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ACETYLCHOLINESTERASE KNOCKOUTS ESTABLISH CENTRAL CHOLINERGIC PATHWAYS AND CAN USE BUTYRYLCHOLINESTERASE TO HYDROLYZE ACETYLCHOLINE

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Abstract—Acetylcholinesterase is one of the most prominent constituents of central cholinergic pathways. It terminates the synaptic action of acetylcholine through hydrolysis and yields the choline moiety that is necessary for transmitter recycling. Despite these pivotal relationships, mice nullizygous for acetylcholinesterase established all principal anatomical components of central cholinergic pathways. No compensatory increase in the distribution of butyrylcholinesterase was detected. However, both the wild-type and nullizygous mice showed that butyrylcholinesterase enzyme activity extended to all parts of the brain receiving cholinergic innervation and that it could hydrolyze the acetylcholine surrogate acetylthiocholine. As opposed to acetylcholinesterase which was mostly of neuronal origin, butyrylcholinesterase appeared to be mostly of glial origin.

These experiments lead to the unexpected conclusion that acetylcholinesterase is not necessary for the establishment of cholinergic pathways. They also show that butyrylcholinesterase can potentially substitute for acetylcholinesterase and that this enzyme is likely to play a constitutive (rather than just back-up) role in the hydrolysis of acetylcholine in the normal brain. The inhibition of butyrylcholinesterase may therefore provide a desirable feature of cholinergic therapies, including those aimed at treating Alzheimer's disease. © 2002 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: basal forebrain, Alzheimer's disease, cerebral cortex, striatum, choline acetyltransferase.

Cholinesterases are ubiquitous constituents of cholinergic pathways. They are necessary for terminating the synaptic action of acetylcholine (ACh) through catalytic hydrolysis. The inhibition of cholinesterases is the major goal of cholinoactive drugs prescribed for Alzheimer's disease, neurotoxins used as insecticides, and nerve gases intended for chemical warfare. When used therapeutically, the purpose of cholinesterase inhibitors is to make more ACh available for neurotransmission. When used as neurotoxins, their purpose is to cause lethal cholinergic hyperactivity (Silver, 1974).

The human CNS contains two cholinesterases: acetylcholinesterase (AChE), encoded by a gene on chromosome 7 (7q22), and butyrylcholinesterase (BChE), encoded by a gene on chromosome 3 (3q26.1–q26.2). AChE (EC 3.1.1.7) is the more prominent of the two, and the only one consistently associated with cholinergic pathways. Cholinergic neurons, identified by the presence of choline acetyltransferase (ChAT), display an AChE-rich enzyme activity in the perikaryon, proximal dendrite, and axon. The only known exception occurs in the rodent cerebral cortex where ChAT-positive (cholinergic) bipolar neurons are not AChE-rich (Levey et al., 1984). Many non-cholinergic (that is ChAT-negative) but cholinoceptive neurons also display an AChE-rich pattern. In contrast to cholinergic neurons where high levels of AChE extend into the axon, the intense AChE activity of non-cholinergic cholinoceptive neurons remains confined to the dendrites and perikarya (Mesulam and Geula, 1992).

BChE (EC 3.1.1.8) is present in much lower concentrations than AChE and is usually thought to have a much more restricted neuronal distribution in the CNS. The number of BChE-rich neurons in the human cerebral cortex is approximately two orders of magnitude less that the number of AChE-rich neurons (Mesulam et al., 1995; Darvesh et al., 1998; Mesulam, 2000). Enzyme histochemistry and immunohistochemistry with monoclonal antibodies indicate that these BChE-rich neurons are found mostly in layer 6 and in the immediately adjacent juxtacortical region containing the U-fibers (Mesulam et al., 1995). The BChE-rich neurons

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Abbreviations: ABC, avidin–biotin–peroxidase complex; ACh, acetylcholine; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; ChAT, choline acetyltransferase; DAB, 3,3′-diaminobenzidine; KF, Koelle–Friedenwald; NADPHd, reduced nicotinamide adenine dinucleotide phosphate diaphorase; PBS, phosphate-buffered saline.

of the cerebral cortex are ChAT-negative and therefore non-cholinergic (Mesulam, 2000). There is almost no axonal BChE in the cerebral cortex. The physiological role of BChE remains unknown since butyrylcholine is not a natural constituent of the brain and since individuals with enzymatically silent forms of BChE do not display any impairment unless challenged by neuromuscular blocking agents, such as succinylcholine (Silver, 1974).

The nearly invariant intensity of AChE activity along cholinergic pathways, the fact that no mechanism other than hydrolysis exists for terminating the action of ACh, and the toxicity of irreversible AChE inhibition would suggest that AChE plays a indispensable role in cholinergic neurotransmission and that its absence would have a devastating impact on the organism. Is the absence of AChE compatible with the development of cholinergic pathways? Does an absence of AChE lead to compensatory changes in BChE? The recent availability of AChE nullizygous (knockout) mice (Xie et al., 1999, 2000) allowed these questions to be addressed. The resultant observations suggest that current concepts concerning AChE and BChE are in need of substantial revision.

EXPERIMENTAL PROCEDURES

Genotype and phenotype

As described previously (Xie et al., 1999), expression of AChE was disrupted by homologous recombination in embryonic stem cells. The mice homozygous for the mutant AChE gene (as determined by the examination of genomic DNA by Southern blotting and polymerase chain reaction) had no detectable AChE enzyme activity or identifiable AChE bands on polyacrylamide gels (Xie et al., 2000). Initial descriptions of the phenotype were reported by Xie et al. (2000). Additional information has been collected since then. The nullizygous mice are indistinguishable from littermates at birth but show a delay of growth in weight and size. Their eyes open 2 days after littermates and thermoregulation is delayed. The nullizygous mice have a fine tremor, decreased grip strength, and an odd gait. Males have sperm but do not mount females. They cannot eat solid food and need to be fed a liquid diet. Gastrointestinal hypomotility and bloating are common. With care to feeding, the nullizygous mice can live for up to 220 days. Death occurs from ileus or startle-induced seizures. Mice heterozygous for the AChE gene are indistinguishable from the wild-type. The colony of mice is maintained at the University of Nebraska Medical Center (Omaha, NE, USA). Animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory animals as adopted by the National Institutes of Health of the United States of America. All animal procedures were reviewed and approved by the University of Nebraska's Institutional Animal Care and Use Committee. All efforts were accordingly made to minimize the number of animals used and their suffering.

Fixation and histology

The studies reported here were based on the brains of 12 mice, four wild-type (two male/two female), five nullizygous (knockout, three male/two female) and three heterozygous (one male/two female). Ten were 32-day-old, and two were 52-day-old mice (one each of wild-type and nullizygous). Brains from the 10 32-day-old mice were collected and fixed by immersion in 4.0% fresh paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at pH 7.4 and 4°C for 21 h. The brains from the two 52-day-old mice were perfused *in situ* for 20 min with the same fixative followed by PBS perfusion. All brains were then placed in ascending concentrations (10–40%) of sucrose in the same buffer, frozen in dry ice, and cut at 30–40 μm in the coronal plane. Cresyl Violet was used as a stain for Nissl substance. Each wild-type animal was matched to at least one nullizygote for age, gender and method of perfusion and was processed at the same time as its match.

AChE histochemistry

Two modified methods were used for obtaining a histochemical reaction product at sites of AChE enzyme activity, one based on the Koelle–Friedenwald (KF) method and the other on the Karnovsky–Roots (KR) direct coloring method (Koelle and Friedenwald, 1949; Karnovsky and Roots, 1964). All staining was done on sections dried on gelatin-coated glass slides. The KF method yields a black reaction product at sites of cholinesterase activity and provides excellent macroscopic identification of areas with high enzyme activity. It is also very effective for the identification of cholinesterase-positive cell bodies. The KR method yields a blue reaction product and provides substantially improved microscopic detail of cholinesterase-positive axons. In these experiments, we employed previously described modifications of both methods (Mesulam and Geula, 1991). Acetylthiocholine iodide (8×10^{-3} M for the KF procedure and 3.6×10^{-4} M for the KR procedure) was used as the substrate with ethopropazine (2×10^{-4} M for the KF procedure and 2×10^{-5} M for the KR procedure) as the inhibitor of non-specific cholinesterases (Silver, 1974). The enzymatic specificity of this reaction is demonstrated by the fact that no reaction product is obtained when the incubation medium contains the selective AChE inhibitor BW-284C51 at 10^{-3}–10^{-2} M (Silver, 1974). All chemicals were purchase from Sigma Chemicals (St. Louis, MO, USA).

