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THYROID STIMULATING HORMONE - RECEPTOR OVEREXPRESSION IN BRAIN OF PATIENTS WITH DOWN SYNDROME AND ALZHEIMER'S DISEASE

Olga Labudova[1)], Nigel Cairns[2)], Thomas Koeck[3)], Erwin Kitzmueller[1)], Hermann Rink[4)], and Gert Lubec[1)]

[1)] University of Vienna, Dpt of Pediatrics, [2)] Brain Bank, Institute of Psychiatry, London, [3)] University of Vienna, Institute of Medical Chemistry, [4)] University of Bonn, Dpt Radiobiology

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Summary

Thyroid hormone abnormalities are strongly associated with Down Syndrome (DS) with elevated thyroid stimulating hormone (TSH) levels as the most consistent finding. Using subtractive hybridization for gene hunting we found significant overexpression of mRNA levels for the TSH - receptor (TSH-R) in brain of a fetus with DS. Based upon this observation we determined TSH-R protein levels in five brain regions of patients with DS(n=8), Alzheimer disease(AD, n=8) and controls (C, n=8). Western blots revealed significantly elevated immunoreactive TSH-R protein(s) 40 kD and 61kD in temporal and frontal cortex of patients with DS and, unexpectedly, in AD. Levels for the 40kD protein in temporal cortex were 1.00±0.036 (arbitrary units±SD) in C, 1.35±0.143 in DS, 1.52±0.128 in AD; in frontal cortex: 1.00±0.046 in C, 1.10±0.03 in DS, 1.10±0.038 in AD. Levels for the 61kD protein in temporal cortex were 1.01±0.015 in C, 1.47±0.013 in DS, 1.623±0.026 in AD; in frontal cortex: 1.02±0.020 in C, 1.18 ±0.123 in DS, 1.48±0.020 in AD. These results show that elevated brain immunoreactive TSH-R is not specific for DS and maybe reflecting apoptosis, a hallmark of both neurodegenerative disorders, as it is well-documented that the thyroid hormone system is involved in the control of programmed cell death.

Key Words: Down syndrome, Alzheimer's disease, TSH-receptor, brain, Western blot

Abnormal thyroid function was shown by many authors in children and adults with Down Syndrome (DS, 1-9 and citations therein). The most consistent finding reported was the presence of elevated thyroid stimulating hormone (TSH) levels, whereas observations

Correspondence: Prof. Dr. Gert Lubec, CChem, FRSC (UK), University of Vienna, Dpt of Pediatrics, Waehringer Guertel 18, A 1090 Vienna, Austria,fax: x43.1.40400 3194

on thyroid hormones, functional / clinical relevance showed inconsistent results. Although it is known that thyroid hormone function is essential for normal brain function and development, this issue has not been appropriately addressed yet in DS. Bhaumik and coworkers (2) who correlated global scores of ability with TSH levels in patients with DS, finding a strong association of elevated TSH with low scores, published the only work on brain function and TSH in DS. Using a gene hunting method (10) to study gene expression in fetal brain of DS we found a 300% overexpressed sequence with very high homology to the TSH-receptor. This finding made us examine immunoreactive TSH-receptor -protein in brains of patients with DS and Alzheimer Disease (AD) in comparison to controls. We intended to study whether our findings on fetal DS brain obtained by gene hunting would be reflected in adult DS brain at the protein level and whether increased TSH-receptor expression in DS brain would be specific for trisomy 21.

Methods

Substractive hybridization protocol [14]:

Fetal brain samples of two fetuses with Down Syndrome and two age and sex matched control, 23 rd week of gestation, were obtained from the Brain Bank of the Institute of Psychiatry (Denmark Hill, London, UK). Hippocampus (gyrus parahippocampalis) was taken into liquid nitrogen and ground for the isolation of mRNA. Isolation of mRNA was performed using the Quick Prep Micro mRNA purification kit (Pharmacia Biotech Inc., Uppsala, Sweden, cat.27 92 55 01). 1 µg of mRNA from each (of the two) preparation was quality - checked by cDNA cloning kit (Gibco,Life Technologies, Eggenstein, Germany, cat. 18248-013) using the incorporation of [alpha - 32P] dATP (Amersham, Buckinghamshire, UK, cat AA0004) with subsequent electrophoresis on 1% agarose followed by autoradiography. The Reflection film (Dupont NEF 496) was exposed to the gel for a period of two hours at room temperature.

