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Alzheimer Amyloid Peptide Aβ42 Regulates Gene Expression of Transcription and Growth Factors

Running title: Aβ42 has a role in gene regulation

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Abstract

The pathogenesis of Alzheimer disease (AD) is characterized by the aggregation of amyloid-beta (Aβ) peptides leading to deposition of senile plaques and a progressive decline of cognitive functions, which currently remains the main criterion for its diagnosis. Robust biomarkers for AD do not yet exist, although changes in the cerebrospinal fluid levels of tau and Aβ represent promising candidates in addition to brain imaging and genetic risk profiling. Although concentrations of soluble Aβ42 correlate with symptoms of AD, less is known about the biological activities of Aβ peptides which are generated from the amyloid precursor protein. An unbiased DNA microarray study showed that Aβ42, at sub-lethal concentrations, specifically increases expression of several genes in neuroblastoma cells, notably the insulin-like growth factor binding proteins 3 and 5 (IGFBP3/5), the transcription regulator inhibitor of DNA binding (ID1-3), and the transcription factor Lim only domain protein 4 (LMO4). Using qRT-PCR, we confirmed that mRNA levels of the identified candidate genes were exclusively increased by the potentially neurotoxic Aβ42 wild-type peptide, as both the less toxic Aβ40 and a non-toxic substitution peptide Aβ42 G33A did not affect mRNA levels. *In vivo* immunohistochemistry revealed a corresponding increase in both hippocampal and cortical IGFBP5 expression in an AD mouse model. Proteomic analyses of human AD cerebrospinal fluid displayed increased *in vivo* concentrations of IGFBPs. IGFBPs and transcription factors, as identified here, are modulated by soluble Aβ42 and may represent useful early biomarkers.

Keywords

Alzheimer disease; amyloid beta; gene regulation; transcription factors; IGFBP; LMO4; ID1-3; immunohistochemistry; CSF proteomics

INTRODUCTION

As life expectancy increases, the growing incidence of Alzheimer disease (AD) makes early prevention or early diagnosis of AD a major public health priority. It is generally accepted that misfolded proteins, such as toxic amyloid-beta42 (Aβ42) peptides, which assemble from monomeric molecules into fibrils and then form diffuse amyloid plaques in the brain, lead to neurodegeneration in patients [1]. However, AD etiology is complex, and brain pathology is advancing rapidly by the time people express Mild Cognitive Impairment (MCI) or even before. The disease includes decades of "silent" but relentless progression known to involve formation of "toxic" Aβ42 peptides [2]. So far, cognitive decline is the clinical benchmark for current diagnosis.

The amyloid cascade hypothesis posits that the formation of toxic Aβ is associated with the pathogenesis although a development of a therapy or even a reliable early diagnosis has not yet been achieved [3, 4]. Aβ peptides are produced by the proteolytic processing of the amyloid precursor protein (APP). Soluble oligomers of Aβ42 in particular are toxic and contribute to progressive neuronal dysfunction and cell death and are likely responsible for AD-related memory deficits [5-9]. Intraneuronal Aβ accumulation in brains of AD patients, in animal models, and in cultured cells has suggested a pathophysiological role for intracellular Aβ40 and Aβ42 [10-12]. Furthermore, as recently shown, intraneuronal Aβ peptides of varying lengths, viz., Aβ38, Aβ40, Aβ42 and Aβ43, are taken up from the medium by the cells and translocated to the nucleus [13]. Notably, however, the Aβ42 G33 substitution variant, which is harmless to neurons even in low-n oligomeric form [5], was also taken up [13]. Moreover, Aβ42 peptides from APP cleavage by the gamma secretase complex were present in hippocampal neuronal nuclei in APP/PS1 mice. While the less- or non-toxic Aβ species did not influence gene expression regulation, Aβ42 specifically associated with AICD-regulated promoters of *LRP1* and *KAI1* [13]. These results agreed with findings showing that Aβ42 can bind to the *APP* promoter sequence [14, 15]. As a consequence, the corresponding mRNA levels have been altered upon Aβ42 treatment, whereas the less- or

non-toxic Aβ species including Aβ38, Aβ40, Aβ42 G33A and Aβ43 did not influence APP mRNA levels [13].

Here, to investigate whether Aβ42 has a specific transcriptional regulatory function – and therefore that deregulation of Aβ target genes could be an alternative pathway for Aβ-induced neurotoxicity – we conducted an unbiased genome-wide DNA microarray study in SH-SY5Y cells. Importantly, we identified genes whose expression was upregulated specifically upon Aβ42 treatment at sub-lethal concentrations but not by the non-toxic Aβ40 peptide nor the non-toxic substitution variant Aβ42 G33A.

Among these genes were those that encoded the transcription regulators ID1-3, transcription factor LMO4 and the insulin-like growth factor binding proteins 3 and 5 (IGFBP3/5). Such data from five independent experiments were confirmed by qRT-PCR. Notably, these changes were exclusively observed upon treatment with sub-lethal concentrations of Aβ42 [13]. Using immunohistochemistry to analyze protein expression we detected an increase of IGFBP5 levels in the TgCRND8 mouse model of AD, carrying the APP KM670/ 671NL (Swedish), APP V717F (Indiana) mutations [16]. We found such increases in neurons of deep cortical layers as well as in and around plaques. Remarkably, IGFBP5 expression was already elevated in pre-plaque stage mice. Finally, high resolution label free quantitative proteomic analyses of CSF from AD patients showed altered protein levels compared to aged non-AD subjects. E.g., we found increased concentrations of IGFBP3 and 5, and transcription regulation by Aβ42 may be an early event in the pathogenic cascade of AD. We therefore hypothesize that IGFBP3/ 5 and other identified candidate proteins in body fluids represent potential targets to develop tools for an early diagnosis.

