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REST/NRSF-induced changes of ChAT protein expression in the neocortex and hippocampus of the 3xTg-AD mouse model for Alzheimer's disease

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ABSTRACT

Aims: The cholinergic system is one of the neurotransmitter systems altered in Alzheimer's disease (AD), the most common form of human dementia. The objective of this work was to determine the REST/NRSF involvement in altered ChAT expression in the neocortex and hippocampus of an AD transgenic mouse (homozygous 3xTg-AD) that over-expresses 3 proteins, amyloid-β precursor protein, presenilin-1, and tau, all of which are associated with AD and cause cellular degeneration.
Main methods: Two groups (WT and 3xTg-AD) of 11-month-old female mice were analyzed and compared. Half of the brains of each group were used for ChAT immunohistochemistry, and Western Blot analyses of ChAT and REST/NRSF were performed on the other half.
Key findings: We observed significant decreases in the number of ChAT-immunoreactive cells in the Meynert nucleus and of fibers in the frontal motor cortex and hippocampal CA1 area in transgenic mice compared with control mice. An increased level of REST/NRSF protein and a reduction of ChAT protein expression in the 3xTg-AD mice compared with their controls were also found in both in the latter two cerebral regions.
Significance: The increased REST/NRSF expression reported here and its effect on the regulatory region for ChAT transcription could explain the decreased expression of ChAT in the 3xTg-AD mouse; these findings may be associated with the degeneration observed in AD.

Introduction

Alzheimer's disease (AD) is associated with a massive loss of cholinergic neurons in the anterior basal brain, the area most vulnerable to the development of the pathological characteristics associated with AD. The process governing the heightened vulnerability of cholinergic neurons in AD and aging depends on their unique metabolic capability, and it is from the anterior basal brain that the cholinergic nuclei project fibers to regions such as the neocortex and the hippocampus (Francis et al., 1999). The main neurotransmitter in these fibers is acetylcholine (Mesulam, 2004; Mufson et al., 2008), whose synthesis is catalyzed by the enzyme choline acetyltransferase (ChAT) (Oda, 1999).

Alterations in cholinergic neurotransmission in AD patients in the neocortex and hippocampus are associated with the early stages of memory loss (Boissière et al., 1997; Kooi et al., 2011), and reductions in ChAT protein expression and activity are strongly correlated with this type of dementia (Wilcock et al., 1982), suggesting that upregulation of ChAT may be caused, at least in part, by the disconnection of the glutamatergic entorhinal cortex input to the hippocampus, which occurs early in the disease process (Hyman et al., 1984; Gómez-Isla et al., 1996; Kordower et al., 2001).

The decreased ChAT mRNA concentration may result from changes in the transcription of the ChAT gene, possibly in relation to accumulated damage by late stages of this disease (Boissière et al., 1997). The transcription of some genes, such as the ChAT gene, is known to be modulated by a consensus element, named Repressor Element-1/Neuron-Restrictive Silencing Element (RE-1/NRSE), which is located in a 5′ promoter region and regulated by a transcriptional repressor named Repressor Element-1 Silencing Transcription Factor (REST)/neuron restrictive silencer factor (NRSF) (Shimojo, 2006). It has been shown that the human ChAT gene locus contains a sequence that is homologous to that of murine NRSE (Hahm et al., 1997).

In vitro and *in vivo* studies show that expression of ChAT, other enzymes, receptors, and ion channels is regulated by REST/NRSF, especially those genes encoding nervous system constituents (Hersh and Shimojo, 2003; Bruce et al., 2004; Kim et al., 2006); REST/NRSF

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contributes to neuronal expression of the ChAT gene by modulating the NRSE region (Lönnerberg et al., 1996).

