Galanin Fiber Hypertrophy within the Cholinergic Nucleus Basalis during the Progression of Alzheimer's Disease

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Key Words

Alzheimer's disease · Basal forebrain hyperinnervation · Galanin hypertrophy

Abstract

Galanin (GAL)-containing fibers enlarge and hyperinnervate remaining cholinergic basal forebrain (CBF) neurons within the anterior nucleus basalis (NB) in late-stage Alzheimer's disease (AD). Whether GAL hypertrophy occurs in the CBF in the prodromal or early stages of AD remains unknown. The present study used GAL immunohistochemistry and an unbiased semiquantitative scoring method to evaluate GAL innervation in the anterior NB of subjects clinically diagnosed as having no cognitive impairment, mild cognitive impairment or early-stage (mild/moderate) AD. There was no difference in GAL fiber staining within the anterior NB across the three clinical groups examined. Furthermore, GAL fiber innervation was not correlated with the number of NB neurons expressing the nerve growth factor receptors p75^{NTR} or TrkA or with cortical choline acetyltransferase activity in the same cases. Single-cell gene expression analysis demonstrated that cholinergic NB neurons express mRNA for the GAL receptors GALR1, GALR2 and GALR3,

yet the levels of these mRNAs were unchanged across the three diagnostic groups. These observations indicate that GAL hypertrophy within the anterior NB subfield is a late-stage AD response, which may play a role in regulating the cholinergic tone of remaining basocortical projection neurons.

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Introduction

The neuropeptide galanin (GAL) regulates the activity of cholinergic basal forebrain (CBF) neurons [1–6]. These cells provide the major cholinergic innervation to the neocortex and hippocampus [7] and play a key role in memory and attention [8–10]. CBF nucleus basalis (NB) neurons undergo selective degeneration during the later stages of Alzheimer's disease (AD), which correlates with clinical severity and disease duration [11–17]. Scattered within the CBF are various peptidergic interneuronal and fiber systems, many of which are reduced in AD [18]. In contrast, several groups have made the striking observation that GAL-containing fibers are enlarged and hyperinnervate surviving anterior NB neurons in late-stage AD [19–21]. Furthermore, in vitro autoradiography demon-

strates that [125 I]GAL binding sites increase threefold within the region of the cholinergic NB in late-stage AD compared to age-matched controls [22]. This concomitant upregulation of GAL receptor (GALR) expression in response to increased GAL within the CBF suggests that GAL may regulate the tone of the basocortical cholinergic projection neurons during the late stages of AD. While the number of NB neurons is stable in early stages of AD [23], recent studies show that these cells undergo significant phenotypic alterations including a loss of the nerve growth factor (NGF) receptors TrkA and p75^{NTR} [24–26]. Whether the prodromal or early stages of AD are also associated with GAL overexpression within the CBF remains to be determined. In the present study, we evaluated GAL overexpression in tissue containing the anterior portion of the cholinergic NB harvested postmortem from subjects clinically diagnosed as having no cognitive impairment (NCI), mild cognitive impairment (MCI), a putative preclinical stage of AD [27] or mild/moderate AD. The degree of GAL innervation within the NB of each case was correlated with clinical and pathological variables, unbiased stereological estimates of total NB neurons immunopositive for the low- (p75^{NTR}) or high-affinity (TrkA) NGF receptors and neocortical levels of choline acetyltransferase (ChAT, the synthetic enzyme for acetylcholine) activity. In addition, we used single-cell gene expression technology to determine whether cholinergic NB neurons express mRNA for the three known GALRs – GALR1, GALR2 or GALR3 – and to test whether the levels of these transcripts are altered in the MCI or AD group.

Methods

Subjects

Tissue samples were evaluated from 42 individuals who were participants in the Religious Orders Study (ROS), a longitudinal clinical pathological study of aging and AD in older Catholic nuns, priests and brothers [24, 27–31]. The clinical and pathological demographics of these cases are presented in table 1. Each participant agreed to an annual detailed clinical evaluation and to brain donation at the time of death. For all subjects, cognitive testing scores were available within the last year of life. The Human Investigations Committee of Rush University Medical Center approved the study.

Clinical Evaluation

Details of the ROS clinical evaluation have been published elsewhere [24, 27–31]. Briefly, a team of investigators performed a complete uniform annual clinical evaluation that included assessments for stroke [32] and parkinsonian signs [28, 33]. Trained neuropsychology technicians administered a battery of tests measuring performance in five cognitive domains: orientation, attention, memory, language and perception [34]. An impaired score for each

domain required impairment on multiple tests within that domain. A board-certified clinical neuropsychologist used these results to summarize impairment in each of the five cognitive domains as not present, possible or probable. Following review of all clinical and neuropsychological test data and examination of the participant, a board-certified neurologist with expertise in the evaluation of the elderly rendered a clinical judgment on the presence of dementia. The diagnosis of dementia and AD followed the recommendations of the joint working group of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA) [35]. Our MCI population was defined as those persons rated as impaired on neuropsychological testing by the neuropsychologist but who were not found to have dementia by the examining neurologist [27, 28]. These criteria are similar to, or compatible with, those used by others in the field to describe persons who are not cognitively normal but do not meet the established criteria for dementia [36–38]. A postmortem examination was conducted at the time of death to identify medical conditions that occurred during the interval between the last clinical evaluation and death. Finally, a consensus conference of neurologists and neuropsychologists reviewed all available data and made a summary clinical diagnosis.

Pathological Evaluation and Tissue Preparation

Brains were removed at autopsy and processed as previously reported [24, 27, 29–31]. The postmortem interval did not differ across groups ($p = 0.15$, table 1). A neuropathologist conducted a gross examination of brain neuropathology and cases were excluded if they exhibited significant non-AD types of pathological conditions (e.g. brain tumors, encephalitis, large strokes, multiple lacunar infarctions). Brains were cut into 1-cm-thick slabs on a plexiglass brain cutting apparatus. Slabs from one hemisphere were immersion-fixed for 24–48 h in 4% paraformaldehyde and cryoprotected [24]. Slabs from the opposite hemisphere were snap-frozen in liquid nitrogen. A complete neuropathological analysis was performed on 8- μ m-thick paraffin-embedded sections with special attention to lesions that might contribute to dementia, including brainstem and cortical Lewy bodies as well as strokes. A pathological diagnosis was made for each case by a neuropathologist blinded to the clinical diagnosis. Designations of 'normal' (with respect to AD or other dementing processes), 'possible' or 'probable AD', or 'definite AD' were based on Consortium to Establish a Registry for Alzheimer's Disease criteria, semiquantitative estimation of neuritic plaque density, an age-adjusted plaque score and presence or absence of dementia [39]. All cases received Braak scores based upon the extent of neurofibrillary tangle pathology [40] and each case was assigned a diagnosis based on NIA-Reagan diagnostic criteria (table 1) [41].

