SHORT REPORT

Alterations of muscarinic acetylcholine receptor subtypes in diffuse Lewy body disease: relation to Alzheimer's disease

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Abstract

Objectives—Dementia associated with Lewy bodies in cortical and subcortical areas is classified as dementia of the non-Alzheimer type and termed diffuse Lewy body disease (DLBD). The generic term “dementia with Lewy bodies (DLB)” was proposed in the international workshop on Lewy body dementia to include the similar disorders presenting Lewy bodies. In DLB, a lower level of choline acetyltransferase (ChAT) activity in the neocortex was found compared with that in Alzheimer's disease. The purpose of the present study was to determine the total amount of muscarinic acetylcholine receptors (mAChRs) and relative proportion of each subtype (m1–m4) of mAChRs in the frontal and temporal cortex of seven DLBD and 11 Alzheimer's disease necropsied brains.

Methods—A [³H]quinuclidinyl benzilate (QNB) binding assay and an immunoprecipitation assay using subtype-specific antibodies were performed. Each antibody was raised against fusion proteins containing peptides corresponding to the third intracellular (i3) loops of the respective mAChR subtype.

Results—The total amounts of mAChRs were significantly lower in the preparations of temporal cortices from DLBD and Alzheimer's disease than in those from dead controls (seven cases). In both diseases, the proportion of the m3 receptor in the frontal cortex was significantly increased and that of the m4 receptor in the temporal cortex was significantly decreased compared with the control specimens. The proportions of the m1 and m2 subtypes were significantly different in the temporal cortex. The proportion of the m1 receptor was significantly greater in the DLBD brains, whereas that of the m2 receptor was significantly greater in the Alzheimer's disease brains than in the controls.

Conclusions—The m1 receptor is the major subtype in the cerebral cortex, and m2 is known to be present at presynaptic

terminals. The higher proportions of m1 in DLBD and m2 in Alzheimer's disease suggest that the manner of degeneration in the cholinergic system is different between the diseases. It is hypothesised that a severe depletion of presynaptic cholinergic projective neurons causes the upregulation of m1 receptor in the temporal cortex in DLBD.

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Keywords: muscarinic receptor; subtype specific antibody; diffuse lewy body disease; cholinergic deficit

Diffuse Lewy body disease (DLBD) is a recently recognised clinicopathological entity associated with Lewy bodies in cortical and subcortical areas, with distinctive clinical features such as fluctuating cognition and recurrent visual hallucinations.¹ Similar terminology has been proposed by some researchers, such as Lewy body dementia, senile dementia of Lewy body type, and Lewy body variant of Alzheimer's disease. There is still some confusion regarding the concept of the disease entity. Among these diseases, the neuropathological criteria for the diagnosis of DLBD are the most strict. In the first international workshop on Lewy body dementia in 1995, the generic term “dementia with Lewy bodies” (DLB) was proposed to include the disorders mentioned above.¹ Dementia with Lewy bodies is not rare²; it is reported to be the most common cause of dementia in elderly people after Alzheimer's disease and cerebrovascular dementia.^{3–5} Dementia with Lewy bodies and Alzheimer's disease were reported to account for 20% and 52% of dementia in elderly people, respectively.⁴

In Alzheimer's disease, a consistent change is found in the cholinergic neurons. The neocortical cholinergic deficit was recently reported to be more extensive in DLB, and greater neuronal loss in the substantia innominata was found in DLB compared with Alzheimer's disease.^{6–8} Perry *et al* reported that choline acetyltransferase (ChAT) activity was lower in DLB compared with Alzheimer's disease in temporal and parietal cortices (10%–20% of controls, compared with 30%–70% of controls).⁸ The

