Choline Acetyltransferase-Containing Neurons in Rodent Brain Demonstrated by Immunohistochemistry

Abstract. *Choline acetyltransferase was demonstrated in neuronal structures of the rodent central nervous system by immunohistochemistry through the application of Fab fragments obtained from monospecific antiserums to human choline acetyltransferase. The specificity of the antiserum for the enzyme was confirmed by the staining of both the ventral horn motor neurons in the rat spinal cord and the neuromuscular junction of the guinea pig diaphragm. Enzyme-containing cell bodies were observed in frontal sections of rat and guinea pig brain in the neostriatum, accumbens, nucleus of the diagonal band, medial septum, and olfactory tubercle. Positively staining fibers and probable nerve terminals were also found in the olfactory tubercle field and other areas of the basal forebrain. The results provide information on the distribution of the cholinergic systems in the rostral forebrain of the rodent.*

Acetylcholine (ACh) was the first neurotransmitter to be discovered. Little is still known about the precise distribution of cholinergic structures in the central nervous system (CNS) and there is no good histochemical technique to demonstrate the presence of ACh. Acetylcholinesterase (AChE) has been demonstrated histochemically and used as a marker for ACh in the brain (*1*). Choline acetyltransferase (CAT) (E.C. 2.3.1.6), however, is a more specific marker for cholinergic neurons than AChE (*2*). When antibodies to CAT were used previously in immunohistochemical studies, the enzyme appeared to be localized in certain neuronal cell bodies, fibers, and possibly terminals in the human brain (*3*).

We have now studied the distribution of CAT in the rostral forebrain of rat and guinea pig by the peroxidase-antiperoxidase (PAP) method (*4*). We have also obtained results, which are in good agreement with those of previous studies (*5*), on the distribution of CAT in the ventral horn motor neurons and shown that specific staining for CAT occurs at the neuromuscular junction.

Some of the pathways in which ACh is believed to be used include (i) ventral horn cells to all voluntary muscles, (ii) neostriatal interneurons, (iii) the habenulo-interpeduncular tract (which may also be derived from the nucleus diagonal band of Broca), and (iv) the septohippocampal pathway. Therefore, prior to mapping the whole brain, we attempted to stain CAT immunohistochemically in the rostral part of the forebrain and in the spinal cord, areas that are concerned with all of the above pathways.

The purification of CAT from human striatal tissue and the preparation of monospecific antibodies to CAT in rabbits is described elsewhere (*6*). The antibodies cross-react with CAT from several other mammalian species. Fab fragments were prepared from the CAT antiserums by previously reported methods (*7*). Cryostat sections of tissues fixed in paraformaldehyde and glutaraldehyde were incubated with the Fab fragments, then the tissues were rinsed and incubated first with goat antibody to rabbit

