Research Article

Neurofibrillary Pathology in the Infundibular Nucleus in Relation to Age and Abnormal Hormone Levels

Patologi Neurofibrilar pada Nukleus Infundibularis Terkait Usia dan Kadar Hormon Abnormal

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Abstract

Objective : To determine whether the decline of testosterone during ageing would make this nucleus more vulnerable for NF changes (i.e.hyperphosphorylated-tau) in men, or that the decline of estrogens in the postmenopausal period would protect the infundibular nucleus in women.

Methods : TWe investigated the infundibular nucleus in postmortem subjects. Brain materials obtained from 29 subjects in the Netherlands Brain Bank were further classified as control subjects and subjects with abnormal hormone conditions. Procedures consisted of tissue collection, immunochemical staining, and analysis of the staining intensity. Results then were collected and concluded using observational methods.

Results : Elderly male subjects with low testosterone conditions showed more severe NF changes in the infundibular nucleus than postmenopausal women. The occurrence of NF changes in elderly subjects was generally accompanied by the presence of basket-like nerve terminals staining for ERβ.

Conclusions : The sex difference in NF changes in the infundibular nucleus in the elderly is due to hyperphosphorylated-tau induction in low testosterone and ageing condition in men, while in postmenopausal women the declining estrogen levels seem to protect against NF changes in this brain area.

Keywords : ageing, estrogen, hyperphosphorylated-tau, infundibular nucleus, testosterone

Abstrak

Tujuan : *Untuk menentukan apakah penurunan level testosteron selama proses penuaan menyebabkan nukleus infundibularis menjadi lebih rentan terhadap perubahan neurofibrilar (NF) (misalnya hyperphosphorylated-tau) pada laki-laki atau apakah penurunan level estrogen selama masa pasca-menopause memiliki efek protektif terhadap nukleus infundibular pada perempuan.*

Metode : *Peneliti memeriksa nukleus infundibular pada subjekpost-mortem. Materi berupa jaringan otak dari 29 subjek dari Netherlands Brain Bank lebih lanjut diklasifikasikan sebagai subjek kontrol dan subjek dengan kondisi hormon abnormal. Prosedur terdiri dari pengumpulan jaringan, pewarnaan dengan teknik imunohistokimia, dan analisis dari intensitas pewarnaan. Hasil yang didapat kemudian dikumpulkan dan disimpulkan sesuai dengan metode observasional.*

Hasil : *Subjek laki-laki lanjut usia dengan testosteron rendah menunjukkan perubahan NF yang lebih buruk pada nukleus infundibular dibandingkan dengan perempuan postmenopause. Kejadian perubahan NF pada subjek lanjut usia secara umum diikuti oleh munculnya pewarnaan pada ujung saraf berbentuk basket-like yang positif untuk Erβ.*

Kesimpulan : *Perbedaan jenis kelamin terkait perubahan NF pada nukleus infundibular pada subjek lanjut usia terjadi akibat induksi hiperfosforilasi tau pada kondisi testosteron yang rendah yang dikombinasi oleh proses penuaan pada pria. Sedangkan pada perempuan pascamenopause, penurunan level estrogen menunjukkan efek protektif terhadap perubahan NF pada area otak ini.*

Kata kunci : *estrogen, hiperfosforilasi protein tau, nukleus infundibularis, penuaan, testosteron.*

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INTRODUCTION

The infundibular nucleus (arcuate nucleus) of the hypothalamus is considered to be the central site of regulation of the Hypothalamus Pituitary Gonadal (HPG) axis and metabolism.[1] Hyper-phosphorylated tau-containing neurofibrillary (NF) pathology, which is often observed in the infundibular nucleus of the hypothalamus of Alzheimer's disease patients, is also present in the hypothalamus of cognitively intact elderly subjects. This NF pathology shows a striking sex difference: it is almost exclusively present in the infundibular nucleus of cognitively intact older men and occurs only rarely in cognitively intact elderly women.[2]

In postmenopausal women, a subset of neurons in the infundibular nucleus becomes strongly activated, as indicated by an increased soma size, larger nuclei containing nuclear spheroids, larger and multiple nucleoli, and increased Nissl substance. In postmenopausal women and in young subjects with a surgical menopause an increased gene expression was found in infundibular nucleus neurons for estrogen receptor (ER), neurokinin-B (NKB), substance-P (SP), and gonadotropin-releasing hormone (Gn-RH). A series of observations strongly suggest that the loss of inhibitory feedback of estrogens on the hypothalamus causes this increased neuronal hyperactivity in postmenopausal women. In addition, some hypertrophied neurons, but to a much lesser degree than in women, were observed in older men.[3] In our previous study in the infundibular nucleus, a shift was observed from a more nuclear localization of ERα in young females to more cytoplasmic localization of ERα in non-demented postmenopausal women.[4] This shift in ERα localization was accompanied by a relative absence in the expression of NF pathology, i.e. hyperphosphorylated-tau stained by AT8, and was considered to be another sign of neuronal activation. Therefore, we considered the sex difference in NF pathology as one of the many examples of neurons that were highly active or strongly activated in the elderly and seemed to be protected against the occurrence of NF pathology, a phenomenon we described as "use it or lose it".[5]

In non-demented older men, only a small increase in cytoplasmic ERα was found, accompanied by NF pathology in the infundibular nucleus. In addition, the occurrence of NF pathology in non-demented older men was accompanied by the presence of more ERβ basket-like nerve terminals in the infundibular nucleus.[4]

In order to determine whether the gradual diminishment of testosterone in men during aging would induce NF pathology or the strong decline of estrogens in postmenopausal women would protect the infundibular nucleus neurons against NF pathology, we investigated this nucleus in postmortem material of patients with abnormal hormone conditions.

