ULTRACYTOCHEMISTRY OF CHOLINE ACETYLTRANSFERASE IN THE NEOSTRIATUM OF THE RAT BRAIN

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The localization site of choline acetyltransferase (ChAc) activity in the neostriatum of the rat brain was ultracytochemically examined. Two of six neuronal types showed ChAc activity, which had previously been reported to be medium-sized interneurons with the Golgi method and an electron microscope study. All types of neurons except Cajal's dwarf cell in the neostriatum were revealed to receive a bouton containing ChAc positive synaptic vesicles and to form an axo-somatic synapse. This evidence was compatible with findings reported elsewhere that all types of neurons in the neostriatum synthesize acetylcholinesterase. A hypothetical schema of neuronal network concerned with neurotransmitters in the neostriatum was proposed.

In the field of neuroscience there has long been a need for a good histochemical method which can clearly demonstrate the cholinergic system. A histochemical procedure for acetylcholinesterase (AChE) has been established, but the mere presence of AChE activity does not distinguish between cholinergic and cholinceptive neurons (31). There is little doubt that choline acetyltransferase (ChAc) is a far more relevant marker for cholinergic neurons than is AChE, because ChAc shows a very similar distribution to acetylcholine (ACh). Although some attempts to localize ChAc have been made enzyme histochemically (6, 9, 23) and immunohistochemically (18), an unequivocal method must be developed. Using a new procedure initiated by Ogawa *et al.* (1, 37), we reported on ChAc neurons in the neostriatum (19), which contains at least cholinergic interneurons (33) and cholinergic afferents (14, 41) from the parafascicular nucleus of the thalamus. In the present study 6 types of neurons and several types of synapses in the neostriatum are examined in detail for fine structural localization of ChAc.

MATERIALS AND METHODS

Fresh tissues were dissected from the caudate head of male Wistar albino rats. Small blocks of the tissues were incubated for 30 min at room temperature in a medium containing 60 mM phosphate buffer, pH 7.0, 0.6 mM acetyl Co A, 10 mM choline chloride, 3 mM sodium citrate, 3 mM cobalt chloride, 0.5 mM potassium ferricyanide, 1 mM tetraisopropylpyrophosphoramidate (cholinesterase inhibitor),

440 mM sucrose, and 0.7 ml distilled water, total volume 10 ml, final pH adjusted to 6.8–7.0. After incubation, sections were fixed in 1% buffered osmium tetroxide and processed for electron microscopy. During dehydration, block staining with uranium acetate was done. Thin sections with or without lead staining were viewed in a JEM T7. In the control study ChAc inhibitor, 3–10 mM *n*-hydroxyethyl-4-(1-naphthylvinyl) pyridinium bromide was used.

Degeneration study of the substantia nigra: Under nembutal anesthesia rats were held in a stereotaxic apparatus. Lesions were placed in the substantia nigra by passing 2 mA current for 10 sec. Coordinates to the Atlas of Pellegrino & Cushman (39) were Fig. 50, V –4, R 2.5 for the substantia nigra. The animals were used for ChAc ultracytochemistry 24 or 48 hr after the operation.

RESULTS

Reaction products indicating ChAc activity were observed in the plasma membrane, especially the synaptic membrane, occasionally in the mitochondria, and rarely in the Golgi apparatus, the endoplasmic reticulum and synaptic vesicles. The ChAc inhibitor, 5 mM *n*-hydroxyethyl-4-(1-naphthylvinyl) pyridinium bromide, destroyed the reaction products.

Two of 6 neuronal types in the neostriatum showed ChAc activity. Type I neuron (Fig. 1) has a round nucleus locating centrally. The karyoplasm has a felt-like appearance with some aggregates of chromatin granules. The cell body ranges up to 12 μm in diameter. The cytoplasm contains many mitochondria having ChAc activity, well developed Golgi apparatus, neurotubules and some dense bodies with or without droplets. A few, short granular endoplasmic reticula are scattered throughout the cytoplasm. This fine structural feature corresponds to the absence of Nissl bodies in light microscopy. The reaction products indicating ChAc are also seen in the cisternal structure and plasma membrane. This type of neuron is sometimes demonstrated to receive a bouton containing ChAc positive synaptic vesicles, forming an axo-somatic synapse. A bouton synapsing at the soma of type I neuron was found to degenerate after coagulation of the ipsilateral substantia nigra (Fig. 2).

