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# Aβ Hinders Nuclear Targeting of AICD and Fe65 in Primary Neuronal Cultures

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**Abstract** The intracellular domain of the Alzheimer's amyloid precursor protein (AICD) has been described as an important player in the transactivation of specific genes. It results from proteolytic processing of the Alzheimer's amyloid precursor protein (APP), as does the neurotoxic Aβ peptide. Although normally produced in cells, Aβ is typically considered to be a neurotoxic peptide, causing devastating effects. By exposing primary neuronal cultures to relatively low Aβ concentrations, this peptide was shown to affect APP processing. Our findings indicate that APP C-terminal fragments are increased with concomitant reduction in the expression levels of APP itself. AICD nuclear immunoreactivity detected under control conditions was dramatically reduced in response to Aβ exposure. Additionally, intracellular protein levels of Fe65 and GSK3 were also decreased in response to Aβ. APP nuclear signaling is altered by Aβ, affecting not only AICD production but also its nuclear translocation and complex formation with Fe65. In effect, Aβ can trigger a physiological negative feedback mechanism that modulates its own production.

**Keywords** Aβ peptide · APP RIP signaling · Alzheimer's disease · Fe65 · CTFs · AICD

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## Introduction

Aβ was originally defined as a pathogenic peptide associated with Alzheimer's disease (AD), but it is now known to be produced during normal intracellular processing of the Alzheimer's amyloid precursor protein (APP; Haass et al. 1992, 1993; Selkoe 1993; da Cruz e Silva et al. 2004). Consecutive APP cleavage by α-secretase (Sisodia 1992; Buxbaum et al. 1998; Allinson et al. 2003) and the γ-secretase complex (Li et al. 2000; Sastre et al. 2001; Esler et al. 2002; Lee et al. 2002; Capell et al. 2005) precludes Aβ production and produces a smaller fragment termed P3, whereas cleavage by β-secretase and γ-secretase results in the production of Aβ peptides, mainly in Golgi and endosomes (Vassar et al. 1999; Yan et al. 2001; Rebelo et al. 2007). The former non-amyloidogenic cleavage pathway also leads to the production of sAPPα, while the latter results in sAPPβ production. Proteolytic sAPP products are typically secreted, although intracellular sAPP (isAPP) production has been detected (Carlson et al. 2000; Henriques et al. 2009). Resulting APP C-terminal fragments (CTFs), the products of α- and β-secretase activities, may be cleaved by γ-secretase at γ- and ε-sites, giving rise to the APP intracellular domain (AICD; Sastre et al. 2001). Cao and Sudhof (2001) have shown that AICD exhibits transcriptional activity, enhanced by the formation of a transcriptional active complex comprising AICD, Fe65, and the histone acetylase Tip60. This trimeric complex was reported to localize to multiple spherical nuclear compartments (von Rotz et al. 2004). AICD and Fe65 localize together at the nucleus (Kimberly et al. 2001; Minopoli et al. 2001; Walsh et al. 2003), and nuclear AICD-containing complexes were reported to activate the transcription of several genes, including *APP* itself, *BACE*, *Tip60* (von Rotz et al. 2004), *GSK3β* (Kim et al. 2003; Ryan and Pimplikar

2005), *KAI1* (Baek et al. 2002), and *Neprilysin* (Pardossi-Piquard et al. 2005). However, it is still unclear how the translocation of Fe65 and AICD from the cytoplasm and/or membrane into the nucleus is accomplished.

APP/Fe65 interaction is also known to modulate APP metabolism, including sAPP secretion and Aβ production (Sabo et al. 1999; Ando et al. 2001). Sabo et al. (1999) reported that in MDCK cells stably expressing $APP_{695}$, Fe65 increased APP translocation to the plasma membrane, which was accompanied by an increase in Aβ and sAPPα secretion. Recently, Xie et al. (2007) showed that Fe65 RNAi silencing leads to an increase in CTF levels and a decrease in Aβ levels, thus suggesting a role for Fe65 as a positive regulator of γ-secretase activity.

