Expression of thyroid hormone transporters in the human hypothalamus, Alkemade et al,

Materials and methods

Subjects

We studied post-mortem hypothalamus specimens obtained from 10 subjects in whom serum thyroid hormone concentrations were determined in a blood sample taken within 24 h before death. In addition, we included one patient with biochemically documented thyrotoxicosis three weeks before death. Clinicopathological data and serum thyroid hormone concentrations of these patients have been published previously and are summarized in Table 1 (supplementary data) (1-3). Brain material was obtained from The Netherlands Brain Bank at The Netherlands Institute for Neuroscience (Director Dr. I. Huizinga) in accordance with the formal permissions for brain autopsy and for the use of human brain material and clinical information for research purposes.

Histology

Hypothalami were fixed in 10% phosphate-buffered formalin at room temperature for 4-5 weeks. After dehydration in a series of graded ethanols, tissues were cleared in xylene and embedded in paraffin. Coronal serial sections (6μm) were cut from the lamina terminalis to the mamillary bodies. Every 100th section was used for Nissl staining for anatomical orientation. MCT10 and OATP1C1 staining was performed on every 100th section of the rostro-caudal axis of the hypothalamus.

Antibodies

For immunocytochemical staining, we used polyclonal rabbit antisera raised by Eurogentec SA (Seraing, Belgium). For MCT10 two immunizing peptides derived from human MCT10 (amino acids 473-487 and C-terminal amino acids 503-515, (4)) were used and for OATP1C1 one peptide (C-terminal amino acids 696-711) conjugated to keyhole limpet hemocyanin. Antisera from the final bleed were used without further purification.
Specificity tests

Specificity of the antisera was supported by: 1) staining with pre-immune serum, 2) pre-adsorption with the homologous peptide, 3) Western blotting, and 4) staining in COS-1 cells transfected with OATP1C1 or empty vector.

1) Pre-immune staining. Staining was performed on hypothalamic sections with the pre-immune serum for MCT10 and OATP1C1. The staining procedure was identical to that of the immunocytochemical staining.

2) Pre-adsorptions. Antisera were pre-adsorbed with the homologous peptide. For MCT10 we added the two immunizing peptides to the antibody diluted in supermix [SUMI, 0.05 M Tris, 0.15 M NaCl, 0.5% Triton X-100 (Sigma, Zwijndrecht, The Netherlands), and 0.25% gelatin (Merck, Darmstadt, Germany) (pH 7.6)] overnight at 4 C. For OATP1C1 we dissolved the peptides (500 ng/µl), spotted and fixed them on gelatin-coated nitrocellulose and subsequently incubated with the antibody diluted in SUMI. Nitrocellulose sheets were immunocytochemically stained to check antigen-antibody binding as described by Van der Beek et al.(5). Both pre-adsorbed antisera were tested on human hypothalamus sections, using the immunocytochemical staining procedure described below. Antiserum preadsorbed with an independent peptide was used as a positive control in adjacent sections.

3) Western blotting. To support antibody specificity we immunoblotted homogenates of COS-1 cells transiently transfected with plasmids containing cDNA coding for human MCT10 (4;6). Samples were run on 10% precast Precise Protein gels (Perbio Science, Etten-Leur, The Netherlands) according to the manufacturer’s protocol and electroblotted onto nitrocellulose membranes (GE Healthcare, Eindhoven, The Netherlands). Blots were blocked with 5% non-fat milk (Campina, Eindhoven, The Netherlands) in PBS with 0.1% Tween 20 (Sigma, PBS-Tween) and subsequently incubated overnight with the antisera (1:500 in PBS-Tween with 1% BSA) at 4C. Blots were washed and incubated with horseradish peroxidase-conjugated goat-anti-rabbit (Sigma, 1:80,000 in PBS-Tween) for 1 h at room temperature (RT). Bands were detected by chemiluminescence, using Western Lightning Plus-ECL enhanced chemiluminescence reagents (PerkinElmer, Groningen, The Netherlands).
4) Staining of COS-1 cells transfected with human OATP1C1. Specificity of the OATP1C1 antibody was further supported by immunocytochemical staining of COS-1 cells that were transiently transfected with cDNA coding for OATP1C1 or empty vector using FuGENE HD transfection reagent (Roche, Diagnostics, Almere, The Netherlands) according to the manufacturer’s guidelines. The expression of functional OATP1C1 was confirmed by T4 uptake studies as described previously (7). Cells were fixated in 4% paraformaldehyde, permeabilized using 0.2% Triton, and stained using the OATP1C1 antibody as described below.

**Immunocytochemical procedures**

Sections were mounted on Superfrost plus slides and dried for at least 2 days at 37°C. After deparaffinization in xylene and rehydration through graded ethanol series, sections were washed in Tris-buffered saline (TBS) and antigen retrieval was performed using microwave treatment (10 min, 700W) in TBS at pH 7.6 for OATP1C1 and in 0.05 M Tris-HCl at pH 9.0 for MCT10. After cooling to room temperature (RT), sections for OATP1C1 staining were pre-incubated for 1 h at RT in TBS-1% non-fat milk (pH 7.6). Sections were then incubated with the first antibody diluted 1:500 in SUMI for MCT10 or OATP1C1 overnight at 4°C in a humidified chamber. Sections were washed in TBS and incubated with the second antibody (biotinylated goat anti-rabbit, 1:400 in SUMI) for 1 h at RT. After washing in TBS sections were incubated for 1h at RT with avidine biotinylated complex (1:800 in SUMI; Vector Laboratories, Burlingame, CA) and subsequently rinsed in TBS. Finally, sections were incubated for approximately 15 min with 0.5 mg/ml 3,3′-diaminobenzidine (Sigma) in TBS containing 0.2% ammonium nickel sulfate (BDH; Brunschwig, Amsterdam, The Netherlands) and 0.01% H₂O₂ (Merck). The reaction was stopped in distilled water. The sections were dehydrated in graded ethanol series, cleared in xylene, and cover-slipped using Entellan (Merck).

