



## Neural restrictive silencer factor and choline acetyltransferase expression in cerebral tissue of Alzheimer's Disease patients: A pilot study

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### Abstract

Decreased Choline Acetyltransferase (ChAT) brain level is one of the main biochemical disorders in Alzheimer's Disease (AD). In rodents, recent data show that the CHAT gene can be regulated by a neural restrictive silencer factor (NRSF). The aim of the present work was to evaluate the gene and protein expression of CHAT and NRSF in frontal, temporal, entorhinal and parietal cortices of AD patient brains. Four brains from patients with AD and four brains from subjects without dementia were studied. Cerebral tissues were obtained and processed by the guanidine isothiocyanate method for RNA extraction. CHAT and NRSF gene and protein expression were determined by reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting. CHAT gene expression levels were 39% lower in AD patients as compared to the control group ( $p < 0.05$ , U test). ChAT protein levels were reduced by 17% ( $p = 0.02$ , U test). NRSF gene expression levels were 86% higher in the AD group ( $p = 0.001$ , U test) as compared to the control group. In the AD subjects, the NRSF protein levels were 57% higher ( $p > 0.05$ , U test) than in the control subjects. These findings suggest for the first time that in the brain of AD patients high NRSF protein levels are related to low CHAT gene expression levels.

**Keywords:** neural restrictive silencer factor, choline acetyltransferase, Alzheimer's disease, protein expression, brain.

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### Introduction

Alzheimer's disease (AD) is a progressive, neurodegenerative disease and the most common type of adult-onset dementia (Cerpa *et al.*, 2008). Although the etiologic and pathogenic events that lead to this disease are still unknown, highly typical neuropathological changes (amyloid plaques, neurofibrillary tangles and an intense glial reactivity) are observed in the patients' brains (Maccioni *et al.*, 2001; Watson *et al.*, 2005; Haroutunian *et al.*, 2008). Cholinergic

dysfunction signaling is an early hallmark of AD (Ikonomovik *et al.*, 2011), one of the most important being a reduction in Choline Acetyltransferase (ChAT) enzyme activity in the cholinergic neurons of the temporal and parietal cortices, the hippocampus, the entorhinal cortex, and the amygdala (Mufson *et al.*, 2003; Abel *et al.*, 2008; Geula *et al.*, 2008)

Decreased ChAT activity in the cerebral cortex has been reported to be related to the severity of Alzheimer's Disease. This reduction is thought to originate from loss or declining function of cholinergic neurons known to be affected in AD (Boissiere *et al.*, 1997; Mufson *et al.*, 2003; Kar *et al.*, 2004; Pakaski and Kalman, 2008). The cholinergic deficit contributes significantly to the neuropsychi-

atric manifestations of the disease (Auld *et al.*, 2002; Kar *et al.*, 2004). Biochemical and *in situ* hybridization studies have shown a significant region-dependent loss in ChAT activity (from 30 to 90%) and ChAT mRNA levels (about 50%) in the temporal, frontal and parietal cortices of AD brains (Wilcock *et al.*, 1982; Coyle *et al.*, 1983; DeKosky *et al.*, 1992; Lehericy *et al.*, 1993; Yan and Feng, 2004; Heese and Akatsu, 2006).

Different studies have shown that the synthesis of the cholinergic neuron-essential molecules involved in cholinergic neurotransmission is controlled by DNA-regulatory elements and DNA-binding proteins (De Gois *et al.*, 2000; Hersh and Shimojo., 2003; Oda *et al.*, 2004). Therefore, the organization of the cholinergic gene locus suggests a coordinated regulation at the transcriptional level (Berrard *et al.*, 1995; Berse and Blusztajn, 1995; Mieda *et al.*, 1997; Tanaka *et al.*, 1998). The mechanism of transcriptional regulation controlling the cholinergic gene is still unclear; however, in cholinergic cells at least two kinds of regulatory elements in the CHAT gene have long been recognized as important for specific gene expression (Lonnerberg *et al.*, 1996; Shimojo *et al.*, 1999).