METHODS

Tissue collection

Postmortem material was obtained from the Netherlands Brain Bank (coordinator Dr. Rivka Ravid). Permission was obtained for a brain autopsy and the use of brain material and clinical information for research purposes. Hypothalami of 13 subjects with abnormal circulating gonadal steroids levels, i.e. castrated, estrogen-treated male-to-female (MF) transsexuals; an ovariectomized testosterone-treated female to male (FM) transsexual; castrated prostate cancer patients; an ovariectomized woman; a subject with complete androgen insensitivity syndrome (CAIS); a subject with an estrogen-producing adrenal tumour, a subject with an androgen-producing adrenal tumour (Table. 2), and 16 age and sex-matched control subjects (Table. 1) were studied immunocytochemically and estimated semi-quantitatively. None of the subjects suffered from a primary neurological or psychiatric disease. All the brains were investigated systematically by a neuropathologist.[6] The distribution of the Alzheimer neurofibrillary changes over the brain was estimated according to the stages of Braak and Braak. Six stages of disease propagation can be distinguished with respect to the location of the intraneuronal cytoskeletal changes stained by AT8, i.e. neuropil threads and neurofibrillary tangles, and the severity of changes in the hippocampal formation, in the transentorhinal and entorhinal regions and the adjoining temporal isocortex.[7] The Braak stages I-II, clinically silent cases, are characterized by the formation of neurofibrillary changes limited to

the transentorhinal region. The Braak stages III-IV, incipient AD, are characterized by the severe changes found only in a few allocortical regions and adjoining areas. Stage III reveals the striking destruction of layer Preα within both the entorhinal and transentorhinal regions and is accompanied by mild changes in the hippocampus and the virtual absence of neocortical lesions. At stage IV, additional changes are found in the deep layer of Priα. The Braak stages V-VI, fully developed in Alzheimer's disease, are characterized by the destruction of neocortical association areas.[8]

The hypothalami were formalin-fixed, paraffin-embedded, and cut serially in 6 µm coronal sections. For anatomical orientation, every 100th section was mounted on chrome-alum sulphate-coated glass slides, deparaffinized, hydrated, and stained with thionine (0.1% w/v thionine in acetate buffer, pH 4). The location of the infundibular nucleus was determined based on the human brain atlas[9], and if necessary, with the help of neuropeptide Y (NPY) immunocytochemical staining.[1] The rostral border of the infundibular nucleus was identified at the level where the nucleus showed its characteristic arcuate shape; the cell-sparse zone separating the infundibular nucleus from the ventromedial nucleus (VMN) indicated the dorsolateral border; the ependymal layer of the third ventricle served as the medial border, and the mamillary bodies were taken as the caudal border. Three series of sections per subject were taken from rostral to caudal at approximately 25%, 50% and 75% of the length of the infundibular nucleus, and mounted onto Super-Frost plus (Menzel, Braunschweig, Germany) slides for immunocytochemistry. We took adjacent sections for estrogen receptor (ER)α, ERβ and hyperphosphorylated-tau protein (AT8) immunocytochemical staining. In addition, we also determined the expression of AT8 staining in other areas in the hypothalamus adjacent to the infundibular nucleus, such as the VMN, the nucleus tuberalis lateralis (NTL), the nucleus basalis of Meynert (NBM), and the tuberomammillary nucleus (TMN).