For the KF procedure, sections were rinsed six times (30 s each) in 0.1 M acetate buffer at pH 5.5, and incubated for 16 h at room temperature (on a shaker) in a solution consisting of 2.1×10^{-4} M ethopropazine, 1.0×10^{-3} M glycine, 2.0×10^{-3} M cupric sulfate, and 0.05 M sodium acetate ($NaC_2H_3{\cdot}3H_2O$) in distilled water. Following the enzymatic incubation, the slides were washed six times (30 s each) in the acetate buffer and placed for 1 min in a developer consisting of 0.16 M sodium sulfide ($Na_2S{\cdot}9H_2O$) in 0.1 M HCl (resultant solution adjusted to pH 7.8 by titrating with 10 N HCl). Sections were rinsed again six times (30 s each) in the acetate buffer and placed in an intensification solution of 1% (w/v) aqueous silver nitrate ($AgNO_3$). The sections were rinsed, dehydrated in graded ethanols, cleared in xylene, and coverslipped with Permount.

For the KR procedure, sections were rinsed for 8 min in 0.1 M maleate buffer at pH 8. They were then incubated for 15 min at room temperature (on a shaker) in a solution consisting of 2.1×10^{-5} M ethopropazine, 5.0×10^{-4} M sodium citrate, 3.0×10^{-4} M cupric sulfate, 5.0×10^{-5} M potassium ferricyanide and 0.1 M maleic acid in distilled water. The solution was titrated to pH 8.0. Following an incubation of 15 min, the sections were rinsed for 8 min in 0.1 M Tris buffer at pH 7.6 and placed for 10 min in an intensification solution containing 3.4×10^{-4} M cobalt chloride in the same Tris buffer. The sections were then rinsed for 16 min in the Tris buffer and incubated for 15 min in a solution containing 1.4×10^{-3} M 3,3′-diaminobenzidine (DAB) tetrahydrochloride and 0.09 M hydrogen peroxide in Tris buffer. After three 3 min rinses in the Tris buffer, the sections were dehydrated in graded alcohols, cleared in xylene, and coverslipped with Permount.

BChE histochemistry

The KF procedure described above was used but with butyrylthiocholine (8×10^{-3} M) as the substrate and BW-284C51 at 10^{-3}–10^{-2} M as the inhibitor of AChE. The specificity of the BChE activity was demonstrated by showing that the reaction product was inhibited upon the addition of ethopropazine to the incubation solution. In order to probe whether BChE could

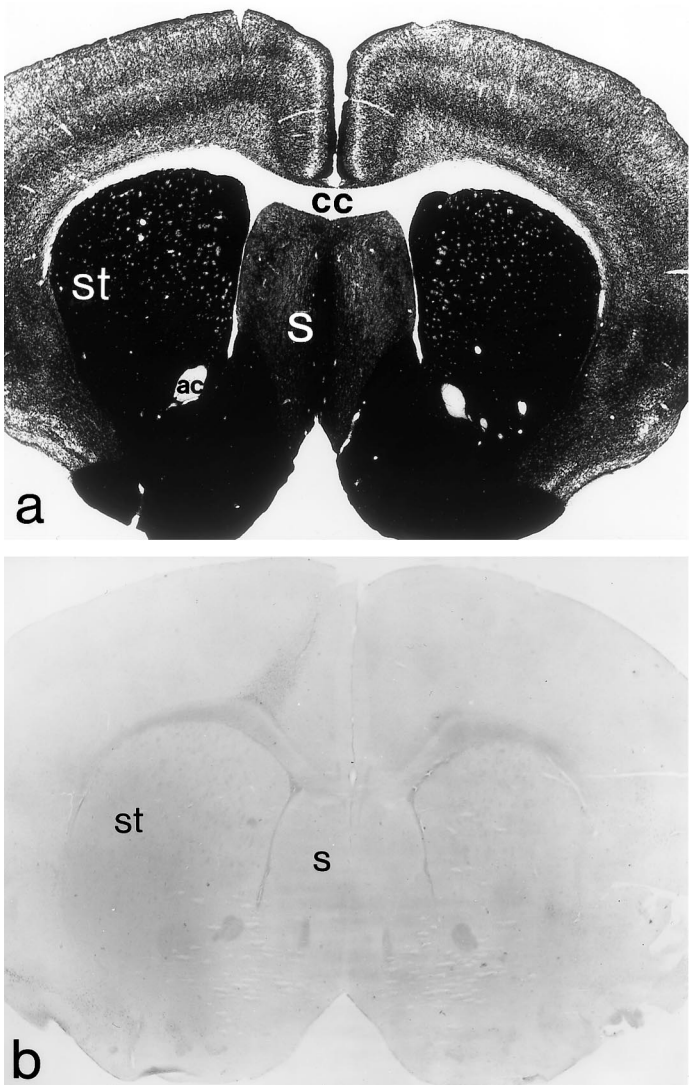

Fig. 1. (a) Coronal section from a wild-type mouse processed for the KF AChE reaction. (b) Same histochemical reaction in a nullizygote mouse. Magnification = 16×. ac – anterior commissure, cc – corpus callosum, s – medial septum, st – striatum.

hydrolyze acetylthiocholine (and, by inference, ACh), the reaction was carried out with acetylthiocholine as the substrate but with BW-284C51 as the inhibitor. If the resultant reaction product was inhibited by the addition of ethopropazine to the solution, it was attributed to the activity of BChE.

Reduced nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd) histochemistry

Free floating sections were rinsed in 0.1 M Tris buffer, pH 8.0. The rinsed sections were incubated in a solution containing 0.8% Triton X-100, 8 mM L-malic acid (monosodium salt), 0.5 mM NADPHd (tetrasodium salt) and 0.8 mM nitroblue tetrazolium in the same Tris buffer at 37°C. The reaction was carried out for 20 min and stopped by rinsing in Tris buffer (Scherer-Singler et al., 1983).

Immunohistochemistry

Immunocytochemistry was done with the avidin–biotin–peroxidase complex (ABC) method, employing the Vectastain Elite kit from Vector Laboratories (Burlingame, CA, USA) (Hsu et al., 1981). The tissue was permeabilized with 0.4% Triton X-100 in PBS at room temperature on a shaker for 30 min. Peroxidases were then blocked with 1% H_20_2 in PBS for 1 h at 4°C. The antibody incubation solution contained 3% normal serum (of appropriate origin), 0.1% Triton X-100, and the relevant antibody in 0.05 M PBS at pH 7.4. The primary antibody incubation occurred for 18–72 h at 4°C. After several buffer rinses, the tissue was treated with the biotinylated secondary antibody of appropriate origin for 2–4 h, rinsed several times, treated with the ABC for 2 h and incubated in 0.05% DAB with 0.01% hydrogen peroxide for 1 min. Control sections were processed in a similar fashion but by substituting the first antibody with an irrelevant IgG of similar origin. We used a polyclonal ChAT antibody (raised in goat against human placenta) at a titer of 1:75, and a polyclonal m1 muscarinic receptor antibody (raised in rabbit) at a titer of 1:250. The specificity of the antibodies for immunolabeling cholinergic and cholinoceptive neurons in the rodent brain was established in previous immunohistochemical investigations (Mesulam et al., 1983; Mufson and Cunningham, 1988; Levey et al., 1991).

Morphometry

Cell area was measured in the dorsal striatum in ChAT- and NADPHd-stained sections using an isotropic nucleator probe within the Stereoinvestigator software environment (Micro-