Type II is a round neuron ranging from 10–15 μm in diameter (Fig. 3). The proportion of nucleus/cytoplasm is very large. The karyoplasm is electron lighter than the cytoplasm. There are many ChAc positive mitochondria and cisternae in the cytoplasm. Some short granular endoplasmic reticula exist without forming Nissl bodies. The dense bodies are the same nature as in type I. A nerve terminal with ChAc positive synaptic vesicles is sometimes observed to make a junction with the soma of type II neuron. In animals in which the substantia nigra had been destroyed 24 hr before preparation, a degenerated bouton making an axo-somatic synapse with this type of neuron was seen.

Type III is an ovoid or triangular, medium-sized neuron, ranging 8–14 μm in diameter (Fig. 4). The nucleus is ovoid and located centrally. Sometimes a clearly noticeable nucleolus exists in the relatively electron light karyoplasm. The granular endoplasmic reticula characteristically have a tendency to wind and pile up in stacks. A granular endoplasmic reticulum relates occasionally to the nuclear membrane. This type of neuron often situates near the myelinated axon bundle. Type III

neuron shows no ChAc activity, but becomes the postsynaptic site of an axo-somatic junction whose terminal contains ChAc positive synaptic vesicles.

Type IV is a neuron with an irregular polygonal cell body about $10 \times 6 \mu\text{m}$ in size (Fig. 5). The roundish nucleus has many indentations. Both the karyoplasm

FIG. 1. Type I neuron of the neostriatum. Reaction products indicating ChAc activity are seen in the mitochondria, the plasma membrane, cisternal structures and synaptic vesicles. The neuron forms an axo-somatic synapse with a nerve terminal containing ChAc positive synaptic vesicles (asterisk). $\times 7,000$

FIG. 2. Type I neuron showing ChAc activity. The experimental animal has undergone coagulation in the substantia nigra 24 hr before preparation. The neuron receives a degenerated nerve terminal, forming an axo-somatic synapse (arrow head). It may be regarded as a nigro-striatal dopaminergic bouton. $\times 12,000$

and the cytoplasm are relatively electron dense due to a rich content of cell organelles. Granular endoplasmic reticula are long and tortuous, and occasionally connect with the nuclear membrane. No ChAc activity was observable in this neuron. A ChAc positive bouton is found to arrive at its soma, making an axo-somatic synapse.

Type V is a large, fusiform neuron with a cell size of up to 40 μm in the long axis and 15 μm in the short axis (Fig. 6). A satellite microglia very often accompanies it. The neuron is usually located in the vicinity of a myelinated axon bundle. Sometimes a nucleolus exists. Nuclear indentations are occasionally observed. The cytoplasm contains large mitochondria, granular endoplasmic reticula forming stack groupings (when identified in glutaraldehyde-perfused materials) and many dense bodies, most of which are homogenous. Type V neuron shows no ChAc activity, but was observed to contact a bouton containing ChAc positive synaptic vesicles and to form an axo-somatic synapse.

It remains unknown whether type VI neuron, Cajal's dwarf cell, has ChAc activity or not.

In the neuropil several types of ChAc positive synapse were found. Some boutons containing ChAc positive synaptic vesicles have a varicose appearance, (Fig. 7). Most synapses containing ChAc positive synaptic vesicles have a smaller presynaptic site than postsynaptic dendrite (Fig. 8). They are characterized by round, uneven synaptic vesicles with a diameter of 40–80 nm. When identified in glutaraldehyde-perfused materials, it was seen that some synaptic vesicles are granular and no postsynaptic membranous thickening exists. In the postsynaptic dendrite large mitochondria are often seen. The symmetrical membranous contact of these ChAc positive synapses sometimes has constricted parts (Figs. 3, 4, 6). Cisternal structures are often seen in the vicinity of the postsynaptic membrane (Figs. 6, 8).

