Increased neuronal nuclear and pericaryal size in the medial mamillary nucleus of vascular dementia and Alzheimer's disease patients: relation to nuclear estrogen receptor α

Running head title "Posterior hypothalamic nuclei in vascular dementia and AD"

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Abstract. The medial mamillary nucleus (MMN) is an important hub in memory circuits. In the present study, we aimed at determining MMN neuronal nuclear and pericaryal sizes that are informative about the neuronal metabolic activity in Alzheimer's disease (AD) in vascular dementia (VD) patients. Both parameters were significantly larger in VD than in control patients ($p<0.01$) and were not different from those in AD subjects. Neuronal nuclei ($p<0.05$), but not pericarya were larger in AD than in control patients. Neuronal nuclei and pericarya were larger if nuclear ERα staining was present. The intensity of ERα in the neuronal nuclei was significantly correlated with both nuclear and pericaryal sizes ($p<0.007$). The only exception was the strongest ERα staining providing a totally black cover over the nucleus that was increased in AD patients. In this case the nuclei and pericarya were smaller than in moderate intensity staining that allowed visualization of the nucleolus and large chromatin clumps. Our data show for the first time prominent activation of MMN neurons in VD and to a lesser degree in AD that may be mediated by nuclear ERα. Our findings are in agreement with the idea that neuronal activation may protect against AD neuropathological alterations.

Key words: vascular dementia, Alzheimer's disease, aging, neuronal nuclei, neuronal size, estrogen receptor α.

INTRODUCTION

The medial mamillary nucleus (MMN) of the mamillary body and the tuberomamillary nucleus (TMN) are essential structures in the caudal hypothalamus for cognitive functions. The MMN is crucial in memory circuits that involve the cingulate gyrus and the hippocampus (1). The TMN is the main source of brain histaminergic projections that play a key role in memory and sleep and wake cycles (2, 3, 4). Both nuclei show metabolic activation in normal aging as shown by an increase of the neuronal nuclei and pericarial sizes reaching maximal values in elderly control patients (60-74 years of age) (5). However, gender differences in age-related changes were opposite with higher activation in the MMN of women (6) and in the TMN of men (7). Although an increased gliosis in the MMN and the TMN was previously demonstrated in both vascular dementia (VD) and Alzheimer's disease (AD) patients indicating their involvement in nerodegeneration (8), these anatomically neighboring nuclei were differentially affected in the two types of dementia. Neuropathological alterations in the MMN are rare or even entirely absent in vascular dementia (VD) patients (9). Although in Alzheimer's disease (AD) this nucleus shows some neuritic plaques, diffuse Aβ plaques and tangles, their extension is significantly less than in the cortex or the adjacent tuberomamillary nucleus (TMN) (10,11). Previous studies that used the Golgi complex (GC) size as a parameter of the neuronal metabolic activity showed decreased metabolic activity of the TMN neurons in both, AD and VD (3,12, 13), while in the MMN no decline in metabolic activity was found, either in VD or AD using this parameter (6,13). We followed these observations up with a morphometric study of the size of neuronal nuclei and pericarya and in the same groups of patients, since these were found earlier to be sensitive parameters for neuronal activity changes (5).

Estrogens affect the activity of many neuronal systems and act by estrogen receptor (ER)α that shows clear changes in aging and AD (6). Another aim of the present study was, therefore, to relate changes in the expression of the nuclear ERα to alterations in the size of neuronal nuclei and pericarya in the MMN of VD and AD patients.