Immunocytochemistry and specificity of the antisera

A polyclonal rabbit anti-ERα antiserum (MC-20) that recognizes the carboxyl-terminus epitope of the ERα (Santa Cruz Biotechnology, Inc., catalogue no. sc-542) and a polyclonal goat anti-ERβ antiserum (N-19), directed against an amino acid sequence mapping at the amino-terminus of human ER β (catalogue no. sc-6820, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used in the present study. The staining procedures and specificity tests for ERα and ERβ antisera have been previously described extensively [10-11]. No staining was observed after omitting the MC-20 antiserum or after adsorption of MC-20 to its blocking peptide.[10] In a spot blot test, MC-20 recognized its blocking peptide on nitrocellulose paper by showing the expected concentration gradient (Santa Cruz Biotechnology; blocking peptide, catalogue no. sc-542, lot no.C059). In addition, two different anti-ERα antisera, C-314 (N-terminus directed; Santa Cruz Biotechnology; Catalog no. sc-786; anti-bovine ERα; lot no.J278) and MC-20 (C-terminus directed) displayed similar distribution patterns in the human hypothalamus. Western blot with the ERα antiserum MC-20 on human hypothalamic tissue showed a specific band around the expected 68 kDa, with no such band around the 54 kDa of ERβ.[11] Western blot with the ERβ antiserum N-19 on human hypothalamic tissue recognized a protein band around expected 54 kDa weight and did not recognize the 68 kDa protein band i.e. ERα.[12] In spot blots it was also confirmed that the antiserum N-19 recognizes the homologous blocking peptides, while an adsorption test with the homologous peptide resulted in elimination of the staining.[10] Moreover, staining of adjacent sections with the antiserum against the C-terminus of the ERβ (L-20, Santa Cruz Biotechnology, Inc, catalogue no. sc-6822)[13] revealed the same staining pattern as the antiserum against the N-terminus of ERβ used in the present study[10]. ERβ cytoplasmic staining was observed in granulosa cells, and follicles of the human ovary, a localization which is consistent with a study in the rat.[14] In human testis, Leydig and connective tissue cells showed nuclear ERβ staining, which is also in agreement with a study in the rat.[15] The differences in distribution shown by the ERα antiserum MC-20 and the ERβ antiserum N-19 in the hypothalamus, pituitary, ovary and testis, as described extensively by [10-11]series of observations demonstrated that the ERα and β antisera used in our study were specific.

For immunocytochemical staining of hyper-phosphorylated-tau, a primary monoclonal antiserum AT8 directed against the phosphorylated-tau

epitopes serine 202 and threonine 205 was used.[16] This antiserum was used to recognize hyperphosphorylated-tau as an early marker for the neurofibrillary AD pathology. The staining procedure was performed.[2]

Earlier studies showed that the variability in fixation and postmortem time does not influence the staining of ERα, Eβ or AT8.[4]

Analysis of the staining intensity

Two independent investigators, blind to the subject's condition, judged the staining intensity of the sections. The staining intensity of ERα and β in the cytoplasmic and nuclear compartment was estimated semi-quantitatively by means of light microscopy, based on the number of stained neuron and basket-like nerve terminals in the infundibular nucleus, and graded according to the following scale: (+++) strong, (++) moderate, (+) weak, (+/-) very weak and (-) absent, according to our previous studies.[16] The semi-quantitative estimation for neuropathological changes in AT8-staining was judged according to the number of stained neurons and neuropil threads in the infundibular nucleus and graded as the following scale: (++++) severe, (+++) marked, (++) moderate, (+) mild, (0) no discernible changes as described.[2]

Control subjects

Female controls did not show NF pathology as stained by AT8 in the infundibular nucleus, while mild changes were only observed in the two oldest males (#15, #16). Staining of hyperphosphorylated-tau was absent in other hypothalamic or adjacent brain areas in controls.

Hypertrophied neurons and nuclear spheroid-containing neurons were present in two elderly female controls (#6, #7), while fewer of such changes were observed in another elderly female (#8) and the two oldest male controls (#15, #16; Figure. 2). Using adjacent sections, we observed that the hypertrophied neurons in the two oldest male controls (#15, #16) did not show NF pathology.

Cytoplasmic ERα was observed more often in the infundibular nucleus of female controls than in male controls, while more nuclear ERα was observed in male controls than in female controls. Only weak staining of both nuclear and cytoplasmic ERβ was observed in the infundibular nucleus of male and female controls (Figure. 1). Basket-like ERβ-staining was observed more in males than in females.

RESULTS

Table 1. AT8, ERα and ERβ Staining in the Infundibular Nucleus of Control Subjects

NBB	Age	Sex	Bw(g)	Pmd(h)	Fix(d)	BS	AT8	ERα (INF)		ERβ (INF)			Cause of death
								N	C	N	C	B	
86032	33	F	1035	41.00	20	0	-	2+	-	+	+	+	AdenoCa metastases to the brain
80008	35	F	1200	08.00	26	0	-	3+	-	-	+	+	Acute lymphoblastic leukaemia
96423	49	F	1253	<17.00	806	0	-	±	2+	2+	+	-	Massive thromboembolism
98125	58	F	991	06.15	41	I	-	+	2+	-	±	-	Multiple organ failure
98035	65	F	ID	<20.00	31	0	-	+	+	-	+	-	Mesenteric ischemia
99085	69	F	1102	<02.30	120	0	-	±	2+	-	+	-	Uremia
93139	78	F	1135	06.25	32	0	-	-	2+	±	+	+	Bronchopneumonia
96084	78	F	1330	07.30	26	II	-	2+	+	-	±	-	Pulmonary emphysema
-	+	2+	-	±	-								
98121	47	M	1420	<82.30	31	0	-	±	±	-	+	-	Cardiac arrest
97159	48	M	1500	05.30	42	0	-	2+	±	-	±	2+	Multiple organ failure
93072	50	M	1573	<09.00	52	0	-	+	+	-	+	+	Hypovolemic shock
97139	59	M	1400	<65.45	180	0	-	±	+	-	±	-	Pulmonary embolism
98122	66	M	1461	<41.00	49	0	-	2+	+	-	+	+	Septic shock
96426	69	M	1222	14.00	728	0	-	2+	2+	±	±	-	Septic shock
97116	80	M	1380	06.56	33	0	+	3+	+	+	±	-	Pulmonary emphysema
94076	78	M	1442	08.25	24	II	+	2+	+	-	±	2+	Myocardial infarction
-	+	2+	-	±	-								