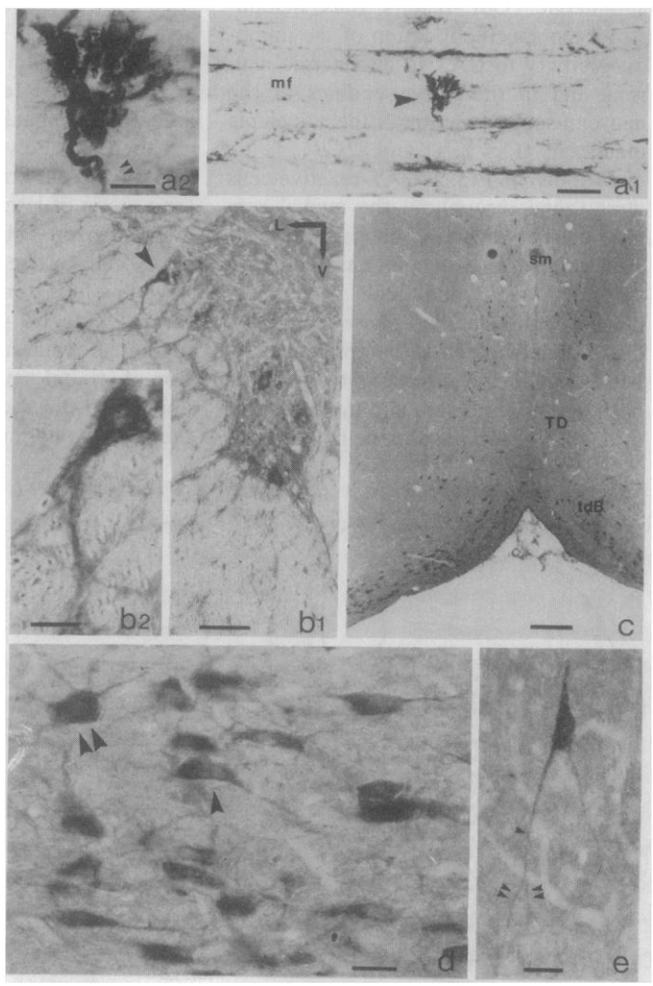

Fig. 1. Immunohistochemical micrograph of CAT stained by the PAP technique. (a1) Low power magnification of CAT-positive staining at the neuromuscular junction (arrowhead) of guinea pig diaphragm (*mf*, muscle fibers). Bar indicates 25 μm. (a2) Higher power magnification of neuromuscular junction of a1. The axon is shown (double arrowhead) just anterior to the nerve endings. Bar indicates 10 μm. (b1) Low power magnification of the ventral horn of the rat spinal cord. Motor neurons are positively stained. The stained reticular network surrounding the cells in the background probably represents cellular processes (*L*, lateral; *V*, ventral). Bar indicates 100 μm. (b2) Higher power magnification of the same cell as in a1 (arrowhead). The nucleus of CAT-staining perikaryon does not stain positively, but the axon passing through the anterior white matter is stained. Bar indicates 25 μm. (c) The basal midline part of a coronal section through the septal nuclei complex of the rat brain. The section shows CAT-positive neurons of the medial septal nucleus (*sm*) and diagonal band of Broca (*tdB*). The positively stained neurons in the area of the diagonal band occur mainly in the basal part of the tdB, which is adjacent to the ventral surface of the brain; few such cells are found in the tractus diagonalis (*TD*). Bar indicates 250 μm. (d) Higher power magnification of the CAT-staining cells in the tdB. No staining is seen in nuclei of these neurons (single arrowhead). The majority of these neurons have large cell bodies with few processes, but large multipolar cells are also occasionally found (double arrowheads). Bar indicates 25 μm. (e) A typical CAT-positive neuron in the guinea pig neostriatum. (Identical results were obtained in the rat neostriatum.) Few long dendrites (single arrowhead) with few branches (double arrowheads) are seen tapering off rapidly from the large cell body. Bar indicates 25 μm.

0036-8075/80/0530-1057$00.50/0

immunoglobulin G and then with PAP (with the antibody being prepared in a rabbit). After each incubation period the sections were washed with phosphate-buffered saline. The tissue-bound PAP was visualized by placing it in a 3,3′-diaminobenzidine solution containing hydrogen peroxide in tris buffer. In control experiments, serum from nonimmunized rabbits and Fab fragments from antibodies to CAT that had been treated with purified human CAT were used instead of the Fab fragments in the above procedure. No positive staining was obtained in these control experiments (*8*).

In the rat spinal cord intense positive reactions occurred in cells of the ventral horn motor neurons (Fig. 1, b1 and b2); no positive-staining cells were found in the apex and head of the dorsal gray column. The nuclei of CAT-staining perikarya in the anterior horn did not stain positively, but the axon bundles of these cells that pass through the anterior white matter on their way to form the ventral roots did show positive staining. These CAT immunoreactive motor neurons, which stain strongly for cholinesterase and have been visualized in CAT histochemical (*9*) and previous immunohistochemical studies (*3*, *5*), are known to be cholinergic. Our results thus demonstrate the specific location of the immunoreactivity to CAT in cholinergic neurons and in the nerve endings at the neuromuscular junction of the guinea pig diaphragm (Fig. 1, a1 and a2).

As shown in Fig. 2, CAT-reactive cells were found throughout the medial septal nucleus, diagonal band area, neostriatum, nucleus accumbens, olfactory tubercle region, and fields of the medial forebrain bundle, These cells were distributed somewhat heterogeneously, with greater concentrations being found both in the septal area and the basal part of the diagonal band and the highest concentrations being found in the diagonal band area adjacent to the ventral surface of the brain (Fig. 1, c and d). The CAT-reactive neurons in the diagonal band region were characterized by their large perikarya (> 25 μm) with few dendritic branches. These cells were usually intensely stained, their axonal processes occasionally being visible (Fig. 1d). In the central portion of the medial septal nucleus, CAT-staining neurons were less densely packed and were distributed in the area bounded by the cells of the septal part of the diagonal band (Fig. 1c). The processes of these neurons and the fibers passing through this cell group were always aligned dorsoventrally. CAT-containing neurons of the medial septum extended ventrally and laterally and seemed to form a group of cells continuous with those in the nucleus of the diagonal band (Fig. 1c).