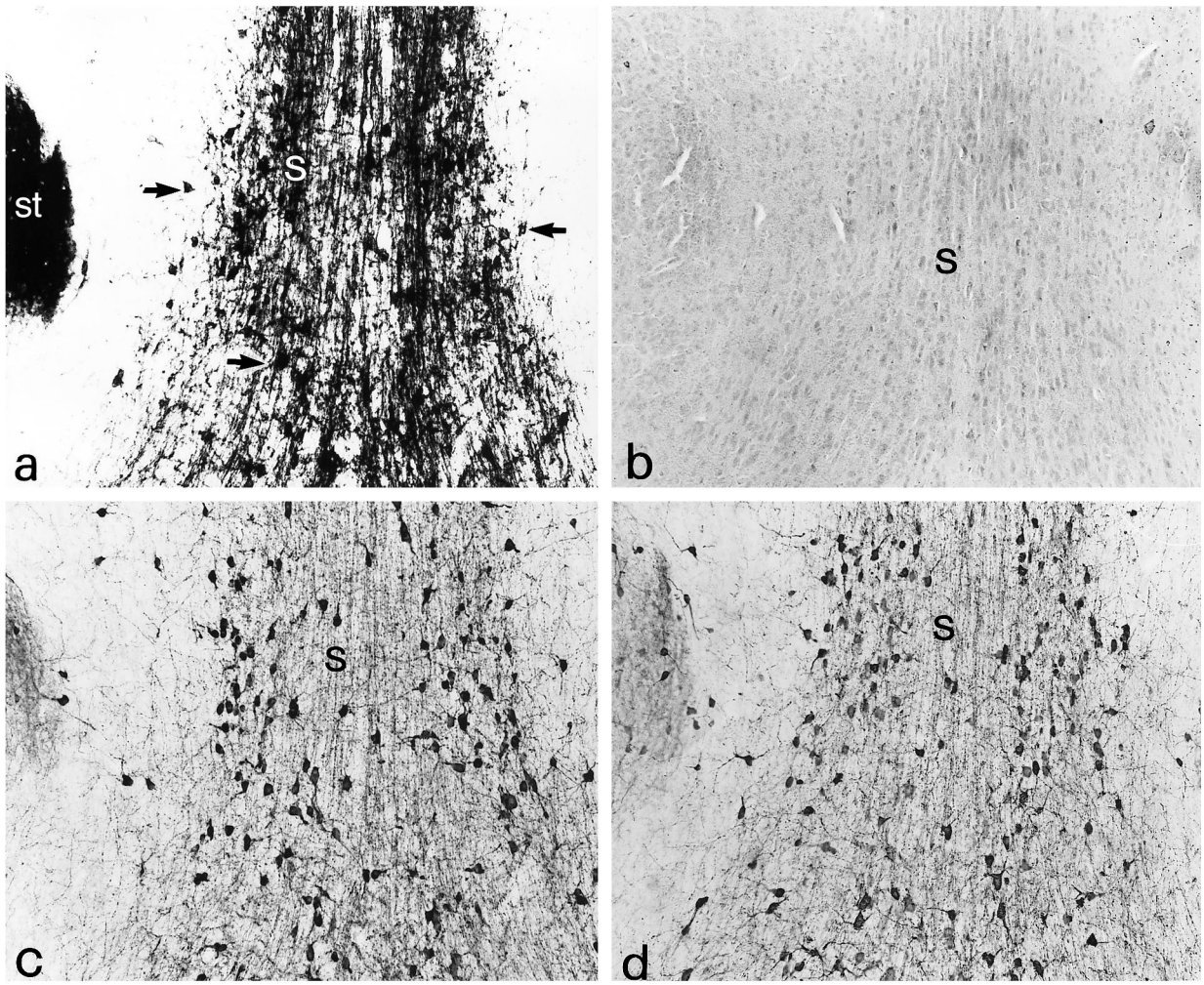

Fig. 2. (a) The KF AChE reaction in the medial septum of a wild-type mouse. Cholinergic neurons of the medial septum (s) are AChE-rich (arrows). (b) Same area, same reaction in a nullizygote mouse. There is no detectable reaction. (c) Matching section to 'a' showing ChAT-positive (and AChE-rich) Ch1 neurons of the medial septum in the wild-type mouse. (d) Matching section to 'b' showing ChAT-positive (but AChE-null) Ch1 neurons in the nullizygote mouse. The differences in staining intensity and cell density are within the range of individual variations. Magnification = 100×.

brightfield™). A nucleator probe with five rays was centered on each cell falling within the counting frame, and the distance from the center of the probe to the intersection of each ray with the cell boundary was measured to compute the surface area of the cell profile (Gunderson et al., 1988).

RESULTS

This investigation was limited to forebrain and upper brainstem regions known to contain prominent cholinesterase activity, cholinergic neurons, and cholinergic innervation. Within the scope of our qualitative survey, no sex-, age- or fixation-related changes were detected.

General survey of AChE distribution

In the wild-type mice, the KF procedure with acetylthiocholine as the substrate yielded an intense AChE reaction product within the striatum, globus pallidus, thalamus, amygdala, hippocampus, septum, diagonal band (vertical and horizontal limbs), nucleus basalis, habenula, substantia nigra, interpeduncular nucleus, superior colliculus, oculomotor–trochlear nuclei, and the pedunculopontine nucleus (Fig. 1a). The cerebral cortex contained an AChE reaction of moderate intensity. The enzymatic specificity of the AChE activity was demonstrated by the fact that it was obtained in the presence of the BChE inhibitor ethopropazine and that it was inhibited by the selective AChE inhibitor BW-284C51. In the cerebral cortex and hippocampus, the vast majority of the reaction product appeared axonal. However, AChE-rich neuronal perikarya were also observed, as previously described (Mufson et al., 1987). All subcortical areas (such as the striatum, medial septum, diagonal band nuclei, nucleus basalis, pedunculopontine nucleus) known to contain cholinergic perikarya displayed a very intense reaction product in the neuropil so that individual neuronal staining could not be identified. The only exception occurred in the lateral boundary of the medial septal nucleus where the neuropil was relatively lightly stained, allowing the AChE-rich perikarya of cholinergic septal neurons to be identified (Fig. 2a).

The AChE reaction product in the heterozygous animals displayed a distribution that was identical to the one seen in the wild-type but could be inhibited by 10 times less BW-284C51. The nullizygous animals displayed a complete absence of the AChE reaction product (Figs. 1b and 2b). These observations confirm previous Southern blotting and polymerase chain reaction experiments which showed that the nullizygotes had no wild-type *AChE* allele and that their brains did not contain biochemically detectable AChE activity (Xie et al., 2000).

Cholinergic perikarya

In the wild-type and heterozygous animals, cholinergic (ChAT-positive) perikarya were seen in the striatum, septal–diagonal band–nucleus basalis complex (Ch1–Ch4), the habenula (Ch7), the parabigeminal nucleus (Ch8), the pedunculopontine nucleus (Ch5), the laterodorsal tegmental nucleus (Ch6), the oculomotor–trochlear nuclei, and the cerebral cortex. Except for the bipolar cholinergic neurons of the cerebral cortex, all of these ChAT-positive neurons are known to be AChE-rich (Mesulam et al., 1983; Levey et al., 1984). Except for a possibly lighter staining in the septal region, the nullizygous animals displayed an overall pattern of perikaryal ChAT immunoreactivity that did not qualitatively differ from the wild-type, indicating that the standard groups of cholinergic perikarya are viable even in the absence of the AChE which is an invariant constituent of these neurons in the normal state (Figs. 2c, d, 3 and 4a, b). A quantitative analysis did not reveal significant differences in the size of striatal cholinergic perikarya when the wild-type was compared to the nullizygous animals (Fig. 5).

Cholinergic axons and terminals

In the normal brain, cholinergic (ChAT-positive) axons and terminals contain intense AChE activity (Mesulam and Geula, 1992). Wild-type and heterozygous animals displayed AChE-rich and ChAT-positive varicose axons in all regions of the brain, each region displaying a characteristic distribution and intensity

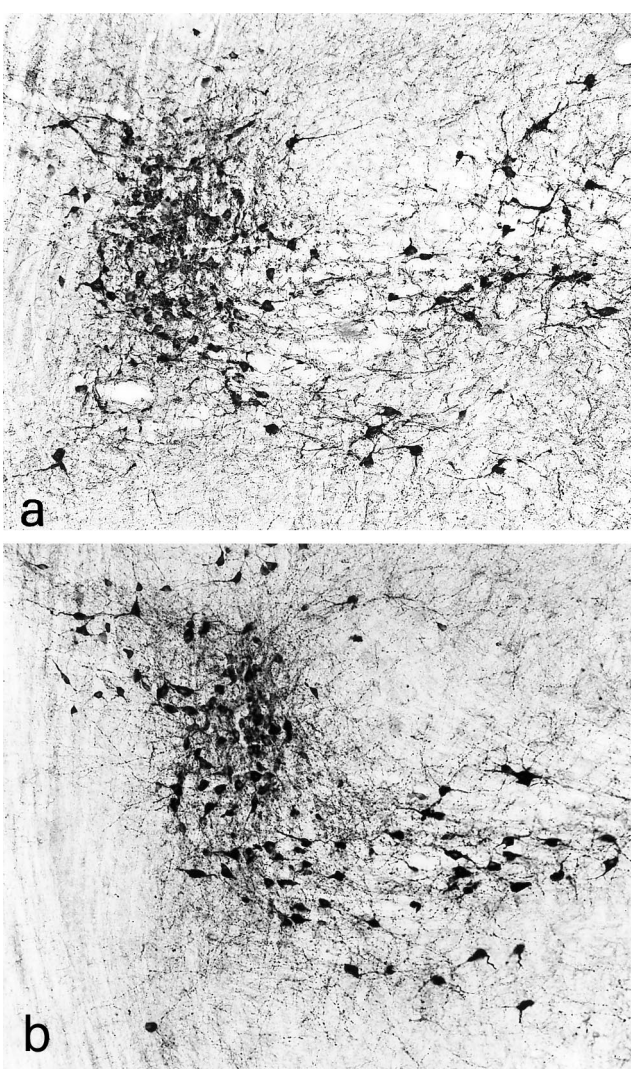

Fig. 3. ChAT-immunoreactive cholinergic Ch5 neurons in the pedunculopontine nucleus of the upper brainstem (a) in the wild-type, (b) in the nullizygote mice. Magnification = 100×.