MATERIALS AND METHODS

Tissue collection

The study was performed with hypothalamic sections containing the medial mamillary and the tuberomamillary nuclei obtained in the framework of the Netherlands Brain Bank (NBB). In all cases permission for the brain autopsy and the use of the material and clinical information for research purposes was obtained by the NBB. The patients with VD (N = 8, 76.7±2.8 years of age for the MMN, N = 11, 76.9±2.7 years of age for the TMN), AD (N = 10, 76.7±3.1 years of age for the MMN and N = 11, 76.6±2.8 years of age for the TMN) and control groups (N = 10, 75.4±2.7 years of age for the MMN, and N = 11, 76.6±2.7 years of age for the TMN) were the same as used in our previous studies (13,14). The differences between the number of patients' in groups for the MMN and the TMN were due to the fact that mamillary bodies were sometimes already completely used in other studies (15). Clinico-pathological information with the details concerning vascular lesions in VD was published in (13, here available as supplementary material Table S1). The demented patients were clinically assessed and diagnosed as 'probable VD" in accordance with the NINDS-AIREN criteria (16) and as 'probable AD' following the NINCDS-ADRDA criteria (17). Controls had no signs of cognitive impairment and no history of any neurological or psychiatric disorder and were matched for age, sex, and fixation time with AD and VD cases. Neuropathological examination of AD patients assessed the distribution of neurofibrillary tangles over the brain according to the classification of Braak (18). **Jellinger and Bancher C (19) showed by multivariant analysis significant correlations between psychostatus and both the CERAD criteria and Braak staging of neuritic Alzheimer-type lesions. In addition, we recently reported a high correlation (0.874, *P*<0.001) between the clinical Reisberg scale and Braak scores (20). On the basis of Braak's analysis a score of 0-6 was assigned to the patients: the AD subjects had a score of 5-6, VD cases of 1-2 and the controls of 0-1. Neuropathological examination of VD patients revealed dilated ventricles, multiple hemorrhages and their remnants, necrotic lesions and atrophy of gyri and adjacent white matter, rarefaction and**

maceration of the white matter, lacunae, état criblé in the caudate nucleus, putamen, pallidum and thalamus. ‘‘Old’’ and ‘‘recent’’ infarctions in the VD group were localized in the temporal, parietal, occipital and occasionally prefrontal cortex, in the cingulate gyrus, insula, caudate nucleus, putamen, pallidum, capsula interna and externa, cerebellum and pons. None of the vascular lesions was found in the studied hypothalamic nuclei. It should also be noted that in all VD cases only slight senile involutive changes were present, and Braak stage did not exceed II in contrast to AD patients. The hypothalami containing the TMN and MMN were dissected, fixed in 4% formaldehyde in phosphate-buffered saline (pH 7.4), dehydrated in graded ethanols, embedded in paraffin and cut serially in 6-μm frontal sections. For anatomical orientation, every 50th section was mounted on chrome-aluminum sulphate-coated glass slides, deparaffinized, hydrated and stained with thionine (0.5%). **Rostro-caudal extension of both the MMN and the TMN was examined in these serial sections. Immunocytochemical and morphometric studies were performed on two sections from the middle of the MMN and two sections from the main ventral group of the TMN (2). The boundaries of the MMN were well distinguished due to the clear fibrous capsule. The TMN ventral group was represented by large neurons that were clearly different from smaller neurons of the adjacent tuberal lateral nucleus neurons typically containing a lot of lipofuscin inclusions. Glial cells were distinguished from neurons based on their smaller size, little cytoplasm and the absence of a nucleolus.**

Immunocytochemical staining

Immunocytochemical staining of the ERα was performed as described earlier (6). Briefly, following deparaffinization in xylene and graded ethanols, rinsing in Tris containing buffered saline (TBS) (pH 7.6), waterbath pre-treatment in 0.05M Tris-HCl buffer (pH 7.6) for 30 min at 90^0C and washing in milk-TBS for 1 hour at room temperature (RT) the sections were incubated with a primary polyclonal rabbit anti-ERα antibody that recognises the carboxy terminus epitope of the ERα (Santa Cruz, cat # sc-542) diluted 1: 100 in sumi-milk (0.25% gelatine and 0.5 ml

Triton X-100 and 5% of milk powder in 100 ml TBS, pH 7.6) for 1 hour at RT and at 4^0C overnight. The next day sections were washed in milk-TBS, incubated with secondary biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) 1:200 in sumi-milk for 1 hour at RT; washed in TBS and incubated with avidin-biotin complex (ABC) (Elite kit, Vector Laboratories, Burlingame, CA) 1:400 in sumi for 1 hour at RT. The final step was the incubation in Tris-HCl containing 0.05% 3,3' diaminobenzidine, 0.01% H_2O_2 and 0.3% nickel ammonium sulphate. Following washing in Tris-HCl buffer sections were dehydrated in graded ethanols, cleared in xylene and coverslipped with Entellan mounting medium.

