Hypothalamic functions in patients with pituitary insufficiency

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Chapter 7

Distribution of serotonin transporters in the human hypothalamus

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ABSTRACT

Background: Serotonergic signalling has been implicated in various hypothalamic functions including circadian rhythmicity, feeding behaviour and neuroendocrine regulation. However, the functional neuroanatomy underlying serotonergic signalling in the human hypothalamus is largely unknown.

Aim and methods: To increase insight in hypothalamic serotonin signalling in humans, we investigated the distribution of serotonin transporters (SERT) systematically over the entire rostro-caudal axis of six post-mortem hypothalami by means of immunohistochemistry, using a monoclonal antibody raised against human SERT. Immunofluorescent double-labelling of SERT with markers for the major cell populations in the suprachiasmatic (SCN) and infundibular nucleus (IFN) was performed to further identify cells that showed basket-like SERT-staining.

Results: SERT-immunoreactivity was ubiquitously expressed in fibers throughout the hypothalamus. A dense track of fibers was seen a) in the perifornical area, b) as a plexus along the ependym and c) in close proximity to the anterior commissure. The suprachiasmatic- and the infundibular nucleus showed strong SERT-immunoreactivity. Clusters of SERT-immunoreactive fibers outlining capillaries and basket cells were present in the SCN and IFN, highly suggestive of synaptic endings. The majority of SERT basket cells did not show double labelling with the major neuropeptidergic markers for SCN and IFN.

Conclusion: We report the distribution of SERT in post-mortem human hypothalamus. The strong SERT-immunoreactivity in the human SCN and IFN suggests a local modulatory role for serotonin although the identity of the SERT-immunoreactive basket cells remains to be identified.
Introduction

Serotonin is a widely expressed neurotransmitter in the central nervous system of vertebrates. Cell bodies of serotonergic neurons are localized in the raphe nucleus and their axons project to numerous areas in the brain, including the hypothalamus (1;2). As the hypothalamus receives dense innervations from serotonergic neurons, it is not surprising that serotonin has been implicated in a broad range of hypothalamic functions, including the regulation of circadian rhythms, feeding, metabolism, temperature, neuroendocrine responses, mood and behaviour (3-8). Axonal serotonin released in the synaptic cleft exerts its effects by actions on various pre- and postsynaptic serotonin receptors (9) or it may diffuse into the extracellular matrix to bind to extrasynaptic receptors (10). The amount of serotonin available for signalling is regulated by the serotonin transporter (SERT) (11). The transport of serotonin from the extracellular space by SERT results in a high affinity reuptake, and is believed to be the most critical element in regulating the signal amplitude and duration of serotonergic transmission in brain (12;13). Therefore, the expression of SERT has been proposed to perform a key role in determining the efficacy and the mode of serotonergic transmission (14).

Very few data are available on the functional neuroanatomy underlying serotonergic signalling in the human hypothalamus. To our knowledge, only the distribution of SERT in the human suprachiasmatic nucleus (SCN) has been described (15). In other species, the majority of the anatomical studies investigating this system in the hypothalamus have been performed with antisera generated against serotonin, and only few studies were targeted to clarify the distribution of SERT expression. In primates, dense SERT-immunoreactivity was observed in paraventricular- and supraoptic nuclei (16), and Moore et al. (15) localized SERT in the SCN and as a plexus along the ependyma of both lateral and third ventricles. These data fit with studies in rodents, identifying a dense plexus of SERT in the SCN (3;17). In the present report, we systematically describe the distribution of SERT-containing cells and fibers in the human hypothalamus by means of immunohistochemistry, using a commercially available monoclonal antibody raised against human SERT.

In a second series of experiments, we attempted to determine which cell types were surrounded by the basket-like SERT-immunoreactive fibers in the SCN and IFN. For this purpose we used immunofluorescent double-labelling of SERT with markers for the major neuropeptidergic cell populations in SCN and infundibular nucleus (IFN), i.e. vasoactive intestinal peptide (VIP) and arginine vasopressin (AVP) in the SCN, and neuropeptide-Y (NPY), agouti-related peptide (AgRP) and alpha-melanocyte stimulating hormone (α-MSH) in the IFN. VIP- and AVP expressing neurons are the main components of the SCN and are highly important in the control of circadian rhythms (18), whereas AgRP-, NPY- and α-MSH expressing neurons in the IFN play a pivotal role in the regulation of energy homeostasis, with anorexigenic properties of α-MSH- and orexigenic properties of NPY-
and AGRP synthesizing neurons (19). Together, these studies provide more insight in the neuroanatomical pathways involved in hypothalamic serotonergic signalling in humans.