The CAT-reactive neurons in the neostriatum and nucleus accumbens had similar if not identical morphological features, with large (> 25 μm) cell bodies with few long dendrites showing few spines and few branches (Fig. 1e). Positively reacting cells were scattered evenly in the gray matter of the striatum and nucleus accumbens. The density of such cells in the nucleus accumbens appeared to be less than that in the striatum. The striatum also appeared to contain dense, fine terminals that gave the area a diffuse background stain.

Kemp and Powell (*10*) classified neurons in the cat striatum into six groups (one group of large cells, four groups of medium-sized cells, and one group of small cells). The giant neurons which have few long dendrites with few spines forms less than 1 percent of the cell population. If we assume that the classification of striatal neurons in the cat can be applied to the rodent we conclude that the CAT-containing neuron observed in the present study belongs to the so-called "aspiny" large-cell type. These results agree with those we obtained in a CAT immunohistochemical study on human brain (*5*) and with those obtained with the use of diisopropylfluorophosphate for AChE histochemistry (*11*). However, there are some discrepancies between previous results from histochemical and immunohistochemical studies on CAT and the present results, since the previous studies (*12*) suggested that some medium-size spiny neurons in the neostriatum may also contain CAT. However, recent evidence indicates that the striatal efferents are derived from one population of medium-sized spiny cells (*13*), while the large neurons, which were at one time thought to be striatal efferent neurons, are probably interneurons (*11*). Inasmuch as the cholinergic neurons in the neostriatum are believed to be intrinsic (*14*), it is reasonable to consider that large CAT-containing aspiny neurons are some of the intrinsic cholinergic neurons of the striatum.

A small number of CAT-positive cells were also present in the olfactory tubercle region and the area of the medial forebrain bundle. In the former area the positive cells were localized mainly in the lamina pyramidalis (pars corticalis) of the olfactory tubercle and distributed laterally to the olfactory tract. Positively stained cells were rarely observed in the lamina plexiformis (pars corticalis) of the olfactory tubercle. Although few positive cells were found in the islands of Calleja, numerous CAT-positive dots, assumed to be nerve terminals, were observed in this structure as well as in the olfactory tubercle. In the medial forebrain bundle a few CAT-reactive neurons were seen in the gaps between the fiber bundles.

In other areas, such as the neo- and piriform-cortex in the anterior-posterior

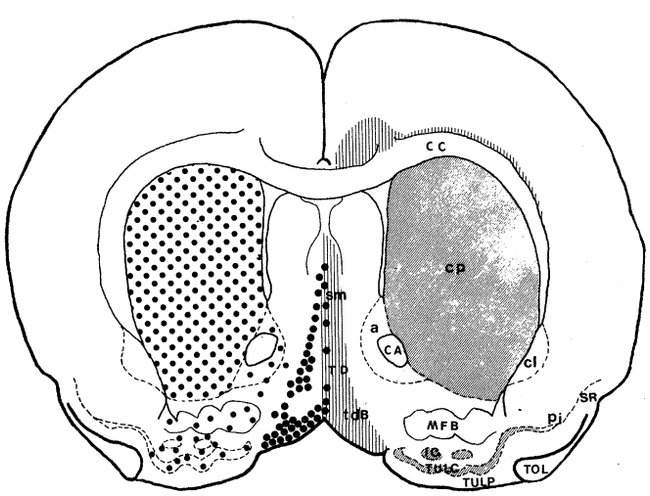

Fig. 2. Mapping of CAT-containing neurons in the rostral forebrain of the rat. The distribution pattern of CAT-positive neuronal structures of the rat is identical with that of the guinea pig at this anterior-posterior plane. The CAT-positive cell bodies (dots), fibers (vertical lines), and probably terminals (diagonal shading) are represented. The relative densities of the cells are designated by the abundance of dots. Abbreviations: *a*, nucleus accumbens; *CA*, commisura anterior; *CC*, corpus callosum; *cl*, claustrum; *cp*, nucleus caudates putamen; *IC*, insulae Calleja; *MFB*, medial forebrain bundle; *pi*, cortex piriformis; *sm*, nucleus medialis septi; *SR*, sulcus rhinalis; *tdB*, nucleus tractus diagonalis Broca; *TD*, tractus diagonalis Broca; *TOL*, tractus olfactorius lateralis; *TULC*, tuberculum olfactorium, pars corticalis, lamina pyramidalis; and *TULP*, tuberculum olfactorium, pars corticalis, lamina plexiformis.

