the observation with fluorescence, the bright-field illumination was turned on the AChE brown reaction products appeared, while, under these circumstances, the fluorescent labeling obviously disappeared. With this simple procedure (i.e. turning on and off the bright-field while observing the fluorescent labeling), the presence of AChE reaction products and the TB or FB retrograde labeling could be easily ascertained in the same neural cell bodies (Figs. 1 and 2); in the cases injected with EB (Fig. 3) the procedure required one additional step in order to exclude the N2 filter system during observation of AChE, as it is discussed further on. The vast majority of the retrogradely labeled SNc neurons also contained AChE reaction products. Quite a few AChE-positive SNc neurons were not retrogradely labeled: their number was dependent upon the larger or smaller size of the fluorescent injection area in the CPu.

As assessed histochemically, the AChE staining was not influenced by the

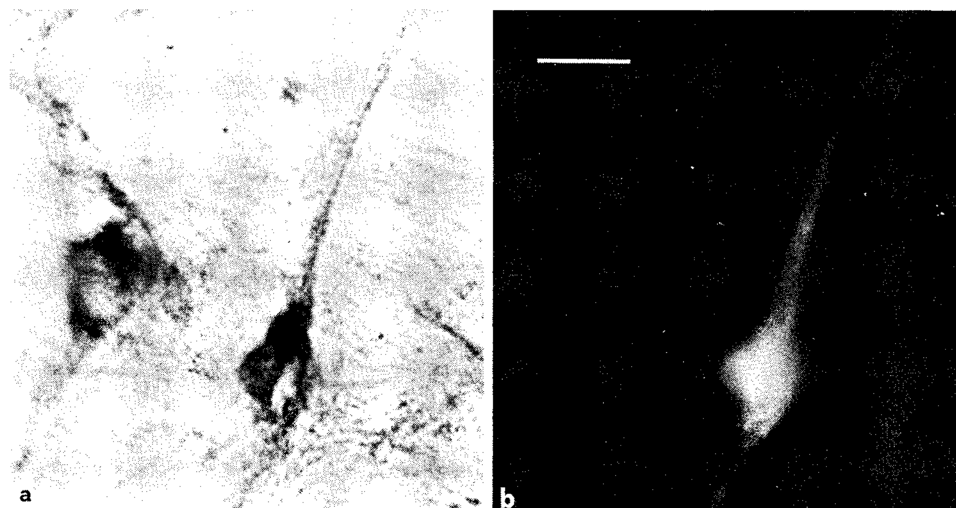

Fig. 1. Photomicrographs of a TB-labeled neuron containing AChE reaction products. a: 2 AChE-containing neurons in the SNc, bright-field illumination. b: same field as in a under fluorescence illumination (360 nm light excitation wavelength); note the TB labeling of one of the AChE-containing cells. Calibration bar: 20 μm.

presence of EB, TB or FB retrograde labeling. Regardless of the tracer injected, in the cases which had survived 4 h after the DFP administration there was no neuropil staining and only the AChE-containing somata and proximal dendrites could be visualized; in the cases which had survived 6 and 8 h after DFP administration the AChE reaction products were also observed in the distal portions of dendrites and in the neuropil. Therefore, since detection of the AChE reaction products in the cell bodies was easier in sections with no neuropil staining, the best results were obtained in the cases which had survived 4 h after DFP poisoning. When the filter-mirror systems A and D were used in the fluorescence study, the AChE reaction products were visible as dark non-fluorescent granules in the neuronal cytoplasm. The dark