REST/NRSF is expressed in both developing and mature neurons; its role may vary from transcription repressor to activator or modulator, depending on the cell type where it is expressed as well as on the temporal and spatial contexts of its own expression and that of its molecular partners. REST/NRSF acts as a genomic platform for integrity, stability, and specific regulation, and it is overexpressed in the murine and human brains during pathology; thus, gene expression is inhibited in neurodegenerative diseases, *e.g.*, brain tumors, Down's syndrome, epilepsy, ischemia, and Huntington's disease. In this latter neuropathology, ChAT immunoreactivity levels are low, and REST/NRSF expression levels are increased, suggesting that REST/NRSF could reduce ChAT gene expression in neurodegenerative pathologies (Calderone et al., 2003; Smith et al., 2006).

Here, we evaluated the levels of REST/NRSF protein in the 3xTg-AD mouse model and compared them with previously reported alterations of ChAT immunoreactivity levels, in order to elucidate the neuronal mechanisms involved in regulating ChAT expression, which could be related to cell degenerative events observed in brain tissue of AD patients.

Materials and methods

The triple-transgenic mouse (3xTg-AD, harboring APP_{Swe} and tau_{P301L} transgenes on a mutant $PS1_{M146V}$ knock-in background) was developed by LaFerla's laboratory (Oddo et al., 2003) and accumulates intracellular and extracellular amyloid-β (Aβ) and tau in an age-dependent manner within the hippocampus. A total of thirty-two, 11-month-old female 3xTg-AD mice were matched with wild-type 129S/C57BL/6 mice of equal number, sex, and age to perform histological (n = 24) and biochemical (n = 8) analyses. These transgenic mice all had the same genetic and strain background (genotyping was performed on each experimental animal) and were drawn from the colony maintained by the Neurobiology Institute of UNAM. Animal management was supervised by a licensed veterinarian in accordance with the principles set forth in the NIH guide for the care and use of laboratory animals, and was approved by the Bioethics Committee of the Neurobiology Institute. All mice were housed 4 per cage with access to food and water *ad libitum* and under optimal *vivarium* conditions (12 h:12 h light–dark cycle, 20 °C, and 40–50% relative humidity).

Genotyping

For DNA extraction, the most caudal tail segments, approximately 0.5 cm long, of the mice were sectioned, placed in Eppendorf® tubes, and kept at −20 °C until use. After alkaline lysis the sample was used immediately in a polymerase chain reaction (PCR) to test for the presence of the amyloid precursor protein (APP) and tau protein genes and for the mutation in the presenilin 1 (PS1) gene. The first PCR detected APP and tau as 500 bp and 350 bp bands, respectively. In the second PCR, PS1 was observed as 2 bands, 350 and 180 bp. The samples were