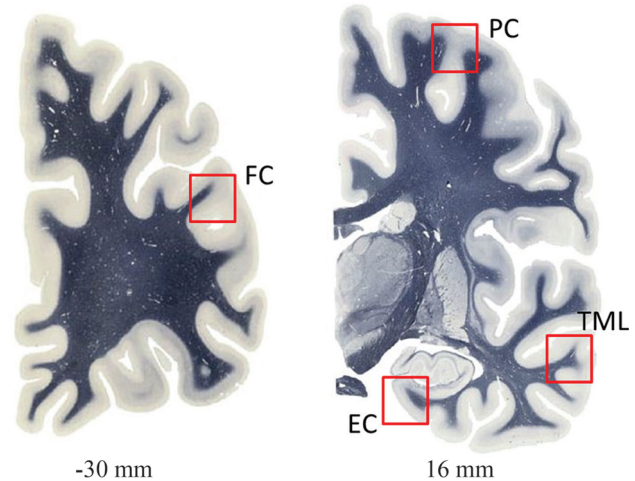
The neuron-restrictive silencer element (NRSE), which comprises ~23 nucleotides, has been involved in silencing the cholinergic gene locus in non-neural cells (Chong *et al.*, 1995; Shimojo *et al.*, 1999). It was reported that a transcription factor called neuron-restrictive silencer factor (NRSF/REST) is recruited to NRSE sites, repressing neuron-specific genes in neural cells (Belyaev *et al.*, 2004; Schoenherr and Anderson, 1995; Palm *et al.*, 1999; Wood *et al.*, 2003). In addition, *in vitro* reports using rodent cell lines have demonstrated that the NRSF binds to rat NRSE to repress the R-type of ChAT in non-neuronal cells (Shimojo *et al.*, 1999; Hersh and Shimojo, 2003). The NRSF/REST gene, *REST*, is located on chromosome 4q12 in humans (OMIM, \*600571), whereas the mouse counterpart is located on chromosome 5 C3.3; 5 (MGI 104897).

It has been shown that the human cholinergic gene locus contains a sequence that is homologous to that of rat NRSE in a corresponding gene region (Hahm *et al.*, 1997). It can therefore be suggested that the cholinergic gene expression is repressed in human neuronal cells by similar mechanisms as in rodents. To date, no comparative studies on CHAT and NRSF expression in patients with Alzheimer's disease were reported. So, we decided to analyze the gene and protein expression in different cortices of Alzheimer's disease patients and of non-demented controls.

## Material and Methods

### Subjects: Tissue preparation

Frontal, temporal, entorhinal and parietal cortices (see Figure 1) were obtained from brains of four autopsied patients with Alzheimer's disease (diagnosed using the DSM-IV and NINCDS-ADRDA criteria) and of four sub-



**Figure 1** - Human brain sections showing the analyzed areas. Numbers indicate the position of the sections relative to the center of the anterior commissure. FC: frontal cortex; PC: parietal cortex; TML: temporal medial lobe; EC: entorhinal cortex.

jects with no clinical history of or pathological findings suggestive of any neurological or psychiatric diseases. This study was approved by the Ethics Committee of the Instituto Mexicano del Seguro Social (IMSS) and carried out after written consent from the patients' relatives obtained prior to the autopsy. The study was also approved by the CNI-IMSS review board and was carried out according to the principles of the Helsinki declaration, as revised in 2000 (World Medical Association, 2008).

### Extraction and quantification of total RNA

Total RNA was isolated according to the method described by Chomczynski and Sacchi (1987). Briefly, cerebral cortical tissue was homogenized using a polytron system in the presence of Trizol (Invitrogen). Chloroform was added and the RNA was precipitated from the resulting aqueous phase with isopropanol at 4 °C for 48 h. RNA was resuspended in DEPC-treated water after ethanol precipitation and stored at -80 °C until use. RNA samples with a 260/280 nm absorption ratio between 1.8 and 2.0 were used for further experiments.

