

Oligomeric Amyloid Decreases Basal Levels of Brain-Derived Neurotrophic factor (BDNF) mRNA via Specific Downregulation of BDNF Transcripts IV and V in Differentiated Human Neuroblastoma Cells

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Alzheimer's disease (AD) is a senile dementia characterized by amyloid plaques, neurofibrillary tangles, and synaptic and cell loss. The "amyloid cascade" hypothesis suggests that amyloid- β ($A\beta$), the peptide deposited as amyloid plaques, is the primary insult in AD. However, debate continues over the mechanism of $A\beta$ toxicity and whether fibrillar or oligomeric $A\beta$ is the active species of the peptide that ultimately causes the synaptic loss and dementia associated with AD. Brain-derived neurotrophic factor (BDNF) is required for survival and function of cells compromised in AD. Decreased BDNF causes defects in long-term potentiation and memory and correlates with cognitive decline. We previously demonstrated that BDNF reduction occurs early in the course of AD, suggesting that decreased BDNF may promote neuronal dysfunction in AD. We also demonstrated that three of seven human BDNF transcripts are specifically downregulated in AD. What pathological feature(s) of AD leads to the decreased BDNF is unknown.

In this study, we administered both fibrillar and oligomeric conformations of $A\beta_{1-42}$ to differentiated SH-SY5Y, a human neuroblastoma cell line, and measured both phosphorylated cAMP response element-binding protein (CREB), a regulator of BDNF transcription, and BDNF total mRNA. We found that oligomeric but not fibrillar preparations of $A\beta_{1-42}$ significantly decrease both phosphorylated CREB and total BDNF mRNA. Furthermore, oligomeric $A\beta_{1-42}$ decreases BDNF transcripts IV and V in these cells, demonstrating that $A\beta_{1-42}$ downregulates the major BDNF transcript decreased *in vivo* in the AD brain. Thus, oligomeric $A\beta_{1-42}$ could compromise neuronal function, causing memory loss and cognitive dysfunction by downregulation of BDNF in AD.

Key words: Alzheimer; neurotrophin; exons; real-time RT-PCR; cell culture; CREB

Introduction

Alzheimer's disease (AD) is marked by progressive decline in cognitive functions and distinct pathology including senile plaques, neurofibrillary tangles, and synaptic degeneration (Glennner and Wong, 1984; Grundke-Iqbal et al., 1986; Geula, 1998; Coleman and Yao, 2003). The amyloid cascade hypothesis identifies amyloid- β ($A\beta$) as the precipitating insult in AD (Selkoe, 1994), although how $A\beta$ produces synaptic degeneration is uncertain. Although fibrillar amyloid deposited as plaques may contribute to AD pathogenesis, oligomeric amyloid is now recognized as the major toxic species (Lambert et al., 1998) and correlates with neurodegeneration in AD (Lue et al., 1999; McLean et al., 1999; Gong et al., 2003).

Brain-derived neurotrophic factor (BDNF) has an established

role in neuronal survival (Yuan and Yankner, 2000) and in long-term potentiation (LTP), a form of synaptic plasticity associated with memory formation and consolidation (McAllister et al., 1999; Lu, 2003; Bramham and Messaoudi, 2005). Transgenic mice with reduced BDNF levels exhibit defects in synaptic transmission, LTP, and memory tests that are rescued by exogenous BDNF administration (Korte et al., 1995; Patterson et al., 1996; Olofsson et al., 2000). BDNF mRNA and protein are significantly decreased in AD and mildly cognitive impaired (MCI) subjects compared with controls (Phillips et al., 1991; Connor et al., 1997; Ferrer et al., 1999; Hock et al., 2000; Holsinger et al., 2000). Moreover, the decrease in BDNF correlates with the severity of cognitive impairment (Peng et al., 2005), identifying BDNF downregulation as an early and possibly underlying event in AD. In rodents, 42 aa amyloid- β ($A\beta_{1-42}$) compromises BDNF production and signaling (Tong et al., 2001, 2004; Wu et al., 2006). Although BDNF downregulation by $A\beta_{1-42}$ has been demonstrated in rodent models, there have been no investigations of human BDNF regulation by $A\beta_{1-42}$.

To investigate the effects of $A\beta_{1-42}$ on human BDNF expression, we used the retinoic acid (RA) differentiated human neuroblastoma cell line SH-SY5Y (SY5Y). Differentiated SY5Y cells express BDNF and TrkB, the high-affinity BDNF receptor, and

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Table 1. PCR primer sequences for human BDNF transcripts

Transcript	Primer sequence	Product (bp)
BDNF transcript I	(F) GCG GAT ATT GCA AAG GGT TA (R) ACC TTG TCC TCG GAT GTT TG	287
BDNF transcript II ^a	(F) GCG GTG ATA GGC TGG AAT AG (R) ACC TTG TCC TCG GAT GTT TG	232
BDNF transcript III	(F) GCG AGT TTC GGG CGC TGG CTT AGA (R) ATT CAC GCT CTC CAG AGT CCC ATG	269
BDNF transcript IV	(F) GAG TAT TAC CTC CGC CAT GC (R) ATT CAC GCT CTC CAG AGT CC	381
BDNF transcript V	(F) AAC CAC GAT GTG ACT CCG C (R) ATT CAC GCT CTC CAG AGT CC	216
BDNF transcript VI	(F) AGT GGA CTT ACA AGT CCG AAG C (R) ATT CAC GCT CTC CAG AGT CC	313
BDNF transcript VII	(F) TTC TGC TGA CAG CAT GAG C (R) ATT CAC GCT CTC CAG AGT CC	515

F, Forward primer; R, reverse primer.