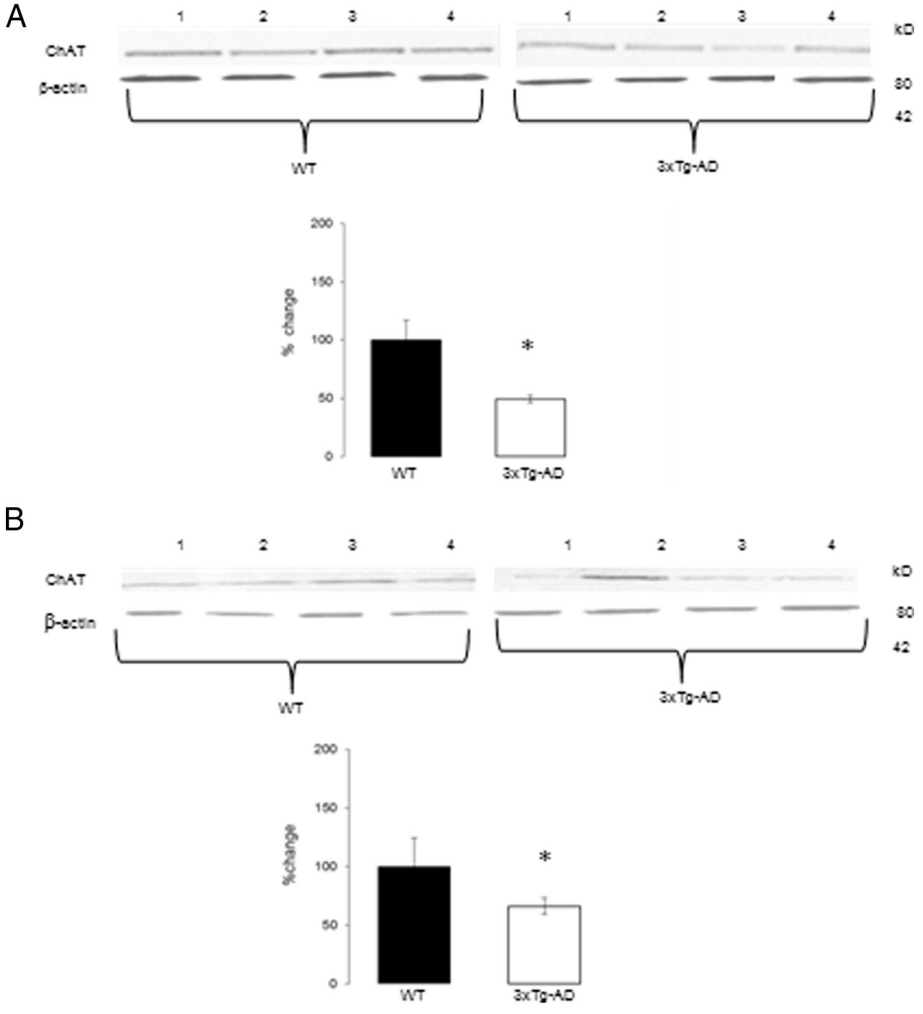

Fig. 1. Western Blot showing ChAT expression in the frontal cortex (A), and the hippocampus (B) of four 3xTg-AD and four control mice. The graphs show the mean ± SEM of ChAT expression in the frontal cortex and hippocampus. A significant (* $P < 0.05$) reduction was found in both the frontal cortex and the hippocampus of the 3xTg-AD mice.

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subsequently loaded onto a 1% agarose gel and monitored under UV light.

Western Blot analysis

A total of 8 mice, 11-month-old female 3xTg-AD (n = 4) and wild-type (129C57/BL6, n = 4) mice, were sacrificed by decapitation to extract their brains and dissect the frontal cortex and hippocampus. The tissues were weighed and immediately frozen at −80 °C until protein extraction and quantification. To obtain the total protein, the tissue was sonicated in 920 μL of lysis buffer (Tris–HCl pH 7.5, 150 mM NaCl, 20 mM NaF, 0.5 mM sodium orthovanadate, 2 mM sodium pyrophosphate) with 80 μL of a protease inhibitor cocktail. The samples were incubated on ice for 30 min and then centrifuged at 13,000 rpm for 30 min at 4 °C. Aliquots of the supernatant were processed with the Bio Rad 163-2089 kit to obtain the cytoplasmic fraction including ChAT and REST/NRSF, and the nuclear fraction containing REST/NRSF.

The protein concentration was estimated in both fractions by the Lowry method (Lowry et al., 1951). Proteins were separated on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and then incubated with primary antibody: goat anti-choline acetyltranferase 1:1000 (cat. AB144P, Millipore) or rabbit anti-NRSF 1:1000 (cat. H-290, Santa Cruz). After washing, the membranes were incubated with the appropriate biotinylated secondary antibody, washed again, incubated with the ABC kit (Vector), and revealed with the diaminobenzidine kit (*peroxidase substrate kit* DAB SK-4100, Vector). The gel was observed by UV trans-illumination, and a photo-documentation image-analyzer system (ChemiDoc XRS, Quantity One Image Analysis Software) was used to semi-quantify the corresponding bands. The optical densities of the various bands were normalized to that of the loading control, either β-actin for the cytoplasmic or laminin B1 for the nuclear fraction.

Immunohistochemical analysis

The 3xTg-AD (n = 12) and control 129SC57/BL6 (n = 12) mice were deeply anesthetized with ketamine/xylazine (85 mg/kg/9.5 mg/kg, i.p.) and transcardially perfused using 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4, 0.1 M) containing 1000 IU of heparin and 0.1% procaine. Brains were removed and postfixed for 24 h in 4% paraformaldehyde at room temperature. Serial sections (50 μm) were sagittally cut with a vibratome (model 3000, tissue sectioning system; Pelco International) and collected in PBS with 0.1% sodium azide (Bregma 1.92–2.28 mm lateral, Franklin and Paxinos 2008).

