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Quantitative pharmacohistochemistry of acetylcholinesterase in neostriatum of inbred strains of mice

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A quantitative pharmacohistochemical technique has been used in the present study to assay acetylcholinesterase (AChE) activity in the neostriatum of C57BL/6 and DBA/2 mice. This technique permits the measurement of enzyme activity into microscopically defined compartments and is suitable for the study of striatal AChE-containing, putatively cholinergic, neurons. Microphotometric measurements have been performed in the cytoplasm of AChE-containing perikarya and in the striatal matrix: in both compartments, AChE activity was significantly higher in DBA/2 than in C57BL/6 mice. The present data show that AChE quantitative pharmacohistochemistry is suitable for studying the enzyme activity in nervous tissue and, particularly, in the cytoplasm of individual AChE-containing neurons. In addition, interstrain comparison indicates the presence of a genetically determined higher AChE content in striatal neurons of the DBA/2 strain.

It is known that C57BL/6 and DBA/2 mice, two inbred strains displaying opposite behavioural responses[4,18], differ significantly in the organization of cholinergic systems. In fact, as compared to C57BL/6, DBA/2 mice possess more cholinergic neurons in the medial septal nucleus, basal forebrain (diagonal band of Broca, basal nucleus of Meynert), and neostriatum[3]. These morphological variations probably account for differences in the activity of choline acetyltransferase and acetylcholinesterase (AChE), which have been demonstrated biochemically in homogenates of whole brain and of selected cortical areas[10,11,17,19,23].

By using Butcher's pharmacohistochemical technique[6], we reported that higher doses of organophosphorus poison are required in DBA/2 than in C57BL/6 mice, in order to produce a comparable decrease of AChE staining in the neostriatal matrix[2,3]. Since no appreciable interstrain differences were seen in the staining pattern of striatal AChE-containing perikarya, we addressed the question as to whether AChE content could be analyzed quantitatively in separate neostriatal components (i.e., neurons and matrix). In the present study we report that photometric analysis can be performed on sections stained according to the AChE pharmacohistochemical protocol, in order to obtain quantitative information on the enzyme content in individual neurons and in extraneuronal regions. This procedure has been used here for a comparative study of neostriatum in C57BL/6 and DBA/2 mice.

Male C57BL/6 and DBA/2 mice were studied at 18 weeks of age. In order to compare histochemically treated specimens, in each experiment the same number of animals from both strains was processed exactly in a parallel fashion. All the animals were injected intramuscularly with an irreversible AChE inhibitor (di-isopropylfluorophosphate, DFP), as 15% (w/v) arachid oil solution, 6 h prior to their euthanasia. Different doses of DFP (2 or 3.5 mg/kg b.wt.) were employed in different experimental groups. The mice were sacrificed under deep general anaesthesia by cardiac perfusion with 0.9% saline followed by 10% phosphate-buffered neutral formalin. The brains were postfixed in the same formalin solution for 48–72 h before being transferred into cold (4 °C)

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30% sucrose for an additional 48-h period. They were cut coronally exactly at 30 μm intervals according to a standard plane, by means of a thermally stabilized freezing microtome. The resulting tissue sections from both strains were processed in parallel according to a standard protocol. They were immersed for 30 min into 30 μM N-N′-bis-(1-methylethyl)-pyrophosphoroamidic anhydride (iso-OMPA), to inhibit butyrylcholinesterase and then incubated exactly 2 h at 21 °C in the AChE incubation medium[6].

Photometric measurements were performed on 5 standard sections of the caudate–putamen, representing all its rostrocaudal extent, by means of a Zeiss Fluoval MFV 4002 microphotometer, under a 550-nm incident light. In animals injected with low DFP doses only 3 rostrocaudal levels, corresponding to the first, third, and fifth level of the standard series, were analyzed. The photometer was used in transmittance mode; it was calibrated considering 100% transmission that of an area of the same microscope slide, which contained no brain specimens, in order that light passed only through slide, mounting medium and coverslip. Each examination was made in a circular area of 8 μm in diameter, delineated by a measuring diaphragm, which was chosen to cover either only the striatal matrix or just the cytoplasm of a single AChE-containing cell body. Areas for photometric assays were chosen at random from different topographic regions of the neostriatum, in order that in each section most striatal territories were analyzed. Samples of the matrix were taken from territories displaying a relatively dark, homogeneous staining, without evidence of fiber bundles or unstained perikarya; samples of perikarya were chosen among darkly stained AChE-containing somata, which best correspond to putatively cholinergic type B neurons, as described by Woolf and Butcher[25]. Only neurons displaying a well-defined, unstained, nucleus were assayed. Ten samples of the matrix were collected from each section in all animals; 10 samples of AChE-containing perikarya were studied in the animals injected with high doses of DFP, where neurons can be clearly distinguished in both strains (see below). Data from the two strains, obtained during a single experimental session, were compared by analysis of variance.