Construction of the substractive library:

10 µg each of mRNA from brain of DS and control were biotinilated by UV irradiation at 360 nm according to the instructions supplied in the subtractor kit (Invitrogen, Leek, Netherlands,cat K4320-01). 1 µg of mRNA - pools each from the DS brain sample was subject to reverse transcriptase reaction (subtractor kit, Invitrogen) and the cDNA - pools were hybridized with the corresponding biotinilated mRNAs from controls. The substractive hybridization mixture was incubated with streptavidin according to the subtractor kit given above and thus the biotinilated molecules (non-induced biotinilated mRNAs and the hybrid [biotinilated mRNAs / cDNAs]) complexed. The streptavidine complexes were removed by repeated phenol-chloroform extraction and subtracted cDNAs were separated from the aquous phase by alcohol precipitation (subtractor kit).

In order to amplify and clone subtracted cDNAs, they were ligated with Not I - linkers followed by Not I - digestion. These Not I linked cDNAs were ligated to Not I site of sPORT 1 cloning vector (cDNA cloning kit, Gibco).

To enable visualization of subtracted cDNAs the cloned cDNAs were amplified using universal primers:

I 5`-GTAAAACGACGGCCAGT-3`

II 5`-ACAGCTATGACCATG-3`

from multiple cloning site of the sPORT -1 vector (cDNA cloning kit, Gibco). Amplified cDNAs were analysed on 1% agarose electrophoresis.

Cloning of subtracted Not I - linked cDNAs

Not I linked cDNAs ligated with sPORT 1 vector were used for the transformation of highly competent INFalpha F' E. coli cells (Invitrogen, Leek, Netherlands, cat C2020-03) and plated clones were analysed by plasmid isolation kit (Quiagen, Hilden, Germany, cat 12245) and digestion with Eco RI / Hind III . Recombinant clones were sequenced by K. Granderath, MWG - Biotech (Ebersberg, Germany). Homologies were determined by computer assisted comparison of data from the genebank sequence library: fastA@ebi.ac.uk (GBALL; EMBL, Heidelberg, Germany).

Subtractive hybridization was performed cross-wise i.e. DS sample mRNA subtraction from control and vice versa.

Western blots for the determination of immunoreactive TSH-receptor protein

The brain regions temporal, frontal, occipital, parietal cortex and cerebellum of patients with DS (n= 8), AD (n= 8) and controls (n= 8) characterized in a previous publication (11) were used for the studies at the protein level.

Shock frozen brains of patients with DS, AD and controls were thawed on ice in the presence of the protease inhibitor PantinolR (Gerot, Austria) 500 KIE/ml homogenization solution and mixed 1:5 (w/v) with homogenization solution (0.25 M sucrose, 1 mM EDTA, 3 mM imidazole, 0.1 % ethanol, pH 7.2). Samples were homogenized for 30 sec (six strokes) at 440 rev/min in a Potter-Elvehjem homogenizer on ice. The homogenate was centrifuged for 5 min at 3000 g and 4°C. The supernatant was used for the determination of protein (12) and immunoblotting. Using ice cold homogenization solution the supernatants were adjusted to equivalent protein concentrations. To these supernatants equal amounts of sample buffer were added. Samples were heated at 95°C and loaded onto a 7.5 % SDS-polyacrylamid gel (Bio Rad 16 10 937) according to the method of Laemmli (13). After separation proteins were transferred electrophoretically to nitrocellulose membranes for 1 hr at 100 V in blotting buffer (25 mM Tris, 192 mM glycine, 20 % methanol). Membranes (Hybond - C-extra, RPN 303E, Amersham Life Science, UK) were washed and blocked in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2H_2HPO_4$ x 12 H_2O, 1.5 mM KH_2PO_4) containing 0.05 % Tween - 20 and 1% BSA for 1 hr at room temperature, followed by probing the membranes with the mouse anti-human recombinant thyrotropin (TSH) - receptor-antibody (Serotec Ltd, Oxford, UK; MCA 1282). This antibody was diluted 1:1000 (v/v) with PBS containing 0.05% Tween-20. Membranes were washed four times for 15 min in PBS containing 0.05 % Tween - 20 and 1% BSA prior to incubation at room temperature with horseradish peroxidase conjugated goat-anti-mouse IgG (Southern Biotechnology Associates Inc, Birmingham, AL; 1070-05), diluted 1:2000 (v/v) in PBS / Tween - 20. Membranes were washed again four times for 15 min and the blots were developed with the ECL Western blotting system (Amersham Life Sciences, UK).

