



UvA-DARE (Digital Academic Repository)

Cholinergic imbalance in the multiple sclerosis hippocampus

Kooi, E.J.; Prins, M.; Bajic, N.; Beliën, J.A.M.; Gerritsen, W.H.; van Horssen, J.; Aronica, E.M.A.; van Dam, A.M.; Hoozemans, J.J.M.; Francis, P.T.; van der Valk, P.; Geurts, J.J.G.

DOI

[10.1007/s00401-011-0849-4](https://doi.org/10.1007/s00401-011-0849-4)

Publication date

2011

Document Version

Final published version

Published in

Acta Neuropathologica

License

CC BY

[Link to publication](#)

Citation for published version (APA):

Kooi, E. J., Prins, M., Bajic, N., Beliën, J. A. M., Gerritsen, W. H., van Horssen, J., Aronica, E. M. A., van Dam, A. M., Hoozemans, J. J. M., Francis, P. T., van der Valk, P., & Geurts, J. J. G. (2011). Cholinergic imbalance in the multiple sclerosis hippocampus. *Acta Neuropathologica*, 122(3), 313-322. <https://doi.org/10.1007/s00401-011-0849-4>

General rights

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations

If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: <https://uba.uva.nl/en/contact>, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

Cholinergic imbalance in the multiple sclerosis hippocampus

Evert-Jan Kooi · Marloes Prins · Natasha Bajic · Jeroen A. M. Beliën ·
Wouter H. Gerritsen · Jack van Horssen · Eleonora Aronica · Anne-Marie van Dam ·
Jeroen J. M. Hoozemans · Paul T. Francis · Paul van der Valk · Jeroen J. G. Geurts

Received: 20 April 2011 / Revised: 9 June 2011 / Accepted: 9 June 2011 / Published online: 21 June 2011
© The Author(s) 2011. This article is published with open access at Springerlink.com

Abstract Hippocampal pathology was shown to be extensive in multiple sclerosis (MS) and is associated with memory impairment. In this post-mortem study, we investigated hippocampal tissue from MS and Alzheimer's disease (AD) patients and compared these to non-neurological controls. By means of biochemical assessment, (immuno)histochemistry and western blot analyses, we detected substantial alterations in the cholinergic neurotransmitter system in the MS hippocampus, which were different from those in AD hippocampus. In MS hippocampus, activity and protein expression of choline acetyltransferase (ChAT), the acetylcholine synthesizing enzyme, was decreased, while the activity and protein

expression of acetylcholinesterase (AChE), the acetylcholine degrading enzyme, was found to be unaltered. In contrast, in AD hippocampus both ChAT and AChE enzyme activity and protein expression was decreased. Our findings reveal an MS-specific cholinergic imbalance in the hippocampus, which may be instrumental in terms of future treatment options for memory problems in this disease.

Keywords Multiple sclerosis · Alzheimer's disease · Hippocampus · Choline acetyltransferase · Acetylcholinesterase

Introduction

It has been shown that 40–65% of patients suffering from multiple sclerosis (MS) experience various degrees of cognitive deterioration [7, 30]. Although different cognitive domains may be affected, processing speed and visuospatial memory are most frequently reported abnormal [2, 4, 7, 30]. The hippocampus is critically important for proper memory function [35], and recent studies have shown that this brain structure is severely affected in MS [16, 26]. In the post-mortem setting, hippocampal lesions were found to be present in a majority of MS patients [12, 16, 26, 37] and several in vivo magnetic resonance imaging studies also showed high numbers of hippocampal lesions [32], as well as reduced volume [17, 33] and connectivity [31] of the MS hippocampus.

The cholinergic neurotransmitter system plays an essential role in learning and memory function [11, 13], and the hippocampus is a major region of cholinergic input from the basal forebrain [23]. Choline acetyltransferase (ChAT) is the enzyme responsible for acetylcholine synthesis and ChAT activity has been shown to be a reliable marker for

E.-J. Kooi (✉) · J. A. M. Beliën · W. H. Gerritsen ·
J. J. M. Hoozemans · P. van der Valk
Department of Pathology (Neuropathology), VU University
Medical Center, De Boelelaan 1117, 1081 HV Amsterdam,
The Netherlands
e-mail: e.kooi@vumc.nl

E.-J. Kooi · M. Prins · A.-M. van Dam · J. J. G. Geurts
Department of Anatomy and Neurosciences, Neuroscience
Campus Amsterdam, VU University Medical Center, van der
Boechorststraat 7, 1081 BT Amsterdam, The Netherlands

N. Bajic · P. T. Francis
King's College London, Wolfson Center for Age-Related
Diseases, Guy's Campus, London SE1 1UL, UK

J. van Horssen
Department of Molecular Cell Biology and Immunology,
VU University Medical Center, Van der Boechorststraat 7,
1081 BT Amsterdam, The Netherlands

E. Aronica
Department of Neuropathology, Academic Medical Center,
University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam,
The Netherlands

cholinergic integrity [8]. In Alzheimer's disease (AD) brains, severe changes in the cholinergic neurotransmitter system have long been recognized [1, 10, 15, 19, 29, 34, 41]. Degeneration of cholinergic neurons in the basal forebrain results in a decreased cholinergic input (as indicated by a decrease in ChAT activity) in the AD hippocampus [1, 10, 15, 40]. Furthermore, acetylcholinesterase (AChE), the acetylcholine degrading enzyme, is also downregulated in the AD hippocampus [19, 28]. Therefore, pharmacological treatment aiming to restore postsynaptic availability of acetylcholine is commonly used as a first-line therapy for symptomatic treatment of memory impairment in AD [3]. Whether similar changes in the cholinergic neurotransmitter system occur in the MS hippocampus is currently unknown.

Therefore, in this post-mortem study, we investigate different components of the cholinergic neurotransmitter system in the MS hippocampus, and compare these findings to hippocampus tissue of AD patients and non-neurological controls.

Materials and methods

Hippocampal tissue

Post-mortem brain material was obtained from the Netherlands Brain Bank, Amsterdam, The Netherlands. From 15 MS patients, as well as 10 donors without neurological disease, and 10 AD patients, frozen, coronally cut hippocampal tissue was selected. From a subset of the cases (i.e., 7 controls, 11 MS, and 8 AD), paraffin-embedded material was also available. Non-neurological controls were carefully matched to the MS patient samples for post-mortem delay, age, sex and Braak stage. Upon neuropathological assessment, the AD cases had Braak scores [5] of 5 or higher and were included to serve as a positive control regarding possible cholinergic changes. Clinical and pathological data of the MS patients, non-neurological controls and AD patients are provided in Table 1.