Abbreviations: AdenoCa: adenocarcinoma, AT8: specific staining for hyperphosphorylated-tau, B: basket-like, BS: Braak score, Bw: brain weight (in grams), C: cytoplasmic staining, ER: estrogen receptor, F: female, Fix: fixation time (in days), ID: incomplete data, INF: the infundibular nucleus, N: nuclear staining, NBB: Netherlands Brain Bank number, M: male, Pmd: postmortem delay (in hours).

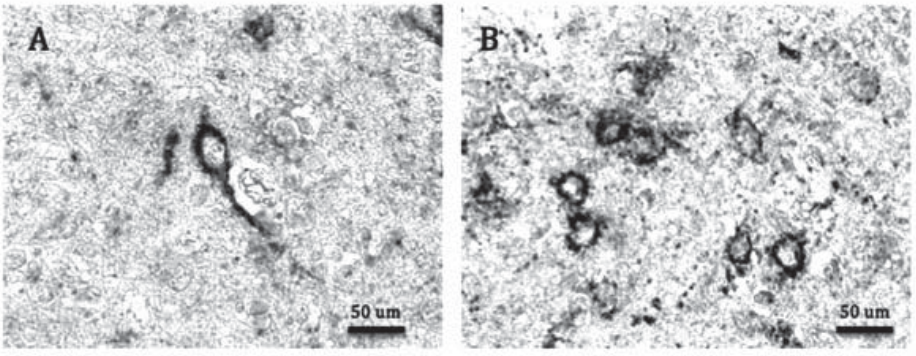

Figure 1. Photomicrograph depicting basket-like nerve terminals containing estrogen receptor (ER)β immunoreactivity (IR) in the infundibular nucleus. Hypogonadal prostate cancer patients (subject #20)(B) showed more of such baskets with a higher intensity of ERβ-IR than elderly male controls (subject #16)(A). Scale bar: 50 µm.

Subjects with abnormal hormone conditions

All subjects that underwent castration because of prostate cancer showed AT8 positive staining in the infundibular nucleus, independent of whether they were treated or not treated with anti-androgen. Five patients in this group (#17, #18, #19, #20, #21) showed more severe NF pathology than observed in elderly male controls (Figure. 2). Two of these patients (#17, #21) also showed hyperphosphorylated-tau in other hypothalamic and adjacent brain areas, i.e. the ventromedial nucleus (VMN), the nucleus tuberalis lateralis (NTL), the nucleus basalis of Meynert (NBM) and the tuberomammillary nucleus (TMN) (Table. 2).

The subject with complete androgen insensitivity syndrome (CAIS) (#22), who had received estrogen substitution, did not show NF pathology in the infundibular nucleus. The 74-year-old male-to-female (MF) castrated, and estrogen-treated transsexual (#25) had moderate AT8-staining in the infundibular nucleus, whereas in two younger MF transsexuals (#23, #24) the presence of hyperphosphorylated-tau was not observed. A female to male (FM) transsexual subject (#27) of 51 years of age who had been treated with testosterone showed no AT8-staining. Negative AT8-staining in the infundibular nucleus was also observed in the 46-year-old ovariectomized female (#26), in a 46-year-old female with an androgen-producing adrenal tumour (#28) and in a 31-year-old male with an estrogen-producing adrenal tumour (#29).

An absent to weak staining of nuclear ERα and a weak to moderate staining of cytoplasmic ERα were observed in prostate cancer patients, MF transsexual subjects, ovariectomized woman and an FM transsexual. A moderate nuclear ERα and a very weak to moderate cytoplasmic ERα-staining were observed in the CAIS subject and in subjects with sex steroid-producing adrenocortical carcinoma.

All subjects with abnormal hormone conditions showed an absent to very weak nuclear ERβ-staining and a very weak to weak cytoplasmic ERβ-staining.

Subjects with low levels of testosterone showed relatively more basket-like ERβ than elderly control subjects (Figure. 1).

The occurrence of hyperphosphorylated-tau in a subset of neurons in the infundibular nucleus of elderly males in low testosterone conditions was generally accompanied by the presence of basket-like ERβ in nerve terminals (#18, #19, #20, and #25).

The hypertrophied neurons were observed more in elderly castrated prostate cancer patients than other subjects with abnormal hormone conditions. These neurons showed histological signs of hyperactivity, i.e. larger cell size, a larger nucleus and nucleolus compared to the surrounding neurons and the presence of nuclear spheroid bodies. They were observed easily, either localized inside or outside basket-like ERβ nerve terminals. Remarkably, these hyperactive neurons never showed NF pathology. Further details regarding subjects' clinical and endocrinology characteristics are presented in Table 3.