MATERIALS AND METHODS

Cell culture

SH-SY5Y cells were purchased from DSMZ (ACC 209) and were routinely cultured as described [5]. Briefly, cells were synchronized in G1 phase of cell cycle by double-thymidine block (2 mM for 15 h; Sigma Aldrich). After first block, cells were washed with PBS (PAA) and grown in fresh medium without thymidine for 8 hours. After the second block, cells were washed and grown for two hours (medium containing 20% (v/v) fetal calf serum) and released to progress through the cell cycle. Medium was replaced by medium containing freshly dissolved Aβ peptides or vehicle control and incubated for the indicated time points.

Aβ peptides and MTT assay

Synthetic Aβ peptides (Peptide Specialty Laboratories, Germany) were monomerized and solubilized as described [5]. Briefly, monomerized peptides were dissolved to 1 mg/ml in deionized water supplemented with ammonia to a final concentration of 0.13% (measured pH9.8). All peptides were used at a sub-lethal concentration of 1 µM [13]. Definition of vehicle control: cell culture medium supplemented with 0.13% ammonia. Briefly, all peptides were used at a sub-lethal concentration of 1 µM [13]. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay was performed as described previously [5].

Real time PCR

Total RNA was isolated with the NucleoSpin RNA kit (Macherey & Nagel). RNA quality was determined by NanoDrop (PeqLab) and Bioanalyzer (Agilent Technologies). For reverse transcription the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and for qRT-PCR the *Taq*Man Gene Expression assays HS00704053_s1 (*id1*), HS00747379_m1 (*id2*), HS00171409_m1 (*id3*), HS00426287_m1 (*igfbp3*), HS01052296_m1 (*igfbp5*) and HS00232488_m1 (*lmo4*) were used.

Illumina microarray hybridization

The RNA quality was determined with the Agilent 2100 Bioanalyzer using RNA 6000 Nano Chips (Agilent Technologies). The RNA integrity numbers of the samples ranged from 9.1-10.

RNA labeling was performed on a Hamilton Star Roboter (Hamilton) using the Illumina Total Prep-96 RNA Amplification kit from Ambion (Applied Biosystems). Briefly, from 500 ng total RNA first and second strand cDNA was synthesized, and cleaned-up using Agencourt magnetic beads CleanKit (Beckman Coulter). After *in vitro* transcription, biotin-labeled cRNA was purified using Agencourt magnetic beads CleanKit. cRNA concentration was determined with Quant-iT RiboGreen (Invitrogen). cRNA (1.5 µg) was mixed and hybridized to Illumina Human HT-12 v3 BeadChips. All washing and staining steps were performed with a Little Dipper Processor for Illumina BeadChips from Scigene (Sunnyvale). Biotin was labeled with streptavidin-Cy3 (Amersham Biosciences).

Bioinformatic Analysis

BeadChip raw data output contains the average signal intensity and the detection *P* value for each probe. Analyses were performed using GeneData's Expression Analyst. For quality control box plots of signal intensity, unsupervised hierarchical clustering and Principal Components Analysis for both unnormalized and LOWESS-normalized data were carried out without identifying any outliers. The analyses revealed even signal distribution within the set of experiments. A two-way ANOVA was used to investigate the influence of the factors "time" and "treatment" on differential gene expression. A total of 225 genes were identified from the ANOVA analysis as significantly differentially expressed (false discovery rate of BH-Q ≤1e-3). These 225 genes clearly separated treatment groups into individual clusters. Five genes showed a fold change ≥ 2 (as referred to Aβ42 versus control treatment in an N-fold regulation analysis), and another 20 genes a ≥ 1.5 fold change.

Immunohistochemistry

All animal work related to this project was approved by the University of Toronto Animal Care committee, in accordance with the guidelines of the Canadian Council on Animal Care (CCAC). Brains from TgCRND8 [16] and aged-matched littermate mice (controls) at the age of 90 and 150 days were fixed in 10% neutral buffered formalin for a minimum of 48 hours and paraffin-embedded. For immunohistochemistry, all sections were blocked in diluted (3%) hydrogen peroxide. Sections were heat-retrieved in a decloaking chamber (Biocare) using Tris-EDTA buffer, pH 9.0, 120 º C for two minutes. Sections were blocked with 2.5 % normal horse serum and incubated over night at room temperature in a moist chamber with anti IGFBP5 antibody (R&D, AF578) at 1:200 dilution. Sections were washed with TBST buffer and stained with ImmPRESS anti-goat Ig (peroxidase) polymer (Vector Labs, MP7405), washed in TBST buffer and developed with DAB solution. Light counterstaining was performed with hematoxylin. Specimens were dehydrated through graded alcohol, cleared in xylenes and mounted with Permount (Fisher).

Human samples

CSF was collected (under approval by the ethics committee of the Charité Berlin, Germany) and quantification of Aβ and tau proteins were performed according to standardized protocols and as described elsewhere [17]. Briefly, Aβ peptides and t-tau samples were quantified as described elsewhere [17]. AD patients and controls were routinely tested by Mini-Mental Status Examination as well as phenotyped for AD pathology using Magnetic Resonance Imaging.

In total, 40 CSF samples were analyzed. Alkylation of CSF proteins was carried out with 55 mM iodacetamide for 20 min. After trypsin digestion over night at RT, samples were diluted with 200 µl buffer A (5 % acetonitrile, 3 % trifluoracetic acid) treated as described elsewhere [18] and vacuum dried. Re-dissolved samples were loaded on a ReprosilPur C18 reverse phase column and peptides were analyzed using a nano-HPLC Dionex Ultimate 3000 system (Thermo scientific) coupled to an Orbitrap Velos mass spectrometer (Thermo

scientific). MS and MS/MS data from each LC/MS run were analyzed with MaxQuant software (Version 1.3.0.5.). Identification of proteins was performed using the MaxQuant implemented Andromeda peptide search engine.