The study was approved by the institutional ethics review board (VU University Medical Center, Amsterdam) and all donors or their next of kin provided written informed consent for brain autopsy and use of material and clinical information for research purposes.

Sampling procedure

The hippocampal region, including Cornu Ammonis (CA) 1–4, identified on sections stained for proteolipid protein (PLP) (see “[Immunohistochemistry](#)”) were traced back in the tissue blocks and carved with a scalpel. In order to ensure that only the correct hippocampal areas were

isolated for an adequate comparison, cryosections were cut before and after tissue isolation and subsequently stained for PLP. Tissue for biochemical assays and western blot analyses were isolated separately and were stored at -80°C until subsequent analyses.

Hippocampal lesion classification

As described previously [16], PLP-stained hippocampal tissue sections were scored for the presence of lesions according to their anatomical localization. Lesions were classified as (1) mixed intrahippocampal-perihippocampal lesions; and (2) isolated intrahippocampal lesions (i.e. specified for CA subfield) and subsequently counted. Furthermore, microglial activation, based on anti-human leukocyte antigen-DR (HLA-DR) staining (see “[Immunohistochemistry](#)”) was also examined.

Choline acetyltransferase (ChAT) activity assay

Hippocampal tissue homogenates were blinded and samples were analyzed for protein content by the Bradford protein assay. Subsequently, a ChAT enzymatic activity assay was performed following the technique used by Fonnum [14] with minor modifications to the protocol [24]. Frozen hippocampal tissue which was stored at -80°C was homogenised in ice cold homogenization buffer (0.87 mM EDTA, 0.1% Triton X-100, pH 7.0). Samples were analyzed for protein content by Bradford protein assay. An incubation mixture (0.4 mM ^{14}C -acetyl CoA, 1 mg/ml BSA, 0.3 mM eserine salicylate, 0.6 M NaCl, 4 mM choline chloride, 0.1 M NaH_2PO_4 , 17.35 mM EDTA) was prepared on ice. The experiments were set up with two time points. Each sample was prepared in triplicate. In addition, a total radioactivity control was set up consisting of incubation buffer only. A blank control was set up with only extraction buffer present. For the first time point brain homogenate was added to incubation buffer with a 1:1 ratio, using equal volumes (e.g. 10 μl) for each sample and incubated at 30°C . Just prior to the end incubation, brain homogenate samples were added to incubation buffer for without incubation. All reactions were then quenched with the addition of ice cold dH_2O . 1 ml of extraction buffer (15% v/v acetonitrile, 85% toluene, 0.5% w/v sodium tetraphenylboron) was added to the experimental samples. Eppendorfs were spun at 13,000 rpm for 2 min. 650 μl of the upper phase was transferred into scintillant tubes. Scintillant tubes for the controls were prepared using the blank and total samples. 2 ml of liquid scintillant (Opti-Phase ‘HiSafe’, Perkin Elmer, UK) was added. Tubes were capped and radioactivity was measured in a beta counter, for 10 min each. The activity of ChAT was measured as

Table 1 Clinical data of MS patients, non-neurological controls, Alzheimer's disease patients

Case	Age (years)	Type of MS	Braak stage	Sex	Post-mortem delay (h:min)	Disease duration (years)	Cause of death
1	66	PP	Braak 0	M	7:30	26	Unknown
2	49	SP	Braak 0	M	8:00	25	Pneumonia
3	71	ND	Braak 0	M	7:00	26	Pneumonia by aspiration
4	47	SP	Braak 0	M	7:15	7	Urosepsis with organ failure
5	76	PP	Braak 1	F	9:45	19	Unknown
6	63	SP	Braak 0	M	7:05	24	Cardiac arrest after rupture abdominal aorta
7	77	ND	Braak 1	F	5:43	Unknown	Respiratory insufficiency with aspiration pneumonia
8	41	SP	Braak 0	F	8:25	11	Unknown
9	43	SP	Braak 0	M	8:30	9	Pneumonia
10	61	SP	Braak 0	M	9:15	18	Euthanasia
11	67	ND	Braak 0	F	9:15	Unknown	Unknown
12	71	SP	Braak 0	F	10:25	24	Respiratory insufficiency
13	76	ND	Braak 0	F	4:40	Unknown	Uremia following a urinary tract infection
14	56	PP	Braak 0	F	6:35	Unknown	Unknown
15	44	PP	Braak 0	M	12:00	13	Pneumonia
Con	56	–	Braak 0	M	9:15	–	Myocardial infarction
Con	80	–	Braak 0	M	7:15	–	Cachexia and dehydration
Con	56	–	Braak 0	M	14:00	–	Unknown
Con	61	–	Braak 0	F	6:50	–	Euthanasia
Con	84	–	Braak 1	M	7:05	–	Exacerbation of COPD
Con	80	–	Braak 1	F	6:58	–	Pulmonary embolism
Con	62	–	Braak 1	F	7:55	–	Euthanasia
Con	66	–	Braak 1	F	7:00	–	Heart failure
Con	66	–	Braak 0	M	7:45	–	Aneurysm
Con	84	–	Braak 1	M	5:35	–	Heart failure
AD	68	–	Braak 6	M	6:30	12	Cachexia
AD	81	–	Braak 6	F	6:00	4	Pneumonia
AD	87	–	Braak 5	F	9:15	8	Congestive heart failure
AD	87	–	Braak 6	F	5:00	20	Cachexia
AD	64	–	Braak 6	M	7:30	9	Dehydration and cachexia in end-stage AD
AD	73	–	Braak 6	F	5:55	16	Unknown
AD	78	–	Braak 5	F	8:25	Unknown	Sepsis
AD	84	–	Braak 6	M	8:05	13	Unknown
AD	82	–	Braak 6	F	4:20	6	Dehydration and cachexia
AD	65	–	Braak 6	M	0:59	Unknown	Pneumonia

PP primary progressive MS, ND MS subtype not determined, SP secondary progressive MS, AD Alzheimer's disease, Con non-neurological control, M male, F female

the amount of acetylcholine produced per hour, per mg of total protein (nmol/h/mg).

Acetylcholinesterase (AChE) enzyme histochemistry

On hippocampal cryosections (8 μ m thick) acetylcholinesterase histochemistry was performed using a slightly modified version of the direct coloring Karnovsky–Roots method [21]. In short, slides were fixed

for 15 min with 8% formaldehyde in 0.1 M CaCl₂ and pre-incubated for 30 min in 0.18 mM tetra-isopropylpyrophosphoramidate in phosphate buffered saline (iso-ompa) to block aspecific staining. Subsequently, sections were incubated in reaction medium (consisting of 6.5 ml iso-ompa, 5 mg acetyl-thiocholine-iodine, 0.5 ml 1 M sodium citrate, 1.0 ml 30 mM copper(II)sulphate, 1.0 ml distilled water and 1.0 ml 5 mM potassium ferricyanide) at 37°C for 6 h to visualise acetylcholinesterase activity.