### Detection of CHAT and NRSF expression by reverse transcription-polymerase chain reaction (RT-PCR)

Two µg of total RNA were adjusted to a volume of 6 µL with DEPC water. Samples were incubated at 70 °C for 10 min and cooled quickly on ice. Reverse transcription reactions were performed using 0.1 µg of random primer in a 20 µL reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 0.2 mM of each deoxynucleotide (Invitrogen), and 4 units of MLV reverse transcriptase (Invitrogen). Reverse transcription was performed at 30 °C for 10 min and 37 °C for 1 h. The reaction was stopped by heating to 99 °C for 5 min. These mixtures were

stored at  $-80^{\circ}\text{C}$  until use as cDNA templates for the reverse transcription-mediated PCR (RT-PCR). The 5' and 3' primer sequences used for amplification were as follows: CHAT, 5'-TAG CCG ATG ACA GCC TGT GAA TGA CC-3' (upper primer, base position 61) and 5'-TTG TTG CCA GGA ACA GAG AGT CCA GA-3' (lower primer, base position 660; GenBank access number: X56585) that amplified a PCR product of 600 bp, total cycles: 32. NRSF, 5'-TGC CGC GAG CTC GCG GCG CAG CAG CG-3' (upper primer, base position 1) and 5'-ATA CAG GCT GAG GTT CTA CGA CGC TG-3' (lower primer, base position 720; Genbank access number: NM\_005612) that amplified a PCR product of 720 bp, total cycles: 36. G3PDH, 5'-CGC TTC GCT CTC TGC TCC TCC TGT TC-3' (upper primer, base position 3) and 5'-GGG GTG CTA AGC AGT TGG TGG TGC AG-3' (lower primer, base position 580; Genbank access number: NM\_002046) that amplified a PCR product of 577 bp; total cycles: 26.

The amplification mixture contained cDNA, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1.75 mM  $\text{MgCl}_2$ , 0.4 mM dNTP, 5  $\mu\text{g}/\text{mL}$  of each of the specific upstream and downstream primers, and 0.175 units of *Taq* DNA polymerase (Invitrogen) in a total reaction volume of 10  $\mu\text{L}$ . The amplification of DNA fragments of each sample was performed in a thermal cycler (Eppendorf Mastercycler) with an initial denaturation step at  $95^{\circ}\text{C}$  for 5 min, followed by various cycles, each consisting of a denaturation step at  $95^{\circ}\text{C}$  for 1 min, an annealing step at  $60^{\circ}\text{C}$  for 1 min, and a final elongation step at  $72^{\circ}\text{C}$  for 1.5 min. PCR products were electrophoresed in a 1.5% agarose gel containing 0.1  $\mu\text{g}/\text{mL}$  ethidium bromide. Gels were photographed under short-wavelength UV. The intensity of the fluorescence level of the product band was determined by densitometry (Alpha Innotech Corporation). The quantities of PCR products of CHAT and NRSF were normalized to the G3PDH level (Figure 5A).

#### Western blotting for ChAT and NRSF protein

Brain tissue was ice-homogenized in lysis buffer containing 920  $\mu\text{L}$  lysis regulator (10 mM), Tris-HCl (pH 7.5), 150 mM, NaCl, 20 mM, NaF, 0.5 mM sodium orthovanadate, 2 mM sodium pyrophosphate, and 80  $\mu\text{L}$  protease inhibitor cocktail (Roche). Samples were incubated on ice for 30 min and centrifuged at 13,000 rpm at  $4^{\circ}\text{C}$ . Protein concentration was estimated according to Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard and 50  $\mu\text{g}$  of protein from each sample, before separation on a 12% SDS-polyacrylamide gel and transfer to nitrocellulose membranes (Sigma). Membranes were blocked with nonfat milk (10%; Svelty) in phosphate buffer saline (PBS) with 0.25% Tween-20 for two hours, to avoid non-specific antibody binding. The membranes were then incubated with anti-ChAT (AB144P, Chemicon) and anti-NRSF (P-18, Chemicon) primary antibodies (1:1000) for 24 h. After

three washes in PBS with 0.05% Tween-20 (PBST), the membranes were incubated for 24 h with donkey anti-goat IgG HRP (1:1000) as the secondary antibody. After three washes with PBST, the membranes were incubated in an ABC Elite Kit (PK 6200; Vector Laboratories) for two hours and, after three washes with PBS of 5 min each, developed with diaminobenzidine (SIGMA). Protein expression was quantitatively determined by densitometry (Figure 5A).