To stain the cholinergic fibers, a primary antibody against ChAT (polyclonal goat anti-ChAT IgG, Chemicon International Inc.; 1:100) and a biotinylated secondary antibody (mouse anti-goat, Vector; 1:500) were used. The ABC and peroxidase substrate kits (Vector) were used to visualize bound antibody. Cholinergic neurons were stained by incubating with the primary antibody (polyclonal goat anti-ChAT IgG, Chemicon International Inc.; 1:100) followed by anti-

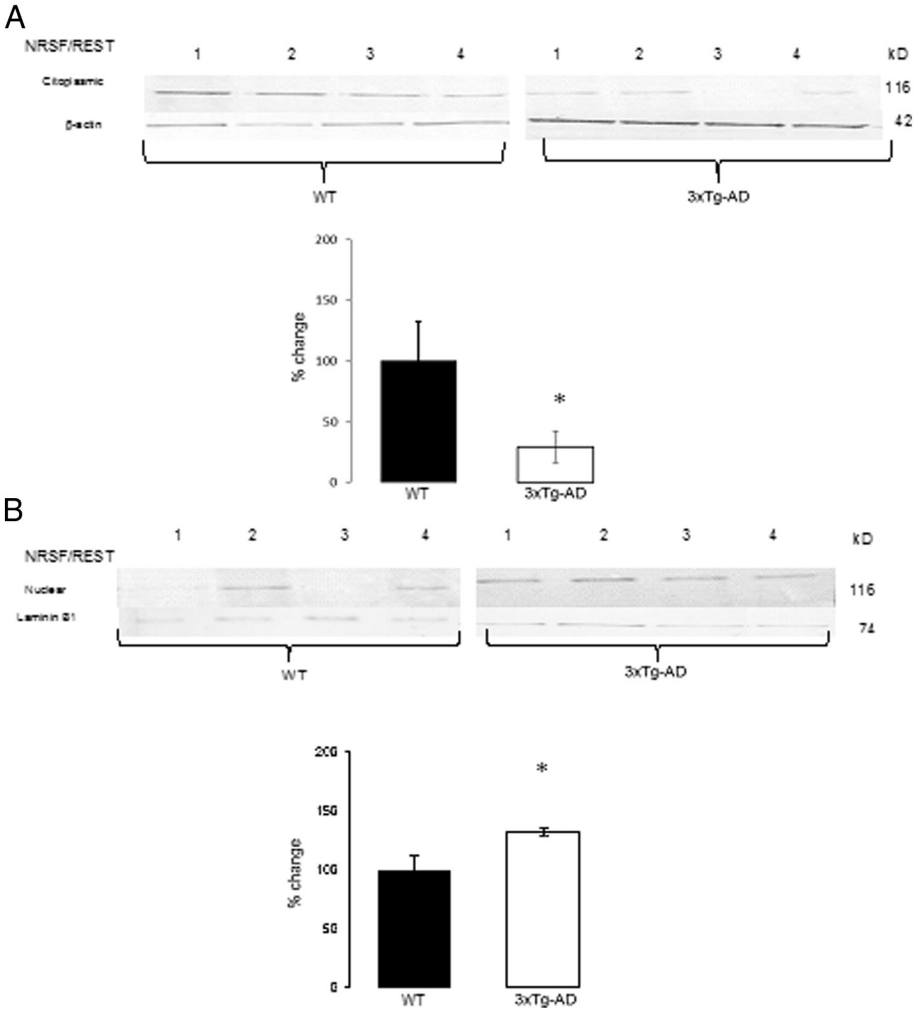

Fig. 2. Western Blot showing REST/NRSF expression in the cytoplasmic (A) and nuclear fraction (B) of samples from the frontal cortex of four control (WT) and four 3xTg-AD mice. Graphs show the mean ± SEM for each group, after normalizing for the amount of laminin B or beta-actin, respectively; expression of REST/NRSF was significantly reduced ($P < 0.05$) in the cytoplasm fraction but significantly increased ($P < 0.05$) in the nuclear fraction of the 3xTg-AD mice compared to the WT mice.

