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Highly conserved and disease-specific patterns of carboxyterminally truncated Aβ peptides 1–37/38/39 in addition to 1–40/42 in Alzheimer's disease and in patients with chronic neuroinflammation

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Abstract

Human lumbar CSF patterns of Aβ peptides were analysed by urea-based β-amyloid sodium dodecyl sulphate polyacrylamide gel electrophoresis with western immunoblot (Aβ-SDS–PAGE/immunoblot). A highly conserved pattern of carboxyterminally truncated Aβ1–37/38/39 was found in addition to Aβ1–40 and Aβ1–42. Remarkably, Aβ1–38 was present at a higher concentration than Aβ1–42, being the second prominent Aβ peptide species in CSF. Patients with Alzheimer's disease (AD, $n = 12$) and patients with chronic inflammatory CNS disease (CID, $n = 10$) were differentiated by unique CSF Aβ peptide patterns from patients with other neuropsychiatric diseases (OND, $n = 37$). This became evident only when we investigated the amount of Aβ peptides relative to their total Aβ peptide concentration (Aβ1–x%, fractional Aβ peptide pattern), which may reflect disease-specific γ-secretase activities. Remarkably, patients with AD and CID shared elevated Aβ1–38% values, whereas otherwise the patterns were distinct, allowing separation of AD from CID or OND patients without overlap. The presence of one or two ApoE ε4 alleles resulted in an overall reduction of CSF Aβ peptides, which was pronounced for Aβ1–42. The severity of dementia was significantly correlated to the fractional Aβ peptide pattern but not to the absolute Aβ peptide concentrations.

Keywords: Alzheimer's disease (AD), β-amyloid protein precursor/metabolism, biological markers, cerebrospinal fluid, 2D-PAGE, western immunoblot.

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Abbreviations used: Aβ peptides, beta-amyloid peptides; Aβ-SDS–PAGE/immunoblot, β-amyloid sodium dodecyl sulphate polyacrylamide gel electrophoresis with western immunoblot; Aβ-IPG-2D-PAGE/immunoblot, β-amyloid immobilized pH gradient electrofocussing with Aβ-SDS–PAGE/immunoblot as second analytic dimension; AD, Alzheimer's disease; AD$\varepsilon4^{plus}$, AD patients carrying one or two ApoE ε4; ApoE, ε4, apolipoprotein E allele ε4; APP, β-amyloid precursor protein; bis, *N*,*N*′-methylenebisacrylamide; bicine, *N*,*N*′-bis-[2-hydroxyethyl]glycine; BSA, bovine serum albumin; C%, percentage (w/w) of bisacrylamide per total acrylamide monomer; CID, chronic inflammatory CNS diseases; Ct-truncated, carboxyterminally truncated; DTT, dithiothreitol; ECL, enhanced chemiluminescence; FAD, familial AD; MALDI-TOF, matrix-assisted laser desorption ionization mass analysis–time-of-flight modus; M_r, molecular mass; NDC, non-demented disease controls (OND plus CID); OND, other non-demented neuropsychiatric diseases; OND$\varepsilon4^{plus}$, OND patients carrying one or two ApoE ε4; OND$\varepsilon4^{minus}$, OND patients without ApoE ε4; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; T%, percentage (w/v) of total acrylamide monomer.

β-Amyloid peptides (Aβ peptides) are generated from β-amyloid precursor protein (APP) by two proteoloytic activities named β- and γ-secretase, whereas cleavage by α-secretase within the Aβ peptide domain of APP precludes their generation (Haass and Selkoe 1993). Aβ peptides comprise a heterogeneous set of peptides, the predominant species starting aminoterminally (Nt) at Asp-1 and ending carboxyterminally (Ct) at Val-40. Regarding the secretases, one recent notable achievement has been the identification of the β-secretase BACE (β-site amyloid cleaving enzyme), a metalloproteinase of 50 kDa (Vassar *et al*. 1999). Another metalloproteinase, ADAM10, is a potential candidate for α-secretase, as are MDC9 and TACE (Black *et al*. 1997; Moss *et al*. 1997; Buxbaum *et al*. 1998; Koike *et al*. 1999; Lammich *et al*. 1999). Even if the γ-secretase has not yet been formally identified, most of the existing *in vivo* and *in vitro* data point to the correspondence of presenilins with an aspartyl γ-secretase activity (De Strooper *et al*. 1998; Wolfe *et al*. 1999; Selkoe and Wolfe 2000).

Aβ peptides are constitutively secreted by APP processing cells and occur as soluble constituents of CSF, blood, and urine (Haass *et al*. 1992; Seubert *et al*. 1992; Shoji *et al*. 1992). Aggregated Aβ peptides form the major constituent of the amyloid fibrils deposited in senile plaques and cerebral blood vessels of patients with Alzheimer's disease (AD) and Down's syndrome (DS) (Glenner and Wong 1984a; Glenner and Wong 1984b; Masters *et al*. 1985). Several findings suggest that Ct-elongated Aβ peptides ending at amino acid 42 (Aβ1–42) are of particular importance in the pathogenesis of AD: (i) familial AD (FAD) mutations in three distinct genes, APP, presenilin-1 (PS1) and presenilin-2 (PS2) were shown to result in increased production of Aβ1–42 (Suzuki *et al*. 1994; Tamaoka *et al*. 1994b; Borchelt *et al*. 1996; Duff *et al*. 1996; Scheuner *et al*. 1996). (ii) Aβ1–42 is the peptide species initially deposited in β-amyloid plaques and forms a major component in all stages of β-amyloid plaque maturation (Miller *et al*. 1993; Roher *et al*. 1993; Iwatsubo *et al*. 1994; Näslund *et al*. 1994; Tamaoka *et al*. 1994a; Gravina *et al*. 1995; Shinkai *et al*. 1995). (iii) Carboxyterminally elongated Aβ peptides (Aβ1–42/43) are more prone to aggregation (Barrow and Zagorski 1991; Hilbich *et al*. 1991; Burdick *et al*. 1992; Jarrett *et al*. 1993).

Previous investigations in blood, CSF or cell culture supernatants have largely focused on total Aβ peptides or on Aβ1–40 and Aβ1–42. Aβ1–40 is the major soluble peptide species, while Aβ1–42 accounts for approximately 10%. Mass spectrometry has indicated the existence of additional Aβ peptides (Vigo-Pelfrey *et al*. 1993; Asami-Odaka *et al*. 1995; Wang *et al*. 1996). However, the absolute and relative quantities of these additional Aβ peptides are not known, nor has it been investigated by mass spectrometry whether they are regularly produced and show disease-specific patterns.

We recently demonstrated electrophoretic baseline separation of Aβ peptides differing in length by only single amino acids (Klafki *et al*. 1996; Wiltfang *et al*. 1997) using the urea-based multiphasic bicine/sulphate SDS–PAGE system of Wiltfang *et al*. (1991). Here, this Aβ-SDS–PAGE/immunoblot was combined with electrofocussing by immobilized pH gradients (IPG) to yield a two-dimensional Aβ-IPG-2D-PAGE/immunoblot.

A constant and highly conserved quintet of the Ct-truncated Aβ peptides 1–37, 1–38, 1–39 in addition to 1–40 and 1–42 was detected by one- and two-dimensional Aβ-SDS–PAGE/immunoblot in human CSF samples. The occurrence and relative amounts of these five Aβ peptides were further investigated in human CSF from patients with various neuropsychiatric disorders. For the relative amounts of Aβ peptides in CSF we observed disease-specific patterns in patients with AD and chronic inflammatory CNS disease (CID). The diagnostic value and the possible pathophysiological importance of these findings are discussed.

Materials and methods

Materials

Acrylamide, *N*,*N*′-methylenebisacrylamide (bis), SDS, TEMED and ammonium peroxydisulphate (AMPS), and Bio-Rad Extra Thick Filter Paper for electroblotting were obtained from Bio-Rad (Richmond, CA, USA); Roti-Block synthetic blocking reagent was obtained from Roth (Karlsruhe, Germany); sucrose, bis-[2-hydroxyethyl]imino-tris-[hydroxymethyl]-methane (bistris), *N*,*N*′-bis-[2-hydroxyethyl]-glycine (bicine), and Tris were purchased from Sigma (St. Louis, MO, USA); H_2SO_4 (Titrisol), was supplied by Merck (Darmstadt, Germany); 2-mercaptoethanol and bromophenol blue were obtained from Fluka (Buchs, Switzerland); urea was obtained from Gibco BRL (Eggenstein, Germany). Immobilon-P polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Bedford, MA, USA).

Synthetic Aβ1–38, Aβ1–40, and Aβ1–42 were obtained from Bachem (Bubendorf, Switzerland). Synthetic Aβ peptides 1–33, 1–34, 1–35, 1–37 and 1–39 were synthesized (see Materials and methods). The purity of synthetic peptides was at least 95%. Magnetic microparticles (Dynabeads M-280) with covalently attached anti-mouse IgG for immunoprecipitation were obtained from German Dynal GmbH (Hamburg, Germany).