Statistical analysis

Statistical significance between treated cells and control groups was determined using the one-way ANOVA Dunnett's multiple comparison test. Number of experiments and p-values are given in the respective figure legend.

For biometric analysis of the identified proteins, data (raw intensity values from MaxQuant) were normalized by total intensity normalization. Statistical significances were calculated using the Welch t-Test with *p<0.05, **p<0.01 and ***p<0.001.

RESULTS

Aβ42 modulates gene transcription

Previously we reported that Aβ peptides can translocate to the nucleus. Thus, Aβ peptides can be detected in neuronal nuclei *in vivo,* and especially Aβ42 can interact with specific gene regulatory elements [13]. To assess the possible effects of intranuclear Aβ species on the regulation of gene expression, we performed an unbiased genome-wide expression analysis in SH-SY5Y cells using the Illumina chip human HT-12 v3. The cells were treated for 12 hours with 1 µM Aβ42 at a sub-lethal concentration. We also treated cells with Aβ40, which is known as less-toxic than Aβ42 and non-toxic substitution peptide Aβ42 G33A (Fig. 1) for 12 hours [5, 13].

After treatment we isolated total RNA from SH-SY5Y cells. The RNA integrity numbers ranged from 9.1-10. We synthesized first and second cDNA strands, which was followed by *in vitro* transcription to generate cRNA, and finally hybridized cRNA to the microarray chip. A total of 225 genes were identified as significantly differentially expressed from the ANOVA

analysis. Solely a small amount of genes revealed as much as a 2-fold increase in expression we have further focused onto. In particular, only the Aβ42-treated cells showed a 2-fold increase in expression levels of IGFBP3 (Fig. 2A) and IGFBP5 (Fig. 2B), as well as the transcription regulator ID2 (Fig. 2D). A 1.5-fold increase in expression was detected of ID1, 3 (Fig. 2C, E) and the transcription factor LMO4 (Fig. 2F). Results from five independent replicate experiments were consistent. However, it was critical that signal intensity of individual time points be considered specifically because of the apparently non-linear temporal gene-expression. The specificity of these results to Aβ42 was confirmed by qRT-PCR, which showed that mRNA levels of the specified genes were increased only by this peptide (Fig. 3A-F). These latter experiments showed that relevant mRNA levels increased significantly after seven hours of incubation, peaked at eight or nine hours, and then returned to basal levels (Fig. 3G). Notably, Aβ42 was the sole peptide capable of altering gene expression; neither Aβ40 nor the non-toxic substitution peptide Aβ42 G33A affected mRNA levels, i.e., gene regulation.

LMO4 belongs to the 'LIM domain only' proteins, a LIM domain being formed by a double zink-finger motif involved in protein-protein interaction [19]. LMO proteins are taking part via their LIM domains in multiple protein complexes involved in transcription regulation during development [20]. It has been shown that LMO4 RNA overexpression interferes with neuritic outgrowth, whereas anti-sense LMO4 RNA expression favors neuritogenesis in SH-SY5Y cells. Consequently, changes in LMO4 RNA expression levels alter the rate of neuritic outgrowth in the developing and adult nervous system [21]. However, functional involvements of LMO4 in the adult human brain and its involvement in AD neurodegeneration have not yet been studied. So far no direct link between AD and ID1-3 has been shown yet. However, it has been reported that presenilin 1 (PS1), a component of the γ-secretase complex, acts independently in controlling myogenesis, which is mediated in part by ID1 [22].

IGFBP5 expression correlates with the pathology in TgCRND8 mice

A large-scale comparative microarray analysis in the TgCRND8 mouse model of AD [16] and

littermate mice has recently unraveled altered expression levels in clusters of genes related to immune/inflammatory pathways [23]. Furthermore, high levels of Aβ42 resulted in dysregulation of genes related to signal transduction and neuroprotection including IGFBP5. This change in expression correlated with increases in Aβ levels and accumulation in mouse brain tissue [23].

To assess a possible relationship of an Aβ42-induced increase in expression of IGFBPs to AD pathology, we performed immunohistochemical staining studies in both wild-type (wt) and TgCRND8 mice. The latter carry transgenic (tg) mutated APP and display progressive accumulation of Aβ in the brain beginning at 70 to 80 days of age [16]. At age 150 days both the tg mice (at mature plaque-stage) and wt controls showed evidence of IGFBP5 expression in hippocampal regions (CA-1, -3, and dentate gyrus). Staining intensity was more pronounced in tg mice, however, when compared to wt mice. Furthermore, only the tg animals showed staining in and around plaques along a synaptic distribution (Fig. 4A). Earlier, at about 70 days of age, the tg mice failed to display significant transcriptomal changes of *IGFBP5* [23], but already by 90 days of age (pre-plaque stage) they showed a striking increase in cortical IGFBP5 expression compared with wt mice (Fig. 4B). Importantly, the increased expression of IGFBP5, in the APP tg mice as they begin to show pathology, is consistent with microarray data from AD patients [24]. Also, other components of the insulin-like growth factor (IGF) system are associated with biomarkers of AD status [25-27]. IGFBP3 correlates inversely with CSF Aβ42 levels in AD patients, and is increased in serum of AD and stable mild cognitive impairment (SMCI) patients compared to controls [26]. Together with previously reported dysregulation of IGFBP5 in an APP tg mouse model and in AD patients [23, 28], these findings suggest that IGFBPs act substantially in the pathophysiology of AD. Interestingly, these proteins can inhibit or potentiate IGF functions in numerous cell types to induce neuroprotection [29, 30], cell proliferation, differentiation, survival, and migration [31].

