FUNCTIONAL ACTIVITY OF THE NOVEL ALZHEIMER'S AMYLOID β–PEPTIDE INTERACTING DOMAIN (AβID) IN THE *APP* AND *BACE1* PROMOTER SEQUENCES AND IMPLICATIONS IN ACTIVATING APOPTOTIC GENES AND IN AMYLOIDOGENESIS

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Abstract

Amyloid β peptide (Aβ) plaque in the brain is the primary (*post mortem*) diagnostic criterion of Alzheimer's disease (AD). Any physiological role of Aβ constituent is poorly understood. We have previously determined an Aβ interacting domain (AβID) in the promoters of AD–associated genes (Maloney and Lahiri, 2011). This AβID interacts in a DNA–sequence specific manner with Aβ. We now demonstrate novel Aβ activity as a possible transcription factor. Herein, we demonstrate Aβ–chromatin interaction in cell culture by ChIP assay. We observed that human neuroblastoma (SK–N–SH) cells treated with FITC conjugated Aβ1–40 localized Aβ to the nucleus in the presence of H_2O_2–mediated oxidative stress. Furthermore, primary rat fetal cerebrocortical cultures were transfected with *APP* and *BACE1* promoter–luciferase fusions, and rat PC12 cultures were transfected with polymorphic *APP* promoter–CAT fusion clones. Transfected cells were treated with different Aβ peptides and/or H_2O_2. Aβ treatment of cell cultures produced a DNA sequence–specific response in cells transfected with polymorphic *APP* clones. Our results suggest the Aβ peptide may regulate its own production through feedback on its precursor protein and BACE1, leading to amyloidogenesis in AD.

Keywords

Alzheimer's disease; amyloid beta; DNA–protein interaction; gene regulation; transcription factor

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1. Introduction

Alzheimer's disease (AD), the most common form of dementia in the elderly (Hebert et al., 2003), is associated with multiple risks that include genetic, epigenetic, dietary, and lifestyle factors (Lahiri and Maloney, 2010b). Neuronal loss, intraneuronal tangles of hyperphosphorylated microtubule–associated τ protein (MAPT), and extracellular deposition of β–amyloid peptide plaque are characteristic of the disorder. Amyloid plaque consists primarily of the 39–42 amino acid amyloid β peptide (Aβ) (Lahiri et al., 2003; De Strooper, 2010), which is cleaved from a larger Aβ precursor protein (APP) by sequenttial actions of the β– and γ–secretase enzymes (Lahiri et al., 2003). Aβ also accumulates within neurons in both human AD cases and transgenic AD models (Gouras et al., 2000; Shie et al., 2003). Non–pathological functions of Aβ include ion channel formation (Jang et al., 2010), kinase activation (Bogoyevitch et al., 2004; Tabaton et al., 2010), cholesterol transport regulation (Yao and Papadopoulos, 2002; Igbavboa et al., 2009), and protection against metal–induced oxidative damage (Zou et al., 2002; Baruch-Suchodolsky and Fischer, 2009). We now report that Aβ may act as a transcription factor with regulatory region(s) of AD–associated genes.

Extracellular Aβ enters the cell under oxidative and heat stress (Ohyagi et al., 2005; Ohyagi and Tabira, 2006; Ohyagi et al., 2007) and is transported to the nucleus by action of the Aβ–related death–inducing protein (AβDIP) (Ohyagi, 2008). Intracellular Aβ induces an increase in levels of the apoptosis–associated tumor protein 53 (p53, gene name *TP53)* (Ohyagi et al., 2005), the transcription factor achaete–scute complex homolog 1 (*ASCL1*) (Uchida et al., 2007), and transcription of the β–amyloid cleaving enzyme 1/β–secretase *BACE1* gene (Giliberto et al., 2009; Tabaton et al., 2010), while reducing levels of oligodendrocyte lineage transcription factor 2 (*OLIG2*) (Uchida et al., 2007) and system A glutamine transporter 1 (*SAT1*/*SLC38A1*) (Buntup et al., 2008). In the case of p53, this induction has been shown to be from direct action of Aβ upon the *TP53* promoter. In that particular case, Aβ activity hinged on binding a known heat shock element (HSE), "GGATTGGGGT" (Ohyagi et al., 2005).

We have recently identified a more general Aβ–interacting domain (AβID) in DNA sequences (Maloney and Lahiri, 2011). The AβID is a decamer with a consensus sequence "KGGRKTGGGG". Substitution of G→A in the seventh nucleotide of the decamer eliminated DNA–peptide interaction. This substitution corresponds to a single–nucleotide polymorphism (SNP) associated with increased AD risk (Lahiri et al., 2005b).

We now turn our attention to investigating functional activity related to Aβ–AβID interaction. We first determined that Aβ associated with DNA *in situ* in an AβID–related fashion. We did this through chromatin immune precipitation (ChIP) assay. Our ChIP for two chromatin regions that each contained an AβID on the APP gene (*APP*) and *BACE1* promoter sequences (Maloney and Lahiri, 2011) demonstrated *in situ* Aβ binding to both of the 200 bp regions. PCR signal did not occur when chromatin was precipitated with antibodies against the N–terminal region of APP or against β–galactosidase (β–gal).

To determine induction of Aβ into cell nuclei under oxidative stress, cell cultures were treated with fluorescein isothiocyanate conjugated Aβ1–40 (FITC–Aβ1–40), added to cell cultures in the presence or absence of a oxidative stress induced by H_2O_2. To determine functional activity of Aβ on 5′–flanking regions of the *APP* and *BACE1* genes, we generated clones containing i) a confirmed AβID within a 3.3 kb *BACE1* fragment and ii) lacking any predicted AβID within a 1.2 kb *APP* fragment. These fragments were each fused to the firefly luciferase coding sequence, and the fusion clones were transfected into primary rat fetal cerebrocortical neuronal (PRCN) cultures. Transfected cultures were

treated with Aβ1–42, 1–40, and 1–28 peptides. We found that oxidative stress induces Aβ localization to the nucleus, and that Aβ increased activity in the *BACE1* promoter.

In addition, we tested the response to Aβ of two clones previously constructed in our laboratory. Specifically, these two clones correspond to an *APP* promoter SNP linked to AD risk at −3829 from the +1 TSS (Lahiri et al., 2005b). This polymorphism is within an AβID. We show that oxidative stress and Aβ treatment significantly increase transcriptional activity of the AD–associated SNP. Taken together, these results suggest that the Aβ peptide may possibly function as a transcription factor or co–factor. In addition to other function(s), the Aβ peptide directs normal apoptosis as well as regulating its own production through feedback on its precursor, APP, and the β–secretase enzyme. This would have pathological consequences relevant to AD. We suggest that under a cytotoxic model, normal cytoprotective activity of Aβ, such as protection against metal–induced oxidative stress, would result in increasing Aβ levels crossing a pathogenic threshold, pushing production of APP and BACE1 to pathological levels, initiating a positive feedback loop. Higher levels of BACE1 protein would favor greater production of Aβ, and higher levels of APP protein would provide more substrate. The combination would result in greater Aβ production, which would stimulate the production of proapoptotic proteins. Thus, Aβ's proposed activity as either TF or cofactor would lead to accumulation of excess Aβ as toxic extracellular amyloid plaque, the hallmark of AD.

2. Materials and Methods

2.1 Chemicals/Reagents

Chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO) and were of "molecular biology" or "analytic" quality unless stated otherwise. DNA modifying and restriction enzymes were purchased from Roche (Indianapolis, IN). Different media and sera and other tissue culture reagents were procured from Invitrogen (Carlsbad, CA).

2.2 Cell culture for transfection and Aβ treatment

Human SK–N–SH neuroblastoma (NB) and rat pheochromocytoma (PC12) cell cultures were acquired from ATCC and cultured in our laboratory as previously described (Ghosh et al., 2000). Briefly, NB cells were seeded into 10 cm culture plates (for ChIP) or 24–well culture plates (for live cell imaging) and maintained in DMEM medium supplemented with 10% fetal bovine serum. PC12 cells were seeded into a collagenated 96–well plate for transfections at 30,000 cells per well and allowed to attach overnight in standard growth medium: RPMI supplemented with 10% horse serum (Invitrogen), 5% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), and an antibiotic cocktail (Invitrogen).

For primary neuronal cultures, timed–pregnancy Sprague Dawley rats were purchased from Harlan Laboratories (Indianapolis). Animals were treated in accordance to the Indiana University School of Medicine Institutional Animal Use Committee guidelines. PRCN cultures were prepared from E16 rat fetal cerebrocortical tissue using slight modifications from previously published procedures (Brewer et al., 1993; Bailey and Lahiri, 2006). The dam was sacrificed in a CO_2 chamber and decapitated. The uterus was removed through an incision in the abdomen and the pups transferred to ice–cold Hanks Buffered Salt Solution (Invitrogen) supplemented with 1 mM sodium pyruvate (Sigma–Aldrich) and antibiotic cocktail (HBSS; Invitrogen). After rinsing, heads were moved to fresh HBSS for dissection. Under a dissecting microscope, overlying tissues and vasculature were removed from the brain surface with fine forceps and whole cortices were separated from subcortical tissue and collected in a separate tube of HBSS on ice. After all cortices were collected, the overlying HBSS was removed and the tissue was homogenized by trituration in 2–3 ml Neurobasal medium supplemented with B27 serum replacement and 5 ng/ml human

recombinant basic fibroblast growth factor (Invitrogen). Cells were counted and seeded into poly–D–lysine (Sigma–Aldrich) coated 6– or 12– well plates (Corning, Lowell, MA) at 2,200 cells/mm^2 (2×10^6 cells per well in 6 well plates).

2.3 Aβ peptides and their fragments

Different forward and reverse peptides, Aβ1–42, 1–40, 1–28, 25–35, 29–40, 31–35, 42–1, 40–1, 35–25, FITC–Aβ1–40 were purchased as trifluoroacetic acid salts from Bachem (Torrance, CA) and resuspended at stock concentrations of 1 mg/ml in different solvents per manufacturer's recommendations. Consultation with the technical staff of the manufacturer indicated that peptides dissolved under these conditions would be dimers or larger aggregates.

