

Evaluation of cholinergic markers in Alzheimer's disease and in a model of cholinergic deficit

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Abstract

Cognitive deficits in neuropsychiatric disorders, such as Alzheimer's disease (AD), have been closely related to cholinergic deficits. We have compared different markers of cholinergic function to assess the best biomarker of cognitive deficits associated to cholinergic hypoactivity. In post-mortem frontal cortex from AD patients, acetylcholine (ACh) levels, cholinacetyltransferase (ChAT) and acetylcholinesterase (AChE) activity were all reduced compared to controls. Both ChAT and AChE activity showed a significant correlation with cognitive deficits. In the frontal cortex of rats with a selective cholinergic lesion, all cholinergic parameters measured (ACh levels, ChAT and AChE activities, "in vitro" and "in vivo" basal ACh release) were significantly reduced. AChE activity was associated to ChAT activity, and even more, to "in vivo" and "in vitro" basal ACh release. Quantification of AChE activity is performed by an easy and cheap method and therefore, these results suggest that determination of AChE activity may be used as an effective first step method to evaluate cholinergic deficits.

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Cognitive deficits in Alzheimer's disease (AD) have been widely associated with dysfunction of the cholinergic system [10] consequent upon degeneration of cholinergic cell bodies in the basal forebrain [12]. Reflecting the loss of cholinergic innervation, reductions in acetylcholinesterase (AChE) and cholinacetyltransferase (ChAT) have been reported in AD brains [13,20,21,37]. It has also been described that in other dementias, such as parkinsonian dementia, cortical cholinergic function is even more severely affected than in AD [2].

Consequent to the cholinergic hypothesis of dementia, animal studies have been used to evaluate the role of cholinergic neurotransmission in learning and memory and selective lesion of cholinergic neurones remains nowadays as a widely used method to mimic some aspects of neurodegeneration in AD (see review [28]). In this sense, the most efficient tool to induce cholinergic hypoactivity is the immunotoxin 192 IgG-

saporin [15,23,30]. The verification and extent of the lesion are commonly assessed by post-mortem measuring of different biochemical markers, such as AChE [1,29], ChAT [24,33] or high-affinity choline uptake (HACU) [26]. All these parameters could be excellent markers of the extent of lesion or the amount of surviving cholinergic neurones subsequent to lesion. However, decreases in cholinergic markers may not reflect the activity of these remaining neurones due to the presence of compensatory mechanisms after long-term situations of cholinergic hypoactivity [17,19]. In addition, total acetylcholine (ACh) levels are labile in post-mortem tissue, and ACh released "in vivo" may not be detected in the absence of AChE inhibitors. Altogether, it is difficult to select the best cholinergic marker to study a situation of cholinergic deficit.

In the present work we sought to study the best marker of cholinergic hypoactivity in relationship to cognitive deficits. We have measured different cholinergic markers in two situations of cholinergic hypofunction: (1) in a human study,

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cholinergic function was assessed in post-mortem frontal cortex from AD patients who had been prospectively assessed with the mini-mental state examination (MMSE) for cognitive impairment and (2) in an animal study, cholinergic parameters were measured in the frontal cortex of rats subjected to a selective cholinergic lesion in the nucleus basalis magnocellularis (NBM). Statistical correlations between cholinergic and cognitive deficits have been also carried out.

Cholinergic functions was assessed in post-mortem frontal cortex (Brodmann area 10, BA10) of 22 patients with clinical diagnosis of dementia and 20 elderly normal controls matched for age, gender, post-mortem delay and brain pH. Those patients with dementia were an autopsied subset of subjects included in a prospective study of behavioural changes in clinically diagnosed as demented patients [16]. Cognitive status was assessed at 4 monthly intervals using the mini-mental state examination (MMSE) [8].

Informed consent had been obtained from relatives before for removal of brain tissue at death. AChE, ChAT and total ACh levels in human brain tissues were measured as described by [11]. To partially mitigate the possible effects of cause of death on neurochemical determinations, brain pH was measured as an index of acidosis associated with terminal coma [18]. Post-mortem delay was also considered.

Male Wistar rats were kept at constant room temperature ($21 \pm 1^\circ\text{C}$) and relative humidity ($55 \pm 5\%$) with a 12-h light/dark cycle (dark from 8 p.m.) and ad libitum access to food and water. A selective cholinergic lesion in the NBM was produced using the selective toxin 192 IgG-saporin. All the experiments were carried out in strict compliance with the recommendations of the EU (DOCE L 358/1 18/2/1986) for the care and use of laboratory animals. At the beginning of the study rats were allocated randomly to one of the experimental groups constituted by controls, sham-operated rats and rats with 192IgG-saporin lesions.