Table 1 List of patients

Patient No	Age	Sex	Postmortem delay (h)	Cause of death
Diffuse Lewy body disease (DLBD):				
1	80	M	7	Suffocation
2	82	F	4	Pneumonia
3	86	F	6	Pneumonia
4	68	M	4	Pneumonia
5	74	M	3	Pneumonia
6	71	M	3	Pneumonia
7	84	M	4	Heart failure
Alzheimer's disease (AD):				
8	73	F	0.5	Heart failure
9	63	M	1.5	Heart failure
10	83	F	2	Bleeding of the alimentary tract
11	86	M	5	Pneumonia
12	63	M	6	Heart failure
13	80	M	1	Pneumonia
14	82	M	3	Heart failure
15	84	F	1	Heart failure
16	86	F	5	Heart failure
17	76	M	2	Heart failure
18	86	F	2	Colon cancer
Controls:				
19	80	M	0.5	Pneumonia
20	85	F	8	Pneumonia
21	91	M	2	Lung cancer
22	84	M	12	Pneumonia
23	87	F	3	Heart failure
24	89	F	18	Heart failure, Sick sinus syndrome
25	91	M	2	Uræmia

number of binding sites of muscarinic acetylcholine receptors (mAChRs) was reported to be raised in the neocortex of patients with DLB.⁹

Systematic measurements of mAChR subtypes in DLBD have never been reported. The genes for five subtypes (m1-m5) of mAChR have been cloned¹⁰⁻¹⁵ and the five subtypes have distinct distributions in the brain.^{16, 17} We have raised subtype-specific antisera against the m1, m2, m3, and m4 receptors as described below. In the present study, we estimated the levels of these mAChR subtypes by using the subtype specific antibodies and compared the values for DLBD and Alzheimer's disease.

Materials and methods

Brain tissue was obtained at necropsy from seven patients with DLBD (mean age 77.9 (SD 6.9), range 68-86; mean postmortem delay 4.4 (SD 1.5) hours, range 3-7 hours), 11 patients with Alzheimer's disease (mean age 78.4 (SD 8.7), range 63-86; mean postmortem delay 2.6 (SD 1.9) hours, range 0.5-6 hours) and seven subjects who had not shown symptoms of either disease (mean age 86.7 (SD 4.0), range 80-91; mean postmortem delay 6.5 (SD 6.5) hours, range 0.5-18 hours, table 1). The dissected brain tissue was immediately frozen in liquid nitrogen, then stored at -80°C until used. All of the patients with DLBD met the following criteria: (1) consensus criteria for the clinical diagnosis of probable and possible "dementia with Lewy bodies"¹¹; and (2) the neuropathological criteria for the diagnosis of DLBD,¹⁸ which are the most strict criteria and demand the presence of more than five or 10 cortical Lewy bodies in the predilection sites in a ×100 visual field in haematoxylin and eosin or ubiquitin immunostained preparations. All of the cases of DLBD were diagnosed as the "common form of diffuse Lewy body disease," because this entity contains the concomitant features of Alzheimer's disease pathology.¹⁹ All

of the cases of Alzheimer's disease met the following inclusion criteria: (1) clinical history of dementia, according to DSM-IV criteria; and (2) consortium to establish a registry in Alzheimer's disease (CERAD) neuropathological criteria for definite Alzheimer's disease.²⁰ Four of the seven patients with DLBD had been treated with anticholinergic drugs. None of the patients with Alzheimer's disease had ever been treated with a cholinesterase inhibitor. The diagnosis of "control" was also confirmed since appreciable senile changes were absent in the brains by histopathological analysis. None of them had had a neurological or psychological disorder.

Segments of porcine (m1, m2) and rat (m3, m4) mAChR genes were subcloned into the bacterial expression vector pGEX3X (Pharmacia, Uppsala, Sweden). Recombinant plasmids were confirmed to be in frame by double stranded dideoxy sequencing. The pGEX3X vector encodes the 26 kDa glutathione S-transferase fused to the N-terminus of the putative third intracellular loop (i3 loop) of the m1 to m4 receptors. The amino acid sequences expressed as fusion proteins were as follows, m1: Q(226)-K(353), m2: N(227)-C(324), m3: S(302)-Q(473), and m4: P(247)-Q(352). The differences in amino acids from human mAChRs were as follows, m1: three, m2: five, m3: 23, m4: 14. Rabbits were immunised with these fusion proteins by a conventional method for preparing antiserum.