The ERα staining was predominantly observed in the neuronal nuclei. **To a significantly lesser extent it was occasionally found in neuronal cytoplasm, glial cells and some nerve fibers.** The intensity of the neuronal nuclear ERα staining was evaluated according to the scale including four categories: 1) strong (+++) – the nuclei are completely black, no distinct chromatin and/or nucleoli visualization, 2) moderate (++) – the staining covers the nucleus, the nucleolus and large clumps of chromatin can be distinguished, 3) weak (+) – weak staining of slender chromatin threads is found, 4) negative (-) – no staining in the nucleus (Fig. 1 and **Supplementary Fig. 1**).

Quantification

Morphometric analysis was performed using the ImageJ program (5). **Microphotographs of the sections made at x400 were retrieved with the help of the "File → open" icons. Neuronal pericarya and their nuclei with the nucleolus were outlined manually with "freehand selections" tool. Their areas were subsequently determined using the "analyse → measure" icons.** The nuclear/cytoplasmic ratio was further calculated. **On average 40±3.5 MMN neuronal nuclei were measured in VD patients, 42±3.4 in controls and 44±3.7 in AD cases (p = 0.635). For the TMN these values were: 39±3.7 (VD), 33±3.3 (controls) and 42±3.5 (AD) (p = 0.190).** At first these parameters were quantified for all neurons independently on the intensity of the ERα staining (Table 1). Subsequently, detailed quantification was made considering the intensity of the ERα staining in the neuronal nuclei in the MMN semi-quantitatively (Table 2). **Differences in the**

intensity of the ERα staining were well apparent at the microscopical examination as shown in microphorographs (Fig.1 and supplementary Fig. 1) and were rechecked by optic measurements. According to the ImageJ measurements, the ratio area/integrated density was 0.012-0,013 for the strong intensity, 0.007-0.008 for the moderate intensity and less than 0.007 (commonly 0.006) for the weak intensity nuclear ERα staining.

Statistical methods

The differences in the size of neuronal nuclei and pericarya were tested using the Kruskall-Wallis and Mann-Whitney U-tests. In order to test for correlations between the different parameters Spearman's linear regression analysis was applied. A p-value < 0.05 was considered to be significant.

RESULTS

Neuronal nucleus and pericaryal size in VD and AD

According to the Kruskall-Wallis test, there were significant differences between the three groups (VD, AD, Controls) in the size (area) of the neuronal nuclei ($p = 0.0068$) and pericarya ($p = 0.023$) in the MMN. The Mann-Whitney test further showed that neuronal nuclei were significantly larger in VD and AD cases compared to non-demented controls ($p<0.01$) (Table 1, Fig. 2). Neuronal pericarya were significantly larger in the VD than in the control group ($p<0.01$).

There were no prominent differences in the studied morphometric parameters between the VD and control groups in the TMN. However, both the neuronal nuclei ($p<0.05$) and pericarya ($p<0.01$) were significantly smaller in AD than in control cases (Table 1).

The nuclear/cytoplasmic ratio did not change in either the VD or the AD in any of the studied nuclei ($p>0.05$). However, in all groups (VD, AD, Controls) this ratio was prominently higher in the MMN than in the TMN ($p<0.01$).

Postmortem delay did not influence the size of neuronal perikarya ($r = -0.189$) or their nucleus ($r = -0.435$) as shown by the absence of a significant correlation in Spearman's linear

regression analysis. Moreover, cases with most prominent postmortem delay had mean values within the main range of their group.

