Cholinergic Markers in Alzheimer Disease and the Autoregulation of Acetylcholine Release

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The status of various cholinergic markers was compared in Alzheimer's and Parkinson's diseases. Rather unexpectedly, similar decrements were observed in choline acetyltransferase (ChAT) activity and in density of muscarinic M_2 and nicotinic receptors in various cortical areas in these two disorders. This may relate to the existence of important functional interactions between cholinergic and dopaminergic systems in cortical and hippocampal areas. Additionally, the parallel decrements in nicotinic and muscarinic M_2 receptor subtypes, with that of ChAT activities in these disorders suggest their presynaptic location. A series of pharmacological data do in fact reveal that nicotinic receptors may act as positive autoreceptors modulating basal acetylcholine release while muscarinic M_2 receptors could act as negative autoreceptors. This information may have significance for the development of new treatment strategies (for example, M_2 antagonists) of disorders associated with cholinergic hypofunction.

Key Words: Alzheimer's disease, pre-synaptic receptors, acetylcholine, memory

INTRODUCTION

Alzheimer's disease (AD) is characterized by neuropathological features including increases in the presence of neurofibrillary tangles and neuritic plaques composed of β-amyloid deposits (Ball 1977; Terry and Katzman 1983; McGeer 1984; Etienne et al 1986; Price 1986; Selkoe 1989). While these structures are not unique, their increased abundance in the brains of subjects with AD, often correlated with memory losses, suggests a possible etiological role in the development of the pathology (Khachaturian 1985; Price 1986; Selkoe 1989; Marx 1993).

Parallel to (or as a consequence of) these neuropathological modifications, various neurotransmitter systems are altered in the brain tissues of subjects with AD. Among those, it is clear that consistent findings apply only to losses of various markers of the cholinergic (Perry 1986; Price 1986; Quirion et al 1986; 1990), somatostatinergic (Davies et al 1980), and possibly corticotropin-releasing factor (CRF) (Bissette et al 1985) innervations. Losses of various other transmitters, including serotonin (5-HT), noradrenaline, dopamine and various neuropeptides, are not reported as consistently and may be related to the existence of "neurochemical" sub-types of AD (Quirion et al 1990). For example, in a given sub-group, in addition to general decrements in cholinergic and somatostatinergic markers, losses in noradrenergic, but not 5-HT, innervation may be seen, while the reverse is observed in other cases. The precise recognition of these putative sub-groups is of clinical relevance, since it could lead to the development of new therapeutic approaches if, for example, it can be demonstrated that depressive behavior observed in some patients with AD is related to alterations of the 5-HT system, for which a variety of clinically effective drugs are already available.

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Table 1

Choline acetyltransferase (ChAT) activity in various areas of the age-matched normal brains and brains of subjects with Alzheimer's disease and Parkinson's disease

	ChAT activity (nmol acetylcholine/mg of protein/hour)[a]		
Area	Normal subjects	Subjects with Alzheimer's disease	Subjects with Parkinson's disease
Frontal cortex	3.8 ± 0.4	1.9 ± 0.2[a]	1.9 ± 0.3[a]
Temporal cortex	7.5 ± 1.0	2.9 ± 0.5[a]	4.0 ± 0.6[a]
Hippocampus	9.2 ± 0.7	4.7 ± 0.6[a]	5.3 ± 0.6[a]
Striatum	90.6 ± 9.9	71.0 ± 7.1	73.2 ± 6.6
Thalamus	3.2 ± 0.2	2.9 ± 0.4	3.6 ± 0.5

[a]$p < 0.05$. Mean ± SEM of eight to 22 brains. Modified from Aubert et al (1992a) with permission.

Cholinergic markers in Alzheimer's disease

After the pioneering work of Davies and Maloney (1976), various groups (Perry et al 1977; Coyle et al 1983; Etienne et al 1986; Quirion et al 1986; 1989b; Araujo et al 1988b; Aubert et al 1992a) reported on the altered status of cortical and hippocampal cholinergic projections in AD (McGeer 1984; Perry 1986; Price 1986; Quirion et al 1990; Gauthier et al 1991). However, the author has been among the few groups to investigate the integrity of various cholinergic projection neurons in AD namely using a large variety of markers, including choline acetyltransferase (ChAT) activity, [^{3}H]hemicholinium-3 binding to the high-affinity choline uptake carrier protein, [^{3}H]vesamicol to the vesicular transporter, selective radioligands of the muscarinic M_1, M_2, M_3, and nicotinic cholinergic receptors, and more recently receptor antibodies and their respective mRNAs. Only the use of a large array of markers will allow for a better understanding of the functional relevance of cholinergic losses in AD. This could then lead to a more rational approach to the development of effective therapeutic strategies.