The insect cell line Sf9, which expresses m1, m2, and m4 receptors, was prepared as described.^{21, 22} Chinese hamster ovary cells expressing m3 receptors were prepared by the same method used for the preparation of D2 receptor.²³

Membranes from the cell lines and brain tissue cells were prepared as follows. The brain tissues were suspended and homogenised in medium A (10 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, and 0.32 M sucrose) containing 1 mM benzamidine, 0.5 mM phenylmethyl sulphonyl fluoride, and 5 µg/ml pepstatin A. After centrifugation at 1000 g for 10 minutes, supernatants were collected and centrifuged at 20 000 g for 30 minutes. The pellets were suspended in medium B (the same as medium A except for the omission of sucrose) and centrifuged again at 20 000 g for 30 minutes. Finally, the pellets were suspended in 10 ml medium B/g original tissue.

The amounts of total mAChRs were estimated as the specific binding sites of [³H]QNB in membrane preparations. The specific binding was defined as the difference between the total and non-specific bindings, which were estimated by incubation with [³H]QNB (1.5 nM) for 90 minutes at 30°C in the absence and presence of 1 µM atropine, respectively. The amounts of proteins were measured by using an assay kit from Bio-Rad (Hercules, CA, USA) and bovine serum albumin as a standard. The specific [³H]QNB binding sites for membrane preparations of each cell line were estimated to be 15, 15, 7.0, and 5.0 pmol/mg protein for the m1, m2, m3, and m4 receptors, respectively.

Table 2 Immunoprecipitation of muscarinic acetylcholine receptor subtypes using subtype specific antiserum

Disease	$^3\text{H}]/\text{QNB}$ bound (fmol/mg)	Proportions of precipitated mAChR			
		m1 (%)	m2 (%)	m3 (%)	m4 (%)
Frontal cortex					
Controls (n=7)	1289 (139)	52.0	9.1	6.9	10.0
DLBD (n=6)	1128 (262)‡	50.5	8.4	9.8*	9.2
AD (n=11)	1407 (181)	51.0	9.4	9.6**	9.4
Temporal cortex					
Controls (n=7)	1934 (188)	53.2	9.1	7.3	10.6
DLBD (n=7)	1120 (95)**†	58.1*‡	8.5‡	8.2	6.8**
AD (n=11)	1371 (298)**	52.1	11.6**	9.3	7.6**

Data are mean (SD) data from a representative experiment. The density of mAChR was estimated as specific binding with $^3\text{H}]/\text{QNB}$, and ranged from 800 to 2200 fmol/mg membrane protein. The proportions of specifically precipitated mAChR subtypes were estimated as the differences in the amounts of bound $^3\text{H}]/\text{QNB}$ precipitated with specific antiserum and those precipitated with non-immune serum.

* $p < 0.05$; ** $p < 0.01$ v controls; † $p < 0.05$; ‡ $p < 0.01$ v AD.

Membrane preparations from postmortem human brains were incubated in KPB (10 mM potassium phosphate buffer (pH 7.0), 1.0 mM EDTA, 0.1 M NaCl) at 30°C for 90 minutes with $^3\text{H}]/\text{QNB}$ (2.3 nM) and centrifuged at 15 000 *g* for 15 minutes to take off free $^3\text{H}]/\text{QNB}$ from the membrane preparation. The labelled membrane was washed in KPB containing 0.1% sodium cholate, and then mAChR was solubilised in KPB containing 1.0% digitonin and 0.1% sodium cholate for at least 4 hours at 4°C. The solubilised mAChR was collected as a supernatant from centrifugation at 15 000 *g* for 20 minutes at 4°C. The proportions of solubilised $^3\text{H}]/\text{QNB}$ labelled receptors were 70%-95% of the $^3\text{H}]/\text{QNB}$ binding sites in each membrane preparation. The supernatant (40 µl) containing 20-100 fmol $^3\text{H}]/\text{QNB}$ -labelled receptors was mixed with 4 µl antiserum before incubation at 4°C for at least 4 hours. After addition of 20 µl Pansorbin (Calbiochem, La Jolla, CA, USA), the mixture was incubated at 4°C with rotation for 1 hour and then precipitated at 15 000 *g* for 10 minutes. The Pansorbin deposit was washed with 200 µl KPB and pelleted again at 15 000 *g* for 10 minutes. The amount of $^3\text{H}]/\text{QNB}$ in the pellet was determined by liquid scintillation counting. The percentage of immunoprecipitation was calculated by dividing the amount of $^3\text{H}]/\text{QNB}$ count in the Pansorbin pellet by the whole count in the total supernatant and Pansorbin pellet.