2.4 Chromatin immune precipitation (ChIP) of Aβ on specific sequences of the APP and BACE1 promoters

Human NB cells were grown to 70%–80% confluency. One set of cultures was treated with 100 μM H_2O_2, and all cultures were fixed with 37% formaldehyde, as specified in the ChIP–IT Express kit protocol (ActiveMotif). Chromatin was sheared enzymatically with the Enzymatic ChIP–IT Express kit. Sheared chromatin was then immunoprecipitated overnight with each of the following antibodies: mAb 4G8 (anti–Aβ), mAb 22C11 (anti N–terminus APP), anti–specificity protein 1 (SP1) transcription factor (Santa Cruz Biotechnology, Santa Cruz, CA), or anti–β galactosidase. After immunoprecipitation, PCR was conducted with oligomers flanking the *APP* –3833 (5′–AGG CCA AAG ATG AGT GAC AGG A–3′, 5′– GGG TTA GGA TCC ATG CTA ATG ACC–3′) or *BACE1* –119 (5′–AAG AAA GAC TGA CAG ACG GGA GGT–3′, 5′–GGC GGC TGT CAA AGC CAA A–3) AβIDs. For the *APP* PCR, the 22C11–precipitated material was used as negative control. For the *BACE1* PCR, the anti–β gal precipitated material was used as negative control.

2.5 Aβ treatment of SK–N–SH cells in presence or absence of H_2O_2

SK–N–SH cells were neuronally differentiated with 10 μM all–trans retinoic acid for 7 days prior to subsequent treatments. Cels were then treated with 1 μM FITC–Aβ1–40 (Bachem) in the presence or absence of 50 μM H_2O_2 in low serum containing medium (MEM with 1% fetal bovine serum and antibiotics) for 48 hours. Cells were washed in Dulbecco's PBS and labeled with 10 μM Hoechst 33342 (Sigma) and 1 μM ethidium homodimer (EtHD; Molecular Probes) for 15 minutes at 37°C. Images were taken using a Leica DM IL microscope with an RT–SE SPOT camera, using SPOT Advanced software (Diagnostic Instruments). Experiments were performed in triplicate. Nuclei of live cells, nuclei of dead cells, and nuclei of live cells with positive FITC–Aβ signal were counted, and results analyzed via Fisher's exact test.

2.6 Construction of promoter–enhancer chloramphenicol transferase (CAT) expression clones

Two clones were previously generated to measure expression effects of a SNP in the *APP* promoter. This polymorphism is associated with some cases of familial AD. Briefly, 24– mers flanking the −3833G and −3833A sequences of the *APP* promoter were inserted into the pCAT3P promoter vector (Promega, Madison, WI), which contains the truncated SV40 promoter, to generate two *APP* polymorphic CAT expression vectors (Lahiri et al., 2005b).

2.6 Construction of luciferase–fusion promoter expression clones

Two clones were generated for evaluation of potential Aβ activity on *APP* and *BACE1* 5′– flanking regions. The human *BACE1* 5′–flanking region clone pBACEP2 (Ge et al., 2004b) was cut with *Hin*dIII and *Xho*I and a 3.3 kb fragment of the *BACE1* 5′–flanking sequence

was gel–purified. This was cloned into the *Hin*dIII/*Xho*I sites of the pGL3–Basic vector (Promega). Additionally, the *APP* 5′–flanking region clone pAmyl1.2 (Lahiri and Robakis, 1991) was digested with *Pst*I and *Bam*HI. This was blunted with T4 polymerase and cloned into the *Sma*I site of pGL3–Basic. Clones were verified by appropriate restriction enzyme digest. Transfections of CAT–based constructs were performed using the Lipofectamine with Plus Reagent per manufacturer's recommended protocol (Invitrogen).

2.8 Aβ exposure of cells transfected with BACE1 or APP promoter constructs

PRCN cells were seeded onto poly–D–lysine coated white–walled 96–well plates (Corning) at 75,000 cells/well in Neurobasal medium supplemented with B27, antibiotic cocktail (Invitrogen) and Normocin (InVivoGen, San Diego, CA). Cells were allowed to differentiate for 14 days prior to transfection, with media changes every fourth day. Cells were transfected with 275 ng/well of the pGL3–Basic plasmid (Promega) containing either the *BACE1* 3.3 kb or *APP* 1.2 kb promoter fragment, each fused to the firefly luciferase reporter gene, using 0.75 μl/well of the Transfectin transfection reagent (BioRad, Hercules, CA). Cells were simultaneously transfected with 25 ng/well of the pRL–SV40 *Renilla* luciferase plasmid (Promega) as an internal control. Twenty–four hours after transfection, 1 μM Aβ1–28, Aβ1–40, or Aβ1–42 was added to the medium. After an additional 24–hour incubation following the addition of Aβ, both firefly and *Renilla* luciferase activities were measured with the Dual–Luciferase kit and Glowmax luminometer (Promega). Firefly luciferase signal was normalized to *Renilla* luciferase signal to account for variations in transfection efficiency.

2.9 Aβ exposure of cells transfected with polymorphic APP promoter constructs in presence and absence of H$_2$O$_2$

PC12 cells were seeded and grown as described herein. Transfections were done with 3 μg total DNA, using the Lipofectamine transfection reagent. The pCAT3P empty vector and clones −3833G–CAT, and −3833A–CAT (Lahiri et al., 2005b), were used. At the end of the recovery period, cells were treated with either vehicle, 1 μM Aβ25–35, 50 μM H$_2$O$_2$, or both Aβ and H$_2$O$_2$ at 1 μM and 50 μM, respectively, in 0.5 ml RPMI with 1% horse serum and 0.25% fetal bovine serum (Table 1) and incubated 24 hours. Cells were harvested and lysed. Total lysate protein was measured by Bradford assay (BioRad). Reporter CAT protein levels were measured by ELISA (Roche). CAT signal was adjusted by protein concentration of the cell lysates. Results were analyzed by generalized linear mixed model (Dean and Nielsen, 2007).

2.10 Data analysis

Transfection assays were performed at N = 4 or N = 5 per treatment. All statistical analysis was carried out using SAS 9.1.3 (SAS Institute, Cary, NC). Expression data was tested for distribution and specific general linear mixed model analysis applied accordingly.

3. Results

3.1 ChIP assay demonstrates Aβ binding to specific sequences within the APP and BACE1 gene promoters on cell culture chromatin

ChIP samples from human NB cells were generated in duplicate, and PCR was carried out. The *APP* region −3899/−3708 (Fig. 1A), overlapping the AβID at −3833, and *BACE1* region −205/−27 (Fig. 1B), overlapping the AβID at −119, were selected for analysis. The *APP* region (Fig. 1C) had *in situ* interaction with Aβ. In addition, the AβID at −3833 overlaps a predicted SP1 binding site. Precipitation with anti–SP1 indicated the likelihood that this site actively binds SP1 in human NB cells. Notably, precipitation with 22C11 revealed no signal by PCR. 22C11 was used as a negative control antibody, since it binds

the opposite end of the APP protein from the Aβ peptide. Lack of PCR signal from the 22C11 precipitation is strongly suggestive of specificity for the 4G8 anti–Aβ and anti–SP1 antibodies. Peroxide treatment of the cells had reduced but did not eliminate SP1–*APP* interaction without altering Aβ–*APP* interaction. For the *BACE1* segment, DNA–protein interaction occurred with both Aβ and SP1, as indicated by ChIP (Fig. 1D), with no signal generated by a negative anti–β–galactosidase control precipitation. Aβ and SP1 binding activity was observed both in the presence and absence of H_2O_2 treatment. The ChIP assay was repeated on separate occasions with different bathes of cells. Results were similar to representative data shown here.

3.2 Treatment of human neuroblastoma cell cultures with H_2O_2 promotes uptake of Aβ into cell nuclei

SK–N–SH cell cultures were treated under low serum conditions (1% FBS) with vehicle + 1 μM FITC–Aβ1–40 or 50 μM H_2O_2 + 1 μM FITC–Aβ1–40. After 48 hours, cells were incubated with Hoechst 33342 dye, washed, and incubated with EtHD. Some quenching of FITC fluorescence by EtHD was observed, however brightness adjustment of individual channels confirmed that in H_2O_2 treated cells, nearly all EtHD positive nuclei observed were also positive for FITC–Aβ. In the vehicle treatment, very few FITC–Aβ1–40 positive cells were observed as well as very few EtHD positive dead cells. Most nuclei in both treatment groups were labeled blue by the Hoechst 33342 dye alone (Fig. 2A, E), indicating that these cells remained intact during this treatment. Nuclei labeled red by EtHD were dead cells with permeabilized membranes (Fig. 2C, G). Nuclei were counted according to EtHD, FITC, and Hoechst 33342 signal presence. Counts were analyzed by Fisher's exact test. The number of dead nuclei was slightly but significantly higher ($p < 10^{-5}$, odds ratio 0.576) with H_2O_2 treatment (Table 2). FITC–Aβ labeling among live cells was significantly more common ($p < 10^{-15}$, odds ratio 3.118) in H_2O_2 treated cells (Fig. 2B, F; Table 2) than in vehicle–treated cells. The odds ratio would correspond to a "moderate" effect size if converted to the scale of Cohen's *d* (Ferguson, 1966; Rosenthal, 1994; Hopkins, 2000).

Signals from all three fluorescent labels are shown overlaid with phase contrast images of the same cells (Fig. 2D, H). Dead nuclei were almost invariably FITC–positive. Given that our criterion for identifying dead cells is membrane permeability, it is not clear in this system whether nuclear localization of FITC–Aβ to the nucleus is part of the apoptotic cascade leading to cell death, or if it leaked into the cell after death. Thus, EtHD positive dead nuclei were excluded from the analysis of FITC–Aβ uptake. We attempted to test the specificity of the Aβ uptake observed in these cells by adding a 5–fold excess of unlabeled Aβ1–40, however this produced increases in cell death as well as apparent aggregation and precipitation, resulting in labeling of non–nuclear structures (data not shown). This phenomenon is the subject of continued investigation. Under the conditions used here, H_2O_2 mediated oxidative stress more than doubled the number of nuclei that incorporated detectable levels of FITC–Aβ1–40.