Immunohistochemistry

Paraformaldehyde-fixed tissue was cut on a freezing sliding microtome into 40- μ m-thick sections through the anterior NB subfield at the level of the crossing of the anterior commissure [7]. The anterior NB subfield shows the greatest degree of GAL hyperinnervation within the CBF in late-stage AD [19–21, 42]. To visualize the putative cellular appositions between GAL-immunoreactive profiles and cholinergic NB neurons, sections were processed according to a previously described double-label procedure that results in an easily identifiable two-colored immunohistochemical profile [21, 43, 44]. Tissue sections were washed several times in

Table 1. Case demographics

	Clinical diagnosis			Total (n = 42)	p value	Pairwise comparisons (p < 0.01)
	NCI (n = 19)	MCI (n = 14)	AD (n = 9)			
Age, years						
Mean $\pm$ SD	81.2 $\pm$ 7.8	83.4 $\pm$ 4.8	90.1 $\pm$ 7.9	83.8 $\pm$ 7.6	0.03 ¹	
Range	66–92	75–92	82–103	66–103		
Males, %	68	43	56	57	0.35 ²	
Education, years						
Mean $\pm$ SD	18.4 $\pm$ 4.4	17.8 $\pm$ 4.9	16.3 $\pm$ 4.6	17.7 $\pm$ 4.6	0.60 ¹	
Range	8–25	8–30	6–21	6–30		
ApoE ϵ 4 allele, %	26	43	44	36	0.54 ²	
MMSE						
Mean $\pm$ SD	27.8 $\pm$ 1.6	25.9 $\pm$ 2.6	17.6 $\pm$ 6.1	25.0 $\pm$ 5.2	<0.0001 ¹	(NCI, MCI) > AD
Range	25–30	20–29	8–25	8–30		
Global cognitive score						
Mean $\pm$ SD	0.46 $\pm$ 0.34	0.06 $\pm$ 0.24	–0.80 $\pm$ 0.53	0.08 $\pm$ 0.6	<0.0001 ¹	NCI > MCI > AD
Range	–0.08 to 1.14	–0.4 to 0.3	–1.6 to –0.14	–1.6 to 1.1		
PMI, h						
Mean $\pm$ SD	10.8 $\pm$ 9.2	8.6 $\pm$ 5.7	5.4 $\pm$ 3.3	8.9 $\pm$ 7.4	0.15 ¹	
Range	2.2–33.5	3.5–23	2.2–12	2.2–33.5		
Braak scores						
0	1	0	0	1	0.026 ¹	
I–II	6	1	1	8		
III–IV	11	9	5	25		
V–VI	1	4	3	8		
Reagan diagnostic criteria						
No	1	0	0	1	0.031 ¹	
Low	11	4	2	17		
Intermediate	6	8	4	18		
High	1	2	3	6		

PMI = Postmortem interval.

¹ Kruskal-Wallis test.² Fisher's exact test.

Tris-buffered saline (TBS, pH 7.4) and incubated for 20 min in TBS/0.1 M sodium periodate to eliminate endogenous peroxidase activity. Sections were blocked for 30 min in TBS/0.25% Triton X-100 with 10% normal goat serum and then incubated overnight at ambient room temperature with rabbit polyclonal GAL antiserum (1:15,000; Peninsula Laboratories, Belmont, Calif., USA) in TBS/Triton X-100 and 1% normal goat serum. Following primary antibody incubation, sections were rinsed and incubated for 1 h at room temperature with biotinylated goat antirabbit IgG (1:200; Vector Laboratories, Burlingame, Calif., USA) followed by a 1-hour incubation with avidin-biotin complex reagent (1:500; Vector Laboratories). The sections were then reacted with 0.05% 3,3'-diaminobenzidine, 0.005% H₂O₂ and 2.5% nickel II sulfate in acetate-imidazole buffer to yield a dark blue-black reaction product labeling GAL-positive profiles. Cholinergic neurons were subsequently

labeled using an antibody to the low-affinity NGF receptor p75^{NTR}, which is an excellent marker for CBF neurons [45]. For this procedure, the sections were blocked in TBS/Triton X-100/3% normal horse serum and incubated overnight at room temperature with a mouse monoclonal p75^{NTR} antibody (1:40,000; Neomarkers, Fremont, Calif., USA). The sections were then rinsed, sequentially incubated for 1 h at room temperature with biotinylated horse antimouse IgG (1:200, Vector Laboratories) and avidin-biotin complex (1:500), and then reacted with a nonmetallic chromogen solution containing 0.05% diaminobenzidine and 0.005% hydrogen peroxide to yield a brown reaction product labeling CBF neurons and fibers. Finally, tissue was mounted onto gelatin-coated slides, dehydrated through a series of graded alcohols (70, 90, 100%), cleared in xylenes and cover-slipped with Permunt (Fisher Scientific, Fairlawn, N.J., USA).

Fig. 1. High-magnification photomicrographs of GAL-immunoreactive fibers within the NB in a late-stage AD case. **A** Example of a GAL-immunoreactive fiber rated semiquantitatively as a score of 1. **B** Example of GAL fiber hypertrophy rated semiquantitatively as a score of 5. Note the increase in GAL fiber density and thickness in **B** compared to **A**. Modified from Mufson et al, 1993 [21].