The monoclonal antibodies 6E10 (Kim *et al*. 1990) and 1E8 directed against the N-terminus of Aβ peptides were obtained from Senetek Drug Delivery Technologies Inc. (St. Louis, MO, USA), and provided by Schering AG (Berlin, Germany), respectively. The monoclonal antibodies 13E9 and 6D5 directed against the C-terminus of Aβ1–40 and Aβ1–42, respectively, were provided by Schering AG (Berlin, Germany). The proteinase inhibitor cocktail Complete™ Mini was purchased from Boehringer Mannheim (Mannheim, Germany). The biotinylated secondary anti-mouse polyclonal antibody IgG (H + L) affinity purified from horse serum was obtained from Vector Laboratories (Burlingame, CA,

USA). The streptavidin-biotinylated horseradish peroxidase complex and the reagent ECLPlus™ were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Preparation of samples

CSF samples

Three to 10 mL of CSF was drawn from patients by lumbar puncture and sampled in polypropylene vials. Following centrifugation (1000 *g*, 10 min, 4°C) CSF samples were processed within 24 h and aliquots of 150 μL were stored at − 80°C for subsequent one- and two-dimensional Aβ-SDS–PAGE/immunoblot and $ELISA_{Aβ1–42}$.

Sample buffer for Aβ-SDS–PAGE

Synthetic Aβ peptides were directly dissolved in sample buffer I containing 0.36 M bistris, 0.16 M bicine, 1% (w/v) SDS, 15% (w/v) sucrose, 0.004% (w/v) bromophenol blue, and heated to 95°C for 5 min. The latter buffer was used for subsequent dilutions of synthetic Aβ peptides. Sample buffer II was composed of 0.12 M bistris, 0.053 M bicine, 5% sucrose, 0.5% SDS, 0.0025% bromophenol blue containing one tablet of proteinase inhibitor cocktail Complete™ Mini per 10 mL. Sample buffer II was dried (SpeedVac, 40°C) in polypropylene cups (Eppendorf, Hamburg, Germany). The equivalent volume of CSF was added to the polypropylene cups, vortexed until complete solubilization of the sample buffer, and heated at 95°C for 5 min after the addition of 2-mercaptoethanol to a final concentration of 2.5% v/v.

Sample buffer for Aβ-IPG-2D-PAGE

Dried samples were solubilized by vortexing for 10 min at room temperature (21°C) in the following IPG sample buffer: 9 M urea, CHAPS 2.0% (w/v), dithiothreitol (DTT) 1.0% (w/v), pharmalyte 3–10 0.8% (v/v), Serdolit MB-1 1% (w/v). The Serdolit ion exchanger had to be removed before adding the sample. Synthetic Aβ peptides were diluted in 0.1% (v/v) of NH_4OH and added to reconstitute the IPG sample buffer, which had been dried (SpeedVac, 40°C) after removing the Serdolit ion exchanger.

Immunoprecipitation of CSF samples

Magnetic microparticles were activated with mAb according to the protocol of the manufacturer (direct IP method). We used 10 μg of mAb 1E8, 10 μg of mAb 13E9, 10 μg of mAb 6D5, and 7.5 μg of mAb 6E10 per 1.68×10^8 beads.

Immunoprecipitation of CSF

Two hundred μL of CSF was added to 200 μL of five-fold concentrated RIPA detergent buffer ($RIPA_{5x}$: 2.5% Nonidet P-40, 1.25% sodium deoxycholate, 0.25% SDS, 750 mM NaCl, 250 mM HEPES, one tablet of Protease Inhibitor Cocktail Complete Mini per 2 mL of $RIPA_{5x}$, pH adjusted to 7.4 with NaOH) and 25 μL of magnetic microparticles coated with the monoclonal antibody 1E8 (1 μg mAb 1E8/1.68×10^7 beads), 600 μL H_2O_{dd}, and 25 μL of activated magnetic microparticles (1 μg mAb 1E8/1.68×10^7 beads). Samples were incubated under rotation for 15 h at 4°C. Beads were washed four times with phosphate-buffered saline (PBS)/0.1% bovine serum albumin (BSA), once with 10 mM Tris/HCl, pH 7.4. For Aβ-SDS–PAGE/immunoblot, bound Aβ peptides were eluted by heating the sample to 95°C for 5 min with 25 μL sample buffer I.

Micropreparative immunoprecipitation of CSF

Sixteen millilitres of CSF of an OND patient were split into aliquots of 800 μL. Two hundred microlitres of $RIPA_{5x}$ and 25 μL of magnetic microparticles (1 μg mAb 1E8/1.68×10^7 beads) were added to each aliquot. Incubation was done as detailed for analytical CSF samples. After two additional washes with $RIPA_{1x}$ washing was done as described. Subsequently, aliquots were pooled and Aβ peptides were eluted in the presence of 400 μL 0.1% (v/v) freshly prepared NH_4OH for 10 min at 37°C in a sonication bath. Samples were dried (SpeedVac, 40°C) and stored at 4°C for subsequent MALDI-TOF mass spectroscopy.

Aβ-SDS–PAGE

For the separation of Aβ peptides we applied the urea version of the bicine/bistris/tris/sulphate SDS–PAGE of Wiltfang *et al*. (1991). This system was used for the separation of Aβ peptides for the first time and without further modification by Klafki *et al*. (1996). Due to urea-induced differential shifts in conformation, Aβ peptides which differ in only one to two amino acids can be separated (Wiltfang *et al*. 1997). The composition of the separation gel initially applied for the analysis of Aβ peptides was modified from 15%T%/5%C/8 M to 12%T%/5%C/8 M urea and gel thickness was reduced to 0.5 mm. Gels were run at room temperature for 2 h at a constant current of 12 mA/gel, using the MiniProtean II electrophoresis unit (Bio-Rad Laboratories, Hercules, CA, USA). Ten microlitres of sample were loaded per lane. All samples were run as quadruplicates and each gel carried a five step dilution series of the synthetic Aβ peptide mix. Mean values were used for subsequent calculations.

Aβ-IPG-2D-PAGE

IPG was performed according to the protocol of the manufacturer (Amersham Pharmacia Biotech, Buckinghamshire, UK) using dry strips (linear pH gradient: 4–7, length: 7 cm). Dry strips were rehydrated overnight at room temperature to 0.5 mm gel height, using the following rehydration solution: 8 M urea, CHAPS 0.5% (w/v), DTT 0.2% (w/v), Serdolit MB-1 1% (w/v), pharmalyte 3–10 0.8% (v/v). Thirty microlitres of sample in IPG sample buffer was applied to the rehydrated dry strip at pH 6.5 (cathodic site) using sample cups and IEF was performed for 30 min/300 V, 30 min/800 V, 30 min/1400 V and 5 h/2000 V (Σ12 500 V/h). Subsequently the strips were equilibrated for the second analytical dimension (Aβ-SDS–PAGE) in the following buffer for 10 min at room temperature: 6 M urea, glycerol 20% (w/v), SDS 2.0% (w/v), bistris 0.36 M, bicine 0.16 M, DTT 1.0% (w/v). DTT was added just prior to equilibration. Equilibrated IPG strips were placed on top of the Aβ-SDS–PAGE stacking gel (1 mm thickness) and embedded by a low gelling temperature agarose solution: agarose 1.0% (w/v), bicine 0.16 M, bistris 0.36 M, SDS 0.25% (w/v), bromophenol blue 0.002% (w/v). A Teflon tooth was inserted next to the IPG strip to form a track for synthetic standard Aβ peptides or a one-dimensional reference separation of the SDS/heat denatured sample. Subsequently, Aβ-SDS–PAGE/immunoblot was performed as described, but separation gels were run at constant voltage for 15 min/60 V and for 1 h 30 min/120 V.

Western blotting, immunostaining and quantification

Aβ peptides were transferred for 30 min at 1 mA/cm^2 and room temperature under semidry conditions (Hoefer Semiphor) onto Immobilon-P PVDF membranes according to Wiltfang *et al*. (1997).

For immunostaining Immobilon-P PVDF membranes were washed for 30 s in H_2O_{dd} and boiled for 3 min in PBS (phosphate buffered saline) using a microwave oven (Ida *et al*. 1996; Wiltfang *et al*. 1997). Blocking was performed for 1 h at room temperature in the presence of RotiBlock (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Incubation with primary mAb, which was diluted 4000-fold (stock: 0.25 mg/mL), was done overnight at 4°C. After a brief wash in PBS-T (0.075% v/v Tween 20) membranes were further washed for 30 min, 15 min and 2 × 10 min. Next, membranes were incubated for 1 h at room temperature with an anti-mouse biotinylated IgG (H + L) antibody (1.5 mg/mL), which was diluted 3000-fold in PBS-T. A second PBS-T wash was done for 3 × 10 min at room temperature. The membranes were then incubated for 1 h at room temperature with streptavidin-biotinylated horseradish peroxidase complex diluted 3000-fold with PBS-T. Following a wash for 3 × 10 min at room temperature, the membranes were developed for 5 min at room temperature with ECLPlus™ solution according to the protocol of the manufacturer. Detection of the emitted light signal was performed by a CCD camera (FluorSMax MultiImager; Bio-Rad), using a series of 1, 5, 20, 60, 120, and 300 s for data acquisition. Band intensities were quantified relative to an internal five-step dilution series of the Aβ peptide standard mix using Quantity One software (version 4.1, Bio-Rad). Detection sensitivity was 0.6 pg and 1 pg for Aβ1–40 and Aβ1–42, respectively (data not shown). Signal acquisition was linear within a range of 3.8 magnitudes of order. The high detection sensitivity was due to the mAb 1E8 and an optimization of the former immunoblot procedure (Wiltfang *et al*. 1997), as we used a synthetic reagent (Roti-Block) instead of non-fat milk powder to block the PVDF membrane. The method allowed quantification of Aβ peptides in only 10 μL of CSF. The inter- and intra-assay coefficients of variation for 80 as well as 20 pg of synthetic Aβ peptides were below 10%.