^aPrimers for BDNF transcript II encompass two of the three splice variants reported by Liu et al. (2005). We redesigned new primers to detect all three splice variants using the new forward sequence CAT TCA GCA CCT TGG ACA GA and the same reverse sequence from Table 1. PCR analysis with the new primers revealed splice variant 2 continued to be the most predominantly expressed transcript, and we were unable to detect the third splice variant in SH-SY5Y cells.

become dependent on BDNF for survival (Kaplan et al., 1993; Encinas et al., 2000; Feng et al., 2001). Furthermore, differentiated SY5Y cells develop a neuronal appearance, with long cell processes and expression of neuron-specific markers (Pahlman et al., 1984, 1990; Encinas et al., 2000) and, like human cortical neurons, are sensitive to A β (Lambert et al., 1994; Datki et al., 2004; Deshpande et al., 2006).

The human BDNF gene consists of seven upstream noncoding exons differentially spliced to the downstream coding exon, producing multiple transcripts (Aoyama et al., 2001; Garzon et al., 2002; Liu et al., 2005) (see Fig. 1A). Upstream of the noncoding exons are promoters governing differential mechanisms of activation and tissue-specific transcript expression in the CNS (Ohara et al., 1992; Metsis et al., 1993; Kokaia et al., 1994; Timmusk et al., 1995). In AD, BDNF transcripts I, II, and IV are specifically decreased (Garzon et al., 2002). However, the mechanism responsible for the observed decrease in these BDNF transcripts is unknown. The aim of this study was to determine whether fibrillar or oligomeric A β_{1-42} downregulates BDNF, and if so, whether it downregulates the transcripts that are decreased in cortical tissue of subjects with AD.

Materials and Methods

SH-SY5Y cells. SH-SY5Y cells were a generous gift from Dr. L. T. Young (Centre for Addiction and Mental Health, Toronto, Ontario, Canada). Cells were incubated in DMEM (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) (Invitrogen), 2 mM L-glutamine (L-Gln) (Invitrogen), and 1% penicillin/streptomycin (Pen/Strep) (Invitrogen). Cells were kept at 37°C and 5% CO₂ and split 1:5 every 5 d. For cell differentiation, 3.25 × 10⁵ cells per well were seeded in six-well plates (Sarstedt, Montreal, Quebec, Canada) in DMEM, 10% FBS, 2 mM L-Gln, and 1% Pen/Strep for 24 h, after which medium was replaced with DMEM, 1% N-2 supplements (Invitrogen), 2 mM L-Gln, 1% Pen/Strep, and 10 μ M all-*trans* RA (Sigma, St. Louis, MO). One-half of the medium was replaced every other day for 9 d. After 9 d of RA differentiation, 5 μ M A β_{1-42} was added in fresh medium and incubated for 48 h.

Amyloid preparation. Amyloid- β was purchased from American Peptide Company (Sunnyvale, CA) or rPeptide (Athens, GA). For initial experiments, A β_{1-42} was dissolved in 0.05 M Tris buffer, pH 10.5, and either A β_{1-42} or vehicle was added to fresh medium (DMEM, 1% N-2 Supplements, 2 mM L-Gln, 1% Pen/Strep, and 10 μ M all-*trans* RA) to a final concentration of 5 μ M.

Preparation of fibrillar or oligomeric amyloid was from Dahlgren et al. (2002). Briefly, both A β_{1-42} and scrambled A β_{1-42} (with the same amino acid composition as A β_{1-42} but in random sequence) (rPeptide) were

dissolved to 1 mM in 100% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP). HFIP was dried in the fume hood and resuspended to 5 mM in anhydrous DMSO. For fibril-forming conditions, amyloid or scrambled peptide was brought to a final concentration of 100 μ M with 10 mM HCl and incubated for 24 h at 37°C. For oligomer-forming conditions, amyloid or scrambled peptide was diluted to a final concentration of 100 μ M with Ham's F-12 (phenol red free; BioSource, Camarillo, CA) and incubated at 4°C for 24 h. After incubation, fibrillar or oligomeric amyloid or vehicle was added to fresh medium (DMEM, N-2 supplements, L-Gln, Pen/Strep, and all-*trans* RA) and incubated with cells at 37°C for 48 h. Amyloid administration was conducted in two independent experiments with three separate samples per group.

RNA isolation, DNase treatment, reverse transcription, and absolute quantitative PCR. After 48 h incubation in the presence of A β_{1-42} , scrambled peptide, or vehicle, cells were lysed

with 1 ml of Trizol (Invitrogen), and RNA was isolated with RNeasy Spin columns (Qiagen, Mississauga, Ontario, Canada). The procedure for RNA isolation with Trizol and RNeasy spin columns was followed as specified by Qiagen. RNA purity was confirmed by spectrophotometry ($A_{260}/A_{280} > 1.7$) and RNA integrity was visualized by agarose gel electrophoresis.

One microgram of RNA was treated with 2 U of Turbo DNase (Ambion, Austin, TX). For reverse transcription (RT), the protocol and reagents for Superscript II of Invitrogen were used. The final volume of 20 μ l contained 250 ng of random primers, 0.5 mM dNTPs (0.5 mM each of dATP, dTTP, dCTP, and dGTP), 1× first-strand buffer, 0.05 mM DTT, 2 U of RNaseOUT, and 200 U of Superscript II (Moloney murine leukemia virus RT). As a control, 1 μ g of RNA was treated according to the same protocol with addition of water instead of the RT enzyme ("no RT" control).

