Abstract

Nitric oxide (NO) plays an important role in cholinergic neurotransmission and has been implicated in the progression of Alzheimer’s disease (AD). Cholinergic neurotransmission has been associated with regulation of NO synthase (NOS) expression, and we determined previously that NO modulates choline acetyltransferase (ChAT) expression. In spite of the important links identified between NOS and ChAT, little is known about the relationship between these two enzymes in AD. Therefore in the present study, we compared the expression levels of ChAT and NOS in necropsy brain of individuals with AD and non-demented controls. ChAT and NOS levels were assessed in control and AD caudate, nucleus basalis of Meynert (nBM), cortex and hippocampus by radioenzymatic assay, immunoblot analysis and RT-PCR. We detected a significant decrease in ChAT activity in the cortex of AD cases, but no alterations in NOS activity were observed in any of the brain regions examined. At the mRNA level, no significant decrease in total ChAT mRNA was detected but the decrease in M-ChAT mRNA levels approached significance in the nBM. No difference in nNOS and iNOS mRNA and protein levels was observed between control and AD tissue in any of the four brain regions sampled, but a statistically significant decrease in eNOS mRNA levels was detected in both the cortex and hippocampus of AD brain. Finally, the levels of ChAT and NOS activity, protein or mRNA isoforms did not correlate in most of the brain regions examined, but a reduction in ChAT activity and eNOS mRNA in basal forebrain projection regions was observed. These data suggest that in general, there is no correlation between the levels of NOS and ChAT in control or AD subjects, but a reduction in eNOS levels in the hippocampus and cortex indicates there could be an interaction between eNOS containing cells and basal forebrain projections neurons in AD.

Keywords: Cholinergic; Nitric oxide; Human brain; Enzyme activity; mRNA levels

Abbreviations: ACh: Acetylcholine; AD: Alzheimer’s Disease; ChAT: Choline Acetyl Transferase; nBM: Nucleus Basalis of Meynert; NGF: Nerve Growth Factor; NO: Nitric Oxide; NOS: Nitric Oxide Synthase; PMI: Post-Mortem Interval; SEM: Standard Error of the Mean; TrkA: Neurotrophic Tyrosine Kinase Receptor, type I.

Introduction

Alzheimer disease’s (AD) is characterized by memory loss and a decline in cognitive and functional abilities. Neuropathological features include β-amyloid (Aβ)-containing neuritic plaques and neurofibrillary tangles (reviewed in [1]), as well as synaptic loss and dysfunction [2]. While abnormalities occur in several neurotransmitter systems, alteration in cholinergic neuronal markers is found early in AD [3,4] with dementia severity correlating best with decreased activity of the cholinergic marker choline acetyltransferase (ChAT) in basal forebrain [5-7], hippocampus [8] and cortex [9,10]. Cholinergic neurons are dependent on the neurotrophin nerve growth factor (NGF) for survival and phenotypic maintenance [11-16]. In AD brains, decreased levels of NGF [17] and mRNA for the high-affinity NGF receptor (TrkA) [18-20] have been observed in surviving basal forebrain neurons, implying that reduced NGF-TrkA signalling could contribute to cholinergic degeneration in AD.

Information regarding NGF-mediated signalling events and enhancement of cholinergic function has come from animal model and cell culture studies. Nitric oxide (NO) has been identified as an important modulator of NGF-mediated neurotrophic responses. NGF increases expression of all three isoforms of nitric oxide synthase (NOS) in PC12 cells [21,22] and the neuronal NOS isoform (nNOS) in cultured rat basal forebrain cholinergic neurons [23,24]. We found that NOS inhibitors attenuate NGF-mediated increases in ChAT mRNA levels and activity in PC12 cells [21]. These studies show that NGF can regulate NOS expression and that NO can modulate ChAT expression, thus linking TrkA signalling and NO production with the maintenance of cholinergic phenotype. It is not known if these signalling events are altered in AD brain, but recent studies have focused on the role of NO in AD and data are emerging regarding the expression levels of NOS isoforms in AD [25-37].

Some studies support the neurotoxic role of NO and depict elevations in NOS isoform expression during AD progression [25-32], whereas others describe reductions in NOS expression and the data are consistent with NO-mediated neuroprotection [33-37]. Hyman et al. [25] found that NOS-containing hippocampal neurons were relatively spared in AD, suggesting that NO produced by these cells was toxic to neighbouring neurons. In support of this, the number of cortical neurons immunoreactive for nNOS and the inducible NOS isoform (iNOS) are increased as AD progresses with the increase linked to neurofibrillary tangle formation [26-28]. As well, increased serum NO levels correlate inversely with Mini-Mental State Examination scores in AD [29-32].

