Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 319 (2004) 733–737
BBRC
www.elsevier.com/locate/ybbrc

Development of Aβ terminal end-specific antibodies and sensitive ELISA for Aβ variant

Yuko Horikoshi,[a,b] Gaku Sakaguchi,[c,1] Amanda G. Becker,[c] Audrey J. Gray,[c] Karen Duff,[d] Paul S. Aisen,[c] Haruyasu Yamaguchi,[b] Masahiro Maeda,[a] Noriaki Kinoshita,[a] and Yasuji Matsuoka[c,*]

[a] *Immuno-Biological Laboratories Co., Ltd. (IBL), Fujioka-shi, Gunma 375-0005, Japan*
[b] *Gunma University School of Health Sciences, Maebashi-shi, Gunma 371-8514, Japan*
[c] *Department of Neurology, Georgetown University Medical Center, Washington, DC 20057, USA*
[d] *Center for Dementia Research, Nathan Kline Institute for Psychiatric Research, New York University School of Medicine, Orangeburg, NY 10962, USA*

Received 5 May 2004

Abstract

Alzheimer's disease (AD) is a neurodegenerative affliction associated with memory dysfunction. Senile plaques are a pathological hallmark of AD, and amyloid β (Aβ) peptides are a major component of these plaques. Aβ peptides are derived from proteolytic cleavage of the Aβ protein precursor (APP) by β- and γ-secretases to generate two principal species, Aβ1–40 and Aβ1–42. We have developed antibodies against the N- and C-termini of these peptides, and an ELISA for accurate and sensitive quantitative assessment. Sandwich ELISA composed of N-terminus (Aβ1) end-specific antibody, clone 82E1, and C-termini end-specific antibodies, and clones 1A10 and 1C3 for Aβ40 and Aβ42, respectively, detects full-length Aβ1–40 and 1–42 with a sensitivity in the sub single digit fmol/ml (equivalent to single digit pg/ml) range with no cross-reactivity to APP. A combination of C-termini antibodies and an antibody against the middle region of Aβ detects mouse Aβ in non-transgenic mouse brains.

Keywords: Alzheimer; Dementia; Amyloid; Aβ; Antibody; β-Secretase; ELISA; Histochemistry; Western

Alzheimer's disease (AD) is a neurodegenerative affliction associated with memory dysfunction. Amyloid β (Aβ) peptides are the major component of senile plaques, a pathologic hallmark of AD. Aβ is generated from a larger precursor protein, the Aβ protein precursor protein (APP), by proteolytic processing at the N and C termini of the Aβ domain by β- and γ-secretases, respectively [1]. Modulating the APP processing cascade is a promising therapeutic approach, and β- and γ-secretase inhibitors are being aggressively pursued (for review [2–4]). To evaluate the efficacy of Aβ-lowering strategies, measurement of full-length Aβ1–40 and 1–42 is crucial. However, the majority of currently available Aβ ELISAs do not use N-terminus end-specific antibody, but rather an antibody against the middle, or N-terminal region (but not specific to the β-cleavage site). These ELISAs quantify Aβx–40 and x–42, which are not the optimal measures for the evaluation of anti-Aβ therapeutic strategies. In this study, we have developed antibodies that specifically detect the N-terminus end (β-cleavage site, i.e., Aβ1) or the C-terminus ends (Aβ40 and 42) and have optimized ELISAs by combination of these antibodies. We have also developed ELISAs which can detect endogenous Aβ from mouse brain homogenate.

Materials and methods

Mice (BALB/c, Charles River, Japan) were immunized weekly with thyroglobulin conjugated Aβ peptides (50 μg/mouse). We used partial human Aβ peptide consisting of amino acid residues of 1–16

* Corresponding author. Fax: 1-202-784-3504.
E-mail address: ym56@georgetown.edu (Y. Matsuoka).
[1] Present address: Shionogi Co. Ltd., Discovery Research Laboratories, Shionogi, Koka, Shiga 520-3423, Japan.