Immunohistochemistry Controls

Tissue sections were processed as described above except that the primary antibody was either omitted or was substituted for an irrelevant IgG matched for protein concentration. In addition, we preadsorbed the GAL antiserum 24 h prior to tissue incubation by adding 1 or 10 mM of the synthetic GAL peptide (Peninsula Laboratories) [44]. These sections showed no positive tissue immunoreactivity. It should be noted that these controls are not absolute and the potential for antibody cross-reactivity with structurally related proteins could not be excluded. Thus, GAL immunostaining in this report refers to GAL-‘like’ immunoreactivity.

Quantitation of GAL Immunostaining

As reported previously, it is extremely difficult to quantify alterations in GAL-containing axonal terminals and fibers in absolute numbers in the human brain [19–21]. Therefore, changes in GAL-immunoreactive profiles within the anterior subfield of the NB have been reported in morphological terms, evaluated using semiquantitative methods and presented using color photomicrography [20,

Fig. 2. Box plots comparing unbiased semiquantitative GAL innervation scores for each clinical group. There was no difference in GAL innervation score among the three groups ($p = 0.83$ via Kruskal-Wallis test).

21, 44]. In the present study, we performed a semiquantitative analysis of GAL fiber innervation within the anterior NB subfield. A computer-operated stage driver mounted to an inverted microscope was driven by Microbrightfield (Williston, Vt., USA) stereological software to select random rectangular ‘counting’ boxes of predefined size within the area of interest. This assured unbiased selection of labeled fibers and eliminated sampling overlap. To determine the degree of innervation regardless of disease progression, two experimenters blinded to clinical category assigned a semiquantitative score indicating the degree of innervation (e.g. 0 = none, 1 = very little, whereas 5 = very dense innervation) to each randomly selected group of GAL-positive fibers [44]. Scores were based on an evaluation of both GAL fiber density and thickness within the area of interest (fig. 1, 2). At least 3 sections throughout the anterior NB subfield from each case were used for the semiquantitative assessment. Independently assigned GAL scores between the two experimenters exhibited good interrater reliability (intraclass correlation coefficient = 0.65).

Stereology

The neuron counts reported in the current project were derived from earlier investigations of the total number of NB neurons containing the low- ($p75^{\text{NTR}}$ [24]) or high-affinity (TrkA [25]) NGF receptor using unbiased optical disector stereological counting methods performed on the same cases examined in the present study.

Neocortical ChAT Activity

The levels of ChAT activity reported in the present study for the anterior cingulate, superior frontal, superior temporal, inferior parietal and visual cortex were derived from previous studies [30, 46] using a modification of the Fonnum method [47] on the same cases analyzed in the current report.

Single-Cell Gene Expression Analysis

To determine whether cholinergic NB neurons express GALR mRNA and whether GALR transcript levels are altered within cho-

linergic anterior NB neurons, we performed single-cell RNA amplification of individual NB neurons aspirated from ROS subjects classified into NCI (n = 12), MCI (n = 10) or early-stage AD (n = 12) groups. Except for 3 additional AD cases, the cases used for this analysis were also used for the semiquantitative analysis of GAL innervation within the NB. Tissue sections containing the anterior NB subfield ventral to the anterior commissure were processed for p75^{NTR} immunocytochemistry and all procedures were carried out in RNase-free conditions [48]. Single p75^{NTR}-immunoreactive NB neurons (n = 174; approx. 5 cells/case) were microaspirated using a micromanipulator and microcontrolled vacuum source (Eppendorf, Westbury, N.Y., USA) attached to a Nikon (Melville, N.Y., USA) inverted microscope. The amplification of RNA from individual NB neurons was performed using a new terminal continuation (TC) RNA amplification methodology [49, 50]. Each neuron was extracted individually in 250 μ l of Trizol reagent (Invitrogen, Carlsbad, Calif., USA). RNAs were reverse transcribed in the presence of the poly-d(T) primer (10 ng/ μ l) and TC primer (10 ng/ μ l) in 1 $\times$ first-strand buffer (Invitrogen), 1 mM dNTPs, 5 mM DTT, 20 units of RNase inhibitor and 5 units reverse transcriptase (Superscript III; Invitrogen). The synthesized single-stranded cDNAs were converted into double-stranded cDNAs by adding into the reverse transcription reaction the following: 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂ and 0.5 units RNase H (Invitrogen) in a total volume of 99 μ l. Samples were placed in a thermal cycler and second-strand synthesis proceeded as follows: RNase H digestion step at 37°C, for 10 min; denaturation step at 95°C, for 3 min, annealing step at 50°C, for 3 min; elongation step at 75°C, for 30 min. Five units (1 μ l) of Taq polymerase (PE Biosystems Foster City, Calif., USA) was added to the reaction at the initiation of the denaturation step (i.e. hot start) [49]. The reaction was terminated with 5 M ammonium acetate. The samples were extracted in phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitated with 5 μ g of linear acrylamide as a carrier. The solution was centrifuged at 14,000 rpm and the pellet washed once with 95% ethanol and air-dried. The cDNAs were resuspended in 20 μ l of RNase-free H₂O and drop dialyzed on 0.025- μ m filter membranes (Millipore, Billerica, Mass., USA) against 50 ml of 18.2 M Ω RNase-free H₂O for 2 h. The sample was collected off the dialysis membrane and hybridization probes were synthesized by in vitro transcription using ³³P incorporation in 40 mM Tris (pH 7.5), 7 mM MgCl₂, 10 mM NaCl, 2 mM spermidine, 5 mM DTT, 0.5 mM ATP, GTP and CTP, 10 μ M cold UTP, 20 units of RNase inhibitor, T7 RNA polymerase (1,000 units, Epicentre, Madison, Wisc., USA) and 40 μ Ci of ³³P-UTP (GE-Amersham, Piscataway, N.J., USA). The reaction was performed at 37°C for 4 h. Radiolabeled TC RNA probes were hybridized to custom-designed cDNA arrays without further purification.