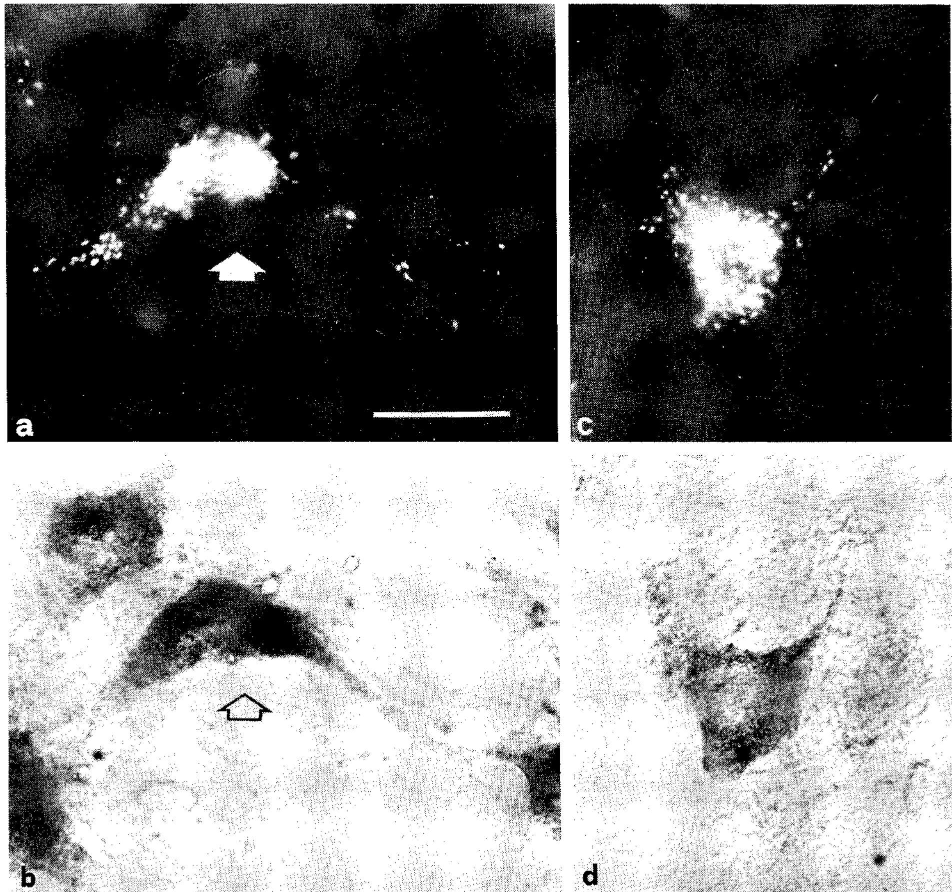

Fig. 2. Photomicrographs of FB-labeled cells containing AChE reaction products. a, c: retrogradely FB labeled fluorescent cells (360 nm light excitation wavelength). b: same field as in a; note the occurrence of AChE reaction products in the FB labeled neuron (arrow) as well as in non-retrogradely labeled neurons (bright-field illumination). d: same field as in c; note the AChE staining (bright-field illumination) in the FB retrogradely labeled neuron. Calibration bar: 20 μm.