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.05.051

(DAEFRHDSGYEVHHQK), 11–28 (EVHHQKLVFFAEDVGS NK), 35–40 (MVGGVV), and 38–42 (GVVIA) as immunogens for Aβ N-terminus, middle region, and C-terminus-specific antibodies, respectively. The cysteine residue was combined in each immunogen beforehand for binding to the carrier protein, bovine thyroglobulin. After four to six immunizations, the spleen was isolated and fused with X63Ag8 myeloma cells. Epitopes and cross-reactivity to human and rodent Aβ of selected clones were determined by a microplate assay using various Aβ fragments (American Peptides, Sunnyvale, CA). A 96-well plate (Maxisorp, Nunc, Denmark) was coated with various Aβ fragments (human Aβ sequence unless otherwise indicated as "r"), such as −11 to −1, −4–8, −3–5, 1–5, 1–7, r1–9, 1–11, r1–15, 1–17, 1–40, r1–40, 1–42, r1–42, 2–40, 3–7, 3–13, 3–40, 7–17, 10–20, 11–28, 13–19, 17–28, 20–29, 35–40, and 38–42 (100 ng/well) and blocked with Block Ace (Serotec) overnight at 4 °C. The plates were incubated with selected antibodies for 4 h, then with an HRP-coupled anti-mouse IgG antibody (Southern Biotechnology, Birmingham, AL), and visualized by TMB substrate (Pierce, Rockford, IL).

For Western blotting analysis, we prepared various Aβ containing samples, such as fibril-free soluble and mixture of soluble and fibril Aβ from synthetic peptides, culture medium from APP overexpressing HEK cells, and brain homogenate from APP overexpressing transgenic mice. Lyophilized Aβ (American Peptide) was sequentially dissolved in trifluoroacetic acid and 1,1,1,3,3,3-hexafluoro-2-propanol followed by complete elimination by exposure to nitrogen gas. Aβ peptide was then re-suspended in dimethyl sulfoxide and used as fibril-free Aβ. Aβ was aggregated at room temperature for 10 days with mixing at 500 rpm and used as fibril-containing Aβ. To assess the N-terminus specificity, HEK293 cells expressing Swedish mutant APP were cultured with or without a gamma-secretase inhibitor, *N*-[*N*-(3,5-difluorophenacetyl-l-alanyl)]-*S*-phenylglycine *t*-butylester (DAPT) [5], for 24 h and cell lysates were analyzed on a 10–20% NuPAGE gel (Invitrogen, Carlsbad, CA). Also, we analyzed overexpressing APP transgenic mice [6], known as Tg2576. At 18 months of age, the brains were homogenized in PBS containing 2% SDS. All samples, except cell lysate, were run on a 16.5% Tris–Tricine gel (Bio-Rad, Hercules, CA) and transferred to a PVDF membrane. After blocking with 5% non-fat dried milk, the membrane was probed with anti-Aβ antibody followed by HRP-coupled anti-mouse IgG (Southern Biotechnology, Birmingham, AL). For a comparison, we also used N-terminus region antibody, 6E10 (Signet, Dedham, MA). Bands were visualized with a kit (Super Signal West Pico, Pierce). For immunohistochemistry, 4 μm-thickness paraffin sections of the temporal cortex from human autopsied AD brain, were incubated with 82E1, 12B2, 1A10, and 1C3 (2, 1, 2, and 2 μg IgG/ml, respectively) overnight after brief pretreatment with formic acid. Immunoreactivity was visualized using an ABC elite kit (ABC elite, Vector Laboratories, Burlingame, CA). Sections were briefly counterstained with hematoxylin.

For sandwich ELISA, 96-well plate (Maxisorp, Nunc, Denmark) was coated with 100 μl of either Aβ40 or Aβ42-specific antibody (5.0 μg IgG/ml each), clones 1A10 and 1C3, respectively, overnight at 4 °C in 100 mM carbonate buffer, pH 9.6, containing 0.05% sodium azide. After blocking with 1% Block Ace in PBS overnight at 4 °C, standards (human or mouse synthetic Aβ peptides 1–40 and 1–42) and samples were loaded and incubated overnight at 4 °C. Human plasma specimens were obtained from the Mount Sinai Alzheimer's Disease Research Center. Mouse brain Aβ was extracted in 0.4% diacetylamine containing buffer, centrifuged, and the resultant supernatant was used. HRP-coupled detection antibody, either 82E1 or 12B2, was incubated for 4 h at room temperature and visualized using a TMB substrate.