Neuronal nucleus and pericaryal size in the MMN of VD and AD patients with respect to the intensity of the nuclear ERα staining

Considering the role of ERα in neuronal activation (5) and the fact that an increase in the neuronal nuclei and pericarya sizes was found in the present study in both dementias in the MMN, a subsequent detailed quantification was performed in this nucleus. According to the Kruskall-Wallis test, neuronal nuclei ($p< 0.00001$) and pericarya ($p<0.007$) differed highly significantly depending on the presence and the amount of ERα in the studied groups.

In VD cases the area of neuronal nuclei did not depend much on the intensity (amount) of nuclear ERα, but rather on its presence in general. Neuronal nuclei were larger if ERα was present ($p<0.01$). Interestingly, the amount of ERα influenced the size of neurons. Moderate and weak ERα staining was associated with larger pericarya ($p<0.01$), whereas in case of the strongest (completely black) staining, the neuronal size did not differ from that in ERα-negative neurons (Fig.1, Table 2, **supplementary Fig. 2**). The scale for this difference can be schematically represented as 2++ (moderate) = 1+ (weak) > 0 (-) (negative) = 3+++ (strong).

In AD cases maximal neuronal nuclei areas were associated with moderate ERα staining. Neuronal nuclei were larger in moderate than in strong nuclear ERα staining and were larger in moderate and strong ERα staining than in case of the weak or negative immunolabelling ($p<0.01$). The schematic scale for these differences looks as 2++ (moderate) > 3+++ (strong) > 1+ (weak) > 0 (-) (negative) ($p<0.01$). The situation with neuronal pericarya was nearly the same. MMN neuronal pericarya were larger in moderate than in strong nuclear ERα staining and were larger in moderate and strong ERα intensity than in the weak or negative ($p<0.01$) (Table 2, **supplementary Fig. 2**). It should be noted that the number of neurons with strong and moderate ERα staining was 2 fold higher in AD than in the VD group. On the contrary, the number of MMN neurons with weak ERα intensity was 2-fold less in AD than in VD cases. This finding confirms the idea that

nuclear ERα is more prominently increased in hypothalamic and basal forebrain areas in AD than in VD or controls (14).

In the control group the number of ERα-negative neurons was 1.5-2 fold higher than in VD or AD cases. Interestingly, strong ERα intensity did not change the size of neuronal nuclei or pericarya in this group. Neuronal pericarya were even smaller than in ERα-negative neurons if strong ERα staining was observed ($p<0.05$). Neuronal nuclei were larger in moderate than in strong, weak or negative ERα staining in controls ($p<0.01$) (Table 2, **supplementary Fig.2**). The schematic scale for these differences looks as 2++ (moderate) > 1+ (weak) > 0 (-) (negative) > 3+++ (strong) ($p<0.01$).

There were no statistically significant gender differences ($p>0.05$) and no significant correlations with age within any of the studied groups ($p>0.05$).

Postmortem delay appeared not to influence the number of neurons with strong (r = -0.451), weak (r = 0.257) or negative (r = 0.382) ERα staining according to Spearman's linear regression analysis.

DISCUSSION

In the present study we found for the first time that neuronal metabolic activity as judged from the size of neuronal nuclei and pericarya is increased in VD and to a lesser extent in AD patients in the MMN. These differences were robust and were not influenced by individual variability that is typical for the human brain (21). In the MMN of VD patients this neuronal activation may be the result of the compensation to the substantial neuronal loss and atrophy of the hippocampus (22, 23). The increased neuronal metabolic activity findings seem to be negatively related to the presence of AD pathology in the TMN and MMN and may represent compensatory changes. Indeed, as far as AD is concerned, in the TMN that has numerous senile plaques, amyloid deposits and multiple neurofibrillary tangles (12) we observed significant decreases in the neuronal nucles and pericaryal size. On the contrary, in the MMN of AD patients where deposits of β-amyloid peptide and neurofibrillary degeneration were minimal (10, 11) and