3.3 Aβ treatment altered transient expression of reporter fused to BACE1 5′–flanking region that contains an AβID

PRCN cell cultures were transiently transfected with a 1.2 kb fragment of the *APP* promoter fused to firefly luciferase (Fig. 3A) and treated with 1 μM Aβ1–28, Aβ1–40, and Aβ1–42. Firefly luciferase activity was measured and adjusted to levels of co–transfected internal control plasmid expressing *Renilla* luciferase. PRCN cells transfected with the proximal *APP* promoter fragment showed little response to Aβ treatment (Fig. 3C). No AβIDs were predicted within the *APP* 1.2 kb fragment. In cells transfected with the *BACE1*–CAT reporter clone (Fig. 3B), treatment with all three Aβ peptides corresponded to higher levels of adjusted luciferase activity (Fig. 3D). Interaction between Aβ and the *BACE1* AβID site

at −119 has been confirmed by electrophoretic mobility shift assay (EMSA) (Maloney and Lahiri, 2011).

3.4 Gene regulatory activity of Aβ on the APP promoter is altered by a single–nucleotide polymorphism

PC12 cell cultures were transfected with different polymorphic promoter/enhancer clones that contained the AβID at *APP*−3833, either the "G" or "A" variant, inserted into the pCAT3P vector or with unmodified pCAT3P vector. Transfected cultures were treated with either vehicle, H_2O_2, Aβ25–35, or both treatments, as described herein. Cells were harvested and protein was extracted. CAT protein levels were measured by ELISA and adjusted for total protein concentration in each respective extract. Adjusted data were analyzed by the SAS GLIMMIX procedure.

Type III tests of fixed effects for the data (Table 3) indicated that each of the three main effects: Clone (pCAT3P, 3833A, 3833G), Aβ treatment (−/+), and H_2O_2 treatment (−/+); exerted significant individual effects ($p < 0.05$) on adjusted CAT levels. In addition, a significant interaction was found between H_2O_2 and Aβ treatment and among all three treatments taken together. Effect sizes were estimated by appropriate generalized ω^2 (ω_G^2) or partial ω_G^2 for a three fixed–effects model, for each individual effect, and for effects taken two at a time (Olejnik and Algina, 2003). Effect sizes were categorized presuming sufficient analogy between η^2 and ω_G^2 (Cohen, 1988; Rosenthal, 1994; Hopkins, 2000). This may have actually underestimated the effect size, since ω^2 is a more conservative estimator than η^2 (Warner, 2008).

Treatments altered expression in all three clones, including pCAT3P in a complex manner (Fig. 4). However, deeper analysis of results showed differential effects by clone and each treatment. Specifically, when analyzed by individual effects, instead of as a whole (Fig. 5). Each insert resulted in higher levels of CAT reporter protein when compared to unmodified pCAT3P (Fig. 5A). The clone identified herein as −3833G–CAT produced greater levels of CAT protein as measured by ELISA than did −3833A–CAT. This agrees with our previous findings regarding transfection of these clones without other treatment (Lahiri et al., 2005b). Treatment with Aβ25–35 (Fig. 5B) or peroxide (Fig. 5C) also resulted in significant single–effect differences.

When considered in interactions of two effects (Fig. 6), the most interesting combination was clone × Aβ. Specifically, while Aβ treatment (holding effects of H_2O_2 treatment constant) significantly increased CAT protein levels for −3833G–CAT transfected cells, no similar response occurred in −3833A–CAT or pCAT3P transfected cells (Fig. 6A). When peroxide treatment vs. clone was examined, H_2O_2 treatment resulted in increased CAT protein levels regardless of specific promoter fragment insert (Fig. 6B). This is a notable contrast to the different effects of Aβ on each clone. DNA sequence specificity for Aβ treatment response was observed, while H_2O_2 treatment produced a nonspecific response that was not altered by changes in the Aβ–binding target sequence. Finally, interaction between Aβ and H_2O_2 treatments (Fig. 6C) had some significance ($p = 0.048$), possibly explained by effect of H_2O_2 in facilitating uptake of external Aβ into the cell.

4. Discussion

In addition to forming extracellular plaque in AD, Aβ is neurotoxic in monomers and oligomers (Walsh et al., 2002; Morgan et al., 2004; Ono et al., 2009). Aβ does play a multifaceted physiological role, including kinase induction (Bogoyevitch et al., 2004), reducing metal–induced oxidative damage (Baruch-Suchodolsky and Fischer, 2009),

regulating cholesterol transport (Igbavboa et al., 2009), and altering glutamatergic transmission in the basal forebrain (Chin et al., 2007).

While Aβ is normally a secreted peptide, oxidative stress can induce intracellular localization of the peptide (Ohyagi et al., 2005; Ohyagi and Tabira, 2006). Structural studies have indicated that Aβ in its non–pathogenic α conformation, may have a structure common to certain transcription factors and Aβ1–42 binds DNA *in vitro* (Durell et al., 1994; Barrantes et al., 2007). Extending such findings, Ohyagi *et al* demonstrated Aβ peptide effects on *TP53* apoptosis–associated protein gene expression (Ohyagi et al., 2005). Aβ enhanced p53 levels by direct interaction with a proximal 5′–region of the *TP53* promoter/ 5′–UTR sequence. Transcription factors ASCL1 and OLIG2 are also regulated in cell culture by Aβ (Uchida et al., 2007). Native *BACE1* gene transcription is upregulated in cell culture by addition of Aβ1–42 (Tamagno et al., 2009). Such activity has been demonstrated specifically with the Aβ25–35 peptide for the *SLC38A1* gene (Buntup et al., 2008). Another cleavage product of the APP protein, APP protein intracellular domain fragment (AICD), forms part of the AICD/KAT5/APBB1 "transcription factory" complex (ATF) (Konietzko et al., 2010). ATF can regulate *APP* gene transcription (von Rotz et al., 2004). However, Aβ stimulation of *BACE1* RNA transcription occurred independently of addition of AICD (Giliberto et al., 2009), suggesting that APP protein processing may generate multiple transcriptionally active peptides. Indeed, Aβ was recently shown to stimulate its own secretion (Marsden et al., 2011), and this study suggested a mechanism involving direct interaction with the *APP* and *BACE1* regulatory regions.

Regulation of APP protein expression occurs at several levels. In addition to regulation through SP1 sites and GC box binding proteins in the proximal promoter region (La Fauci et al., 1989; Pollwein et al., 1992; Dosunmu et al., 2009), and through other proximal (Ge et al., 2004a) and distal promoter elements (Lahiri et al., 1999), APP mRNA translation is regulated at both the 5′–UTR (Ge et al., 2004b; Lahiri et al., 2005a) and 3′–UTR (Long and Lahiri, 2010). In addition, promoter–like activity has been found within the CDS regions of the *APP* gene (Vostrov et al., 2010). Our work suggests an additional mechanism of *APP* regulation that involves polymorphism–specific feedback between Aβ levels and transcriptional activity of its precursor gene.

We have recently demonstrated the existence of a generalized AβID that has a consensus of "KGGRKTGGGG", primarily through electrophoretic mobility shift assays (Maloney and Lahiri, 2011). However, binding of Aβ to DNA *in vitro* would be moot if the peptide did not exist intracellularly and interact with chromatin. Intracellular Aβ has been previously shown to exist (Ohyagi et al., 2005; Ohyagi and Tabira, 2006). We confirmed such presence further when comparing cells treated with FITC–Aβ1–40 + H_2O_2 to cells treated with FITC–Aβ alone. FITC signal was observed in a minority of nuclei in either case, but it was significantly more common in H_2O_2 treated cells. We also observed that dead cells treated with H_2O_2 were almost invariably labeled with FITC–Aβ1–40. This suggests that Aβ internalization could be involved in the process of oxidative stress mediated cell death. Previous observations of *TP53* activation by intracellular Aβ (Ohyagi et al., 2005) and the antioxidant role of p53 (Sablina et al., 2005) are consistent with a cell death pathway involving Aβ and the p53 pathway.

Investigation of chromatin–protein interaction within live cells via ChIP suggested that the Aβ peptide interacts with the chromatin of SK–N–SH human neuroblastoma cells, which strongly supports the intra-nuclear localization of Aβ1–40 suggested by the live-cell imaging data. More specifically, it may bind within two 200 bp regions of chromatin corresponding to −3899/−3708 in the *APP* gene promoter and to −205/−27 in the *BACE1* gene promoter. Both of these regions include Aβ binding oligomer sites, at 3833 in the *APP*

promoter at and at −119 in the *BACE1* promoter. We also determined that Aβ binding signal within the *APP* promoter is unlikely to result from binding of by full–length APP to DNA, as immunoprecipitation with the N–terminal binding negative control antibody 22C11 did not produce a PCR signal. To date, no positive ChIP result has been reported using the 22C11 antibody. The *APP* and *BACE1* promoter regions found to contain AβIDs that have *in situ* activity in human neuroblastoma cells are also produced a signal consistent with SP1 binding, which suggests the possibility that Aβ and SP1 may interact. The "G" vs. "A" SNP status at −3829 (within the AβID at −3833/−3824) in the *APP* promoter of SK–N–SH cells is currently unknown. However, *in situ* transcription factor interaction with a potential target site is more complex than is indicated by the "naked DNA" model of EMSA. We, therefore, consider our ChIP data to be strongly supportive rather than conclusive.

To demonstrate activity associated with Aβ binding to selected promoter sequences, we constructed luciferase fusion clones with a 3.3 kb fragment of the *BACE1* 5′–flanking region and a 1.2 kb fragment of the *APP* 5′–flanking region. The *BACE1* sequence contained a single confirmed AβID while the *APP* sequence contained no predicted AβIDs. PRCN cultures were transiently transfected with each of these luciferase fusion reporter clones, and the transfected cells were treated with Aβ peptides 1–28, 1–40, and 1–42. In our system, all three peptides had some apparent effect on *BACE1* promoter activity, as measured by reporter activity levels. This is in contrast to Ohyagi et al, who determined that Aβ1–42 altered expression levels of the *TP53* promoter but Aβ1–40 did not (Ohyagi et al., 2005). In addition, our Aβ1–28 did not show DNA binding capacity as measured by EMSA (Maloney and Lahiri, 2011) but was associated with changes in reporter gene activity levels. The apparent effect on promoter activity without direct DNA binding suggests that, in addition to direct Aβ–DNA interaction, Aβ may regulate *BACE1* promoter activity through other mechanisms.