Table 2. AT8, ERα and ERβ Staining in the Infundibular Nucleus and other Brain Areas of Subjects with Abnormal Hormone Conditions

NBB	Diagnosis	Age	Sex	Bw(g)	Pmd(h)	Fix(d)	BS	AT8 (INF)	AT8 in NTL, TMN, VMN, NBM	ERα (INF)		ERβ (INF)			Assumption androgen status as compared to controls	Assumption estrogen status as compared to controls
										N	C	N	C	B		
89103	Prostate cancer	67	M	1290	24:00	28	ID	2+	NTL(2+), TMN(2+), VMN(2+), NBM(2+)	-	+	-	±	±	↓	↓
97157	Prostate cancer	69	M	1475	05:55	45	0	4+	-	±	+	±	+	2+	↓	↓
95062	Prostate cancer	80	M	1400	04:30	24	II	4+	-	±	+	-	±	2+	↓	↓
94109	Prostate cancer	82	M	1110	05:35	32	II	4+	-	±	+	-	±	3+	↓	↓
94090	Prostate cancer	86	M	1663	03:00	93	II	3+	TMN(+), VMN(+), NBM(2+)	+	2+	-	±	+	↓	↓
'02089	CAIS	75	M	1484	06:30	34	I	-	-	2+	2+	-	±	-	↓	↑
84020	MF Transsexual	50	M	1380	ID	30	0	-	-	±	-	-	±	±	↓	↑
93070	MF Transsexual	53	M	1500	<100	34	0	-	-	-	±	-	±	±	↓	↑
98141	MF Transsexual	74	M	1118	06:35	33	I	2+	-	±	2+	-	+	2+	↓	↑
80002	Surgical menopause	46	F	1300	02:30	36	0	-	-	-	+	-	±	-	↓	↓
98138	FM Transsexual	51	F	1171	04:15	32	0	-	-	+	+	-	+	±	↑	↓
83004	Androgen-producing adrenal tumour	46	F	1360	<10:50	34	ID	-	-	2+	±	-	±	+	↑ (measured)	(*)
91005	Estrogen-producing adrenal tumour	31	M	1377	<34:00	35	ID	-	-	2+	+	-	±	2+	Slightly ↑ (measured)	↑ (measured)

Abbreviations: AT8: staining for hyperphosphorylated-tau, B: basket-like, BS: Braak score, Bw: brain weight (in grams), CAIS: complete androgen insensitivity syndrome, C: cytoplasmic staining, ER: estrogen receptor, F: female, FM: female to male transsexual, Fix: fixation time (in days), ID: incomplete data, INF: the infundibular nucleus, N: nuclear staining, NBB: Netherlands Brain Bank number, M: male, MF: male to female transsexual, NBM: the nucleus basalis of Meynert, NTL: the nucleus tuberalis lateralis, ORX: orchidectomy, OVX: ovariectomy, Pmd: postmortem delay (in hours), TMN: the tuberomammillary nucleus, VMN: the ventromedial nucleus, (*): cannot be assumed.

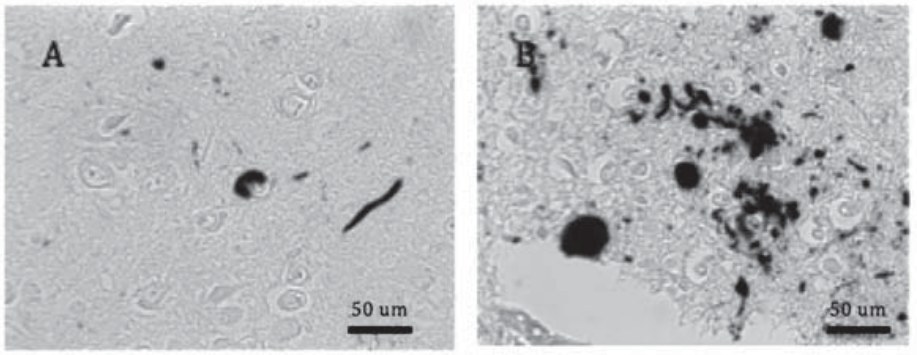

Table 3. Clinical and Endocrine History of Patients with Abnormal Hormone Conditions

Case	No.	Age (yr)	Age of hormonal treatment (yr)	Age of Orchid Ovari-ectomy (yr)	Clinical data and endocrine history	Cause of death
Prostate cancer patients	#17	67	-	67	Orchidectomy 3 months before death, the patient did not receive anti-androgen treatment	Carcinoma of the pancreas with metastases; cachexia
	#18	69	67	67	Orchidectomy 3 years before death, anti-androgen, anandron 150mg 1 dd during three years before death	Prostate cancer with advances metastases
	#19	80	-	75	Orchidectomy 5 years before death, the patient did not receive anti-androgen treatment	Renal insufficiency
	#20	82	-	82	Orchidectomy 20 years before death, the patient did not receive anti-androgen treatment	Prostate cancer, respiratory failure, renal insufficiency
	#21	86	85	85	Memory problem started six years before death. Diagnosis at hospitalization: subcortical dementia. Neuropathological diagnosis: slight alzheimerization, Braak for tangle = II. Orchidectomy 1 year before death. The patient received CPA, anti-androgen, (50mg 4 dd) during the first 14 months, (50 mg 2 dd) during the last six months	Prostate cancer, lung cancer and septic shock
CAIS	#22	75	70	55	Orchidectomy 20 years before death, the patient received 17β-estradiol (2mg 1 dd) during the last five years before death and stopped two months before death.	Advance state of squamose cell vagina carcinoma

