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ORIGINAL ARTICLE

Estrogen receptor α splice variant TADDI in the human supraoptic nucleus: an effect on neuronal size and changes in pneumonia

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Abstract

BACKGROUND: Estrogens mediate various effects in the brain not only via classical estrogen receptors (ERs) but also through their splice variants. We showed earlier that the ERα splice variant TADDI is abundantly expressed in the human hypothalamic supraoptic nucleus (SON).
METHODS: In the present study we aimed at determining a possible effect of TADDI on human SON neuronal morphometric parameters in 58 control patients from 20 to 94 years old and in 26 patients with Alzheimer's disease (AD) aged 54-94 years old. The size of neuronal nuclear and perikaryal profiles was determined as measure of the neuronal metabolic activity in relation to the intensity of TADDI immunocytochemical staining. The size of SON neuronal nuclei and perikarya were also measured with respect to the wild type (wt) ERα nuclear staining in the group of 11 elderly patients.
RESULTS: Independently of gender, age or AD status SON neuronal nuclei and perikarya were significantly smaller in neurons with moderate and strong TADDI staining than in neurons that did not express this ERα splice variant. On the contrary, neuronal nuclei and perikarya were considerably larger in SON neurons with moderate and strong nuclear staining for wt ERα as compared to neurons that showed an absence of the classic receptor. It is noteworthy that TADDI immunoreactivity was increased in control patients with pneumonia and/or respiratory insufficiency.
CONCLUSIONS: We showed for the first time the association of the ERα splice variant TADDI with neuronal morphometric parameters in the human post-mortem brain tissue.

INTRODUCTION

Estrogen is known for its neuroprotective role (Phung *et al.* 2010; Arevalo *et al.* 2015; Lan *et al.* 2015). Its effects through canonical ERα and β receptors are well documented (Lan *et al.* 2015) However, a number of ER splice variants are also present in the brain and may affect neuronal functioning (Perlman *et al.* 2005; Ishunina and Swaab, 2012). In a series of previous studies we

identified in the human brain around 62 ERα splice variants, including 50 novel variants, and confirmed that they may be present there at the protein level (Ishunina and Swaab, 2012). One of the novel variants, TADDI, was isolated from the human hippocampus. It lacks 31 bp from the junction between exons 3 and 4, and 13 nucleotides from exon 2 are inserted into this splice site (Ishunina and Swaab, 2009; Ishunina *et al.* 2007). TADDI protein has a deletion of 11 amino acids 253–263 of the canonical ER (gb/AAA52399.1) that are replaced by 5 novel amino acids DWPVP. The altered region is inside the hinge domain and the excluded sequence overlaps with the nuclear localization signal that may be important for the induction of a directed bend in DNA regions enclosing estrogen responsive element (Picard *et al.* 1990; Schultz *et al.* 2002). With the help of polyclonal antibody T2.2 that was raised in a rabbit by Eurogentec S.A. (Seraing, Belgium) against the sequence YEVGMMKDWPVPMLKH that covers the unique splice site of TADDI predicted protein, we further showed that this ERα splice variant is present in the human brain at the protein level and changes in aging and Alzheimers disease (AD) (Ishunina and Swaab, 2009). The largest amount of TADDI protein was found in the hypothalamic supraoptic nucleus which is the main source of plasma arginine vasopressin (AVP) (Swaab, 2003). In the present study we determined by a morphometric analysis SON neuronal nuclei and perikarya in relation to the intensity of TADDI and canonical ERα immunocytochemical staining to estimate their possible effects on neuronal metabolic activity of these neuroendocrine neurons.

MATERIALS AND METHODS

The study was performed with hypothalamic sections containing the SON obtained in the framework of the Netherlands Brain Bank (NBB). In all cases written consent for the brain autopsy and the use of the material and clinical information for research purposes was received by the NBB. Fifty eight patients without neurological or psychiatric disorders (control cases) from 20 to 94 years of age and twenty six patients with AD from 54 to 94 years of age were used in the same way as in our previous study (Ishunina and Swaab, 2009). Clinico-pathological information is available in (Ishunina and Swaab, 2009). Immunocytochemical and morphometric studies were performed on sections from the mainly (90-95%) vasopressinergic dorsolateral part of the SON. The procedure of immunocytochemical staining of the ERα splice variant TADDI and the specificity of the staining were described in detail earlier (Ishunina and Swaab, 2009).