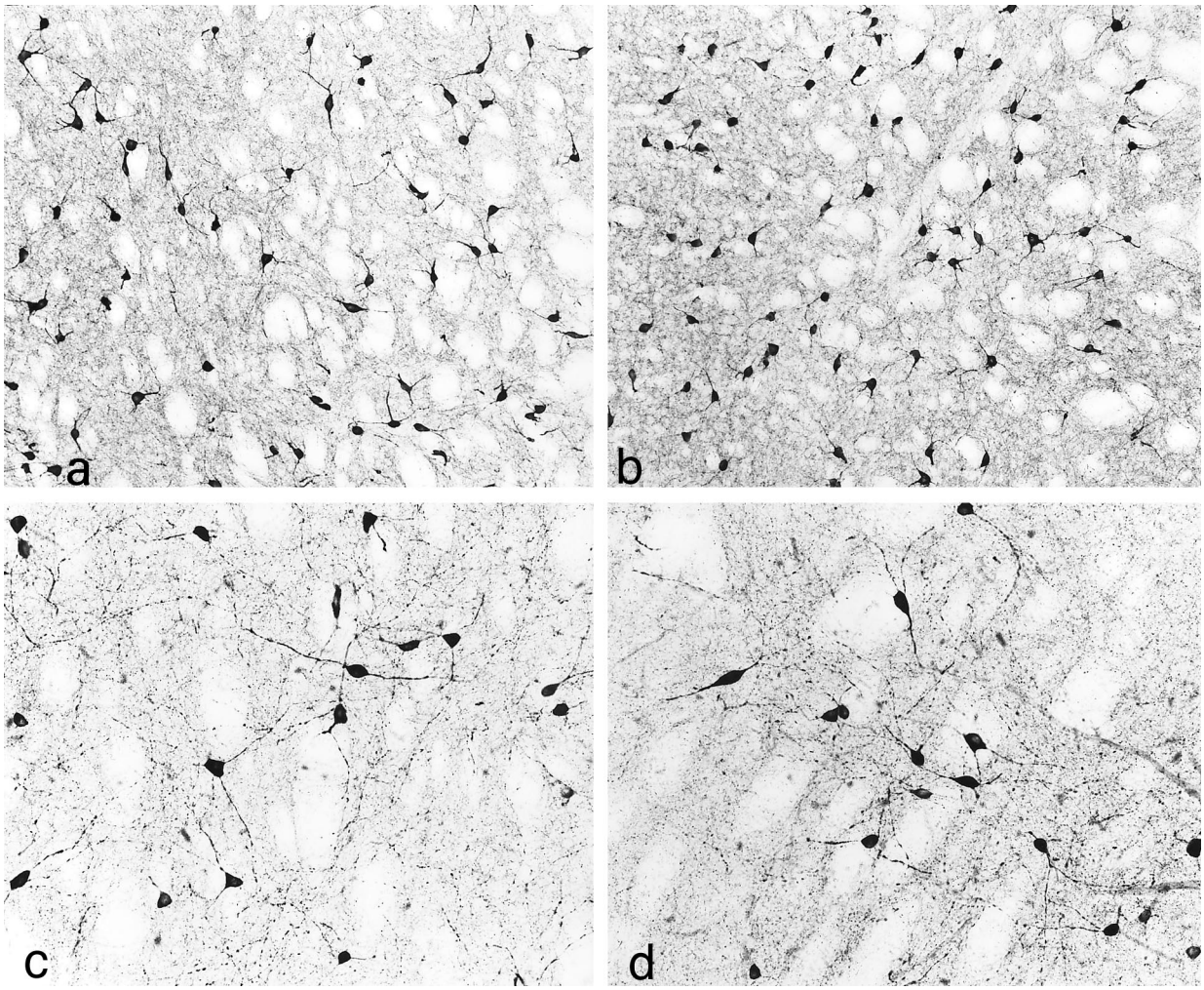

Fig. 4. (a) ChAT-immunoreactive cholinergic striatal neurons in the wild-type mouse. (b) Same reaction as in 'a' in the nullizygote mouse. (c) NADPHd-reactive striatal neurons in the wild-type mouse. (d) Same reaction as in 'c' in the nullizygote mouse. Magnification = 100×.

(Fig. 6a). The nullizygous animals displayed no AChE-positive axons in any of these regions (Fig. 6b). However, they displayed a pattern of ChAT-positive axons qualitatively similar to the one seen in the wild-type (Fig. 6c vs d). In other words, cholinergic axons survive even in the absence of their normally occurring high AChE levels.

Cholinoceptive receptors and perikarya

The fate of cholinoceptive neurons in the nullizygous animals is of considerable interest since AChE is thought to provide the major means through which these neurons can terminate the effect of ACh released by cholinergic axons. Cholinoceptive neurons can be of the muscarinic or nicotinic receptor subtypes, each of which is further divided into several subtypes. The muscarinic m1 receptor subtype is the predominant muscarinic receptor in the cerebral cortex. In keeping with previous studies in the rat and mouse (Levey et al., 1991; Hohman et al., 1995), we found m1 immunoreactivity in many cortical, hippocampal and amygdaloid neurons. Immunostaining in the cerebral neocortex was somewhat inconsistent and appeared less intense in the nullizygotes. In the amygdala and pyriform cortex, however, the pattern of m1-positive neurons appeared unchanged when the wild-type mice were compared to the nullizygous (Fig. 7). We are currently conducting quantitative investigations of muscarinic receptor subtypes in the AChE nullizygotes.

Many cortical and subcortical neurons express NADPHd (Geula et al., 1993; Smiley et al., 1998). In the striatum, NADPHd-reactive neurons also express m2 receptors and are therefore cholinoceptive (Smiley et al., 1999). These neurons did not show a change of size, density or distribution in the nullizygous mice (Figs. 4c, d and 5). All cholinoceptive neurons are likely to express AChE. However, only a subset of these are AChE-rich (Mesulam and Geula, 1991). Although all AChE-rich neurons are likely to be cholinoceptive, immunohistochemical proof for this is particularly strong for two groups of neurons: ChAT-positive striatal neurons and the NADPHd-reactive juxtacortical polymorphic neurons, both of which express muscarinic m2 receptors (Hersch et al., 1994; Smiley et al., 1998, 1999). These

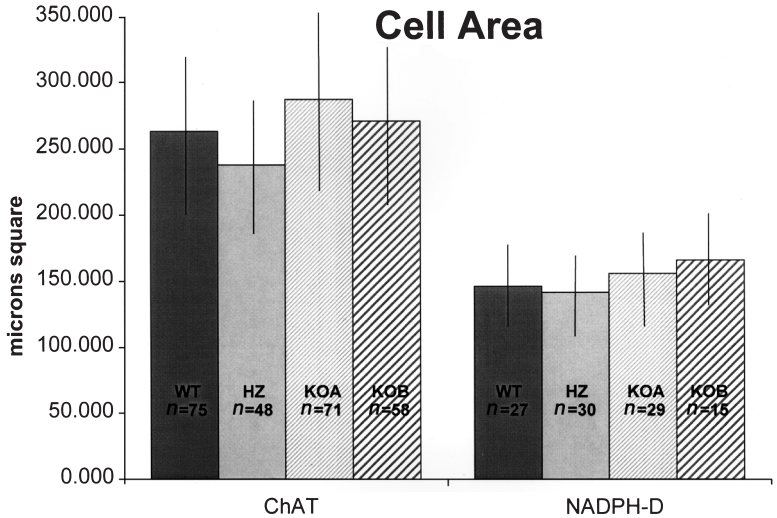

Fig. 5. Cell areas of striatal ChAT-positive cholinergic and NADPHd-positive cholinoceptive neurons in a wild-type (WT), a heterozygote (HZ) and two nullizygote (KOA, KOB) mice. There were no significant differences across groups. n = number of neurons. The bars indicate the standard deviations.