Results and discussion

We selected clones that showed high and selective affinity to the Aβ peptide used for immunization. We identified four potential clones, 82E1, 12B2, 1A10, and 1C3, which react with different regions of Aβ peptide. The specific epitope for each of these antibodies was determined by plate assays using various Aβ fragments. We examined human and rodent cross-reactivity using human and rodent Aβ1–40 and 1–42. Clone 82E1 showed higher selectivity for human Aβ (approximately 10% cross-reactivity to rodent Aβ), probably because the human-specific 5th amino acid is a part of the epitope. Clones 12B2, 1A10, and 1C3 reacted with both human and rodent Aβ to a similar degree because their epitopes do not include a human-specific residue. Table 1 summarizes epitopes, specificity, cross-reactivity to human and rodent Aβ, and applications of these antibodies.

Clone 82E1 reacted with both soluble and fibrillar Aβ to a similar degree (Fig. 1A). Clone 82E1 detected Aβ in

Table 1
Antibodies and applications

Clone	Selectivity, human/rodent cross-reactivity	Applications			
		WB	IHC	ELISA	IP
82E1 (IgG1)	N-terminus end specific (Aβ1–5) No cross-reactivity to non-cleaved APP fragments Preference to human Aβ (≈10% cross-reactivity to mouse Aβ)	+++	+++	+++	+++
12B2 (IgG1)	Middle region-specific (Aβ17–28) Cross-reactive to human and rodent Aβ at similar degree	+[a]	+++	+++	+[b]
1A10 (IgG1)	C-terminus end specific (Aβ40) Cross-reactive human and rodent Aβ at similar degree <1.8% cross-reactivity to Aβ 42	+++	+++	+++	+++
1C3 (IgG1)	C-terminus end specific (Aβ42) Cross-reactive human and rodent Aβ at similar degree <0.1% cross-reactivity to Aβ40	–	+++	+++	+[b]

The utility of the antibodies for various applications is indicated as follows: +++, very useful; +, useful with limitations; and –, not useful under our testing conditions.

[a] 12B2 did not work in tissue homogenates, but worked with synthetic Aβ peptides (>50 ng Aβ).

[b] 12B2 and 1C3 worked in IP but not as well as 82E1 and 1A10. WB, Western blotting; IHC, immunohistochemistry; IP, immunoprecipitation.

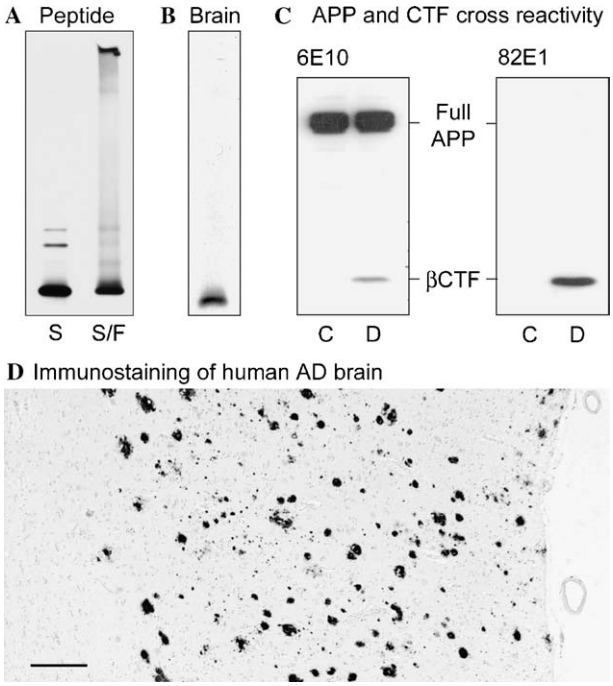

Fig. 1. Characterization of β-cleavage site-specific antibody, clone 82E1. (A) Fibril-free soluble (S) and soluble and fibril-containing (S/F) Aβ were prepared from synthetic Aβ peptide, run on a gel, and probed with 82E1. Clone 82E1 detects both soluble and fibril Aβ. (B) APP transgenic mouse (Tg2576) brain extract was run on a gel and probed with 82E1. 82E1 detects Aβ, but not with full-length APP. (C) β-cleavage site specificity was further confirmed using the transfected cells. 82E1 does not react with full-length APP (lane C), although non-β site-specific antibody 6E10 detects (lane C). βCTF is strongly detectable after treatment with a γ-secretase inhibitor, DAPT (lane D). (D) Human AD brain was stained with 82E1. Bar = 200 μm.

