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Galanin-like immunoreactivity within Ch2 neurons in the vertical limb of the diagonal band of Broca in aging and Alzheimer's disease*

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Summary. The neuropeptide galanin is known to inhibit the evoked release of acetylcholine in ventral hippocampus of the rat. Co-localization of this peptide with choline acetyltransferase in neurons of the cholinergic septal nuclei has been demonstrated in the rat and non-human primate. The severe deficiency of the cholinergic hippocampal projection system arising mainly from the vertical limb nucleus of the diagonal band of Broca, also referred to as Ch2 region, is a constant finding in Alzheimer's disease, a disorder which is neuropathologically characterized by the appearance of senile plaques, neurofibrillary tangles and congophilic angiopathy in neo- and archicortical structures. In the present study for the first time galanin immunoreactivity in the human Ch2 region is morphologically investigated and related to the severity of hippocampal plaques and neurofibrillary tangles in Alzheimer's disease. An inverse relationship between decreasing galanin immunoreactivity in the Ch2 region and amounts of senile plaques and neurofibrillary tangles in the hippocampus is indicated. Considering the cholinergic deficiency in Alzheimer's disease as a secondary phenomenon to primary cortical and hippocampal lesions, and realizing the inhibitory effect of galanin upon acetylcholine release in hippocampus, this preliminary study suggests that a decreased galanin immunoreactivity in Ch2 in Alzheimer's disease reflects a possible negative feedback mechanism to a degenerating cholinergic projection system.

Key words: Galanin – Immunochemistry – Nucleus basalis of Meynert complex – Hippocampus – Alzheimer's disease

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Galanin (GA) is a 29-amino acid peptide, isolated from the porcine intestine [27]. GA-like immunoreactivity (GA-LI) was found to be widely distributed in the animal central nervous system of different species [12, 15, 22, 25, 29], including human [5, 11]. This neuropeptide may be of special interest because the presence of choline acetyltransferase-like immunoreactivity (ChAT-LI) and GA-LI have been shown in the same basal forebrain neurons in rat [13] and monkey [12, 29].

Co-localization of ChAT-LI and GA-LI in a large population of cholinergic neurons in the rat vertical limb nucleus of the diagonal band of Broca has been demonstrated [13] as well as GA-LI emerging as faintly labeled spots within the rat hippocampus [14].

The existence of a GA-like neuropeptide in putative cholinergic somata in the septum-basal forebrain was found to be much more pronounced in the owl monkey than in the rat, and within the hippocampus and dentate gyrus an extensive network of GA-immunoreactive (GA-IR) fibers could be demonstrated, closely resembling the distribution of putative cholinergic fibers [12]. This more extensive distribution of the GA-like peptide in the cholinergic system of the monkey as compared to the rat may reflect an increased importance of this peptide in cotransmission processes in higher animals and raises the possibility of a modulatory role for the GA-like peptide(s) on functions associated with the hippocampus, such as memory and learning. Recently it was demonstrated that one of the effects of GA involves inhibition of the evoked release of acetylcholine in the rat ventral hippocampus [7].

The cholinergic nature of the projection from the vertical limb nucleus of the diagonal band of Broca, better referred to as the Ch2 region [17], to the hippocampal formation is now well established in rat [2, 23, 30] and monkey [10, 16].

Degeneration of the cholinergic system is the most consistently found abnormality in Alzheimer's disease (AD), which is characterized by memory impairment and intellectual decline [3, 6]. Moreover, in AD the degree of dementia correlates with both the number of senile plaques (SP) and neurofibrillary tangles (NFT) in neocortex and hippocampus [4] and with the deficiency of the acetylcholine synthetizing enzyme ChAT [19]. Therefore, it is relevant to analyze to what degree GA-LI is present in the cholinergic system in normal brain and also whether or not the GA-LI content is changed in AD, senile dementia of Alzheimer type (SDAT) and other dementing disorders.

Very recently GA-LI has been described in the cholinergic nucleus basalis of Meynert [5], also known as the Ch4 region, which has cholinergic projections to neocortex and amygdale [17]. It was claimed that GA-LI increases in AD and in Parkinson's disease accompanied with dementia.

The present preliminary study describes the existence and changes of GA-LI in the Ch2 region (vertical limb nucleus of the diagonal band of Broca, which has cholinergic projections to the hippocampus) of two human control brains, two AD brains and one senile atrophy (SA) brain.