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goat, Alexa 594 (Invitrogen, 1:500). Cholinergic fibers and neurons were identified in both control and transgenic mice, and evaluated using a NIKON microscope (Eclipse 50i). The images were digitalized with a DS-U2 S Camera at 100× (Plan-Apochromat®, 1.25 NA/160). The density of ChAT-positive neurons was measured in the Meynert nucleus in its full extension (262.5 × 125 μm) in one focal plane (50 μm thickness); the density of ChAT fibers was measured in the frontal motor cortex and hippocampus (CA1) areas. The latter quantifications were performed on gray-scale images with a 100× objective lens and computer-assisted imaging analysis system (Image J) software (National Institutes of Health, USA). All these measurements, including the density of ChAT-positive neurons as well as the ChAT-immunpositive fibers, were performed by an experimenter who was blind to the identity of the groups.

Statistical analyses

The results of the Western Blot assays for ChAT and REST/NRSF as well as morphometric density analyses for fibers and cells (cholinergic neurons) are presented as mean ± standard error of the mean (SEM). All data were evaluated with a Student's t-test in each of the experimental conditions. Results were considered to be statistically significant if $P \leq 0.05$. StatView software was used for the analyses.

Results

Increased expression of REST/NRSF protein and reduced expression of ChAT protein in the frontal neocortex and hippocampus of 3xTg-AD mice.

Expression of the ChAT and REST/NRSF proteins in both the frontal neocortex and the full hippocampus of 3xTg-AD mice was evaluated by Western Blot. An expression analysis of ChAT showed it to be lower in the 3xTg-AD than in the control group (WT), in both cerebral regions.

In the frontal neocortex of 3xTg-AD mice, ChAT protein decreased by 50% and in the hippocampal region it decreased by 30% compared to WT control mice (Fig. 1).

Interestingly, compared to the control mice, REST/NRSF expression increased significantly in the nuclear fraction, but it decreased in the cytoplasmic fraction in the transgenic mice (Figs. 2–3) after normalization.

The expression analysis of REST/NRSF in the frontal neocortex, showed an increase of 30% in the nuclear fraction and a decrease of 60% in the cytoplasmatic fraction from the 3xTg-AD mice compared to the controls (Fig. 2).

In the hippocampus, the analysis of REST/NRSF expression indicated an increase of 60% in the nuclear fraction, as well as a decrease of 70% in the cytoplasmatic fraction, in the 3xTg-AD mice with respect to the controls (Fig. 3).

