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Muscarinic Cholinoceptive Neurons in the Frontal Cortex in Alzheimer's Disease[1]

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SCHRÖDER, H., E. GIACOBINI, R. G. STRUBLE, P. G. M. LUITEN, E. A. VAN DER ZEE, K. ZILLES AND A. D. STROSBERG. *Muscarinic cholinoceptive neurons in the frontal cortex in Alzheimer's disease.* BRAIN RES BULL **27**(5) 631–636, 1991.—The cellular distribution of muscarinic acetylcholine receptor protein in the frontal cortex of Alzheimer (AD) patients, age-matched and middle-aged controls was assessed quantitatively by means of immunohistochemistry using the monoclonal antibody M35. As shown previously in biopsy cortices, mainly layer II/III and V pyramidal neurons were immunolabeled. Neither distribution nor numbers of labeled cells displayed significant differences between the groups investigated. This is in accordance with the results of ligand binding studies that mostly failed to reveal different binding characteristics in AD compared to controls. Muscarinic and nicotinic receptor proteins have been shown to be colocalized in many cholinoceptive pyramidal neurons. Since nicotinic receptors—in contrast to muscarinic receptor proteins—are severely reduced in AD, this indicates a selective impairment of nicotinic receptor expression and not a significant death of cholinoceptive neurons per se.

Muscarinic acetylcholine receptor Alzheimer's disease Cerebral cortex Human brain Immunohistochemistry M35

ALZHEIMER'S DISEASE (AD) (1) is characterized neurochemically by a pronounced cortical cholinergic dysfunction (5,14). At the presynaptic level, the reduction of cholineacetyltransferase (ChAT) activity indicates the impairment of acetylcholine (ACh) synthesis in the cholinergic basal forebrain projection system (3, 6, 14) [cf. (13, 24, 42)]. As to the receptor sites, binding studies have shown the concentration of nicotinic ACh receptors (nAChRs) in the frontal cortex to be markedly reduced (12, 14, 15, 26, 44–46) as compared to age-matched controls. Findings on muscarinic receptors (mAChRs) are equivocal describing increased, reduced or—in the majority of studies—unchanged numbers of binding sites [cf. (14)]. Receptor autoradiography has shown the preferential labeling of the superficial cortical layers for muscarinic binding sites (9,48) to be no longer detectable in AD (18).

No information, however, is available so far about the cellular distribution of mAChRs in AD. Recently, immunocytochemical techniques have enabled the visualization of cholinergic receptors at the cellular level (23, 32–34, 40). Using a monoclonal antibody, M35 (2, 23, 34), directed against the mAChR, we here report on the qualitative and quantitative evaluation of immunoreactive (ir) neurons in the frontal cortex of AD patients as compared to age-matched and middle-aged controls.

METHOD

Selection of Cases

In the present study, the frontal cortices of three middle-aged controls [55 ± 5 years, two females, one male, postmortem delay (range) (PM): 5–22 h], three age-matched controls (73 ± 6 years, two males, one female, PM: 3–8 h) and six AD patients (74 ± 5, four females, two males, PM: 6–24 h) were studied. As far as known, the subjects selected for this study were not treated with centrally active drugs or drugs interfering with cholinergic receptors, nor did the control subjects show evidence of dementia on chart review.

All brains had been subjected to neuropathological examination. For control brains, the diagnosis of a neurological disease was ruled out. All AD cases met the clinical and neuropathological working criteria for the definite diagnosis of AD (16). There were clinical reports of dementia combined with large numbers of senile plaques and neurofibrillary tangles. In the control

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group, senile plaques and neurofibrillary tangles were seen occasionally but in no case were enough for the neuropathological diagnosis of AD [cf. (16)].

Tissue Preparation

Cortical tissue was obtained from the brain bank of the Southern Illinois University Center for Alzheimer's Disease and Related Disorders. Upon autopsy, tissue was fixed in 10% buffered formalin. For the present study, samples of the frontal cortex (area 10 according to Brodmann) were dissected and rinsed for at least 12–15 h in 0.1 M phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS) at 4°C.