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AD patients [29], and it is suggested that Aβ-induced NO release from neurons [28] and iNOS expression in astrocytes [31,32] contribute to neuron damage in AD. In contrast, Norris et al. [33] found reduced nNOS-containing neurons in the frontal cortex and hippocampus of AD brains and in the end-stage of the disease, the expression of constitutive endothelial NOS (eNOS) [34] and nNOS [35] in cortical neurons was decreased. In addition, cortical plaque and tangle burden has been correlated with a reduction in capillary ENOS levels [36]. It has also been suggested that elevations in NOS expression occurring early in the disease process are a protective response to early neurodegenerative events [37]. While NO likely contributes to both neuroprotective and neurotoxic events in AD, the changes described in NOS expression could play a role in the phenotypic maintenance of cholinergic neurons. In addition, nNOS-containing cortical neurons and micro vessels receive input from basal forebrain cholinergic neurons [38-40], and loss of this input may contribute to the reduction in cortical blood flow and decreased cognitive abilities in AD [39]. These data suggest that cholinergic neuron degeneration could lead to alterations in NOS expression in AD. Taken together, these studies support the interaction between cholinergic neurotransmission and NOS expression.

Therefore, we set out to investigate whether there was a correlation between cholinergic deficit and NOS expression in post-mortem tissue from AD subjects and age-matched controls. Since the predominant form of AD is late onset, we only examined tissues from individuals over the age of 65 years. In this more common form of the disease, inheritance of the ε4 allele of apolipoprotein (apo) E is a major risk factor [41,42]. In fact, greater cholinergic deficits have been detected in tissues obtained from AD subjects that express apoE4 [43-45] as a factor [41,42]. In fact, greater cholinergic deficits have been detected in tissues obtained from AD subjects that express apoE4 [43-45] as compared with individuals that express ApoE2 and/or 3. In addition, ApoE4 has been reported to increase NO release, enhance arginine (NO precursor) transport and increase oxidative stress [46-51], which could make ApoE4 carriers more vulnerable to neuronal damage. We therefore also investigated whether the levels of ChAT and NOS in necropsy brain tissue differed between ApoE4 carriers and non-carriers.

Materials and Methods

Tissue samples

Necropsy brain samples used in this study were obtained from the University of California, Irvine, Institute for Brain Aging and Dementia. Brains were collected at autopsy of control and AD subjects with written consent of next-of-kin and in compliance with Research Ethics Review Boards. The present studies were carried out in compliance with Canadian Institutes for Health Research (CIHR) guidelines for handling of human tissues, and received local institutional Biosafety Committee approval. Neuropathological evaluation of tissues was carried out at UC Irvine and used to confirm the diagnosis of AD through the presence of Aβ-containing neuritic plaques and neurofibrillary tangles, or to document the absence of these AD markers in control subjects. Brain samples from 6 age-matched controls (81.8 ± 3.6 years) and 13 AD patients (78.8 ± 1.9 years) were used in this study. For the experiments described, when available, the frontal cortex, hippocampus, caudate and nucleus basalis of Meynert (nBM) were dissected and the tissue was frozen at −80°C until preparation of samples for enzyme assays, immunoblots or RNA isolation.

Materials

Trizol and reverse transcriptase (RT)-polymerase chain reaction (PCR) reagents were from Invitrogen Life Technologies (Burlington, ON, Canada). NOS isoforms were detected by immunoblot analysis using a polyclonal rabbit anti-rat nNOS (Santa Cruz Biotechnology Inc.), rabbit polyclonal iNOS antibody (a gift from Dr. David W. Mercer, University of Texas-Houston Medical School), and mouse monoclonal anti-eNOS (Biomol International). Acetylthiocholine (acetylCoA) trilithium salt was obtained from Boehringer Mannheim (Mannheim, Germany), acetylCoA [acetyl-3H], specific activity 7.2 Ci/mmol, was purchased from ICN (East Hill, NY, USA) and [14C] arginine, specific activity 0.319 Ci/mmol, was purchased from New England Nuclear Research Products (Mississauga, ON, Canada) Liquid scintillation cocktail and anti-rabbit IgG-horse radish peroxidase secondary antibody was from Amersham Pharmacia Biotech (Piscataway, NJ, USA). All other chemicals were obtained from Sigma Chemical Company or VWR (Mississauga, ON, Canada).