#### Statistical analyses

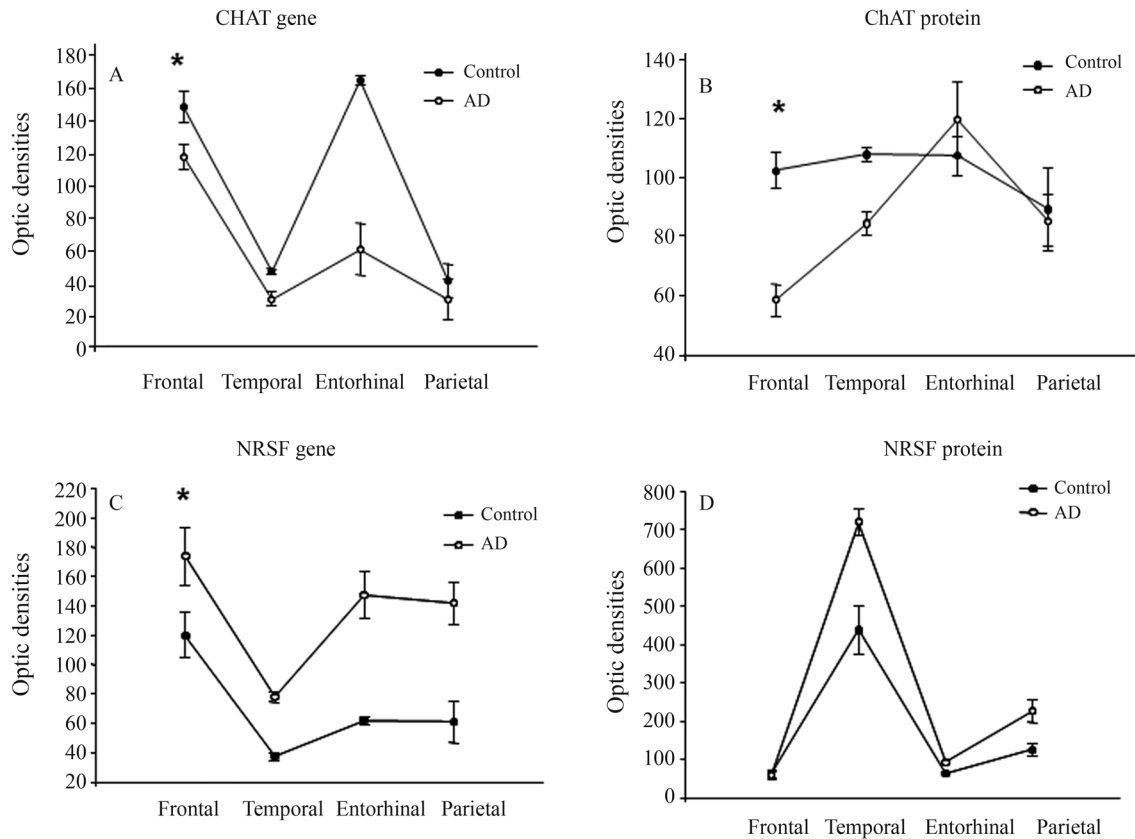
CHAT and NRSF expression differences between groups were analyzed by means of Mann Whitney's U test and the Wilcoxon test. Data are shown as mean  $\pm$  standard error of the mean (S.E.M.). Statistical significance was set at  $p < 0.05$ .

#### Results

The brains of four patients with sporadic, severe AD and four control subjects were studied (mean age  $64 \pm 8$  vs.  $54 \pm 4$  yrs,  $p > 0.05$ ). None of the patients had been in a coma or shown any other CNS lesions (*i.e.*, infections, tumors). The time frame for autopsy and brain processing was less than 6 h.