As shown in Table 1, the activity of the synthetic enzyme responsible for the transformation of choline into acetylcholine, ChAT, is markedly reduced in cortical and hippocampal areas in AD (Araujo et al 1988b; Aubert et al 1992a). In contrast, striatal and thalamic ChAT activities are mostly normal, revealing their relative sparing in AD. This demonstrates that only certain cholinergic neurons are affected in AD, namely the relatively long projection neurons originating from the basal forebrain. Intrinsic striatal cholinergic neurons are apparently mostly spared in AD (Table 1). It is therefore clear that AD is not a disease necessarily associated with the cholinergic phenotype, since not all cholinergic neurons are affected, at least on the basis of the activity of the pre-synaptic marker, ChAT.

When [^{3}H]QNB was used as a universal muscarinic receptor ligand, no clear evidence of significant changes in receptor affinity (K_d) and maximal capacity (B_{max}) in cortical and sub-cortical regions of the AD brain was found (Araujo et al 1988b; Aubert et al 1992a). B_{max} values may even increase slightly in some patients (Araujo et al 1988b). These results are in agreement with most (Perry 1986; Quirion et al 1986; 1990) and indicate that more selective probes must be used in order to determine the respective status of each muscarinic receptor sub-type in AD. Accordingly, [^{3}H]pirenzepine (PZ) was used as a fairly selective M_1 ligand

Table 2

Maximal binding capacity of [^{3}H]pirenzepine/putative M_1 binding sites in various areas of the age-matched normal brains and brains of subjects with Alzheimer's disease and Parkinson's disease

	M_1 muscarinic binding (fmol/mg protein)		
Area	Normal subjects	Subjects with Alzheimer's disease	Subjects with Parkinson's disease
Frontal cortex	289 ± 55	325 ± 57	259 ± 38
Temporal cortex	320 ± 49	327 ± 57	390 ± 53
Hippocampus	283 ± 71	319 ± 38	248 ± 33
Striatum	497 ± 83	762 ± 73[a]	556 ± 89
Thalamus	57 ± 7.3	71 ± 8.3	54 ± 6.9

[a]$p < 0.05$. Mean ± SEM of six to 12 brains. K_d values were not altered. Modified from Aubert et al (1992a) with permission.

Table 3

Maximal binding capacity of [^{3}H]AF-DX 116/putative M_2 binding sites in various areas of the age-matched normal brains and brains of subjects with Alzheimer's disease and Parkinson's disease

	M_2 muscarinic binding (fmol/mg protein)		
Area	Normal subjects	Subjects with Alzheimer's disease	Subjects with Parkinson's disease
Frontal cortex	37.3 ± 4.5	16.8 ± 2.3[a]	18.1 ± 2.8[a]
Temporal cortex	33.5 ± 4.8	19.9 ± 3.3[a]	20.5 ± 3.2[a]
Hippocampus	68.4 ± 9.4	27.7 ± 4.1[a]	22.6 ± 3.8[a]
Striatum	55.7 ± 12.8	44.3 ± 7.0	30.4 ± 4.6
Thalamus	34.3 ± 5.0	27.1 ± 7.0	22.9 ± 8.7

[a]$p < 0.05$. Mean ± SEM of five to eight brains. K_d values were not altered. Modified from Aubert et al (1992a) with permission.

(Hammer et al 1980), and either [^{3}H]acetylcholine (under muscadnic conditions; Araujo et al 1988b; Quirion et al 1989a), [^{3}H]AF-DX 116 (Hammer et al 1986; Regenold et al 1989; Araujo et al 1989) or [^{3}H]AF-DX 384 (Aubert et al 1992b) as preferential M_2 radioligands.