RNA Isolation and RT-PCR amplification

Total RNA was isolated from frozen human tissue using Trizol. Extracted RNA was reverse transcribed with Thermoscript, using oligo-dt as the primer for 60 min at 55°C and the cDNA generated was used for PCR with Platinum Taq DNA polymerase. All PCR reactions were performed in a final volume of 50 μL, with an initial strand separation at 94°C for 2 min and a final elongation for 2 min at 72°C after the last cycle. Primer pairs and cycling conditions are presented in Table 1.

PCR products were separated on 1.5% agarose gels and visualized with ethidium bromide under UV light. Semi-quantitative evaluation of relative amounts of PCR product formed was carried out by densitometric analysis [BioRad Multi-Analyt/PC version 1.1] and the value obtained for each sample was compared with the corresponding β-actin sample. The specificity of PCR products was verified by Southern blot analysis or DNA sequencing.

Determination of nitric oxide synthase activity

NOS activity in tissue samples was measured by a radiolabelled arginine to citrulline conversion assay using modifications of the method of Breit and Snyder [52]. Frozen brain tissue was homogenized in 5 vols of ice-cold 50 mM HEPES buffer (pH 7.4) containing 0.1 mM EDTA and 10 μg/mL each of leupeptin, AEBSF and pepstatin. Duplicate aliquots of homogenates (25 μL) were incubated for 30 min at 37°C with 100 μL of reaction buffer consisting of 25 mM arginine, 5 nCi [14C] arginine, 5 μM each of FAD and FMN, 10 μM tetrahydrobiopterin, 1 μM NADPH, 1.2 mM MgCl₂, 1 mM CaCl₂, 1 mM vinal and 10 μg/mL calmodulin in 50 mM HEPES buffer (pH 7.4). Reactions were stopped by the addition of 500 μL ice-cold 20 mM HEPES buffer containing 2 mM EDTA (pH 5.5). Each sample was passed through a 1 mL Dowex 50WX8–400 (Na⁺) column to separate [14C] arginine

| Primer Pair | Primer Sequence (F=forward, R=reverse) | Cycle number | Annealing (°C) |
|-------------|----------------------------------------|--------------|----------------|
| ChAT (total) | (F) 5'-caagggccagctagcccctggc-3' (R) 5'-cctggagaggaagaggtcag-3' | 30 | 62 |
| M-ChAT specific | (F) 5'-gaggaggagaggaaggaggaaag-3' (R) 5'-cggfittgacacggagggaaglg-3' | 37 | 57.5 |
| nNOS | (F) 5'-cccacgtggtcctcattctg-3' (R) 5'-catcilttttinggggagcttt-3' | 35 | 60 |
| iNOS | (F) 5'-cgcttggtatggctgcctct-3' (R) 5'-cccatccctgcgcctctccct-3' | 37 | 60 |
| eNOS | (F) 5'-gcccagcagctgccctct-3' (R) 5'-gaaaccacagctgccctctgaa-3' | 37 | 62.5 |
| TrkA | (F) 5'-aaccaccccaacaccctgcaaggc-3' (R) 5'-cgctccctgacctgacact-3' | 36 | 65 |
| β-actin | (F) 5'-atggtgagctctctcaacccagc-3' (R) 5'-gaagagctgctgccagggcag-3' | 25 | 65 |

Table 1: Primer sequences and cycling conditions used in the present study.
from $[^{14}C]$ citrulline. The eluate and two 0.5 mL washes were collected, 10 mL of scintillation cocktail added and the resulting $[^{14}C]$ citrulline counted. Protein content of tissue homogenates was determined by the method of Bradford [53] and NOS activity was expressed as nmol $[^{14}C]$ citrulline formed/mg protein/h.