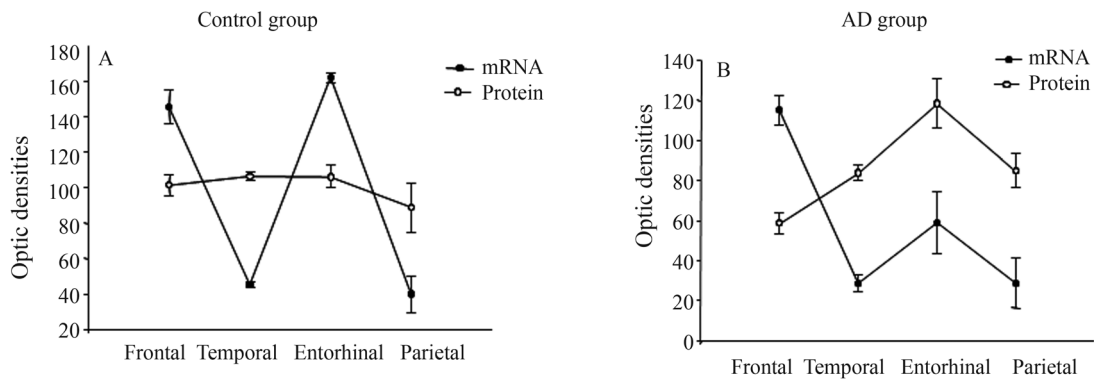
When comparing CHAT expression among brain regions, in both the frontal and entorhinal cortices it is higher than the parietal and temporal cortices in both groups. However, when comparing between groups, in all brain regions it is lower in the AD group than in the control group (Figure 2A). Despite these variations, in the control group the ChAT protein levels were constant, whereas in the AD group a peak in the entorhinal cortex and a remarkable decrease in the frontal region were found (Figure 2B,  $p > 0.05$ , Wilcoxon test). In the AD group, ChAT protein expression in both the temporal and parietal regions was similar although slightly lower than in the control group (Figure 2B,  $p > 0.05$ , Wilcoxon test). On the other hand, regional analysis of the NRSF gene showed an expression pattern similar to that of the CHAT gene (higher in the frontal and lower in the temporal cortices,  $p > 0.05$ ; Wilcoxon test). Unlike the greater CHAT expression in the control group, the NRSF expression was greater in the AD group, while the NRSF protein expression was not constant (greater in the temporal region, with similar levels in the frontal, entorhinal and parietal regions;  $p > 0.05$ , Wilcoxon test, see Figure 2C, D).

Average analysis of both ChAT and NRSF gene and protein expression showed the CHAT gene expression to be 39% lower in the AD than in the control group ( $p < 0.05$ , U test), and its protein expression to be 17% lower in the AD than in the control group ( $p = 0.02$ , U test). In contrast, the NRSF gene expression was increased by 86% in the AD group as compared to the control group ( $p = 0.001$ , U test)



**Figure 2** - CHAT and NRSF gene and protein expression. A. The CHAT expression curve in both groups is similar, although lower in the AD group. B. The ChAT curve is constant in the control group, whereas in the AD group it presents a peak in the entorhinal cortex and a decrease in the frontal region. C. The NRSF expression curve is similar in both groups, but higher in the AD group. D. The NRSF protein is higher in the AD group. Data are shown as mean  $\pm$  S.E., \* statistically significant, U test;  $p \leq 0.05$ , Wilcoxon test.

ChAT gene and protein expression



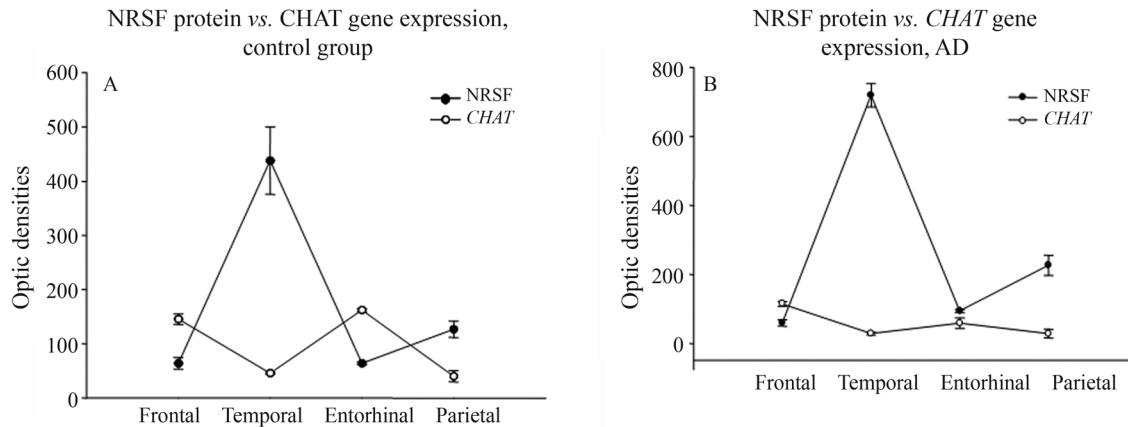
**Figure 3** - CHAT gene and protein expression in the frontal, temporal, entorhinal and parietal regions of: A. the control group; B. the AD group. Data are shown as mean  $\pm$  S. E.,  $p \leq 0.05$ , Wilcoxon test.