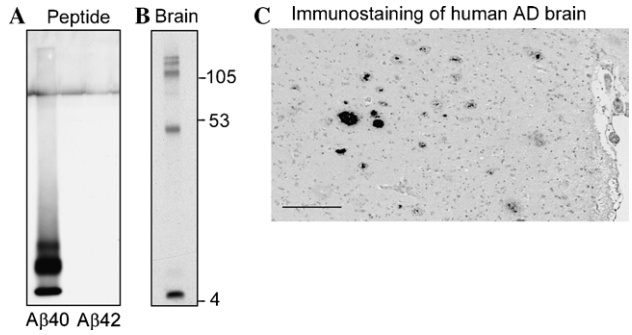

Fig. 2. Characterization of Aβ40-specific antibody, clone 1A10. Aβ40 and Aβ42 were run on a gel and probed with 1A10. 1A10 specifically detected Aβ40 and did not show cross-reactivity to Aβ42 (A). Aβ was also detected in brain Aβ extract from an APP transgenic mouse, Tg2576 (B). Immunohistochemistry stained Aβ plaques in AD brain (C). Bar = 200 μm.

the SDS extract from APP-expressing transgenic mice (Tg2576), but did not detect non-cleaved APP (Fig. 1B). N-terminus specificity was further confirmed using HEK cells expressing Swedish mutant APP. 82E1 did not react with full-length APP (Fig. 1C, lane C), although a control antibody, 6E10 (epitope within 3–10), detected full-length APP (Fig. 1C, lane C). β-secretase (BACE) generates β-cleaved C-terminus fragment (βCTF) from the full-length APP, but βCTF was not detectable in this transfected cell probably due to high γ-secretase activity. After treatment with a γ-secretase inhibitor, DAPT, for 24 h, βCTF is strongly detectable with 82E1 (Fig. 1C, lane D). 82E1 stained senile plaques in AD patients with brief formic acid treatment (Figs. 1D and 3C) Thus, 82E1 specifically detected fragments generated by β-secretase cleavage.

Clone 12B2 was raised against the middle region of Aβ peptide using Aβ11–28 as an immunogen. This antibody is cross-reactive to human and rodent Aβ in a plate assay, and a sandwich ELISA made with a combination of C-terminus-specific antibodies, 1A10 and 1C3, detects mouse endogenous brain and CSF Aβ (see below).

C-terminus-specific antibodies, clones 1A10 and 1C3 (Aβ40 and Aβ42 specific, respectively), showed virtually no cross-reactivity to each other (<1.8 and <0.1%, respectively). The Aβ40-specific antibody, 1A10, worked well in immunoblotting (Figs. 2A and B), immunohistochemistry (Figs. 2C and 3B), immunoprecipitation, and ELISA, although Aβ42-specific antibody, 1C3, worked well only in immunohistochemistry (Fig. 3D) and ELISA.

We developed sandwich ELISA by a combination of these antibodies. We selected the C-terminus antibodies, either Aβ40 (1A10) or 42 specific (1C3), as the capturing antibody, and another antibody, either 82E1 or 12B2, as a detection antibody (reporter). We did not consider other combinations (82E1/12B2 for capturing and 1A10/1C3 for detection), because Aβ40 is much more abundant than Aβ42 in biological specimens and Aβ40 might monopolize the capturing antibody and reduce the sensitivity of Aβ42 detection. A combination of 1A10/82E1 and 1C3/82E1 gave a linear standard curve in the range of 1–1000 pg/ml (typical correlation factor >0.98) and detected human Aβ as low as 1.5 pg/ml (equivalent to sub fmol/ml range). We measured human Aβ in the plasma of AD patients and non-AD controls (Table 2). Previous publications showed that human plasma has a wide range of Aβ, and there is no significant difference between levels in AD and non-AD controls [7–11]. Our sandwich ELISAs using 1A10/82E1 and 1C3/82E1 similarly revealed no significant difference between AD and non-AD control cases ($p = 0.442$).