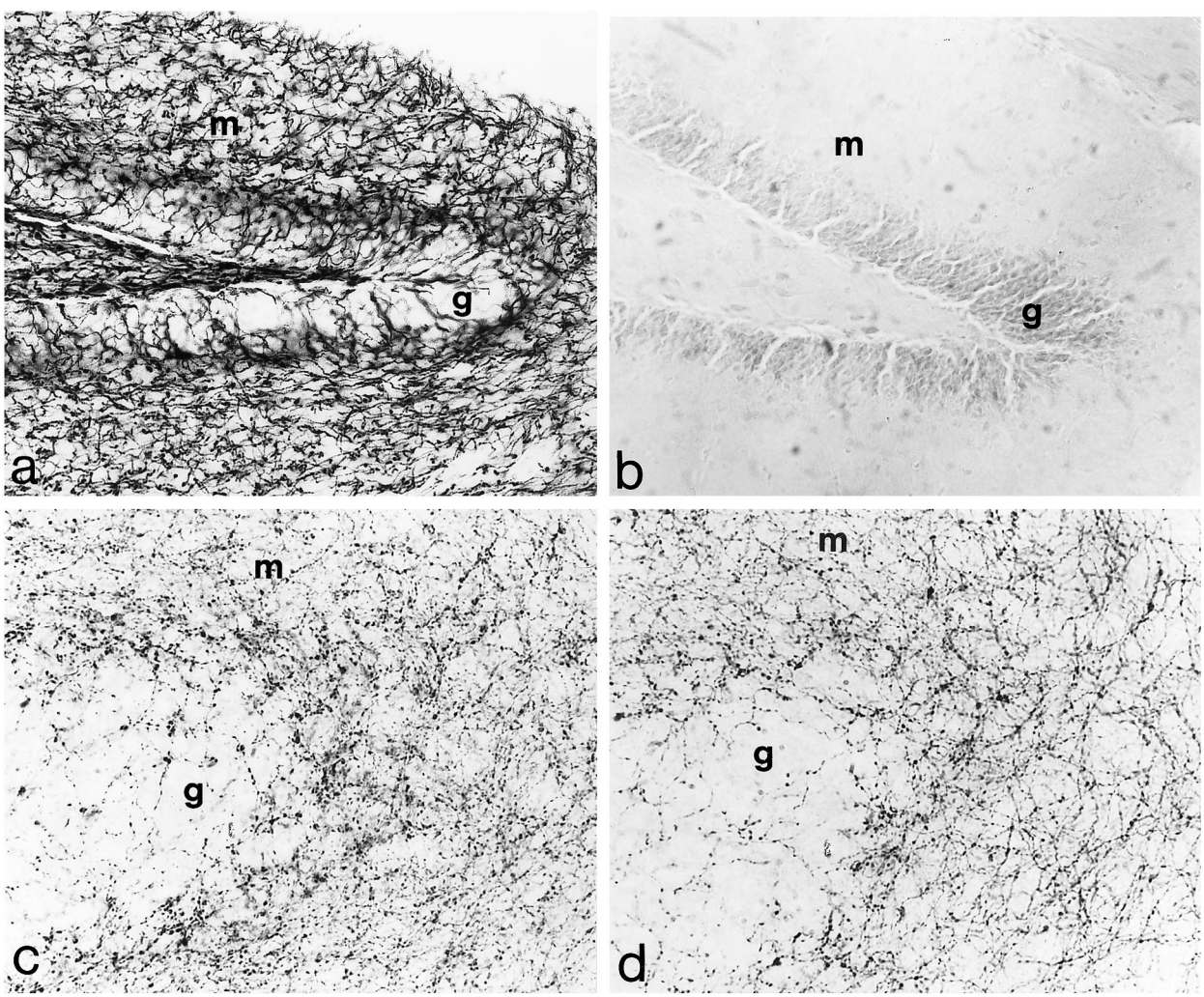

Fig. 6. (a) The KR AChE reaction to show AChE-rich axons in the hippocampal formation. (b) Same area, same procedure as in the nullizygote mouse. (c) ChAT immunoreactivity in matching section to 'a' showing that the AChE-rich axons are ChAT-positive. (d) ChAT immunoreactivity in matching section to 'b' showing that the cholinergic axons do not show major differences despite the lack of AChE. Magnification = 200×. g – granule cell layer of dentate gyrus, m – molecular layer of dentate gyrus.

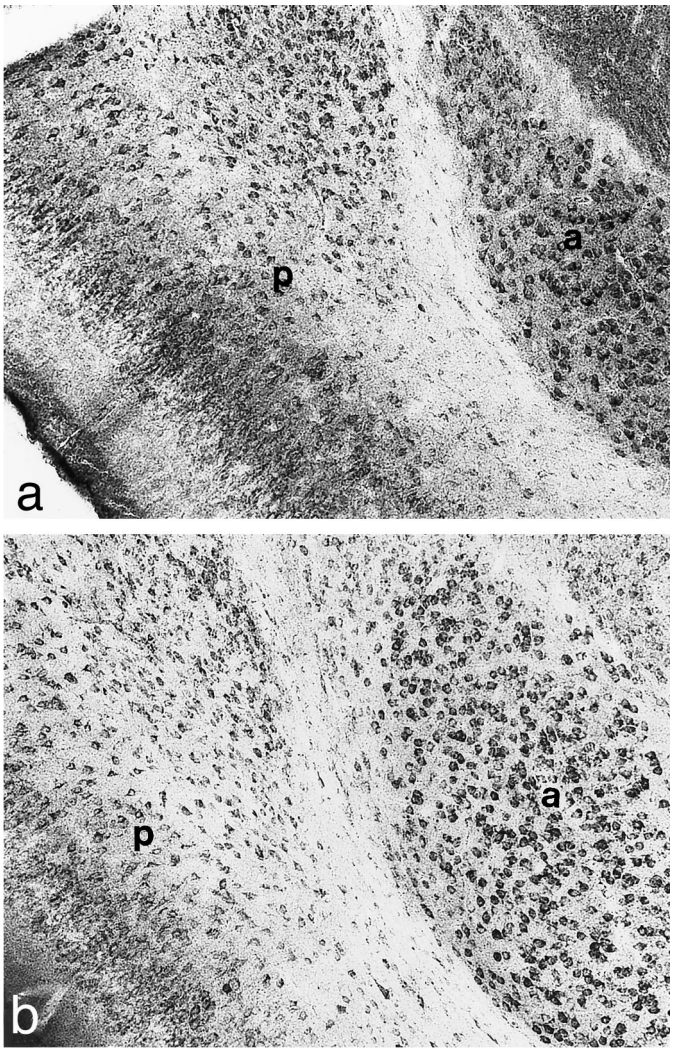

Fig. 7. (a) Muscarinic m1 receptor immunoreactivity in the wild-type mouse. (b) Same reaction in the nullizygote mouse. Magnification = 100×. a – amygdala, p – pyriform cortex.

subsets of AChE-rich cholinoceptive neurons did not display any major change in the nullizygous animals. This was determined quantitatively for the striatal neurons (Fig. 5a) and qualitativeley for the juxtacortical neurons.

BChe

The distribution of BChE was investigated by incubating tissue in the presence of the AChE inhibitor BW-284C51 and with the butyrylcholine analogue butyrylthiocholine as the substrate. The specificity of the resultant reaction product was shown by its complete inhibition by the specific BChE inhibitor ethopropazine. The distribution of the BChE enzyme activity was markedly different from that of AChE (Fig. 8a). In the wild-type, intense BChE activity was confined to white matter bundles such as the corpus callosum, anterior commissure, fornix, and the internal capsule fibers traversing the striatum. The thalamus and upper brainstem were also intensely reactive. In the cerebral cortex and subcortical areas containing cholinergic neurons, the BChE reaction product was located in capillaries, few (mostly non-pyramidal) perikarya, and numerous neuroglia-like structures. There was widespread neuropil reactivity. In contrast to AChE, however, where the neuropil activity was distinctly axonal (Figs. 6a and 9a), the BChE activity was more diffuse in a way that was compatible with a localization in glial processes (Fig. 9b). In contrast to their AChE-rich pattern, the cholinergic neurons of the basal forebrain, striatum and brainstem were not associated with intense neuronal BChE reactivity.

Dark-field microscopy showed that the BChE enzyme activity in the neuropil was widely distributed throughout the brain and that it reached all parts of the cerebral cortex, hippocampus and striatum (Fig. 10). In the hippocampal formation, for example, neuropil-bound BChE activity was particularly intense in the molecular layer of the dentate gyrus and in the strata radiatum, oriens and lacunosum-moleculare of the Ammonic fields, regions of the hippocampus which receive intense cholinergic innervation. The BChE patterns were qualitatively indistinguishable in the wild-type, heterozygous and