It was occasionally observed that a bouton containing ChAc positive synaptic vesicles makes an axo-spinous synapse, whose postsynaptic membrane also shows ChAc activity (Fig. 9). The bouton contains pleomorphic synaptic vesicles and one or two small mitochondria. The postsynaptic membranous thickening gives the appearance of an asymmetrical synaptic contact in glutaraldehyde-perfused materials. It is noteworthy that not all synaptic vesicles in these boutons show reaction products indicating ChAc activity.

The ChAc positive process is the postsynaptic site of an axo-spinous (Fig. 10),

FIG. 3. Type II neuron showing ChAc activity. The proportion of nucleus/cytoplasm is very large.

The neuron receives a bouton with ChAc positive synaptic vesicles at the soma(asterisk). $\times 15,000$ Inset: the axo-somatic synapse.

FIG. 4. Type III neuron having no ChAc activity. The ovoid cytoplasm contains more or less long and tortuous endoplasmic reticula. A nerve terminal with round synaptic vesicles, some of which show ChAc activity, makes a junction with the neuronal soma. The symmetrical synaptic membrane has constricted parts. $\times 7,400$ Inset: enlargement of the axo-somatic synapse.

FIG. 5. Type IV neuron with polygonal cytoplasm, which contains numerous ribosomes and other cell organelles, and appears electron dense. No ChAc activity is seen in the neuron, but the ChAc positive bouton contacts the soma, making an axo-somatic synapse (asterisk). $\times 25,000$

FIG. 6. Type V large, fusiform neuron in the neostriatum. The neuron shows no ChAc activity, while an adjacent type I neuron is ChAc positive. Type V neuron also receives a bouton containing ChAc positive synaptic vesicles (asterisk). $\times 5,200$ Inset: enlargement of the axo-somatic synapse.

FIG. 7. Varicose boutons with ChAc positive synaptic vesicles. $\times 26,000$

FIG. 8. An axo-dendritic synapse with round synaptic vesicles, most of which show ChAc activity. A large mitochondrion is seen in the postsynaptic dendrite. A cisternal structure is seen in the vicinity of the postsynaptic membrane (arrow). $\times 33,000$

FIG. 9. An axo-spinous synapse. The bouton which contains ChAc positive round synaptic vesicles and small mitochondria makes a junction with the dendritic spine whose plasma membrane also shows reaction products for ChAc. $\times 28,000$

or sometimes an axo-dendritic synapse (Fig. 11). The nerve terminal is compactly packed with uniformly round vesicles about 60 nm in diameter without ChAc activity. Usually no mitochondria are seen in the bouton. This type of synapse appears asymmetric in the glutaraldehyde-perfused materials.

FIG. 10. An axo-spinous synapse. The bouton is compactly filled with synaptic vesicles without ChAc activity. The reaction products for ChAc locate in the synaptic cleft, mainly, postsynaptic site. $\times 28,000$

FIG. 11. An axo-dendritic synapse. The bouton is of the same nature as that in Fig. 10. ChAc activity is postsynaptically seen. $\times 21,000$

FIG. 12. Mitochondria in a myelinated axon show ChAc activity. $\times 21,000$

A myelinated axon shows ChAc activity in some mitochondria (Fig. 12).

DISCUSSION

The pattern of subcellular localization of ChAc corresponds to that found in a

previous study on the rat cerebrum (37). In contrast to our study, Kása (24) detected enzyme activity with copper-lead-thiocholine method in ribosomes, and only in the outer surface of synaptic vesicles and of mitochondria. The controversy about the localization of ChAc in synaptic vesicles between DeRobertis *et al.* (8) and Whittaker *et al.* (43) might be due to species variation of the experimental animals (32). It seems that the presence of ChAc in synaptic vesicles is more acceptable evidence explaining the synthesis and uptake of ACh by vesicles (40).