Real-time PCR was performed in the Stratagene (La Jolla, CA) MX3000P using the DNA binding dye SYBR green (Platinum SYBR Green qPCR SuperMix UDG; Invitrogen). The 20 μ l PCR mix contained 1× qPCR SuperMix, forward and reverse primers, 30 nM ROX reference dye (Stratagene), and cDNA from 50 ng of RNA or reference standard for absolute quantification. A "no template" control lacking RNA was included. For BDNF detection, 300 nM forward and reverse primers were used (forward primer, 5'-AAA CAT CCG AGG ACA AGG TG; reverse primer, 5'-AGA AGA GGA GGC TCC AAA GG; product, 249 bp), and 150 nM forward and reverse primers were used for β -actin (forward primer, 5'-CTC TTC CAG CCT TCC TTC; reverse primer, 5'-TGT TGG CGT ACA GGT CTT; product, 109 bp). Levels of BDNF and β -actin were determined using absolute quantification. Standards for BDNF were made in our laboratory from a pGEM plasmid containing the entire BDNF coding region of 755 bp (National Center for Biotechnology Information accession no. AY890649) and were run in triplicate with six 10-fold dilutions starting at 2.4 × 10⁶ copies of pGEM plasmid. β -Actin standards were from a plasmid obtained from Invitrogen and were run in duplicate with six 10-fold dilutions starting at 1.0 × 10⁷ copies of plasmid. Standards for individual BDNF transcripts were run in triplicate and obtained from purified PCR products using the primers listed in Table 1. Only experiments with $R^2 > 0.990$ and a PCR efficiency between 90 and 100% were used for analysis. All unknowns, no RT, and no template controls were run in triplicate. The following thermal profile was used for total BDNF and β -actin: 2 min at 50°C, 2 min at 95°C followed by 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s. For individual BDNF transcripts, the 40 cycles had the following thermal profile: 95°C for 30 s, 58°C for 30 s, and 72°C for 45 s. After PCR, a dissociation curve was added to verify that no secondary products had formed. Results were expressed as copies per nanogram of total RNA.

Western blotting. To confirm fibrillar and oligomeric speciation of

$A\beta_{1-42}$, Western blotting was performed following the protocols of Dahlgren et al. (2002) and Stine et al. (2003) with the following modifications: $A\beta_{1-42}$ samples were diluted in 4× loading buffer (0.25 M Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 20% β -mercaptoethanol, 0.004% bromophenol blue) and separated by SDS-PAGE in a 12% Tris/glycine gel.

For determination of cAMP response element-binding protein (CREB), phosphorylated CREB (P-CREB), caspases-3 and -7, and β -actin, RA-differentiated SY5Y cells subjected to different treatments were lysed in buffer containing 50 mM Tris, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, pH 8, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM PMSF, and 1 μ g/ml each of aprotinin, leupeptin, and pepstatin A. Samples were centrifuged for 5 min at 300 × g. Protein concentration of the supernatant was determined using a DC protein assay (Bio-Rad, Hercules, CA). Twenty micrograms of total protein in 4× loading buffer was separated by SDS-PAGE in a 12% Tris/glycine gel.

After transfer to polyvinylidene fluoride membranes and blocking for 1 h at room temperature in 1× Tris buffered saline (TBS)–0.1% Tween 20 (TBS/T) with 5% nonfat dry milk (MLK) [or PBS containing 3% nonfat dry milk (PBS-MLK) for CREB and P-CREB], blots were incubated overnight at 4°C with primary antibodies for $A\beta_{1-42}$ (6E10; Signet Laboratories, Dedham, MA; diluted 1:3000 in TBS/T), β -actin (Sigma; diluted 1:5000 in TBS/T), caspases-3 and -7 (Cell Signaling Technology, Danvers, MA; diluted 1:1000 in TBS/T-MLK), or CREB and P-CREB (Upstate, Lake Placid, NY; diluted 1:1000 in PBS-MLK). Secondary antibodies anti-mouse IgG HRP (GE Healthcare, Buckinghamshire, UK) diluted 1:5000 for $A\beta_{1-42}$, 1:5000 for β -actin, and 1:2000 for caspase-7, or anti-rabbit IgG HRP (Cell Signaling) diluted 1:2000 for caspase-3 and 1:5000 for CREB and P-CREB, were incubated for 1 h at room temperature. Blots were developed using enhanced chemiluminescence (GE Healthcare) and visualized using CL-X Posure film (Pierce Biotechnology, Rockford, IL). Pixel density was determined using Scion Image software (Scion, Frederick, MD).

Cell viability assay. Amyloid-induced cytotoxicity was determined by measuring release of cytosolic lactate dehydrogenase (LDH) into the medium (Cytotox 96 nonradioactive cytotoxicity assay; Promega, Madison, WI). The protocol was followed as specified by the manufacturer. Each sample was tested in triplicate, and the LDH release was measured at 492 nm using a Titertek Multiscan Plus (MP Biomedicals, Irvine, CA) plate reader.

Quantitative and statistical analysis. Samples for BDNF mRNA exposed to different species of amyloid- β and LDH cytotoxicity were assayed in triplicate in each of two independent experiments. Data from Western blots for total CREB (T-CREB), P-CREB, and caspases-3 and -7 were obtained from three independent experiments. The data were analyzed by one-way ANOVA with *post hoc* Tukey's test for pairwise group comparison or Student's *t* test where indicated (SPSS, version 13, software; SPSS, Chicago, IL).

Results

Retinoic acid-differentiated SH-SY5Y cells express all BDNF transcripts

RA-differentiated SY5Y cells after 9 d *in vitro* express all seven BDNF transcripts (Fig. 1B). The BDNF transcript expression pattern in SH-SY5Y cells is very similar to that of human cortical tissue. A comparison of levels of each BDNF transcript demonstrates that BDNF transcript IV is the most highly expressed transcript in both the human cortex and SY5Y cells, representing over one-half of the total BDNF (Table 2). Both the human cortex and SY5Y cells predominantly express BDNF transcripts I, IV, and V, whereas transcripts II, VI, and VII are minor contributors to the total, and transcript III is not detectable (Table 2).