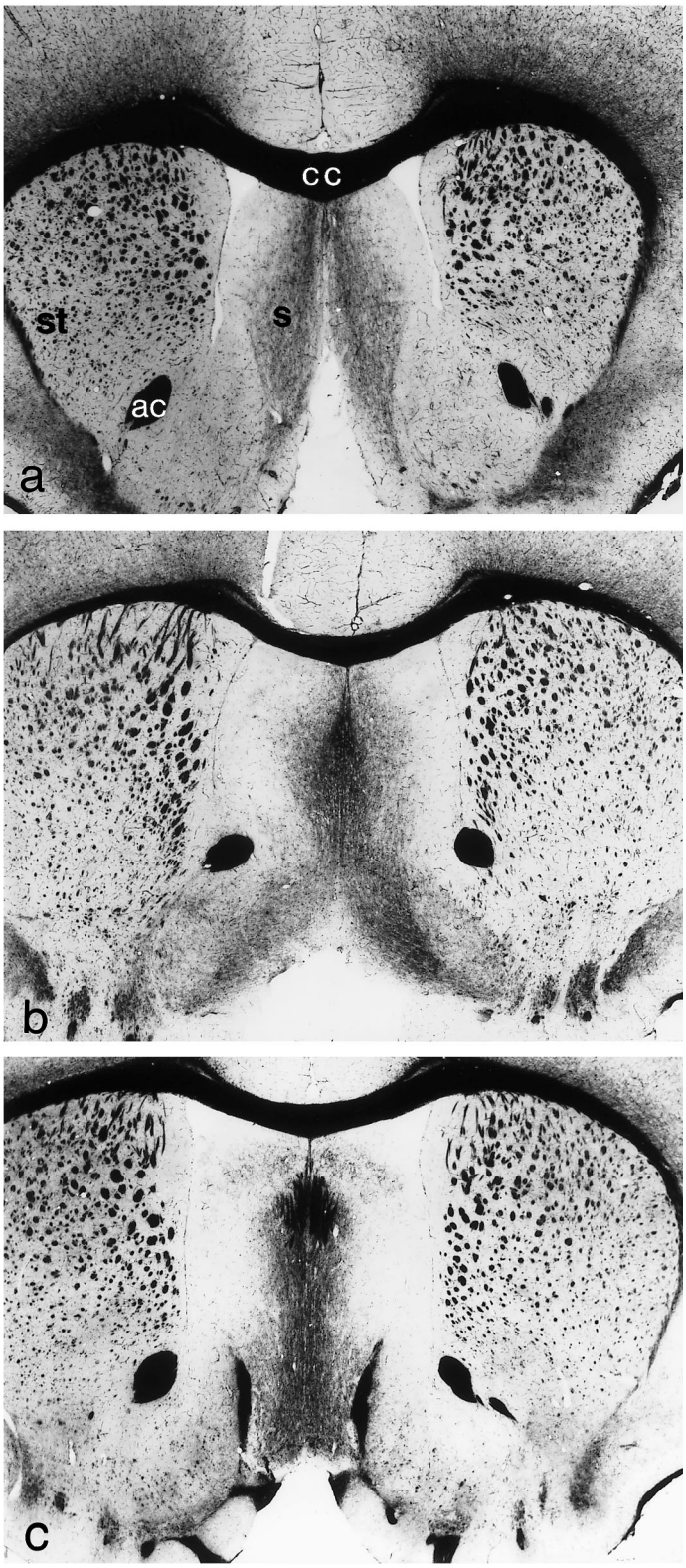

Fig. 8. (a) BChE reaction in the wild-type mouse. The distribution is distinctly different from that of AChE. (b) BChE reaction in the nullizygote mouse. (c) BChE enzyme hydrolyzing acetylthiocholine (an ACh surrogate) in the presence of the specific AChE inhibitor BW-284C51 in the wild-type mouse. This reaction was inhibited by the selective BChE inhibitor ethopropazine. This experiment shows that BChE can hydrolyze ACh in the wild-type. The slight differences in the exact pattern of septal staining in the three cross-sections reflect the slightly different antero-posterior levels. The BChE activity of the nullizygote did not show up-regulation. Magnification = 20×. ac – anterior commissure, cc – corpus callosum, s – medial septum, st – striatum.

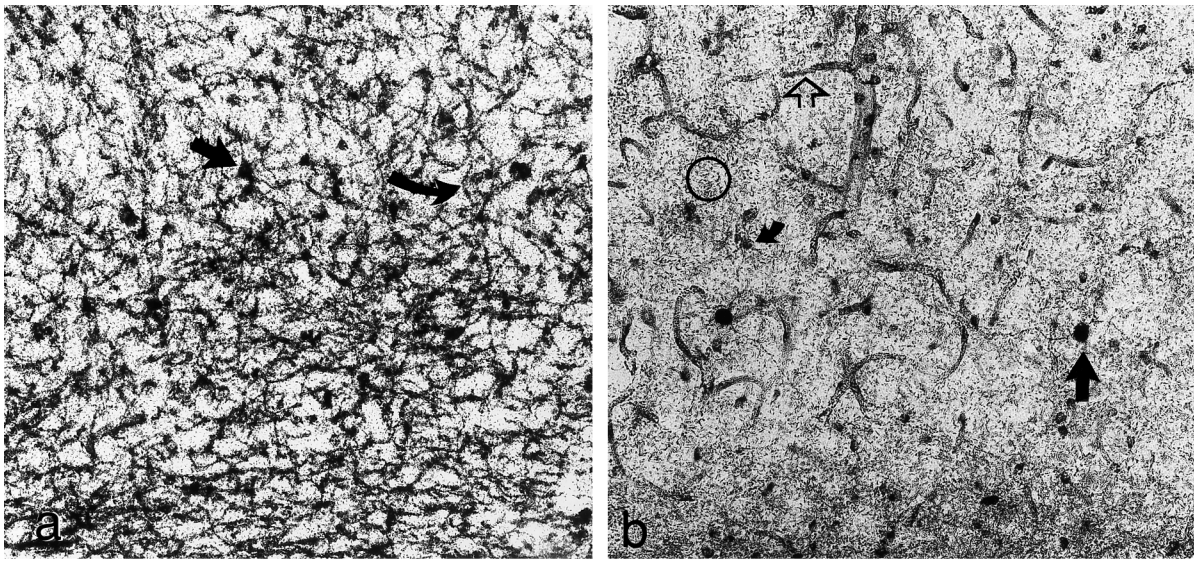

Fig. 9. (a) The KF AChE reaction in the frontal cortex of the wild-type mouse. The reaction product is predominantly in axons (curved arrow) and a few neurons, many of which are pyramidal (arrow). (b) The BChE reaction in the frontal cortex of the wild-type mouse. The reaction product is in few non-pyramidal neurons (arrow), in capillaries (open arrow) and in numerous glia-like profiles (curved arrow). The dense neuropil staining (e.g. inside the circle) does not have an axonal pattern and may reflect staining in glial processes. Magnification = 200×.

nullizygous animals, with no evidence of compensatory increases in the intensity or distribution of BChE in the nullizygous (Fig. 8a vs b).

Whether the widespread BChE activity could be used to hydrolyze ACh was addressed by incubating tissue in the presence of the AChE inhibitor BW-284C51 but with the ACh analogue acetylthiocholine (instead of butyrylthiocholine) as the substrate. For these reactions, we used the higher concentration of BW-284C51 (10^{-2}) which was necessary for the total inhibition of acetylthiocholine hydrolysis by AChE at these incubation parameters in the wild-type animals. The resultant reaction product, reflecting the cross-hydrolysis of the ACh surrogate acetylthiocholine by BChE, was nearly identical to the reaction product obtained with butyrylthiocholine as the substrate, demonstrating that BChE has little substrate selectivity and that it can hydrolyze ACh (Figs. 8c and 10). This reaction was completely inhibited by the addition of ethopropazine to the incubation medium, providing further evidence that it represents the action of BChE. An identical pattern of BChE hydrolysis of acetylthiocholine was seen in the heterozygous and nullizygous animals. These results indicate that the brain normally contains widespread BChE activity which can be used to hydrolyze ACh.

Tissue was also incubated with butyrylthiocholine as the substrate in the presence of ethopropazine to see if the converse cross-hydrolysis of butyrylthiocholine by AChE could be demonstrated. The resultant reaction product was very faint, orders of magnitude less intense than the reaction product in Fig. 1a. This result confirms the well-known fact that AChE has greater substrate specificity than BChE and shows that the cross-hydrolysis of acetylthiocholine by BChE reflects an intrinsic property of the enzyme rather than an artifact of our histochemical manipulations.

DISCUSSION

Central cholinergic pathways play critical roles in the regulation of numerous vital functions including sleep, arousal, attention, emotion, and memory (see Karczmar, 1975; Mesulam, 1996; Sarter and Bruno, 2000 for review). AChE is found in the presynaptic (cholinergic) and postsynaptic (cholinoceptive) components of these pathways where it terminates the synaptic action of ACh through catalytic hydrolysis. The choline which is liberated in the course of this process is taken up by presynaptic cholinergic neurons and used to synthesize new ACh. An absence of AChE would presumably create a situation where the effect of ACh could not be terminated and where the pathway for ACh recycling would become interrupted. Such circumstances would be expected to undermine the integrity of both cholinergic and cholinoceptive neurons and to have lethal consequences, similar to those seen in response to nerve gases (which irreversibly inhibit AChE) or hemicholinium (which interferes with the reuptake of choline). Furthermore, the putative influence of AChE on synaptogenesis, axonal targeting, and dendritic branching (Mesulam and Geula, 1991; Layer et al., 1993; Robertson and Yu, 1993; Beeri et al., 1997) would independently suggest that an absence of this enzyme should have devastating consequences on development. In fact, deletion of AChE activity in *Drosophila* leads to embryonic lethality (Greenspan et al., 1980).