MALDI-TOF mass spectrometry of immunoprecipitated Aβ peptides

Samples micropreparatively immunoprecipitated from CSF were resolved in 60% acetonitrile, 0.1% trifluoroacetic acid. An aliquot of 0.5 μL was mixed with 0.5 μL saturated α-cyano-hydroxycinnamic acid, 50% acetonotrile, 0.1% trifluoroacetic acid on the target, dried and analysed using a REFLEX III MALDI-TOF mass spectrometer (Bruker Daltoniks, Germany) in the positive reflectron mode.

Aβ peptide synthesis

The peptides investigated were synthesized automatically using the Fmoc-chemistry according to Janek *et al*. (2001).

Patients

We investigated 59 patients with various neuropsychiatric disorders under the guidelines and regulations of the Institutional Review Board of the University of Göttingen. Neuropsychiatric diagnosis was established by ICD-10 and DSM-IV criteria [American Psychiatric Association (APA), 1994]. Patients with probable Alzheimer's dementia (AD) had to satisfy DSM-IV criteria for dementia of the Alzheimer's type and the NINCDS-ADRDA criteria (McKhann *et al*. 1984).

A group of patients with a broad range of neuropsychiatric disorders but without dementia (non-demented disease controls, NDC; $n = 47$; age: 45.2 ± 15.8 y; mean ± SD) were differentiated from patients with AD (AD, $n = 12$; age: 73.0 ± 7.9 years; mean ± SD). In five of the NDC patients analysis of the CSF samples by immunoprecipitation (IP) with Aβ-SDS–PAGE/immunoblot was compared with SDS-heat denaturation with direct loading and Aβ-SDS–PAGE/immunoblot. The two respective NCD groups were termed as IP-CSF and SDS-CSF (see Table 1). The latter two NDC subgroups allowed us to compare the effects of different sample pretreatment (immunoprecipitation versus SDS-heat denaturation) on the concentrations of CSF Aβ peptides.

Mini mental state examination (MMSE) results (Folstein *et al*. 1975) at the time of sampling, ApoE genotyping, and routine CSF parameters (cell count, total protein, albumin, presence of oligoclonal bands, IgG, IgM, and CSF/serum ratios for albumin and immunoglobulins) were available for 12/12, 11/12, 10/12, and 42/47, 46/47, 46/47 of the AD and NDC patients, respectively.

The NDC group was further subdivided into patients with symptoms of chronic inflammatory CNS disease (CID, $n = 10$; age: 44.9 ± 14.2 years; mean ± SD) and patients with other neuropsychiatric disorders (OND, $n = 37$; age: 45.3 ± 16.4 years; mean ± SD). The heterogeneous diagnostic group of patients with CID was defined by clinical (e.g. prompt response to glucocorticoid therapy), technical (electrophysiology, cerebral MRI) and/or neurochemical parameters (intrathecal IgG synthesis). The CID group included five patients with multiple sclerosis and five patients with unknown aetiology of the chronic neuroinflammation.

The OND group included patients with cerebral transient ischaemic attacks ($n = 6$), subcortical arteriosclerotic encephalopathy ($n = 5$), epilepsy ($n = 4$), Meniere's disease ($n = 1$), benign paroxysmal positioning vertigo ($n = 1$), head trauma ($n = 2$), brain metastasis ($n = 1$), motor neuron disorder ($n = 2$), tension headache ($n = 1$), hemicrania ($n = 1$), unspecified neurological condition without dementia ($n = 1$), major depressive disorder ($n = 5$), bipolar I disorder ($n = 2$), psychotic disorder not otherwise specified ($n = 1$), anxiety disorder ($n = 2$), somatoform (conversion) disorder ($n = 1$), and benzodiazepine dependence ($n = 1$).

To control for a pronounced effect of the ApoE ε4 allele on the pattern of Aβ peptides we further subdivided the OND group into patients with one or two alleles of ε4 (OND$\varepsilon4^{plus}$, $n = 6$) and without ε4 (OND$\varepsilon4^{minus}$, $n = 30$). Since 11/12 AD patients had one or two ApoE ε4 alleles, the effect of ApoE genotype could not be eliminated in this group of patients, but to identify AD-specific effects on the Aβ peptide pattern in CSF we compared patients with AD, who carried the ε4 allele (AD$\varepsilon4^{plus}$, $n = 11$) to the OND$\varepsilon4^{plus}$ group ($n = 6$). MMSE scores, ApoE ε4 allele frequencies, absolute and relative Aβ peptide CSF concentrations for groups NDC, AD, IP-CSF, and SDS-CSF are summarized in Table 1.

Statistics

Individual Aβ peptides were expressed as absolute values (ng/mL) and as a percentage of the total Aβ peptide concentration (Aβ1-x%, fractional Aβ peptide values). We characterized patient groups by median and percentile range (p25-p75 or p5-p95) and analysed data

Table 1 Aβ peptides in the CSF of several patient subgroups relative to their MMSE performance and ApoE genotyping

Groups	Subjects number M F	MMSE* median p25 p75	ApoE no ε4 1 or 2 ε4 n.a.	Aβ1–37 (ng/mL) median p25 p75	Aβ1–38 (ng/mL) median p25 p75	Aβ1–39 (ng/mL) median p25 p75	Aβ1–40 (ng/mL) median p25 p75	Aβ1–42 (ng/mL) median p25 p75	total Aβ[1] (ng/mL) median p25 p75	Aβ1–37[2] (%) median p25 p75	Aβ1–38[2] (%) median p25 p75	Aβ1–39[2] (%) median p25 p75	Aβ1–40[2] (%) median p25 p75	Aβ1–42[2] (%) median p25 p75
NDC[a]	47	30	37	1.23	2.78	1.42	11.62	2.07	18.66	6.72	15.02	7.31	59.79	10.93
	28	29	9	1.12	2.44	1.11	9.32	1.56	15.66	6.44	14.37	6.86	58.30	9.98
	19	30	1	1.60	3.49	1.77	14.24	2.88	24.45	7.27	15.97	7.67	60.91	11.58
AD[b]	12	16.0	1	1.30	3.08	1.35	12.81	1.49	19.93	7.13	16.22	7.78	62.42	6.73
	3	9.5	11	1.02	2.41	1.15	10.32	0.91	16.07	6.38	15.43	6.80	61.54	5.80
	9	22.5	0	1.81	4.11	2.13	15.74	1.57	25.36	7.33	16.57	8.40	64.11	7.65
IP-CSF[c]	5	30	4	1.35	2.80	1.93	13.23	2.73	22.04	6.49	13.73	8.73	60.03	12.39
subgroup of NCD	3	29	1	1.27	2.69	1.54	12.19	2.04	19.59	6.10	13.58	7.88	53.35	10.77
	2	30	0	2.30	4.74	2.54	15.25	3.76	28.58	8.04	16.05	8.75	62.25	13.16
SDS-CSF[d]	5	30	4	1.62	4.53	2.28	18.40	3.40	30.23	5.93	14.99	8.04	60.87	11.24
subgroup of NCD	3	29	1	1.23	3.30	1.94	14.24	2.38	23.02	5.35	14.33	7.56	58.54	10.43
	2	30	0	1.93	5.09	2.58	18.81	3.72	32.13	6.01	15.83	8.44	60.95	11.58

*MMSE scores not available in 5 of 47 NDC patients; n.a., not available; [1]total Aβ peptide concentration; [2]percentage of Aβ peptide of total Aβ peptides; [a]non-dementive disease controls; [b]Alzheimer's disease; [c]immunoprecipitation and Aβ-SDS-PAGE/immunoblot of CSF from five patients of group NDC; [d]SDS/heat denaturation and Aβ-SDS-PAGE/immunoblot of CSF from the same five patients of group NDC.

distribution by the Shapiro–Wilks *W*-test (Royston 1982). To evaluate significant group differences we applied the Mann–Whitney *U*-test and chi-squared analysis according to Fisher's exact test. The Mann–Whitney *U*-test was adjusted for small sample size. Regression functions were obtained by linear regression analysis and Spearman's rho (*r*) was used for correlation analysis. Multiple group comparisons were compensated by the sequentially rejective Bonferroni test (Holm 1979). The two-sided level of significance was taken as $p < 0.05$. Computations were performed using the statistical software package Statistica for Windows, version 5.1 F.