We have noted that a potential Aβ binding motif starting at −3833 in the *APP* promoter had Aβ binding activity, and this activity was greatly reduced by a naturally occurring G→A SNP. Response to Aβ treatment differed between polymorphic insert variants, suggesting specific differences in Aβ binding activity at the polymorphic site. Both variants and the pCAT3P vector responded to H_2O_2 stimulation, suggesting that response to Aβ treatment is specific to Aβ–DNA interaction at the polymorphic site and not a general oxidative stress reaction from Aβ activity as an oxidizing species. We, therefore, suggest that this particular site in the *APP* promoter may be an active site for Aβ activity as a transcription factor *in vivo* and that differences in Aβ interaction at the site may correspond to differential risk of AD depending on the SNP. The peptide used for this experiment was Aβ25–35, which induces transcription of the *BACE1* gene promoter (Tabaton et al., 2010) and reduces transcription of the *SLC38A1* gene (Buntup et al., 2008). Our own work did not show that this peptide had different binding affinity based on the polymorphism in EMSA (Maloney and Lahiri, 2011), but our assay used a great excess of Aβ25–35, which could have overcome an incomplete reduction in DNA–protein affinity. Alternately, segments of the Aβ peptide outside the 25–35 range could also contribute to sequence specificity such that part of the peptide that might interfere with binding to the −3833A version of the SNP is excluded from this truncated peptide.

The −3833G variant is associated with reduced AD risk in a test population (Lahiri et al., 2005b). Our *in vitro* and cell culture work, herein, has indicated that this allele confers both greater affinity for and activity of full–length 1–40 and 1–42 Aβ peptide than with −3833A. While it is tempting to associate increased transcription of the *APP* gene with greater risk of AD, APP is neuroprotective when processed through the α–secretase cleavage pathway (Fahrenholz and Postina, 2006; Postina, 2008). Since the end products of APP metabolism can be either neurotrophic or neurotoxic, these data alone do not fully explain the protective

effect of the −3829G SNP but rather add to our expanding knowledge of the varied function of Aβ. Potential pathological effects of Aβ activity may, therefore, include stimulation of *BACE1* overexpression and stimulation of other apoptotic pathways, rather than through upregulation of *APP* gene expression.

The many functions of the Aβ peptide may include acting as a transcription factor that directs apoptosis and regulates its own production through feedback on expression of its precursor protein and the β–secretase enzyme (Fig. 7A). Aβ production may be stimulated as a neuroprotective mechanism under cytotoxic conditions (Fig. 7B), until a pathogenic threshold is crossed, and in turn increasing transcription of *APP* and *BACE1* genes to pathological levels, further stimulating its own production in a positive feedback loop. Additionally, increased Aβ would result in a "cytotoxic pair response" involving increased *ASCL1* and decreased *OLIG2* gene products. The combination would result in greater Aβ production, stimulating the production of apoptotic proteins such as p53

Current treatments for AD concentrate upon remediation of cholinergic loss or other specific receptor–based treatments (Lahiri et al., 2003). However, even if restricting AD etiology to effects of Aβ, evidence exists to suggest a wide variety of pathways that are worth exploration (Lahiri and Maloney, 2010a). Furthermore, other pathways, such as those that lead to loss of synaptic markers, may not be directly caused by Aβ and could be targeted through separate mechanisms (Bailey and Lahiri, 2010). Several promising compounds with varied modes of action have recently failed in clinical trials, including phenserine, metrifonate, tarenflurbil, dimebon, and most recently, semagacestat, highlighting the difficulties in slecting effective targets for potential AD treatments (Sambamurti et al., 2011). These failures highlight both the difficulty in translating a compound from pre–clinical conceptualization to clinical use, and the potential need to re–examine the methodologies applied in these clinical trials (Becker and Greig, 2010a; Becker and Greig, 2010b).

Nevertheless, given our data and the work of others cited herein, we propose that Aβ's very broad range of potential functions could be extended to include activity as a DNA transcription factor, modifying expression of AD–related genes, such as *APP* and *BACE1* and glutamatergic pathway genes, such as *SLC38A1*. We have confirmed that oxidative stress can induce uptake of extracellular Aβ peptide into neuronal cells. Finally, we have determined that the Aβ peptide alters promoter–reporter clone activity in a DNA sequence–specific manner. Our own work has concentrated on gene sequences intimately tied with pathogenesis of AD. However, preliminary examination of sequences downloaded from the Eukaryotic Promoter Database (Schmid et al., 2006) produced multiple instances in other gene promoters of high–quality matches for the AβID weight matrix that we developed (Maloney and Lahiri, 2011). Determination of functional or structural gene families in which the AβID may be over– or underrepresented will serve to further elucidate the overall role of Aβ as a transcription factor both within a healthy organism and potentially point to pathogenic activity of Aβ in associated disorders.

Acknowledgments

Abbreviations: The abbreviations used are

AD   Alzheimer's disease

AICD	APP protein intracellular domain fragment
APP	Aβ–precursor protein
APP	Aβ–precursor protein gene
ASCL1	achaete–scute complex homolog 1 gene
ATF	AICD/KAT5/APBB1 transcription factory complex
Aβ	amyloid beta–peptide
BACE1	β–amyloid cleaving enzyme 1/β–secretase gene
EMSA	electrophoretic mobility shift assay
FITC	fluorescein isothiocyanate
HSE	heat shock element
OLIG2	oligodendrocyte lineage transcription factor 2 gene
p53	tumor protein 53
PC12	rat pheochromocytoma cells
PRCN	primary rat cerebrocortical neuronal (culture)
SNP	single–nucleotide polymorphism
SLC38A1	solute carrier family 38 member 1 gene
SP1	specificity protein 1
TP53	tumor protein 53 gene

References

Bailey JA, Lahiri DK. Neuronal differentiation is accompanied by increased levels of SNAP-25 protein in fetal rat primary cortical neurons: implications in neuronal plasticity and Alzheimer's disease. Ann N Y Acad Sci. 2006; 1086:54–65. [PubMed: 17185505]

Bailey JA, Lahiri DK. A novel effect of rivastigmine on pre-synaptic proteins and neuronal viability in a neurodegeneration model of fetal rat primary cortical cultures and its implication in Alzheimer's disease. J Neurochem. 2010; 112:843–53. [PubMed: 19912467]

Barrantes A, Rejas MT, Benitez MJ, Jimenez JS. Interaction between Alzheimer's Abeta1–42 peptide and DNA detected by surface plasmon resonance. J Alzheimers Dis. 2007; 12:345–55. [PubMed: 18198421]

Baruch-Suchodolsky R, Fischer B. Abeta40, either soluble or aggregated, is a remarkably potent antioxidant in cell-free oxidative systems. Biochemistry. 2009; 48:4354–70. [PubMed: 19320465]

Becker RE, Greig NH. Lost in translation: neuropsychiatric drug development. Sci Transl Med. 2010a; 2:61rv6.

Becker RE, Greig NH. Why so few drugs for Alzheimer's disease? Are methods failing drugs? Curr Alzheimer Res. 2010b; 7:642–51. [PubMed: 20704560]

Bogoyevitch MA, Boehm I, Oakley A, Ketterman AJ, Barr RK. Targeting the JNK MAPK cascade for inhibition: basic science and therapeutic potential. Biochim Biophys Acta. 2004; 1697:89–101. [PubMed: 15023353]

Brewer GJ, Torricelli JR, Evege EK, Price PJ. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J Neurosci Res. 1993; 35:567–76. [PubMed: 8377226]

Buntup D, Skare O, Solbu TT, Chaudhry FA, Storm-Mathisen J, Thangnipon W. Beta-amyloid 25–35 peptide reduces the expression of glutamine transporter SAT1 in cultured cortical neurons. Neurochemical Research. 2008; 33:248–56. [PubMed: 18058230]

Chin JH, Ma L, MacTavish D, Jhamandas JH. Amyloid beta protein modulates glutamate-mediated neurotransmission in the rat basal forebrain: involvement of presynaptic neuronal nicotinic acetylcholine and metabotropic glutamate receptors. J Neurosci. 2007; 27:9262–9269. [PubMed: 17728440]

Cohen, J. Statistical Power Analysis for the Behavioral Sciences. 2. Erlbaum; Hillsdale, NJ: 1988.

De Strooper B. Proteases and proteolysis in Alzheimer disease: a multifactorial view on the disease process. Physiol Rev. 2010; 90:465–94. [PubMed: 20393191]

Dean CB, Nielsen JD. Generalized linear mixed models: a review and some extensions. Lifetime Data Anal. 2007; 13:497–512. [PubMed: 18000755]

Dosunmu R, Wu J, Adwan L, Maloney B, Basha MR, McPherson CA, Harry GJ, Rice DC, Zawia NH, Lahiri DK. Lifespan profiles of Alzheimer's disease-associated genes and products in monkeys and mice. J Alzheimers Dis. 2009; 18:211–30. [PubMed: 19584442]

Durell SR, Guy HR, Arispe N, Rojas E, Pollard HB. Theoretical models of the ion channel structure of amyloid beta-protein. Biophys J. 1994; 67:2137–2145. [PubMed: 7535109]

Fahrenholz F, Postina R. Alpha-secretase activation--an approach to Alzheimer's disease therapy. Neurodegener Dis. 2006; 3:255–61. [PubMed: 17047365]

Ferguson, GA. Statistical Analysis in Psychology & Education. McGraw-Hill; New York: 1966.