Materials and methods

Two brains from clinically diagnosed AD patients (case 1 and 2) were selected. The neuropathological diagnosis was performed on paraffin-embedded sections stained for Nissl, Bodian, Yamamoto-Bielschowsky, and fluorescence Congo red [20, 21] and thioflavine S. These two AD cases differed in duration and severity of illness: case 1 had a duration-time of 8 years and showed clinically advanced aphaso-apraxo-agnostic symptoms; case 2 lasted for 4 years with less severe dysfunctions.

In case 1 NFT were abundantly present in neocortex and hippocampus, especially in the subiculum area; the latter region was also interspersed with neuritic plaques as was the whole neocortex. Case 2 showed less NFT in the hippocampus and there was a predominance of small immature plaques in moderate numbers only. One case of familiar frontal lobe dementia clinically imposing as Pick's disease (case 3) could not be neuropathologically confirmed as such, but was diagnosed as SA: general atrophy of the hippocampus and whole neocortex was present especially in frontal and temporal lobes, showing a severe loss of neurons; however, SP and NFT were absent. Autopsy revealed no clues for extracranial causes of dementia. The two control cases (case 4 and 5) showed no clinical or neuropathological evidence of neurological or psychiatric symptoms (see also Table 1).

Tissue blocks containing the vertical limb nucleus of the diagonal band of Broca (Ch2 region) were obtained and fixed 24 h in formaldehyde sublimate (cases 2, 3 and 4) or formaldehyde (cases 1 and 5). After dehydration the slices were embedded in Ralwax and sections of 8 μm were cut using a Jung 2050 microtome. The sections were incubated with GA antiserum (Cambridge Research Biochemicals, Cambridge, UK) in a dilution of 1:500 for 48 h at room temperature. Staining was performed using the avidin-biotin-peroxidase complex technique (ABC) [9] according to the manufacture instructions (Vector). Immunoreactivity was demonstrated by 0.05% diaminobenzidine (DAB) – 0.01% H_2O_2 to which 0.01 M imidazole was added [26]. Sections were counterstained with hematoxylin. With regard to the specificity of the GA antiserum, control experiments were carried out by using a nonimmune serum, and by preadsorbing GA antiserum with an excess of purified GA-peptide (Cambridge Research Biochemicals).

Sections were investigated at a 200×, 400× and 1000× magnification. In each case GA-LI was evaluated on ten randomly chosen test fields of 250 × 250 μm^2. Three quantitative and three qualitative criteria were used for this purpose:

1. Mean total neuron number per mm^2; all neurons regardless of size and staining properties were counted at 400× magnification
2. Mean GA-IR neuron number per mm^2 at 400× magnification
3. Percentage GA-IR neuron number of total neuron number
4. Estimation of amount of GA-IR granules per GA-IR neuron
5. Estimation of staining intensity of GA-IR granules
6. Description of neuron processes

Data concerning the percentage GA-IR neuron number of total neuron number were statistically analyzed using the two-sided Wilcoxon test.

Table 1. Data of selected cases

Case	1	2	3	4	5
Sex	F	M	M	F	M
Age (y)	85	64	73	41	46
Duration of illness (y)	8	4	8.5		
Post-mortem delay (h)	4	3	3	24	9
Fixation	Fa	FaS	FaS	FaS	Fa
Diagnosis					
clinical	AD	AD	Pick	N	N
neuropathological	AD	AD	SA	N	N
SP and NFT					
neocortex	++++	+	–	–	–
hippocampus	++++	++	–	–	–

F, Female; M, male; y, year; Fa, formaldehyde; FaS, formaldehyde sublimate; AD, Alzheimer's disease; SA, senile atrophy; N, normal; SP, senile plaque; NFT, neurofibrillary tangle; ++++, numerous; ++, moderate; +, few; –, occasional or absent

Table 2. Quantitative and qualitative results of GA-Li in Ch2 neurons

Case number	1	2	3	4	5
Diagnosis NPA	Severe AD	Mild AD	SA	N	N
Mean neuron number/mm^2	98	102	107	112	104
Mean GA-IR number/mm^2	53	80*	83*	91**	85**
% Ga-IR neurons	54	78	78	81	82
GA-IR granules/neuron	+	++	+++	+++	+++
Intensity GA granules	+	+/++	++/+++	+++	++/+++
Description neuron processes: amount	Seldom	Few	Few	Some	Some
length	Short	Short + long	Short + long	Short + long	Short + long

NPA, Neuropathological; AD, Alzheimer's disease; SA, senile atrophy; N, normal brain; GA-IR, galanin-like immunoreactive; +, low; ++, moderate; +++, high
* $P < 0.05$ and ** $P < 0.01$ in two-sided Wilcoxon test as compared to case 1