[^{3}H]PZ/M_1-like receptor binding parameters (K_d and B_{max}) in the brains of normal controls are the same as those in patients with AD, except for significant increases in B_{max} in striatal and potentially, hippocampal (Araujo et al 1988b) areas (Table 2). In contrast, putative M_2 receptor binding capacities (not K_d) are markedly decreased in cortical and hippocampal areas of the AD brain (Table 3). As for ChAT activity (Table 1), M_2 receptor binding parameters are not significantly altered in sub-cortical regions such as the striatum and the thalamus (Table 3). Rather, similar decrements in putative M_2 receptors were detected using either [^{3}H]acetylcholine (Araujo et al 1988b); [^{3}H]AF-DX 116 (Aubert et al 1992a) or [^{3}H]AF-DX 384 (Quirion et al 1993). Moreover, preliminary data using m_1, m_2 and m_4 receptor antibodies suggest that the m_2 (but not the m_1 and m_4) receptor protein is decreased in the hippocampal formation of patients with AD; this supports results of binding assays (Quirion et al 1993; Hersi et al, unpublished data). The concomitant decreases in both ChAT activity and putative M_2 binding sites in cortical, but not sub-cortical, areas of the brain of a subject with AD suggests that a certain proportion of M_2 receptors are located on cholinergic nerve terminals in these regions (Mash et al 1985). It is also possible that some M_2 receptors are located on cortical nerve terminals and/or intrinsic neurons of non-cholinergic nature that degenerate in AD (Davies et al 1980; Bissette et al 1985); this may explain in part the decrements observed here.

Regarding nicotinic receptors, various groups of researchers (Flynn and Mash 1986; Whitehouse et al 1986; Nordberg and Winblad 1986; Perry et al 1987; Araujo et al 1988b; Aubert et al 1992a) have reported marked losses in their densities in cortical and hippocampal regions of the brain of subjects with AD using either [^{3}H]acetylcholine (under nicotinic conditions), [^{3}H]nicotine or [^{3}H]N-methylcarbamylcholine (Boksa and Quirion 1987; Boksa et al 1989) (Table 4). In fact, with decreases in ChAT activities, decrements in nicotinic receptors is one of the most consistent neurochemical findings in AD brains. As for ChAT and M_2 binding, nicotinic binding parameters are not altered in subcortical areas such as the thalamus and the striatum in AD (Table 4). This may also indicate that a certain proportion of

Table 4

Maximal binding capacity of [^{3}H]N-methylcarbamylcholine/nicotinic binding sites in various areas of the age-matched normal brains and brains of subjects with Alzheimer's disease and Parkinson's disease

	Nicotinic binding (fmol/mg protein)		
Area	Normal subjects	Subjects with Alzheimer's disease	Subjects with Parkinson's disease
Frontal cortex	8.5 ± 1.6	3.5 ± 0.5[a]	3.8 ± 0.6[a]
Temporal cortex	12.9 ± 3.3	3.5 ± 0.6[a]	4.5 ± 1.1[a]
Hippocampus	7.9 ± 1.2	3.1 ± 0.5[a]	3.9 ± 0.8[a]
Striatum	24.0 ± 6.2	18.4 ± 6.9	5.8 ± 1.7[a]
Thalamus	14.3 ± 1.6	10.7 ± 1.1	6.5 ± 1.5[a]

[a]$p < 0.05$. Mean ± SEM of five to 12 brains. K_d values were not altered. Modified from Aubert et al (1992a) with permission.

nicotinic receptor sites is located on cholinergic nerve terminals. Among the other cholinergic markers studied, reproducible results could not be obtained with [^{3}H]hemicholinium-3, a marker of the pre-synaptic, high-affinity choline uptake carrier protein, while this probe was most useful in other mammalian brain tissues (Quirion 1987). This may be related to the agonal states and/or post-mortem delays inevitably associated with studies of human brains. However, Pascual and colleagues (1991) recently reported the successful use of [^{3}H]hemicholinium-3 in post-mortem human brains and demonstrated that losses in binding are correlated, as expected, with decrements in ChAT activities. It therefore appears that [^{3}H]hemicholinium-3, under optimal conditions, can be a useful marker of the integrity of cholinergic nerve terminals in AD. [^{3}H]vesamicol binding, a marker of the vesicular transport protein, is apparently spared in the brains of subjects with AD (Ruberg et al 1990; Kish et al 1990).