Changes in protein expression in CSF of AD patients

To test this last notion, we examined CSF from 20 AD patients and 20 aged controls using a label-free quantitative proteomics approach with high-resolution mass-spectrometry. Subjects' cognitive impairment state was assessed using the Mini-Mental Status Examination (MMSE) (Fig. 5A), and, the disease stage of AD patients was estimated by ELISAs for Aβ42 and total-tau CSF concentration (Fig. 5B, C). Although there was no apparent change in CSF total protein concentrations of AD subjects (Fig. 5D), we identified 108 proteins that were significantly increased or decreased in CSF from AD patients (Suppl. table 1). Notable among these were increases in IGFBP3 and 5 (Fig. 6A, B), but AD-associated changes fell more broadly into three groups indicated by stars (Suppl. table 1). In figure 6C these groups are categorized according to their biological and molecular functions: (i) extracellular matrix interaction, cell adhesion, and cytoskeleton formation; (ii) inflammatory response, neuroprotection, and tumor suppressors; (iii) oxidative stress, phosphorylation, and oxidation/reduction. Interestingly, most (76%) of the proteins and/or their individual functions had been linked to AD in the past. For example CD44 and syndecan2 were elevated in AD CSF (Suppl. table1). CD44 expression is increased in lymphocytes from AD patients [32], and is a substrate of the gamma secretase complex [33]. Syndecan2 is likely co-deposited with Aβ40 [34], and is involved in the formation of cerebral amyloid angiopathy [35]. Reelin, which was reduced here in AD CSF is apparently decreased in brain of APP-overexpressing mice [36, 37], and is thought to be neuroprotective, perhaps by delaying fibril formation through a physical interaction with soluble Aβ42 species [38].

DISCUSSION

Recent studies of dominantly inherited and sporadic AD have provided quantitative insights into biomarker changes in preclinical AD that start decades before onset of clinical symptoms [39, 40]. Intra-neuronal accumulation of Aβ is among the earliest known events in AD

pathogenesis, preceding the appearance of both neurofibrillary tangles and amyloid plaques [10-12]. Our results support the hypothesis of a pathophysiological cascade starting with processes such as Aβ accumulation in the nucleus which we recently found to affect gene expression [13]. Here we show that 1) Aβ42 uniquely induces expression of genes and that such gene expression specifically includes synthesis of mRNA for ID1-3, LMO4 and IGFBP3/5, 2) that these latter proteins are overexpressed in TgCRND8 strain of APP-overexpressing mice before these animals develop mature amyloid plaques, and 3) that they are increased in CSF of AD.

Together with our recent findings that Aβ accumulates in neuronal nuclei in proportion to its concentration in cytosol, and that intra-nuclear Aβ42 provokes gene expression [13], these findings support the notion that the AD pathophysiological cascade may begin with intra-nuclear accumulation of Aβ species. Overexpression of ID1-3, LMO4, IGFBP3/5 is resulting as well as of other proteins listed in Suppl. Table 1. Importantly, the observed *in vitro* effects of Aβ42 occur at extracellular concentrations that are lower than toxic Aβ42 levels *in vivo* [41]. They appear to be distinct from effects of other, less- or non-neurotoxic Aβ species including Aβ40 and Aβ42 G33A [5, 13, 42, 43], even though the latter can assemble into cross-β fibrils [44] and enter the nucleus [13]. Today, it is unknown whether intra-neuronal Aβ42-induced expression of other genes including ID1-3 and LMO4 also have a role in these events. Our study is novel as Aβ42 was used at sub-lethal concentrations and markedly affected gene expression in the absence of overt neurodegeneration. Also, post-mortem AD brain analyses have yielded controversial results, upregulation of tumor suppressor genes, downregulation of retromer trafficking complex genes and alterations in expression of genes related to synaptic activity in cultivated slices from post-mortem AD brain. In a rather empirical approach LMO4 was previously found differentially expressed in AD brain [45] when immunoreactivity was consistently and specifically found diminished. This finding is in contrast to our observations in SH-SY5Y cells where we find a 1.5-fold increase in LMO4 expression. Changes in gene expression detected in such studies may represent a late consequence of cerebral inflammation and neurodegeneration rather than a direct or early

effect of Aβ oligomers as seen for several pathways important for neuronal physiology [48]. Other transcription factors identified earlier in brain sections of AD patients were NF-κB [46] and transcription regulators of the human presenilin 1 gene promoter [47]. Here, we present evidence that Aβ42 wt, at sub-lethal concentrations, markedly affects gene expression in the absence of overt neurodegeneration. Thus, alterations in gene expression have previously been reported as part of AD pathology but largely relied on analysis of post-mortem material. Recent microarray data demonstrated an increased gene expression of IGFBP5 in TgCRND8 mice expressing human APP, compared to wild-type control mice [23]. We confirmed these results on the protein level by immunohistochemistry showing that IGFBP5 is increased in the hippocampus as well as in deep cortical layers in and around amyloid plaques in TgCRND8 mice. Expression starts at pre-plaque stages and together with our SH-SY5Y cell-based microarray data upregulation of IGFBP3/5 is likely a direct and early effect of Aβ42 low-n oligomers in the nucleus [13]. Also, overall alterations in gene expression observed in post-mortem studies [28] differ from changes observed following Aβ42 treatment, further supporting the argument that Aβ42 treatment can create a condition of overt neurodegeneration. Possibly, late changes seen in post-mortem studies may rather reflect a consequence of cerebral inflammation and neurodegeneration [26].