For orientation cell nuclei were stained with 1% methylgreen.

Immunohistochemistry

Five-micrometer thick frozen sections were collected on Superfrost Plus glass slides (VWR international; Leuven, Belgium), dried overnight at room temperature (RT) and subsequently fixed in acetone for 10 min. Then, sections were rinsed for 3×10 min with 0.01 M phosphate buffered saline (PBS; pH 7.4) followed by incubation with mouse-anti-human primary antibodies against myelin proteolipid protein (PLP; Clone: Plpc1; mouse IgG2a; 1:500; Serotec, Oxford, UK) diluted in PBS containing 1% bovine serum albumin (BSA) (Roche Diagnostics; Mannheim, Germany) for 1 h. Subsequently, sections were rinsed with PBS (3×10 min) and incubated with biotin-labeled rabbit anti-mouse immunoglobulins F(ab')₂ (1:500) (DAKO, Glostrup, Denmark) diluted in PBS with 1% BSA for 30 min. Then, sections were rinsed in PBS (3×10 min) and incubated for 1 h at RT with streptavidin–biotin-peroxidase complexes (1:100; Vectastain; Vector Laboratories Inc., Burlingame, CA, USA). Finally, sections were rinsed with PBS (3×10 min) and peroxidase reaction was developed with 3,3' diaminobenzidine-tetrahydrochloridedihydrate (DAB; DAKO, Glostrup, Denmark) as a chromogen. After a short rinse with tap water, sections were counterstained with hematoxylin for 1 min and intensely washed with tap water for 5 min.

Eight-micrometer thick paraffin sections were deparaffinized in a series of xylene (4×5 min), 100% ethanol, 96% ethanol, 70% ethanol. Endogenous peroxidase activity was blocked by incubating the sections in methanol with 0.3% H₂O₂ for 30 min. In case of primary immunostaining with anti-proteolipid protein (PLP) (mouse IgG2a; 1:3,000; Serotec, Oxford, UK) sections were rinsed for 3×10 min with PBS followed by incubation with primary antibodies diluted in PBS containing 1% BSA for 1 h. In case of primary immunostaining with anti-HLA-DR, sections were pretreated with microwave antigen retrieval (3 min at 900 W and 30 min at 180 W). After pretreatment, sections were cooled to room temperature, rinsed with PBS (3×10 min) and incubated with anti-HLA-DR (mouse IgG2b; 1:50, generous gift from Dr. Hilgers, VUMC, Amsterdam) diluted in PBS containing 1% BSA for 1 h. Then, sections were rinsed again with PBS (3×10 min) and incubated with EnVision horseradish peroxidase (HRP) complex (DAKO, Glostrup, Denmark) and finally with 3,3' diaminobenzidine-tetrahydrochloridedihydrate (DAB; DAKO, Glostrup, Denmark) as a chromogen. After a short rinse with tap water, sections were counterstained with hematoxylin for 1 min and intensely washed with tap water for 5 min.

For immunostaining with anti-ChAT (AB144P, goat polyclonal, 1:400, Millipore, Billerica, MA, USA) sections were deparaffinized and rehydrated as described above. Subsequently, sections were incubated with 37% formaldehyde for 10 min and then rinsed with distilled water (2×5 min). Then, sections were rinsed with citrate buffer (pH 6.0) (3×10 min) and finally rinsed overnight in citrate buffer at 4°C. The other day the slides were again rinsed for 2×10 min with citrate buffer and then 10 min in tris-buffered saline (TBS; pH 7.6). Hereafter, endogenous peroxidase activity was blocked by incubating the sections with TBS with 3% H₂O₂ for 30 min. Then, sections were rinsed with TBS (10 min) and citrate buffer (2×10 min). Subsequently, sections were pretreated by microwave antigen retrieval (3 min at 900 W and 30 min at 180 W) in citrate buffer. After pretreatment, sections were cooled to room temperature and rinsed with TBS (3×10 min). Then, sections were incubated with 5% normal rabbit serum (DAKO, Glostrup, Denmark) in TBS with 0.5% Triton X-100 (TBS-Tx) for 30 min and then with primary antibodies diluted in TBS-Tx containing 2% normal rabbit serum for 2 h at RT and subsequently 48 h at 4°C. After 2 days, sections were rinsed again with TBS (3×10 min) followed by 1 h incubation with biotin-labeled rabbit-anti-goat antibodies (DAKO, Glostrup, Denmark) diluted in TBS-Tx. Subsequently, sections were rinsed in TBS (3×10 min) and incubated for 1 h at RT with streptavidin–biotin-peroxidase complexes (1:100; Vectastain; Vector Laboratories Inc., Burlingame, CA, USA) diluted in TBS-Tx. After two rinse steps (10 min) in TBS, sections were rinsed with Tris-hydrochloride (pH 7.6) for 10 min and then incubated with 3,3' diaminobenzidine-tetrahydrochloridedihydrate (DAB; DAKO, Glostrup, Denmark) as a chromogen. After a short rinse in TBS-hydrochloride ($3 \times$), sections were counterstained with hematoxylin for 1 min and intensely washed with tap water for 5 min. For all immunostainings performed, negative controls were prepared by incubation without primary or secondary antibody and gave no immunoreactivity.

Western blot analysis

For protein analysis, 30 hippocampal sections of 10- μ m thick were isolated from 7 controls, 9 MS and 7 AD patients as described in “[Sampling procedure](#)”. Subsequently, tissue was homogenized by incubating the samples with radio-immunoprecipitation assay (RIPA) buffer and protease inhibitors (Sigma-Aldrich, Zwijndrecht, The Netherlands) on ice for 30 min followed by sonification (Branson sonifier, Danbury, CT, USA). The protein concentration was measured with the bicinchoninic acid (BCA) protein assay (Pierce, Perbio Science, Etten-Leur, The Netherlands) as indicated in the user manual, using

bovine serum albumin for standard curve. Samples were stored at -80°C until assayed.