Results

Three additional Aβ peptides in human CSF and their identification

Human lumbar CSF samples from non-demented patients were treated by SDS-heat denaturation. Aliquots were subsequently analysed by Aβ-SDS–PAGE/immunoblot in the presence of 8 M urea (Fig. 1a) or by conventional SDS–PAGE/immunoblot without urea (Fig. 1b). Surprisingly, we observed a highly conserved pattern of three further Aβ peptides in addition to Aβ1–40/42, if the samples were separated in the presence of urea, whereas otherwise they migrated as a single band. Accordingly, separation was not achieved by pure differences in molecular mass, which were too small to allow separation by conventional SDS–PAGE. Apparently, addition of urea to the separation gel induced Aβ peptide specific conformational shifts, which differentially affected their effective molecular radii and thus provided baseline separation. Using mAbs 1E8 and 6E10, which are specific for the aminoterminus of Aβ peptides (Kim *et al*. 1990; Wiltfang *et al*. 2001) all five Aβ peptide species were immunoprecipitated (data not shown). However, using mAbs 13E9 and 6D5, which are specific for the carboxytermini of Aβ1–40 and Aβ1–42, respectively, only Aβ1–40 or Aβ1–42 were immunoprecipitated, but not the three additional Aβ peptide species (data not shown). Moreover, during Aβ-SDS–PAGE the Ct-elongated Aβ1–42 consistently migrated faster than Aβ1–40, the opposite being observed for the three additional Aβ peptide species. Taken together our data suggested that the three additional Aβ peptides might correspond to the Ct-truncated Aβ peptides 1–37/38/39, which was supported by comigration of the three additional Aβ peptide bands in human CSF with synthetic Aβ peptides 1–37/38/39 (Figs 1a and 2b).

To further support this assignment, we established an Aβ-IPG-2D-PAGE/immunoblot. Since Aβ peptides 1–37/38/39 and 1–40/42 differ only carboxyterminally in non-charged (hydrophobic) amino acids, their isoelectric points should be identical. Correspondingly, Aβ-IPG-2D-PAGE/immunoblot revealed an isoelectric point of 5.37 for the synthetic Aβ peptides (Fig. 2a), as well as for the five Aβ peptide species

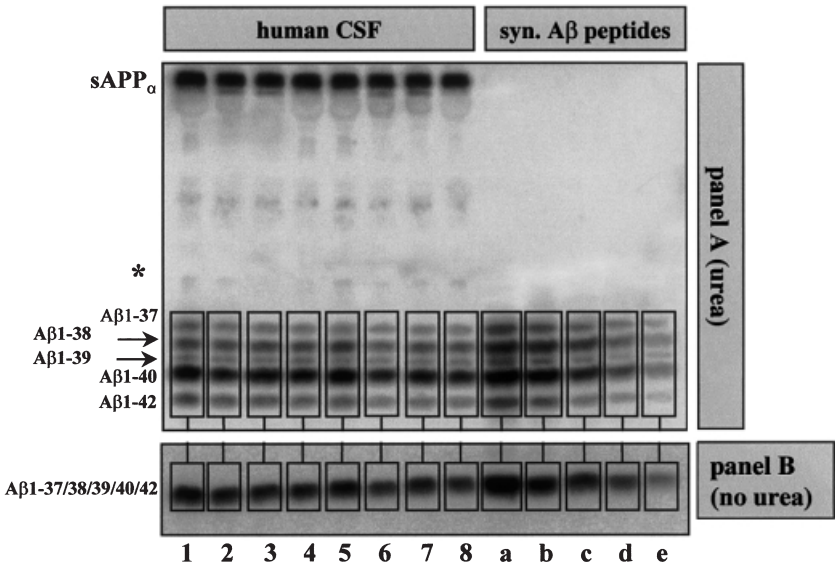

Fig. 1 Aβ-SDS–PAGE/immunoblot of human lumbar CSF in separation gels with (a) and without (b) urea. (a) 10 μL of SDS/heat-denaturated CSF samples from non-demented patients (NDC; lanes 1–8) and a five-step dilution series (lanes a–e) of a mix of the synthetic Aβ peptides 1–37/38/39 and 1–40/42 (lanes a–e) were analysed. The concentration of synthetic Aβ peptides ranged from (a) Aβ1–37/80 pg, Aβ1–38/120 pg, Aβ1–39/60 pg, Aβ1–40/300 pg, Aβ1–42/80 pg to (e) Aβ1–37/5 pg, Aβ1–38/15 pg, Aβ1–39/5 pg, Aβ1–40/25 pg, Aβ1–42/5 pg. The CSF was not concentrated prior to analysis. At the upper (cathodic) end of the separation gel sAPPα is separated, corresponding to the soluble ectodomaine of APP, which is generated after cleavage by α-secretase. The asterisk indicates the migration position of Aβ peptides 1–33/34 (non-resolved) and 1–35, which were not consistently observed (see also Fig. 2, inset). (b) Aβ peptides 1–37/38/39 and 1–40/42 were not resolved by separation gels without urea.

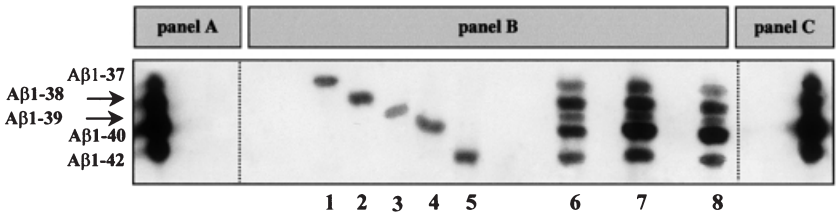

Fig. 2 Aβ-IPG-2D-PAGE/immunoblot and Aβ-SDS–PAGE/immunoblot of CSF and synthetic Aβ peptides. (a and c) 30 μL of a mix of the synthetic Aβ peptides 1–37/38/39 plus 1–40/42 (a; 60 pg/peptide) and 30 μL of human CSF of a non-demented patient (c) were analysed by Aβ-IPG-2D-PAGE/immunoblot. (b) 10 μL (20 pg/peptide) of synthetic Aβ peptides 1–37 (lane 1), 1–38 (lane 2), 1–39 (lane 3), 1–40 (lane 4), and 1–42 (lane 5), 10 μL of a mix of the latter synthetic Aβ peptides (lane 6), 10 μL of a CSF sample from a non-demented patient (lane 7), and 10 μL of the latter CSF sample spiked with the mix of synthetic Aβ peptides (lane 8) were separated by Aβ-SDS–PAGE/immunoblot.

from human CSF (Fig. 2c). Comigration of individual synthetic Aβ peptides and spiking of CSF samples with a mix of the five synthetic Aβ peptides (Fig. 2b) confirmed the identification of the latter Aβ peptide species in human CSF as 1–37/38/39 and 1–40/42. Moreover, MALDI-TOF analysis of Aβ peptides selectively enriched from human lumbar CSF by Nt-selective immunopreciptitation (mAb 1E8) proved the presence of Aβ peptides 1–37/38/39 and 1–40 (Fig. 3). Significant amounts of Aβ1–42 were not detected due to methodological reasons (cf. Discussion). In addition, the Aβ peptide species 1–33/34/35 were immunoprecipitated. Therefore, we also synthesized the latter three Ct-truncated Aβ peptides to exclude comigration with the other Aβ peptide species. During Aβ-SDS–PAGE these Aβ peptides migrate ahead (cathodically) of Aβ1–37, in the order 1–33/34/35 [cathodically (*r*) anodically] (Fig. 3, inset). Aβ1–35 was separated from Aβ1–33/34, which migrate as a single band. However, Aβ peptides 1–33/34/35 were not consistently observed in human CSF and, if present, their concentrations were close to the level of detection (Fig. 1a, see asterisks; Fig. 3, inset). Interestingly, the actual CSF concentrations of Aβ peptides were not adequately reflected by the MALDI-TOF mass spectrum, since the hydrophobic Aβ1–42 was close to the detection limit, whereas the much