did only appear in Brak stage 3 (24), we found the neuronal nuclei to be enlarged. The fact that neuronal nuclei and pericarya were more prominently increased in VD than in AD cases tallies well with a generally lower expression of AD pathology in VD (stages I or II according to the Braak classification) than in AD. Moreover, volumetric changes in the MMN are related to the progression of AD pathology. In MCI (mild cognitive impairment, Braak stages III and IV) no decrease in the mamillary body volume was found, whereas significant reduction in the volume of the mamillary body and fornix was apparent at the conversion of the MCI to AD (25). Functional magnetic resonance imaging study in MCI patients also showed hypermetabolism in the cerebello-limbic system involving the MMN using cerebral blood volume measures which the authors related to a cognitive reserve protecting against dementia (26). This means that whereas the volume of the MMN becomes smaller due to the death of some MMN cells, the remaining ones compensate by an enlargement. It is relevant to mention here that clear differences in neuronal metabolic activity estimated on the basis of the Golgi complex size in relation to Braak staging were previously described in the NBM (the nucleus basalis of Meynert). An increase in the Golgi complex size was found in MCI followed by a decrease in advanced AD (stages V-VI) (27). In addition, some 800 genes showed similar contrasting expression changes from upregulation in earlier Braak stages to downregulation in advanced AD. This pattern was called the UP-DOWN (28) and included, among others, genes involved in ribosome biogenesis and assembly that are clearly related to the neuronal nuclear size studied in the present work. Riudavets et al. (29) observed nuclear hypertrophy in the neurons of the anterior cingulate gyrus and CA1 region of the hippocampus in asymptomatic AD patients and suggested this to be a compensatory phenomenon that may prevent dementia progression. It should be noted that nuclear hypertrophy in the MMN and TMN occurred in the course of aging and was prominent in the elderly (60-74 years of age) group (5). The present study shows that this activation proceeds in dementias in the MMN with lower neuropathology but is replaced by atrophy in the TMN with higher extension of senile plaques and neurofibrillar tangles. Thus, extra activation of the MMN neurons in dementia may,

indeed, protect against progression of AD neuropathology. In this respect, increased gliosis in the MMN of VD and AD cases (8) also looks as a compensatory mechanism that is switched on between 40-50 years. The number of glial cells in the MMN gradually increases in aging and reaches its maximum in the group of elderly patients (60-74) (30) for which we also showed maximal metabolic activation (5). Increased sizes of neuronal nuclei and pericarya (5) and gliosis (30) in aging can also be related to the decrease in the density of blood vessels in this nucleus (30) and, consequently, to the diminished blood supply. Consistent with this, compensatory metabolic changes resulting in elevated neuron specific enolase were also described in human cerebral cortex neurons in chronic ischemia (31). Together, these data indicate that the elevated neuronal metabolic activity in aging and dementia accompanied by increased gliosis is likely to be a compensatory phenomenon that may be a key to the question of dementia prevention or postponing.

In our previous studies we showed that an increase in the MMN neuronal metabolic activity in normal aging, as determined by the size of the Golgi-apparatus, is accompanied by an increase in estrogen receptor α (ERα) expression and that nuclear ERα expression is prominently enhanced in AD (6). These findings suggested a role for ERα in the aging-related activation that was more apparent in women (6) and was related to a drop in estrogen levels around the menopause. In the present investigation we aimed at determining whether the ERα was related to the size of neuronal nuclei and pericarya in VD, AD and elderly controls (60-86 years of age). As expected, the number of nuclear ERα-positive neurons with the strongest and moderate staining intensity was the highest in the AD group. In VD cases moderate staining was abundant. In the control group moderate staining was predominant, and the number of ERα- negative neurons was 2-fold higher than in the VD or AD group. The interesting and important conclusion was that, indeed, the ERα may have an effect on the neuronal nuclear and pericaryal size and that this effect depends on the amount of the ERα in the nucleus. In most cases neuronal nuclei and pericarya were larger if the ERα was present in the nucleus (Supplementary Fig. 2). The higher was the expression of the nuclear ERα,