MATERIALS AND METHODS

Subjects
Post-mortem hypothalamic tissue was studied of 6 subjects (3 men and 3 women) without neurodegenerative disease ranging in age between 67 and 86 years. Clinicopathological data are presented in table 1. Brain material was obtained from The Netherlands Brain Bank at The Netherlands Institute for Neuroscience (director Dr. I. Huitinga) on the basis of written informed consent of the subject according to the Declaration of Helsinki and in accordance with the formal permissions for brain autopsy and for the use of human brain material and clinical information for research purposes, approved by the Medical Ethics Committee of the VU Medical Centre.

Table 1. Brain material

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age</th>
<th>PMD</th>
<th>Fix</th>
<th>Cause of death, clinical diagnoses</th>
</tr>
</thead>
<tbody>
<tr>
<td>99101</td>
<td>M</td>
<td>69</td>
<td>19</td>
<td>41</td>
<td>Respiratory insufficiency, infarction distal brainstem, alcohol abuse, hypertension</td>
</tr>
<tr>
<td>00007</td>
<td>M</td>
<td>85</td>
<td>15</td>
<td>35</td>
<td>Myocardial infarction, bladder carcinoma</td>
</tr>
<tr>
<td>03054</td>
<td>M</td>
<td>67</td>
<td>4.5</td>
<td>50</td>
<td>Cardiogenic shock, multi organ failure, type II diabetes, COPD</td>
</tr>
<tr>
<td>98056</td>
<td>F</td>
<td>83</td>
<td>5</td>
<td>41</td>
<td>Respiratory insufficiency, colon carcinoma, type II diabetes, cataract, arthrosis</td>
</tr>
<tr>
<td>95016</td>
<td>F</td>
<td>86</td>
<td>13.5</td>
<td>30</td>
<td>Decompensatio cordis, type II diabetes, angina pectoris, nephropathy, retinopathy</td>
</tr>
<tr>
<td>01005</td>
<td>F</td>
<td>76</td>
<td>20</td>
<td>36</td>
<td>Respiratory insufficiency, Non-Hodgkin lymphoma, hypertension, basalar cell carcinoma</td>
</tr>
</tbody>
</table>

Fix: duration of fixation in days; PMD: post mortem delay in hours

Histology
Brains were dissected at autopsy and the hypothalamus was fixed in 10% phosphate-buffered formalin at room temperature (RT) for 1-2 months. After dehydration in graded ethanol series, tissues were cleared in toluene and embedded in paraffin. Coronal serial sections (6mm) were cut over the entire rostro-caudal axis of the hypothalamus. For anatomical orientation, every 100th section was collected and mounted on chrome alum-gelatine coated glass slides and subsequently dried for two days at 37°C, followed by Nissl staining.
Antibody characterization

Mouse monoclonal anti-human SERT antibody was purchased from Millipore, MAb Technologies Inc. (Stone Mountain, GA; catalogue no Mab5618). Antibody specificity has been reported before and was supported using Western blotting (20-23). Rabbit polyclonal anti-human AgRP antibody was obtained from Phoenix Pharmaceuticals (Belmont, CA; catalogue no. H-003-53). AgRP staining disappeared after preadsorption with AgRP, and was not affected by cross adsorption using the NPY peptide (24). The α-MSH antibody was raised against the α-MSH C-terminal, which is modified in α-MSH free acid, and absent in ACTH, minimizing cross reaction with other POMC products. Staining was abolished after pre-adsorption with the α-MSH peptide (25).

The AVP antibody (Truus) was raised in rabbit against vasopressin in its processed form, although this antibody has been reported to cross react with oxytocin (26;27). NPY (Niepke) and VIP (Viper) antibodies were raised in rabbit against NPY and VIP in its processed form and specificity was previously tested by pre-immune staining and antibody pre-adsorption (28) (24;29). No staining was seen after omission of the primary antibodies. Optimal antibody concentrations were determined experimentally by testing of dilution curves. Characteristics of the antibodies are given in Table 2.