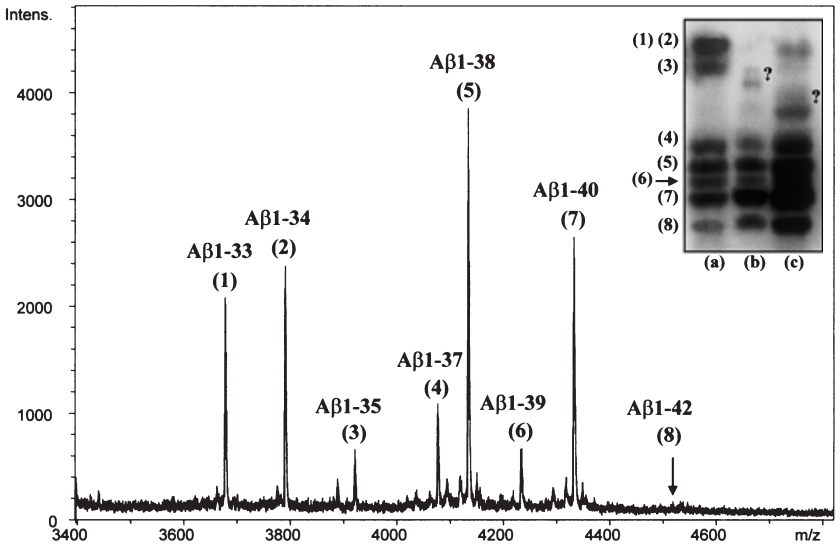

Fig. 3 MALDI-TOF mass spectroscopy of Aβ peptides from human lumbar CSF. Prior to analysis Aβ peptides were enriched from 16 mL of CSF of a non-demented patient by Nt-selective immunoprecipitation (mAb 1E8). The neutral monoisotopic masses (M_r) were determined as follows (theoretical M_r in brackets): (1) Aβ1–33, 3672.44 (3671.78); (2) Aβ1–34, 3784.93 (3784.86); (3) Aβ1–35, 3915.64 (3915.09); (4) Aβ1–37, 4072.16 (4071.99); (5) Aβ1–38, 4129.07 (4129.01); (6) Aβ1–39, 4228.18 (4228.08); (7) Aβ1–40, 4327.26 (4327.15); (8) Aβ1–42 was close to the level of detection (arrow); calculated mass 4511.34 (4511.27). Inset: Aβ-SDS–PAGE/immunoblot of Aβ peptides from the same CSF sample, which was used for MALDI-TOF mass spectroscopy; Aβ peptide species are indicated by Arabic numbers; (a) mix of synthetic Aβ peptides (Aβ1–33/34 migrate as a single band); (b) 10 μL of the SDS/heat denaturated CSF sample; (c) 2.5 μL of immunoprecipitated Aβ peptides, which corresponds to the amount of Aβ peptides present in 100 μL of the native CSF. Unknown bands are indicated by '?'. Evidently, the mass spectrum does not adequately reflect the relative CSF concentrations of the Aβ peptides. The more hydrophilic Ct-truncated Aβ peptides are overrepresented, as compared with Aβ1–40 or the Ct-elongated Aβ1–42.

more hydrophilic Aβ peptides Aβ1–33/34/35 were over-represented.

A highly conserved Aβ peptide quintet in human CSF

The latter findings prompted us to investigate whether the Ct-truncated species were regularly expressed in human CSF, and led us to look for disease-specific Aβ peptide patterns.

Analysis of a group of non-demented disease controls (NDC) proved that the CSF Aβ peptide quintet was highly conserved, and showed the following order of abundance in absolute and relative terms (Table 1): Aβ1–40 > Aβ1–38 > Aβ1–42 > Aβ1–39 ≥ Aβ1–37. The latter differences in absolute and relative abundances were significant ($p < 0.00025$), except for the absolute concentrations of Aβ1–37 and Aβ1–39, which were almost of the same magnitude ($p = 0.411$). Accordingly, next to Aβ1–40 the second prominent Aβ peptide in human CSF was Aβ1–38 and not Aβ1–42.

Absolute and relative Aβ peptide quantities did not differ significantly by age and sex and were not correlated to parameters of routine CSF analysis. For the NDC group, normality was rejected for the distribution of CSF concentration of Aβ peptides, as well as for Aβ1–42%, but accepted for Aβ1–37/38/39% and Aβ1–40%.

Aβ peptides in the CSF of NDC patients were closely and significantly ($p < 1.0 \times 10^{-16}$) correlated and their coefficients of regression ranged between 0.89 and 0.96 (Fig. 4, inset). Accordingly, the content of the five Aβ peptides in CSF was controlled within narrow limits, which became most prominent for their relative abundances (Table 1). Thus NDC patients had coefficients of variation for the Ct-truncated Aβ1–37/38/39% and Aβ1–40/42% of only 10.3, 7.2, 8.3, 3.4, and 13.8, respectively.

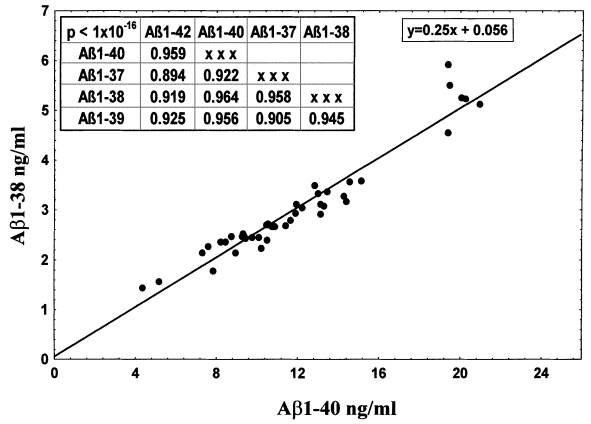

Fig. 4 Aβ-SDS–PAGE/immunoblot of CSF from patients with non-dementive neuropsychiatric diseases (NDC): covariation of Aβ1–38 and Aβ1–40 is shown relative to the correlation matrix of all Aβ peptides (inset). Individual coefficients of correlation were close to or even beyond 0.9, i.e. the Aβ peptide quintet in CSF was controlled within surprisingly narrow limits.

Ct-truncated Aβ peptides were detectable in human CSF directly following lumbar puncture, and were not generated during storage of native CSF at room temperature for three days (data not shown). Taken together the latter findings indicate close enzymatic control of their processing.

CSF sample pretreatment: evidence of carrier-mediated epitope masking of Aβ1–42

The measured CSF Aβ peptide concentrations were strongly dependent on sample pretreatment. SDS-heat denaturation of CSF samples from a subgroup of five NDC patients (SDS-CSF, Table 1) yielded slightly higher concentrations as compared with immunoprecipitations of the same samples (IP-CSF, Table 1) in the presence of a low concentration of a detergent mixture (RIPA; cf. Materials and methods). This difference was most pronounced in absolute and relative terms for Aβ1–38 and Aβ1–42, but failed to achieve the level of significance. By contrast, Aβ1–42 concentrations determined by an $ELISA_{Aβ1-42}$ (Hulstaert *et al.* 1999) in a subgroup of NDC patients ($n = 27$, median: 0.719 ng/mL, p25-p75: 0.545–0.813 ng/mL) were 3.0-fold lower ($p < 1.0 \times 10^{-9}$) as compared with the corresponding concentrations determined by SDS-heat denaturation with subsequent Aβ-SDS–PAGE/immunoblot ($n = 27$, median: 2.16 ng/mL, p25-p75: 1.63–3.40 ng/mL). The ELISA did not use detergents during the capture of antigens (1 h incubation time) and we also observed CSF concentrations of Aβ1–42 at the level determined by the ELISA when the immunoprecipitation prior to Aβ-SDS–PAGE/immunoblot was performed without detergents (data not shown). Moreover, we obtained almost identical concentrations by $ELISA_{Aβ1-42}$ and Aβ-SDS–PAGE/immunoblot when the two synthetic Aβ1–42 standard preparations were quantified by both methods vice versa (data not shown). In line with this observation, even higher concentrations for CSF Aβ1–42 were determined by SDS-heat denaturation and Aβ-SDS–PAGE/immunoblot when the latter sample pretreatment was performed prior to freezing (Wiltfang *et al.*; paper submitted). These data suggest that a considerable fraction of CSF Aβ1–42 is transported with high affinity binding to the carrier. This carrier seems to be prone to precipitate in the cold and to mask Aβ1–42 epitopes.

CSF Aβ peptides in neuropsychiatric diseases

According to diagnosis several subgroups of patients were created and tested for significant differences in absolute or relative Aβ peptide quantities.

Patients with Alzheimer's disease (AD, $n = 12$) and a subgroup of NDC patients with chronic inflammatory diseases of the CNS (CID, $n = 10$) were differentiated by a characteristic CSF Aβ peptide pattern from the remaining NDC patients with other neuropsychiatric diseases (OND, $n = 37$).

AD patients were characterized by a significant reduction of Aβ1–42 (AD versus OND, $p = 0.0016$; AD versus CID,

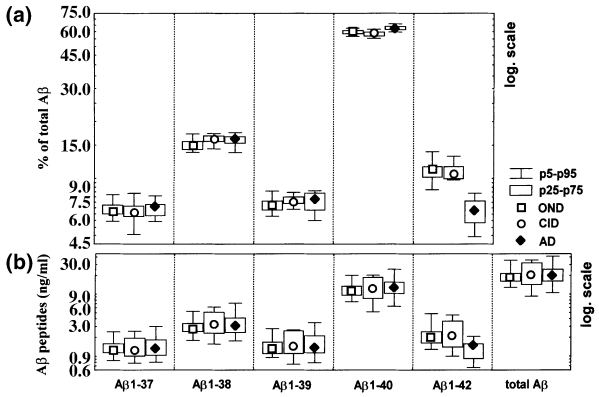

Fig. 5 Aβ-SDS–PAGE/immunoblot of CSF from patients with Alzheimer's disease (AD), chronic inflammatory CNS disease (CID) and other non-dementive neuropsychiatric diseases (OND). The logarithmic scale was chosen to allow the simultaneous comparison of all Aβ peptide species, however, highly significant differences (e.g. compare the relative quantities (a) for OND versus AD: Aβ1–38%, $p = 0.0056$; Aβ1–40%, $p = 7.3 \times 10^{-6}$; Aβ1–42β, $p = 2.2 \times 10^{-11}$) are not adequately highlighted by this way of data presentation. (a) Box plot of the concentrations (ng/mL) of Aβ peptides 1–37/38/39 and 1–40/42 and of total Aβ peptides (total Aβ). (b) Box plot of the amount of single Aβ peptide species relative to total Aβ (percentages). Interestingly, group differences were much more pronounced for the amounts of Aβ peptides relative to total Aβ (b).