Immunoperoxidase Procedure

From all specimens, 50-μm thick cortical vibratome sections were prepared and treated for visualization of ir sites as described previously (34) with some modifications. Briefly, sections were preincubated in PBS containing 20% normal goat serum (NGS) for 1 h at room temperature (RT). Subsequently, the sections were rinsed in PBS (2 × 10 min) and then incubated with the primary antiserum, the monoclonal mAChR antibody M35 (48–72 h, 4°C). Production and characterization of the antibody have been described in detail elsewhere (2, 23, 34, 40). The antibody was used in a working dilution of 1:1000 in PBS, containing 1% NGS. After primary antibody incubation, sections were washed in PBS (2 × 10 min). A biotinylated anti-mouse IgM (Amersham) was used as secondary antibody (1 h, RT). After another wash in PBS (2 × 10 min), the samples were incubated in a streptavidin-peroxidase complex (Amersham; 1:50, 1 h, RT) followed by a wash in PBS. Visualization of the immunoprecipitate was obtained by immersion of the sections in a solution containing 5 mg diaminobenzidine (DAB, Sigma) per 10 ml PBS and 130 μl 0.3% hydrogen peroxide (Merck) per 10 ml PBS (10 min, RT). The reaction was stopped by transferring the specimens into PBS. The sections were mounted onto glass slides using Permount (Fisher Scientific) as mounting medium. For control purposes, sections otherwise treated as described above were incubated omitting 1) the primary antibody, 2) the biotinylated anti-mouse IgM antibody, or 3) were exclusively incubated with the streptavidin-peroxidase complex to exclude unspecific adherence of the detection systems to the sections. All controls revealed negative results. From each sample, cresylviolet-stained sections (Nissl) were prepared. Photomicrographs were taken using an Olympus Vanox photomicroscope.

Quantitative Evaluation

Immunohistochemical and Nissl-stained samples of each patient were examined using an Olympus BH2 light microscope equipped with a drawing tube (magnification 1.25 ×). Camera lucida drawings were prepared of a cortical strip of 250 μm width, extending from the pial surface to the border with the white matter, documenting ir neurons or histologically stained neurons respectively (40 × objective). In the latter case, only neurons with a visible nucleolus were counted (7). Numbers of neurons per strip were assessed and the height of the cortex was determined for each sample (7). One-way analyses of variance (ANOVAs) were performed to compare statistically the means of the three groups (middle-aged controls, age-matched controls and AD cases) with respect to immunolabeled and Nissl-stained neurons. Results were considered statistically significant if p values were less than 0.05.

RESULTS

Qualitative Results

As described previously for biopsy specimens (34), mainly pyramidal perikarya in layers II/III and V and their apical dendrites were labeled. This becomes obvious in camera lucida drawings of the immunostained neurons (Fig. 1). It can be recognized that there are no major differences as to the distribution pattern of labeled neurons between middle-aged controls, age-matched controls and AD cases. It is apparent that the density of labeled neurons in the cortices of aged controls (Fig. 1B) is somewhat lower than that of middle-aged controls (Fig. 1A) and AD cases (Fig. 1C). At higher magnification, the histochemical findings are shown paradigmatically in micrographs of the superficial cortical layers of all three groups (Fig. 2A–C). Again, the arrangement of immunolabeled perikarya is comparable between the different groups. The density of immunoreactive neurons appears somewhat less in age-matched controls (Fig. 2B) as compared to the other groups (Fig. 2A,C).

Quantitative Results

The results of the quantitative evaluation are shown in Fig. 3. For the middle-aged controls, 4673 ± 1364 labeled neurons/mm^3 were counted (mean ± s.e.m.), for the age-matched controls, 2393 ± 499, and for the AD brains, 5070 ± 1008. The differences between the three groups were not statistically significant, $F(2) = 1.6765$, $p > 0.05$. A t-test comparing age-matched controls and AD patients did not reveal statistically significant differences, $F(9) = 1.3541$, $p > 0.05$.

Densities of neurons, as revealed by counting Nissl-stained neurons, were not significantly different between all three groups [$p > 0.05$; mean ± s.e.m (middle-aged controls: 6526 ± 566; age-matched controls: 5973 ± 731; AD cases: 6723 ± 845) $F(2) = 0.027$].