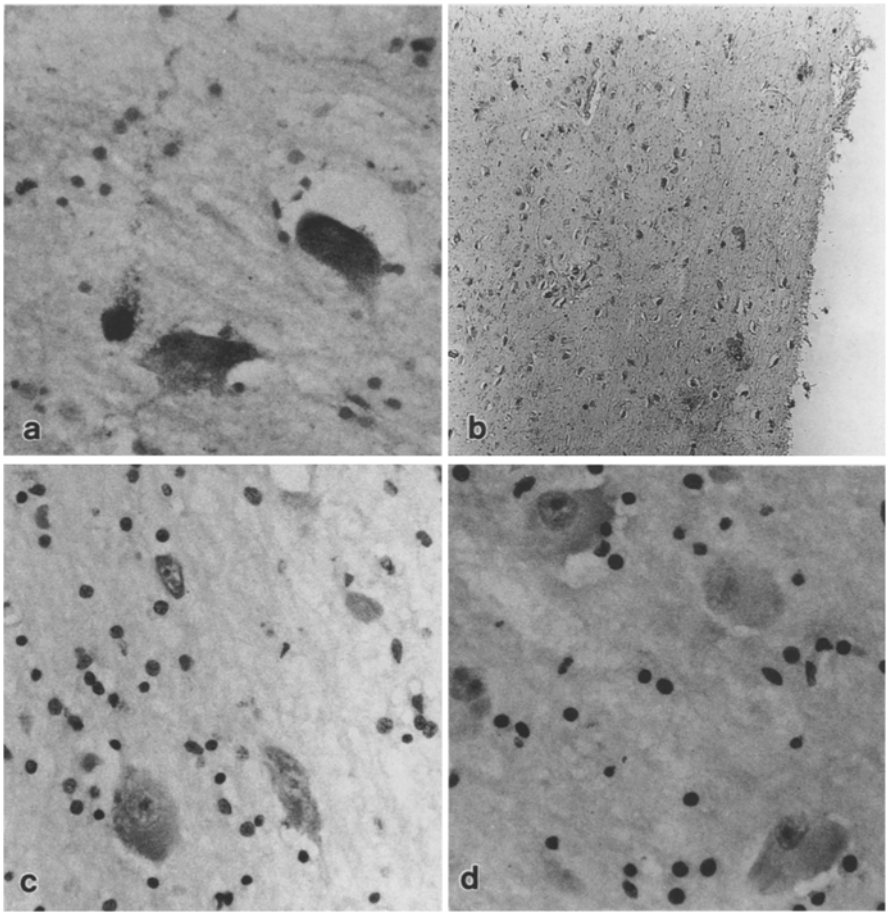

Fig. 1 a–d. Extensive galanin-like immunoreactivity (GA-LI) in the vertical limb nucleus of the diagonal band of Broca in normal aging (**a** and **b**, *top*); strongly decreased GA-LI in advanced form of Alzheimer's disease (AD) (**c**, *bottom left*); and absence of GA-LI in control case with excess of GA peptide in antiserum (**d**, *bottom right*). **a, c, d** ×280; **b** ×28

Results

Using the quantitative and qualitative criteria described above, the following results were obtained (see Table 2). The control brains (case 4 and 5) exhibited extensive GA-LI in the cytoplasm of over 80% of all large and small Ch2 neurons (Fig. 1a, b). An almost uniform distribution of many small, intensively stained granules was present throughout the entire cytoplasm (Fig. 2a). However, at the very periphery of the cytoplasm an increased densitiy of granules gave the impression of GA-LI on the outside surface of the soma as well. Some fusiform neurons showed a very dense, evenly intensive immunoreactivity resembling the GA-1 type interneurons as described by Chan-Palay [5]. The GA-IR neuronal processes could be