Retinoic acid induces BDNF mRNA expression

BDNF mRNA levels increased significantly over time when cells were grown in serum free medium with N-2 supplements and 10

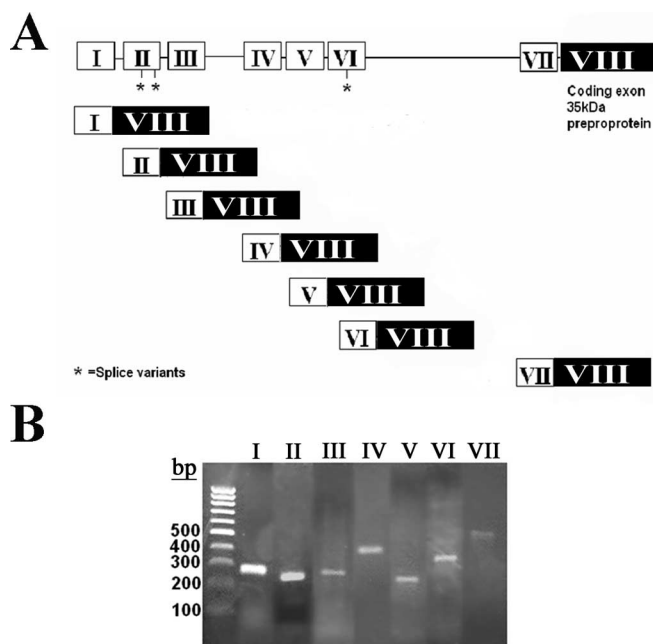


Figure 1. Human BDNF gene with mRNA transcripts. **A**, Diagram showing the entire BDNF gene with the noncoding exons in white spliced to the coding exon in black. The asterisk (*) indicates splice variants, of which three are known, two on exon II and one on exon VI for a total of 10 transcripts. **B**, PCR products for all seven BDNF transcripts from retinoic acid-differentiated SH-SY5Y cells at 9 d *in vitro*. Lanes numbered I–VII correspond to the transcripts in **A**. PCR primer sequences and size of fragments are found in Table 1. The splice variants, although present, are not significant products in lanes 2 and 6. A 100 bp ladder is shown on the left.

Table 2. BDNF transcript abundance in human parietal cortex and RA-differentiated human neuroblastoma SH-SY5Y cells

Human parietal cortex		Human neuroblastoma SH-SY5Y	
BDNF transcript order	Copies ^a	BDNF transcript order	Copies ^b
Transcript IV	625	Transcript IV	643
Transcript I	332	Transcript V	215
Transcript V	130	Transcript I	121
Transcript VI	70	Transcript II	95
Transcript VII	16	Transcript VI	N/D
Transcript II	15	Transcript VII	N/D
Transcript III	N/D	Transcript III	N/D

^aDetermination of BDNF transcript copy number for human parietal cortex was done using relative quantitative RT-PCR with incorporation of ³³P (Garzon et al. 2002). The ³³P isotope was detected by pixelation intensity on a phosphorimage cassette, and pixel values were averaged for all control subjects for each BDNF transcript. One pixel was assigned a value of 65,000 and was used as the denominator in the following ratio: total averaged pixel value/65,000. The value of the ratio appears in the 'Copies' column (under 'Human parietal cortex'). BDNF transcript III was not detectable (N/D) in human parietal cortex.

^bDetermination of BDNF transcript copies after 9 d of retinoic acid differentiation of SH-SY5Y cells, using absolute quantitative real-time PCR. Three samples per group were assayed in triplicate in each of two independent experiments. The value of 'Copies' (under 'Human neuroblastoma SH-SY5Y') is copies of BDNF per 50 ng of total input RNA. BDNF transcripts III, VI, and VII were below quantifiable levels (N/D, <40 copies/50 ng total RNA).

μ M RA (Fig. 2). BDNF mRNA was significantly increased at 3 d (one-way ANOVA and *post hoc* Tukey's test, $p < 0.01$), 5 d ($p < 0.001$), and 9 d ($p < 0.001$) of growth in N-2 and RA compared with serum free medium with N-2 supplements alone or with medium with 10% FBS (Fig. 2).

$A\beta_{1-42}$ downregulates BDNF mRNA in human neuroblastoma SH-SY5Y cells

The effect of 5 μ M $A\beta_{1-42}$ administration on BDNF mRNA in SY5Y cells was examined after incubation for 6, 12, 24, and 48 h. Five micromolar $A\beta_{1-42}$ is a concentration previously reported to interfere with cellular signaling without induction of apoptosis

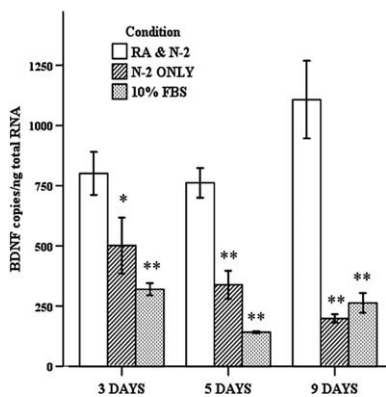


Figure 2. Measurement of total BDNF mRNA after 3, 5, and 9 d of growth in different media. BDNF is significantly increased in N-2 supplements and 10 μ M RA compared with N-2 supplements alone or 10% FBS at 3, 5, and 9 d (* $p < 0.01$; ** $p < 0.001$). In addition, comparing BDNF mRNA levels for N-2 and RA across the time points, there is a significant increase in BDNF mRNA between days 3 and 9 (* $p = 0.036$) and between days 5 and 9 ($p = 0.022$). Error bars represent SEM; $n = 3$.