The observations on the AChE nullizygotes run somewhat counter to these expectations. The presence of tremor, weakness, and ileus in the nullizygous mice implies a dysfunction of central and peripheral cholinergic pathways. Nonetheless, with care to feeding, the nullizygotes did survive to adulthood and displayed all the standard components of cholinergic pathways in the

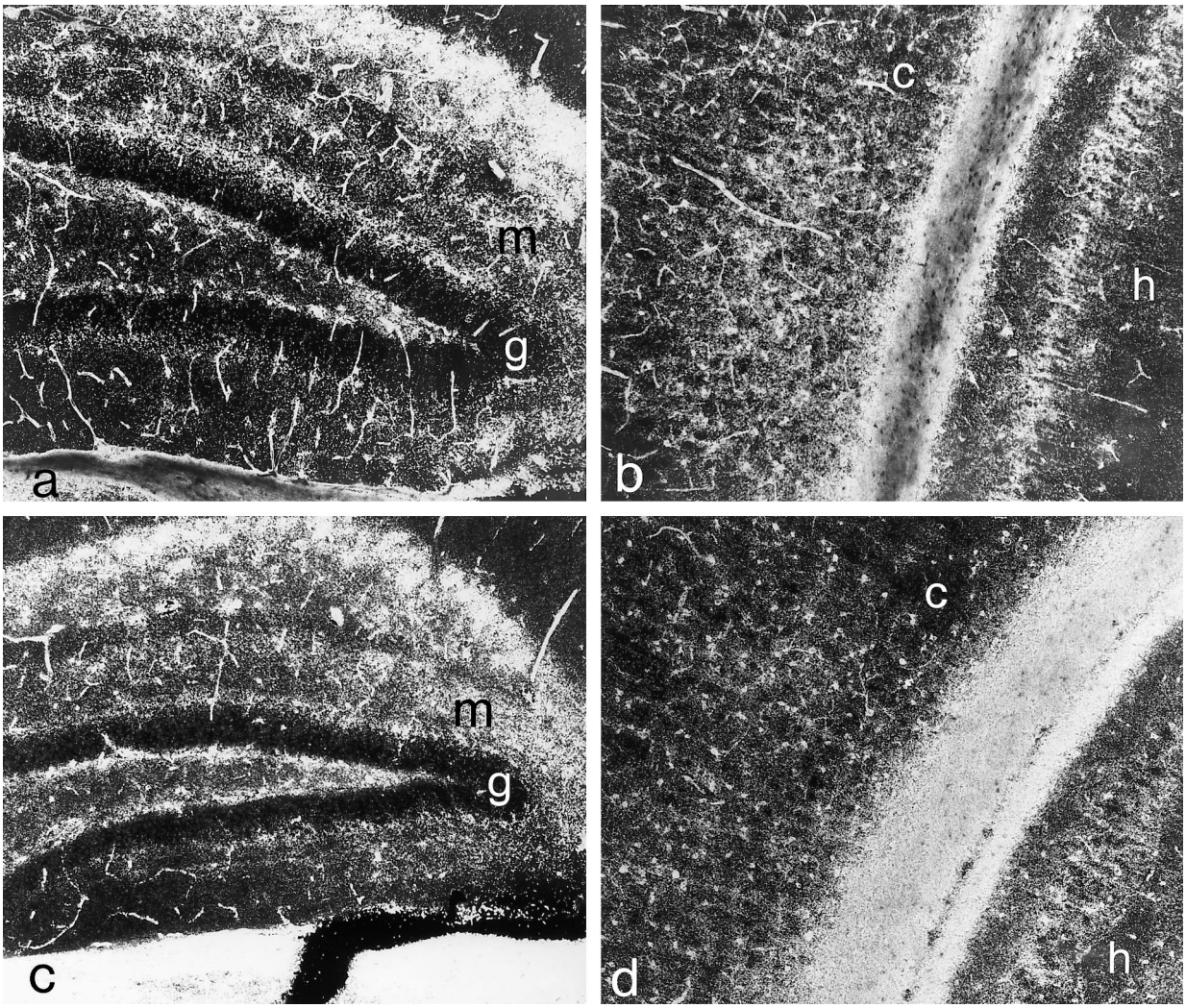

Fig. 10. Dark-field photomicrography. (a and b) The BChE reaction in the hippocampal formation (a) and parietal cortex (b) of a nullizygote mouse. BChE reactivity covers areas that receive cholinergic innervation. A similar distribution is seen in the wild-type mouse. (c and d) The hydrolysis of acetylthiocholine in the presence of the AChE inhibitor BW-284C51 in the hippocampal formation (c) and parietal cortex (d) of a wild-type mouse, showing that BChE can hydrolyze the ACh surrogate. A similar reaction is obtained in the nullizygote mouse. The specificity of this reaction for BChE is based on its inhibition by ethopropazine. Magnification 100×. c – cerebral cortex, g – granule cell layer of the dentate gyrus, h – hippocampus, m – molecular layer of the dentate gyrus.

CNS. It appears, therefore, that AChE is not absolutely essential for the development or maintenance of cholinergic pathways. This study provides a general survey of cholinergic markers. Relatively more subtle quantitative abnormalities in ultrastructure, dendritic branching, cell number, cell size or receptor density cannot be ruled out. In fact, our unpublished observations show that the nullizygous mice are hypersensitive to atropine, suggesting that muscarinic receptors may, in fact, have undergone a compensatory down-regulation.

BChE may modulate cholinergic transmission in smooth muscle and may substitute for AChE in the neuromuscular junction of AChE nullizygotes (Norel et al., 1993; Li et al., 2000). However, its role in the CNS remains poorly understood. Our experiments provide the first histochemical evidence that the BChE of the normal brain can hydrolyze ACh. That this is more than a 'back-up' process and that it is likely to contribute to normal cholinergic transmission was shown in the rat where cortical perfusion with a selective BChE inhibitor led to a 15-fold increase in the extracellular concentration of ACh (Giacobini, 2000). Such BChE-mediated hydrolysis of ACh may have allowed the nullizygous mice to escape the otherwise devastating consequences of cholinergic hyperactivity, and to establish central cholinergic pathways even in the absence of AChE. In fact, the BChE inhibitor bambuterol was lethal to these animals at doses that had no toxicity for the wild-type, further emphasizing the heightened role of BChE in the cholinergic function of these animals (Xie et al., 2000).

In *Drosophila* where AChE deletion is lethal, there is no BChE to provide this parallel mechanism for ACh hydrolysis (Greenspan et al., 1980; Xie et al., 2000). Individuals with enzymatically silent BChE seem to have no difficulty with CNS development or cholinergic function, unless challenged with certain pharmacological

agents. It appears therefore that neither AChE nor BChE is, by itself, necessary for maintaining the functional integrity of cholinergic pathways but that both enzymes may simultaneously participate in this process. In fact, nerve gases (e.g. Soman, Tabun) inhibit not only AChE but also BChE (Silver, 1974), implying that lethal neurotoxicity may require the inhibition of both enzymes.

The histochemical parameters that were used in these experiments demonstrated a distribution of BChE activity that was more widespread than previously surmised. In fact, BChE activity extended to all regions known to have cholinergic neurotransmission. However, the cellular distribution was different from that of AChE. There were relatively few BChE-rich neurons and almost no axons that displayed a BChE-rich pattern of enzyme activity. Much of BChE activity appeared glial. Such glial BChE has been described in the human brain (Wright et al., 1993a,b). The intimate association of astrocytic processes with synaptic sites is well-known. In analogy to the AChE of *Torpedo*, at least some of the glial BChE is likely to act as an ectoenzyme with its catalytic site on the extracellular side of the plasma membrane. Synaptically released ACh could reach these glial catalytic sites of BChE to undergo hydrolysis and to have its choline moiety returned to the synaptic region for reuptake into cholinergic neurons. The participation of neuroglia in cholinergic transmission could thus be analogous to their crucial participation in glutamatergic transmission (Daikhin and Yudkoff, 2000).

Alzheimer's disease is associated with an early and severe depletion of cholinergic innervation (Geula and Mesulam, 1999; Beach et al., 2000). Currently available pharmacological interventions for Alzheimer's disease focus on the inhibition of cholinesterases in order to increase ACh availability at synaptic sites. The development of these anticholinesterase drugs has engendered a debate between those who advocate the selective inhibition of AChE (also known as the 'specific' or 'neuronal' enzyme) and those who advocate the combined inhibition of AChE and BChE (Weinstock, 1999). The observations reported above support the desirability of inhibiting BChE for enhancing cholinergic neurotransmission and suggest that selective BChE inhibitors may also have cholinergic properties.

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REFERENCES

Beach, T.G., Kuo, Y.-M., Spiegel, K., Emmerling, M.R., Sue, L.I., Kokjohn, K., Roher, A.E., 2000. The cholinergic deficit coincides with Aβ deposition at the earliest histopathologic stages of Alzheimer disease. J. Neuropathol. Exp. Neurol. 59, 308–313.