Ge YW, Ghosh M, Song W, Maloney B, Lahiri D. Mechanism of promoter activity of the beta-amyloid precursor protein gene in different cell types. Identification of a specific 30 bp fragment in the proximal promoter region. J Neurochem. 2004a; 90:1432–44. [PubMed: 15341527]

Ge YW, Maloney B, Sambamurti K, Lahiri DK. Functional characterization of the 5′ flanking region of the BACE gene: identification of a 91 bp fragment involved in basal level of BACE promoter expression. FASEB J. 2004b; 18:1037–1039. [PubMed: 15059977]

Ghosh C, Song W, Lahiri DK. Efficient DNA transfection in neuronal and astrocytic cell lines. Mol Biol Rep. 2000; 27:113–121. [PubMed: 11092558]

Giliberto L, Borghi R, Piccini A, Mangerini R, Sorbi S, Cirmena G, Garuti A, Ghetti B, Tagliavini F, Mughal MR, Mattson MP, Zhu X, Wang X, Guglielmotto M, Tamagno E, Tabaton M. Mutant presenilin 1 increases the expression and activity of BACE1. J Biol Chem. 2009; 284:9027–38. [PubMed: 19196715]

Gouras GK, Tsai J, Naslund J, Vincent B, Edgar M, Checler F, Greenfield JP, Haroutunian V, Buxbaum JD, Xu H, Greengard P, Relkin NR. Intraneuronal Abeta42 accumulation in human brain. Am J Pathol. 2000; 156:15–20. [PubMed: 10623648]

Hebert LE, Scherr PA, Bienias JL, Bennett DA, Evans DA. Alzheimer disease in the US population: prevalence estimates using the 2000 census. Arch Neurol. 2003; 60:1119–22. [PubMed: 12925369]

Hopkins, WG. A new view of statistics. Internet Society for Sport Science. 2000. http://www.sportsci.org/resource/stats/

Igbavboa U, Sun GY, Weisman GA, He Y, Wood WG. Amyloid beta-protein stimulates trafficking of cholesterol and caveolin-1 from the plasma membrane to the Golgi complex in mouse primary astrocytes. Neuroscience. 2009; 162:328–38. [PubMed: 19401218]

Jang H, Arce FT, Ramachandran S, Capone R, Azimova R, Kagan BL, Nussinov R, Lal R. Truncated beta-amyloid peptide channels provide an alternative mechanism for Alzheimer's Disease and Down syndrome. Proc Natl Acad Sci USA. 2010; 107:6538–43. [PubMed: 20308552]

Konietzko U, Goodger ZV, Meyer M, Kohli BM, Bosset J, Lahiri DK, Nitsch RM. Co-localization of the amyloid precursor protein and Notch intracellular domains in nuclear transcription factories. Neurobiol Aging. 2010; 31:58–73. [PubMed: 18403052]

La Fauci G, Lahiri DK, Salton SR, Robakis NK. Characterization of the 5′-end region and the first two exons of the beta-protein precursor gene. Biochem Biophys Res Commun. 1989; 159:297–304. [PubMed: 2538123]

Lahiri DK, Farlow MR, Sambamurti K, Greig NH, Giacobini E, Schneider LS. A critical analysis of new molecular targets and strategies for drug developments in Alzheimer's disease. Curr Drug Targets. 2003; 4:97–112. [PubMed: 12558063]

Lahiri DK, Ge YW, Maloney B. Characterization of the APP proximal promoter and 5′-untranslated regions: identification of cell-type specific domains and implications in APP gene expression and Alzheimer's disease. FASEB J. 2005a; 19:653–655. [PubMed: 15703276]

Lahiri DK, Maloney B. Beyond the signaling effect role of amyloid-beta(42) on the processing of APP, and its clinical implications. Exp Neurol. 2010a; 225:51–54. [PubMed: 20451519]

Lahiri DK, Maloney B. The "LEARn" (Latent Early-life Associated Regulation) model integrates environmental risk factors and the developmental basis of Alzheimer's disease, and proposes remedial steps. Exp Gerontol. 2010b; 45:291–6. [PubMed: 20064601]

Lahiri, DK.; Maloney, B.; Bailey, J.; Ge, Y-W. Role of alzheimer's amyloid-beta peptide as a putative transcription factor. In: Lebl, M., editor. Peptides: Breaking Away, Proceedings of the Twenty-First American Peptide Symposium; Bloomington, Indiana, USA. 2009. p. 185-186.

Lahiri DK, Nall C, Ge YW. Promoter activity of the beta-amyloid precursor protein gene is negatively modulated by an upstream regulatory element. Brain Res Mol Brain Res. 1999; 71:32–41. [PubMed: 10407184]

Lahiri, DK.; Robakis, NK. Characterization of two blocks of cis–acting regulatory elements modulating the expression of the gene encoding the Alzheimer's Amyloid Precursor Proteins. In: Iqbal, K., editor. Alzheimer's Disease: Basic Mechanisms, Diagnosis and Therapeutic Strategies. J. Wiley & Sons; New York: 1991. p. 444-450.

Lahiri DK, Wavrant De-Vrieze F, Ge YW, Maloney B, Hardy J. Characterization of two APP gene promoter polymorphisms that appear to influence risk of late-onset Alzheimer's disease. Neurobiol Aging. 2005b; 26:1329–1341. [PubMed: 16243604]

Long JM, Lahiri DK. MicroRNA-101 downregulates Alzheimer's amyloid-β precursor protein levels in human cell cultures and is differentially expressed. Biochem Biophys Res Commun. 2010 IN PRESS.

Maloney B, Lahiri DK. The Alzheimer's amyloid β–peptide (Aβ) binds a specific DNA Aβ–interacting domain (AβID) in the APP, BACE1, and MAPT promoters in a sequence–specific manner: characterizing a new regulatory motif. Gene. 2011 IN PRESS.

Marsden IT, Minamide LS, Bamburg JR. Amyloid-beta-Induced Amyloid-beta Secretion: A Possible Feed-Forward Mechanism in Alzheimer's Disease. J Alzheimers Dis. 2011; 24:681–91.

Morgan C, Colombres M, Nunez MT, Inestrosa NC. Structure and function of amyloid in Alzheimer's disease. Prog Neurobiol. 2004; 74:323–49. [PubMed: 15649580]

Ohyagi Y. Intracellular amyloid beta-protein as a therapeutic target for treating Alzheimer's disease. Curr Alzheimer Res. 2008; 5:555–61. [PubMed: 19075582]

Ohyagi Y, Asahara H, Chui DH, Tsuruta Y, Sakae N, Miyoshi K, Yamada T, Kikuchi H, Taniwaki T, Murai H, Ikezoe K, Furuya H, Kawarabayashi T, Shoji M, Checler F, Iwaki T, Makifuchi T, Takeda K, Kira JI, Tabira T. Intracellular Abeta42 activates p53 promoter: a pathway to neurodegeneration in Alzheimer's disease. FASEB J. 2005; 19:255–257. [PubMed: 15548589]

Ohyagi Y, Tabira T. Intracellular amyloid beta-protein and its associated molecules in the pathogenesis of Alzheimer's disease. Mini Rev Med Chem. 2006; 6:1075–80. [PubMed: 17073707]

Ohyagi Y, Tsuruta Y, Motomura K, Miyoshi K, Kikuchi H, Iwaki T, Taniwaki T, Kira J. Intraneuronal amyloid beta42 enhanced by heating but counteracted by formic acid. J Neurosci Methods. 2007; 159:134–8. [PubMed: 16860394]

Olejnik S, Algina J. Generalized eta and omega squared statistics: measures of effect size for some common research designs. Psychol Methods. 2003; 8:434–47. [PubMed: 14664681]

Ono K, Condron MM, Teplow DB. Structure-neurotoxicity relationships of amyloid beta-protein oligomers. Proc Natl Acad Sci U S A. 2009; 106:14745–50. [PubMed: 19706468]

Pollwein P, Masters CL, Beyreuther K. The expression of the amyloid precursor protein (APP) is regulated by two GC-elements in the promoter. Nucleic Acids Res. 1992; 20:63–68. [PubMed: 1738605]

Postina R. A closer look at alpha-secretase. Curr Alzheimer Res. 2008; 5:179–86. [PubMed: 18393803]

Rosenthal, R. Parametric measures of effect size. In: Cooper, H.; Hedges, LV., editors. The Handbook of Research Synthesis. Sage; New York: 1994. p. 239

Sablina AA, Budanov AV, Ilyinskaya GV, Agapova LS, Kravchenko JE, Chumakov PM. The antioxidant function of the p53 tumor suppressor. Nat Med. 2005; 11:1306–13. [PubMed: 16286925]

Sambamurti K, Greig NH, Utsuki T, Barnwell EL, Sharma E, Mazell C, Bhat NR, Kindy MS, Lahiri DK, Pappolla MA. Targets for AD treatment: conflicting messages from gamma-secretase inhibitors. J Neurochem. 2011; 117:359–74. [PubMed: 21320126]

Schmid CD, Perier R, Praz V, Bucher P. EPD in its twentieth year: towards complete promoter coverage of selected model organisms. Nucleic Acids Res. 2006; 34:D82–5. [PubMed: 16381980]

Shie FS, LeBoeuf RC, Jin LW. Early intraneuronal Abeta deposition in the hippocampus of APP transgenic mice. Neuroreport. 2003; 14:123–9. [PubMed: 12544843]

Song W, Lahiri DK. Functional identification of the promoter of the gene encoding the Rhesus monkey beta-amyloid precursor protein. Gene. 1998; 217:165–176. [PubMed: 9795200]

Tabaton M, Zhu X, Perry G, Smith MA, Giliberto L. Signaling effect of amyloid-beta(42) on the processing of AbetaPP. Exp Neurol. 2010; 221:18–25. [PubMed: 19747481]

Tamagno E, Guglielmotto M, Giliberto L, Vitali A, Borghi R, Autelli R, Danni O, Tabaton M. JNK and ERK1/2 pathways have a dual opposite effect on the expression of BACE1. Neurobiol Aging. 2009; 30:1563–73. [PubMed: 18255190]

Uchida Y, Nakano S, Gomi F, Takahashi H. Differential regulation of basic helix-loop-helix factors Mash1 and Olig2 by beta-amyloid accelerates both differentiation and death of cultured neural stem/progenitor cells. J Biol Chem. 2007; 282:19700–9. [PubMed: 17488716]

von Rotz RC, Kohli BM, Bosset J, Meier M, Suzuki T, Nitsch RM, Konietzko U. The APP intracellular domain forms nuclear multiprotein complexes and regulates the transcription of its own precursor. J Cell Sci. 2004; 117:4435–48. [PubMed: 15331662]

Vostrov AA, Taheny MJ, Izkhakov N, Quitschke WW. A nuclear factor-binding domain in the 5′-untranslated region of the amyloid precursor protein promoter: Implications for the regulation of gene expression. BMC Res Notes. 2010; 3:4. [PubMed: 20205906]

Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, Rowan MJ, Selkoe DJ. Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. Nature. 2002; 416:535–9. [PubMed: 11932745]

Warner, RM. Applied Statistics: From Bivariate through Multivariate Techniques. Sage Publications; Thousand Oaks, CA: 2008.