The significance of upgregulated IGFBP3 and 5 was supported by increases in these proteins in a synaptic distribution in brain tissue of APP tg mice, and in CSF from AD patients. The identification of upregulated *IGFBP3/5 per se* suggests the insulin signaling pathway is important in AD pathogenesis [27, 49, 50]. IGF 1 and IGF2 are thought to play a role in the pathogenesis of sporadic AD [51]. Furthermore, IGF1 has been linked to neuroprotection [29, 30] although there is also evidence that it may promote amyloid deposition [52, 53]. Importantly, the bioavailability and actions of IGF1 are influenced by IGFBPs [31]. Thus, our findings of Aβ42-induced expression of the latter, together with their elevated accumulation in brain and CSF of AD patients support previous findings that suggest the IGF system is associated with AD biomarkers. IGFBP3 negatively correlated with CSF Aβ42 levels in AD patients and was found increased in serum of AD and SMCI

patients compared to controls [26], whereas another study concluded that low IGFBP3 serum levels were associated with AD in men, but not in women [54]. Early changes in the insulin signaling pathway are subtle, occurring before neuronal injury and alteration in biomarkers such as CSF Aβ42 and tau [3, 4]. Our findings of elevated IGFBP3/5 in AD CSF using a hypothesis-free proteomic screening method let ELISA findings of increased IGFBP3/5 in CSF from AD patients *vs*. controls appear in a new light and give it a new significance [55]. The link between AD and IGFBP regulation is still not fully understood, however, and requires further investigation. Though, we are confident that novel potential biomarkers identified from Aβ42-mediated gene expression profiles might open up new avenues for an early diagnosis of AD.

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The authors declare that they have no conflict of interest.

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Legends

Figure 1: Viability of SH-SY5H cells determined by the MTT assay after incubation of cells with Aβ peptides 40, 42 and 42G33A. A treatment with a concentration of 1 µM of individual peptides for 12 hours incubation left cell viability unimpaired (ctrl, cell culture medium supplemented with 0.13% ammonia), n = 6 - 8.

Figure 2: Gene identification by DNA microarrays. Gene expression in SH-SY5Y cells was analyzed after treatment with Aβ40, Aβ42 and Aβ42 G33A (1 µM) for indicated times. After 8 hours incubation with Aβ42, expression of IGFBP3 (A), IGFBP5 (B), ID1 (C), ID2 (D), ID3 (E) and LMO4 (F) were approximately twofold increased compared to untreated controls (ctrl). Changes in expression levels were determined by bioinformatics analyses at individual time points (n = 5, independent experiments). Treatment with Aβ40 and Aβ42 G33A did not alter gene expression at any of the indicated time points (compare fluorescence units of Aβ42 with Aβ40 and Aβ42 G33A values within one time point).

Figure 3: Validation and quantification of mRNA levels of identified genes by microarray using qRT-PCR. Treatment with Aβ42 peptides for 8 hours led to a significant increase of the mRNA levels of IGFBP3 (A), IGFBP5 (B), ID1 (C), ID2 (D), ID3 (E) and LMO4 (F). Note, treatment with Aβ40 and Aβ42 G33A did not alter mRNA levels at the indicated time points (*p<0.05, **p<0.005, ***p<0.0005), n = 6 - 8.

(G) The mRNA levels of ID2, IGFBP5 and LMO4 in SH-SY5Y cells that were incubated with Aβ42 for 2, 6, 7, 8, 9 and 12 hours are significantly found increased between 7 and 9 hours treatment (ID2 and IGFBP5) and for LOM4 at 8 hours time points (*p<0.05, **p<0.005), n = 3 - 8.

Figure 4: Immunohistochemistry of IGFBP5 in TgCRND8 mice. (A) Immunohistochemical staining of IGFBP5 in TgCRND8 and wt littermate mice brain tissue revealed that IGFBP5 was expressed both in 150 day-old TgCRND8 (plaque stage) and in wt mice in the hippocampal regions CA-1 and -3, along a synaptic distribution as well as in the dentate gyrus (DG). The other area of expression in TgCRND8 mice is selectively stained in neurons of mid and deep cortical layers (layer V, VI) as well as in and around plaques (arrows). Inset displays an amyloid plaque with IGFBP5 staining indicated by arrows (scale bar: 400 μm). (B) In 90 day-old TgCRND8 mice (pre-plaque stage), expression of IGFBP5 was increased in cortex. Inset shows IGFBP5 staining in cortical neurons (scale bar: 150 μm).

Figure 5: Clinical parameters used for AD diagnosis from CSF and MMSE are shown. Indicated are MMSE-values (A), Aβ42 concentrations (B), and total-tau concentrations (C). No significant differences in protein concentration in CSF from AD patients and aged controls were determined (D), n = 20 subjects per group.

Figure 6: Label free quantitative proteomics approach with high-resolution mass-spectrometry. CSF samples from AD patients showed significant upregulation of IGFBP3 (A) and IGFBP 5 (B), (*p<0.05, **p<0.01), n = 20 subjects per group. Categories of proteins

changed in CSF of AD patients after total intensity normalization out of ***p<0.001 and **p<0.01 (C) (see also Suppl. table1).

Suppl. table 1: Summary of data sets comparing identified and quantified proteins of 40 individual CSF samples obtained by highly sensitive nanoLC-MS proteome analysis. A total of 108 proteins revealed significantly changed levels when CSF samples from AD (n=20) were compared to non-demented controls (n=20). The statistical analysis after total intensity normalization showed that 15 CSF-proteins were significantly changed with a ***p-value <0.001, 23 with **p<0.01, and 73 proteins with *p<0.05.