The ERα splice variant TADDI staining was predominantly observed in the neuronal cytoplasm. The intensity of the immunocytochemical staining for TADDI was estimated semi-quantitatively at the microscopical examination as: 1) strong (+++), 2) moderate (++), 3) weak (+), 4) negative (-) – no staining, and was verified by optic measurements. According to the ImageJ measurements, the ratio area/integrated density was more than 0,008 for the strong intensity, 0.007-0.008 for the moderate intensity and in the range of 0.006-0.007 for the weak intensity. It should be noted that these values agree well with the intensity scale used in our previous study (Ishunina *et al.* 2019) and that the differences in the intensity of the staining were well apparent at the microscopical examination (Fig. 1).

A total of 7673 morphometric measurements on microphotographs of the SON containing sections made at x400 magnification were performed using the ImageJ program (Ishunina *et al.* 2019). Neuronal perikarya, their nuclei and nucleoli were perfectly detectable following TADDI staining. Glial cells were differentiated from neurons based on their smaller size, little cytoplasm and the absence of a nucleolus. Randomly chosen neuronal perykaryal and nuclear profiles with nucleoli were outlined manually and their areas were subsequently determined using the "analyse → measure" tools. The differences in the size of neuronal nuclei and perikarya were tested using the Mann-Whitney U-test. A p-value < 0.05 was considered to be significant.

In addition, SON nuclei and perikaryal sizes were estimated with respect to canonical ERα staining in 11 control cases (from 61 to 89 years of age) that were used in our previous study (Ishunina *et al.* 2019). SON neuronal nuclei and perikarya were compared between neuronal groups with either strong or moderate staining for canonical ERα and neurons with the absence or very weak staining. Wild type (wt) ERα was localized predominantly in the SON neuronal nuclei.

RESULTS

Morphometric analysis of SON neurons in relation to TADDI staining

Morphometric data from control and AD cases were presented in relation to the intensity of TADDI staining to determine the effect of TADDI on neuronal nuclear and perikaryal sizes. Within each group the subgroups with weak or no staining, moderate and strong intensity of immunocytochemical staining for TADDI were defined. There were 9 cases with negative or weak staining and 17 cases with moderate to strong staining in the AD group and 13 cases with negative or weak staining and 45 cases with moderate to strong staining among control patients (Table 1). This approach was completely different from the methodology of our previous study where we measured total TADDI-immunoreactivity in the SON independently on the intensity of staining (Ishunina and Swaab, 2009). In both control and AD cases the profile areas of neuronal nuclei and perikarya were significantly larger if TADDI-immunoreactivity was low or absent than in cases with moderate and strong staining (p<0,001) (Table 1,

Tab. 1. Neuronal nuclear and perikaryal profile areas in the human supraoptic nucleus in relation to ERα splice variant TADDI immunoreactivity (mean±SEM).

Groups	Nuclear profiles, μm²		Perikaryal profiles, μm²	
	Negative or weak staining	Moderate and strong staining	Negative or weak staining	Moderate and strong staining
AD cases, n=26	120,8±5,0[1] n=9	97,4±3,3[1] n=17	436,5±18,5[2] n=9	383,4±21,1[2] n=17
AD men, n=12	116,6±8,4	97,9±4,8	466,3±34,9	409,5±32,1
AD women, n=14	124,1±6,7[5]	96,9±4,7[5]	412,7±13,5	360,1±27,3
Control cases, n=58	113,8±5,0[3] n=13	87,5±2,2[3] n=45	417,2±24,7[4] n=13	329,7±11,4[4] n=45
Control men, n=25	116,3±7,4[6]	88,1±2,3[6]	433,8±38,4[7]	340,7±14,6[7]
Control women, n=33	110,9±7,1[8]	87,1±3,4[8]	397,8±31,1[9]	322,4±16,3[9]

1,3,4,6,7 – $p<0{,}001$; **2,9** – $p<0{,}05$; **5,8** – $p<0{,}01$.

Fig. 1). These differences were independent of either the gender or the age of human subjects.