the larger were the neurons and their nuclei. The only exception was the “very strong” ERα staining intensity, when neuronal nuclei looked completely black. In this case neuronal nuclei and pericarya were smaller than in “moderate” intensity. This means that overwhelming accumulation of nuclear ERα is associated with a decrease in neuronal metabolic activity. Consistent with this, neuronal nuclei were smaller in AD with the strongest ERα intensity than in VD with moderate immunolabelling. In the control and VD groups the effect of the strong ERα intensity on the size of neurons did not differ from that of the moderate or weak immunolabelling, but the number of neurons with this kind of staining was 1.5-2 fold less than in AD. These results demonstrate that nuclear ERα may either mediate trophic or stimulatory (32) effects on neurons both in normal aging and dementias or be a part of the compensatory machinery in estrogen deficiency state. Consistent with this, in our previous study we found a decrease in aromatase immunoreactivity in the MMN in aging and AD (33**). In addition to extra brain estrogen deficiency, this phenomenon may also be related to the alterations in receptor proteasomal degradation that is also located in the nucleus. On the other hand, the prolonged nuclear stimulation that might be the case in “strong intensity” neurons through ERα remains to be determined in vitro.** The survey of the literature suggests a number of neuroprotective effects of the ERα that might be relevant for the AD prevention. Those include the inhibition of Death domain-associated protein translocation, the ERα association with caveolin or with the voltage-dependent anion channels, blocking Aβ glutamate excitotoxic injury, co-activation with the IGF-IR and growth factors (34). ERα may also prevent VD since it mediates beneficial effects of estrogens on the cerebral microvasculature (33), their protective effects against cerebral ischemia (36) and ability to reduce postischemia inflammation (37). Our data show that in VD not only vascular but also neuronal ERα should be considered.

In controls the smaller neuronal perikarya and nuclei in “strong intensity” ERα staining were explained by higher levels of the dominant del. 7 ERα variant which inhibits classical ERα transcriptional activity. Based on our previous research, we may suggest that

splice variant/wild ERα del. 7 ratio may play a role in the mentioned events. Del. 7 is a dominant negative splice variant that is abundant in the human brain and can severely inhibit binding of wild-type ER α and β receptors to their responsive elements and reduce the estrogen-dependent transcriptional activation by classical ERs. As shown earlier, expression levels of the wild type ERα were higher in the MMN of AD patients, whereas in controls the splice variant del. 7/wild ERα ratio was increased (15). The abundance of del.7 may explain the lack of the relationship between strong ERα staining and the size of neurons and their nuclei in the control group.

Taken together, our data show for the first time neuronal metabolic activation in the MMN of VD and to a lesser extent of AD patients related to the expression of nuclear ERα and suggest these findings to represent compensatory phenomena accompanying early neuropathological alterations and preventing their progression.

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LIST OF ABBREVIATIONS

AD – Alzheimer's disease

MMN – the medial mamillary nucleus

TMN – the tuberomamillary nucleus

VD – vascular dementia

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LEGENDS

Figure 1. Microphotographs of the ERα staining in vascular dementia (VD) (A,B,C), Alzheimer's disease (AD) (D,E,F) and non-demented control (NDC) (G,H,I) groups. Note differences in the size of neuronal nuclei and pericarya in relation to the intensity of the nuclear ERα staining: strong +++ (A,D,G), moderate (B,E,H) and negative (C,F,I). Objective x 40.

Figure 2. Graphs demonstrating differences in the size (in square micrometers) of neuronal nuclei (A) and pericarya (B) among vascular dementia (VD), Alzheimer's disease (AD) and non-demented controls (NDC) groups. Neuronal nuclei are larger in VD than in NDC cases ($p < 0.01$) and are larger in AD than in NDC cases ($p < 0.05$). Neuronal pericarya are larger in VD than in NDC patients ($p < 0.01$).

Supplementary materials

Fig. 1. Microphotographs of the ERα staining demonstrating the examples of the weak intensity staining.

Fig. 2. Graphs demonstrating the effect of the nuclear ERα staining and its intensity on the size of neuronal pericarya and their nuclei in VD, AD and control groups. Black columns show "strong" intensity, dark gray – "moderate" intensity, light gray – "weak intensity, white – nnegative for ERα. Mean values of neuronal nuclei and pericarya are indicated above the columns.