plane shown in Fig. 2, we could not detect any positive cell staining, but a few fibers appeared in the indusium griseum which covers the superior surface of the corpus callosum. Some of these fibers pass parallel with the dorsal corpus callosum; others radiate, rapidly taper off, and fade away into the cingulate cortex.

We also examined the CAT-positive neurons of the guinea pig and found their distribution and morphological features to be similar to those of the rat.

Our results indicate that CAT, a reliable marker for cholinergic neurons, occurs in certain neurons of the rostral forebrain as well as the spinal cord. Cell bodies, fibers, and probably nerve terminals were stained. These findings in the rostral forebrain provide the morphological basis for the view that ACh may be the transmitter substance in some of the neuronal pathways postulated as a result of biochemical, neurophysiological, and histochemical studies (*2*). CAT-reactive cells in the medial septum and a part of the diagonal band seem to be the source of the cholinergic septo-hippocampal pathway. Some of the positive neurons in the diagonal band may be the origin of part of the presumed cholinergic pathway to the interpeduncular nucleus via the fasciculus retroflexus of Meynert. The morphological evidence for these pathways was obtained by testing histochemically for the presence of AChE, but that method is not a sufficient criterion for the identification of cholinergic neurons. Our results strongly indicate that these pathways are truly cholinergic. Cholinergic pathways originating from cells in the area of the olfactory tubercle and medial forebrain bundle have not yet been reported, but may be part of the sources for the cholinergic input to the olfactory bulb. It is noteworthy that those areas that contain a rich distribution of CAT terminals are also well known to receive a dense dopaminergic innervation.

HIROSHI KIMURA, PATRICK L. MCGEER
FRANK PENG, EDITH G. MCGEER
Kinsmen Laboratory,
Department of Psychiatry,
University of British Columbia,
Vancouver, Canada V6T 1W5

References and Notes

1. M. J. Karnovsky and L. Roots, *J. Histochem. Cytochem.* **12**, 219 (1964); G. B. Koelle, *J. Comp. Neurol.* **100**, 211 (1964).
2. P. L. McGeer and E. G. McGeer, in *Nutrition and the Brain*, A. Barbeau, J. H. Growdon, R. J. Wurtman, Eds. (Raven, New York, 1979), vol. 5, pp. 177–199.
3. H. Kimura, E. G. McGeer, P. L. McGeer, F. Peng, *Neurosci. Abstr.* **5**, 240 (1979).
4. L. A. Sternberger, P. H. Hardy, J. J. Cuculis, H. G. Myer, *J. Histochem. Cytochem.* **18**, 315 (1970).
5. P. L. McGeer, E. G. McGeer, V. K. Singh, W. H. Chase, *Brain Res.* **81**, 373 (1974); K. S. Kaaren-Kan, L. P. Chao, L. F. Eng, *ibid.* **146**, 221 (1978).
6. J. H. Peng, P. L. McGeer, H. Kimura, S. C. Sung, E. G. McGeer, *Neurochem. Res.*, in press.
7. S. Hjerten, *Biochim. Biophys. Acta* **31**, 216 (1959); R. R. Porter, *Biochem. J.* **73**, 119 (1959).
8. For the fixation and staining procedures, see H. Kimura, E. G. McGeer, J. H. Peng, P. L. McGeer, in preparation.
9. A. M. Burt and A. Silver, *Brain Res.* **62**, 509 (1973).
10. J. M. Kemp and T. P. S. Powell, *Philos. Trans. R. Soc. London Ser. B* **262**, 383 (1971).
11. J. Lehmann, H. C. Fibiger, L. L. Butcher, *Neuroscience* **4**, 217 (1979).
12. T. Hattori, V. K. Singh, E. G. McGeer, P. L. McGeer, *Brain Res.* **102**, 164 (1976); H. Kaiya, T. Iwata, M. Namba, Y. Ago, H. Mayahara, K. Ogawa, *Exp. Brain Res.* **34**, 395 (1979).
13. J. D. Kocsis, R. J. Preston, S. T. Kitai, *Neurosci. Abstr.* **2**, 65 (1976); A. M. Grabiel, C. W. Ragsdale, S. M. Edley, *Exp. Brain Res.* **34**, 189 (1979); B. S. Bunney and G. K. Aghajanian, *Brain Res.* **117**, 423 (1976); I. Kanazawa, G. R. Marshall, J. S. Kelley, *ibid.* **115**, 485 (1976).
14. P. L. McGeer, E. G. McGeer, H. C. Fibiger, V. Wickson, *Brain Res.* **35**, 308 (1971).
15. This work was supported by the Medical Research Council of Canada. We thank S. J. Suzuki for excellent technical assistance and S. R. Vincent and H. C. Fibiger for reading the manuscript.