Custom-Designed cDNA Array Platforms

Array platforms consisted of 1 μ g of linearized cDNA purified from plasmid preparations adhered to high-density nitrocellulose (Hybond XL, GE-Amersham) [48, 50, 51]. Each cDNA and/or expressed sequence-tagged cDNA was verified by sequence analysis and restriction digestion. cDNA clones and expressed sequence-tagged cDNAs from mice, rats and humans were employed. Approximately 220 cDNAs/expressed sequence-tagged cDNAs were utilized on the current array platform. The probe used for GALR1 detection corresponds to sequences 648–1050 of the human GALR1 gene (Genebank accession No. L34339). The GALR2 probe cor-

responds to sequences from 994 to 1326 of the human GALR2 gene (AF040630), and the GALR3 probe corresponds to sequences 518–845 of the human GALR3 gene (AF129514). Arrays were prehybridized (2 h) and hybridized (12 h) in a solution consisting of 6 $\times$ SSPE, 5 $\times$ Denhardt's solution, 50% formamide, 0.1% sodium dodecyl sulfate (SDS) and denatured salmon sperm DNA (200 μ g/ml) at 42°C in a rotisserie oven. Following hybridization, arrays were washed sequentially in 2 $\times$ standard saline citrate (SSC)/0.1% SDS, 1 $\times$ SSC/0.1% SDS and 0.5 $\times$ SSC/0.1% SDS for 20 min each at 42°C. Arrays were exposed to a phosphor screen for 24 h and developed on a phosphor imager (GE-Amersham). Hybridization signal intensity was quantified by subtracting background using empty vector (pBS Bluescript; Stratagene, La Jolla, Calif., USA). Expression of TC-amplified RNA bound to each linearized cDNA was expressed as a ratio of the total hybridization signal intensity of the array (a global normalization approach). Global normalization effectively minimizes variation due to differences in the specific activity of the synthesized TC probe as well as the absolute quantity of probe present [50].

Statistical Analysis

Demographic and clinical pathological characteristics of the diagnostic groups were compared using the Kruskal-Wallis test for continuous and ordinal variables and Fisher's exact test for categorical variables. The Kruskal-Wallis test was used to compare GAL scores (degree of NB innervation) across the clinical diagnostic categories. As GAL innervation was scored by two independent observers, interrater reliability was assessed by the intraclass correlation coefficient and the average of the two scores was used in the analyses. Relative changes in GALR gene expression in single NB neurons were analyzed by one-way analysis of variance with a post hoc Neumann-Keuls test. Spearman rank correlation and Wilcoxon rank sum tests were used to test for associations between demographic and neuropathological variables and GAL innervation scores. The level of statistical significance was set at 0.01 (two-sided) to account for the number of comparisons made.

Results

Case Demographics

The NCI, MCI and AD individuals did not differ significantly in age, sex, years of education, apolipoprotein E status or postmortem interval (table 1). The last Mini Mental State Examination (MMSE) scores ranged from 8 to 30 and no significant differences were found in MMSE scores between the NCI (27.8 $\pm$ 1.6) and MCI (25.9 $\pm$ 2.6) groups. The AD group had a significantly lower mean MMSE (17.6 $\pm$ 6.1) than the NCI or MCI subjects (table 1).

ROS subjects in the different diagnostic groups displayed considerable heterogeneity with respect to pathological diagnosis based upon Braak or NIA-Reagan criteria (table 1). Neuropathological examination revealed that 95% of NCI cases were classified as Braak stages I–IV, whereas 93% of MCI cases and 89% of the AD cases

Fig. 3. Photomicrographs of sections dually immunostained for GAL fibers (dark blue-black) and cholinergic NB neurons labeled using p75^{NTR} (brown) from an NCI (**A**), MCI (**B**) and AD (**C**) case. Note that GAL-immunoreactive profiles were typically similar in NCI and MCI cases with occasional thickened fibers in MCI (**B**). A modest increase in GAL fiber staining was found in some AD cases (**C**), but most AD cases were similar to NCI and MCI. The arrow in **C** shows a thickened GAL-immunoreactive fiber in the AD case.

were classified as Braak stages III–VI. Using NIA-Reagan criteria, 89% of NCI and 86% of MCI cases were classified as low or intermediate likelihood of AD, whereas 78% of the AD cases were classified as intermediate to high likelihood of AD (table 1).

General Characteristics of GAL Immunoreactivity within the NB

Dual immunohistochemistry revealed GAL-positive fibers (dark blue-black) in close association with p75^{NTR}-immunoreactive neurons (brown) within the anterior NB across all clinical groups (fig. 3). In NCI, a fine network of GAL-immunoreactive fibers was seen coursing through the NB and often appeared in direct apposition to cholinergic (p75^{NTR}) soma or dendrites (fig. 3A). In MCI, a similar dual staining pattern was observed with an occasional thickened GAL-positive fiber (fig. 3B). Although 1 AD case with an MMSE of 8 was rated with a GAL score of 4 (fig. 3C), the more mild to moderate cases displayed GAL-immunoreactive innervation similar to the NCI or MCI groups.

Quantitative Analysis of GAL Fiber Innervation within the NB Compared to Clinical and Neuropathological Variables

The degree of GAL innervation within the NB of the three ROS clinical diagnostic groups was evaluated using a semiquantitative scoring method. There were no sig-

nificant differences in the GAL innervation scores among the clinical groups [fig. 2; NCI = 3.0 ± 0.9 (mean $\pm$ SD); MCI = 2.9 ± 1.2 ; AD = 2.7 ± 1.3 ; $p = 0.83$]. Furthermore, GAL innervation scores were similar when cases were categorized by Braak stage or NIA-Reagan diagnosis (data not shown).

GAL Innervation Compared to NB Neuronal Number and Cortical ChAT Activity

GAL fiber innervation scores did not correlate with the number of low- (p75^{NTR}; fig. 4) or high-affinity (TrkA; not shown) NGF-receptor-containing NB neurons [24, 25]. In addition, the GAL innervation score did not correlate with ChAT activity measured in any of the five neocortical regions [anterior cingulate (fig. 5), superior frontal, superior temporal, inferior parietal and visual] examined [30, 46].

GAL Innervation Compared to Cognitive Test Scores

There was no correlation between the GAL innervation score within the NB and the MMSE (Spearman $r = 0.08$, $p = 0.61$) or Global Cognitive Score ($r = 0.28$, $p = 0.084$) for the cases examined.

GALR mRNA Expression in Cholinergic NB Neurons

Single-cell gene expression analysis revealed that cholinergic NB neurons express GALR1, GALR2 and GALR3 mRNA (fig. 6). However, there were no changes

Fig. 4. Scattergram showing a lack of correlation between GAL-immunoreactive fiber score and the total number of cholinergic NB neurons immunopositive for p75^{NTR} in each case examined ($r = 0.05$; $p = 0.8$ via Spearman rank correlation).