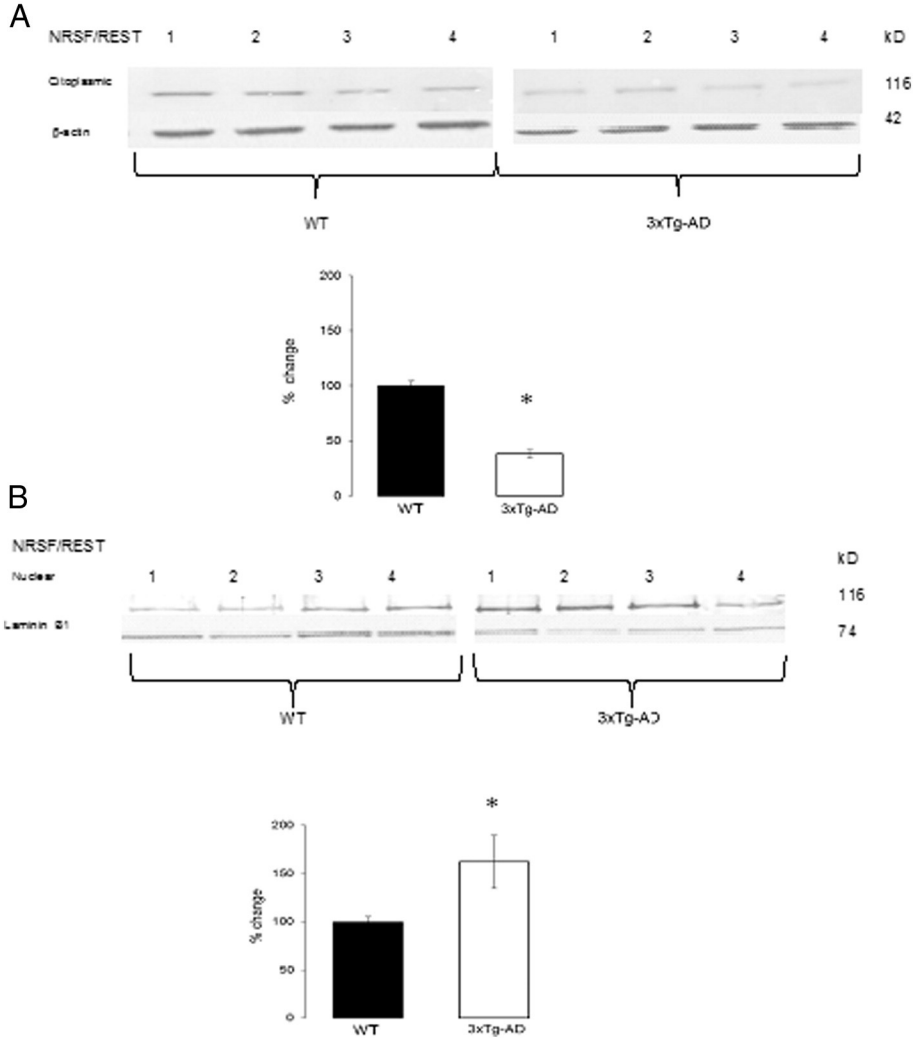

Fig. 3. Western Blot showing REST/NRSF expression in the cytoplasmic (A) and nuclear fraction (B) of samples from the hippocampus of four control (WT) and four 3xTg-AD mice. Graphs show the mean ± SEM for each group, after normalizing for the amount of laminin B or β-actin, respectively; expression of REST/NRSF was significantly reduced ($P < 0.05$) in the cytoplasm fraction but significantly increased ($P < 0.05$) in the nuclear fraction of the 3xTg-AD mice.

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Decreases in the number of ChAT-immunoreactive cells in the Meynert nucleus in the anterior basal brain and in fiber density in the frontal motor cortex and hippocampal CA1 area in transgenic mice (3xTg-AD).

Quantitative analysis of these ChAT immunoreactive fibers revealed a significantly diminished fiber density in the 3xTg-AD mouse compared to the (129C57/BL6) WT control. The decrease was 57.2% in the primary motor cortex and 59.2% in the hippocampal CA1 (Fig. 4).

Decrease of ChAT-immunoreactive cell number in the transgenic mice (3xTg-AD) compared to control mice (129C57/BL6) in the Meynert nucleus was observed (Fig. 5).

Conclusion

The increase of REST/NRSF expression has an effect on the decrease of ChAT transcription found in several degenerative diseases, AD included, as observed in the 3xTg-AD mouse model.

Discussion

A dysfunction in the interaction between REST/NRSF and the ChAT gene has been implicated in a number of disorders, including different types of cancer (Lawinger et al., 2000; Coulson, 2005; Fuller et al., 2005; Westbrook et al., 2005; Lv et al., 2010), Down's syndrome (Bahn et al., 2002; Canzonetta et al., 2008) cardiac hypertrophy (Bingham et al., 2007), and cerebral ischemia (Calderone et al., 2003; Noh et al., 2012). Moreover, an increase in REST/NRSF expression and a decline in ChAT immunoreactivity were reported in a transgenic mouse model (R6/1) for Huntington's disease (Smith et al., 2006). Taken together, these findings suggest that REST/NRSF could down-regulate expression of the ChAT gene in neurodegenerative pathologies. Increased REST/NRSF leads to decreased ChAT expression, and therefore less ACh synthesis, which in turn, is progressively diminished in AD and has clinical implications such as learning and memory loss in these patients.