Figure 1

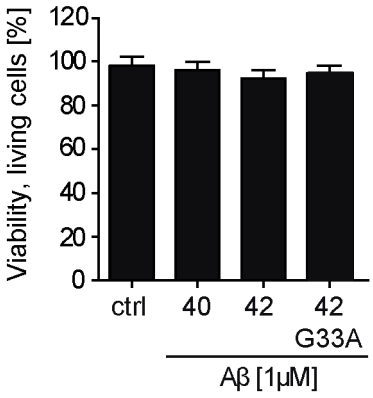


Figure 2

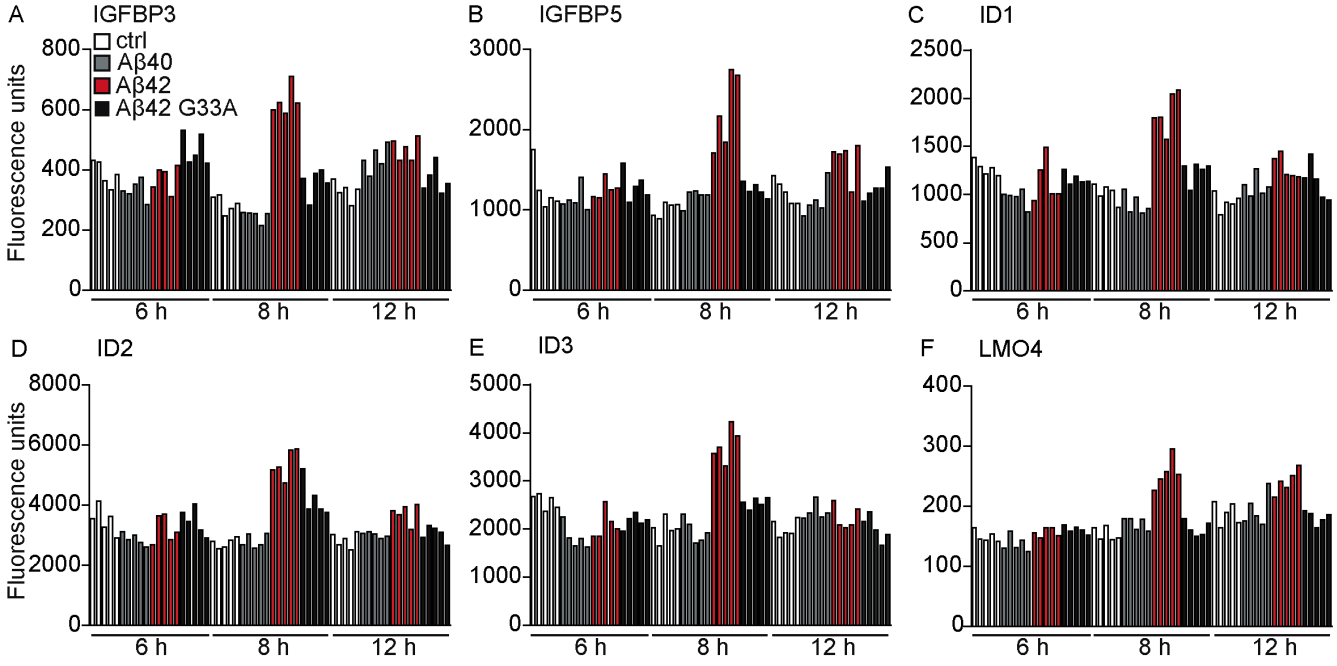


Figure 3

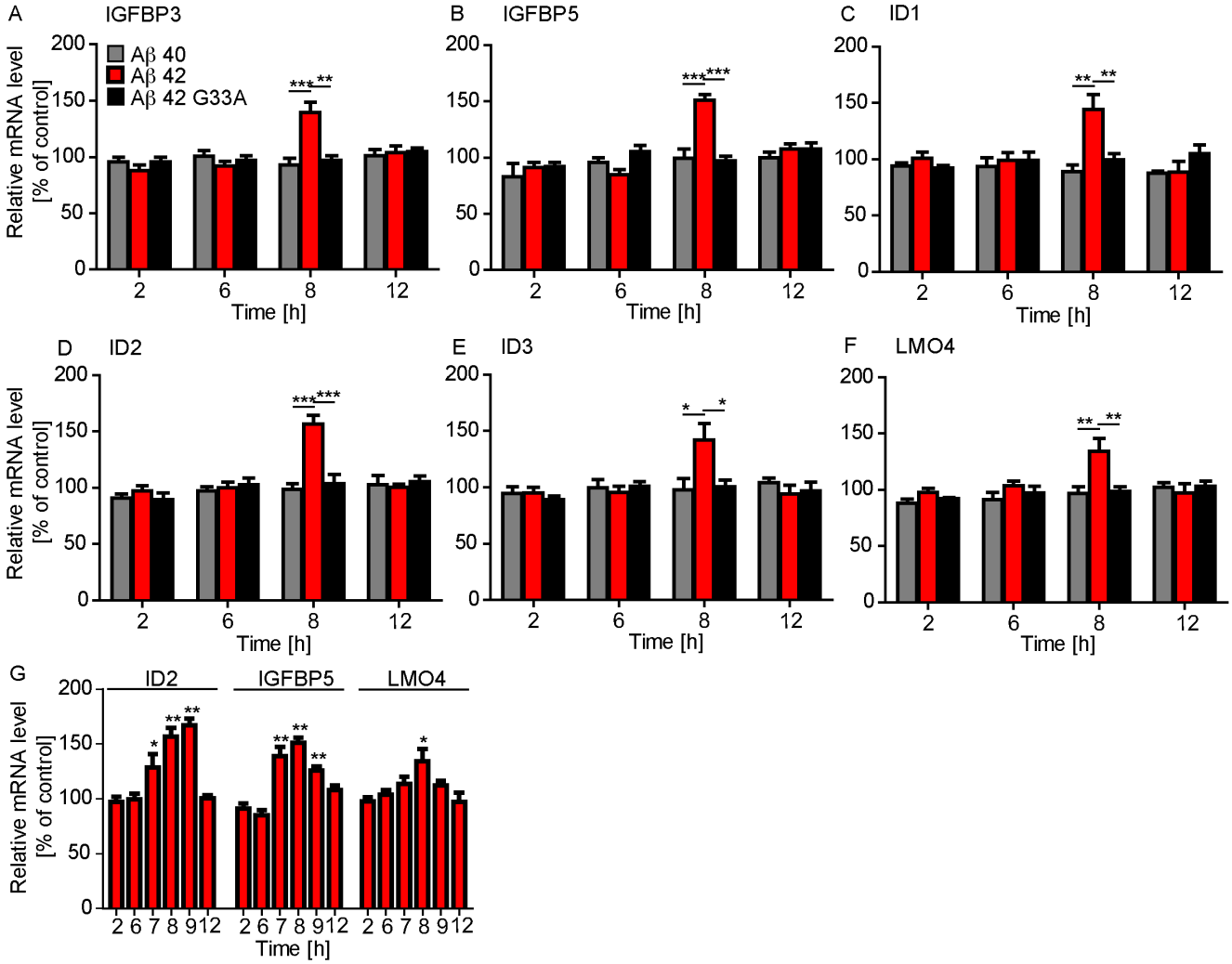

A
IGFBP3
Aβ 40
Aβ 42
Aβ 42 G33A
Relative mRNA level [% of control]
Time [h]
B
IGFBP5
C
ID1
D
ID2
E
ID3
F
LMO4
G
ID2
IGFBP5
LMO4