Fig. 5. Representative scattergram showing a lack of correlation between GAL-immunoreactive fiber score and ChAT activity in the anterior cingulate cortex in each case examined ($r = -0.39$, $p = 0.027$ via Spearman rank correlation).

in the relative expression levels of any of the GALRs in NB neurons across the clinical groups. GAL mRNA expression was not detected in NB neurons from any of the diagnostic groups examined (data not shown) supporting previous observations that these cholinergic neurons do not co-express GAL in humans [52].

Discussion

The present study revealed that the well-characterized GAL plasticity response observed within the anterior NB subfield in late-stage AD [19–22] does not occur in the prodromal or early stages of AD. Previous studies demonstrated that anterior NB tissue from end-stage AD patients was densely innervated by hypertrophic GAL-immunoreactive fibers [20, 21], which were often in direct apposition to magnocellular CBF neurons [19]. However, we found that the degree of GAL innervation within the NB was similar in subjects clinically diagnosed as having NCI, MCI or mild/moderate AD. Previous studies also revealed an upregulation in GAL binding sites within the NB of end-stage AD patients [22], although the identity of the receptors mediating GAL binding was not established. Here we report for the first time that human cholinergic NB neurons express GALR1, GALR2 and GALR3 mRNA and that the levels of these receptor transcripts remained stable across the three clinical groups examined. Taken together, our observations suggest that

Fig. 6. Expression of GALR1, GALR2 and GALR3 mRNA derived from single p75^{NTR}-immunopositive anterior cholinergic NB neurons processed for single-cell RNA amplification and custom cDNA array analysis. Quantitation of normalized hybridization signal intensities on the arrays revealed expression of GALR1, GALR2 and GALR3 mRNA. The histogram shows that levels of GALR subtype mRNAs within single NB neurons were unchanged across clinical diagnoses.

the mechanism(s) initiating GAL system plasticity within the CBF are not activated until the later stages of AD.

The neuropathological events that trigger GAL hyperinnervation within the NB in late AD but not MCI or early AD are a matter of speculation. It was originally suggested that GAL fiber hypertrophy was activated by CBF neuron degeneration in late-stage AD [20]. Support-

ing this suggestion are findings that GAL overexpression occurs in several experimental models of CBF neuronal injury, including cholinergic immunotoxin treatment [53] and neocortical infarction [54]. The present study demonstrates that GAL overexpression does not occur early in the disease process when cholinergic NB neuron numbers are preserved [23]. Interestingly, GAL innervation did not correlate with the number of NB neurons expressing the NGF receptors p75^{NTR} or TrkA. The number of NB neurons expressing these receptors is reduced in MCI and early-stage AD cases [24, 25] in the absence of frank cholinergic NB cell loss [23]. These observations suggest that GAL plasticity within the NB is not initiated by cholinergic NB phenotypic changes with putative long-term pathogenic consequences (e.g. loss of cholinergic neurotrophic support) per se, but perhaps by the extensive NB cellular degeneration seen in the later stage of AD.

Alternatively, GAL hyperinnervation may occur in response to the formation of neurodegenerative lesions such as neurofibrillary tangles and amyloid plaque formation in the AD brain. GAL scores did not correlate with Braak stage, which ranged from 0 (no neurofibrillary tangles) to stage V–VI in the cases examined. Since CBF tangle-bearing neurons are found as early as Braak stage I–II [40, 55], it remains to be determined what role, if any, neurofibrillary tangle formation plays in the onset of GAL plasticity within this region. In this regard, we have found that GAL fibers innervate NB neurons during different stages of tangle evolution [56]. On the other hand, GAL-immunoreactive neurites have also been found in association with amyloid plaques in the AD brain [57]. Transgenic mice that overexpress amyloid-plaque-like deposits in the hippocampus and entorhinal cortex exhibit an increase in GAL fiber density in these areas [56, 58]. Therefore, amyloid deposition, which becomes prominent within the cholinergic NB and its cortical projection sites during late-stage AD [59], may also play a role in triggering the GAL plasticity response.

A caveat to these hypotheses is that GAL hypertrophy does not occur within the NB of patients with Down's syndrome [44], a disease that displays brain amyloidosis due to triplication of the amyloid- β protein precursor gene [60]. The brains of subjects with Down's syndrome are characterized by an age-related loss of CBF neurons and significant NB and cortical amyloid deposition. However, the majority of amyloid deposits in these patients are diffuse with less frequent senile plaques like those that predominate in the end-stage AD brain [61]. Thus, despite the pathological similarities between Down's syndrome and AD cases, other genetic or sporadic etiologies

involved in amyloid deposition or other pathogenic pathways may play a part in the initiation of the GAL plasticity response in AD.

The physiological consequences of GAL overexpression in late-stage AD remain unknown [42]. In vivo and in vitro studies indicate that GAL inhibits acetylcholine release in hippocampal preparations and disrupts cognitive performance [2–4]. In contrast, GAL promotes neurogenesis following injury to cholinergic sensory neuron preparations [62, 63] and is protective in several models of hippocampal neurotoxicity [64]. Interestingly, GAL fiber plasticity is most prominent in the anterior subregion of the NB [19–22], which displays the least degree of cell loss of all the cholinergic subfields of the NB [14, 15], despite a significant reduction in ChAT activity in its cortical projection sites in severe AD [11, 13, 17]. These findings suggest that GAL may alter acetylcholine production [4] within NB neurons in an attempt to regulate the survival of the remaining cholinergic neurons in late-stage AD.

The function of GAL overexpression in late-stage AD will likely depend on the repertoire of GALR-mediated signaling upon the remaining cholinergic NB neurons. Although previous GALR studies have shown an upregulation of GAL binding sites within the anterior NB subfield [22] and its cortical projection sites [65] in late-stage AD, it was unclear which GALR subtypes were expressed. The present single-cell gene expression analysis shows that GALR1, GALR2 and GALR3 mRNAs are found in NB neurons. However, the levels of these transcripts remained unchanged across the three clinical groups. It remains to be determined whether any of these GALRs are upregulated in NB neurons in late-stage AD. If so, a change in GALR phenotype may determine the physiological consequences of GAL hyperinnervation upon NB neurons. In general, GALR1 and GALR3 are coupled to adenylyl cyclase inhibition while GALR2 is coupled to phospholipase C activation [66–68]. Thus, putative increases in GAL signaling may be inhibitory or excitatory with regard to cholinergic neuronal activity in late-stage AD depending on the relative expression levels of the GALR subtypes.