The decline of ChAT expression agrees with the finding that the promoter region of the ChAT gene contains the NRSE consensus region (Lönnerberg et al., 1996), which is recognized by the REST/NRSF transcription factor; thus, increased REST/NRSF expression could inhibit the transcription (data not shown) and translation of ChAT in the 3xTg-AD mice. We found an increase in the expression of REST/NRSF protein in the cell nucleus in the neocortex and the hippocampus; this is consistent with studies that show that the nuclear localization of the different transcription factors is crucial for the regulation of genes associated with the installation of the damage process (Shimojo, 2006).

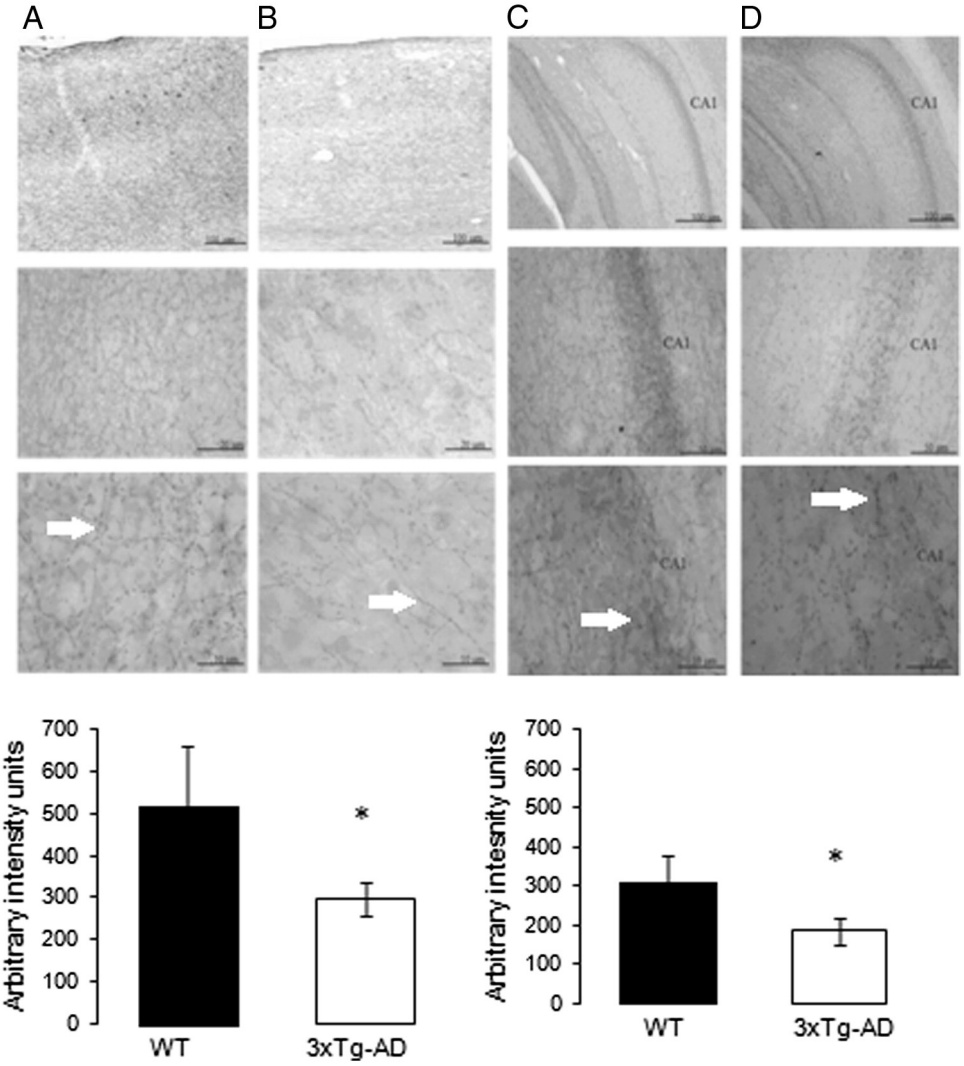

Fig. 4. Photomicrographs show representative sagittal sections of the frontal motor cortex (A, B) and hippocampal CA1 (C, D) from WT (A,C) and 3xTg-AD (B, D) mice. Bars represent 250, 100, and 25 μm in the top, middle, and bottom panels, respectively. Graphs show the mean ± SEM of arbitrary intensity units/0.1 mm^2 and indicate significant (* $P < 0.05$) reductions in ChAT fibers in both structures from 3xTg-AD mice. The arrows indicate ChAT immunoreactivity.

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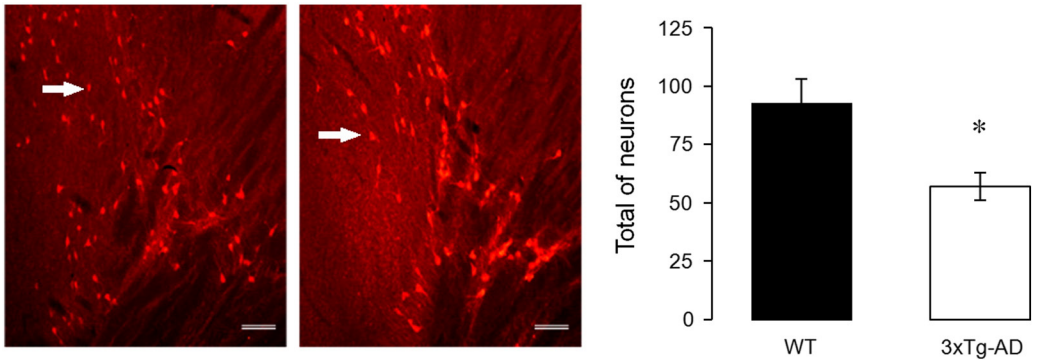

Fig. 5. Representative sagittal sections of the Meynert nucleus showing the ChAT-positive cells from WT (left) and 3xTg-AD (right) mice. Bars = 25 μm. The graph shows the mean + SEM of the total number of ChAT-immunoreactive neurons in both groups and indicates a significant (* $P < 0.001$) reduction in the number of ChAT-immunoreactive cells in the 3xTg-AD mice. The arrows indicate ChAT immunoreactive cells.