Figure 4

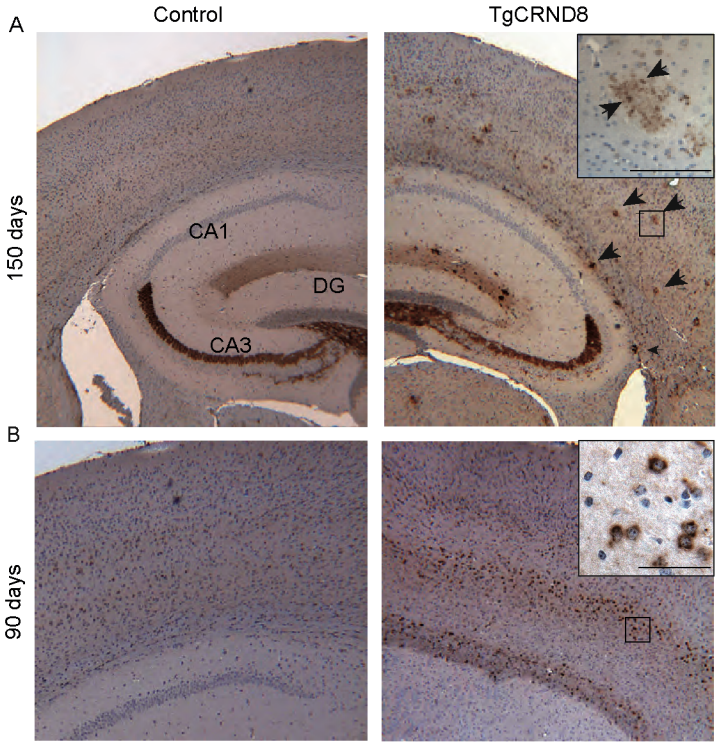


Figure 5

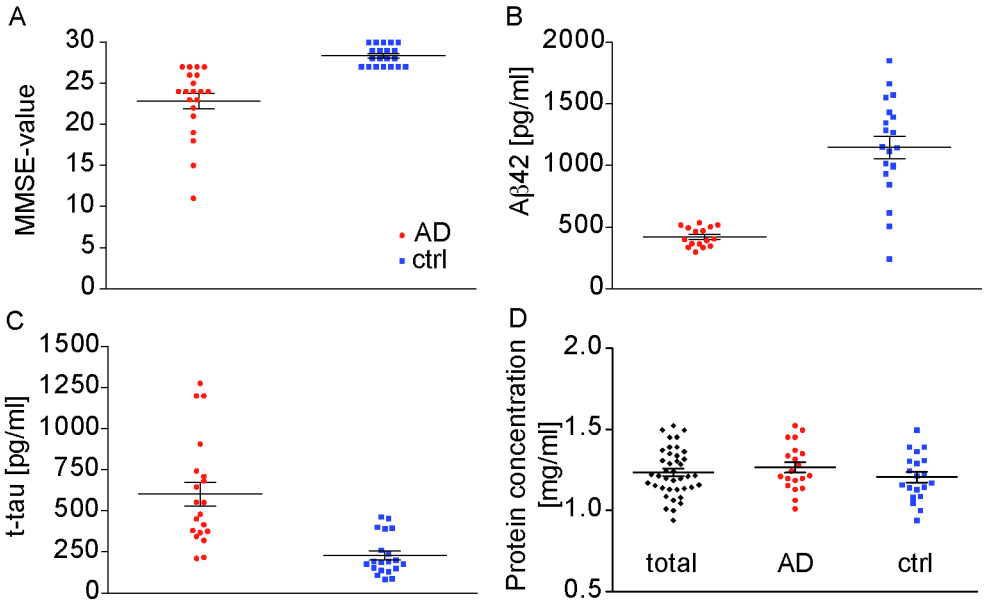
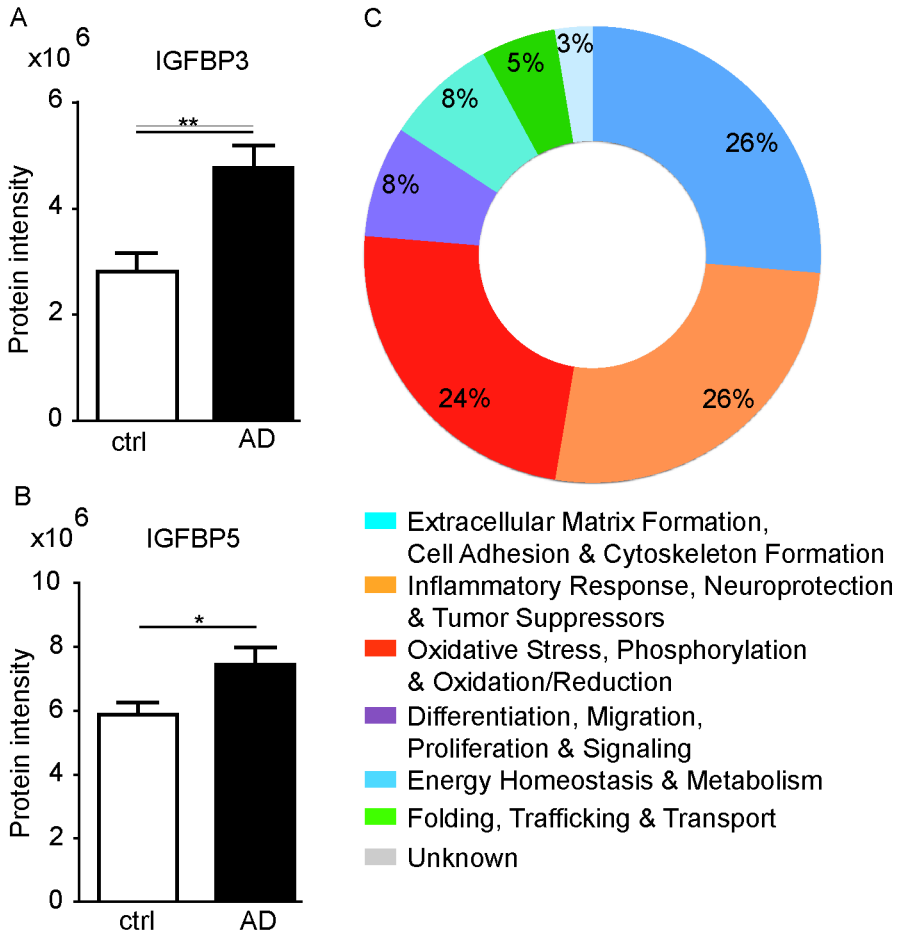

A
MMSE-value
30
25
20
15
10
5
0
AD
ctrl
B
Aβ42 [pg/ml]
2000
1500
1000
500
0
C
t-tau [pg/ml]
1500
1250
1000
750
500
250
0
D
Protein concentration [mg/ml]
2.0
1.5
1.0
0.5
total
AD
ctrl

Figure 6


A
x10 6
IGFBP3
Protein intensity
6
4
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ctrl
AD
B
x10 6
IGFBP5
Protein intensity
10
8
6
4
2
0
*
ctrl
AD
C
3%
5%
8%
26%
8%
24%
26%
Extracellular Matrix Formation, Cell Adhesion & Cytoskeleton Formation
Inflammatory Response, Neuroprotection & Tumor Suppressors
Oxidative Stress, Phosphorylation & Oxidation/Reduction
Differentiation, Migration, Proliferation & Signaling
Energy Homeostasis & Metabolism
Folding, Trafficking & Transport
Unknown

Suppl. table 1: List of proteins identified differentially in human AD CSF compared to controls

Protein Name	Signifi-cance	Direction Change AD	Protein ID
Extracellular Matrix Formation, Cell Adhesion & Cytoskeleton Formation			
Cadherin 8, type 2	**	Down	IPI00024036
Cartilage oligomeric matrix protein	**	Up	IPI00028030