Mice injected with 2 mg/kg of DFP did not show relevant signs of organophosphorus intoxication: they only appeared slightly hyporeactive and displayed poor responsiveness to environmental stimulation. On the other hand, administration of 3.5 mg/kg of DFP evoked in both strains the typical signs of organophosphorus intoxication, which included salivation, lacrimation, hyperreactivity to touch, diarrhea, respiratory tract secretions and tremors. Under our experimental conditions, no animal died during the 6-h DFP-euthanasia interval, indicating that either the doses employed are not lethal, or that lethality occurs more than 6 h after poisoning.

When mice were injected with low doses of DFP, the AChE-containing cell bodies were clearly detected in the C57BL/6, but not in the DBA/2 strain, where AChE deposits in the striatal matrix were so intensely stained that individual cell bodies could not be clearly visualized (Fig. 1). The administration of DFP, at the dose of 3.5 mg/kg, allowed to reduce AChE staining in the striatal matrix of both strains, thus producing a clear morphological detail of AChE-rich neurons. Comparative interstrain microphotometric measurements were performed on animals injected with low and high doses of DFP (Fig. 2).

In all cases, AChE staining in the neostriatal matrix was higher in the DBA/2 strain by 8–12%. In fact, measurements performed in animals injected with lower doses of DFP revealed a mean transmittance of 54.97 in this strain and 61.85 in the C57BL/6 ($P < 0.001$); assays performed in animals injected with higher doses of DFP indicated a mean transmittance of 73.27 in the DBA/2 strain and of 80.86 in the C57BL/6 one ($P < 0.001$). Therefore, under these experimental conditions, parenteral administration of 3.5 rather than 2 mg/kg of DFP reduced AChE staining in the striatal matrix by 25% in the DBA/2 strain and by 23.5% in the C57BL/6 one.

Microphotometric analysis, performed in animals injected with higher doses of DFP, revealed that AChE staining in striatal perikarya was significantly higher in the DBA/2 strain by ca. 6% ($P < 0.001$; Table I). In each animal, the intensity of AChE staining in perikarya was compared to the intensity of staining in the matrix: it was seen that neural AChE content was 23.7% higher in the DBA/2 strain and 34.9% higher in the C57BL/6 one ($P < 0.001$ in each case).