**Determination of choline acetyltransferase activity**

Aliquots of tissue homogenates prepared for the NOS assay were further solubilized by addition of an equal volume of ice-cold 0.1M sodium phosphate buffer (pH 7.4) containing 0.87 mM EDTA, 0.1% Triton X-100 and 0.15 mM eserine and incubated for 30 min at 4°C. ChAT activity was measured by monitoring the conversion of $[^{3}H]$ acetylCoA to $[^{3}H]$ acetylcholine (ACh) using a modification of the method of Fonnum [54]. Each supernatant sample (10 μL) was incubated with 10 μL of reaction buffer for 30 min at 37°C. The reaction buffer consisted of 0.285 M NaCl, 0.095% BSA, 0.19 mM eserine, 3.8 mM choline, 0.2 mM acetylCoA and 37 μM $[^{3}H]$ acetylCoA (7.2 Ci/m mole) in 0.1 M sodium phosphate buffer, pH 7.4. Each reaction was stopped by the addition of 300 μL of 3-heptanone containing 20 mg/mL sodium tetraphenylboron. Samples were vortex mixed for 30 s and centrifuged for 5 min at 5700 g. Subsequently, 200 μL of the upper organic layer was added to 3.5 mL scintillation cocktail and the radioactivity measured. The amount of protein present in tissue homogenates was determined [53] and ChAT activity was expressed as nmol $[^{3}H]$ ACh formed /mg protein/h.

**Immunoblots**

Aliquots of tissue homogenate prepared for the NOS assay were mixed with an equal volume of ice-cold RIPA buffer containing (final concentration): 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM each of EDTA, sodium fluoride, AEBSF and sodium orthovanadate and 1 μg/mL each of leupeptin, aprotinin and pepstatin and shaken on ice for 30 min. Samples were centrifuged at 17 500 g for 15 min and protein content of the supernatant measured [53]. SDS sample buffer was added to aliquots containing 100 μg of protein, boiled for 5 min and loaded onto 7.5% SDS-PAGE gels for electrophoresis. Separated proteins were transferred to nitrocellulose membranes that were then blocked in 8% non-fat milk in PBS containing 0.05% Triton X-100 for 1 h at room temperature. Primary antibody (1:2000 rabbit anti-human anti-ChAT antibody [55]) was added into fresh blocking solution and the blots incubated for 2 h at room temperature. Antibody detection was achieved using 1:2500 donkey anti-rabbit IgG-horse radish peroxidase conjugated secondary antibody followed by chemiluminescence detection (ECL, Amersham). Blots were stripped and probed for iNOS (1:2000, rabbit polyclonal anti-iNOS), nNOS (1.25 μg/mL rabbit polyclonal anti-rat nNOS) and eNOS (1:2000, mouse monoclonal anti-eNOS).

**Data analysis**

Enzyme activity for ChAT and NOS is presented as mean ± standard error of the mean (SEM) and parametric statistical analysis was performed using Student's t-test to compare samples obtained from AD brain to those from age-matched controls. Semi-quantitative analysis of steady-state mRNA levels was obtained using MultiAnalyst image analysis software (BioRad). Data were calculated as the ratio of arbitrary densitometric units of ChAT, NOS or TrkA transcripts normalized to values obtained for β-actin from the same sample. Data are presented as the mean ± SEM and parametric statistical analysis was performed using Student’s t-test. Mean values were considered different if p<0.05. Correlations were assessed using Pearson's Linear correlation and were considered significant if p<0.05.

**Results**

Analysis of ChAT and NOS expression was performed on samples of frontal cortex, hippocampus, caudate and nbM from post-mortem brain of confirmed AD subjects and age-matched controls. Neuropathological identification of AD along with age, sex, post-mortem interval (PMI) and ApoE genotype was determined by the University of California, Irvine, Institute for Brain Aging and Dementia and these data are presented in Table 2.

**Expression of ChAT in Alzheimer's and control brains**

ChAT levels were assessed in brain samples by radioenzymatic assay, immunoblot analysis and RT-PCR. Figure 1A illustrates a significant decrease in ChAT activity in frontal cortex samples from individuals with confirmed AD as compared to age-matched controls. A trend towards decreased ChAT activity was found in the other brain regions, but as there was substantial variability in the activity measured from caudate samples and the sample size for hippocampal tissues from AD cases was smaller (n=6), ChAT activity values for these tissues from control and AD cases did not achieve statistical significance (p=0.07). We also evaluated if age or PMI correlated with ChAT activity, but did not find significant correlations in any of the brain regions examined for either control or AD cases (data not shown). Similar findings were observed when ChAT protein levels were assessed by immunoblot analysis. Densitometric analysis revealed similar amounts of ChAT in caudate samples obtained from control and AD brain and, while levels were variable in the nbM and hippocampus, no difference was observed between tissues obtained from control or AD brain. Of note, in contrast to what is observed in samples from control subjects, ChAT protein was generally below detectable levels using immunoblot in the frontal cortex in tissue obtained from AD brains.