DISCUSSION

No significant differences were detectable in the number of cortical mAChR-ir and of Nissl-stained neurons between the cortices of AD patients, age-matched and middle-aged controls. On the cellular level, this finding indicates a comparable extent of mAChR-protein expression in all three groups, consonant with the results of muscarinic receptor binding studies. The majority of these did not reveal any differences in receptor density in AD patients as compared to age-matched controls using radiolabeled quinuclidylbenzilate (QNB)- or N-methylscopolamine (NMS) as ligands (15, 18, 20, 29, 37). There have been, however, some reports on a slight increase (10,26) or decrease (22, 28, 47) of muscarinic binding sites in the frontal cortex of AD patients. Binding studies discriminating between the M1 and M2 mAChR subtypes, in part, have shown a tendency for M2 receptors to be reduced in AD (3,22), while others reported unchanged M2-binding sites (31,39). The presently used antibody does not allow for a discrimination of the M1 or M2 mAChR. Recently, the production of subtype-specific antibodies has been reported (19). These will be useful tools to study the differential expression of subtype-specific receptor proteins.

Using receptor autoradiography [(^{3}H)-QNB], the laminar labeling pattern with highest density of silver grains in the superficial layers disappeared in AD (18). In our material, AD cortices did not differ in the distribution pattern of ir neurons as compared to controls (cf. Figs. 1, 2). Altered binding patterns in AD brains may be due to receptor changes at the synaptic level. Studies on the ultrastructural distribution of AChR proteins in

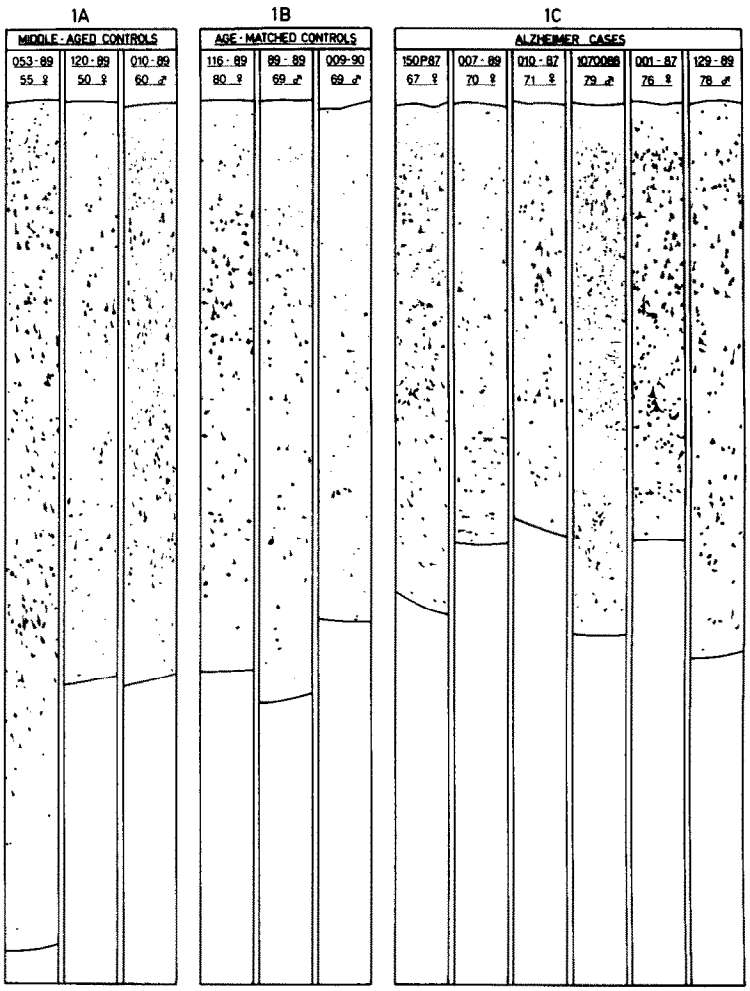

FIG. 1. Camera lucida drawings of the middle-aged (A) and age-matched control cases (B) and of the AD patients (C) studied depicting the distribution of M35-immunoreactive neurons in the frontal cortex (A 10). Protocol numbers (first row), age and sex (second row) are given for each column. Width of cortical strip, 250 μm. Note the apparently lower density of labeled neurons in the age-matched control group (B).