Densitometry was performed using an ElscriptTM 400 densitometer (Hirschmann, Germany).

Between-group-differences were calculated by non-parametric Mann-Whitney-U test. The level of significance was considered at $p<0.05$.

Results and Discussion

Subtractive hybridization:

The corresponding sequence found by subtractive hybridization is given in fig.1 and reveals the high homology with the rat TSH-R, which is 90% identical with the human TSH-R (14) .

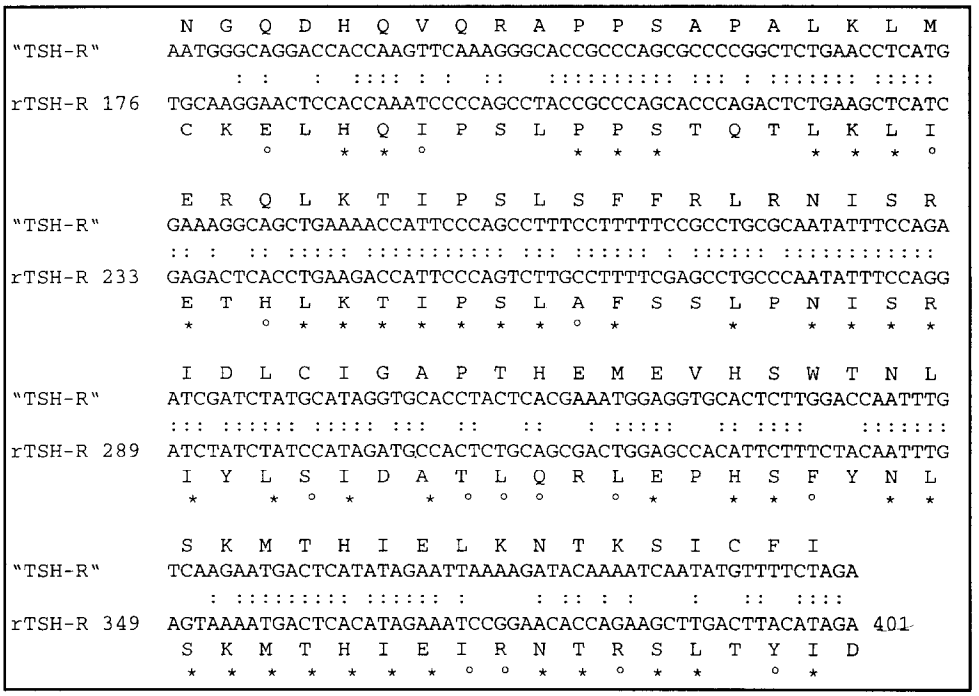

Fig.1

Alignment of the nucleic acid sequence obtained from subtractive hybridization ("TSH-R", above) in our studies with nucleic acid sequence of rat thyroid stimulating hormone receptor (rTSH-R; 14) obtained from Genbank. Sequence homology was 67% within 235 bp. Asterisks show identical and open circles show conserved amino acids (87% of homology).

Immunoreactive TSH-R proteins:

Immunoreactive TSH-R protein was detectable in all five regions of DS, AD and controls. The presence of two bands at 61kD and 40 kD was a consistent finding in all groups and all brain regions and these bands were subject to densitometry (fig. 2).

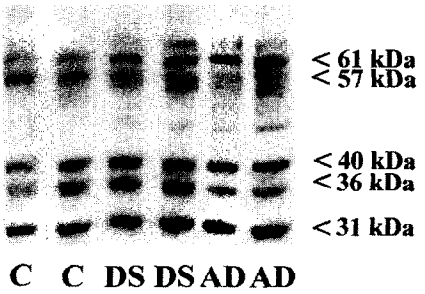

Fig. 2
Western blot pattern revealing immunoreactive TSH-R proteins: Immunoreactive TSH-R protein was detectable in all five regions of controls (lanes 1,2), DS (lanes 3,4) and AD (lanes 5,6). The presence of two bands at 61kD and 40 kD was a consistent finding in all groups and all brain regions and these bands were subject to densitometry. Bands assigned to 57 kD, 36 kD, 31 kD were found frequently but inconsistently and were not quantified.