We also compared steady-state levels of total ChAT mRNA (Figure 1B), as well as the M-ChAT transcript that encodes a form of the enzyme that is unique to humans [56-58]. Surprisingly, there were no significant differences between control and AD subjects when comparing total ChAT mRNA levels, but comparison of the mean densitometric values of M-ChAT mRNA levels revealed a trend towards a decrease in the nbM of AD subjects (p=0.06) (Figure 1C and D). Moreover, there was

| Case | Age (yrs) | Sex | PMI (h) | ApoE genotype |
|------|----------|-----|---------|---------------|
| C1   | 87       | F   | 4.5     | 3 / 3         |
| C2   | 86       | F   | 9.5     | 3 / 3         |
| C3   | 87       | F   | 6.5     | 3 / 3         |
| C4   | 71       | F   | 4.0     | 3 / 3         |
| C5   | 73       | M   | 10.5    | 2 / 2         |
| AD1  | 92       | M   | 2.7     | 3 / 3         |
| AD2  | 72       | F   | 12.0    | N/A           |
| AD3  | 75       | M   | 3.0     | 3 / 4         |
| AD4  | 91       | M   | 5.0     | 3 / 3         |
| AD5  | 76       | M   | 16.0    | N/A           |
| AD6  | 75       | F   | 8.5     | N/A           |
| AD7  | 72       | M   | 4.25    | 3 / 3         |
| AD8  | 79       | M   | 7.0     | 3 / 3         |
| AD9  | 83       | F   | 6.0     | N/A           |
| AD10 | 83       | M   | 7.0     | N/A           |
| AD11 | 75       | F   | 4.25    | 3 / 4         |
| AD12 | 70       | M   | 5.0     | 3 / 3         |
| AD13 | 81       | M   | 5.0     | 3 / 4         |

Table 2: Patient information integrated in the present study.
a correlation between increased age and decreased levels of M-ChAT mRNA in this brain region in tissues from both age-matched controls and AD cases (Figure 1E). No other correlations were found between total ChAT or M-ChAT mRNA and PMI or age in any of the other brain regions examined for either control or AD cases (data not shown). We compared mRNA levels and activity for ChAT and found that while total ChAT mRNA did not correlate with ChAT activity in any of the brain regions, relative M-ChAT mRNA levels correlated with ChAT activity in the caudate. We also compared ChAT activity in hippocampus and cortex to ChAT mRNA levels in nbM and again, there was no correlation between total ChAT mRNA and ChAT activity. However importantly, in AD samples, ChAT activity in frontal cortex correlated with the levels of M-ChAT transcript in nbM with the same correlation almost achieving statistical significance for hippocampus. A summary of the changes in ChAT expression in AD is presented in Table 3.

### Expression of NOS in Alzheimer’s and control brains

Both elevations and reductions in NOS expression in AD brain have been reported in the literature. In our study, we measured NOS activity and steady-state levels of NOS mRNA from necropsy samples obtained from the cortex, caudate, nbM and hippocampus of individuals with confirmed AD and age-matched controls, first to evaluate NOS expression and secondly to compare the levels of NOS with ChAT. As seen in Figure 2A, there was no difference in total NOS activity between control and AD tissue in any of the brain regions examined. We also assessed levels of iNOS activity (in the absence of calcium), but did not find detectable levels in any of the brain regions in samples from either control or AD brain (data not shown).

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**Table 3.**

| Brain Region | Relative ChAT expression | Relative M-ChAT expression |
|--------------|--------------------------|----------------------------|
| Caudate      | control                  | AD                         |
| NbM          |                          |                            |
| FCtx         |                          |                            |
| Hippo        |                          |                            |
| C            |                          |                            |
| NbM          |                          |                            |
| FCtx         |                          |                            |
| Hippo        |                          |                            |
| CB           |                          |                            |
| H            |                          |                            |
| C            |                          |                            |
| NbM          |                          |                            |
| FCtx         |                          |                            |
| Hippo        |                          |                            |
| Age (years)  | 60                       | 80                        |
| Relative M-ChAT expression | AD | control |
| Caudate      | 0.5                      | 1.0                       |
| NbM          | 1.5                      | 2.0                       |
| FCtx         | 2.0                      | 2.5                       |
| Hippo        | 2.5                      | 3.0                       |