$p = 0.029$; Fig. 5a). However, characteristic group differences became much more evident when instead of CSF concentrations the relative amounts of Aβ peptides were compared (Fig. 5b). For AD relative to OND patients, we observed significantly elevated values of Aβ1–38% ($p = 0.0056$), a substantial increase in Aβ1–40% ($p = 7.3 \times 10^{-6}$), and a pronounced decrease of Aβ1–42% ($p = 2.2 \times 10^{-11}$). Aβ1–37% ($p = 0.4833$) and Aβ1–39% ($p = 0.2633$) remained virtually unchanged. AD relative to CID was characterized by elevated Aβ1–40% ($p = 9.28 \times 10^{-5}$) and reduced Aβ1–42% ($p = 3.09 \times 10^{-6}$). Interestingly, AD and CID patients shared a significant increase in Aβ1–38%, as compared with OND patients. In contrast, relative to OND Aβ1–40% was significantly elevated in AD, but moderately reduced in CID patients ($p = 0.1050$), whereas %Aβ1–42 was significantly reduced in AD, but unchanged in CID patients (Fig. 5b).

For CID versus OND we observed significantly elevated values of Aβ1–39% ($p = 0.043$) and a pronounced increase in Aβ1–38% ($p = 0.0022$), which was paralleled by a moderate non-significant decrease of Aβ1–40% ($p = 0.1050$) at virtually unchanged Aβ1–37% ($p = 0.75$) and Aβ1–42% ($p = 0.55$) (Fig. 5b). We observed the latter Aβ peptide pattern independently of the ApoE4 genotype, i.e. it was still evident after omitting the three CID patients with ε4 alleles and was not correlated to glucocorticoid medication. By contrast, other diagnostic groups such as cerebrovascular disorders, or affective disorders did not show characteristic changes in the relative and absolute abundances of CSF Aβ peptides.

The impact of ApoE genotype on the Aβ peptide pattern in CSF

To control for an effect of ApoE genotype on the CSF Aβ peptide pattern, OND patients ($n = 37$) were separated according to the presence of one or two ε4 alleles. The OND$\varepsilon4^{plus}$ group comprised six patients with one ε4 allele, whereas 30 patients had no ε4 allele (OND$\varepsilon4^{minus}$). For one OND patient, data on ApoE genotyping were not available. Additionally, we compared the AD patients with one ($n = 9$) or two alleles ($n = 2$) of ε4 (AD$\varepsilon4^{plus}$, $n = 11$) to OND$\varepsilon4^{minus}$ and OND$\varepsilon4^{plus}$ patients (Figs 6a and b). This comparison revealed a pronounced effect of the ε4 allele on the CSF Aβ peptide concentration. For OND$\varepsilon4^{plus}$ relative to OND$\varepsilon4^{minus}$ all CSF Aβ peptides were reduced (total Aβ ↓, $p = 0.0202$), but to a different extent for the single Aβ peptides species (Fig. 6a): a striking decrease of Aβ1–42 ($p = 0.00082$) was accompanied by a moderate reduction of Aβ1–40 ($p = 0.0230$), and a minor decrease of the Ct-truncated Aβ peptides 1–37 ($p = 0.0464$), 1–38 ($p = 0.0575$) and 1–39 ($p = 0.0464$).

Comparing AD$\varepsilon4^{plus}$ and OND$\varepsilon4^{plus}$, we were surprised, that Aβ1–42 was reduced to almost the same extent in both patient groups. However, the reduction of Aβ1–42 in

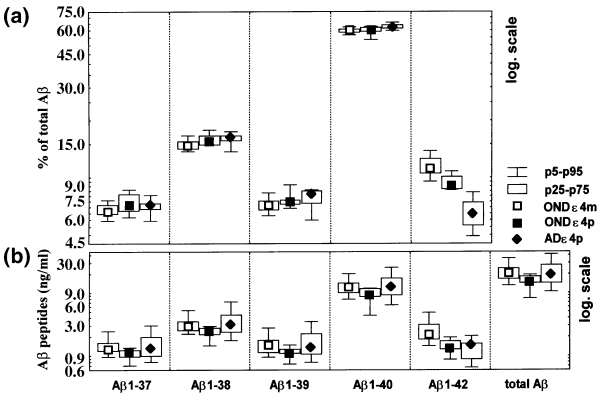

Fig. 6 Aβ-SDS–PAGE/immunoblot of CSF from patients with one or two ApoEε4 alleles and Alzheimer's disease (AD$\varepsilon4^{plus}$), other non-dementive neuropsychiatric diseases with one or two ApoEε4 alleles (OND$\varepsilon4^{plus}$), and OND patients without the ε4 allele (OND$\varepsilon4^{minus}$). The logarithmic scale was chosen to allow the simultaneous comparison of all Aβ peptide species, however, highly significant differences (e.g. compare the elevated relative quantities (a) in AD patients for Aβ1-38%, Aβ1-39%, Aβ1-40%) are not adequately highlighted by this way of data presentation. (a) Box plot of the concentrations (ng/mL) of Aβ peptides 1–37/38/39 and 1–40/42 and of total Aβ peptides (total Aβ). (b) Box plot of the amount of single Aβ peptide species relative to total Aβ (percentages). Interestingly, group differences were much more pronounced for the amounts of Aβ peptides relative to total Aβ, e.g. compare the concentrations (a) and relative amounts (b) of Aβ1–42 for groups OND$\varepsilon4^{plus}$ and AD$\varepsilon4^{plus}$.

ADε4^{plus} was more selective as compared with ONDε4^{plus} (ONDε4^{minus} versus ADε4^{plus}, $p = 0.00021$), since in this case it was not paralleled by an overall reduction of other Aβ peptides (total Aβ ↓). Thus in ADε4^{plus}, as opposed to ONDε4^{plus}, low concentrations of Aβ1–42 were compensated by comparatively high concentrations of other Aβ peptides. This effect was most pronounced for Aβ1–40, which was significantly elevated in ADε4^{plus} as compared with ONDε4^{plus} ($p = 0.0365$). This explains why, in spite of almost identical concentrations of Aβ1–42 in ONDε4^{plus} and ADε4^{plus}, both patient groups were differentiated without overlap by Aβ1–42% (Fig. 6b, $p = 0.00016$). We obtained the same results when the patient groups NDCε4^{plus} ($n = 9$) and ADε4^{plus} were compared (data not shown).

Next we compared the patient groups ADε4^{plus} and ONDε4^{plus} to ONDε4^{minus}. Both groups shared a significant elevation Aβ1–38%, as compared with ONDε4^{minus}. However, this difference was more pronounced for ε4-positive AD patients (ONDε4^{minus} versus ONDε4^{plus}, $p = 0.0371$; ONDε4^{minus} versus ADε4^{plus}; $p = 0.0016$). Moreover, both groups shared a reduction of Aβ1–42%. Again, this drop was much more pronounced in AD (ONDε4^{minus} versus ONΔe^{4plus}, $p = 0.0004$; ONDε4^{minus} versus ADε4^{plus}; $p = 6.3 \times 10^{-10}$).

Taken together our data show that ε4-positive patients with or without AD share elevated fractions of Aβ1–38% and reduced fractions of Aβ1–42%, as compared with ε4-negative non-demented patients. However, both changes were more pronounced in AD, where the striking reduction of Aβ1–42% was additionally paralleled by an increase in %Aβ1–40.

Disease-specific CSF patterns of Aβ1–38%, Aβ1–40%, and Aβ1–42% in AD and CID

A scatterplot of the individual values for Aβ1–42% and Aβ1–38% showed that only AD patients presented with Aβ1–42% below 8.5 (Fig. 7). Additionally, a strong correlation became evident for Aβ1–38% and Aβ1–42% in AD patients ($r = -0.91$; $p = 0.0001$). One AD patient (number 143) was defined as an outlier and not included in the non-parametric correlation analysis. This patient presented with a very early stage of AD (MMSE = 27/30). Interestingly, the ε4-positive OND patients were aligned adjacent to a hypothetic curvilinear cut-off line, which may be drawn by connecting the fractional Aβ peptide values of the OND patients neighbouring the AD group (data not shown).