TADDI immunoreactivity in relation to pneumonia/ respiratory insufficiency

In the control group TADDI immunoreactivity was prominent in people who died from pneumonia or respiratory insufficiency. 89% of cases with these diagnoses demonstrated moderate to strong staining for this ERα splice variant. And only 11% of patients with pneumonia/respiratory insufficiency showed negative or weak staining for TADDI ($p=0.003$, according to chi-square test). Total TADDI immunoreactivity per SON area was significantly (by 31%) higher in control patients with pneumonia/respiratory insufficiency than in people without this pathology ($p<0.05$) (Table 2) showing that hypoxia may stimulate the expression of this ERα splice variant which in turn may decrease the AVP synthetic activity of SON neurons. In the AD group this link was less clear as AD patients had decreased levels of TADDI as shown previously (Ishunina and Swaab, 2009).

Morphometric analysis of SON neurons in relation to classical ERα staining

In the group of elderly control cases, SON neuronal nuclei ($p < 0{,}001$) and perikarya ($p < 0{,}01$) were significantly larger if strong or moderate nuclear staining for classical ERα was present as compared to neurons with the absence or very weak staining for the wt ERα (Table 3).

AD-related changes in SON neuronal nuclear and perikaryal sizes

Both nuclear and perikaryal areas were significantly larger in AD patients (n=26) than in the entire control group (n=58) ($p<0{,}01$) (Table 1). When these morphometric parameters were compared between AD (n=26) and age-matched (54-94 years of age) control cases (n=30), the difference was less clear and showed only

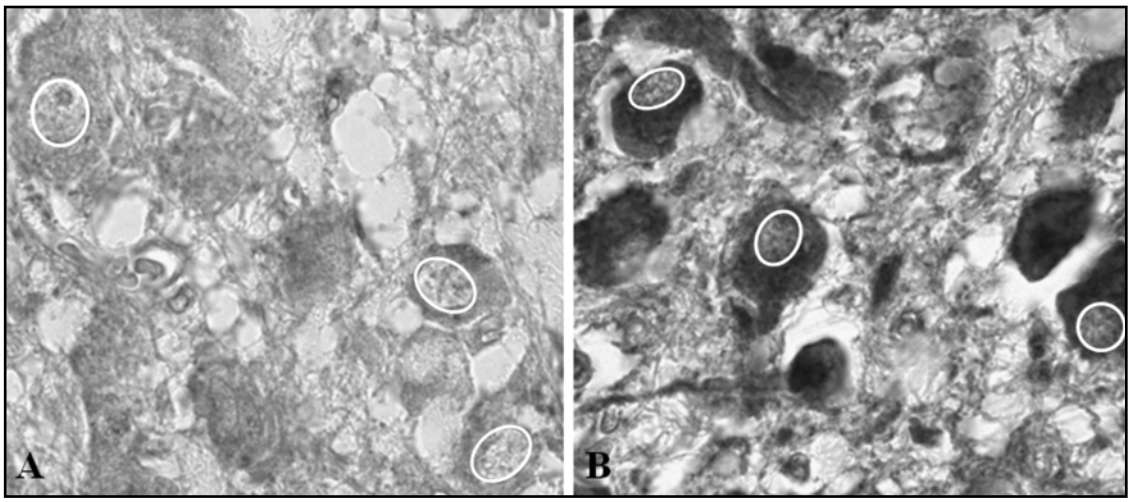

Fig. 1. Microphotographs demonstrating larger nuclear sizes in the human SON with weak immunostaining for TADDI (A) as compared to the SON neurons with strong immunoreactivity for TADDI (B) in 60 year old AD (A) and control (B) women. Original magnification x630.

Tab. 2. TADDI immunoreactivity in control cases with and without pneumonia/respiratoty insufficiency (mean±SEM).

Pneumonia/respiratoty insufficiency status	+ (17 cases)	– (40 cases)
% of the SON area covered by TADDI immunoreactivity	3,97±0,6	2,75±0,4

Tab. 3. Neuronal nuclear and perikaryal profile areas in the human supraoptic nucleus in relation to the wtERα in control cases (mean±SEM).

Groups	Nuclear profiles, μm²		Perikaryal profiles, μm²	
	Negative or weak staining	Moderate and strong staining	Negative or weak staining	Moderate and strong staining
Control cases, n=11	68,4±5,3[1]	92,4±6,2[1]	273,3±31,1[2]	312,9±25,5[2]

1 – $p < 0{,}001$; **2** – $p < 0{,}01$.

a trend towards statistical significance (p=0,07-0,08). Interestingly, this slight SON neuronal activation in AD was apparent in case of moderate or strong staining for TADDI. The differences in nuclear and perikaryal profile areas between AD and age-matched controls were completely absent if TADDI immunoreactivity was absent or weak. Independently of the intensity for TADDI staining, SON neuronal nuclei in AD women were about 30% smaller than in the age-matched control women (p<0,05).