In summary, we report that GAL plasticity within the anterior NB is not an early event during the progression of AD. GAL hyperinnervation of the NB neurons appears to be a late-stage event when cholinergic cells are undergoing extensive degeneration and the brain has accumulated significant amounts of plaque and tangle pathology. The utility of GALR-specific ligands for the treatment of AD will likely depend on the relative levels of GALR subtype expression in the remaining cholinergic NB neurons in the later stages of the disease.

Acknowledgements

We are indebted to the altruism and support of the hundreds of nuns, priests and brothers participating in the ROS. A list of participating groups can be found at the website <http://www.rush.edu/rumc/page-R12394.html>. We are also indebted to the dedication of: Dr. David A. Bennett, MD, Director of the ROS and Rush AD

Center; Drs. Elizabeth J. Cochran, MD, and Julie A. Schneider, MD, past and present Director of the ROS and Rush AD Center Neuropathology Core, respectively; Traci Colvin, MPH, ROS Coordinator, and Greg Klein, Wenqing Fan and Woojeong Bang for data retrieval. This work was supported by NIH AG14449, AG10161, AG10688, AG09466, AG26032, NS43939, the Alzheimer's Association and the Illinois Department of Public Health.

References

- McDonald MP, Willard LB, Wenk GL, Crawley JN: Coadministration of galanin antagonist M40 with a muscarinic M₁ agonist improves delayed nonmatching to position choice accuracy in rats with cholinergic lesions. *J Neurosci* 1998;18:5078–5085.
- Crawley JN: Minireview – Galanin-acetylcholine interactions: relevance to memory and Alzheimer's disease. *Life Sci* 1996;58:2185–2199.
- Dutar P, Lamour Y, Nicoll RA: Galanin blocks the slow cholinergic EPSP in CA1 pyramidal neurons from ventral hippocampus. *Eur J Pharmacol* 1989;164:355–360.
- Fisone G, Wu CF, Consolo S, Nordstrom O, Brynne N, Bartfai T, Melander T, Hokfelt T: Galanin inhibits acetylcholine release in the ventral hippocampus of the rat: histochemical, autoradiographic, in vivo, and in vitro studies. *Proc Natl Acad Sci USA* 1987;84:7339–7343.
- Jhamandas JH, Harris KH, MacTavish D, Jassar BS: Novel excitatory actions of galanin on rat cholinergic basal forebrain neurons: implications for its role in Alzheimer's disease. *J Neurophysiol* 2002;87:696–704.
- Steiner RA, Hohmann JG, Holmes A, Wrenn CC, Cadd G, Jureus A, Clifton DK, Luo M, Gutshall M, Ma SY, Mufson EJ, Crawley JN: Galanin transgenic mice display cognitive and neurochemical deficits characteristic of Alzheimer's disease. *Proc Natl Acad Sci USA* 2001;98:4184–4189.
- Mesulam MM, Mufson EJ, Levey AI, Wainer BH: Cholinergic innervation of cortex by the basal forebrain: cytochemistry and cortical connections of the septal area, diagonal band nuclei, nucleus basalis (substantia innominata), and hypothalamus in the rhesus monkey. *J Comp Neurol* 1983;214:170–197.
- Bartus RT: On neurodegenerative diseases, models, and treatment strategies: lessons learned and lessons forgotten a generation following the cholinergic hypothesis. *Exp Neurol* 2000;163:495–529.
- Baxter MG, Chiba AA: Cognitive functions of the basal forebrain. *Curr Opin Neurobiol* 1999;9:178–183.
- Sarter M, Bruno JP, Turchi J: Basal forebrain afferent projections modulating cortical acetylcholine, attention, and implications for neuropsychiatric disorders. *Ann NY Acad Sci* 1999;877:368–382.
- Bartus RT, Dean RL III, Beer B, Lippa AS: The cholinergic hypothesis of geriatric memory dysfunction. *Science* 1982;217:408–414.
- Coyle JT, Price DL, DeLong MR: Alzheimer's disease: a disorder of cortical cholinergic innervation. *Science* 1983;219:1184–1190.
- DeKosky ST, Harbaugh RE, Schmitt FA, Bakay RA, Chui HC, Knopman DS, Reeder TM, Shetter AG, Senter HJ, Markesbery WR: Cortical biopsy in Alzheimer's disease: diagnostic accuracy and neurochemical, neuropathological, and cognitive correlations. Intraventricular Bethanecol Study Group. *Ann Neurol* 1992;32:625–632.