CD44 molecule (Indian blood group)	**	Up	IPI00305064
Collagen, type XVIII, alpha 1	***	Up	IPI00783931
Cysteine-rich secretory protein LCCL domain containing 1	**	Up	IPI00027806
Latent transforming growth factor beta binding protein 2	***	Up	IPI00292150
Moesin	***	Up	IPI00219365
Profilin 1	**	Up	IPI00216691
Syndecan 2	**	Up	IPI00300407
Versican	***	Up	IPI00009802
Inflammatory Response, Neuroprotection & Tumor Suppressors			
colony stimulating factor 1 (macrophage)	**	Up	IPI00015881
complement component 7	**	Up	IPI00296608
glucosidase, alpha; neutral AB	**	Down	IPI00383581
insulin-like growth factor binding protein 2	***	Up	IPI00297284
insulin-like growth factor binding protein 3	**	Up	IPI00556155
inter-alpha (globulin) inhibitor H1	**	Up	IPI00383338
peptidylprolyl isomerase A (cyclophilin A); peptidylprolyl isomerase A (cyclophilin A)-like 3	***	Up	IPI00419585
reelin	**	Down	IPI00241562
secreted phosphoprotein 1	**	Up	IPI00921882
transgelin	**	Up	IPI00216138
Oxidative Stress, Phosphorylation & Oxidation/Reduction			
carbonyl reductase 1	**	Up	IPI00295386
creatine kinase, brain	**	Up	IPI00022977
glutathione peroxidase 3 (plasma)	***	Up	IPI00026199