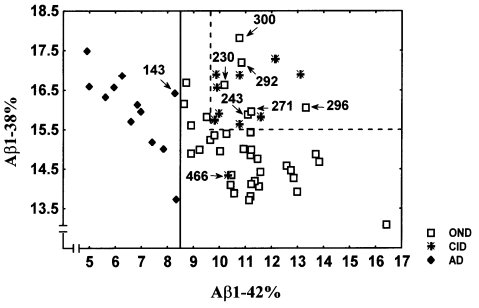

Fig. 7 Aβ-SDS–PAGE/immunoblot of CSF from patients with Alzheimer's disease (AD), chronic inflammatory CNS disease (CID) and other non-dementive neuropsychiatric diseases (OND). Aβ1–38% is shown in dependence of Aβ1–42%. The solid cut-off line (Aβ1–42% = 8.5) is given for AD patients. The dashed cut-off lines (Aβ1–38% = 15.5, Aβ1–42% = 9.6) are shown for CID patients. Individual patients are identified by Arabic numbers.

CID patients were characterized by values for Aβ1–38% of greater than 15.5 and a value of greater than 9.6 for Aβ1–42%. Applying the latter cut-off values 9/10 CID patients were classified as CID, whereas only 6/49 non-CID patients (OND & AD) were misclassified as CID ($p < 0.0001$).

One CID patient was identified as an outlier (number 466). This 25-year-old male patient presented with optic neuritis and local intrathecal IgG synthesis, but without additional clinical symptoms and no MRI manifestations of other inflammatory CNS lesions. The subset of six OND patients (Fig. 7, numbers 230, 243, 271, 292, 296 and 300) showing a fractional Aβ peptide pattern otherwise typical for CID, will be presented below.

There was a clear-cut differentiation between AD and NDC patients concerning Aβ1–40%, since NDC patients did not present Aβ1–40% values greater than 63.0 (data not shown). Opposite to AD, CID patients were characterized by reduced Aβ1–40%, usually below 60.0. Only two CID patients presented with Aβ1–40% above 60.0, again including patient number 466. Applying a cut-off value of Aβ1–40% = 60.0 in addition to those identified for Aβ1–38% and Aβ1–42%, 8/10 CID patients were classified as CID, whereas the same 6/49 non-CID patients (OND and AD) as indicated in Fig. 7 were misclassified as CID ($p < 0.0001$).

Remarkably, a re-evaluation of the patient records revealed that neuroinflammation due to immune vasculitis or glia activation was implicated in the CNS disease of at least three of these six patients. In a 38-year-old female (number 230) with known epilepsy, systemic lupus erythematodes (sLE), anticardiolipin antibodies retrospectively a CNS manifestation of the sLE cannot be excluded.

A 70-year-old female (number 292) with known breast cancer, hyperlipoproteinemia type IIa, arterial hypertension, and hypothyroidism substituted with thyroxine was admitted because of cranial nerve palsy and haemianopia due to disseminated cerebral metastasis. Her routine CSF analysis was normal except for a mild impairment of the blood–brain barrier.

A 31-year-old female (number 300), who had the most pronounced fractional CID Aβ peptide pattern of all patients investigated, had experienced cerebral sinus vein thrombosis at the age of 25 and pre-eclampsia at the age of 23. She was admitted due to recurrent transient ischaemic attacks affecting predominantly the left medial cerebral artery. A thromboembolic aetiology of the symptomatology was ruled out.

Retrospectively, her clinical symptoms, EEG abnormalities, and cerebral MRI findings indicated an immune vasculitis of unknown aetiology. Interestingly, her absolute and relative Aβ peptide pattern abnormalities closely resembled that of a male patient with CID of unknown aetiology (cf. materials and methods), but her changes were more pronounced.

The remaining three non-CID patients (numbers 243, 271 and 296) who were misclassified as CID had lower Aβ1–38% values as compared with the former three non-CID patients. These patients showed no obvious evidence of a neuroinflammatory process as part of their CNS disease.

Fractional CSF Aβ peptide values indicate the severity of dementia in AD

Next we investigated whether the CSF Aβ peptide pattern was correlated to the severity of dementia, as measured by the MMSE. Overall MMSE scores were negatively correlated to Aβ1–40% ($r = -0.660$, $p = 0.020$), but positively to the percentages of Ct-truncated Aβ peptides (Aβ1–37%: $r = 0.650$, $p = 0.022$; Aβ1–38%: $r = 0.552$, $p = 0.063$; Aβ1–39%: $r = 0.573$, $p = 0.051$). Correspondingly, we observed a significant correlation between the ratio of the sum of Ct-truncated Aβ peptides to the sum of Aβ1–40 and Aβ1–42 ($r = 0.657$, $p = 0.020$). By contrast, we did not observe significant correlations between MMSE scores and absolute CSF Aβ peptide concentrations.

Furthermore, we separated the AD patients according to the cut-off value Aβ1–40% = 63.0. This cut-off value was selected, since all NDC patients had values of Aβ1–40%, which were below 63.0. AD patients with a value for Aβ1–40% equal to or exceeding 63.0 scored a median MMSE of only 8.0, whereas otherwise they scored a median MMSE of 20.0 ($p = 0.018$). When AD patients were separated according to %Aβ1–38 = 16.0 they scored a median MMSE of 19.0, whereas otherwise they obtained a median MMSE score of 8.0 ($p = 0.048$). Finally, we split the group of AD patients according to both cut-off values. Patients with Aβ1–40% > 63.0 and Aβ1–38% < 16.0 scored a median MMSE of 19.5, otherwise a median MMSE score of only 6.5 ($p = 0.0081$).

Discussion

High-resolution separation and sensitive detection of Aβ peptides

The original urea version of the bicine/tris SDS–PAGE of Wiltfang *et al.* (1991) was used for the electrophoretic separation of Aβ peptides, which was applied for the electrophoretic separation of Aβ1–40 and Aβ1–42 for the first time and without further modification by Klafki *et al.* (1996). Here, we further improved the sensitivity of the western-immunoblot procedure and used a CCD camera for signal acquisition, which allowed us to study the Aβ peptide expression in human CSF by a quantitative Aβ-SDS–PAGE/immunoblot. Separation gels containing 8 M urea allowed baseline separation of Aβ peptides differing by not more than a single hydrophobic amino acid. This can only be explained by highly reproducible and discrete urea-induced shifts in the conformation of Aβ peptides, which affect their effective molecular radii during SDS–PAGE separation (Wiltfang *et al.* 1997). Furthermore, the Aβ-SDS–PAGE/immunoblot was combined with electrofocussing by immobilized pH gradients (IPG) to yield a two-dimensional Aβ-IPG-2D-PAGE/immunoblot. This method allows a high-resolution and sensitive expression profiling of APP metabolites and their post-translational modifications.

Three additional Aβ peptides in human CSF

Aβ1–40 and Aβ1–42 are generally believed to be the major Aβ species in biological fluids (Seubert *et al.* 1992; Shoji *et al.* 1992; Motter *et al.* 1995; Ida *et al.* 1996; Galasko 1998; Hulstaert *et al.* 1999). However, Aβ-SDS–PAGE/immunoblot revealed that additionally the Ct-truncated Aβ peptides 1–37/38/39 are regularly found in human CSF. The relative abundances of these five Aβ peptides were surprisingly constant, suggesting regulation of their production and/or degradation in narrow limits. Interestingly, in humans CSF Aβ1–38 was found to be present at a higher concentration than 1–42, and thus it was the second prominent Aβ species next to Aβ1–40.

Affinity-purification of human CSF followed by MALDI-MS has indicated the existence of a large panel of additional Nt- and Ct-truncated Aβ peptide species, including Aβ1–38, but not Aβ1–37 or Aβ1–39 (Vigo-Pelfrey *et al.* 1993). However, the absolute and relative quantities of these additional Aβ peptides were not investigated, nor is it known whether they are regularly produced. Surprisingly, the authors did not detect Aβ1–42. It is known that the $[M + H]^+$ signal intensity of the β-sheet forming Aβ1–42 during MALDI-MS is significantly decreased, as compared with non-β-sheet forming peptides of similar molecular masses, due to a relationship between secondary structure and MALDI-MS signal intensity (Wenschuh *et al.* 1998). This may also explain the low level of Aβ1–42 we observed in human CSF by MALDI-MS following Nt-selective immunoprecipitation.

Aβ1–37, Aβ1–38 and Aβ1–39 together with Aβ1–42 and most predominantly Aβ1–40 were also detected by mass spectroscopy in the supernatants of neuroblastoma cells (Asami-Odaka *et al.* 1995; Wang *et al.* 1996). Other groups who applied urea-based Aβ-SDS–PAGE/immunoblot for the analysis of secreted Aβ peptides may have missed Aβ1–37/38/39 due to misinterpretation as cross-reactive proteins (Cescato *et al.* 2000) or aggregated Aβ peptides (Beck *et al.* 2000).

We recently demonstrated that Aβ1–37/38/39 in addition to Aβ1–40/42 are regularly produced by a primary neuronal

cell culture and a human neuroglioma cell line (Wiltfang *et al*. 2001). The fractional pattern of the Aβ peptide quintet in the cell culture supernatants does closely resemble the pattern we identified in the human CSF samples. Moreover, analysis of presenilin-1 (PS-1) knockout cells revealed that the generation of the carboxyterminally truncated Aβ1–37/38/39 was strongly dependent on PS-1, as opposed to the aminoterminally truncated Aβ2–42. The same study demonstrated that Aβ1–38 was elevated in the brain of patients with sporadic AD or familial AD due to a PS-1 mutation, but not Aβ1–37 or Aβ1–39 (Wiltfang *et al*. 2001).