- Mufson EJ, Bothwell M, Kordower JH: Loss of nerve growth factor receptor-containing neurons in Alzheimer's disease: a quantitative analysis across subregions of the basal forebrain. *Exp Neurol* 1989;105:221–232.
- Vogels OJ, Broere CA, ter Laak HJ, ten Donkelaar HJ, Nieuwenhuys R, Schulte BP: Cell loss and shrinkage in the nucleus basalis Meynert complex in Alzheimer's disease. *Neurobiol Aging* 1990;11:3–13.
- Whitehouse PJ, Price DL, Clark AW, Coyle JT, DeLong MR: Alzheimer disease: evidence for selective loss of cholinergic neurons in the nucleus basalis. *Ann Neurol* 1981;10:122–126.
- Wilcock GK, Esiri MM, Bowen DM, Smith CC: Alzheimer's disease: correlation of cortical choline acetyltransferase activity with the severity of dementia and histological abnormalities. *J Neurol Sci* 1982;57:407–417.
- Bierer LM, Haroutunian V, Gabriel S, Knott PJ, Carlin LS, Purohit DP, Perl DP, Schmeidler J, Kanof P, Davis KL: Neurochemical correlates of dementia severity in Alzheimer's disease: relative importance of the cholinergic deficits. *J Neurochem* 1995;64:749–760.
- Bowser R, Kordower JH, Mufson EJ: A confocal microscopic analysis of galaninergic hyperinnervation of cholinergic basal forebrain neurons in Alzheimer's disease. *Brain Pathol* 1997;7:723–730.
- Chan-Palay V: Galanin hyperinnervates surviving neurons of the human basal nucleus of Meynert in dementias of Alzheimer's and Parkinson's disease: a hypothesis for the role of galanin in accentuating cholinergic dysfunction in dementia. *J Comp Neurol* 1988;273:543–557.
- Mufson EJ, Cochran E, Benzing W, Kordower JH: Galaninergic innervation of the cholinergic vertical limb of the diagonal band (Ch2) and bed nucleus of the stria terminalis in aging, Alzheimer's disease and Down's syndrome. *Dementia* 1993;4:237–250.
- Mufson EJ, Decher DC, Basile M, Izenwasse S, Mash DC: Galanin receptor plasticity within the nucleus basalis in early and late Alzheimer's disease: an in vitro autoradiographic analysis. *Neuropharmacology* 2000;39:1404–1412.
- Gilmor ML, Erickson JD, Varoqui H, Hersh LB, Bennett DA, Cochran EJ, Mufson EJ, Levey AI: Preservation of nucleus basalis neurons containing choline acetyltransferase and the vesicular acetylcholine transporter in the elderly with mild cognitive impairment and early Alzheimer's disease. *J Comp Neurol* 1999;411:693–704.
- Mufson EJ, Ma SY, Dills J, Cochran EJ, Leurgans S, Wu J, Bennett DA, Jaffar S, Gilmor ML, Levey AI, Kordower JH: Loss of basal forebrain P75(NTR) immunoreactivity in subjects with mild cognitive impairment and Alzheimer's disease. *J Comp Neurol* 2002;443:136–153.
- Mufson EJ, Ma SY, Cochran EJ, Bennett DA, Beckett LA, Jaffar S, Saragovi HU, Kordower JH: Loss of nucleus basalis neurons containing trkA immunoreactivity in individuals with mild cognitive impairment and early Alzheimer's disease. *J Comp Neurol* 2000;427:19–30.
- Counts SE, Mufson EJ: The role of nerve growth factor receptors in cholinergic basal forebrain degeneration in prodromal Alzheimer's disease. *J Neuropathol Exp Neurol* 2005;64:263–272.
- Bennett DA, Schneider JA, Bienias JL, Evans DA, Wilson RS: Mild cognitive impairment is related to Alzheimer disease pathology and cerebral infarctions. *Neurology* 2005;64:834–841.
- Bennett DA, Wilson RS, Schneider JA, Evans DA, Beckett LA, Aggarwal NT, Barnes LL, Fox JH, Bach J: Natural history of mild cognitive impairment in older persons. *Neurology* 2002;59:198–205.
- Counts SE, Nadeem M, Wu J, Ginsberg SD, Saragovi HU, Mufson EJ: Reduction of cortical TrkA but not p75(NTR) protein in early-stage Alzheimer's disease. *Ann Neurol* 2004;56:520–531.
- DeKosky ST, Ikonomic MD, Styren SD, Beckett L, Wisniewski S, Bennett DA, Cochran EJ, Kordower JH, Mufson EJ: Upregulation of choline acetyltransferase activity in hippocampus and frontal cortex of elderly subjects with mild cognitive impairment. *Ann Neurol* 2002;51:145–155.