Our results are the first indication that increased expression of REST/NRSF could explain the decreased ChAT expression in 3xTg-AD mice: a decrease in both ChAT-immunoreactive fibers in the frontal motor cortex and hippocampal areas and in ChAT-positive neuron number in the Meynert nucleus compared with control mice.

Consistent with our data in the 3xTg-AD model, a reduced density of ChAT-positive cholinergic fibers was found in post-mortem tissue of AD patients (Wu and Xie, 2006) and correlated with cognitive dysfunctions (Billings et al., 2005).

Previous studies showed that the presence of amyloid plaques and neurofibrillary tangles correlated with alterations in neuronal populations localized in areas affected by AD in the anterior basal brain, *i.e.*, the Meynert nucleus (primary source of cholinergic neurons), where we found a reduction of ChAT-immunopositive neurons. Histopathological brain changes in AD start in the temporal lobe and extend to the Meynert nucleus that projects to the hippocampus and to the frontal, parietal, and occipital cortices, all of which have an important role in learning and memory (Whitehouse et al., 1981; Mufson et al., 2008). This is consistent with our finding of a reduction in fibers projecting to the primary motor cortex and the hippocampus CA1 area of 3xTg-AD mice.

Our results suggest that REST/NRSF expression is associated with cellular changes in the neocortex and hippocampus in this 3xTg-AD mouse model. It will be necessary to quantitatively explore the expression of REST/NRSF in other regions of the brain, such as the amygdala, and enthorrinal and prefrontal cortices of animals with advanced stages of Alzheimer's pathology.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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