Immunohistochemistry

For SERT-immunohistochemistry, a series of coronal sections at 100-section intervals over the entire rostro-caudal axis of the hypothalamus was mounted on Superfrost plus slides

Table 2. List of primary antibodies used for immunolabelling

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Immunogen</th>
<th>Host</th>
<th>Manufacturer, Catalogue#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serotonin Transporter (SERT)</td>
<td>Synthetic peptide from the human serotonin transporter</td>
<td>Mouse monoclonal</td>
<td>Millipore, MAb Technologies Inc. Stone Mountain, GA: Mab5618</td>
</tr>
<tr>
<td>Vasoactive Intestinal Polypeptide (VIP)</td>
<td>VIP coupled to thyroglobulin (Sigma) with glutaraldehyde (Merck, Rahwah, NJ)</td>
<td>Rabbit polyclonal</td>
<td>Netherlands Institute for Neuroscience, (Amsterdam): #Viper, 180886</td>
</tr>
<tr>
<td>Arginine Vasopressin (AVP)</td>
<td>Synthetic peptide Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH2 (V-9879, Sigma, St. Louis, MO) coupled to thyroglobulin (Sigma) with glutaraldehyde (Merck, Rahwah, NJ)</td>
<td>Rabbit polyclonal</td>
<td>Netherlands Institute for Neuroscience, (Amsterdam): #Truus, C.P.180985</td>
</tr>
<tr>
<td>Neuropeptide-Y (NPY)</td>
<td>porcine NPY(1–36) (Sigma, St. Louis, MO) coupled to thyroglobulin (Sigma) with glutaraldehyde (Merck, Rahwah, NJ)</td>
<td>Rabbit polyclonal</td>
<td>Netherlands Institute for Neuroscience, (Amsterdam): #Niepke C.P.261188</td>
</tr>
<tr>
<td>Alpha-Melanocyte-Stimulating-Hormone (α-MSH)</td>
<td>α-MSH coupled to bovine thyroglobulin</td>
<td>Sheep polyclonal</td>
<td>Millipore, MAb Technologies Inc. Stone Mountain, GA: Mab5087</td>
</tr>
<tr>
<td>Agouti-Related Protein (AgRP)</td>
<td>human AGRP(83–132)</td>
<td>Rabbit polyclonal</td>
<td>Phoenix Pharmaceuticals Inc., Belmont, CA: #H-003-57</td>
</tr>
</tbody>
</table>
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(Menzel Glaser, Germany) and dried for at least 2 days at 37°C. Next, antigen retrieval was performed using microwave treatment (30) and sections were stained using the avidine biotinylated complex method (31), according to the following protocol: Sections were deparaffinised in xylene and rehydrated through graded ethanol series. After rinsing in distilled water, the sections were washed in TBS (pH 7.6) and antigen retrieval was performed using microwave treatment (10 min 700W) in TBS (pH 7.6). After adjustment to RT, sections were incubated in the primary antibody diluted 1:5000 in SUMI [supermix, 0.05M Tris, 0.15M NaCl, 0.5% Triton X-100 (Sigma, Zwijndrecht, The Netherlands), and 0.25% gelatine (Merck Darmstadt, Germany) (pH 7.6)] overnight at 4°C. The slides were rinsed in TBS (pH 7.6, 3x5min) and incubated for 1h at RT in the second antibody (biotinylated horse anti-mouse 1:400 in SUMI; Vector Laboratories, Burlingame, CA). After rinsing in TBS (pH 7.6, 3x5min), the sections were incubated 1h at RT in avidine biotinylated complex (1:800 in SUMI; Vector Laboratories, Burlingame, CA) and subsequently rinsed in TBS (pH 7.6, 3x5 min). Finally, sections were incubated in 0.5 mg/ml 3,3’-diaminobenzidine (Sigma) in TBS containing 0.2% ammonium nickel sulphate (BDH; Brunschwig, Amsterdam, The Netherlands) and 0.01% H2O2 (Merck) for approximately 15 min. The reaction was stopped in distilled water. The sections were dehydrated in ascending series of ethanol, cleared in xylene, and coverslipped using Entellan (Merck, Darmstadt, Germany).