The present study confirms that microphotometry

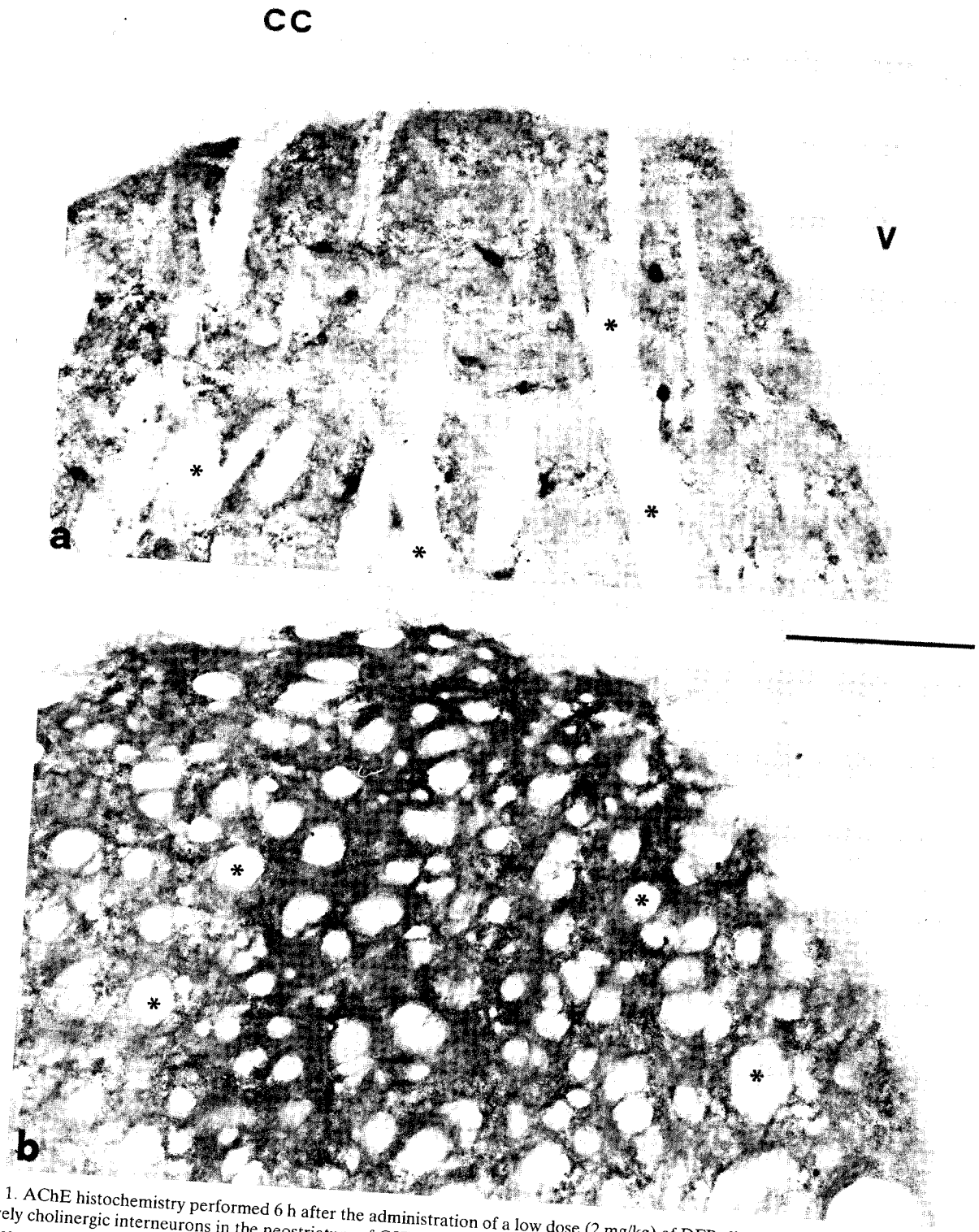

Fig. 1. AChE histochemistry performed 6 h after the administration of a low dose (2 mg/kg) of DFP allows a clear visualization of putatively cholinergic interneurons in the neostriatum of C57BL/6 (a), but not of DBA/2 (b) mice. In the latter strain AChE-containing perikarya are present, but their identification is made difficult by the intensity of background staining. These corresponding brain sections were treated exactly in the same way during a single experimental session. CC, corpus callosum; V, lateral ventricle; *, fiber bundle. Bar, 0.2 mm.