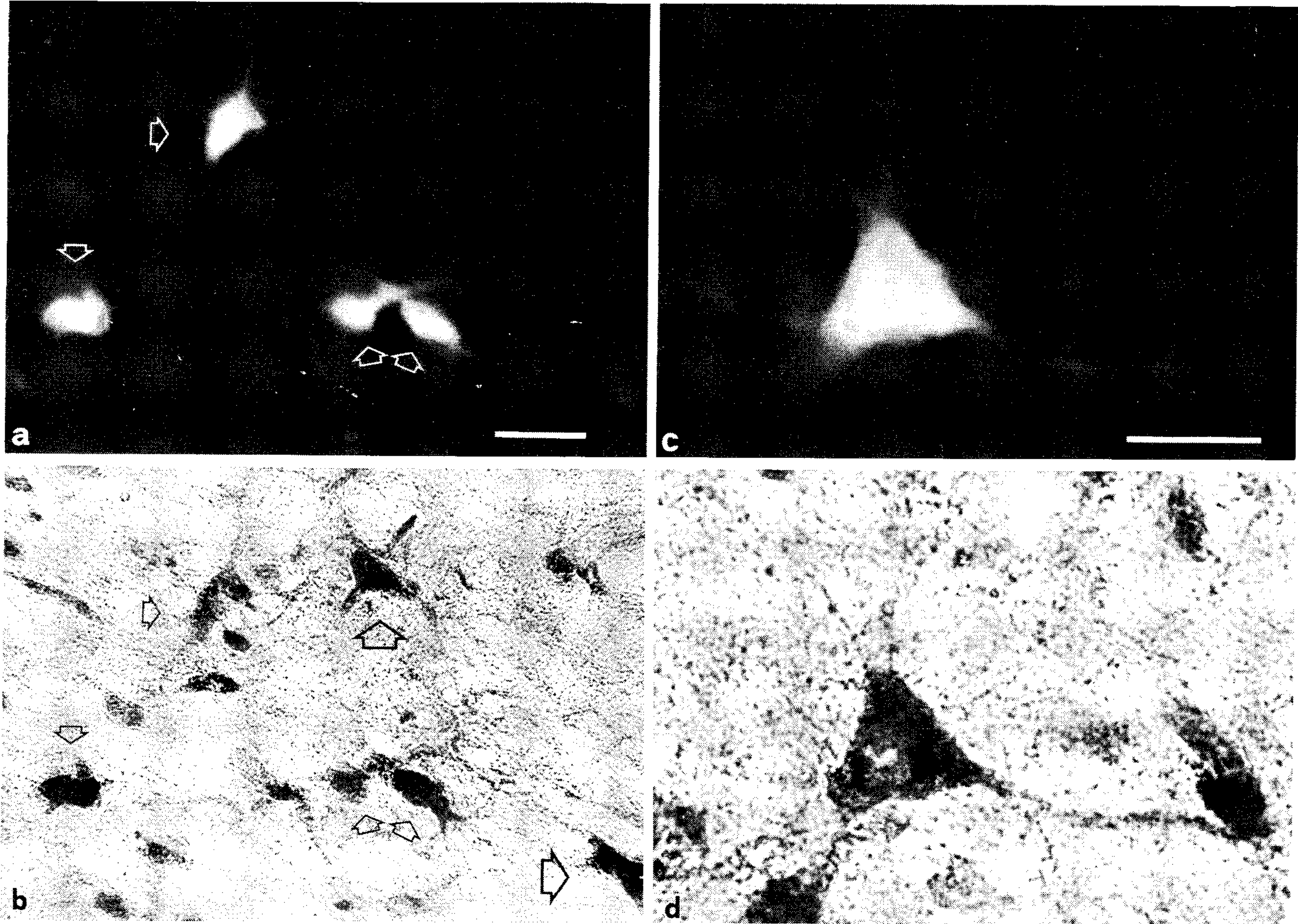

Fig. 3. Photomicrographs of EB-labeled fluorescent cells (550 nm excitation wavelength) containing AChE reaction products. a: EB retrogradely labeled neurons in the SNc. b: the same area as in a illuminated with bright-field reveals AChE-stained cell bodies; the small arrows point to the EB labeled neurons (a and b), larger arrows (b) point to AChE-containing somata which are not EB retrogradely labeled; note that both lightly and darkly stained AChE-containing somata were EB retrogradely labeled. c: EB retrogradely labeled neuron. d: the bright-field illumination shows AChE reaction products in the EB-labeled cells. Calibration bars: 20 μm.

AChE granules were particularly easy to detect under system D.

In the cases injected with the tracers TB and FB, while turning slowly on the bright-field illumination the AChE reaction products gradually appeared more clearly. With an adequate balance between fluorescence and bright-field, the fluorescent labeling and the AChE dark granules could be easily simultaneously seen in the same neurons. This "shining through" effect with simultaneous fluorescence–bright-field microscopy was not observed in the sections containing the EB labeling, because filter-mirror system N2 (which has been used to elicit the EB fluorescence) contains a red (580 nm) barrier filter that reduces image contrast when observing the russet-coloured AChE-containing neurons; thus, before turning the bright-field on, it had first to be switched to the D or A systems. Therefore, each field containing EB labeled neurons had to be screened first with fluorescence and then with bright-field, or vice versa.

In the sections processed for AChE, the TB, FB or EB fluorescent retrograde labeling was clearly detectable. However, the TB, FB and EB retrograde labeling was in general less brilliant in sections stained for AChE than in the unstained ones. This diminution of the intensity of the fluorescence was especially evident in the cases injected with TB and FB with the shortest survival times, i.e. 3 and 2 days respectively. A slight prolongation of the survival time (5 and 4 days respectively) gave better results in that the fluorescent labeling, although less intense than in the sections not processed for AChE histochemistry, was still quite brilliant. Further prolongation of the survival time after the TB and FB injections resulted in fluorescent labeling of glial nuclei around retrogradely labeled neurons, which revealed the diffusion of the fluorescent compounds out of retrogradely labeled cells into the adjacent glial cells (Bentivoglio et al., 1979, 1980); in these cases, the fluorescent glial nuclei were well evident also after incubation for AChE. Moreover, after the AChE histochemistry procedure, the FB labeling somewhat changed, such that some of the FB labeled cells displayed a brilliant orange, rather than blue, granular fluorescent labeling. Furthermore, even though in the material not processed for AChE EB diffusely labeled the cell bodies (Kuypers et al., 1977), after incubation for AChE in some neurons fluorescent labeling of the nucleus was slightly more brilliant than fluorescent labeling of the cytoplasm (Fig. 3c).

Conclusions

The method described in the present investigation indicates that retrograde fluorescent tracers can effectively be used in the study of the efferent projections of AChE-containing neurons. The combination of retrograde fluorescent tracing with AChE histochemistry is extremely simple, does not require any further manipulation of the sections, and allows a simultaneous observation of retrograde labeling and AChE reaction products in the same neural cell bodies. The sensitivity of the technique mainly depends upon two factors: (1) the survival time after injection of the fluorescent tracer (especially in the use of TB and FB), in order to obtain a very brilliant fluorescent labeling, since the procedure for AChE histochemistry slightly

interferes with the intensity of the fluorescence; and (2) the survival time after DFP administration, in order to obtain a clear morphological picture of the AChE-containing neural cell bodies.

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