- 31 Kordower JH, Chu Y, Stebbins GT, DeKosky ST, Cochran EJ, Bennett D, Mufson EJ: Loss and atrophy of layer II entorhinal cortex neurons in elderly people with mild cognitive impairment. *Ann Neurol* 2001;49:202–213.
- 32 Goldstein LB, Samsa GP: Reliability of the National Institutes of Health Stroke Scale. Extension to non-neurologists in the context of a clinical trial. *Stroke* 1997;28:307–310.
- 33 Bennett DA, Shannon KM, Beckett LA, Goetz CG, Wilson RS: Metric properties of nurses' ratings of parkinsonian signs with a modified Unified Parkinson's Disease Rating Scale. *Neurology* 1997;49:1580–1587.
- 34 Pittman J, Andrews H, Tatemichi T, Link B, Struening E, Stern Y, Mayeux R: Diagnosis of dementia in a heterogeneous population. A comparison of paradigm-based diagnosis and physician's diagnosis. *Arch Neurol* 1992;49:461–467.
- 35 McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM: Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* 1984;34:939–944.
- 36 Flicker C, Ferris SH, Reisberg B: Mild cognitive impairment in the elderly: predictors of dementia. *Neurology* 1991;41:1006–1009.
- 37 Petersen RC, Smith GE, Waring SC, Ivnik RJ, Tangalos EG, Kokmen E: Mild cognitive impairment: clinical characterization and outcome. *Arch Neurol* 1999;56:303–308.
- 38 Rubin EH, Morris JC, Grant EA, Vendegna T: Very mild senile dementia of the Alzheimer type. I. Clinical assessment. *Arch Neurol* 1989;46:379–382.
- 39 Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, Vogel FS, Hughes JP, van Belle G, Berg L: The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). II. Standardization of the neuropathologic assessment of Alzheimer's disease. *Neurology* 1991;41:479–486.
- 40 Braak H, Braak E: Neuropathological staging of Alzheimer-related changes. *Acta Neuropathol* 1991;82:239–259.
- 41 The National Institute on Aging: Consensus recommendations for the postmortem diagnosis of Alzheimer's disease. *Neurobiol Aging* 1997;18:S1–S2.
- 42 Counts SE, Perez SE, Ginsberg SD, De Lacalle S, Mufson EJ: Galanin in Alzheimer disease. *Mol Interv* 2003;3:137–156.
- 43 Kordower JH, Mufson EJ: Galanin-like immunoreactivity within the primate basal forebrain: differential staining patterns between humans and monkeys. *J Comp Neurol* 1990;294:281–292.
- 44 Sendera TJ, Ma SY, Jaffar S, Kozlowski PB, Kordower JH, Mawal Y, Saragovi HU, Mufson EJ: Reduction in TrkA-immunoreactive neurons is not associated with an overexpression of galaninergic fibers within the nucleus basalis in Down's syndrome. *J Neurochem* 2000;74:1185–1196.
- 45 Mufson EJ, Bothwell M, Hersch LB, Kordower JH: Nerve growth factor receptor immunoreactive profiles in the normal, aged human basal forebrain: colocalization with cholinergic neurons. *J Comp Neurol* 1989;285:196–217.
- 46 Ikonomic MD, Mufson EJ, Wu J, Bennett DA, DeKosky ST: Reduction of choline acetyltransferase activity in primary visual cortex in mild to moderate Alzheimer's disease. *Arch Neurol* 2005;62:425–430.
- 47 Fonnum F: A rapid radiochemical method for the determination of choline acetyltransferase. *J Neurochem* 1975;24:407–409.
- 48 Mufson EJ, Counts SE, Ginsberg SD: Gene expression profiles of cholinergic nucleus basalis neurons in Alzheimer's disease. *Neurochem Res* 2002;27:1035–1048.
- 49 Che S, Ginsberg SD: Amplification of RNA transcripts using terminal continuation. *Lab Invest* 2004;84:131–137.
- 50 Ginsberg SD, Elarova I, Ruben M, Tan F, Counts SE, Eberwine JH, Trojanowski JQ, Hemby SE, Mufson EJ, Che S: Single-cell gene expression analysis: implications for neurodegenerative and neuropsychiatric disorders. *Neurochem Res* 2004;29:1053–1064.
- 51 Ginsberg SD, Che S: RNA amplification in brain tissues. *Neurochem Res* 2002;27:981–992.
- 52 Walker LC, Rance NE, Price DL, Young WS III: Galanin mRNA in the nucleus basalis of Meynert complex of baboons and humans. *J Comp Neurol* 1991;303:113–120.
- 53 Harton I, Mufson EJ, de Lacalle S: Long-term plastic changes in galanin innervation in the rat basal forebrain. *Neuroscience* 2002;115:787–795.
- 54 Barbelivien A, Vaussy C, Marchalant Y, Maubert E, Bertrand N, Beley A, Roussel S, Mackenzie ET, Dauphin F: Degeneration of the basolateral pathway from the cortex induces a functional increase in galaninergic markers in the nucleus basalis magnocellularis of the rat. *J Cereb Blood Flow Metab* 2004;24:1255–1266.
- 55 Sassini I, Schultz C, Thal DR, Rub U, Arai K, Braak E, Braak H: Evolution of Alzheimer's disease-related cytoskeletal changes in the basal nucleus of Meynert. *Acta Neuropathol (Berl)* 2000;100:259–269.
- 56 Mufson EJ, Counts SE, Perez S, Binder L: Galaninergic plasticity in the cholinergic basal forebrain in Alzheimer's disease and transgenic mice. *Neuropeptides* 2005;39:233–237.
- 57 Kowall NW, Beal MF: Galanin-like immunoreactivity is present in human substantia innominata and in senile plaques in Alzheimer's disease. *Neurosci Lett* 1989;98:118–123.
- 58 Diez M, Koistinaho J, Kahn K, Games D, Hokfelt T: Neuropeptides in hippocampus and cortex in transgenic mice overexpressing V717F β -amyloid precursor protein – Initial observations. *Neuroscience* 2000;100:259–286.
- 59 Arendt T, Taubert G, Bigl V, Arendt A: Amyloid deposition in the nucleus basalis of Meynert complex: a topographic marker for degenerating cell clusters in Alzheimer's disease. *Acta Neuropathol (Berl)* 1988;75:226–232.
- 60 Rumble B, Retallack R, Hilbich C, Simms G, Multhaup G, Martins R, Hockey A, Montgomery P, Beyreuther K, Masters CL: Amyloid A4 protein and its precursor in Down's syndrome and Alzheimer's disease. *N Engl J Med* 1989;320:1446–1452.
- 61 Lemere CA, Blusztajn JK, Yamaguchi H, Wisniewski T, Saido TC, Selkoe DJ: Sequence of deposition of heterogeneous amyloid β -peptides and APO E in Down syndrome: implications for initial events in amyloid plaque formation. *Neurobiol Dis* 1996;3:16–32.
- 62 Holmes FE, Mahoney S, King VR, Bacon A, Kerr NC, Pachnis V, Curtis R, Priestley JV, Wynick D: Targeted disruption of the galanin gene reduces the number of sensory neurons and their regenerative capacity. *Proc Natl Acad Sci USA* 2000;97:11563–11568.
- 63 Mahoney SA, Hosking R, Farrant S, Holmes FE, Jacoby AS, Shine J, Iismaa TP, Scott MK, Schmidt R, Wynick D: The second galanin receptor GalR2 plays a key role in neurite outgrowth from adult sensory neurons. *J Neurosci* 2003;23:416–421.
- 64 Elliott-Hunt CR, Marsh B, Bacon A, Pope R, Vanderplank P, Wynick D: Galanin acts as a neuroprotective factor to the hippocampus. *Proc Natl Acad Sci USA* 2004;101:5105–5110.
- 65 McMillan PJ, Peskind E, Raskind MA, Leverenz JB: Increased galanin receptor occupancy in Alzheimer's disease. *Neurobiol Aging* 2004;25:1309–1314.
- 66 Smith KE, Forray C, Walker MW, Jones KA, Tamm JA, Bard J, Branchek TA, Linemeyer DL, Gerald C: Expression cloning of a rat hypothalamic galanin receptor coupled to phosphoinositide turnover. *J Biol Chem* 1997;272:24612–24616.
- 67 Smith KE, Walker MW, Artymyshyn R, Bard J, Borowsky B, Tamm JA, Yao WJ, Vaysse PJ, Branchek TA, Gerald C, Jones KA: Cloned human and rat galanin GALR3 receptors: pharmacology and activation of G-protein inwardly rectifying K^+ channels. *J Biol Chem* 1998;273:23321–23326.
- 68 Wang S, Hashemi T, Fried S, Clemmons AL, Hawes BE: Differential intracellular signaling of the GalR1 and GalR2 galanin receptor subtypes. *Biochemistry* 1998;37:6711–6717.