AD and control brains are presently under way in our laboratory.

The postmortem stability of mAChR has repeatedly been studied: in the rat brain, QNB binding was reported not to change up to 72 h postmortem at a storage temperature of 4°C (38). At 25°C, a 25% decrease of binding was observed after 48 h and 47% after 72 h. NMS binding decreases by 23% after 24 h at 4°C (43). In the human brain, QNB binding was reduced by 10% 70 h postmortem (27). No major differences in the immunohistochemical picture could be observed comparing biopsy specimens of temporal and occipital cortices (34) with the presently studied autopsy cortices. In the light of these findings and the PMs of our patients, it appears unlikely that autolysis may have been a major factor influencing the presently obtained results.

Rinne (30) showed the binding of tritiated QNB in the human frontal cortex to decrease by 7% per decade. Applied to our patients, one would expect a decrease of approximately 12%. Numbers of ir neurons showed a decrease of about 49% in age-matched vs. middle-aged controls. Immunocytochemistry and ligand binding techniques, however, reveal partly different aspects of receptor expression. While the former visualizes individual receptor-producing neurons, the latter reflects the receptor affinity for the applied ligand. Therefore, one would have to take into account the cellular mAChR density. Unfortunately, no data are available on this topic. It is, however, known that the ratio of nicotinic vs. muscarinic binding sites is approximately 1:2–4 [cf. (21)]. Using immunohistochemical methods, we found a ratio of nAChR- vs. mAChR-ir cortical neurons of about 1.7:1 (Schröder, unpublished observation), pointing at a relatively higher density of mAChR sites, since this ratio is the contrary of that calculated for the binding sites. The relatively small number of muscarinergic neurons then could well bear the 88% muscarinic binding sites predicted to be left in aged as compared to middle-aged subjects [cf. (30)]. Additional information on this issue might be obtained by microspectrofluorimetric measurements of single immunolabeled neurons.

Immunofluorescent double-labeling in the human cerebral cortex shows a colocalization of mAChR and nAChR in at least 30% of cholinoceptive neurons, the majority of these being py-