Equal amounts of protein (10 μg) were separated on 10% SDS-PAGE gels (Bio-Rad Laboratories, CA, USA) and transferred to PVDF membranes (Bio-Rad). After blocking in PBS and Odyssey blocking buffer (1:1) (LI-COR Biosciences, Lincoln NE, USA), membranes were incubated with primary antibody anti-AChE (PAB6747, goat polyclonal, 1:2,000, Abnova, Walnut, CA, USA) overnight at 4°C . Then, membranes were rinsed in PBS containing 0.1% Tween-20 (4×5 min) and incubated with donkey-anti-goat-IRDye[®] 800CW infrared dye (1:15,000) (LI-COR Biosciences) diluted in PBS, Odyssey blocking buffer (1:1), 0.1% Tween-20 and 0.01% SDS for 1 h at RT. Finally, the membranes were rinsed in PBS and 0.1% Tween-20 (4×5 min), 3 times in PBS and then membranes were analyzed using a Odyssey Infrared Imaging system (LI-COR Biosciences) and relevant signal intensity was quantified with LI-COR analysis software. Molecular weight and predicted band size of AChE is 68 kDa. After this, the membranes were incubated (overnight at 4°C) with the primary antibody anti-lactate dehydrogenase (1:20,000) (LDH; ab52448, rabbit monoclonal, Abcam, Cambridge, UK). Molecular weight and predicted band size of LDH is 37 kDa. After this, membranes were incubated with goat-anti-rabbit-IRDye[®] 800CW infrared dye (1:15,000) (LI-COR Biosciences) diluted in PBS, Odyssey blocking buffer (1:1), 0.1% Tween-20 and 0.01% SDS for 1 h at RT and analyzed as describe above and subsequently relative AChE protein expression was quantified.

Quantification of (immuno)histochemical stainings

(Immuno)histochemically stained sections were scanned with a Mirax slide scanner system (3DHISTECH, Budapest, Hungary). Digital images scanned with a $20\times$ objective were captured in three randomly selected areas of each of the hippocampal subfields (CA1, CA3-2 and CA4) for ChAT immunostainings and AChE histochemical stainings. As already indicated above, paraffin-embedded material was available from 7 control, 8 AD and 11 MS hippocampi and this material was therefore immunohistochemically stained for ChAT. For each investigated hippocampal subfield (i.e. CA1, CA2-3 and CA4), three randomly selected areas were captured for subsequent quantification. This means that for controls a total of 21 (3×7), for AD 24 (3×8) and for MS 33 (3×11) randomly chosen areas were captured. For the control, AD and MS hippocampal sections (frozen) on which AChE enzyme histochemistry was performed, respectively 30 (3×10), 30 (3×10) and 45 (3×15) randomly selected areas from each hippocampal subfield were captured for subsequent

quantification. The (immuno)reactivity of the stainings was scored in a blinded fashion with respect to the presence of demyelinated lesions in order to avoid potential bias (e.g. by the expectation that lesional areas should show more pronounced cholinergic system changes). However, differences in terms of cholinergic alterations between lesional and perilesional or non-lesional areas was retrospectively assessed. The relative amount of staining was analyzed using a, for this staining, tailor-made batch mode ImageJ macro as previously described [18]. ImageJ is freely available at: <http://rsb.info.nih.gov/ij/index.html> (US National Institutes of Health, Bethesda, MD, USA).

Statistics

GraphPad Prism software (San Diego, CA, USA) was used for the statistical analyses. In case of normal distribution of data, analysis of variance (ANOVA) with Dunnet's correction for multiple comparisons was used to compare differences among the control, MS and AD groups, with the control group as a reference point. When normality was not found, the non-parametric Kruskal–Wallis test was used with a Dunn's correction for multiple comparisons. Results were considered significant when $p < 0.05$.

Results

Hippocampal demyelination

None of the control or AD hippocampus samples showed any sign of demyelination (Fig. 1a, b). As for MS, 12 out of 15 MS hippocampus samples were partly or completely demyelinated (Fig. 1c). More specifically, a total number of 25 lesions were found of which 14 lesions were mixed intrahippocampal-perihippocampal lesions and 11 lesions were isolated intrahippocampal lesions. Only three MS hippocampi showed no demyelination (Fig. 1d). This is in line with our previous observations [16] and with post-mortem findings of other groups [12, 26]. In addition, as described in detail before [16], our MS samples indicated slightly enhanced microglia activation, as reflected by increased HLA-DR immunopositivity, in hippocampal areas where the myelin was still intact (data not shown). Furthermore, as expected based on the literature [16, 26, 37], no lymphocyte/macrophage infiltration was found in the MS hippocampal lesions.

Hippocampal ChAT activity and protein expression

The biochemical assay revealed differences in mean hippocampal ChAT activity levels among the control, MS and

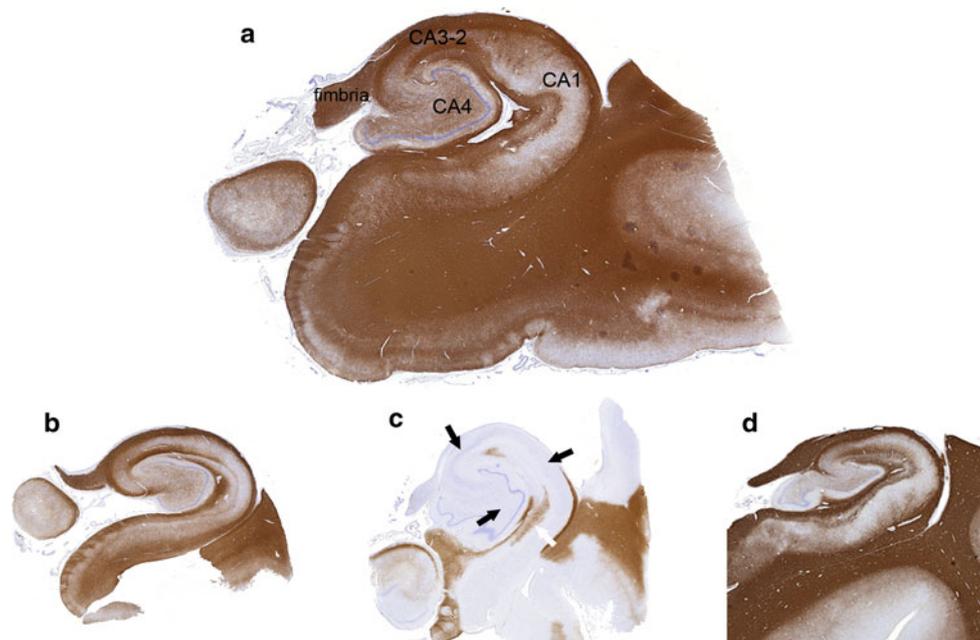


Fig. 1 Hippocampus anatomy and hippocampal demyelination of the MS samples. Hippocampus tissue from controls, AD and MS patients were immunohistochemically stained for anti-proteolipid protein and revealed **a** as expected, no sign of demyelination in any of the control hippocampus samples. Cornu Ammonis subfields of the hippocampus (CA4, CA3-2, CA1), as well as the fimbria, are indicated. **b** None of