and the expression of its protein increased by 57% ( $p > 0.05$ , U test).

In addition to CHAT gene expression, we determined the ChAT protein concentrations, in order to evaluate the genetic information corresponding to the total amount of protein produced. As can be seen in Figure 3, in the AD group the ChAT protein concentrations varied in all the

brain regions, being higher than the CHAT gene expression in the temporal, entorhinal and parietal cortices, albeit with the same distribution pattern. Thus, except for the frontal region, the ChAT protein expression was observed to correspond to the CHAT gene expression. In contrast, the opposite was found in the control group.





**Figure 4** - CHAT gene expression and NRSF protein levels in the frontal, temporal, entorhinal and parietal regions of: A. the control group; B. the AD group. Data are shown as mean  $\pm$  S. E.,  $p > 0.05$ , Wilcoxon test.

Comparing NRSF protein concentration and CHAT gene expression, an inverse relationship (high NRSF with low CHAT and vice versa) was observed in the control group, with lower CHAT levels in both the temporal and parietal cortices ( $p > 0.05$ ; Wilcoxon test, Figure 4A). The same was found in the AD group ( $p > 0.05$ , Wilcoxon test, Figure 4B).

## Discussion

In this pilot study, we found a decreased expression of both CHAT gene and protein levels, and an overexpression of the NRSF gene in AD patients as compared to the control group. This is the first study showing such a relationship in the human brain.

Increasing evidence indicates that the CHAT gene expression can be regulated by the NRSF protein (Shimojo *et al.*, 1998; Hersh and Shimojo, 2003). NRSF is a transcription factor that regulates neuronal activity in the adult brain. This is consistent with a role for the NRSF-NRSE system in modulating gene expression rather than simply acting as an ON/OFF switch under different circumstances (Schoenherr and Anderson, 1995; Schoenherr *et al.*, 1996).

In the present study, the increased NRSF levels and the decreased CHAT expression in the cerebral regions studied are in agreement with the behavior of this complex (*i.e.*, the negative regulation of CHAT expression by the NRSF protein). This NRSF/CHAT interaction is similar to that observed in both the murine model and cell cultures (Chong *et al.*, 1995; Chen *et al.*, 1998; Jones and Meech, 1999; Hersh and Shimojo, 2003).

The higher expression of CHAT in the frontal region of the control group may be explained by the presence of cholinergic fibers coming from several areas, mainly from the Nucleus Basalis of Meynert (Selden *et al.*, 1998). Considering the presence of motor, memory and behavior control circuits in the frontal cortex (Karczmar, 1990, 1993; Pepeu, 1993), we speculate that these are acetylcholinergic