Although the histochemical method used here demonstrates only the particulate form of ChAc, other methods for ChAc (18, 24) have also reported the soluble form as diffusely stained throughout the cytoplasm. According to Fonnum (10), the binding of ChAc to membrane was a reversible process primarily dependent on pH and ionic strength. The enzyme binds more easily to membrane as pH and ionic strength decrease, and *vice versa*. The emergence form of the enzyme in a histochemical demonstration is, therefore, thought to depend on the conditions of the incubation medium used. After reviewing recent studies on biochemistry of ChAc and ACh, Rossier (40) reopened the debate concerning the subcellular localization of ChAc. We were only able to demonstrate histochemically the enzyme localization on the cellular level, and the precise fine structural localization still requires further biochemical and histochemical investigation. In the present study we were able to estimate the specificity of histochemical reaction of ChAc, because; 1. A SH-inhibitor, *n*-hydroxyethyl-4(1-naphthylvinyl) pyridinium bromide, has counteracted the reaction products; 2. The reaction products selectively appeared in certain types of neuron and synapses, i.e., only in type I and II neuron.

Electron microscopical features of 6 neuronal types in the present study are correlated with those reported by other authors in Table 1. Most neostriatal neurons are type I (over 95%). A Golgi study (26) showed that this type is interneuron, having a short axon and many dendritic spines. Most reports, except one

TABLE 1. *Correlation of neuronal types in the neostriatum with the nomenclature of other authors*

Present Study	ChAc activity	Mori (36)	Kemp & Powell (26)	Fox <i>et al.</i> (11, 12)	Adinolfi (2)	Pasik <i>et al.</i> (38)
Type I medium	+	small nerve cell	medium spiny short axon	spiny neuron	small neuron	medium-sized neuron spiny I type
Type II medium	+		medium smooth long dendrite short axon			medium-sized neuron aspiny III type
Type III medium	—		medium long axon			medium-sized neuron spiny II type
Type IV submedium	—	large type II nerve cell?	medium varicose dendrite short axon	spidery aspiny neuron		medium-sized neuron aspiny I type?
Type V large	—	large type I nerve cell	giant long axon	large aspiny neuron	large neuron	large neuron aspiny II type
Type VI small	—		small	aspiny neuron with somatic spines		small neuron neurogliform

suggest that type I is intrinsic to the neostriatum (11, 26, 34). Recently, an electrophysiological study (30) combined with a horseradish peroxidase (HRP) technique confirmed that the caudate medium-sized spiny neuron receives monosynaptic excitatory inputs from the cerebral cortex, the intralaminar thalamus and the substantia nigra. In agreement with the present findings, an immunocytochemical study (18) in the neostriatum revealed type I neuron to have ChAc activity. Type II ChAc positive neuron has also been shown to be an interneuron in a Golgi study (26). Type III is considered to have a long efferent according to the Golgi method (26). This is probably a striatal neuron projecting to the substantia nigra, which Grofová (13) demonstrated to be a round or triangular medium-sized nerve cell with 4–6 thick dendrites, by means of retrograde axonal transport of HRP. Recently, Bak *et al.* (4) described a nerve cell similar to this type as an efferent neuron of the striato-nigral pathway, by using retrograde axonal flow of herpes simplex virus. We have electron microscopically observed HRP granules in the neuronal perikaryon of this type after injection in the substantia nigra (22). It can not be ascertained under the present conditions whether type III striato-nigral neuron contains GABA (29) or substance P (25). Type IV was determined to be a neuron with a short axon and varicose dendrite in a Golgi study (26). Some authors (5, 36, 38) described two types of large nerve cells in the neostriatum, but we could only identify one, namely type V. The Golgi method indicates that the large neuron has a long axon extending out of the neostriatum (26).

All I–V type neurons were demonstrated to receive a bouton containing ChAc positive synaptic vesicles and to form an axo-somatic contact. The evidence implies that all I–V types are cholinceptive. This is compatible with our other ultracytochemical study (20) showing that all types of neurons in the neostriatum synthesize AChE.