Yao ZX, Papadopoulos V. Function of beta-amyloid in cholesterol transport: a lead to neurotoxicity. FASEB J. 2002; 16:1677–9. [PubMed: 12206998]

Zou K, Gong JS, Yanagisawa K, Michikawa M. A novel function of monomeric amyloid beta-protein serving as an antioxidant molecule against metal-induced oxidative damage. J Neurosci. 2002; 22:4833–41. [PubMed: 12077180]
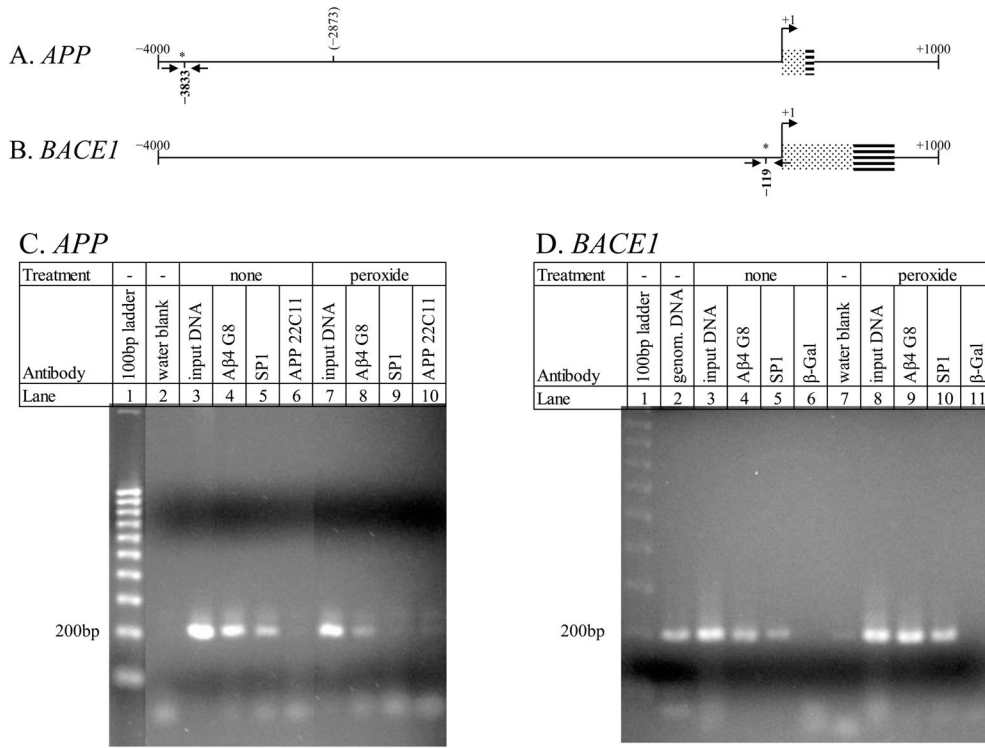
Fig. 1. Chromatin Immunoprecipitation (ChIP) assay of Aβ in NB cell nuclei

ChIP and PCR were carried out as described in the text. <u>A.</u> ChIP for Aβ binding a 200 bp region containing the predicted *APP* −3833 AβID. <u>B.</u> ChIP for Aβ binding a 200 bp region containing the *BACE1* −119 AβID. <u>C.</u> Precipitated, cross–linked chromatin was probed for presence of Aβ peptide (lanes 4, 8), SP1 (lanes 5, 9), and the N–terminus of the APP protein (lane 6) within the indicated region of the *APP* promoter. <u>D.</u> Precipitated, cross–linked chromatin was probed for presence of Aβ peptide (lanes 4, 9), SP1 (lanes 5, 10), and β–galactosidase (lanes 6, 11) within the indicated region of the BACE1 promoter. Treatment conditions in the cells and other appropriate controls in the assay were as indicated in the figure.
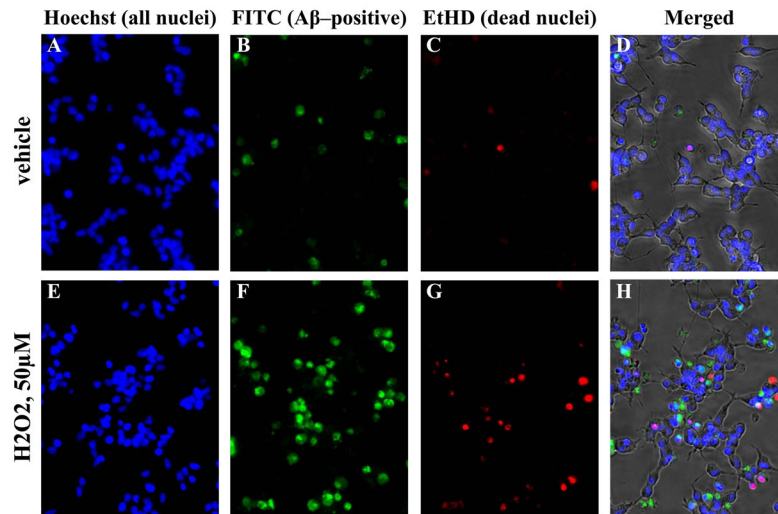
Fig. 2. Treatment of SK–N–SH cells with Aβ in the presence or absence of H$_2$O$_2$
Human SK–N–SH neuroblastoma cells were treated with FITC–Aβ1–40 in presence or absence of H$_2$O$_2$ as described in the text. Cells were incubated with these treatments for 48 hours and visualized with Hoechst 33342 dye, and EtHD, corresponding to all cell nuclei and dead nuclei, respectively. Cells were fluorescently imaged at appropriate wavelengths for FITC, Hoechst treatment, and EtHD treatment. *A and E.* with all nuclei labeled with Hoechst 33342. *B and F.* FITC–Aβ1–40 labeled nuclei. *C and G.* Dead cells labeled with EtHD. *D and G.* Composite of all three fluorescent signals overlaid on a phase contrast image of the cells.
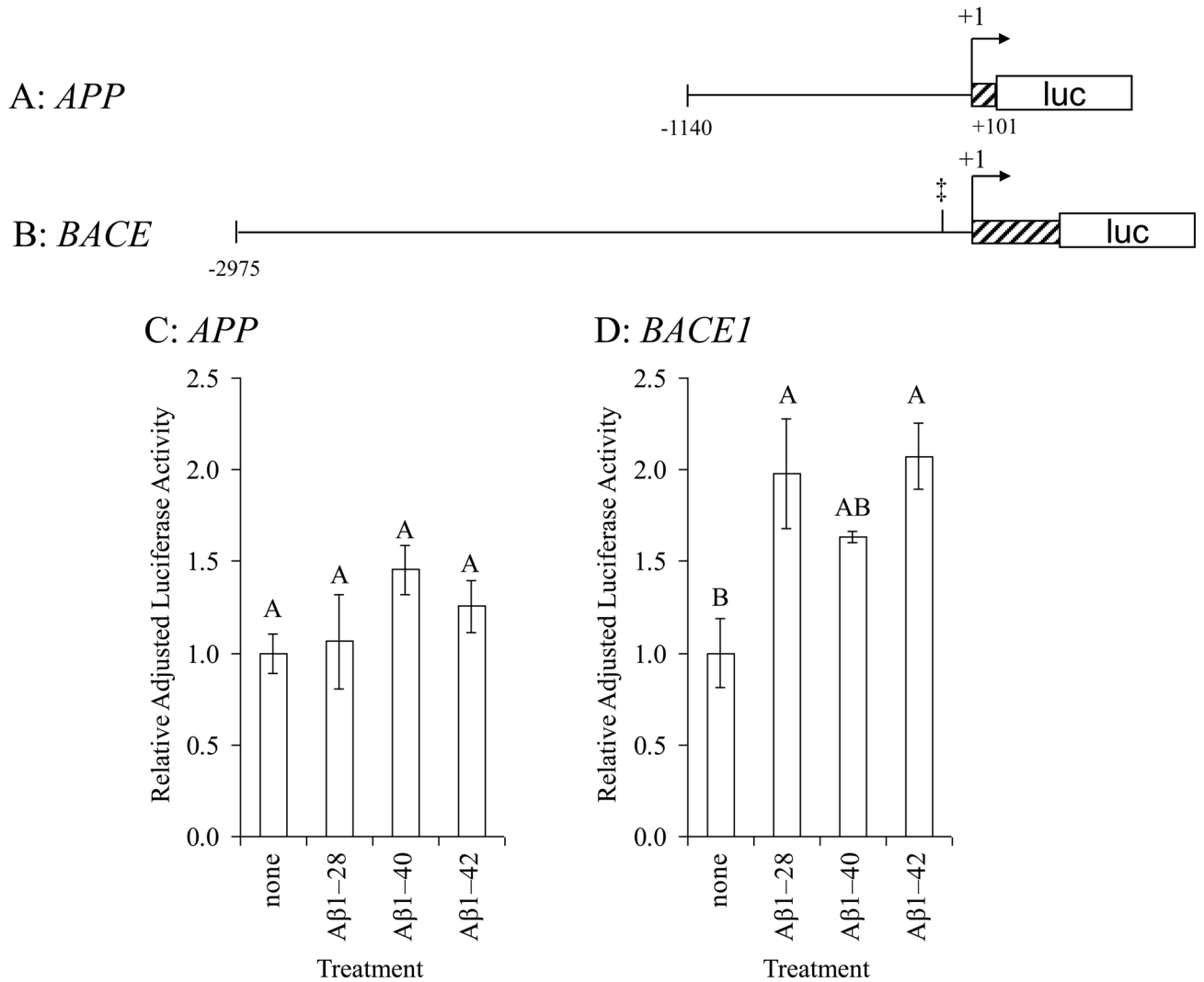
Fig. 3. Effects of Aβ treatment on APP and BACE1 5′–flanking sequence activity