The production of the lesion was performed as described by [14]. Briefly, rats were anaesthetised with a mixture of ketamine (10 mg/kg, i.p.) and xylazine (0.3 mg/kg, i.p.). One microliters of the immunotoxin 192 IgG-Saporin ($0.067 \mu\text{g}/\mu\text{l}/\text{hemisphere}$) was infused bilaterally into the nucleus basalis magnocellularis (NBM) of the basal forebrain at the following coordinates (from bregma): AP -0.9 mm , ML $\pm 2.9 \text{ mm}$, DV -6.5 mm . Post-lesion survival times were established at 1 week and 1 month.

AChE activity, ChAT activity, total ACh levels, and ACh release both “in vivo” and “in vitro” were measured in the frontal cortex according to the methods described by [14].

Data were analysed by SPSS for Windows, release 11.0. Normality was checked by Shapiro–Wilks’s test ($p > 0.05$). Student’s *t*-test (human studies) or ANOVA test (animal studies) were used to study decreases in cholinergic parameters. The effects of demographic factors (age, post-mortem delay and brain pH) on neurochemical variables, intercorrelation between neurochemical variables or relationships between severity of dementia (MMSE score at last interview before death) and neurochemical measures were determined

by Pearson’s or Spearman’s rank correlation coefficients, according to the normality of variables. Significance was defined as $p \leq 0.05$.

The following compounds were used: ketamine (Ketastol, Parke-Davis, USA), xylazine (Rompun, Bayer, Germany) neostigmine bromide, hemicholinium-3, acetylthiocholine iodide (Sigma, UK); [methyl- ^3H]choline chloride ($86 \text{ Ci}/\text{mmol}$, PerkinElmer, USA); [$1\text{-}^{14}\text{C}$]acetyl-coenzyme A ($59 \text{ mCi}/\text{mmol}$, Amersham Biosciences, UK); 192IgG Saporin (lot number 21071083, Chemicon International, Inc., USA). Inorganic salts and other reagents were from Merck and Sigma.

Demographic details of subjects are shown in Table 1.

In controls, mean value for ACh levels, and AChE and ChAT activity were $0.36 \pm 0.04 \text{ nmol}/\text{g}$ tissue, 99.75 ± 2.80 (absorbance) and $100.01 \pm 9.82 \text{ nmol ACh}/\text{h g}$ tissue, respectively. In the frontal cortex of AD patients, concentration of both ACh was significantly reduced (58%) compared to controls. Similarly, AChE and ChAT activities in frontal cortex from AD patients were significantly lower (28.5 and 72.3%, respectively) when compared to control values. ACh value, in AD patients, was significantly correlated with brain pH ($n = 22$; Spearman’s $\rho = -0.861^*$; $p = 0.001$) and post-mortem delay ($n = 22$; Spearman’s $\rho = -0.503^*$; $p = 0.033$). There were no significant correlations between brain pH, post-mortem delay, length of tissue preservation time and AChE or ChAT activity Table 1.

In AD patients, cortical AChE and ChAT activities were significantly intercorrelated ($n = 21$; $r = 0.530^*$; $p = 0.019$), while ACh levels did not correlate with either AChE or ChAT activities.

The mean MMSE score before death was 5 ± 1 in AD patients. A statistically significant positive correlation between last MMSE score and both AChE ($n = 21$, Spearman’s $\rho = 0.453^*$; $p = 0.045$) and ChAT activities ($n = 21$, Spearman’s $\rho = 0.473^{**}$; $p = 0.030$) was found. However, ACh levels failed to show an association with the MMSE score.

In these studies, as there were no statistical differences and for simplicity purposes, control non-lesioned and sham animal data were combined in one same control group. Similarly, the two lesioned groups (corresponding to 1 week and 1 month post-lesion time) were combined in a single lesioned group. As expected, significant decreases were found in all cholinergic markers measured, as shown in Table 2.

Correlations between all cholinergic parameters are represented in Fig. 1. In the frontal cortex of animals with a selec-

Table 1
Demographic features of patients

	Control	Alzheimer
Age (years)	74.75 ± 2.67	81.06 ± 1.60
Gender (male/female)	11/9	11/13
Post-mortem delay (h)	39.28 ± 5.40	51.18 ± 6.44
pH	6.28 ± 0.16	6.42 ± 0.54

pH, standard chemical symbol, negative log of hydrogen ion concentration. (Control $n = 19\text{--}20$, Alzheimer $n = 20\text{--}22$). Values are mean \pm S.E.M.