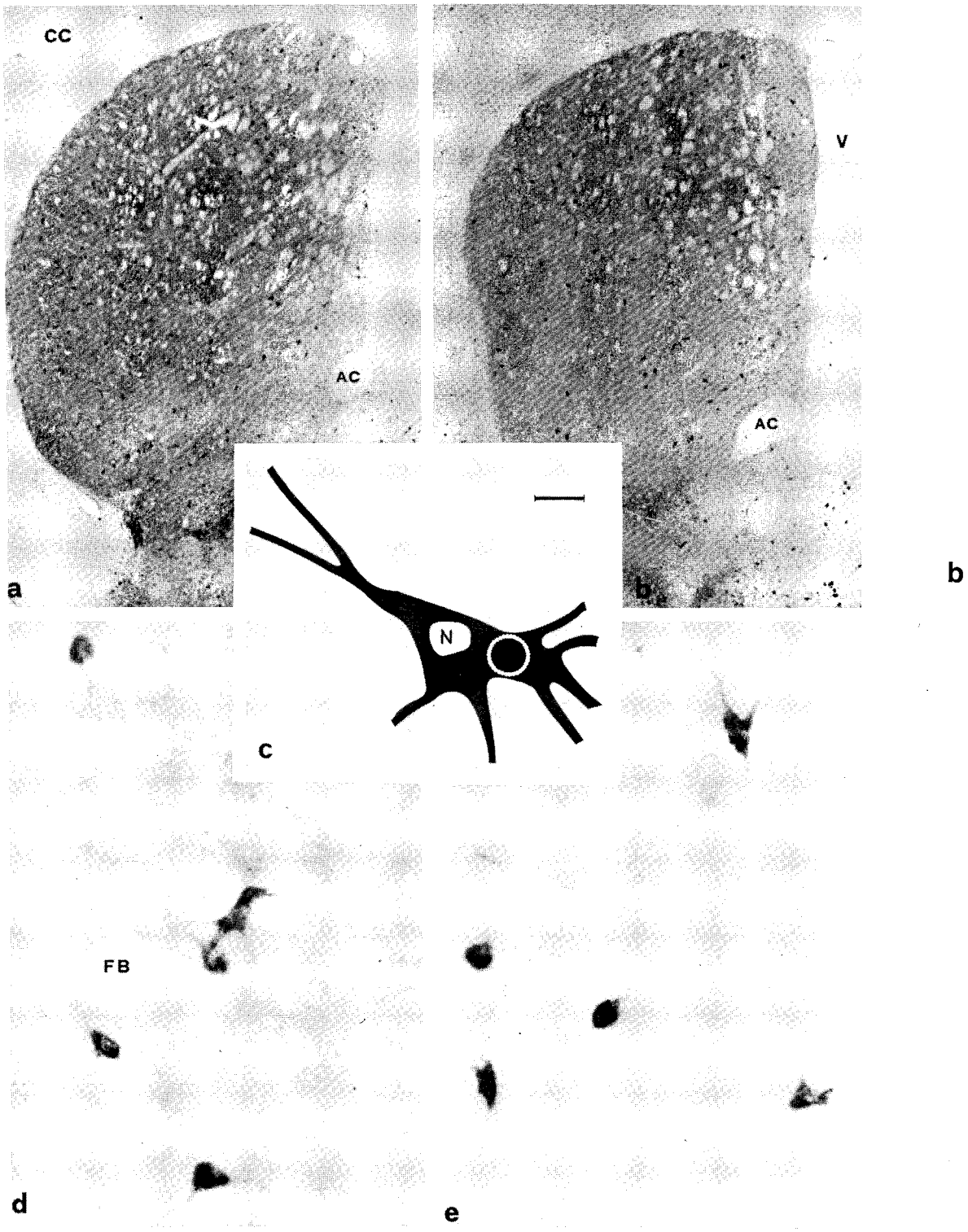

Fig. 2. AChE histochemistry performed 6 h after the administration of 3.5 mg/kg of DFP allows a clear visualization of perikarya in both C57BL/6 (right column) and DBA/2 mice (left column). a, b: low-power microphotographs of two corresponding brain sections, depicting the entire extent of neostriatum. c, camera lucida drawing of a large AChE-containing striatal neuron, from which somatal AChE activity has been assayed by centering the microphotometric diaphragm (white circumference) on the cytoplasm. d, e: high-power microphotographs of two corresponding striatal regions: it can be observed that AChE staining in the matrix is more intense in the DBA/2 strain (d). AC, anterior commissure; CC, cerebral cortex; FB, fiber bundle of the internal capsule; N, nucleus; V, lateral ventricle. Bar, for a and b, 90 μm; for c, 10 μm; for d and e, 30 μm.

TABLE I

Summary of mean values for microphotometric measurements of AChE in different neostriatal compartments

Number of samples in each animal and of experimental sessions are indicated in parentheses.

DFP pretreatment	*Striatal compartment*	*DBA/2 strain (mean value ± S.E.M.)*	*C57BL/6 strain (mean value ± S.E.M.)*	*P*
2 mg/kg	matrix	54.97 ± 1.33 (30,2)	61.85 ± 0.85 (30,2)	< 0.001
3.5 mg/kg	matrix	73.27 ± 0.91 (50,4)	80.86 ± 0.83 (50,4)	< 0.001
3.5 mg/kg	perikarya	55.90 ± 0.82 (50,4)*	52.68 ± 0.71 (50,4)**	< 0.001