*Figure 1: ChAT expression measured in lysates of caudate, nbM, frontal cortex and hippocampus obtained from AD and age-matched control brains. Panel A. ChAT activity is expressed in nmol/mg protein/h. Statistical differences were found for ChAT activity in the cortex of AD brains compared to control [*p* < 0.05]; differences in ChAT activity measured in control and AD hippocampal extract did not achieve statistical significance [*p* = 0.07]. Panel B. Steady-state levels of ChAT mRNA analysed densitometrically, with values expressed as a ratio of β-actin mRNA from the same sample. Panel C. Representative ethidium bromide-stained gels showing amplification of M-ChAT and β-actin transcripts from RNA extracted from control or AD tissue. Lane 1 in both panels is a 100-bp DNA ladder with the brightest marker at 600 bp. Samples were taken from frontal cortex (FCtx), cerebellum (CB), hippocampus (H), caudate (C) and nucleus basalis of Meynert (NbM) of control (lanes 2 to 6) and AD (lanes 7 to 15) brains. Panel D. Steady-state levels of M-ChAT mRNA were analysed densitometrically with values expressed as a ratio of β-actin mRNA from the same sample. Panel E. Steady-state levels of M-ChAT mRNA as a ratio of β-actin mRNA from the same sample for NbM graphed relative to age. In each Panel, data are presented as mean±SEM.
Figure 2: Pattern of NOS expression in tissue lysates of caudate, nbM, cortex and hippocampus obtained from AD and age-matched control brains. Panel A. NOS activity is expressed in nmol/mg protein/h and each bar represents the mean±SEM. There was no statistically significant difference in NOS activity between control and AD tissue in any of the brain regions examined. Panel B. Representative data showing RT-PCR amplification products of specific NOS isoform transcripts from RNA extracted from control and AD tissue. Primer sets were used for nNOS, eNOS, iNOS and β-actin. Lane 1 in all panels is a 100-bp DNA ladder with the brightest marker at 600 bp. Samples were taken from frontal cortex (FCtx), cerebellum (CB), hippocampus (H), caudate (C) and nucleus basalis of Meynert (NbM) of control (lanes 2 to 6) and AD (lanes 7 to 15) brain. Steady-state levels of mRNA for nNOS (Panel C) and eNOS (Panel D) were analysed densitometrically with values expressed as a ratio of β-actin mRNA from the same sample; data are presented as mean ± SEM. Statistical differences were found for eNOS levels in the cortex and hippocampus of AD brains compared to control, denoted by *p < 0.05.
To examine if age or PMI affected NOS activity, we evaluated the relationship between these factors in all four brain regions from both control and AD tissue samples. There was no correlation between age and NOS activity levels, but increasing PMI correlated with a decrease in NOS activity in the caudate and approached statistical significance in the frontal cortex (p=0.06). Expression of NOS protein isoforms was investigated by immunoblot analysis. In both control and AD, nNOS was present in tissue from all four brain regions, with more variable expression observed in the AD samples. Similarly, iNOS was present in all tissue samples but levels did not appear to differ between control and AD subjects. A comparison of nNOS protein levels in control and AD samples was difficult as the bands observed were either weak or not present in several of the tissue lysates. Interestingly, eNOS appeared to be absent from the frontal cortex and hippocampus from most of the AD cases studied.

Finally, NOS isoform mRNA levels were assessed with representative data depicting steady-state levels of nNOS (top panel), eNOS (upper middle panel), iNOS (lower middle panel) and β-actin (bottom panel) mRNA in frontal cortex, hippocampus, caudate and nbM shown in Figure 2B. The level of nNOS mRNA from AD tissues was similar to that of controls (Figure 2C), but the levels of eNOS were decreased in both the hippocampus and frontal cortex. There was no correlation between age or PMI and the level of nNOS or eNOS, with the exception of the caudate where increased PMI correlated with increased eNOS expression. In some samples, iNOS mRNA was not detected and the number of samples in which iNOS was observed in each of the brain regions, for control or AD tissue samples, was not large enough to make valid statistical comparisons. Interestingly, when we compared NOS mRNA isoform levels within the various brain regions, we observed an inverse correlation between the expression of nNOS and iNOS; when levels of nNOS appeared lower, iNOS bands of greater density were observed (see Figure 2B). The only significant correlation between NOS activity and steady-state mRNA levels was between eNOS expression and NOS activity in the frontal cortex. A summary of changes in NOS expression in AD is presented in Table 3.