MF-trans sexuals	#23	50	42	44	Age 42: stilbestrol (5 mg 1 dd); after 2 months to (5 mg 2 dd); age 44: CPA (50 mg 2 dd); treatment lasted 4 years; stopped 2 years before death; ethinylestradiol (50 µg 2 dd); treatment lasted 8 years until death	Suicide
	#24	53	40	50	Age 40: stilbestrol treatment (stopped after 1 yr); at age 43–47: Premarin (0.625 mg dd); at age 47–50: Premarin (3.75 mg dd); at age 50–53: Premarin (2.5 mg 3 dd); CPA (50 mg 1 dd); topical estrogen cream (estrogen treatment stopped 3 months before death)	Acute fatty liver due to alcohol abuse
	#25	74	64	64	Age 64: received CPA treatment (50 mg 2 dd) and ethinyl estradiol (50 µg 2 dd) treatment; at age 67: received Estraderm (100 µg 1 dd); at age 74 received spironolactone (50 mg 1 dd) and Estraderm (100 µg 1 dd)	Coma post-appendicitis, pneumonia, lung embolism, and cerebral occipital infarction
Surgical Menopause	#26	46	-	45	Bilateral ovariectomy 22 months before death, the patient did not receive hormone treatment	Septicemia and ovarian cancer
FM-transsexual	#27	51	27	32	Bilateral ovariectomy at age 32. At age 27 testosterone, Sustanon (250 mg), twice a month injections; at age 30 testosterone undecanoate (40 mg 3 dd); at age 34 testosterone undecanoate (40 mg 2 dd); at age 36 testosterone undecanoate (40 mg 4 dd); at age 44 testosterone, Sustanon (250 mg) twice a month injections; at age 47–48 testosterone, Sustanon (250 mg) every 3 weeks. No testosterone replacement therapy during the last three years before death	Cachexia
Androgen-producing adrenal tumour	#28	46	-	-	Female patient with a virilizing adrenocortical carcinoma for 1 yr that produced high levels of cortisol, androstenedione, and testosterone levels; latest androstenedione serum level before death was 48.0 ng/ml (normal range for women 0.4–3.5 ng/ml); the latest serum testosterone level before death, 26.82 nmol/L (normal range for women is 1.04–3.30 nmol/L).	Adrenocortical carcinoma; postoperative haemorrhage
Estrogen-producing adrenal tumour	#29	31	-	-	Male patient with the recurrent of feminizing adrenocortical carcinoma for 3 yr that produced high levels of DHEA-S, DHEA, 17-hydroxyprogesterone, and estradiol levels; the latest estradiol serum levels 1 yr before death was around 689-732 pmol/L (normal range for men is 50-200 pmol/L); the latest testosterone levels 1 year before death was around 28.9-41.3 nmol/L (normal range for men is 10-30 nmol/L)	Advance metastasis of recurrent adrenocortical carcinoma

Abbreviations: CAIS: complete androgen insensitivity syndrome, CPA: cyproterone acetate, DHEA: dehydroepiandrosterone, DHEA-S: dehydroepiandrosterone-sulphate, No: numbers corresponding with table 2.

DISCUSSION

The infundibular nucleus, a key structure in the regulation of reproduction and metabolism[1] shows remarkable neurofibrillary (NF) changes in cognitively intact subjects (with Braak stage 0-II). The NF pathology in the infundibular nucleus is characterized by neurofibrillary tangles, a network of neuropil threads and terminal-like portal vessel-associated processes. This NF pathology shows a striking sex difference. From 60 years onwards the prevalence of neurofibrillary changes in the infundibular nucleus of cognitively intact elderly males rises from 20% up to 90% around the age 80-85 years, while in only 6-10% of cognitively intact elderly females such changes were observed.[2, 4] These sexually dimorphic NF alterations should be related to changes in reproduction and metabolism, rather than to cognitive deficits, as observed in AD in other brain areas. Earlier we found, e.g. more NF alterations in the nucleus basalis of Meynert (NBM) in AD women as compared to AD men[17], a difference that may be related to cognition and a sex difference in this disease.