The epitope of clone 12B2 is 17–28 which is an identical sequence in human and rodent Aβ, and 12B2 reacted to human and rodent Aβ to a similar degree. We used this antibody as a reporter antibody and developed an ELISA to detect endogenous mouse brain Aβx–40 and x–42. A combination of 1A10/12B2 and 1C3/12B2 gave a linear standard curve in the range of 1–500 pg/ml (typical correlation factor >0.96). We measured

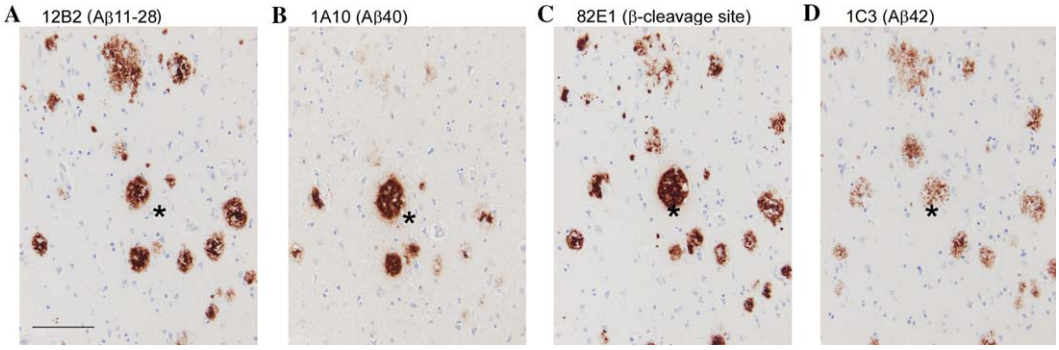

Fig. 3. Immunostainng of AD brains. Serial sections (A–D) from AD patient were stained with antibodies generated in this study. An asterisk indicates an identical Aβ plaque. Bar = 100 μm.

Table 2
Levels of Aβ40 in human plasma

Case No.	Gender	Age (year)	MMSE	ApoE1/E2	Aβ40 (fmol/ml)
(A) Non-AD controls					
052	m	71.8	29	3, 3	52
2065	f	69.2	30	3, 4	4
9415	f	74.4	29	3, 3	798
4019	m	67.4	30	3, 3	n.d.
5487	m	70.5	28	3, 4	33
0505	m	74.5	30	3, 3	224
3138	m	70.9	30	3, 3	182
		71.2 ± 2.4	29 ± 1		184 ± 264
(B) AD					
8640	m	73.7	27	3, 3	27
7564	f	84.3	8	3, 4	60
6270	f	85.7	8	3, 3	n.d.
9029	m	74.4	27	4, 4	120
5207	f	76.3	8	4, 4	6
2502	f	75.5	24	3, 3	19
6625	m	83.2	9	3, 4	475
4379	f	78.8	9	3, 3	66
6003	f	79.3	13	3, 4	33
		79.0 ± 4.2	15 ± 8		89 ± 141

Levels of Aβ in the human plasma were compared between non-AD controls (A) and AD patients (B). AD patients met NINDS-ADRDA criteria [12] for probable AD, and had lower MMSE scores [13] than controls, $p = 0.001$. No significant difference in Aβ levels between AD patients and controls was detected ($p = 0.442$).

endogenous brain and CSF Aβ in wild type (non-transgenic) mice, finding 25.2 ± 4.8 fmol Aβ40/ml (means ± SE, $n = 4$) and 817 ± 73 fmol Aβ40/ml (means ± SE, $n = 2$), respectively.

In conclusion, we have generated a series of anti-Aβ antibodies, including those that are specific both for the N-terminus β-cleavage site (clone 82E1) and for C-terminus γ-cleavage sites (clones 1A10 and 1C3). Because 82E1 is not cross-reactive with APP and β-cleavage site-specific, 82E1 is a critical tool in investigation of full-length Aβ. Combinations of these antibodies were used in ELISA to quantify full-length Aβ1–40 and 1–42, and rodent endogenous Aβx–40 and x–42. These assays will allow for very sensitive and accurate evaluation of anti-Aβ therapeutic approaches.