A varicose dendrite containing ChAc positive vesicles (Fig. 7) is compatible with varicose axon collaterals of interneurons in the caudate nucleus observed in a Golgi study (26, 38). Furthermore, the varicose bouton seems to correspond to intrinsic 'en passant' terminals forming both asymmetrical and symmetrical junctions which remained viable following deprivation of caudate tissue of all afferent connections (42).

Most synapses containing ChAc positive synaptic vesicles in the neostriatum belong to the axo-dendritic type shown in Fig. 8. This type is equivalent to the intrinsic synapse demonstrated by isolation of the caudate nucleus (symmetrical type of Tennyson *et al.* (42), and type IX bouton of Hassler *et al.* (16)). The postsynaptic site of the ChAc positive axo-dendritic junction was seen occasionally to contain a very large ovoid mitochondrion (Fig. 8), the presence of which Kemp and Powell (27) considered as a sign indicating that the dendritic process belongs to a large efferent neuron in the caudate nucleus. A cisternal structure in the vicinity of the postsynaptic membrane of the ChAc positive axo-dendritic synapse (Fig. 8) is thought to be a localization site for AChE.

A ChAc positive axo-spinous synapse shown in Fig. 9 appears similar to the type IV boutons of Hassler *et al.* (7). They considered that this type is a bouton from the centro-median-parafascicular complex of the thalamus, which is suggested to be cholinergic (41) and to terminate on spines of a medium-sized neuron (28, 30) (type I ChAc positive neuron in the present study). If this is the case, the presence

of ChAc activity in the pre- and postsynaptic site may represent extrinsic ACh-intrinsic ACh connection in the neostriatum (Fig. 13).

It was reported that the striatal ChAc level was reduced 40–70% seven days after unilateral coagulation of the centro-median nucleus of the thalamus (14). The authors assumed that about 60% of the ChAc in the neostriatum depends on the thalamo-striatal pathway. This speculation does not accord with the present observation that the ChAc positive axo-spinous synapse regarded as thalamo-striatal terminal is less frequently observed.

ChAc activity was only postsynaptically found in some synapses (Figs. 10, 11). Axo-spinous (Fig. 10) and axo-dendritic (Fig. 11) synapses correspond to type III and VII boutons of Hassler *et al.* (17), respectively. They mentioned that these two types of bouton undergo a degeneration after cerebral ablation. A morphometric study with Golgi method (27) and a neurophysiological investigation combined with HRP technique (30) confirmed that caudate medium-sized interneurons which were demonstrated to have ChAc activity in the present study receive afferents from the cerebral cortex on the dendritic spines. This evidence means that cortico-striatal fibers terminate on cholinergic interneurons in the same fashion as axo-spinous and axo-dendritic asymmetrical synapses.

The result of the present study with nigral lesions that type I and II ChAc positive neurons receive degenerated terminals establishes morphologically the dopamine-Ach connection in the neostriatum (21). As mentioned above, type I ChAc positive neuron is the postsynaptic site not only of the nigro-striatal dopamine pathway but also of cortico-striatal and thalamo-striatal projection. This evidence

FIG. 13. A hypothetical schema of neuronal network concerned with neurotransmitters in the neostriatum.

makes it reasonable to find dopamine receptor-related enzyme, cyclic nucleotide phosphodiesterase activity, at sites postsynaptic to the degeneration terminals of the cortico-striatal and thalamo-striatal pathway (3).

The present study has shown that type I and II neurons have ChAc activity and receive simultaneously boutons containing ChAc positive synaptic vesicles. Because the thalamo-striatal cholinergic fibers form an axo-spinous (type IV of Hassler *et al.* (15)) and in part an axo-dendritic synapse (type VII of Hassler *et al.* (15)), the cholinergic axo-somatic synapse seen in the present study can possibly be regarded as intrinsic. These findings prompt the speculation that type I and II neurons connect reciprocally in cholinergic transmission (Fig. 13). This hypothesis is supported by a neurophysiological finding (35) that stimulation of the substantia nigra not only activates neostriatal input cells, but also interneurons, which in turn inhibit input cells. A hypothetical neuronal network in the neostriatum concerned with neurotransmitters is summarized in Fig. 13.

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