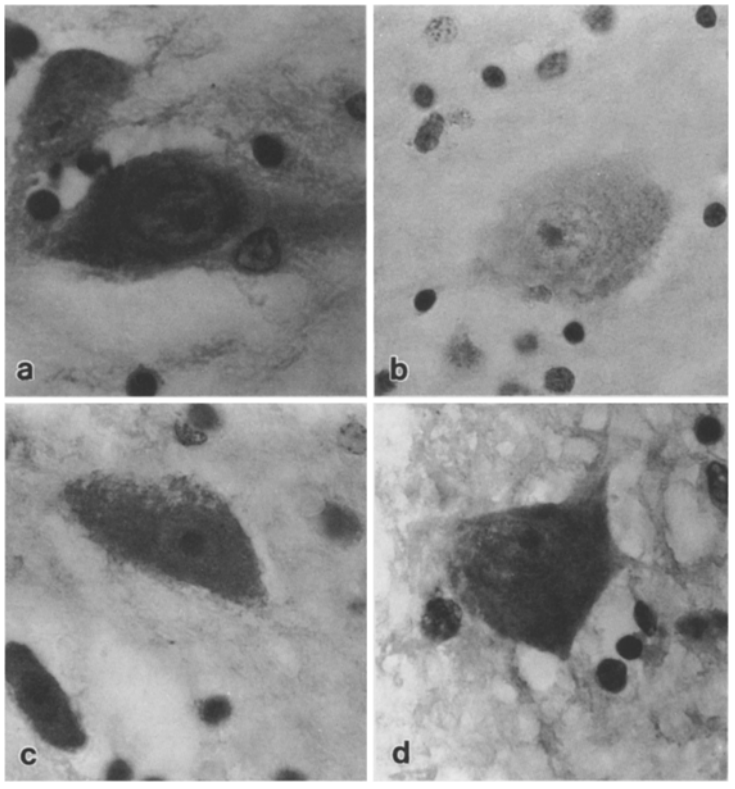

Fig. 2 a–d. GA-LI in the vertical limb nucleus of the diagonal band of Broca: extensive in both normal aging (**a**, *top left*) and senile atrophy (**d**, *bottom right*); strongly decreased in advanced form of AD (**b**, *top right*); intermediate in mild form of AD (**c**, *bottom left*). ×600

traced only along a small distance, but were sometimes prominently present (Fig. 1a).

In contrast to the control brains, the AD cases showed less GA-LI (Fig. 1c). GA-LI was most decreased in the advanced AD case (case 1), in which only 53% of all Ch2 neurons exhibited GA-LI with low amounts of weakly stained granules per neuron. Applying the two-sided Wilcoxon test, this decrease in percentage of GA-IR neurons in case 1 proved to be significant at the 0.01 level compared to both control cases 4 and 5, and at the 0.05 level compared to cases 2 and 3. All other comparisons between the cases 2–5 demonstrated no significant differences. Especially, the non-GA-IR neurons appeared to be shrunken and showed hardly any visible processes. Only the larger neurons showed low amounts of weakly stained granules mainly located at the periphery of the cytoplasm, and sometimes GA-IR dots over the soma surface could be pointed out (Fig. 2b).

In the AD case with less-pronounced neuropathological cortical changes (case 2) the number of GA-IR neurons (78%) equaled that of the control brains. However, the amount of GA-IR granules per neuron was clearly less in comparison with the control brains, but still more prominent than in the advanced AD case (Fig. 2c). The difference in clinical severity between the two AD cases is also reflected in the numbers of SP and NFT in the neocortex and hippocampus, see Table 1. Neuronal processes were hardly visible in both AD cases. The fusiform GA-1 type interneurons were still present but less easily detectable because of decrease in number as well as in staining intensity.

Surprisingly, case 3, that could be readily diagnosed according to neuropathological criteria as SA without SP or NFT, showed GA-LI as extensively as compared to the control cases: almost 80% of all Ch2 neurons were GA positive showing numerous intensively stained granules throughout the entire cytoplasm with an increase in density at the periphery of the cell. GA-IR processes were comparable to control brains (Fig. 2d).

Another interesting finding is the stability of mean neuron number/mm^2 in the Ch2 region for all five cases. Regarding to the specificity of the GA antiserum, control experiments both with a nonimmune serum as well as with an antiserum preadsorbed with an excess of GA-antigen showed completely no immune reaction (Fig. 1d).

These results demonstrate the presence of GA-LI in the Ch2 region of the human brain. The large putative cholinergic neurons show both cytoplasmic GA-IR granules and extracellular GA-IR dots along the cellular surface. This latter strongly suggests the possibility of GA-IR axon endings terminating upon the neuronal somata. Smaller fusiform neurons showed very dense, evenly intensive GA-LI. In AD the GA-LI

decreases: the number of GA-IR neurons diminishes significantly and/or the staining intensity decreases as does the amount of GA-IR granules per neuron, showing an inverse relationship to the numbers of NFT and SP in hippocampus. The SA brain showed GA-LI comparable to the control brains.