For double staining, sections were pretreated as described for single staining. Nitro blue tetrazolium (NBT)\ 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and 3-amino-9-ethylcarbazole (AEC) were used as substrate for the alkaline phosphatase and peroxidase reactions for the visualization of the proteins. NeuN was used as a neuronal marker and GFAP as a glial marker. Double labeling was assessed using spectral analysis of the staining, allowing objective interpretation of the colors.
**Supplemental Table 1:**

*Expression of thyroid hormone transporters in the human hypothalamus.* Alkemade et al,

<table>
<thead>
<tr>
<th>Subject #</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>PMD (h)</th>
<th>Fix (days)</th>
<th>Clinicopathological diagnosis</th>
<th>TSH (0.40-4.00 mU/L)</th>
<th>T4 (70-150 nmol/L)</th>
<th>T3 (1.30-2.70 nmol/L)</th>
<th>Cort (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>95-117</td>
<td>M</td>
<td>70</td>
<td>24</td>
<td>28</td>
<td>Chronic pancreatitis, endocarditis, renal insufficiency</td>
<td>0.50</td>
<td>25</td>
<td>&lt;0.30</td>
<td>1.15</td>
</tr>
<tr>
<td>95-130</td>
<td>F</td>
<td>77</td>
<td>16</td>
<td>31</td>
<td>Basilary artery thrombosis, brain stem infarction</td>
<td>0.09</td>
<td>55</td>
<td>0.55</td>
<td>0.68</td>
</tr>
<tr>
<td>95-128</td>
<td>F</td>
<td>88</td>
<td>66</td>
<td>31</td>
<td>Mitral valve insufficiency, atrial fibrillation, shock, probably sepsis</td>
<td>2.80</td>
<td>125</td>
<td>0.70</td>
<td>4.04</td>
</tr>
<tr>
<td>95-121</td>
<td>F</td>
<td>43</td>
<td>24</td>
<td>32</td>
<td>Intracerebral haemorrhage, ischaemic heart disease</td>
<td>0.42</td>
<td>85</td>
<td>0.75</td>
<td>nd</td>
</tr>
<tr>
<td>95-127</td>
<td>M</td>
<td>76</td>
<td>70</td>
<td>28</td>
<td>Metastatic renal carcinoma, renal insufficiency, intracerebral haemorrhage</td>
<td>0.62</td>
<td>85</td>
<td>0.80</td>
<td>1.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Myocardial infarction</td>
<td>1.70</td>
<td>70</td>
<td>0.85</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Acute myocardial infarction, resuscitation</td>
<td>4.60</td>
<td>70</td>
<td>1.15</td>
<td>0.76</td>
</tr>
<tr>
<td>95-124</td>
<td>F</td>
<td>71</td>
<td>22</td>
<td>35</td>
<td>Trauma, massive subdural haematoma</td>
<td>1.30</td>
<td>120</td>
<td>1.35</td>
<td>1.09</td>
</tr>
<tr>
<td>95-131</td>
<td>M</td>
<td>74</td>
<td>29</td>
<td>28</td>
<td>Massive intracerebral haemorrhage</td>
<td>0.95</td>
<td>115</td>
<td>1.40</td>
<td>0.91</td>
</tr>
<tr>
<td>95-123</td>
<td>M</td>
<td>34</td>
<td>58</td>
<td>31</td>
<td>Insulin-dependent diabetes mellitus, cardiac arrest, resuscitation</td>
<td>4.50</td>
<td>70</td>
<td>1.55</td>
<td>1.33</td>
</tr>
<tr>
<td>93-121</td>
<td>M</td>
<td>78</td>
<td>17</td>
<td>28</td>
<td>Thyrotoxicosis, massive pulmonary embolism</td>
<td>&lt;0.01</td>
<td>fT4 44.2*</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

*reference range FT4: 10-23 pmol/L, nd=not determined, PMD=Post mortem delay*
Supplemental Figure 1:
Expression of thyroid hormone transporters in the human hypothalamus, Alkemade et al,
Results Antibody specificity

Figure 1: A) Western blot using rabbit MCT10 antibody 1758 (1:500) with or without blocking peptides (10μM), B) Staining of OATP1C1 in COS-1 cells transfected with OATP1C1 or empty vector. C) Confirmation of functional expression of OATP1C1 in COS-1 cells by T4 uptake. C=μ-crystallin.
Supplemental Figure 2, continued:
Expression of thyroid hormone transporters in the human hypothalamus, Alkemade et al,
Results Antibody specificity

Fig 2: PVN staining with the OATP 1C1 antibody pre-adsorbed with independent (A), or immunizing peptide (B), PVN staining with the MCT10 antibody pre-adsorbed with independent (C) or immunizing peptides (D). Bar represents 100μm.
Supplemental Figure 3, continued:
Double labelling of thyroid hormone transporters in the human hypothalamus with the glial marker GFAP or the neuronal marker NeuN. Alkemade et al.

Fig 3: Left: GFAP (red) staining combined with OATP1C1 (blue), right: NeuN (red) combined with MCT10 (blue) Note the clear double labeling of OATP1C1 with GFAP and of MCT10 with NeuN. OATP1C1 double labeling with NeuN was also observed (data not shown).