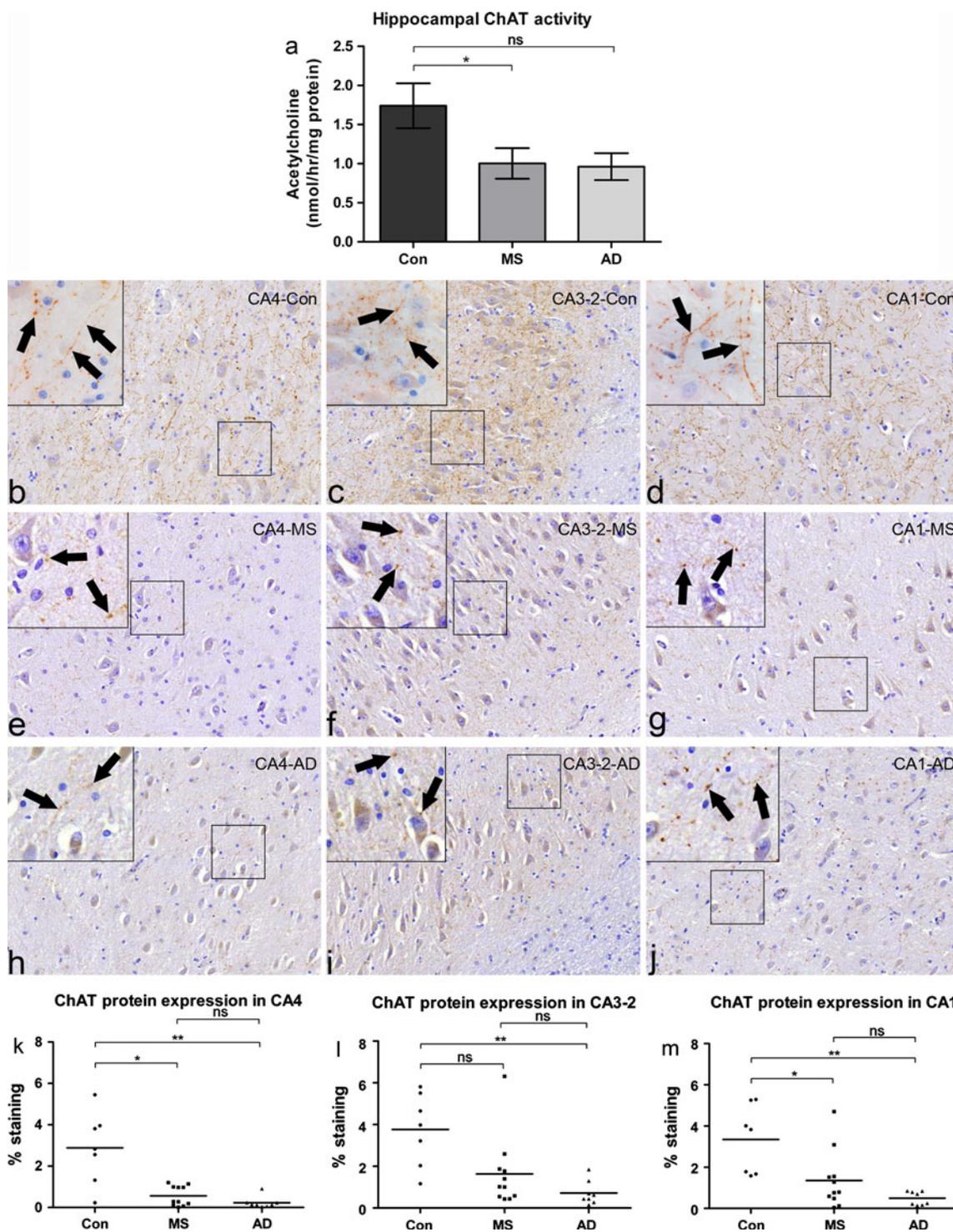
the AD hippocampus samples showed any demyelination, **(c)** while 12 out of 15 MS patients showed demyelinated lesions. *Black arrows* indicate demyelination in CA4, CA3-2 and CA1. The *white arrow* indicates a small preserved area of normal myelination in CA1. **d** 3 out of 15 MS hippocampi were not affected by demyelination

AD groups (Kruskal–Wallis; $p = 0.026$). Post hoc, a mean reduction in ChAT activity level of 43% was found in MS hippocampi compared to controls (Fig. 2a; MS vs. Con, mean of 1.00 vs. 1.74 nmol acetylcholine/h/mg protein; $p < 0.05$). A mean reduction of 45% was found in AD hippocampi compared to controls (Fig. 2a; AD vs. Con, mean of 0.96 vs. 1.74 nmol acetylcholine/h/mg protein; not significant after Dunn's correction).

Compared to control hippocampus (Fig. 2b–d), immunohistochemical staining for ChAT was reduced in the different hippocampal subfields (CA4, CA3-2 and CA1) in MS (Fig. 2e–g) and AD (Fig. 2h–j). Subsequently, detailed quantification of digitized images revealed a significant and substantial decrease in ChAT immunoreactivity in CA4 in MS (mean 80% reduction) and AD hippocampi (mean 92% reduction) (Fig. 2k, Kruskal–Wallis; $p = 0.0023$; after correction: Con vs. MS: $p < 0.05$ and Con vs. AD: $p < 0.01$). Although less prominently, ChAT immunoreactivity was also decreased in CA3-2 in MS (mean 57% reduction) and AD hippocampus (mean 81% reduction) (Fig. 2l, Kruskal–Wallis; $p = 0.0035$; after correction: Con vs. MS: not significant and Con vs. AD: $p < 0.01$). Finally, ChAT immunoreactivity was decreased in CA1 in MS (mean 59% reduction) and in AD (mean 85% reduction) (Fig. 2m, Kruskal–Wallis; $p = 0.0019$; after correction:

Con vs. MS: $p < 0.05$ and Con vs. AD: $p < 0.01$). Retrospective examination of the MS samples showed that the decrease in ChAT immunoreactivity was present irrespective of demyelination (data not shown).