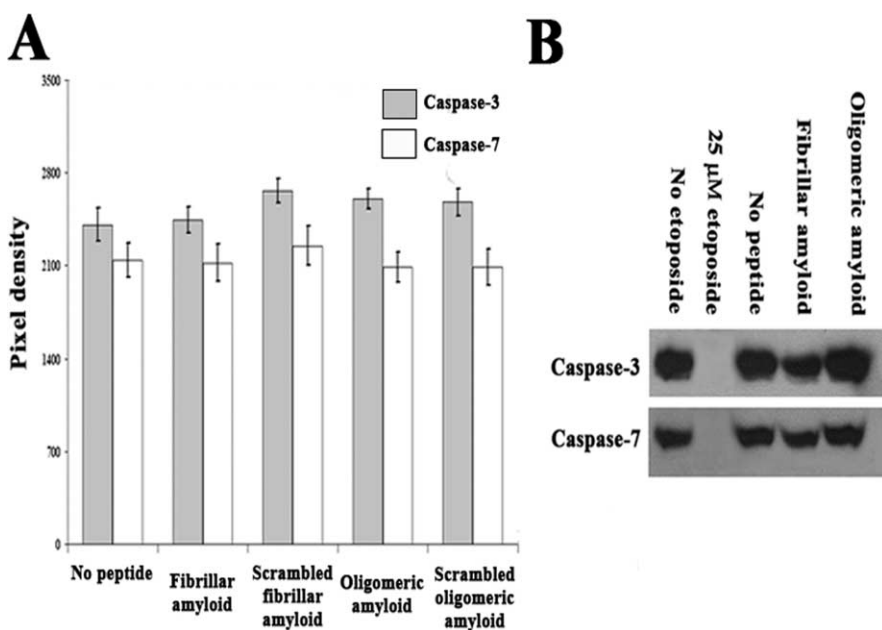


Figure 3. Western blotting of inactivated caspase-3 and -7. **A**, No difference in levels of inactivated caspase-3 and -7 among differently treated RA-differentiated SY5Y cells (one-way ANOVA, $p = 0.354$ for caspase-3, and $p = 0.921$ for caspase-7). Analysis is based on three independent experiments; $n = 9$. Error bars represent SEM. **B**, Western blot shows only inactivated caspase-3 and -7 were detected after treatment with $A\beta_{1-42}$. As a positive control, SY5Y cells were treated with 25 μ M etoposide, an apoptosis-inducing reagent. After 24 h, no inactivated caspase-3 or -7 was detected.

Table 3. Percentage cytotoxicity after 48 h 5 μ M $A\beta_{1-42}$ administration, determined by LDH assay

Condition	Percentage cytotoxicity ^a
Control (no peptide)	17.82
Fibrillar $A\beta_{1-42}$	11.77
Scrambled fibrillar $A\beta_{1-42}$	17.92
Oligomeric $A\beta_{1-42}$	17.36
Scrambled oligomeric $A\beta_{1-42}$	17.17

^aOne-way ANOVA, $p = 0.84$.

(Tong et al., 2001, 2004). Although total BDNF mRNA (exon VIII) was decreased at all time points, the greatest decrease occurred at 48 h (data not shown). The 48 h time point was subsequently used for all additional experiments. Administration of 5 μ M $A\beta_{1-42}$ for 48 h to SY5Y cells resulted in a 33% decrease in total BDNF mRNA compared with vehicle-treated cells (Student's t test, $p = 0.002$). In contrast, amyloid- β did not downregulate BDNF in undifferentiated SY5Y cells (data not shown).

$A\beta_{1-42}$ (5 μ M) in fibrillar or oligomeric conformations is not cytotoxic to SH-SY5Y cells

To confirm that $A\beta_{1-42}$ is not cytotoxic to SY5Y cells under these conditions, a colorimetric assay measuring the release of cytoplasmic LDH and Western blots measuring levels of caspases-3 and -7 were used. There was no change in levels of inactivated caspases-3 and -7 or in LDH release from cultures exposed to fibrillar or oligomeric $A\beta_{1-42}$ for 48 h in comparison with the scrambled peptide or no peptide controls (one-way ANOVA, $p > 0.05$) (Fig. 3, Table 3).

Oligomeric but not fibrillar $A\beta_{1-42}$ significantly decreases BDNF mRNA

The 5 μ M $A\beta_{1-42}$ applied for 48 h was an undefined, heterogeneous mixture of fibrillar and oligomeric amyloid. To determine which amyloid species was responsible for BDNF downregulation, homogeneous fibrillar and oligomeric amyloid samples were prepared. Western blotting with an antibody to $A\beta$ confirmed the efficacy of the $A\beta_{1-42}$ oligomeric and fibrillar speciation procedure (Fig. 4). The oligomeric preparation (lane 1) contained monomers (4 kDa), small oligomeric trimers (~12 kDa), and the larger oligomeric amyloid (40–98 kDa), but no fibrils. The fibrillar amyloid (lane 2) contained both monomeric amyloid and fibrillar amyloid (>250 kDa), but no detectable oligomers. The procedures for amyloid speciation were also performed on the scrambled peptide, containing the same amino acid composition as $A\beta_{1-42}$, which served as a negative control and did not cross-react with the amyloid antibody (lanes 3 and 4). After confirmation of the amyloid species, oligomeric or fibrillar $A\beta_{1-42}$ was added to the SY5Y cells. Scrambled $A\beta_{1-42}$ treated identically to oligomeric and fibrillar preparations and vehicle without added peptide served as negative controls. Total BDNF mRNA was decreased 62% (ANOVA and *post hoc* Tukey's test, $p < 0.001$) in cultures exposed to oligomeric preparations of $A\beta_{1-42}$ compared with scrambled oligomeric peptide and control without peptide (Fig. 5). SY5Y cells treated with fibrillar and oligomeric scrambled peptide controls showed no change in BDNF levels compared with controls without added peptide ($p > 0.05$) (Fig. 5). Furthermore, cells treated with fibrillar $A\beta_{1-42}$ showed no change in BDNF levels compared with scrambled controls or controls without added peptide ($p > 0.05$) (Fig. 5).

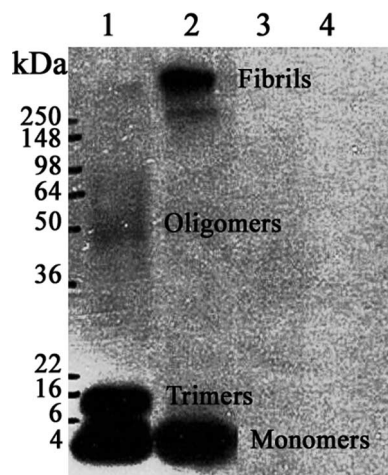


Figure 4. Western blot of fibrillar and oligomeric $A\beta_{1-42}$ speciation. The oligomeric preparation (lane 1) contains monomers (4 kDa), small oligomeric trimers (~12 kDa), and the larger oligomeric amyloid (40–98 kDa), but no fibrils. The fibrillar amyloid (lane 2) contains both monomeric amyloid and fibrillar amyloid (>250 kDa), but no oligomers. The procedures for amyloid speciation were also performed on the scrambled peptide, containing the same amino acid composition as $A\beta_{1-42}$, which served as a negative control and did not cross-react with the amyloid antibody (lanes 3 and 4).