Cholinergic markers in Parkinson's disease

For comparison, an exhaustive study was undertaken on the status of various cholinergic markers in Parkinson's disease (PD), with or without AD-type dementia (Aubert et al 1992a). Surprisingly, it was found that ChAT activities, as well as M_2 muscarinic and nicotinic receptor binding parameters, in cortical and hippocampal areas of the idiopathic PD brain were as affected as they were in AD (Tables 1 to 4). Similar results were recently reported by two other groups of researchers (Perry et al 1991; Lange et al 1993), although ChAT activity in the entorhinal cortex was altered differentially in the brains of subjects with PD and AD (Perry et al 1991). It thus appears that cortical cholinergic deficits may be more prominent than first expected in idiopathic PD without apparent dementia. Could it be that the dementia process believed to be associated with cortical and hippocampal cholinergic deficits is related mostly to a more extensive loss in the entorhinal cortex of the patient with AD which could be relatively spared in PD? This hypothesis is currently under investigation and has some merit from a neuroanatomical perspective, the entorhinal cortex being a key input area for the hippocampal formation. In fact, one of the best animal models of synaptic plasticity occurring in AD is the entorhinal cortex lesioning model, known to induce a "major" reorganization of the chemical neuroanatomy of the hippocampus in the rat (Steward 1986).

Another possibility is related to the fact that most of the brains of subjects with PD used in the three studies described above were from patients having a long history of treatment with dopaminergic drugs, such as L-dopa. Could it be that these treatments somehow either mask or ameliorate symptoms of dementia? While cholinergic and dopaminergic systems may be mostly antagonistic in the striatum, recent evidence suggests that dopamine and dopaminergic drugs, acting via D_1 receptors, can stimulate the *in vivo* release of acetylcholine in the cortex (Casamenti et al 1987; Day and Fibiger 1992) and the hippocampal formation (Hersi et al, unpublished results). Accordingly, could it be that treatments with L-dopa (producing dopamine then acting on D_1 receptors) facilitate the release of acetylcholine from remaining cholinergic nerve terminals still functional in cortical and hippocampal areas of the brains of subjects with PD? If this can be demonstrated, L-dopa treatments could indirectly improve cognition by stimulating the release of acetylcholine in relevant cortical and hippocampal regions. Already, it has been suggested that certain dopaminergic-related agonists should be considered for the treatment of demented patients (McGurk et al 1992).

Autoreceptors on cholinergic neurons

On the basis of data obtained from the brains of subjects with AD and PD, the author investigated whether or not parallel decrements in ChAT activities, M_2 and nicotinic binding sites could be related to the presence of these two receptor classes on cholinergic nerve terminals acting as autoreceptors to regulate acetylcholine release (Raiteri et al 1984; Marchi and Raiteri 1985; Mash et al 1985).

Naturally, it is rather difficult to assess transmitter release in post-mortem human brains; hence, *in vitro* rat brain slice preparations and *in vivo* dialysis in behaving animals were used to investigate the respective effects of muscarinic and nicotinic-related drugs on acetylcholine release.

Muscarinic M_2 (AF-DX 116, AF-DX 384 and BIBN-99) (Hammer et al 1986; Doods et al 1993)) but not M_1 (pirenzepine) (Hammer et al 1980) receptor antagonists potently stim-

Table 5

Effect of various muscarinic receptor agonists and antagonists on 25 mM K^+-evoked acetylcholine release from three-month old rat hippocampal slices

Drug	Acetylcholine release (% control)
Antagonists	
• atropine	148 ± 8.0[a]
• pirenzepine (M_1)	97 ± 5.0
• AF-DX 116 (M_2)	152 ± 14[a]
Agonists and antagonists	
• oxotremorine	63 ± 6.0[a]
• oxotremorine + atropine	98 ± 7.0
• oxotremorine + pirenzepine	65 ± 6.0[a]
• oxotremorine + AF-DX 116	98 ± 5.0

[a]$p < 0.05$ from baseline values. Mean ± SEM of five to nine determinations. All drugs were tested at an equimolar concentration (0.1 mM). Modified from Lapchak et al (1989b) with permission.