A. A 1.2 kb fragment of the *APP* 5′–flanking region (Song and Lahiri, 1998) was cloned into the pGL3 luciferase expression vector as described in the text. The *APP* fragment contains a putative site for Aβ binding. *B.* A 3.3 kb fragment of the human *BACE1* 5′–flanking region (Ge et al., 2004b) was cloned into the pGL3 luciferase expression vector as described in the text. The *BACE1* fragment contains a confirmed site for Aβ binding, determined herein. Primary rat cerebrocortical neuronal (PRCN) cultures were transiently transfected with a *Renilla* luciferase control vector and *C.* *APP*/pGL3 firefly luciferase fusion clone or *D.* *BACE1*/pGL3 luciferase fusion clone. Transfected cells were left untreated or treated with Aβ1–28, 1–40, or 1–42, all 1 μM. Cells were harvested and activities of firefly and *Renilla* luciferases were measured. Firefly luciferase activity within each treatment was normalized to corresponding *Renilla* luciferase activity. All within each cell type was then normalized to the average firefly/*Renilla* ratio for untreated *APP*/ luciferase fusion activity. Letters above data bars indicate general linear mixed model multiple range categories. Samples sharing a letter do not significantly differ at p < 0.05. Thus, no differences in *APP* promoter activity were found between any treatment group, while there were significant increases in BACE1 promoter activity in the Aβ1–28 and Aβ1–42 treatment groups relative to untreated cells and Aβ1–40 was similar to vehicle.
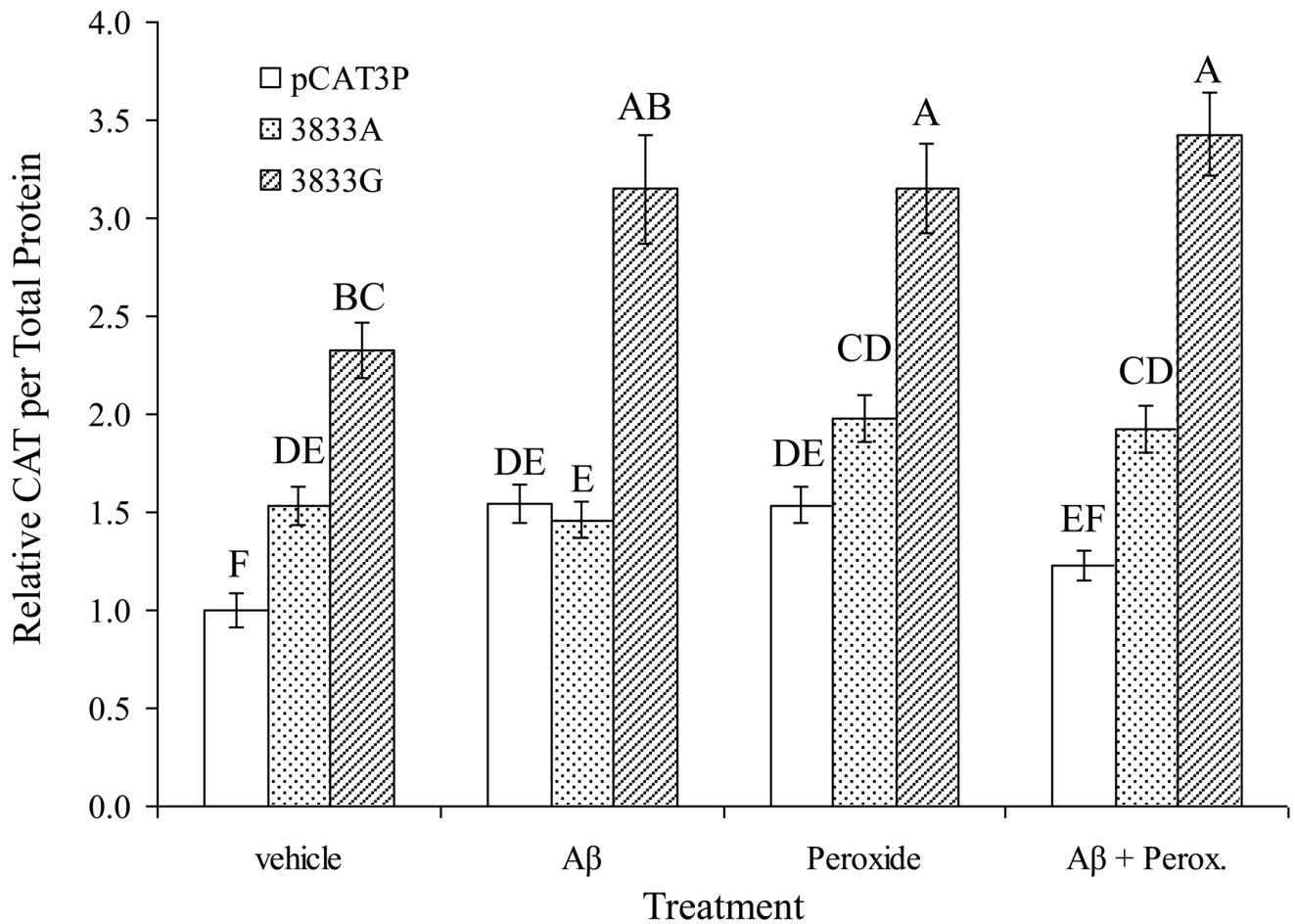
Fig. 4. Differential response of a single–nucleotide polymorphism of the APP promoter to Aβ treatment, all three effects combined
Polymorphic promoter/enhancer CAT expression clones and pCAT3P were transfected into PC12 cells and the transfected cells were treated with H_2O_2 and Aβ, singly or in combination, as described in the main text. Reporter CAT ELISA signal was normalized to total cell protein. Results are presented as proportional to untreated pCAT3P vector transfected cells. Letters above data bars indicate general linear mixed model multiple range categories. Statistical significance is indicated by letters above the data bars, with samples sharing a letter do not significantly differ at $p < 0.05$, and conversely, samples not sharing a letter are significantly different at $p < 0.05$.
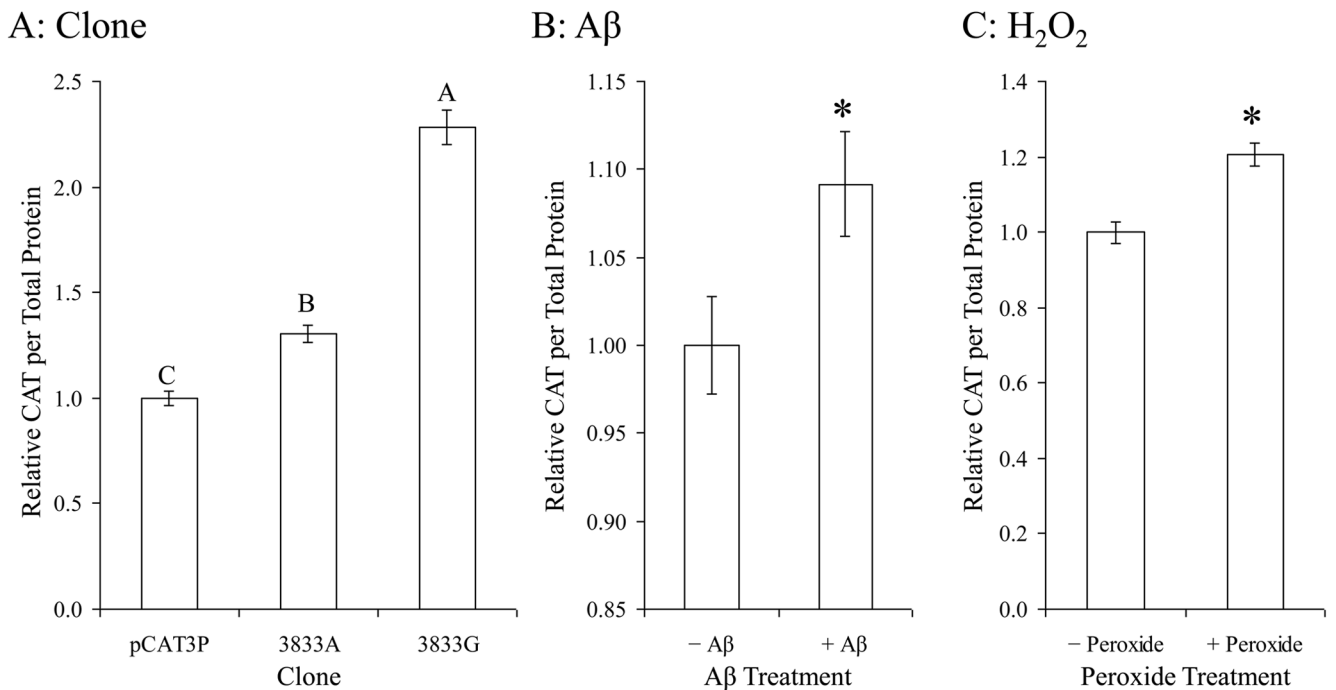
Fig. 5. Differential response of a single–nucleotide polymorphism of the APP promoter to Aβ treatment taken as single effects