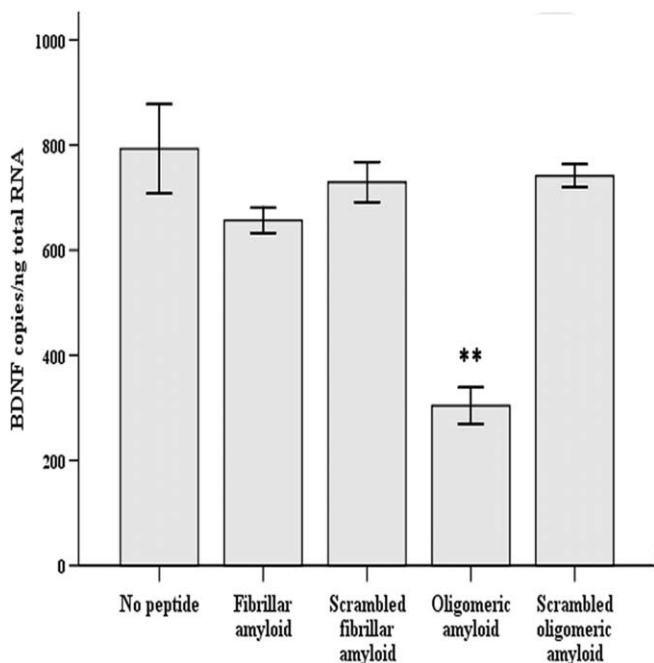


Figure 5. BDNF mRNA levels in RA-differentiated SY5Y cells exposed to oligomeric or fibrillar $A\beta$. Five micromolar $A\beta_{1-42}$, treated to form either oligomeric or fibrillar species, was added to RA-differentiated SH-SY5Y cells for 48 h. Only oligomeric amyloid caused a significant decrease (** $p < 0.001$) in BDNF mRNA (exon VIII) compared with no peptide and scrambled oligomeric amyloid controls. There was no change in BDNF exposed to fibrillar amyloid compared with either no peptide or scrambled fibrillar controls. The analysis is based on two independent experiments; $n = 6$. Error bars represent SEM.

Oligomeric $A\beta_{1-42}$ decreases BDNF transcripts 4 and 5

Examination of the levels of each of the seven BDNF transcripts demonstrated oligomeric $A\beta_{1-42}$ decreased BDNF transcripts IV and V by 45% (ANOVA and *post hoc* Tukey's test, $p = 0.001$) and 54% ($p = 0.015$), respectively (Fig. 6C,D), increased BDNF transcript I by 70% ($p = 0.034$) (Fig. 6A), and did not change BDNF transcript II levels (Fig. 6B). BDNF transcripts III, VI, and VII

were below the level of the standard curve both before and after $A\beta_{1-42}$ administration (<40 copies/50 ng total RNA).

Oligomeric but not fibrillar $A\beta_{1-42}$ significantly decreases levels of phosphorylated CREB

Levels of phosphorylated CREB were decreased by 38% in samples treated with oligomeric amyloid compared with all other groups (one-way ANOVA and *post hoc* Tukey's test, $p = 0.005$) (Fig. 7). The levels of total CREB protein did not differ among amyloid and control groups (one-way ANOVA, $p > 0.05$) (Fig. 7).

Discussion

In this study, we showed that $A\beta_{1-42}$ significantly decreases BDNF mRNA and P-CREB, a transcriptional regulator of BDNF. We demonstrated that oligomeric, not fibrillar, species of $A\beta_{1-42}$ are responsible for the observed effects. Furthermore, oligomeric $A\beta_{1-42}$ significantly decreases BDNF transcripts IV and V. Transcript IV is the most abundantly expressed BDNF transcript in human cortical tissue and in SY5Y cells and is decreased in AD (Garzon et al., 2002). Therefore, our results suggest that $A\beta_{1-42}$ is responsible for the majority of BDNF downregulation in AD.

RA-differentiated SY5Y cells demonstrate human neuronal characteristics, making their use as a model system ideal. They exhibit neuronal morphology and express neuron-specific markers including neurofilaments, neuron-specific enolase, and growth-associated protein-43 as well as the neuronal polarity markers tau and microtubule-associated protein 2 (Pahlman et al., 1984, 1990; Encinas et al., 2000). We show that, unlike previously reported human and murine cell culture systems, RA-differentiated SY5Y cells produce endogenous BDNF, increasing BDNF expression over the course of RA administration. A previous study in rat demonstrated that membrane depolarization-induced BDNF upregulation is disrupted by $A\beta_{1-42}$ administration (Tong et al., 2001). Our findings are the first to report that $A\beta$ administration significantly decreases basal levels of BDNF mRNA and to identify oligomeric amyloid as responsible for the observed decrease.

In addition, we report here that the expression of BDNF transcripts in RA-differentiated SY5Y cells correlates closely with the expression pattern of BDNF transcripts in postmitotic human CNS tissue. Furthermore, RA-differentiated SY5Y cells closely resemble human cortical neurons in their response to $A\beta$ (Deshpande et al., 2006). Primary murine neuronal cultures were not used in this study, because the number and the expression patterns of BDNF transcripts in the brain differ between human and mouse (Garzon et al., 2002; Liu et al., 2005, 2006).

The amyloid cascade hypothesis proposes that $A\beta$ production and toxic action on neurons and tissues cause AD. SY5Y cells have been used extensively to study mechanisms of $A\beta$ toxicity. $A\beta$ binds to the neurites of differentiated SY5Y cells, promoting neurite degeneration and induction of tau hyperphosphorylation (Datki et al., 2004). Similar data are reported for rat and human cultured neurons (Gong et al., 2003; Lacor et al., 2004; Deshpande et al., 2006).