Table 2

Percentage of remaining activity/levels of different markers after cortical cholinergic denervation

Post-lesion time	AChE activity	ChAT activity	Total ACh levels	"In vivo" basal ACh levels	"In vitro" basal ACh levels
1 week	52.89 ± 4.25	50.96 ± 4.4	45.91 ± 4.20	65.57 ± 3.38	43.33 ± 1.88
1 month	55.06 ± 2.73	54.99 ± 3.93	63.15 ± 6.21	67.52 ± 3.51	48.53 ± 1.91
Total lesion	54.21 ± 2.93	52.57 ± 3.26	52.63 ± 3.88	66.56 ± 3.45	46.24 ± 1.80

Data shown as mean ± S.E.M. Percentage was calculated in relation to control and sham values. Control values were: AChE (absorbance) = 0.28 ± 0.032; ChAT = 139.92 ± 16.21 nmol ACh/h g tissue; total ACh = 3.82 ± 0.73 nmol/g tissue; "in vivo" basal ACh levels = 464.15 ± 24.76 fmol/15 µl; "in vitro" ACh levels = 2443.08 ± 115.49 dpm. The two lesioned groups (corresponding to 1 week and 1 month post-lesion time) were combined in a single lesioned group (total lesion) for simplicity purposes, as there were no statistical difference between them.

tive cholinergic lesion, AChE activity significantly correlated with ChAT activity ($n = 19$; Pearson's $r = 0.556^*$; $p = 0.014$), ACh levels ($n = 18$; Pearson's $r = 0.505^*$; $p = 0.033$), and ACh release "in vivo" ($n = 28$; Spearman's $\rho = 0.557^*$; $p = 0.002$).

Disruption of basal forebrain cholinergic pathways and consequent cortical cholinergic denervation is one of the hallmarks of AD, together with histopathological changes such as neurofibrillary tangles and senile plaques [20,21,37]. As expected, profound depletions in all cholinergic markers