Effect of different sample pretreatments on the CSF concentration of Aβ peptides: evidence of carrier-mediated epitope masking of Aβ1–42

When different methods for the detection and quantification of Aβ peptides in CSF where compared, we observed a strong influence of the sample pretreatment on the measured Aβ peptide concentrations. This was most pronounced for the quantification of Aβ1–42. For the latter peptide, we determined approximately three-fold higher values by SDS-heat denaturation and Aβ-SDS–PAGE/immunoblot as compared with a commercially available ELISA (Hulstaert *et al*. 1999). Our finding that immunoprecipitation of the samples prior to Aβ-SDS–PAGE/immunoblot yielded results comparable to SDS-heat denaturation only if the immunoprecipitation was done in the presence of detergents (RIPA buffer), suggested that a fraction of Aβ peptides is not accessible to antibodies due to binding to carrier proteins (epitope masking). Aβ peptides in CSF were shown to bind to several carrier proteins including apolipoprotein J and apolipoprotein E (Koudinov *et al*. 1996).

Published values of CSF-concentrations of total Aβ peptides, Aβ1–40 and Aβ1–42 show large variations (Motter *et al*. 1995; Nitsch *et al*. 1995; Ida *et al*. 1996; Southwick *et al*. 1996; Tamaoka *et al*. 1997; Hock *et al*. 1998; Pirttila *et al*. 1998; Shoji *et al*. 1998; Andreasen *et al*. 1999; Hulstaert *et al*. 1999). Presumably, these differences are due to different antibodies that were used, and to variations in the experimental protocols that were applied in the respective studies.

In general, ELISA methods for the determination of Aβ peptides in human CSF samples reported lower values (Motter *et al*. 1995; Tamaoka *et al*. 1997; Shoji *et al*. 1998; Andreasen *et al*. 1999; Hulstaert *et al*. 1999) than we obtained by Aβ-SDS–PAGE/immunoblot. This discrepancy is selectively pronounced for Aβ1–42 and it is most prominent in AD patients. Accordingly, this finding indicates the presence of a CSF pool of Aβ1–42 in AD, which is detectable by Aβ-SDS–PAGE/immunoblot after SDS-heat denaturation, but which is not accessible to the antibodies used in conventional ELISA methods. Most probably, this is due to enhanced epitope masking in AD.

The striking ApoE ε4-associated reduction of CSF Aβ1–42 observed in our study seems to be associated with the large pool of CSF Aβ1–42, which we were able to access by SDS-heat denaturation and Aβ-SDS–PAGE/immunoblot. The smaller ELISA-accessible pool of CSF Aβ1–42 may not reflect this ε4-associated decrease of Aβ peptides to the same degree (Galasko *et al*. 1998; Hulstaert *et al*. 1999).

Reduced CSF concentration of Aβ1–42 in AD: is this due to clearance by nucleation to intracerebral β-amyloid plaques?

In agreement with previous studies we observed reduced CSF levels of Aβ1–42 in AD patients compared with non-demented patients with other neuropsychiatric diseases.

As one probable explanation for the decrease of Aβ 1–42 levels in AD, it has been suggested, that the peptide becomes increasingly insoluble and forms deposits in the form of diffuse and neuritic plaques. However, we previously showed that CSF Aβ1–42 levels are reduced in patients with Creutzfeldt–Jakob disease (CJD) to the same degree as in AD (Otto *et al*. 2000). Since these CJD patients did not develop β-amyloid plaques, we conclude that Aβ deposition is very unlikely to be the reason for this drop of Aβ1–42 in CSF.

Pitschke *et al*. (1998) demonstrated by fluorescent correlation spectroscopy that specifically the CSF of AD patients contain Aβ1–42 binding complexes, which can serve as a nucleus for seeded polymerization of synthetic fluorescent Aβ1–42. Seeded multimerization was also demonstrated by fluorescent correlation spectroscopy for prion protein (Post *et al*. 1998). Hence, both amyloid diseases may share a common pathological chaperone(s), which may induce complexation of Aβ1–42 within the CSF compartment.

Disease specific patterns of Aβ1–38%, Aβ1–40%, and Aβ1–42% in AD and CID

AD patients showed a significant reduction of Aβ1–42%, which was accompanied by increases of Aβ1–40% and Aβ1–38%. In CID patients, we observed a reduction of Aβ1–40% and elevated Aβ1–38/39%, as compared with the OND group. Aβ1–37% was unchanged in both patient groups, as compared with OND patients.

Taken together our data demonstrate that disease-associated changes of Aβ peptides in CSF were reflected with superior sensitivity and specificity by the fractional Aβ peptide pattern, as compared with the absolute Aβ peptide values. This may be explained by the following line of evidence: Results from cell culture experiments with cells expressing mutant human APP or presenilin-1 indicate that variation of the β-secretase activity strongly influences the total amount of Aβ1–40 and Aβ1–42, whereas variation of the γ-secretase activity predominantly modulates their relative abundances. Accordingly, the fractional CSF Aβ peptide pattern might reflect disease-associated changes in

γ-secretase activity with superior sensitivity, as compared with the absolute CSF levels of Aβ peptides.

The pathophysiological mechanisms underlying the characteristic fractional Aβ peptide pattern in the clinically heterogeneous group of CID patients remain obscure so far, but may be correlated to disease-specific cytokine expression patterns affecting enzymatic neuronal APP processing or altered APP processing by activated astroglia and microglia. Interestingly, patients with evidence of chronic neuroinflammation and those with AD share an elevation of Aβ1–38%. This further stresses the role of neuroinflammation as part of the pathophysiology of AD.

Remarkably, a re-evaluation of the patient records showed that chronic neuroinflammation due to immune vasculitis or glia activation was implicated in the CNS disease of at least three of six patients OND patients, who presented with a fractional Aβ peptide pattern otherwise typical for CID. Accordingly, the fractional Aβ peptide pattern may become of clinical relevance for the neurochemical diagnosis of CID in as much as the routine neurochemical CSF analysis, including oligoclonal bands, was normal in several of those patients.

The pathophysiological role of Aβ1–38

So far only limited data are available on the physiological and pathophysiological role of Aβ1–38. Aβ1–38 can form fibrils of 70–90 Å in diameter (Fraser *et al*. 1991), activate the plasma kinin-forming cascade (Shibayama *et al*. 1999), destabilize neuronal calcium regulation (Mattson *et al*. 1992), and it can stimulate cultured rat microglia to release matrix metalloproteinase-9 (Gottschall 1996). Moreover, Weggen *et al*. (2001) most recently found that non-steroidal anti-inflammatory drugs (NSAIDs) can lower amyloidogenic Aβ42 independently of cyclooxygenase activity, which was paralleled by a selective increase of Aβ1–38. Their findings indicate that in contrast to the current generation of γ-secretase inhibitors, NSAIDs do not perturb APP or Notch processing but rather seem to induce a subtle shift in γ-secretase activity.

Most interestingly, Aβ1–38 binds to NACP (Yoshimoto *et al*. 1995) and specifically induces its oligomerization (Paik *et al*. 1998). NACP is the presynaptic precursor protein of the low molecular mass non-amyloid Aβ peptide component of Alzheimer's disease amyloid (NAC), which is the second major constituent of β-amyloid plaques (Ueda *et al*. 1993). NACP is also known as α-synuclein, which is not only implicated in the formation of abnormal protein depositions in senile plaques in AD, but also found in the Lewy bodies of Lewy body dementia (LBD) and Parkinson's disease (Iwai *et al*. 1995; Heintz and Zoghbi 1997; Spillantini *et al*. 1997; Baba *et al*. 1998; Clayton and George 1998; Takeda *et al*. 1998). Thus, the interaction between α-synuclein and Aβ1–38 may be of pathophysiological relevance since it may generate a nucleation centre for subsequent amyloidogenesis and formation of Lewy bodies.

The fractional Aβ peptide pattern correlates with the severity of dementia

Whereas no individual absolute Aβ peptide value showed a significant correlation to the severity of dementia, this was observed for the fractional Aβ peptide pattern. Interestingly, the fractional amounts of the Ct-truncated Aβ peptides showed a tendency to decrease with increasing severity of dementia, whereas Aβ1–40% showed a positive correlation with the degree of dementia. There is recent evidence from post mortem studies that the soluble fraction of Aβ peptides is much more closely correlated to the severity of dementia, as compared with the insoluble Aβ peptides aggregated as β-amyloid plaques (Lue *et al*. 1999; McLean *et al*. 1999). This finding was most pronounced for soluble Aβ1–40, as opposed to soluble Aβ1–42. The positive significant correlation of Aβ1–40% and the missing covariation of Aβ1–42% with the severity of dementia show an interesting analogy with the latter observation from post mortem studies.

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