Double-labelling by immunohistochemistry

To identify cell types showing basket-like staining of SERT-immunoreactive fibers, we performed immunofluorescent double labelling followed by confocal laser scanning microscopy for SERT (1:5000) with respectively AVP (1:800) and VIP (1:600) in hypothalamic sections containing the SCN of all 6 subjects. In addition, we performed immunofluorescent double staining of respectively SERT with αMSH (1:20000), NPY (1:1000) and AgRP (1:3000) in hypothalamic sections containing the IFN of all 6 subjects.

After overnight primary antibody incubation at 4°C the slides were rinsed in TBS (pH 7.6, 3x5min) and incubated in the secondary antibodies (biotinylated horse anti-mouse 1:400 in SUMI; Vector laboratories, Burlingame, CA) for 1h at RT. Following rinsing in TBS (pH 7.6, 3x5min), the sections were incubated 1h at RT in avidine biotinylated complex (1:800 in SUMI; Vector Laboratories, Burlingame, CA), subsequently rinsed in TBS (pH 7.6, 3x5min) and incubated in biotinylated tyramide (1:750 in SUMI, 0.01% H2O2 (Merck, Darmstadt, Germany)) for 15 min at RT followed by rinsing in TBS (pH 7.6, 3x5 min). SERT was detected in green by streptavidin-Alexa488 (1:1000; Invitrogen, Eugene, Or). The other peptides were visualized in red by respectively anti-rabbit Alexa594 (1:1000; Invitrogen, Eugene, Or) for AVP, VIP, NPY or AgRP and anti-sheep Alexa594 (1:1000; Invitrogen, Eugene, Or) for αMSH. This fluorochrome-conjugated antibody incubation was performed for 1h at RT, followed by overnight incubation at 4°C. Vectashield with DAPI (Vector laboratories, Inc, Burlingame, CA) was used for nuclear staining and cover slipping. The sections were stored under dark conditions at 4°C until further analysis.
Photomicrographs

The immunoperoxidase images were captured with a Zeiss Axioplan 2 microscope with 5 megapixel colour Evolution MP camera (MediaCybernetics, Bethesda, MD, USA) and the double labelling immunofluorescence images were taken on a confocal laser scanning microscope (Leica TCS-SP5, Wetzlar, Germany). The emission signals of Alexa 488 (SERT) and Alexa 594 (AVP, VIP, NPY, AgRP and αMSH) were assigned to green and red, respectively. All photomicrographs were then opened in Adobe Photoshop. The immunoperoxidase images were converted to black-white images and blue and green channels were switched off.

RESULTS

SERT expression in the human hypothalamus

Many SERT-immunoreactive fibers and few scattered SERT-containing cells were found throughout the entire hypothalamus, with the exception of white matter tracts (figure 1 and figure 2A). The general distribution of SERT was similar in all subjects studied. There was, however, strong interindividual variation in staining intensity.

The SERT-positive fibers were present throughout the entire hypothalamus, with a denser track of SERT-immunoreactive fibers in the perifornical area and in close proximity to the anterior commissure. A plexus along the ependym of the third ventricle wall showed also strong SERT-immunoreactivity (figure 2B). In addition, the highest fibre density was observed in the SCN (figure 2C) and IFN, which is the human equivalent of the

Figure 1. Schematic illustration of the distribution of immunoreactive serotonin transporter (SERT) fibers in coronal sections of the human hypothalamus. Abbreviations: see page 155.
arcuate nucleus (figure 2D). In these areas, cell bodies and capillary vessels were directly surrounded by clusters of SERT-immunoreactive fibers, highly suggestive of SERT-positive nerve endings in contact with SERT-negative perikarya and capillaries (high power inserts of figure 2C and 2D).

The supraoptic nucleus, paraventricular nucleus, lateral tuberal nucleus and tuberomamillary nucleus contained relatively small numbers of SERT-immunoreactive fibers.

**Double labelling of SERT with AVP, VIP, NPY, AgRP, and αMSH**

To further characterize the cell types showing basket-like staining of SERT-immunoreactive fibers in the SCN and IFN, we performed immunofluorescent double staining of SERT with AVP or VIP on sections containing the SCN, and with NPY, AgRP or αMSH on sections containing the IFN.