A one way analysis of variance (ANOVA) followed by a least significant difference (LSD) test was used to compare the values of total QNB binding and the proportions of subtypes (m1 to m4). Statistical evaluations were performed with the SPSS package.

Results

The specificity of antisera raised against GST fusion proteins incorporating the i3 loops was tested by the immunoprecipitation of mAChR subtypes. The results indicated that each of the antisera specifically recognised and precipitated only one of the m1-m4 receptors (figure).

The amounts of $^3\text{H}]/\text{QNB}$ binding sites in membrane preparations ranged from 0.8 to 1.8 pmol/mg protein (frontal cortex) and from 0.8 to 1.9 pmol/mg protein (temporal cortex). In the temporal cortex, the $^3\text{H}]/\text{QNB}$ binding

sites of the DLBD and Alzheimer's disease tissues were significantly lower than those of the control tissue. The $^3\text{H}]/\text{QNB}$ binding sites in DLBD were significantly lower than those in Alzheimer's disease. In the frontal cortex, there were no significant differences in $^3\text{H}]/\text{QNB}$ binding sites comparing DLBD or Alzheimer's disease with controls, whereas these binding sites were significantly lower in DLBD than in Alzheimer's disease.

Membrane preparations from the brains were used for precipitation with each of the antisera against m1-m4 receptors. The proportions of $^3\text{H}]/\text{QNB}$ -receptor complex precipitated with each of the antisera in solubilised $^3\text{H}]/\text{QNB}$ -receptor complex were estimated and are summarised in table 2. The proportions of $^3\text{H}]/\text{QNB}$ -receptor complex precipitated with the antiserum against m1, m2, m3, and m4 receptors were estimated to be 44.4%-60.6%, 6.4%-15.8%, 3.3%-14.8%, and 4.7%-14.7%, respectively. In the frontal cortex, the proportion of $^3\text{H}]/\text{QNB}$ -receptor complex precipitated with anti-m3 antibodies was significantly higher in both DLBD and Alzheimer's disease than in controls. In the temporal cortex, the $^3\text{H}]/\text{QNB}$ -receptor complex precipitated with anti-m4 antibodies was significantly lower in DLBD and Alzheimer's disease than in controls. By contrast, $^3\text{H}]/\text{QNB}$ -receptor complex precipitated with anti-m1 antibodies was significantly higher in DLBD than in controls, whereas the precipitate by m2 antiserum was significantly higher in Alzheimer's disease than in controls. The level of m1 in DLBD was significantly higher than that in Alzheimer's disease, whereas the level of m2 in DLBD was significantly lower than that in Alzheimer's disease.

Discussion

Our results show common and different characteristics of mAChR in Alzheimer's disease and DLBD. The saturated bindings of $^3\text{H}]/\text{QNB}$ were lower in DLBD and Alzheimer's disease tissues than in control tissues in the temporal area. Perry *et al* reported that ChAT activity was seriously reduced in temporal and parietal cortices whereas low affinity mAChR binding was significantly increased in DLB but not in Alzheimer's disease.⁹⁻²⁴ We do not know the reason for the differences in the total binding sites of mAChR among the tissues examined here. The major finding of the present study was that the total binding sites of mAChR in DLBD and Alzheimer's disease were decreased in the temporal area, but not in the frontal area. We suspect that the depletion of the cholinergic system is more severe in the temporal area than in the frontal area.

It is not possible to determine the absolute proportion of m1 to m4 receptors strictly by our method of immunoprecipitation, because the efficiencies of solubilisation or precipitation may vary among these receptors. As shown in the figure, the efficiency of immunoprecipitation of m3 receptor was lower than that of the other subtypes. m3 Receptor was expressed in Chinese hamster ovary cells, whereas m1, m2,