Acknowledgments

This work was supported by Grants from the National Institutes of Health (Y.M., K.D.), and the Alzheimer's Association (Y.M.), and a grant for priority areas from Ministry of Education, Science, Culture and Sports, Japan (H.Y.).

References

[1] J. Hardy, D.J. Selkoe, The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics, Science 297 (2002) 353–356.
[2] S.B. Roberts, Gamma-secretase inhibitors and Alzheimer's disease, Adv. Drug Deliv. Rev. 54 (2002) 1579–1588.
[3] K. Sambamurti, N.H. Greig, D.K. Lahiri, Advances in the cellular and molecular biology of the beta-amyloid protein in Alzheimer's disease, Neuromol. Med. 1 (2002) 1–31.

[4] S. Roggo, Inhibition of BACE, a promising approach to Alzheimer's disease therapy, Curr. Top. Med. Chem. 2 (2002) 359–370.
[5] H.F. Dovey, V. John, J.P. Anderson, L.Z. Chen, A.P. de Saint, L.Y. Fang, S.B. Freedman, B. Folmer, E. Goldbach, E.J. Holsztynska, K.L. Hu, K.L. Johnson-Wood, S.L. Kennedy, D. Kholodenko, J.E. Knops, L.H. Latimer, M. Lee, Z. Liao, I.M. Lieberburg, R.N. Motter, L.C. Mutter, J. Nietz, K.P. Quinn, K.L. Sacchi, P.A. Seubert, G.M. Shopp, E.D. Thorsett, J.S. Tung, J. Wu, S. Yang, C.T. Yin, D.B. Schenk, P.C. May, L.D. Altstiel, M.H. Bender, L.N. Boggs, T.C. Britton, J.C. Clemens, D.L. Czilli, D.K. Dieckman-McGinty, J.J. Droste, K.S. Fuson, B.D. Gitter, P.A. Hyslop, E.M. Johnstone, W.Y. Li, S.P. Little, T.E. Mabry, F.D. Miller, J.E. Audia, Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain, J. Neurochem. 76 (2001) 173–181.
[6] K. Hsiao, P. Chapman, S. Nilsen, C. Eckman, Y. Harigaya, S. Younkin, F. Yang, G. Cole, Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice, Science 274 (1996) 99–102.
[7] Y.M. Kuo, M.R. Emmerling, H.C. Lampert, S.R. Hempelman, T.A. Kokjohn, A.S. Woods, R.J. Cotter, A.E. Roher, High levels of circulating Abeta42 are sequestered by plasma proteins in Alzheimer's disease, Biochem. Biophys. Res. Commun. 257 (1999) 787–791.
[8] P.D. Mehta, T. Pirttila, B.A. Patrick, M. Barshatzky, S.P. Mehta, Amyloid beta protein 1–40 and 1–42 levels in matched cerebrospinal fluid and plasma from patients with Alzheimer disease, Neurosci. Lett. 304 (2001) 102–106.
[9] A. Olsson, E. Vanmechelen, H. Vanderstichele, P. Davidsson, K. Blennow, Unaltered plasma levels of beta-amyloid(1–40) and beta-amyloid(1–42) upon stimulation of human platelets, Dement. Geriatr. Cogn. Disord. 16 (2003) 93–97.
[10] L.A. Farrer, L.A. Cupples, J.L. Haines, B. Hyman, W.A. Kukull, R. Mayeux, R.H. Myers, M.A. Pericak-Vance, N. Risch, C.M. van Duijn, Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. APOE and Alzheimer disease meta analysis consortium, JAMA 278 (1997) 1349–1356.
[11] J. Naslund, V. Haroutunian, R. Mohs, K.L. Davis, P. Davies, P. Greengard, J.D. Buxbaum, Correlation between elevated levels of amyloid beta-peptide in the brain and cognitive decline, JAMA 283 (2000) 1571–1577.
[12] G. McKhann, D. Drachman, M. Folstein, R. Katzman, D. Price, E.M. Stadlan, Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA work group under the auspices of department of health and human services task force on Alzheimer's disease, Neurology 34 (1984) 939–944.
[13] M.F. Folstein, S.E. Folstein, P.R. McHugh, "Mini-mental state." A practical method for grading the cognitive state of patients for the clinician, J. Psychiatr. Res. 12 (1975) 189–198.