Acceptance of the original amyloid cascade hypothesis is not pervasive, because data support no correlation between plaque deposition and neurodegeneration (Cummings and Cotman, 1995; Irizarry et al., 1997; Chui et al., 1999; Naslund et al., 2000). However, oligomeric $A\beta_{1-42}$ is a potent neurotoxin at nanomolar concentrations and inhibits LTP and synaptic plasticity (Lambert et al., 1998; Walsh et al., 2002). Oligomeric $A\beta_{1-42}$ levels are increased in areas of the brain compromised in AD and are correlated with areas of neurodegeneration; oligomeric $A\beta_{1-42}$ is

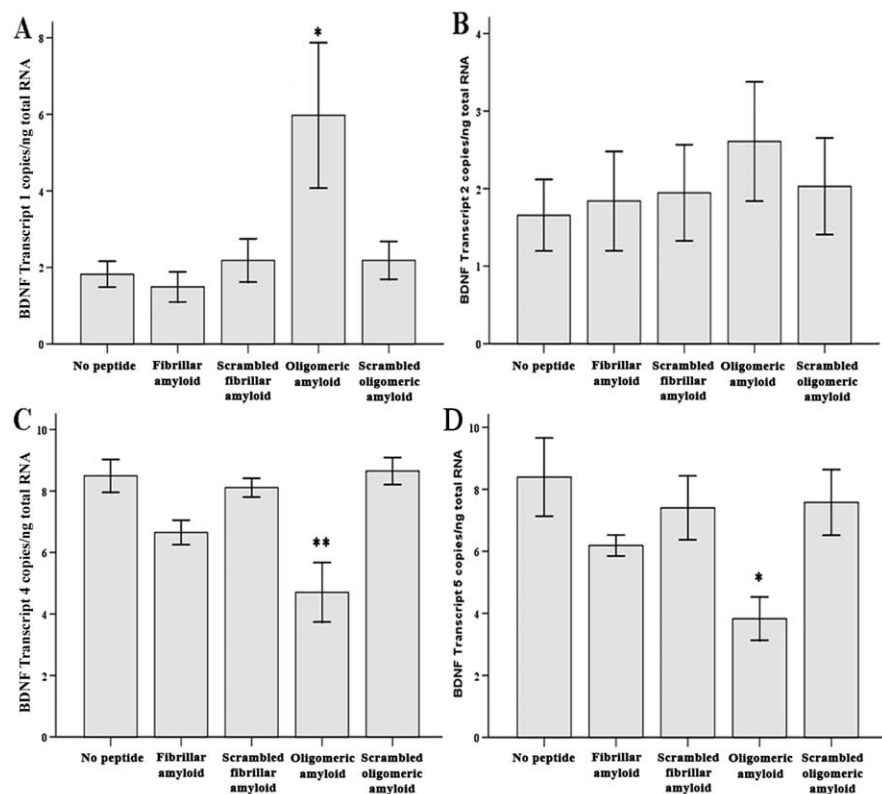


Figure 6. Measurement of BDNF transcripts I, II, IV, and V in RA-differentiated SY5Y cells exposed to oligomeric or fibrillar A β . **A**, A significant increase ($*p = 0.034$) in BDNF transcript I after oligomeric amyloid administration compared with both control conditions. **B**, No change in BDNF transcript II levels among the five experimental conditions. **C, D**, Significant decreases in BDNF transcripts IV and V ($**p = 0.001$ and $*p = 0.015$, respectively) after oligomeric amyloid administration compared with both controls. There was no change in any BDNF transcript level after fibrillar amyloid administration compared with either control. Transcripts III, VI, and VII were below the levels of detection. The analysis is based on two independent experiments; $n = 6$. Error bars represent SEM.

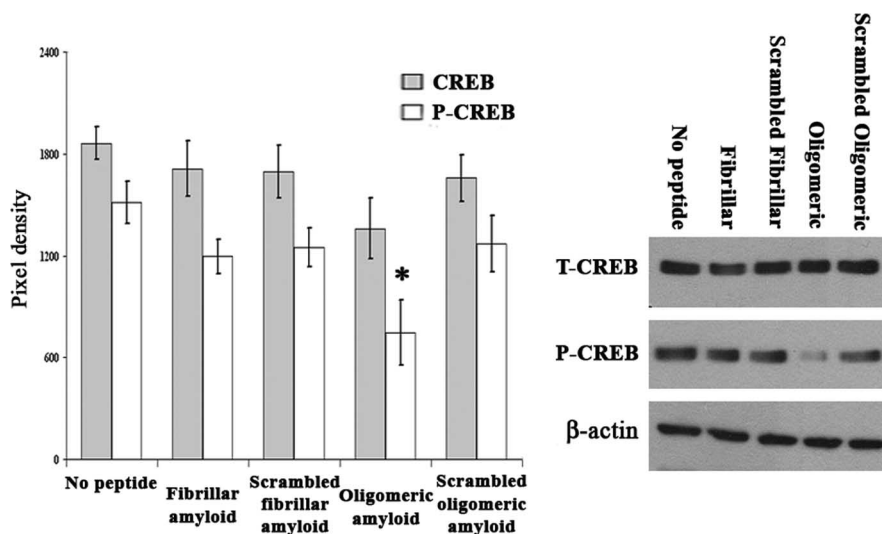


Figure 7. Western blots of T-CREB and P-CREB levels in RA-differentiated SY5Y cells treated with oligomeric or fibrillar A β . RA-differentiated SY5Y cells administered oligomeric amyloid demonstrate significantly decreased phosphorylated CREB levels ($*p = 0.005$). Levels of total CREB were unchanged among all five conditions (one-way ANOVA, $p = 0.237$). The analysis is based on three independent experiments; $n = 9$. Error bars represent SEM.

more neurotoxic than fibrillar amyloid and is found intracellularly, in the absence of amyloid plaques and neurofibrillary tangles, suggesting the presence of oligomeric A β_{1-42} early in the progression of AD (Funato et al., 1999; Lue et al., 1999; McLean et

al., 1999; Walsh et al., 2000; Dahlgren et al., 2002; Gong et al., 2003). Our findings of BDNF downregulation by oligomeric A β_{1-42} support the growing literature suggesting oligomeric A β_{1-42} is the first neurotoxic insult in AD. Together with our recent findings of decreased BDNF in prodromal AD subjects (Peng et al., 2005), we propose that oligomeric amyloid-induced downregulation of BDNF in the brain is one of the primary and earliest mechanisms of neurodegeneration in AD.