* Significantly different from matrix in 3.5 mg/kg pretreated DBA/2 mice ($P < 0.001$)
** Significantly different from matrix in 3.5 mg/kg C57BL/6 mice ($P < 0.001$)

is a suitable means for quantifying AChE content in histochemically stained brain tissue. Although quantitative histochemistry of AChE has been previously described in the hippocampus[22] and in the cerebral cortex[24] to our knowledge this methodology has been applied here for the first time in sections prepared according to the pharmacohistochemical technique for AChE: this quantitative pharmacohistochemical procedure allows combination of the main advantages of biochemical and morphological techniques, as it provides a quantification of the enzyme content into microscopically defined striatal compartments. In fact, pretreatment with DFP is required in order to reveal the striatal AChE-containing neurons, which are not seen in pharmacologically untreated animals[6]. Due to the elevated concentration of AChE in the neostriatum of DBA/2 mice, 3.5 mg/kg of DFP (i.e., ca. half the LD_{50} value[21]) were required in order to obtain a clear morphological detail of striatal AChE-positive neurons in this strain. Microphotometric data allowed quantification of this dose-dependent reduction of extra-somatal AChE in neostriatum of both strains, by indicating an increase in light transmittance (Table I). Percent transmittance inversely correlates with AChE concentration in sample areas, because: (1) it has been shown that microphotometry of AChE is almost as sensitive in detecting enzyme activity as biochemical analysis[22]; (2) the amount of final precipitated products (i.e., essentially copper ferrocyanide) and, consequently, the intensity of staining, directly depend upon AChE activity[6].

The present findings complete earlier biochemical studies indicating that, in whole brain and in selected cortical regions, AChE activity is higher in DBA/2 than in C57BL/6 mice[11,19,23]. In addition, they are consistent with a previous report[10] indicating that acetylcholine turnover in the striatum (and in other forebrain territories) is higher in the DBA/2 strain. Finally, the present data are also supported by a recent report[21], showing that the LD_{50} value for DFP is significantly higher in DBA/2 than in C57BL/6 mice, and also that residual AChE after acute poisoning with DFP is higher in the striatum of DBA/2 mice. Taken together, previous biochemical and pharmacological reports suggest that DBA/2 mice contain more AChE in neural tissue than C57BL/6, and that they can resist to correspondingly higher doses of organophosphorus poisons. Such interpretation is also corroborated by the present findings, which show in fact that increasing doses of DFP (2–3.5 mg/kg) are capable to reduce AChE content in the neostriatal matrix of both strains, while each dose reduces AChE content in the neostriatum of C57BL/6 mice more than it does in the DBA/2 strain.

Interstrain comparisons of AChE content in different striatal compartments revealed that DBA/2 mice possess higher enzymatic levels both in somata and in the matrix. It is well known that AChE is synthesized in the perikaryon before being transported somatofugally into neuropil[5]; therefore, since staining in the matrix derives essentially from AChE contained into neuropil, the present data suggest that both AChE synthesis and its subsequent somatofugal transport are more active in striatal neurons of the DBA/2 strain. Accordingly, the metabolic (and, possibly, functional) activity of the AChE-containing neostriatal neurons may also be higher in DBA/2 than in C57BL/6 mice.

AChE in the striatal matrix derives mainly from the putatively cholinergic striatal AChE-containing

neurons, and in addition from the non-cholinergic AChE-containing mesencephalostriatal[8,14,16] and thalamostriatal[15] fibers. Thus, variations of AChE content in the striatal matrix depend upon interstrain differences (1) in the concentration of enzyme contained into striatal neurons, as shown in the present study, and (2) in the number of these neurons, which have been shown to be more numerous in the DBA/2 strain[3]; in addition, they may also indicate the presence of still unknown differences in the organization of AChE-containing mesencephalostriatal and thalamostriatal fibers (see, also, Ragsdale and Graybiel[20]).

Inhomogeneities in the staining pattern of striatal AChE have been shown both in the matrix and in somata. The first are present in different species, and are best seen when AChE histochemistry is performed in pharmacologically untreated animals[12,13,15]. This patchy staining is barely visible in DFP-treated rodents (see, also, Butcher and Hodge[7]), while some degree of uneven staining is observed in all sections. In our experiments we did not study areas of poor cholinesterase content, as we chose to measure only matricial regions displaying a rich and homogeneous AChE staining: transmittance values, obtained by applying such criterion, displayed a very low degree of variability (Table I). Heterogeneities of AChE staining in somata have been demonstrated in DFP-pretreated animals[25]. As reported above, only darkly staining perikarya, which represent putatively cholinergic interneurons, were analyzed in the present study. AChE-containing perikarya, as shown by means of Butcher's pharmacohistochemistry, are usually classified as lightly, moderately or intensely staining on a pure subjective basis (e.g., see Albanese and Butcher[1]; Butcher and Woolf[9]). This criterion, which relies mostly on the observer's experience, may probably be replaced by photometric determination of AChE, especially in those regions (such as the neostriatum) where the discrimination of staining intensity is more crucial.

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