Fig. 2 Reduced ChAT activity and ChAT protein expression in MS hippocampus. **a** Statistically significant differences among the three different groups were found regarding the hippocampal choline acetyltransferase (ChAT) activity (Kruskal–Wallis: $p = 0.026$). In MS hippocampus compared to control hippocampus, a mean reduction in ChAT activity of 43% was found (after Dunn's correction: $p < 0.05$). In AD hippocampus compared to control hippocampus, a mean reduction of 45% was found in ChAT activity (after Dunn's correction: not significant). Non-neurological control hippocampus immunohistochemically stained with anti-ChAT, revealed ChAT immunopositive neurites (*arrows in the insets*) in CA4 (**b**), CA3-2 (**c**) and CA1 (**d**). In all MS hippocampal subfields (**e** CA4, **f** CA3-2, **g** CA1), ChAT immunopositivity was clearly decreased as compared to control tissue, although bead-like varicosities were occasionally observed (*arrows in the insets*). In AD hippocampus, a decrease of ChAT immunopositivity was also found in CA4 (**h**), CA3-2 (**i**) and CA1 (**j**), with occasional immunopositive bead-like varicosities (*arrows in the insets*). Quantification of ChAT immunostainings revealed reductions of the amount of staining in MS and AD hippocampus in (**k**) CA4 (Con vs. MS: $p < 0.05$; Con vs. AD: $p < 0.01$), (**l**) CA3-2 (Con vs. MS: not significant; Con vs. AD: $p < 0.01$) and (**m**) CA4 (Con vs. MS: $p < 0.05$; Con vs. AD: $p < 0.01$). Original magnifications: (**b–j**) $\times 200$ and $\times 630$ (*insets*). Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$, ns not significant



Hippocampal AChE activity and protein expression

Enzyme histochemistry for AChE revealed no differences in the reaction product formed in the different hippocampal subfields when comparing control (Fig. 3a) and MS (Fig. 3b) hippocampus, indicating an unaltered AChE

activity in MS hippocampus. In line with previous results [19], the reaction product was decreased in AD hippocampus (Fig. 3c). Digital analyses confirmed no differences in the reaction product in MS and control hippocampus in all hippocampal subfields (Fig. 3d–f). Retrospectively, examination of the MS samples indicated that lesional areas did not

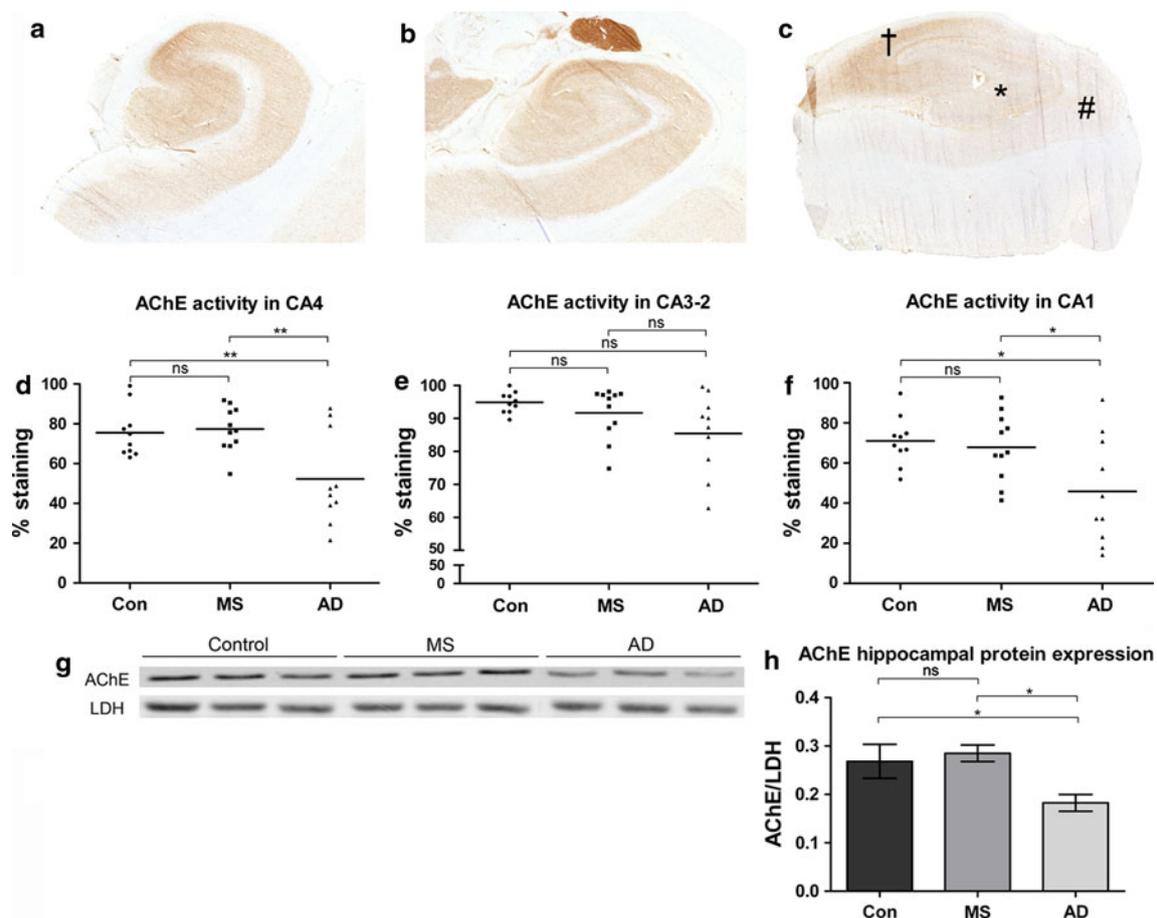


Fig. 3 Unchanged AChE activity and protein expression in MS hippocampus. The activity of AChE as reflected by the amount of reaction product formed was equal in **a** non-neurological control hippocampus and **b** MS hippocampus in all hippocampal subfields. In contrast, in **c** AD hippocampus, the formed reaction product was clearly decreased in CA4 (*asterisk*) and CA1 (*hash*), but not in CA3-2 (*dagger*), indicating a decreased activity of AChE in these areas. Quantification of the reaction product formed by AChE in the (**d**) CA4 hippocampal subfield indicated differences among the three groups (ANOVA: $p = 0.003$, Dunnet correction revealed Con vs. AD and MS vs. AD: $p < 0.01$; Con vs. MS: no significant difference). For the CA3-2 subfield (**e**), no differences were found between the three

differ from non-lesional areas in terms of AChE enzyme activity (data not shown). In hippocampus tissue of AD patients, decreased AChE activity was found in CA4 and CA1 (Fig. 3d, f; $p < 0.01$ and $p < 0.05$, respectively). Subsequent western blot analysis confirmed decreased protein expression levels of AChE in AD hippocampus (Fig. 3g, h; $p < 0.05$), but no differences in AChE protein expression in MS and control hippocampus (Fig. 3g, h).