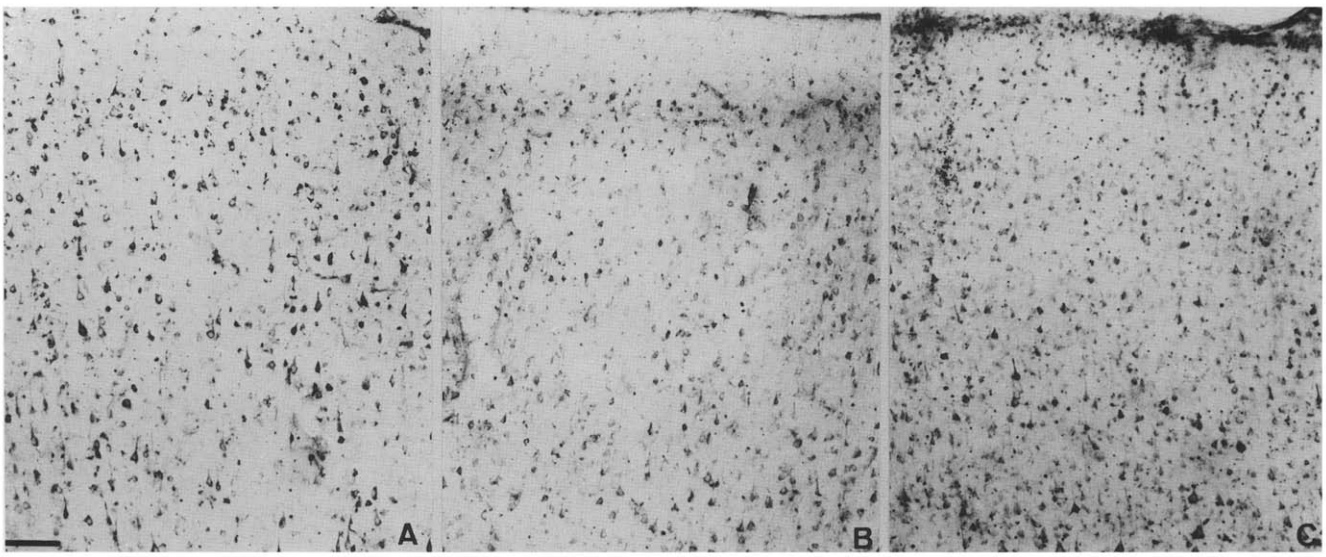

FIG. 2. Photomicrographs of representative control cases and AD patients showing the distribution of M35-immunoreactive neurons in the superficial layers (II/III) of the frontal cortex (A 10). Calibration bar 100 μm. (A) Middle-aged control. (B) Age-matched control. (C) AD case. Note that the normal distribution pattern is present in all three specimens.

ramidal cells (33). In AD, the number of cortical nAChR-ir neurons is only 33% of that of age-matched controls (35). This is not due to a neuron loss, since no significant differences in the density of Nissl-stained neurons were detected, which is in keeping with morphometric studies on the frontal cortex [cf. (8,36)].

Biochemical findings show a decrease in AD patients of nAChR binding sites ranging from 44 to 77% (14, 15, 26, 45). These findings raise the question of functional sequels of selective nAChR impairment on the cellular level and of the selective vulnerability of nAChR in AD.

As to the first point, it appears likely that, in AD, *relatively more* of the remaining cholinergic input into pyramidal neurons is mediated by mAChR. In terms of cell pharmacology, the propagation of ACh-elicited signals would then be predominantly achieved by cyclic adenosine monophosphate (cAMP)- or guanosine monophosphate (cGMP)-dependent and/or phosphoinositol-related processes [cf. (17,21)]. To our knowledge, however, no data are available on the functional properties of neurons following the loss of one type of its receptors.

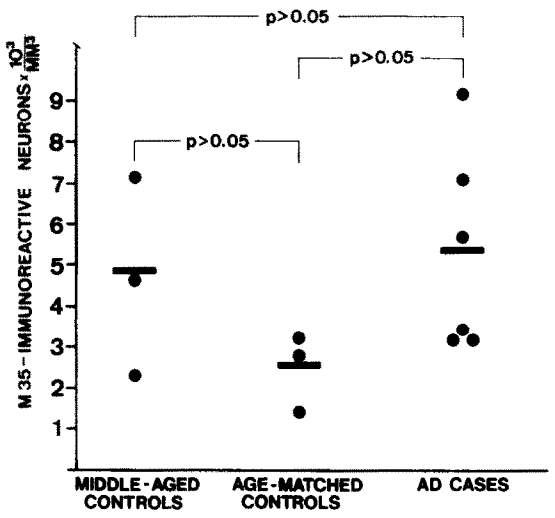

FIG. 3. Quantitative results of neuronal counting showing individual and mean numbers of M35-immunoreactive cortical neurons per mm^3 for the three different groups studied. There are no significant differences between the three groups ($p>0.05$).

As to the second issue, the loss of nicotinic binding sites in AD might be due to a long-term downregulation of receptors following presynaptic cholinergic degeneration. At the time being, it is not understood why mAChR are not affected in a similar way.

Animal studies trying to model some of the neuropathology associated with AD by lesions of the basal nucleus have revealed partly equivocal results. While the activity of ChAT (4,25) and acetylcholinesterase (AChE) (11) was, as expected, reduced, nicotinic binding was unaltered (4,41), and mAChR binding sites were either unchanged (4,41) or decreased (11).

With regard to possible treatment strategies of AD, the present findings might point to mAChR protein as a possible target of pharmacologically active compounds. The stimulation of mAChRs by specific agonists might be a possibility to increase cholinergic input into cortical neurons.

In conclusion, at the cholinoceptor site, AD appears to be characterized by the persistence of mAChR in pyramidal neurons and a selective loss of nAChR. To evaluate pharmacological intervention strategies, it would be desirable to create an animal model mimicking this situation. Toxins selectively destroying different types of cholinergic receptors might be a useful tool to this end.

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