5 November 1979

M-Statistics and Morphometric Divergence

Cherry, Case, and Wilson (*1*) compared morphological divergence between humans and chimpanzees to that of various taxa of frogs. They suggested that morphological differences between humans and chimpanzees were greater than between suborders of frogs although the divergence in structural genes in humans and chimpanzees is known to be small (*2*). Cherry *et al.* concluded that morphological evolution and biochemical evolution in structural genes can proceed at independent rates. Their work warrants scrutiny in view of its importance to current dialogue in evolutionary biology.

The distance statistic used by Cherry *et al.* to estimate the degree of morphological divergence may give erroneous results. Humans and chimpanzees may be so different morphologically that the results reported by Cherry *et al.* would also be obtained if the widely recommended Mahalanobis (*3*) distance were used. In view of the problems described below these data should be reanalyzed.

Cherry *et al.* used nine seemingly comparable continuous characters measured on frogs, humans, and chimpanzees. Each measure was expressed as a fraction of the combined length of all nine measurements; differences between means of the scaled variables were divided by the standard error of the difference between the means and the results for all characters were summed. Cherry *et al.* defined this M statistic as the average number of standard deviations by which two taxa differ.

An important deficiency of the M statistic is that it ignores correlations between characters. Multidimensional "divergence" statistics such as the M statistic must incorporate information not only about the means and variances of the characters but also about the intercorrelations between characters. In biologically divergent taxa, one would expect not only differences in means and variances but also in correlations as well. Serious statistical mistakes as well as biological misinterpretations may result from ignoring intercorrelations in morphometric data.

To understand the effect of character correlations, consider the Pythagorean distance (D_P^2) for characters X_1 and X_2 and taxa A and B

$$D_P^2 = d_1^2 + d_2^2 \quad (1)$$

where

$$d_1^2 = (\bar{X}_{1A} - \bar{X}_{1B})^2 \text{ and } d_2^2 = (\bar{X}_{2A} - \bar{X}_{2B})^2 \quad (2)$$

Equation 1 can be rearranged to produce

$$D_P^2 = d_1^2 + d_1^2 f^2 \quad (3)$$

where $f = d_2/d_1$. The Pythagorean distance does not take into account intercorrelations between characters. A distance measure that accounts for intercharacter correlations is the Mahalanobis distance (*3*). If r is the nonzero within taxon correlation between X_1 and X_2, then the Mahalanobis distance for the two-dimensional case can be written as

$$D_M^2 = d_1^2 + \frac{d_1^2(f - r)^2}{1 - r^2} \quad (4)$$

Figure 1 shows the change in D_M^2 as r varies from -0.9 to 0.9 for various positive values of f. Figure 1 indicates that, when d_1 and d_2 are positive, negative correlation always increases the distance; however, positive correlations can have a manifold effect in that they augment the distance in some instances and decrease it in others.

Distance statistics that do not account for intercorrelated characters have been proposed in the past for morphometric data; for example, the coefficient of racial likeness of Pearson (*4*) which is very similar to the M statistic. However, these methods have also been heavily criticized by statisticians since the 1930's (*3*, *5*–*8*). Fisher (*5*, p. 62) stated that "the effect [of not accounting for intercorrela-

0036-8075/80/0530-1059$00.50/0