Discussion

In the current study, a clear reduction of ChAT activity and ChAT protein expression was found in the MS

groups (Kruskal–Wallis: $p = 0.175$). For the CA1 subfield (**f**) differences among the three groups were found (ANOVA: $p = 0.013$, Dunnet correction revealed Con vs. AD and MS vs. AD: $p < 0.05$; Con vs. MS: no significant difference). **g** Representative western blot of 3 control, 3 MS and 3 AD samples indicated decreased protein expression of AChE in AD hippocampus, but not in MS hippocampus tissue. **h** Quantification of protein expression levels of AChE of all samples included indicated significant differences among the three different groups (ANOVA: $p = 0.015$, Dunnet correction revealed Con vs. AD and MS vs. AD: $p < 0.05$; Con vs. MS: no significant difference). *Error bars* indicate SEM. * $p < 0.05$, ** $p < 0.01$, *ns* not significant

hippocampus, with unchanged AChE protein expression levels and enzyme activity, which is in line with a recent positron emission tomography study [38]. Our findings suggest that a selective imbalance exists in the hippocampal cholinergic neurotransmission of MS patients, with reduced synthesis of acetylcholine but an unaltered degradation of the neurotransmitter. This imbalance was not found in AD.

Cholinergic neurons in se are not localized within the human hippocampus, and this brain structure receives its main cholinergic input from the basal forebrain [23]. The basal forebrain consists of several nuclei: the cholinergic neurons in the nucleus basalis of Meynert and the horizontal limb of the diagonal band nucleus mainly innervate

the olfactory bulb, the amygdala and the cerebral cortex [23], whereas the cholinergic neurons associated with the vertical nucleus of the diagonal band and the medial septal nucleus mainly innervate the hippocampal complex [23]. The cholinergic pathways extending from the vertical nucleus and the medial septal nucleus predominantly travel within the fornix. Of interest, in a post-mortem study by Huitinga et al. [20] it was reported that the hypothalamus and adjacent fiber bundles, including the fornix, were affected by (active) demyelination in 16 out of the 17 MS patients studied. It is, therefore, tempting to speculate that demyelinating processes in these areas, co-occurring with axonal damage and loss [36], lead to a cholinergic deficit within the MS hippocampus. However, we were unfortunately not able to investigate lesions in the fornix of the MS patients included in this study, therefore future studies are warranted to investigate this possible correlation. Interestingly, hippocampal cholinergic deficits were found both in demyelinated and non-demyelinated MS hippocampi, although our sample size of non-demyelinated MS hippocampi was low ($n = 3$) to definitively conclude that cholinergic changes are completely lesion-independent. The fact that demyelination and degenerative changes in the neuropil are not necessarily coupled processes in MS was also shown by a post-mortem study by Wegner et al. [39] who found an overall lesion-independent neocortical thinning of $\sim 10\%$ in MS brains indicating that other mechanisms than cortical demyelination may contribute to grey matter pathology in MS.

As reported extensively in AD literature [1, 10, 15, 19, 29, 34, 41], and replicated in this study (although not statistically significant for ChAT activity, probably due to a small AD sample size), our study now also implicates a decreased cholinergic input in the hippocampus of MS patients. As the cholinergic neurotransmitter system has an important role in learning and memory function [11, 13], decreased cholinergic function within the MS hippocampus may be expected to contribute to these memory complaints as experienced by a significant proportion of the MS patients [2, 4, 7, 30]. Indeed, although medical information was sparse for our post-mortem study, cognitive deficits had been specifically reported in the majority of the MS cases included in this study. Future studies investigating hippocampus-dependent cognitive disturbances in MS patients should now be set up in order to study direct causal relationships with the cholinergic abnormalities found in the current study. Interestingly, a magnetic resonance imaging study indicated that cholinergic pathways are more heavily affected in MS patients who scored worse on visuospatial memory tests [6].

Pharmacological manipulation of the cholinergic system, by elevating the postsynaptic acetylcholine content using acetylcholinesterase inhibitors, has repeatedly been proven

to be successful in AD [3] in (temporarily) restoring learning and memory function. First tentative explorations in an animal model of MS [9, 25] and in a small clinical trial on MS patients [22] showed beneficial effects in cognitive performance following the use of acetylcholinesterase inhibitors. Additionally, the use of the acetylcholinesterase inhibitor rivastigmine was found to enhance functional brain adaptive changes related to cognitive processing in MS patients [27]. Our neuropathological and neurochemical findings indeed verify that extensive, and possibly treatable, abnormalities are present in the MS hippocampus. However, our data also show that, compared to AD, MS cases have an imbalance of acetylcholine producing and degrading enzymes.

In MS, hippocampal lesions lack lymphocyte/macrophage infiltration [16, 26, 37]. Hence, it is unlikely that our western blot and enzyme activity results have been influenced by major differences in cellularity. What causes this difference in the cholinergic system between the two neurodegenerative diseases, and how it may possibly affect treatment schemes, is at present unclear and should be the focus of future investigation. Furthermore, as a recent microarray-based study [12] found changes in the glutamate neurotransmitter system in the MS hippocampus, it will also be interesting to see how abnormalities in different neurotransmitter systems may cooperate in the development of cognitive decline in MS.

Acknowledgments E-JK and the MS Center Amsterdam are supported by the Dutch MS Research Foundation (grant numbers 02-498MS, 06-587MS and 09-358c). Furthermore, we thank Prof. Dr. H.J. Groenewegen for reading the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

Open Access This article is distributed under the terms of the Creative Commons Attribution Noncommercial License which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

References

1. Bartus RT, Dean RL, Beer B, Lippa AS (1982) The cholinergic hypothesis of geriatric memory dysfunction. *Science* 217: 408–414
2. Benedict RH, Cookfair D, Gavett R et al (2006) Validity of the minimal assessment of cognitive function in multiple sclerosis (MACFIMS). *J Int Neuropsychol Soc* 12:549–558
3. Birks J (2006) Cholinesterase inhibitors for Alzheimer's disease. *Cochrane Database Syst Rev* 25(1), Art No: CD005593
4. Bobholz JA, Rao SM (2003) Cognitive dysfunction in multiple sclerosis: a review of recent developments. *Curr Opin Neurol* 16:283–288
5. Braak H, Braak E (1991) Neuropathological staging of Alzheimer-related changes. *Acta Neuropathol* 82:239–259
6. Chamelian L, Bocti C, Gao FQ, Black SE, Feinstein A (2005) Detecting cognitive dysfunction in multiple sclerosis with a