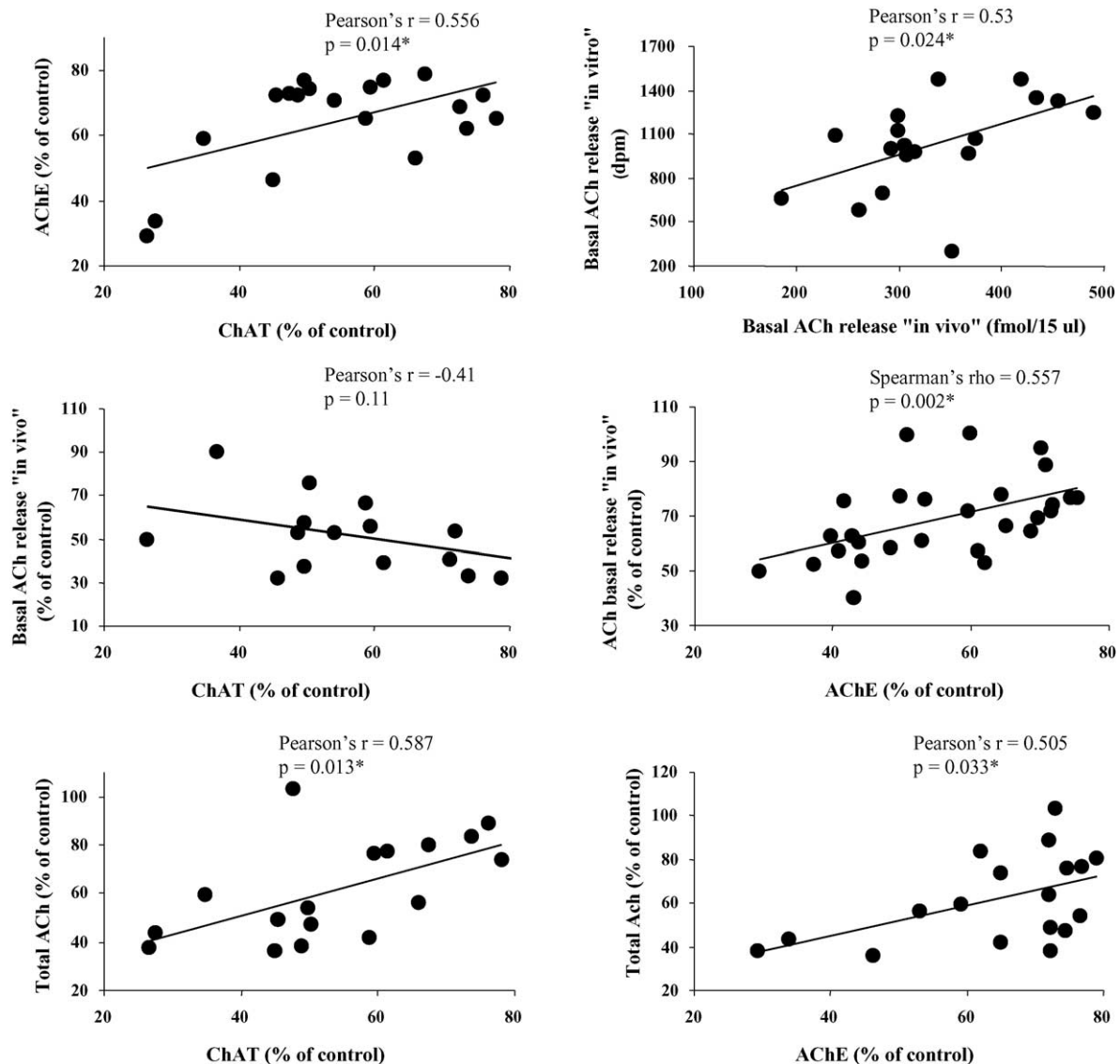


Fig. 1. Correlations between different cholinergic parameters measured in the frontal cortex of rats after a selective cholinergic lesion in the nucleus basalis magnocellularis. AChE, acetylcholinesterase; ChAT, coline acetylcholinesterase; ACh, acetylcholine. AChE activity is expressed as % absorbance relative to controls; ChAT activity is expressed as nmol ACh/h g tissue; ACh levels are expressed as nmol/g tissue.

measured (ACh, ChAT, AChE) were found in frontal cortex from AD patients in the present study. Data on total levels of ACh, which are more directly related to neural activity, due to the degradation of ACh by AChE in post-mortem tissue, should be taken cautiously. Cholinergic degeneration in AD has been widely associated with cognitive impairments in the illness [28]. In our study not only ChAT, but also AChE levels in AD patients showed to be correlated to final MMSE scores. Decreases in AChE activity have also been described in several dementing conditions, such as parkinsonian dementia [2] or subcortical ischemic vascular dementia [34]. As the traditional treatment of AD are based on the use of AChE inhibitors, determination of the remaining AChE activity could be a good marker of the expected efficacy of the treatment to delay the progression of cognitive deficit. Supporting these data, AChE inhibitors such as rivastigmine and donepezil have been shown to maintain MMSE scores for up to 52 weeks in placebo-controlled study [27].

With respect to the animal studies, cholinergic neurones in the NBM give rise to a dense network of cholinergic fibres innervating the entire cortical mantle [32]. Partial cortical cholinergic deafferentation was achieved by intraparenchymal infusion of the selective cholinergic immunotoxin, 192 IgG-Saporin, into the NBM [15,30]. It has been described that this immunotoxin, at the concentration used in the present work, produces maximal depletion of cortical cholinergic markers, sparing other non-cholinergic neuronal populations [3,7,22,30]. Under this experimental condition, a significant decrease in cholinergic function was achieved, as reflected by significant decreases in different cholinergic markers in the frontal cortex of lesioned animals, similar to those previously described after this type of lesion [4,25,36]. Furthermore, as expected, a decrease in “in vivo” [6] and “in vitro” [25] basal cortical ACh level was found.

ChAT activity has been long used as a marker of the loss of cholinergic neurones after a cholinergic lesion [4,25,36]. Since ChAT enzyme is not the rate-limiting step of ACh synthesis [31], it cannot be assumed that post-mortem assay of ChAT might be representative of the extracellular levels of ACh, and therefore, of the cholinergic neural activity. There is a large body of evidence (e.g., [33]) that, at least 1 week after cholinergic lesion, surviving neurones may compensate the loss of terminals increasing the neural activity. If these compensatory mechanisms do not imply a regulation of some enzyme activities, then the post-mortem ChAT assay might be underestimating the real state of neurones after a cholinergic lesion. Accordingly to this, we did not find an association between ChAT activity and extracellular levels of ACh measured by microdialysis, thus, it appears to strengthen the hypothesis that ChAT may be a good marker for loss of cholinergic terminals but perhaps not for neural activity.

Traditionally, ChAT activity has been considered as the best marker of cholinergic function, as AChE appears to be located also in non-cholinergic post-synaptic neurones. However, in our study, decreases in ChAT and AChE activity seem to be correlated. Not only that, but AChE activity was cor-

related to extracellular levels of ACh, reinforcing AChE, as an excellent candidate to evaluate the effects of a cholinergic lesion. The AChE activity assay is a fast, cheap and simple colorimetric method [5,35] compared to the radioenzymatic assay used to assess ChAT activity [9]. Thus, AChE activity assay described by [35] might be utilised as a rapid and first step assay to characterise a situation of cholinergic hypoactivity.

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