**Relationship between ChAT and NOS expression in Alzheimer’s and control brains**

To investigate the potential link between ChAT and NOS in the frontal cortex, hippocampus, caudate and nbM of AD and age-matched control brain, we compared the levels of ChAT and NOS activity and mRNA in these regions. A comparison of the ratios of ChAT to NOS activity between control and AD tissues revealed no difference in these values in any of the brain regions examined, but a trend towards a decrease in this ratio was observed in the hippocampus of individuals with AD (p=0.06).

As well, there was no correlation between the level of ChAT and NOS activity in the caudate, nbM or hippocampus, but in samples of frontal cortex from both control and AD cases, there was a significant correlation between the measured enzyme activity levels. To determine if the NOS isoforms expressed in the various brain regions correlated with ChAT expression, protein levels were compared following immunoblot analysis. As shown in Figure 3 (upper panel), levels of the 160 kDa splice variant of nNOS appeared to correlate with ChAT protein levels in the caudate from both control and AD samples. Levels of 160 kDa nNOS protein did not correlate with ChAT protein expression in the nbM (Figure 3, lower panel) cortex or hippocampus. As seen in Figure 3, a second band was consistently observed on blots probed for nNOS. There are four splice variants of human nNOS (reviewed in [59]). The most abundant is nNOSα which corresponds with the 160 kDa band observed in the nNOS standard and with the upper band depicted on the nNOS blots in Figure 3. Although the identity of the lower band was not determined, it is possible that it could be made up of one or both of the two smaller molecular mass splice variants, nNOSβ and nNOSγ, which are 136 and 125 kDa respectively. Levels of this smaller band did not correlate with nNOSα or with ChAT expression in either the caudate or nbM, and the levels of this protein were variable in both control and AD samples. For all other NOS isoforms in all other brain regions, there was no correlation between the levels of NOS and ChAT protein. At the level of mRNA expression, the only correlations detected were in the caudate, where increasing levels of steady-state ChAT mRNA corresponded to increasing levels of eNOS transcript.
Reports. In frontal cortex, ChAT activity was significantly lower in AD patients when compared to controls. A similar trend was observed in hippocampus, but this was not statistically significant (p=0.07) likely due to the smaller number of hippocampal samples available relative to the other brain regions examined. ChAT expression was also evaluated at the mRNA level. A critical finding is that, while there was no change detected in total ChAT mRNA levels in nbm, lower levels of M-ChAT transcript were observed (p=0.06) and the level of M-ChAT mRNA correlated with the loss of enzyme activity in frontal cortex. We showed recently that primate-specific 82-kDa ChAT is found in the nuclei of cholinergic neurons in human brain and spinal cord, and compared the subcellular localization patterns and temporal expression of this cholinergic protein in necropsy brain of control subjects of varying ages and AD patients [68]. Importantly, the decline in M-ChAT transcript found in the present study extends our previous findings as we observed an age-related decline in 82-kDa ChAT protein encoded by the M-ChAT mRNA in brain cholinergic neurons between birth and the eighth decade of life and in mild cognitive impairment and AD; this age-related change was associated with reduced nuclear localization of 82-kDa ChAT [68]. A larger sample size would be needed to determine whether M-ChAT transcript levels in the nbM correlate with the expression of 69 and 82-kDa ChAT protein in the hippocampus and cortex of control individuals and AD patients.