Each antiserum can immunoprecipitate a single cloned mAChR subtype (m1-m4) in a subtype specific manner. The m1, m2, and m4 receptors were expressed in Sf9 cells, and the membrane preparations were labelled with [3 H]QNB and then solubilised with 1% digitonin and 0.1% sodium cholate. The m3 receptors were treated in the same way except that they were expressed in Chinese hamster ovary cells instead of Sf9 cells, because the m3 receptors expressed in Sf9 cells were reported not to be solubilised with digitonin. The m3 receptor in Chinese hamster ovary cells could be solubilised for precipitation. Solubilised receptors were precipitated with antisera. Roughly 85, 82, 58, and 90% of solubilised m1, m2, m3, and m4 receptors, respectively, were precipitated with the specific antiserum, whereas less than 10% of solubilised receptors were precipitated with non-immune serum (control serum) or antiserum against other subtypes.

and m4 were expressed in Sf9 cells. The solubility of receptors may be changed when the composition of the cell membrane is different. We were able to compare the relative changes of mAChR subtypes in Alzheimer's disease, DLBD, and controls because the efficiencies of solubilisation and immunoprecipitation are thought to be essentially the same among these states. We can only discuss alterations of mAChR subtypes in the comparison of DLBD, Alzheimer's disease, and controls.

In the frontal cortex, the levels of the m3 subtype in the present cases of DLBD and Alzheimer's disease were higher than in the control cases. In the temporal cortex, the level of the m4 subtype in DLBD and Alzheimer's disease was lower than that in controls. Flynn *et al* reported increases of immunoprecipitated m4 receptor protein in frontal, temporal, and parietal cortices of Alzheimer's disease.²⁵ We cannot explain the opposite results of m4 subtypes. We suspect that the increase of m3 in the frontal cortex and the decrease of m4 in the temporal cortex are the results of cholinergic destruction, which is common in both types of degenerative dementia.

In the temporal cortex, the differences were found in the m1 and m2 receptor levels between DLBD and Alzheimer's disease. The m1 level was higher in DLBD than in controls,

whereas the m2 level was higher in Alzheimer's disease than in controls. Comparing the two disease states, m1 receptor was somewhat higher in DLBD and m2 was higher in Alzheimer's disease. The m1 receptor was shown to be the most abundant subtype present in the cerebral cortices and hippocampus of the human brain by immunoprecipitation assay.²⁵ The m2 receptor has been shown to exist at the presynapses of the cholinergic system, in an examination of the presence of m2 mRNA.²⁶ A pharmacological properties analysis²⁷ and electron microscopic studies²⁸⁻³⁰ showed that m2 is located at the presynaptic termini of the cholinergic system.

The mAChR binding sites in DLBD and Alzheimer's disease were decreased in the temporal cortex more than in controls, and in DLBD the mAChR binding sites were significantly lower than in Alzheimer's disease. The ChAT activity was decreased in both diseases more than in controls,^{6,8} and temporal ChAT activity was significantly lower in DLBD than in Alzheimer's disease.⁸ The cholinergic deficit is considered to be more severe in DLBD than in Alzheimer's disease. We suspect that the mechanism of cholinergic depletion in DLBD is different from that in Alzheimer's disease. The higher level of m1 in the present cases of DLBD suggests the possibility that m1 (the major subtype of postsynaptic mAChR) is preserved or upregulated in this disease. We suspect that the destruction of cholinergic projection neurons precedes the loss of cholinergic neurons in the temporal area in DLBD; this possibility seems to be in good agreement with the reduction of ChAT activity in DLBD reported previously.⁴ On the other hand, the immunoprecipitated m2 levels were higher in the present cases of Alzheimer's disease than in the controls and cases of DLBD. We speculate that the loss of cholinergic neurons precedes the loss of presynaptic cholinergic projection neurons in Alzheimer's disease. The higher m2 level in Alzheimer's disease suggests that the presynaptic m2 receptors are preserved or upregulated in this disease.

Our present findings provide the first description of the alteration of molecular subtypes of mAChR in DLBD and its difference from Alzheimer's disease. Our findings suggest differences in cholinergic deficits between DLBD and Alzheimer's disease. In recent trials of cholinergic replacement therapies, DLBD was indicated to be an entity which is more responsive to cholinesterase inhibitor (tacrine) compared to Alzheimer's disease.^{5,31,32} We suspect that a severe deficit of cholinergic projections in conjunction with the relative preservation of m1 in DLBD at the temporal cortex is one of the reasons for the good response to tacrine.

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