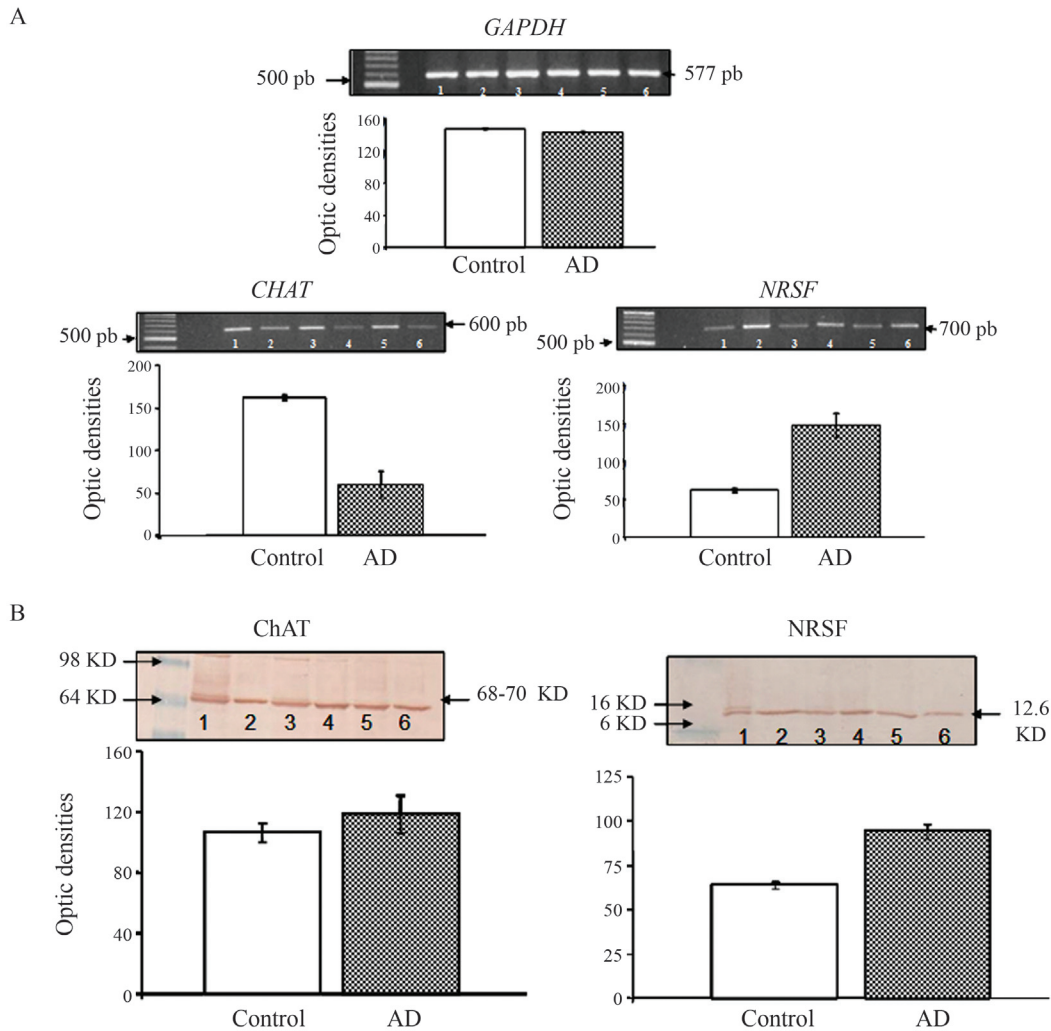
circuits and need ChAT to recycle choline in order to maintain the chemical signal to the circuit.

The findings in the entorhinal cortex may be attributed to the presence of numerous cholinergic afferents in this region, mainly coming from the Vertical Diagonal Band of Broca and the Basal Nucleus of Meynert (Mesulam, 1996; Mesulam and Geula, 1988; Semba *et al.*, 1989). The proximity of the entorhinal cortex to the hippocampus, the amygdala and the cingulate gyrus is correlated with the cholinergic pathways (Squire and Zola-Morgan, 1991; Wible *et al.*, 1992; Price, 2000).

We found that the NRSF expression in the control group was practically constant, but diminished in the temporal cortex. Furthermore, the NRSF protein expression was significantly increased in this region. This may be due to a post-transcriptional regulation of the NRSF expression (Lewin, 2000; Luque, 2002).

Neither the expression of CHAT nor its protein diminished in the same proportion to the increase of NRSF/NRSF suggesting that, at least in the temporal region, the protein NRSF regulates more than a single gene. This gene regulates the expression of other genes, such as BDNF (Timmusk *et al.*, 1999), synapsin I (Thiel *et al.*, 1994), SCG10 (Mori *et al.*, 1992; Mori *et al.*, 1990), Na<sup>+</sup> channel (Mori *et al.*, 1992), nicotinic receptor (Bessis *et al.*, 1995), muscarinic receptor, and others (Roopra *et al.*, 2000). The role of NRSF protein in this region is probably not limited to the regulation of CHAT expression only, but also to some other genes as those described above. A co-regulation of CHAT by any of the above mentioned genes may also be involved.