In postmenopausal women, the abrupt decline in the circulating estrogen levels[18] and diminished negative feedback of estrogens on the infundibular nucleus neurons[19] is accompanied by a strong activation of neurons in this brain area as shown by neuronal hypertrophy and increased amounts of estrogen receptors (ER), neurokinin B (NKB) or substance P (SP) gene transcripts in this nucleus.[3] In older men more gradually decreasing plasma testosterone levels are observed[20] accompanied by a lesser degree of neuronal hypertrophy.[8] We hypothesized that the activation of infundibular nucleus neurons that is most pronounced in women would make this sub-population of neurons less vulnerable to the process leading to NF pathology.[4]

The present study indicates that the sex difference in NF changes in the infundibular nucleus in older adults may be due to hyperphosphorylated-tau induction in older men as a result of low testosterone levels. The gradual decline of testosterone in the course of normal aging[20], as also assumed to be present in two elderly male controls (#15, #16), was accompanied by a mild to moderate NF pathology in the infundibular nucleus, confirming earlier studies.[2, 4] In elderly prostate cancer patients, the strong and abrupt decline of serum testosterone following orchidectomy (#18, #19, #20, #21) was accompanied by a much stronger NF pathology in the infundibular nucleus as compared to controls. This NF pathology occurred independently of the fact whether subjects did (#18, #21) or did not (#17, #19, #20) receive anti-androgen treatment following orchidectomy (Tables. 2, 3). Hyperphosphorylated-tau as stained by AT8 was also observed in other brain areas in low testosterone conditions such as the ventromedial nucleus (VMN), the nucleus tuberalis lateralis

(NTL), the nucleus basalis of Meynert (NBM) and the tuberomammillary nucleus (TMN) (#17, #21) and was accompanied in one patient (#20) with subcortical dementia symptoms, suggesting that low testosterone levels may be involved in the development of NF pathology in cognition-related area in the hypothalamus, i.e. NBM. From our ongoing study, AT8 positive staining was also observed in the infundibular nucleus in two Prader-Willi syndromes (PWS) subjects, a 49-year-old male and a 64-year-old female, who generally have low estrogen and testosterone levels, and hypogonadism since early life.[21] In addition, NF pathology was present in the infundibular nucleus of a 74-year-old castrated-MF transsexual subject, who was treated with a combination of estrogens and cyproterone acetate. This also suggests, but of course does not prove, that estrogens do not protect against the formation of NF changes in this brain area.

The observation that two relatively young male orchidectomized patients (#23, #24) did not show any NF pathology in the infundibular nucleus, in contrast to the six elderly castrated men, suggests that the process of ageing is another requirement for the formation of NF pathology. The importance of age in the development of NF pathology in the infundibular nucleus is reinforced by the increase in this neuropathology in the course of normal aging in men[2,4], which is accompanied by a gradual decline of testosterone levels[20] and by the mild NF pathology that was present in the 2 elderly control subjects in the present study (#15, #16). Indirect evidence that lower circulating androgen levels were present in the older men, as compared to the younger ones, comes from the observation that less staining for androgen receptors was observed in the elderly patients in the medial mamillary nucleus. This hypothalamic area is very sensitive to circulating androgen levels as reported earlier.[22] In Kreb's cycle, mitochondria use testosterone for enzymatic reactions in aerobic metabolism and lead to the production of high-energy phosphate compounds.[23] In addition, aging is also related to mitochondrial DNA mutation. Since the brain is highly dependent on aerobic metabolism, it suggests that aging and low testosterone levels may reduce energy and metabolic activities of neurons in the infundibular nucleus, thus increase the risk of neurons to have NF pathology.[24]However, the exact contributions for the factors age and low testosterone levels on the formations of NF pathology have to be studied further.

In a previous study, we found that a subset of proopiomelanocortin (POMC) neurons in the infundibular nucleus co-expresses hyperphosphorylated-tau as stained by AT8, both in cognitively intact elderly males and in AD patients.[4] In the rodent, the cellular POMC mRNA content in the arcuate nucleus was significantly lower in old males than in young males.[25] Moreover, the abruptly decreased testosterone levels following castration of adult male rhesus monkeys results in a strong suppression of the mRNA production of POMC neurons.[26] This indicates that indeed both ageing and low testosterone conditions may contribute to reduced metabolic activity of POMC neurons in the hypothalamus and so increase the risk of these neurons to develop NF pathology in males. α-Melanocyte-Stimulating Hormone (MSH), derived by post-translational processing of the POMC gene product, facilitates penile erection in males.[27] The observation that AT8-positive staining in the infundibular nucleus is only found in a subset of α-MSH producing neurons4 points to the possibility that the occurrence of hyperphosphorylated-tau in the infundibular nucleus may be related to the incidence of erectile dysfunction in elderly males.[28]In our present study, low testosterone conditions also seemed to increase the risk for elderly males to get NF pathology in areas which are related to memory and attention (i.e. the NBM and TMN) (#17, #21).