Table 1

		40 kD	
	Controls	DS	AD
frontal lobe	1.000±0.046	1.105±0.030**	1.109±0.038*
cerebellum	1.000±0.013	1.043±0.062	1.081±0.041**
parietal lobe	1.000±0.010	1.027±0.047	0.931±0.081
temporal lobe	1.000±0.036	1.358±0.143**	1.528±0.126**
occipital lobe	1.000±0.018	1.060±0.060	1.000±0.049
		61 kD	
	Controls	DS	AD
frontal lobe	1.000±0.020	1.184±0.123*	1.480±0.020**
cerebellum	1.000±0.049	1.034±0.031	1.082±0.058
parietal lobe	1.000±0.037	1.030±0.070	0.990±0.028
temporal lobe	1.000±0.015	1.479±0.013**	1.623±0.026**
occipital lobe	1.000±0.091	0.956±0.098	0.943±0.051

Means ±SD of immunoreactive TSH-R-protein kD 40 and kD 61 in brain of patients (n=8) with DS, AD and controls. (arbitrary units)
* Statistically significant different from controls ($p<0.025$)
** Statistically significant different from controls ($p<0.01$)

The results of densitometry are given in table 1 showing that immunoreactive TSH-R - protein kD 40 was significantly increased in temporal and frontal lobe of DS and AD patients and in cerebellum of patients with AD. The immunoreactive TSH-R - protein kD 61 was significantly increased in temporal and frontal lobe of patients with AD and DS.

Subtractive hybridization applied on a pair of normal and DS fetal brain provided first evidence for differences in TSH-R gene expression. As the thyroid hormone system plays a major role in brain development and function , we pursued this clue and found significantly increased immunoreactive TSH-R proteins 40 kD and 61 kD in temporal and frontal lobe of brain from patients with DS. Surprisingly, these proteins were also significantly increased in identical brain regions of AD patients, which suggests an involvement of the TSH-R in neurodegeneration rather than specifically in DS.

The TSH-R antibody used recognizes both, native and denatured TSH-R epitope 125-369 and was raised against recombinant human TSH-R (15). In our detection system it recognized a series of immunoreactive TSH-R proteins, which cannot be further identified as no information on the human brain TSH-R protein is available. Data on human TSH-R from the thyroid may explain the presence of multiple bands: Russo and coworkers showed that subunits of the TSH-R are linked by disulfide bridges and as we run the electrophoretic separation under reducing conditions their findings may well be taken into account (16). Furthermore, a series of different molecular weights was expected and described for species differences which may be also due to differences in the carbohydrate moiety (17 - 21) and in addition, the TSH-R is highly susceptible to proteolytic cleavage (22). Harfst, using an antibody against an epitope structurally comparable to ours (313-330) also found an immunoreactive human recombinant TSH-R protein of approximately 60 kD which may well correlate with our brain isoform (20) and Huang and coworkers detected a human TSH-R-polypeptide of 63 kD among other target antigens of Grave's disease consisting of the large extracellular region, expressed in insect cells (23). These findings along with the consistent pattern of expression of the 61 kD (and 40 kD) immunoreactive TSH-R - pattern made us select them for quantification.

The biological or pathological meaning of overexpressed immunoreactive TSH-R in brain regions of patients with DS and AD remains unclear. Although the strong association of thyroid hormone abnormalities with DS is well-documented and antibodies against the TSH-receptor in DS have been described (24), attempts to interprete our findings would remain speculative and any suggestion of a role in pathogenesis would be challenged by the finding of Saunier and coworkers who demonstrated that the rat brain TSH-R (in astroglia) was not coupled to adenylyl cyclase and phospholipase C but to phospholipase A2 only (25). The findings of TSH-R overexpression in the same brain regions in AD would demand at least one additional explication. Percy and coworkers compared autoimmune thyroid disease in adult DS patients with and without manifestations of AD, as most DS patients develop AD brain pathology in the fourth decade (26). They found that hypothyroidism is common in adults with DS and more pronounced in patients with AD manifestations (6). This may suggest a common role for the thyroid hormone system in the development of neurodegeneration, or, more specifically, in the process of apoptosis, a hallmark of both disorders (27). This speculation is supported by well-documented observations that thyroid hormones control programmed cell death (28-30). Muller and Clos clearly outlined and reviewed the role of thyroid hormones for regulation of apoptosis in the brain (31): temporal regulation of proliferation, differentiation and restructuring during

development of the central nervous system is mediated by programmed cell death. An upregulated TSH-R may well be interfering with the apoptotic equilibrium found in DS and AD brain.

This is the first report on the immunoreactive human brain TSH-R in health and neurodegenerative disease. We are aware of the preliminary nature of the report but our study may form the basis for understanding and challenge further studies on this intriguing subject.

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