In contrast to ChAT, NOS activity was not altered in AD patients in any of the brain regions examined. However, increasing PMI correlated with decreasing NOS activity in tissue obtained from both AD patients and age-matched controls indicating that caution must be taken when interpreting NOS activity data from cases with very different PMIs. When examining the specific isoforms of NOS, we did not detect differences between control and AD tissue for expression of nNOS or iNOS at either the mRNA or protein level in the brain regions examined. These data support the findings of Hyman et al. [25] who reported that in AD, hippocampal NOS-containing neurons were relatively spared, but appear to contrast several others that found alterations in NOS isoform expression. A number of studies observed an increase in the number of nNOS and iNOS positive neurons [25–27], while Norris et al. [33] and Thorns et al. [69] detected a reduction in nNOS-containing neurons in cortex and hippocampus of AD brains. A similar decrease in nNOS immunoreactive neuron number was also reported by Yew et al. [35] but an increase in staining intensity was observed, suggesting that in AD, NOS levels in surviving neurons may be increased. While our results indicate there was no overall change in the tissue levels of nNOS or iNOS expression, it is possible that the changes in the level of these isoforms occur within individual neurons, astrocytes and glia and that these alterations are a consequence of or contribute to AD progression. In contrast to nNOS and iNOS, we observed a significant decrease in eNOS expression in hippocampus and cortex of tissue from AD patients. These data support the findings of de la Monte and Bloch [34] who detected a decrease in cortical neuronal eNOS levels in end-stage AD, but these authors also detected elevations in eNOS in glial cells. While we do not know whether the reduction in eNOS detected in our samples results from changes in neuronal, endothelial or glial cells, these results indicate that eNOS levels are altered in late-onset AD. Since the reduction was only detected in the projection areas of basal forebrain cholinergic neurons, this suggests an interaction between basal forebrain cholinergic neurons and eNOS-containing cells of the hippocampus and cortex.

We examined the relationship between NOS and ChAT in AD further by comparing the mRNA and protein levels of these enzymes in frontal cortex, hippocampus, caudate and nbM in AD and age-matched control brain. With respect to NOS and ChAT activity, we found no...
difference in the ratio of these enzymes between control and AD tissue in the brain regions examined and the only significant correlation between the activity levels of the two enzymes was observed in frontal cortex samples of both control and AD brain. As well, few correlations were observed at the RNA and protein level for specific NOS isoforms and ChAT. Finally, since NGF-mediated TrkA activation increases NOS and ChAT expression, we compared the steady-state mRNA levels of TrkA, ChAT and NOS isoforms. We found no difference in the level of TrkA mRNA in tissue from AD and control brains, and the only correlation observed was between TrkA and eNOS mRNA levels in cortex. Taken together, these data suggest that ChAT and NOS are not regulated together in cholinergic neurons of the nbM, but we did not examine the location of the NOS isoforms and levels of expression within the cholinergic neurons themselves.

In the basal forebrain of rodents, colocalization of ChAT, NOS and TrkA has been reported, with the proportion of colocalization varying between regions. The majority of NOS-containing neurons in rat basal forebrain are also immunopositive for TrkA, but many of the TrkA-positive neurons do not stain for NOS [70]. The proportion within the nbM is lower, with approximately one-third of NOS-positive cells exhibiting TrkA immunoreactivity [70]. Similarly, a proportion of basal forebrain cholinergic neurons also express NOS, with higher levels of colocalization occurring in the substantia innominata and medial septum as compared to the nbM [71-73]. Since the proportion of TrkA-positive cholinergic neurons in the nbM that also express NOS is small, co-regulation of these enzymes by NGF will only occur in a limited subpopulation of cells. This could explain the lack of correlation between ChAT and NOS levels in the nbM in our study. While the majority of cholinergic neurons in the nbM do not express NOS, the coordinated regulation of ChAT and NOS in a small population of neurons in this brain region may be important for the modulation of cortical neuron function and blood flow [38,39].

This subpopulation of cholinergic neurons may also exhibit differences with respect to neurodegeneration and alterations ChAT isoform levels. We did not detect a difference in overall expression of ChAT mRNA but, compared to control, the decrease in steady-state levels of M-ChAT in the nbM of AD samples approached significance (p=0.06). We reported previously that in PC12 cells, NO modulates metabolism and acetylcholine synthesis in relation to neuronal activity in Alzheimer’s disease. Lancet 1: 333-336.

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Since NO plays a role in cholinergic neuron function and is implicated in AD pathogenesis, it is important to understand the relationship between ChAT and NOS in the brain. The results from this study suggest that the levels of NOS and ChAT isoforms are regulated independently in cortex, hippocampus and nbM in AD. However, a reduction in eNOS levels in the projection regions of basal forebrain cholinergic neurons suggests that loss of cholinergic input could affect eNOS levels, ultimately altering local cerebral blood flow. Also, this study evaluated NOS and ChAT levels in specific brain regions. Modest changes in NO levels in neurons and surrounding cells may have important influences on cholinergic neuron function. Examining the levels of NOS and ChAT isoforms within cells could yield critical information regarding the role of NO in the neurodegeneration observed in AD.

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