SERT-expressing fibers were present at all levels in all studied subjects. Many single labelled cells expressing AVP or VIP were found in the SCN. Likewise, a large number of NPY, AgRP- and αMSH-immunoreactive cells were found in the IFN in all subjects. However, only sporadic basket-like SERT staining was observed surrounding neurons which were immunoreactive for AVP, αMSH and AgRP. As SERT did not co-localize with VIP or NPY,

![Figure 2](image-url)  
*Figure 2. A series of high magnification photomicrographs of coronal hypothalamic sections showing immunoreactivity of the serotonin transporter (SERT). (A) SERT staining, overview. Note a large number of darkly stained SERT-positive fibers. (B) Dense SERT-immunoreactivity was seen along the ependym of the third ventricle (C) SERT-staining of the suprachiasmatic nucleus. Note darkly stained SERT immunoreactive fibers surrounding unstained cell body and capillary (high power insert). (D) SERT-staining of the infundibular nucleus. Note darkly stained SERT immunoreactive fibers surrounding unstained cell body and capillary (high power insert). Bars: 100μm for A and B; 250μm for C; 500μm for D. Asterisks indicate third ventricle.*
Distribution of hypothalamic SERT

Figure 3. A series of immunofluorescent photomicrographs showing serotonin transporter (SERT)-immunostaining (shown in green) combined with markers for major cell types (shown in red) of suprachiasmatic nucleus (SCN) and infundibular nucleus (IFN). Cell nucleoli are shown in blue. (A) The SCN of subject 03054, SERT-staining with arginine vasopressin-staining. Arrow shows one of the sporadic AVP-expressing neurons surrounded by SERT-immunoreactive fibers. (B) The SCN of subject 00007, SERT-staining with vasoactive intestinal polypeptide-staining. Note that VIP-expressing cells are not surrounded by SERT-positive fibers. (C) The IFN of subject 00007, SERT-staining with neuropeptide Y-staining. (D) The IFN of subject 00007, SERT-staining with α-MSH-staining. Arrow shows one of the sporadic α-MSH-expressing neurons surrounded by SERT-immunoreactive fibers.

Figure 4. Immunofluorescent photomicrographs showing serotonin transporter (SERT)-immunostaining (shown in green) combined with immunostaining for Agouti Related Peptide (AgRP) (shown in red). Cell nucleoli are shown in blue. (A) The IFN of subject 00007. Note heavily stained SERT-positive fibers. The high power insert shows basket-like SERT staining surrounding neurons which are not immunoreactive for AgRP. (B) The IFN of subject 010005. The high power insert shows an AgRP immunoreactive cell surrounded by SERT-positive fibre, suggesting nerve ending.
neurons showing basket-like SERT-staining remained largely unidentified (figure 3 and figure 4). Of note, the labelling of SERT in the IFN was predominantly localized more lateral to the NPY-, AgRP- and αMSH-immunoreactive cells. Together, our results indicate that SERT axons project to currently unidentified subgroup of neurons both in the SCN and in the IFN.

**DISCUSSION**

In the present study we describe the distribution of SERT in the post-mortem human hypothalamus. Our results show ubiquitous SERT-staining throughout the hypothalamus with denser immunoreactivity in the perifornical area, SCN and IFN, in close proximity to the anterior commissure and along the ependymal lining of the third ventricle. We observed strong interindividual variation in staining intensity for which we found no clear explanation. This may be partly due to illness-related and therapy-related pre-mortem factors that are known to alter post-mortem observed protein expression (32-34). Additionally, nuclear imaging studies have reported a significant association between the duration of sunlight exposure and SERT availability in the human brain, considered to be responsible for seasonal serotonin changes (35). Also age (36), body mass index (37) and gender (38) might affect the SERT availability in human brain. Therefore, it is conceivable that these factors have influenced the observed staining intensities. However, the present qualitative study in a small sample does not allow for a quantitative comparison of SERT-expression levels between subjects.