- magnetic resonance imaging rating scale: a pilot study. *CNS Spectr* 10:394–401
7. Chiaravalloti ND, Deluca J (2008) Cognitive impairment in multiple sclerosis. *Lancet Neurol* 7:1139–1151
 8. Contestabile A, Ciani E, Contestabile A (2008) The place of choline acetyltransferase activity measurement in the “cholinergic hypothesis” of neurodegenerative diseases. *Neurochem Res* 33:318–327
 9. D’Intino G, Paradisi M, Fernandez M et al (2005) Cognitive deficit associated with cholinergic and nerve growth factor down-regulation in experimental allergic encephalomyelitis in rats. *Proc Natl Acad Sci USA* 102:3070–3075
 10. Davies P, Maloney AJ (1976) Selective loss of central cholinergic neurons in Alzheimer’s disease. *Lancet* 2:1403
 11. Drachman DA, Leavitt J (1974) Human memory and the cholinergic system. A relationship to aging? *Arch Neurol* 30:113–121
 12. Dutta R, Chang A, Doud MK et al (2011) Demyelination causes synaptic alterations in hippocampi from multiple sclerosis patients. *Ann Neurol* 69:445–454
 13. Everitt BJ, Robbins TW (1997) Central cholinergic systems and cognition. *Annu Rev Psychol* 48:649–684
 14. Fonnum F (1975) A rapid radiochemical method for the determination of choline acetyltransferase. *J Neurochem* 24:407–409
 15. Francis PT, Palmer AM, Snape M, Wilcock GK (1999) The cholinergic hypothesis of Alzheimer’s disease: a review of progress. *J Neurol Neurosurg Psychiatry* 66:137–147
 16. Geurts JJ, Bø L, Roosendaal SD et al (2007) Extensive hippocampal demyelination in multiple sclerosis. *J Neuropathol Exp Neurol* 66:819–827
 17. Gold SM, Kern KC, O’Connor MF et al (2010) Smaller cornu ammonis 2–3/dentate gyrus volumes and elevated cortisol in multiple sclerosis patients with depressive symptoms. *Biol Psychiatry* 68:553–559
 18. Hadi AM, Mouchaers KT, Schaliq I et al (2011) Rapid quantification of myocardial fibrosis: a new macro-based automated analysis. *Cell Oncol (Dordr)* (e-publication)
 19. Henke H, Lang W (1983) Cholinergic enzymes in neocortex, hippocampus and basal forebrain of non-neurological and senile dementia of Alzheimer-type patients. *Brain Res* 267:281–291
 20. Huitinga I, De Groot CJ, van der Valk P, Kamphorst W, Tilders FJ, Swaab DF (2001) Hypothalamic lesions in multiple sclerosis. *J Neuropathol Exp Neurol* 60:1208–1218
 21. Karnovsky MJ, Roots L (1964) A “direct-coloring” thiocholine method for cholinesterases. *J Histochem Cytochem* 12:219–221
 22. Krupp LB, Christodoulou C, Melville P, Scherl WF, MacAllister WS, Elkins LE (2004) Donepezil improved memory in multiple sclerosis in a randomized clinical trial. *Neurology* 63:1579–1585
 23. Mesulam MM (2004) The cholinergic innervation of the human cerebral cortex. *Prog Brain Res* 145:67–78
 24. Minger SL, Esiri MM, McDonald B et al (2000) Cholinergic deficits contribute to behavioral disturbance in patients with dementia. *Neurology* 55:1460–1467
 25. Nizri E, Irony-Tur-Sinai M, Faranesh N et al (2008) Suppression of neuroinflammation and immunomodulation by the acetylcholinesterase inhibitor rivastigmine. *J Neuroimmunol* 203:12–22
 26. Papadopoulos D, Dukes S, Patel R, Nicholas R, Vora A, Reynolds R (2009) Substantial archaeocortical atrophy and neuronal loss in multiple sclerosis. *Brain Pathol* 19:238–253
 27. Parry AM, Scott RB, Palace J, Smith S, Matthews PM (2003) Potentially adaptive functional changes in cognitive processing for patients with multiple sclerosis and their acute modulation by rivastigmine. *Brain* 126:2750–2760
 28. Perry EK, Perry RH, Blessed G, Tomlinson BE (1978) Changes in brain cholinesterases in senile dementia of Alzheimer type. *Neuropathol Appl Neurobiol* 4:273–277
 29. Perry EK, Tomlinson BE, Blessed G, Bergmann K, Gibson PH, Perry RH (1978) Correlation of cholinergic abnormalities with senile plaques and mental test scores in senile dementia. *Br Med J* 2:1457–1459
 30. Rao SM, Leo GJ, Bernardin L, Unverzagt F (1991) Cognitive dysfunction in multiple sclerosis. I. Frequency, patterns, and prediction. *Neurology* 41:685–691
 31. Roosendaal SD, Hulst HE, Vrenken H et al (2010) Structural and functional hippocampal changes in multiple sclerosis patients with intact memory function. *Radiology* 255:595–604
 32. Roosendaal SD, Moraal B, Vrenken H et al (2008) In vivo MR imaging of hippocampal lesions in multiple sclerosis. *J Magn Reson Imaging* 27:726–731
 33. Sicotte NL, Kern KC, Giesser BS et al (2008) Regional hippocampal atrophy in multiple sclerosis. *Brain* 131:1134–1141
 34. Sims NR, Bowen DM, Allen SJ et al (1983) Presynaptic cholinergic dysfunction in patients with dementia. *J Neurochem* 40:503–509
 35. Squire LR, Stark CE, Clark RE (2004) The medial temporal lobe. *Annu Rev Neurosci* 27:279–306
 36. Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mork S, Bø L (1998) Axonal transection in the lesions of multiple sclerosis. *N Engl J Med* 338:278–285
 37. Vercellino M, Masera S, Lorenzatti M et al (2009) Demyelination, inflammation, and neurodegeneration in multiple sclerosis deep gray matter. *J Neuropathol Exp Neurol* 68:489–502
 38. Virta JR, Laatu S, Parkkola R, Oikonen V, Rinne JO, Ruutinen J (2011) Cerebral acetylcholinesterase activity is not decreased in MS patients with cognitive impairment. *Mult Scler* (e-publication)
 39. Wegner C, Esiri MM, Chance SA, Palace J, Matthews PM (2006) Neocortical neuronal, synaptic, and glial loss in multiple sclerosis. *Neurology* 67:960–967
 40. Whitehouse PJ, Price DL, Struble RG, Clark AW, Coyle JT, Delon MR (1982) Alzheimer’s disease and senile dementia: loss of neurons in the basal forebrain. *Science* 215:1237–1239
 41. Wilcock GK, Esiri MM, Bowen DM, Smith CC (1982) Alzheimer’s disease. Correlation of cortical choline acetyltransferase activity with the severity of dementia and histological abnormalities. *J Neurol Sci* 57:407–417