Table 6

Effect of various muscarinic receptor agonists and antagonists on *in vivo* cortical acetylcholine release in three-month-old freely moving rats

Drug	Acetylcholine release (% control)
Atropine (non-selective)	
• 1 μM	422 ± 44[a]
AF-DX 116 (M_2)	
• 1 μM	136 ± 14
• 10 μM	438 ± 49[a]
• 40 μM	582 ± 111[a]
AF-DX 384 (M_2)	
• 0.1 μM	186 ± 29[a]
• 1 μM	458 ± 84[a]
• 10 μM	543 ± 151[a]
• 40 μM	711 ± 113[a]
AQ-RA 741 (M_2)	
• 40 μM	600 ± 91[a]
Pirenzepine (M_1)	
• 40 μM	349 ± 83[a]

[a]$p < 0.05$ from baseline values. Mean ± SEM of four to eight determinations. All drugs were infused directly into the probes at the indicated concentrations.

ulated the *in vitro* release of acetylcholine in the cortical, hippocampal and striatal slice preparations of young adult rats (Pohorecki et al 1988; Lapchak et al 1989a; 1989b; Hoss et al 1990; Richards 1990) (Table 5). In contrast, agonists such as oxotremorine inhibited acetylcholine release in these preparations; this effect was reversed by sub-threshold doses of M_2 antagonists. M_2 modulatory effects were not blocked by tetrodotoxin (Lapchak et al 1989a). These results suggest the presence of negative muscarinic autoreceptors of the M_2 sub-type on cholinergic nerve terminals in various cortical and subcortical regions of the brain of the rat.

Data on *in vivo* dialysis also support this hypothesis. A variety of M_2-related blockers were shown to potently increase acetylcholine release (six- to ten-fold over baseline) in the cortex (Richard et al 1989; 1991) and the hippocampus (Wilson et al 1992) of freely moving rats (Table 6). In contrast, agonists blunted acetylcholine release according to a M_2-like profile of activity (Richard et al 1991). Thus, both *in vitro* and *in vivo* functional results suggest the presence of "negative" M_2 autoreceptors, affecting cholinergic nerve terminals. This may explain the concomitant decreases in both ChAT activities and M_2 binding sites in the cortex and hippocampus of patients with AD (Mash et al 1985; Araujo et al 1988b).

In contrast to the effects observed with muscarinic drugs, nicotinic *agonists* stimulate the release of acetylcholine in cortical and hippocampal (but not striatal) areas of rats' brains (Richardson and Szerb 1974; Szerb et al 1977; Rowell and Winkler 1984; Beani et al 1985; Wonnacott 1987; Araujo et al 1988a; De Sarno and Giacobini 1989) (Table 7). Nicotine and the selective nicotinic agonist N-methylcarbamylcholine (Boksa et al 1989) increased the basal release of acetylcholine in cortical and hippocampal slice preparations of rats. These effects were not sensitive to tetrodotoxin but were blocked by CNS-type nicotinic antagonists, such as dihydro-beta-erythroidine and D-tubocurarine, but not by α-bungarotoxin (Araujo et al 1988a), demonstrating the specificity of the observed potentialization. Similar results were observed using *in vivo* dialysis, a combination of a nicotinic agonist and an M_2 antagonist producing more than just an additive effect, suggesting a possible synergism between these two classes of autoreceptors in the modulation of acetylcholine release (Richard et al 1991). Interestingly, it appears that the capacity

Table 7

Effects of the nicotinic agonist N-methylcarbamylcholine (MCC) on basal acetylcholine release from brain slices of three-month-old rats

Area	Acetylcholine release (% control)
Hippocampus	
• MCC (10 μM)	211 ± 33[a]
• MCC (10 μM), tetrodotoxin (1 μM)	191 ± 16[a]
• MCC (10 μM), Ca^{2+}-free	103 ± 13
• MCC (10 μM), D-tubocurarine (1 μM)	102 ± 3
Frontal cortex	
• MCC (10 μM)	177 ± 25[a]
• MCC (10 μM), tetrodotoxin (1 μM)	164 ± 12[a]
• MCC (10 μM), Ca^{2+}-free	98 ± 8
• MCC (10 μM), D-tubocurarine (1 μM)	102 ± 16
Striatum	
• MCC (up to 10 mM)	98 ± 120[b]

[a]$p < 0.05$ from baseline values. [b]non-significant. Mean ± SEM of four to eight determinations. Similar results were obtained using nicotine. No effects were observed on 25 mM K^+-stimulated acetylcholine release. Modified from Lapchak et al (1989b) with permission.

of the positive nicotinic autoreceptors to modulate hippocampal acetylcholine release is mostly unimpaired in aged rats (Araujo et al 1990).