The serotonergic system has been extensively studied in other species using antibodies against serotonin itself (39). As neurotransmitters dissipate rapidly from neurons after death in human post-mortem tissue, we used an antibody against SERT, and compared their distribution with known other SERT distribution studies. Our findings are in general agreement with those reported on rodent- (3;17), primate- and human SCN (15). The SCN is the biological clock of the brain and known to control circadian rhythmicity (40). The abundant expression of SERT in this area suggests that the serotonergic system may play an important role in the regulation of day- and night rhythms. Indeed, a variety of pharmacological studies in rodents have implicated the serotonergic system as a direct regulator of circadian rhythm phase (see for review (41)). Moreover, it has been suggested that SERT distribution in human overlaps with that of the VIP neuronal population in SCN (15). Interestingly, we showed that basket cells in the SCN surrounded by SERT-expressing fibers were not immunoreactive for VIP and only sporadic for AVP, the two key neuropeptides of the SCN which are highly important in circadian timekeeping (18). Therefore, we did not find evidence supporting an important direct effect of the serotonergic system on VIP- or AVP-expressing neurons.
Interestingly, we found strongest SERT-immunoreactivity in the IFN. Immunoreactivity for serotonin has been reported before in this nucleus, especially in the immediate vicinity of capillaries and cells (42;43). Our double labelling study indicates that SERT-immunoreactive fibers in the IFN were mostly situated lateral from the neuronal populations expressing NPY, AgRP and αMSH, although occasionally AgRP and αMSH was expressed in basket cells. Future research is needed to identify the majority of SERT-positive basket cells in the IFN, targeting e.g. substance P, neurotensin, galanin, and dopamine (44). Moreover, it would be interesting to know whether the cells surrounded by basket-like SERT-immunoreactive fibers are neurons. Theoretically, neuronal staining can be achieved using the NeuN antibody (45). A complication is that both NeuN and SERT are monoclonal antibodies to mouse antigens.

High concentrations of SERT-immunoreactive fibers were also observed along the ependymal lining of the third ventricle. Except for minor disparities regarding the density of immunoreactive fibers, this pattern was also reported in rodents (3;17), primates and humans (3). The function of SERT-immunoreactive fibers that constitute this network is not known, but one could speculate that these fibres are involved in exchange of substances between the cerebrospinal fluid and nervous tissue of the brain.

In contrast to dense SERT-staining in supraoptic nucleus and paraventricular nucleus in rodents (16), we did not see an intense SERT-staining in these areas. A species-related difference may explain this discrepancy, but it might also be related to pre-mortem disease. Several studies reported that serotonin influences endocrine functions, such as the regulation or secretion of growth hormone, thyrotropin-releasing hormone, adrenocorticotropic hormone, prolactin, renin, oxytocin and corticosterone (6). Those studies implicate the paraventricular nucleus and supraoptic nucleus as major sites of serotonergic actions. The presence of SERT-immunoreactivity as described here in humans indicates that the wiring for similar effects in humans is present, but that these actions may be impaired at the end stage of life or that in humans other signalling systems may be of more importance.

It was long believed that serotonin is released and taken up by SERT exclusively in terminal endings at the synaptic cleft. However, Zhou et al (46) showed with electron microscopy that a great majority of SERT are distributed on axonal plasma membranes far beyond synaptic junctions. Indeed, we observed that SERT-immunoreactivity is prominent in long fibers and not in close proximity to cells exclusively. This supports the notion that SERT may regulate serotonin through extrasynaptic transmission in addition to synaptic termination.

The clinical importance of SERT is evident from the role of specific serotonin reuptake inhibitors in the treatment of psychiatric and neurological disorders, in particular major depression. These widely prescribed drugs are believed to act on SERT, thereby inhibiting the reuptake of serotonin (47). Interestingly, side effects of specific serotonin reuptake inhibitors include changes in sleep, appetite, bodyweight, autonomic dysfunction and mood (48). These functional domains show major overlap with the diverse functions of the
hypothesis, which harbours control centres for diurnal rhythmicity, energy homeostasis and autonomic regulation. Our description of SERT distribution in the human hypothalamus provides an anatomical framework for future investigations on the role of the serotonergic system in the human hypothalamus in health and disease.

ACKNOWLEDGEMENTS

Brain material was obtained from the Netherlands Brain Bank (coordinator I. Huitinga). We wish to acknowledge Bart Fisser and Joris Coppens for their excellent technical assistance.
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