Circulating total testosterone in men results both from testicular Leydig cell secretion and from peripheral conversion of dehydroepiandrosterone (DHEA) and dehydro¬epi¬androsterone sulphate (DHEA-S), that are produced by the adrenals.[29] Approximately 90% of the serum testosterone in men originates from the testes, whereas only 67% of the serum testosterone in women is secreted by the ovaries.[30] Whether there is a change in free testosterone levels in postmenopausal women is a controversial topic in literature. Some cross-sectional studies indicate that menopause is associated with decreased testosterone, both in total and free testosterone levels.[31] The decline in testosterone levels around menopause would occur over the first three years of the postmenopausal period, while the levels would remain relatively

constant over the following five years.[32] However, another longitudinal study reported that the total testosterone levels might remain stable across the menopausal transition, while the amount of bioavailable testosterone would even increase as Sex Hormone-Binding Globulin (SHBG) levels decrease in postmenopausal women.[33] In addition, a drop in testosterone levels during peri-menopause with increasing testosterone levels in the following two years was observed in another study.[18] Moreover, increased levels of both total and bioavailable testosterone were observed in intact postmenopausal women after adjusting for Body Mass Index (BMI). Most of the increase occurred after 50-59 years, reaching premenopausal levels in the 70-79 decade with relatively stable levels thereafter.[34] Concluding, probably no major decline occurs in free testosterone levels in postmenopausal women. Moreover, the infundibular nucleus in men has more intense staining for the Androgen Receptor (AR) than in women[35], which suggests a more pronounced direct effect of androgens on neuronal functioning in men than in women in this brain area.

In a previous study, the shift from more nuclear localization of ERα in young females to more cytoplasmic ERα in the infundibular nucleus neurons in cognitively intact post¬menopausal women were found to be related to neuronal hyperactivity and lower vulnerability of these neurons to develop NF pathology.[4] In the present study, this observation was confirmed in the controls, while there was no consistent shift of ERα localization found in the patients with abnormal hormone conditions.

In a previous study, we observed the occurrence of basket-like nerve terminals staining for ERβ in the infundibular nucleus of older men and AD patients in relation to NF pathology.[4] In the rodent, the cellular POMC mRNA content in the arcuate nucleus was significantly lower in old males than in young males.[25] Moreover, the abruptly decreased testosterone levels following castration of adult male rhesus monkeys results in a strong suppression of the mRNA production of POMC neurons.[26] This indicates that indeed both ageing and low testosterone conditions may contribute to reduced metabolic activity of POMC neurons in the hypothalamus and so increase the risk of these neurons to develop NF pathology in males. α-Melanocyte-stimulating hormone (MSH), derived by post-translational processing of the POMC gene product, facilitates penile erection in males.[27] The observation that AT8-positive staining in the infundibular nucleus is only found in a subset of α-MSH producing neurons[4] points to the possibility that the occurrence of hyperphosphorylated-tau in the infundibular nucleus may be related to the incidence of erectile dysfunction in elderly males.[28]In our present study, low testosterone conditions also seemed to increase the risk for elderly males to get NF pathology in areas which are related to memory and attention (i.e. the NBM and TMN) (#17, #21).

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In the present study, the occurrence of hyperphosphorylated-tau in elderly hypogonadal men was, however, generally found to be accompanied by the presence of basket-like nerve terminals staining for ERβ in the infundibular nucleus. Our ongoing study showed that such basket-like ERβ containing terminals might co-express Glutamic-Acid Decarboxylase (GAD) immunoreactivity as a marker for the inhibitory transmitter γ-aminobutyric acid (GABA).[36] The neurons inside the ERβ staining basket tended to be larger and to have a larger nucleus and nucleolus than the surrounding neurons, which suggests that the neuron inside a basket is strongly activated and remains free of NF changes in the middle of NF neuropathology, as we also observed in AD patients.[4] We hypothesized, therefore, that ERβ-mediated inhibition of the GABAergic terminals may induce an increased activity of the neurons inside the basket and thus prevent the formation of hyperphosphorylated-tau in this subpopulation of neurons, which is in line with the phenomenon we described as "use it or lose it".[5]

CONCLUSION

In conclusion, the sex difference in NF changes in elderly controls may be due to hyperphosphorylated-tau induction by low testosterone conditions such as observed here in the combined conditions of castration and ageing in males. The levels of SHBG reinforce the decrease in serum concentration of free testosterone in older men.[37] Since testosterone substitution may prevent the formation of heat shock-induced hyperphosphorylation of tau in rats[38], further investigation of the efficacy of testosterone substitution therapy for delaying or preventing the occurrence of NF pathology in the hypothalamus of elderly males seems worthwhile. The increased activity of some neurons in the infundibular nucleus of hypogonadal elderly males, probably mediated by surrounding basket-like nerve terminals containing ERβ, is accompanied by an absence of neurofibrillary pathology, even in the middle of NF changes in this nucleus. This phenomenon, which is also present in postmenopausal women[5] indicates a new mechanism in the local prevention of NF pathology.

CONFLICT OF INTEREST

Nil.

ACKNOWLEDGEMENT

Brain material was obtained from the Netherlands Brain Bank Amsterdam (coordinator Dr. Rivka Ravid). The authors want to thank Unga Unmehopa and Bart Fisser for their technical help and advice; Joop van Heerikhuize and Gerben van der Meulen for photography and Wilma T.P. Verweij for secretarial help. Financial support was obtained from the Hersenstichting Nederland, the Internationale Stichting Alzheimer Onderzoek, the Nederlandse Alzheimer Stichting, and from the Research Institute for Diseases in the Elderly, funded by the Ministry of Education and Science and the Ministry of Health, Welfare and Sports, through the Netherlands Organization for Scientific Research (NWO).

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