Polymorphic promoter/enhancer CAT expression clones and pCAT3P vector plasmid were transfected into PC12 cells and the transfected cells were treated with H_2O_2 and Aβ, singly or in combination, as described in the main text. Reporter CAT ELISA signal was normalized to total cell protein. _A._ Both of the clones tested (A and G) had significantly higher activity than the vector, and the G allele was significantly more active than the A allele. _B and C._ Both Aβ and H2O2 treatment significantly increased activity of this site, independent of the polymorphism. Results are presented as proportional to untreated pCAT3P vector transfected cells. Letters above data bars indicate generalized linear mixed model multiple range categories. Samples sharing a letter do not significantly differ at p < 0.05. For Aβ treatment and H_2O_2, "*" indicates significant difference at p < 0.05.
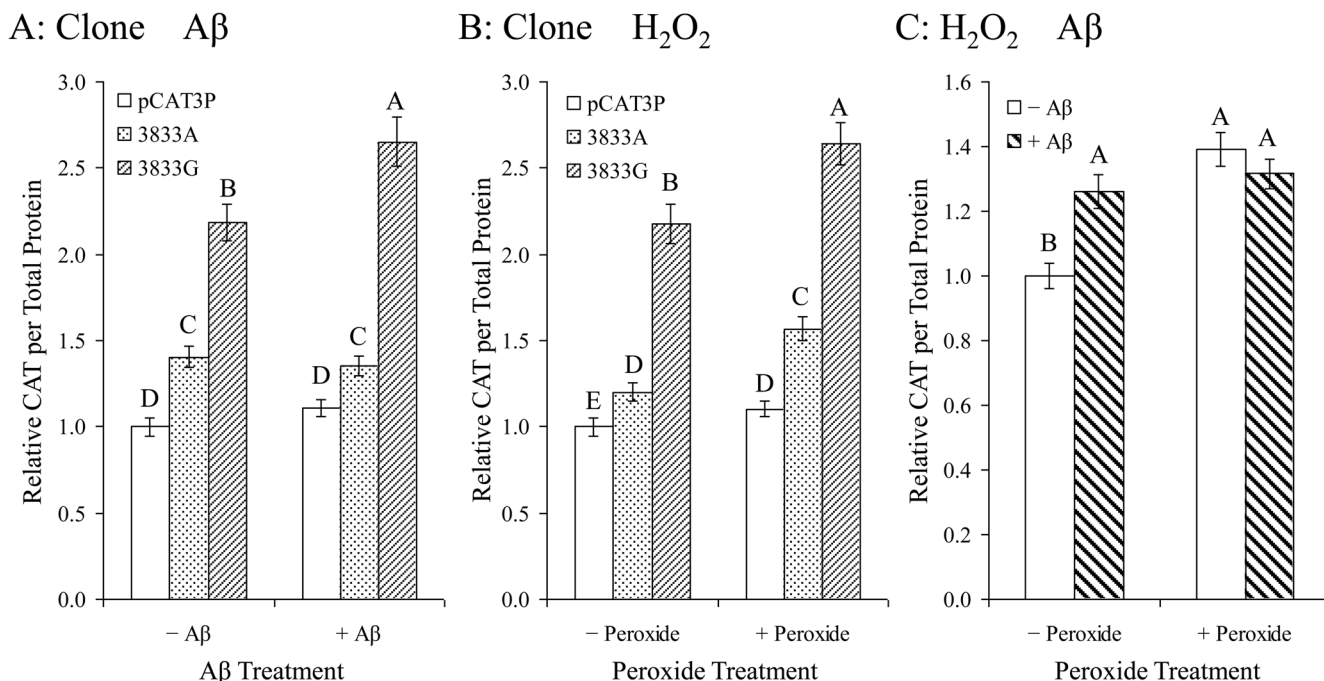
Fig. 6. Differential response of a single–nucleotide polymorphism of the APP promoter to Aβ treatment analyzed as pairs of effects

Polymorphic promoter/enhancer CAT expression clones and pCAT3P were transfected into PC12 cells and the transfected cells were treated with H_2O_2 and Aβ, singly or in combination, as described in the main text. CAT ELISA signal was normalized to total cell protein. Results were then normalized to adjusted signal for untreated pCAT3P transfected cells and expressed as marginal means estimated by holding a single effect (clone, Aβ treatment, or H_2O_2 treatment) constant. *A.* Aβ treatment increased transcriptional activity of the G but not the A clone. Both clones were significantly more active than the pCAT3P vector. *B.* H_2O_2 treatment increased the activity of both clones, indicating that this effect is not sequence–specific. *C.* Aβ treatment in the absence of H_2O_2 increased promoter activity, and H_2O_2 increased promoter activity significantly both in the presence or absence of Aβ. Letters above data bars indicate generalized linear mixed model multiple range categories. Samples sharing a letter do not significantly differ at $p < 0.05$. Although the $-H_2O_2/-Aβ$ treatment had a significant difference from combinations that were treated with H_2O_2, Aβ, or $H_2O_2 + Aβ$, when examining Aβ × H_2O_2, the interaction was not significant overall by Type III test.
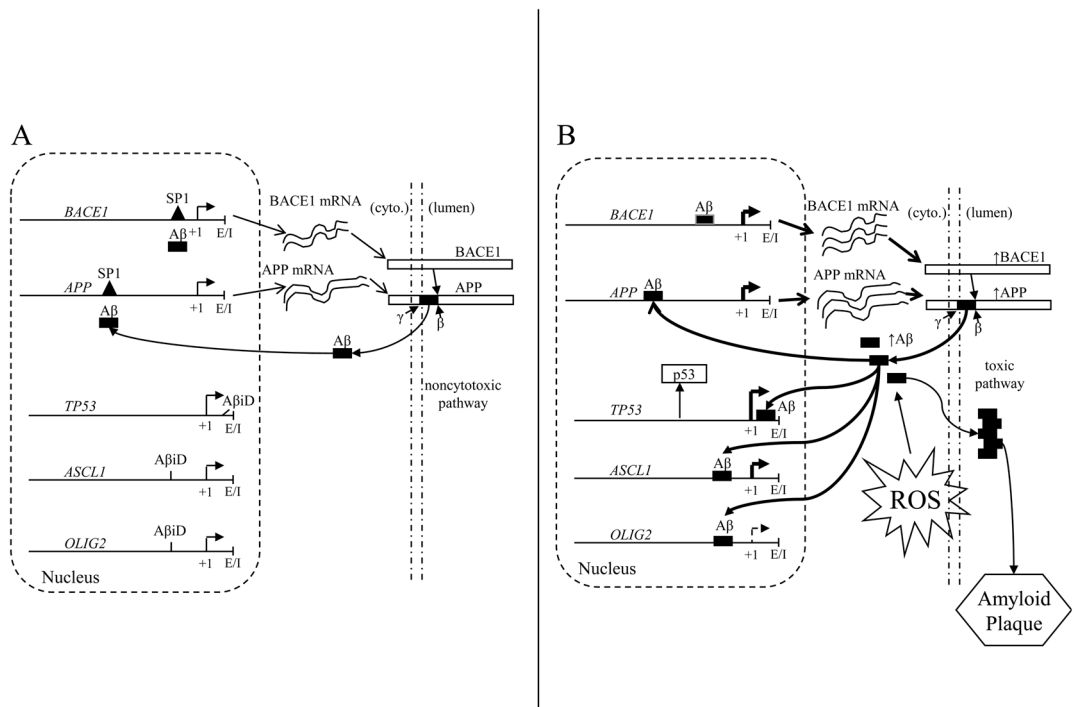
Fig. 7. Models of Aβ feedback and activity as a transcription factor
A. Non–cytotoxic pathway. Aβ levels are insufficient to significantly activate apoptotic genes such as *TP53* and the "cell death pair response" of upregulated *ASCL1* and downregulated *OLIG2* through their AβID sequences. These levels maintain *APP* and *BACE1* at non–pathogenic levels. *B.* Cytotoxic pathway. Aβ levels and resulting *APP* and *BACE1* production are stimulated by conditions such as metal–induced oxidation (ROS), which ultimately results in increased production of Aβ peptides. The levels of Aβ are sufficient to significantly increase levels of p53 and induce the "cell death pair response" of upregulated *ASCL1* and downregulated *OLIG2*. Additional Aβ is produced beyond what is required for physiological function, and the excess Aβ becomes deposited as amyloid plaque.

Table 1

Treatment levels for analysis of Aβ and peroxide (H_2O_2) effects on two different polymorphic *APP* promoter–CAT reporter fusion clones in neuronal (PC12) cells

Clone	pCAT3P (Vector alone)		− 3833A		− 3833G	
Treatment	**Aβ**	**H_2O_2**	**Aβ**	**H_2O_2**	**Aβ**	**H_2O_2**
vehicle	0	0	0	0	0	0
Aβ	1 μM	0	1 μM	0	1 μM	0
peroxide	0	50 μM	0	50 μM	0	50 μM
Aβ + perox	1 μM	50 μM	1 μM	50 μM	1 μM	50 μM

Table 2

Treatment of human neuroblastoma (SK–N–SH) cultures with peroxide (H_2O_2) + Aβ vs. treatment with vehicle +Aβ

	All Nuclei[a]	All Live Nuclei[b]	Live, with FITC signal[c]
Vehicle	1873	1736 (92.7%)[d]	239 (13.8%)[e]
H_2O_2[f]	1727	1519 (88.0%)	505 (32.2%)

[a]Counts of nuclei stained by Hoechst 33342 dye.

[b]Counts of nuclei stained by Hoechst 33342 dye minus counts of nuclei stained by Hoechst and EtHD. Fisher's exact test $p < 10^{-5}$, odds ratio 0.576 ("small" effect size).

[c]Counts of nuclei not stained by EtHD but positive for Hoechst 33342 and FITC fluorescence. Fisher's exact test $p < 10^{-15}$, odds ratio 3.118 ("moderate" effect size).

[d]Percent is live nuclei vs. all nuclei.

[e]Percent is live with FITC vs. live without FITC.

[f]50 μM H_2O_2.

Table 3

Type III analysis of treatment by H_2O_2 and $A\beta$ on polymorphic APP promoter–CAT reporter fusion clones in PC12 cells

Effect	Num DF[a]	Den DF[b]	F Value	Pr > F	ω_G^2	Effect Size
Clone	2	43	149.70	<0.001	0.874	extremely large
$A\beta$	1	43	5.26	0.027	0.090	moderate
H_2O_2	1	43	23.93	<0.001	0.348	large
$A\beta \times H_2O_2$	1	43	14.04	<0.001	0.233	moderate
Clone $\times$ $A\beta$	2	43	3.25	0.048	0.095	moderate
Clone $\times$ H_2O_2	2	43	1.71	0.193	0.032	small
Clone $\times$ $A\beta \times H_2O_2$	2	43	7.13	0.002	0.222	moderate

[a]Numerator degrees of freedom

[b]Denominator degrees of freedom

[c]Effect size category is based on Cohen's categories for effect sizes, as refined by Hopkins, presuming strong analogy between ω_G^2 and η^2.