It was previously unknown which species of A β_{1-42} downregulates BDNF. Here, we demonstrate that oligomeric but not fibrillar A β_{1-42} significantly decreases BDNF mRNA levels in differentiated SY5Y cells. Furthermore, undifferentiated, amyloid-treated SY5Y cells do not downregulate BDNF, consistent with previous reports that undifferentiated SY5Y cells are not responsive to A β (Lambert et al., 1994).

To confirm that the effects of amyloid administration on BDNF expression occur before any cytotoxic effects, we demonstrated the absence of LDH release and of caspase activation, a proapoptotic marker, in amyloid-treated RA-differentiated SY5Y cells during the time course of BDNF mRNA measurement. Our results are in agreement with recent data demonstrating that A β -derived diffusible ligands (of similar molecular weight to our oligomeric amyloid) induce subtle mitochondrial changes, but not overt cell death, in human cortical neurons over a period of 1 week (Deshpande et al., 2006). Therefore, oligomeric amyloid-induced BDNF downregulation is not a result of cell death.

In our previous study, we reported BDNF transcripts I, II, and III were significantly decreased in the parietal cortex of AD compared with control subjects (Garzon et al., 2002). Recently, there has been a revision to both the human and the rodent BDNF genes with the discovery of an additional exon between exons II and III (Liu et al., 2005, 2006). The newly discovered exon is called exon III and the previous exons III and IV are renamed exons IV and V, respectively. Based on this revision, our previous data show a significant decrease in human BDNF transcripts I, II, and IV in the parietal cortex of AD compared with control subjects. In this study, consistent with Alzheimer's subjects, RA-differentiated SY5Y cells exposed to oligomeric A β_{1-42} exhibit significantly decreased transcript IV (Garzon et al., 2002). BDNF transcript IV is the most abundantly expressed transcript in SY5Y cells and in human cortical tissue, exhibiting a 2- to 16-fold greater level of expression than the other transcripts (Table 2) and accounting

for more than one-half of the total BDNF mRNA. Therefore, downregulation of transcript IV is likely the primary driver behind downregulation of total BDNF mRNA by $A\beta_{1-42}$ in both SY5Y cells and in human cortical tissue.

In differentiated SY5Y cells, BDNF transcript V is decreased 54% by oligomeric $A\beta_{1-42}$. In human cortical tissue, BDNF transcript V showed a similar trend in AD subjects compared with controls, but the finding was not statistically significant ($p = 0.062$) (Garzon et al., 2002). The discrepancy between these findings is likely a result of insufficient sample numbers in our human cortical tissue analysis, diminished accuracy based on our previous use of relative RT-PCR versus real-time RT-PCR in this study, and intersubject variability in the human cortical tissue samples.

Interestingly, in differentiated SY5Ys, oligomeric amyloid increases BDNF transcript I and does not change BDNF transcript II, whereas in AD, transcripts I and II are both decreased. The discrepancy between amyloid-treated SY5Y cells and human AD cortical samples suggests that factor(s) other than $A\beta$ are responsible for BDNF transcript I and II downregulation in AD. BDNF transcripts III, VI, and VII are minor contributors to total BDNF mRNA levels; in SY5Y cells, they are expressed at levels below our standard curve (<40 copies/50 ng total RNA) and at least 16-fold lower than transcript IV.

The effect of $A\beta$ on LTP and memory is postulated to be mediated by inhibition of CREB phosphorylation. Activation of CREB by phosphorylation is essential for learning and memory (Silva et al., 1998; Tully, 1998); P-CREB levels are decreased in AD hippocampus (Yamamoto-Sasaki et al., 1999) and in $A\beta$ -treated rat primary cortical neurons (Tong et al., 2001). $A\beta$ reduces adenylate cyclase and protein kinase A (PKA) activity and hence P-CREB levels in hippocampal neurons (Vitolo et al., 2002). Consistent with the role of P-CREB as a transcription factor for activity-dependent regulation of BDNF transcript IV (Shieh et al., 1998; Tao et al., 1998; Tong et al., 2001, 2004) and with our results demonstrating oligomeric amyloid-specific downregulation of BDNF transcript IV, we find only oligomeric, not fibrillar amyloid decreases the levels of P-CREB in SY5Y cells. Our results suggest that decreased CREB phosphorylation induced by oligomeric $A\beta$ is responsible for downregulation of BDNF transcript IV in SY5Y cells and in AD.

BDNF is a well known mediator of LTP and memory. Transgenic mice with reduced BDNF levels exhibit defects in synaptic transmission, LTP, and memory tests that are rescued by exogenous BDNF administration (Korte et al., 1995; Patterson et al., 1996; Olofsson et al., 2000), and reduced BDNF protein levels are correlated with cognitive impairment in MCI and AD subjects (Peng et al., 2005). Therefore, we propose a mechanism for the effects of $A\beta$ on cognitive and memory function in AD, which is that oligomeric $A\beta$ inhibits adenylate cyclase activity and reduces PKA activation, reducing CREB phosphorylation and subsequent BDNF transcript IV expression. Because transcript IV represents over one-half of the total BDNF mRNA, this mechanism may explain reduced BDNF in both RA-differentiated SY5Y cells and in human neurons exposed to $A\beta$, and identifies BDNF downregulation as an underlying event in AD. Furthermore, because oligomeric amyloid appears before plaque deposition and because BDNF reduction occurs early in the course of AD and is correlated with cognitive dysfunction, oligomeric amyloid may produce synaptic dysfunction and learning and memory deficits by downregulation of BDNF before the clinical onset of Alzheimer's disease.

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