We determined ChAT protein concentrations besides CHAT gene expression to evaluate the genetic information corresponding to the total amount of the produced protein. We found that (by exception of the frontal region in the AD group) ChAT protein expression was closely associated to CHAT gene expression, whereas in the control group an inverse relationship was found. The fact that ChAT protein



**Figure 5** - A. Agarose gel electrophoresis of the RT-PCR products of GDPH, CHAT and NRSF. B. ChAT and NRSF protein detection by Western Blot in the entorhinal cortex of the AD and Control groups. Molecular weights are indicated in kDa. 1, 3 and 5: control group; 2, 4 and 6: AD group. Graphs show the relative expression quantification in the entorhinal cortex of both the AD and control groups. Data are shown as mean  $\pm$  S. E.,  $p=0.05$ , Wilcoxon test.

levels highly overpass the expression of CHAT in the parietal and temporal region may be explained by the numerous cholinergic afferents originated in the Ventral Tegmental Nucleus (for the temporal region) and the Medial Septal Nucleus (for the parietal region) (Fibiger, 1982; Gaykema *et al.*, 1990; Mesulam, 1996). Consequently, ChAT may be produced in distant places and transported toward the synaptic button (Martinez-Murillo *et al.*, 1989; Oda *et al.*, 1992, 1996; Jones, 2004).

High CHAT expression associated with decreased ChAT levels may indicate that the frontal region receives afferents from distal connectivity and with probably little local circuitry (Oda *et al.*, 1992, 1996). Production of the protein in the frontal and entorhinal cortex or from fibers coming directly from the Nucleus Basalis of Meynert probably contributes to the high values of ChAT enzyme in the parietal and temporal regions, maintaining circuits with these zones. To verify these circuits, subsequent studies are

required in order to confirm the cerebral distribution sites of Ach and ChAT enzyme produced in the frontal and entorhinal regions.

ChAT expression in the AD group showed a similar tendency to that of the control group: higher in the frontal and entorhinal regions and lower in the parietal and temporal lobes, but in all of the cases CHAT expression was lower in the AD group. This is in line with previous reports describing a decrease in the cholinergic activity in different cerebral regions of patients with AD (Boissiere *et al.*, 1997; Garcia-Alloza *et al.*, 2005; Ikonovic *et al.*, 2005, 2011).

NRSF expression was higher in the AD group than in the control group: as compared to the control group, NRSF expression and NRSF production were increased in the AD group by 86% and 57%, respectively. An increased NRSF protein production was also observed in the temporal region in both groups. In the AD subjects, this proportion was maintained although with higher expression, showing an

important regulatory effect. These findings could be a consequence of neuronal damage (Palm *et al.*, 1998; Timmusk *et al.*, 1999). The temporal region is one of the most vulnerable to damage produced by the accumulation of the  $\beta$ -amyloid protein as well as of the tau protein (Pakaski and Kalman, 2008), which may increase NRSF expression. Since there was also a NRSF overexpression, a negative feedback may act at the nuclear level. In other words, there seems to be a mechanism of direct signposting between the damaged neuronal tissue and the gene expression. It would be necessary to study how this process participates in the neurodegeneration present in AD and whether this is a direct or a mediated effect.

The lower ChAT levels in the frontal lobe may be due to neurodegeneration of cholinergic afferents (Nunes-Tavares *et al.*, 2012). Therefore, high ChAT expression may be due to the persistence of cholinergic afferents. Thus, the deafferentation seems to be presented in long axons cholinergic systems than in short axons.

In conclusion, our data suggest that NRSF is related to a decreased CHAT expression in human neural tissue. This CHAT expression has a topographical distribution, being more important in areas neurodegenerated by AD. The influence of the loss of cholinergic regulation on some of the symptoms of AD still needs to be assessed.

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