Beeri, R., Le Novere, N., Mervis, R., Huberman, T., Grauer, E., Changeux, J.-P., Soreq, M., 1997. Enhanced hemicholinium binding and attenuated dendrite branching in cognitively impaired AChE-transgenic mice. J. Neurochem. 69, 2441–2451.

Daikhin, Y., Yudkoff, M., 2000. Compartmentation of brain glutamate metabolism in neurons and glia. J. Nutr. 130, 1026S–1031S.

Darvesh, S., Grantham, D.L., Hopkins, D.A., 1998. Distribution of butyrylcholinesterase in the human amygdala. J. Comp. Neurol. 393, 374–390.

Geula, C., Mesulam, M.-M., 1999. Cholinergic systems in Alzheimer's disease. In: Terry, R.D., Katzman, R., Bick, K.L., Sisodia, S.S. (Eds.), Alzheimer Disease, 2nd edn. Lippincott, Williams and Wilkins, Philadelphia, PA, pp. 269–292.

Geula, C., Schatz, C.R., Mesulam, M.M., 1993. Differential localization of NADPH-diaphorase and calbindin-D28k within the cholinergic neurons of the basal forebrain, striatum and brainstem in the rat, monkey, baboon and human. Neuroscience 54, 461–476.

Giacobini, E., 2000. Cholinesterase inhibitors: from the Calabar bean to Alzheimer therapy. In: Giacibini, E. (Ed.), Cholinesterases and Cholinesterase Inhibitors. Martin Dunitz, London, pp. 181–226.

Greenspan, R.J., Finn, J.A., Hall, J.C., 1980. AChE mutants in *Drosophila* and their effects on the structure and function of the central nervous system. J. Comp. Neurol. 189, 741–774.

Gunderson, H.J.G., Bagger, P., Bendtsen, T.F., Evans, S.M., Korbo, L., Marcussen, N., Moller, A., Nielsen, K., Nyengaard, J.R., Pakkenberg, B. et al., 1988. The new stereological tools: Disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. Acta Pathol. Microbiol. Immunol. Scand. 96, 857–881.

Hersch, S.M., Gutekunst, C.-A., Rees, H.D., Heilman, C.J., Levey, A.I., 1994. Distribution of m1–m4 muscarinic receptor proteins in the rat striatum: light and electron microscopic immunocytochemistry using subtype-specific antibodies. J. Neurosci. 14, 3351–3363.

Hohman, C.F., Potter, E.D., Levey, A.I., 1995. Development of muscarinic receptor subtypes in the forebrain of the mouse. J. Comp. Neurol. 358, 88–101.

Hsu, S.M., Raine, L., Fanger, H., 1981. Use of avidin–biotin–peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) procedures. J. Histochem. Cytochem. 29, 577–580.

Karczmar, A.G., 1975. Cholinergic influences on behavior. In: Waser, P.G. (Ed.), Cholinergic Mechanisms. Raven Press, New York, pp. 501–529.

Karnovsky, M.J., Roots, L., 1964. A 'direct coloring' thiocholine method for cholinesterases. J. Histochem. Cytochem. 12, 219–221.

Koelle, G.B., Friedenwald, J.S., 1949. A histochemical method for localizing cholinesterase activity. Proc. Soc. Exp. Biol. Med. 70, 617–622.

Layer, P.G., Weikert, T., Alber, R., 1993. Cholinesterases regulate neurite growth of chick nerve cells *in vitro* by means of a non-enzymatic mechanism. Cell Tissue Res. 273, 219–226.

Levey, A.I., Wainer, B.H., Rye, D.B., Mufson, E.J., Mesulam, M.M., 1984. Choline acetyltransferase-immunoreactive neurons intrinsic to rodent cortex and distinction from AChE-positive neurons. Neuroscience 13, 341–353.

Levey, A.I., Kitt, C.A., Simonds, W.F., Price, D.L., Brann, M.R., 1991. Identification and localization of muscarinic ACh receptor protein in brain with subtype-specific antibodies. J. Neurosci. 11, 3218–3226.

Li, B., Stribley, J.A., Ticu, A., Xie, W., Schopfer, L.M., Hammond, P., Brimijoin, S., Hinrichs, S.H., Lockridge, O., 2000. Abundant tissue butyrylcholinesterase and its possible function in the AChE knockout mouse. J. Neurochem. 75, 1320–1331.

Mesulam, M.-M., 1996. The systems-level organization of cholinergic innervation in the cerebral cortex and its alterations in Alzheimer's disease. Prog. Brain Res. 109, 285–298.

Mesulam, M.-M., 2000. Neuroanatomy of cholinesterases in the normal human brain and in Alzheimer's disease. In: Giacobini, E. (Ed.), Cholinesterases and Cholinesterase Inhibitors. Martin Dunitz, London, pp. 121–138.

Mesulam, M.-M., Geula, C., 1991. AChE-rich neurons of the human cerebral cortex: cytoarchitectonic and ontogenetic patterns of distribution. J. Comp. Neurol. 306, 193–220.
Mesulam, M.-M., Geula, C., 1992. Overlap between AChE-rich and choline acetyltransferase-positive (cholinergic) axons in human cerebral cortex. Brain Res. 577, 112–120.
Mesulam, M.M., Mufson, E.J., Wainer, B.H., Levey, A.I., 1983. Central cholinergic pathways in the rat: an overview based on an alternative nomenclature (Ch1–Ch6). Neuroscience 10, 1185–1201.
Mesulam, M.-M., Geula, C., Brimijoin, S., Smiley, J.F., 1995. Butyrylcholinesterase immunochemistry in human cerebral cortex. Soc. Neurosci. Abstr. 21, 1976.
Mufson, E.F., Cunningham, M.G., 1988. Observations on choline acetyltransferase containing structures in the CD-1 mouse brain. Neurosci. Lett. 84, 7–12.
Mufson, E.J., Kehr, A.D., Wainer, B.H., Mesulam, M.M., 1987. Cortical effects of neurotoxic damage to the nucleus basalis in rats: persistent loss of extrinsic cholinergic input and lack of transsynaptic effect upon the number of somatostatin-containing cholinesterase-positive, and cholinergic cortical neurons. Brain Res. 417, 385–388.
Norel, X., Angrisani, M., Labat, C., Gorenne, I., Dulmet, E., Rossi, F., Brink, C., 1993. Degradation of ACh in human airways: role of butyrylcholinesterase. Br. J. Pharmacol. 108, 914–919.
Robertson, R.T., Yu, J., 1993. AChE and neural development: New tricks for an old dog? News Physiol. Sci. 8, 266–272.
Sarter, M., Bruno, J.P., 2000. Cortical cholinergic inputs mediating arousal attentional processing and dreaming: differential afferent regulation of the basal forebrain by telencephalic and brainstem afferents. Neuroscience 95, 933–952.
Scherer-Singler, U., Vincent, S.R., Kimura, H., McGeer, E.G., 1983. Demonstration of a unique population of neurons with NADPH-diaphorase histochemistry. J. Neurosci. Methods 9, 229–234.
Silver, A., 1974. The Biology of Cholinesterases. American Elsevier Publishing, New York.
Smiley, J.F., Levey, A.I., Mesulam, M.-M., 1998. Infracortical interstitial cells concurrently expressing m2-muscarinic receptors. AChE, and NADPH-d in the human and monkey cerebral cortex. Neuroscience 84, 755–769.
Smiley, J.F., Levey, A.I., Mesulam, M.-M., 1999. m2 muscarinic receptor immunolocalization in cholinergic cells of the monkey basal forebrain and striatum. Neuroscience 90, 803–814.
Weinstock, M., 1999. Selectivity of cholinesterase inhibition. Clinical implications for the treatment of Alzheimer's disease. CNS Drugs 12, 307–323.
Wright, C.I., Geula, C., Mesulam, M.M., 1993a. Neuroglial cholinesterases in the normal brain and in Alzheimer's disease: relationship to plaques, tangles, and patterns of selective vulnerability. Ann. Neurol. 34, 373–384.
Wright, C.I., Geula, C., Mesulam, M.M., 1993b. Protease inhibitors and indoleamines selectively inhibit cholinesterases in the histopathologic structures of Alzheimer disease. Proc. Natl. Acad. Sci. USA 90, 683–686.
Xie, W., Wilder, P.J., Stribley, J., Chatonnet, A., Rizzino, A., Taylor, P., Hinrichs, S.H., Lockridge, O., 1999. Knockout of one AChE allele in the mouse. Chem.-Biol. Interact. 119–120, 289–299.
Xie, W., Stribley, J.A., Chatonnet, A., Wilder, P.J., Rizzino, A., Mccomb, R.D., Taylor, P., Hinrichs, S.J., Lockridge, O., 2000. Postnatal developmental delay and supersensitivity to organophosphate in gene-targeted mice lacking AChE. J. Pharmacol. Exp. Ther. 293, 896–902.

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