These results suggest the existence of two types of autoreceptors on cholinergic nerve terminals in cortical and hippocampal areas of the brains of rats. If a similar organization exists in the human brain, it may explain the concomitant losses of ChAT activities and M_2 muscarinic and nicotinic receptors in neurological disorders, such as AD. The "positive" nicotinic autoreceptor is apparently responsible for the maintenance of a basal release of acetylcholine in the synaptic cleft, while the M_2 muscarinic sub-type would negatively regulate an impulse-driven release to avoid over-stimulation.

Behavioral significance of muscarinic M_2 and nicotinic receptors in learning paradigms

The possible significance of potential M_2 muscarinic and nicotinic autoreceptors in learning paradigms was evaluated next. It was reported that the M_2 blocker AF-DX 116 facilitated learning in the eight-arm radial maze WIN/STAY, WIN/SHIFT task, suggesting the potential involvement of this receptor sub-type in hippocampal and striatal components of the learning process (Packard et al 1990). However, the rather poor lipophilicity of this drug hampered a detailed investigation of its CNS effects. Accordingly, BIBN-99, a more selective M_2 antagonist with a better brain partition coefficient (Doods et al 1993), was investigated. This molecule markedly improved learning performances in both young rats with scopolamine-induced amnesia as well as in 24-month aged-memory impaired rats (Doods et al 1993; Wilson et al 1992). It therefore appears that the blockade of purported M_2 negative autoreceptors by relatively selective antagonists can facilitate mnesic processes in a variety of learning tasks rats. This finding is of interest for AD, since the blockade of the remaining M_2-negative autoreceptors (at least 40% to 60%) (Araujo et al 1988b) may increase acetylcholine release from still functioning cholinergic nerve terminals thereby improving learning and memory.

As for nicotinic agonists and the possible activation of positive nicotinic autoreceptors, various groups of researchers have proposed that nicotine-like agents can facilitate certain aspects of learning and memory in humans and animals (Newhouse et al 1988; Sunderland et al 1988; Clarke 1990). In fact, it appears that nicotine and related agents may be most relevant for attention-related behaviors (Newhouse et al 1988; Sunderland et al 1988; Clarke 1990).

Accordingly, a variety of nicotinic drugs were tested in the latent inhibition paradigm, a model of attentional behavior in the rat (Sen et al 1993). In this model, nicotine and related agonists, such as lobeline and cytisine, facilitate attention in a dose-dependent manner. The mechanism by which these various agonists produced their effects is now under investigation, as is the potential synergism with muscarinic drugs (for example, M_2 blockers). Interestingly, few pilot trials have already performed with nicotinic agonists on patients with AD (Newhouse et al 1988; Sunderland et al 1988). Some beneficial effects have been observed, and further studies are in progress to confirm these preliminary findings and the application of this approach to a large number of patients.

CONCLUSION

It appears that it is possible to markedly facilitate the release of acetylcholine by modulating the activity of both positive and negative cholinergic autoreceptors present in the cortical and hippocampal areas of the brain of mammals. This likely stimulates various processes associated with learning and cognition, even in the aged-memory impaired animal (for example, BIBN-99). While cortical cholinergic nerve terminals are markedly altered in disorders such as AD, highly significant functional capacities (40% to 60% of age-matched control values on the basis of ChAT activity) are still observable, even in very advanced cases (Etienne et al 1986). It may be that by properly activating (nicotinic) or inhibiting (M_2) (or both) the autoreceptors that are present on remaining cholinergic nerve terminals, memory deficits observed in AD could be improved. This hypothesis deserves clinical investigation; a selective M_2 antagonist, in combination with a potent esterase inhibitor (for example, tacrine) (Summers et al 1986; Davis et al 1992) having the potential of significantly improving AD conditions by relieving the negative feed-back inhibition is likely associated with the maintenance of high levels of acetylcholine in the synaptic cleft. Finally, the recent demonstration that cholinergic agonists can regulate the processing and secretion of the Alzheimer β/A4 amyloid protein precursor (Buxbaum et al 1992) further demonstrates that a dysregulation of cholinergic functions may have broad, unexpected effects on normal brain functions, including β-amyloid deposition.

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