Discussion

The study presented here demonstrates the presence of GA-LI in the Ch2 region (vertical limb nucleus of the diagonal band of Broca) in two human control brains, two AD brains and one SA brain.

In the control brains GA-LI was present in most of the small and large putative cholinergic Ch2 neurons showing GA-IR granules in the cytoplasma and extracellular GA-IR dots along the outer cell membrane; the latter could probably be interpreted as GA-IR axon endings terminating upon neuronal somata.

In AD a prominent decrease in both intra- and extracellular GA-LI could be demonstrated, however, this decrease was absent in the SA brain that was clinically characterized by dementia, but neuropathologically showed no features of AD or Pick's disease.

The Ch2 region is well known to have cholinergic projections to the hippocampus [2, 10, 16, 23, 30], a limbic structure that is functionally involved in memory and learning processes. AD is clinically characterized by memory impairment and intellectual decline [6]. In AD the degree of dementia correlates with both the numbers of SP and NFT in the neocortex and hippocampus [4] as well as with the deficiency of the acetylcholine synthetizing enzyme ChAT [19]. This cholinergic deficiency is most pronounced in the hippocampus [8, 24].

Co-localization of ChAT- and GA-LI has been described in the Ch2 region of the rat [12, 15] and non-human primate [12, 29]. This co-localization was more pronounced in the higher species. Recently it was demonstrated that one of the functions of GA involves inhibition of the evoked release of acetylcholine in the rat ventral hippocampus in vivo and in vitro [7]; GA, therefore, acts as an inhibitory modulator of cholinergic transmission in the hippocampus.

The cholinergic basal forebrain system can be subdivided into four regions (Ch1, Ch2, Ch3 and Ch4) with different projection patterns and different amounts of cholinergic neurons [17]. The major cholinergic regions are Ch2, that projects to the hippocampus, and Ch4 also known as the nucleus basalis of Meynert, that projects to the neocortex and amygdala. Degeneration of the cholinergic system in AD is probably due to axonal damage as a result of cortical and hippocampal lesions such as SP and NFT. Recent morphometric studies support this view of secondary degeneration: many of the magnocellular cholinergic neurons are not completely lost, but persist in a shrunken state [1, 18, 28]. Moreover, in AD a differential involvement of the Ch2 and Ch4 region can be indicated: in Ch4 secondary cell shrinkage occurs as well as primary cell loss to an extent of 20% of all cells regardless of size properties; in Ch2 no cell loss could be observed, however a shift from large neurons into shrunken ones was prominently present [28].

In the present study no cell loss in the Ch2 region was seen in any of the five cases (see Table 2) and this finding strongly supports the hypothesis of secondary degeneration of the Ch2 system due to hippocampal lesions.

Our results suggest an inverse relationship between decreased GA-LI in Ch2 in AD and the numbers of neuropathological lesions in the hippocampus; the more severe these lesions, the lower the GA-LI in Ch2 (see Table 2). Because it has been shown that GA inhibitis the evoked release of acetylcholine in the hippocampus [7], a decreased GA-LI in Ch2 in AD suggests the possibility of a negative feedback mechanism to a degenerating cholinergic system.

Interestingly, our results indicating a GA-LI decrease in Ch2 in AD, are inconsistent with the recently reported increase in GA-LI in Ch4 in AD and Parkinson's disease accompanied with dementia [5]. These contradictory results may be due to the differential involvement of Ch2 (only secondary) and Ch4 (both primary and secondary degeneration) in AD [28]. Differences in fixation and immunohistochemical procedures can be an additional reason.

However, three variables could have influenced our results: age, post-mortem delay and differences in fixation (see Table 1). Concerning age and post-mortem delay, the GA-LI in cases 3, 4, and 5 (no presence of hippocampal SP and NFT) were comparable, although ranging in age from 41 to 73 years and in post-mortem delay from 3 to 24 h (no significant differences could be demonstrated applying the two-sided Wilcoxon test). Difference in fixation procedure slightly favored the formaldehyde-sublimate fixation; however, this impression demands further clarification based on objective groups. Although many immunopositive and immunonegative neurons are located within surrounding holes, this does not suggest neuron shrinkage and as a consequence possible nonspecific staining, but has to be considered as tissue shrinkage due to fixation artefacts.

In conclusion, our preliminary results demonstrate a decreased GA-LI in Ch2 in AD patients, but probably not in patients with dementia of other origin. This decrease in GA-LI is inversely related to the

number of SP and NFT in hippocampus in AD. This is in full agreement with the functional effect of GA, inhibition of the acetylcholine release in the hippocampus.

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