DISCUSSION

In the present study we found for the first time that the size of SON neuronal nuclei and perikarya indicative of the neuronal metabolic activity may depend on the presence of ERα splice variants. To our knowledge, this is the first study to demonstrate the effect of ERα splice variants on human neurons ex vivo. Both SON neuronal nuclei and perikarya were larger when TADDI immunoreactivity was low or absent. This observation clearly points to the inhibitory role of this ERα splice variant on SON neuronal metabolic activity and plasma AVP synthesis. In our previous functional study in HeLa cells TADDI showed the presence of (17β)-estradiol-stimulated transcriptional activity and demonstrated pronounced dominant negative function (Ishunina *et al.* 2013). In the present study this inhibitory effect was confirmed in the human brain. It should be noted that TADDI was not related to the menopausal changes in the SON neuronal metabolic activity (Ishunina and Swaab, 2009). However, its diminished amount in the SON of AD cases (predominantly women) (Ishunina and Swaab, 2009) could be attributed to the larger AVP neuronal size. We found that TADDI was related to decreased morphometric parameters of SON neurons, a result that was largely independent of gender, age or AD. This effect of TADDI is opposite to that of the canonical ERα since nuclei and perikarya were larger in SON neurons with strong and moderate nuclear wt ERα staining as compared to neurons that did not contain wt ERα. It should be noted here that the presence of the wt ERα was also associated with larger neuronal nuclei and perikarya in the human medial mamillary nucleus (MMN) (Ishunina *et al.* 2019).

When SON neuronal nuclear and perykaryal profile areas were compared between AD patients and all control subjects, AD-related activation of SON neuronal metabolic activity was noted. It was also suggested by other researchers that the SON vasopressinergic neurons are generally not affected by AD-related pathology, such as amyloid plaque or neurofibrillary tangles and may even show some activation as judged from the increased nucleolar and Golgi apparatus size (Fliers *et al.* 1985; Lucassen *et al.* 1994; Baloyannis *et al.* 2015). The association of increased neuronal metabolic activity with the lower expression of AD-related neuropathology was also reported in the MMN of AD and vascular dementia patients (Ishunina *et al.* 2019), in the nucleus basalis of Meynert of patients with mild cognitive impairment (Dubelaar *et al.* 2014) and was apparent as the UP-DOWN pattern of some 800 genes that showed contrasting expression changes from upregulation in earlier Braak stages to downregulation in advanced AD (Bossers *et al.* 2010). To note, this list included, among others, genes involved in ribosome biogenesis and assembly that are clearly related to the neuronal nuclear size studied in the present and previous work. Although the AVP gene was suggested to play a role in dementia development in Down syndrome and AD (Labudova *et al.* 1998), no change in AVP mRNA levels were detected in the SON of AD and age-matched controls (Lucassen *et al.* 1997). It is also unclear whether minor SON AVP neuronal activation in AD could be reflected in AVP plasma levels and/or AVP-related kidney function (Albert *et al.* 1995).

Thus, the main conclusion of this study is that TADDI is associated with a clear decrease in morphometric parameters of SON neurons while canonical ERα mediates stimulatory effects as suggested earlier based on gender- and age-related differences in the ERα immunoreactivity. Consequently, people expressing TADDI splice variant in the SON will be expected to have lower plasma vasopressin levels and larger volumes of less concentrated urine.

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STATEMENT OF ETHICS

Donors provided informed consent for brain autopsy and for the use of material and clinical data for research purposes in compliance with national ethical guidelines. The NBB autopsy procedures were approved by the Ethical Committee of the VU University Medical Center in Amsterdam, the Netherlands. The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki.

DISCLOSURE STATEMENT

The authors have no conflicts of interest to declare.

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AUTHOR CONTRIBUTIONS

Dr. T.A. Ishunina performed immunocytochemical staining, morphometric and statistical analysis and wrote the paper. Prof. Dr. D.F. Swaab offered the hypothalamic tissues and contributed to the data analysis and writing the manuscript.

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