The present work focuses on the effect of exogenously added Aβ on APP metabolism in primary neuronal cultures and its effects on AICD/Fe65 nuclear signaling. The data obtained support the hypothesis that Aβ plays a role in APP processing and RIP signaling by altering APP intracellular proteolytic cleavage and by decreasing both APP and Fe65 intracellular and nuclear levels. The intracellular Aβ effects appear to include decreased AICD production, given the increase in CTFs production and decreased targeting and nuclear co-localization of AICD/Fe65.

## Materials and Methods

### Preparation and Maintenance of Primary Neuronal Cultures

Rat cortical and hippocampal cultures were established from embryonic day 18 embryos as previously described (Henriques et al. 2007). After dissociation with trypsin (0.45 or 0.75 mg/ml for cortical or hippocampal cultures, respectively, for 5–10 min at 37°C) and deoxyribonuclease I (0.15 mg/ml) in Hank's balanced salt solution, cells were plated on poly-D-lysine-coated dishes at a density of $1.0\times10^5$ cells/$cm^2$ in B27-supplemented Neurobasal medium (GIBCO), a serum-free medium combination (Brewer et al. 1993). The medium was supplemented with glutamine (0.5 mM), gentamicin (60 μg/ml), and with or without glutamate (25 μM) for hippocampal or cortical cultures, respectively. Cultures were maintained in an atmosphere of 5% $CO_2$ at 37°C for 9 days before being used for experimental procedures.

### Incubation with Aβ Peptide

$Aβ_{25–35}$ peptide (Sigma Aldrich) was dissolved in distilled water to prepare a 1 mM stock. Rat primary neuronal cultures were incubated for 24 h in Neurobasal medium free of B27 containing 20 μM $Aβ_{25–35}$, with the medium being replaced during the last 3 h of incubation by fresh medium with or without $Aβ_{25–35}$.

### Sample Collection and Immunoblotting

Following exposure to Aβ, conditioned media and cells were collected in boiling 1% sodium dodecyl sulfate (SDS) and the lysates were homogenized as previously described (Amador et al. 2004). Protein determination was carried out using the BCA kit (Pierce). Samples normalized for protein content were separated on 7.5% or 5–20% gradient SDS polyacrylamide gels and then electrophoretically transferred onto nitrocellulose membranes for immunoblotting. Intracellular APP/isAPP and extracellular sAPP detection was carried out using the 22C11 mouse monoclonal antibody directed against the APP N terminus (Boehringer), while for holoAPP and endogenous C-terminal fragments, an APP C-terminal antibody was used (rabbit polyclonal anti-β-APP C terminus, Zymed). Detection of total GSK3 was achieved using a rabbit polyclonal anti-glycogen synthase kinase 3 antibody (Chemicon). For Fe65 detection, the antibody clone 3H6 (Uspstate) was used, and tubulin detection was carried out using the monoclonal anti-β-tubulin antibody (Zymed). Following incubation with the primary antibodies, immunodetection made use of horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgGs secondary antibodies (Amersham Pharmacia), and for visualization, enhanced chemiluminescence detection (ECL) was employed (Amersham Pharmacia). The ECL Plus reagent was used for extracellular sAPP, CTFs, and Fe65 detection.

### Quantification

Quantity One densitometry software (Bio-Rad) was used to quantify band intensity and correlate it to protein levels. Data are expressed as mean±SEM of at least three independent experiments. Statistical analysis was carried out using one-way analysis of variance. When significantly different, the Dunnett test was applied to compare all groups to the control. The level of statistical significance accepted was $P<0.05$.

### Northern Blot Analysis

Total RNA was isolated from control primary cortical cultures ($3.0\times10^6$ cells) following Aβ treatment (TRI REAGENT, Sigma). Normalized total RNA aliquots (10 μg) were separated by formaldehyde gel electrophoresis and transferred to nitrocellulose membranes using standard laboratory protocols (da Cruz e Silva et al. 2009). The blot was then hybridized with a [$^{32}$P]-labeled APP cDNA probe (25 ng, $1\times10^6$ cpm/ng) to evaluate APP expression levels. The APP probe used (756 bp) was obtained by *Age*I/*Bam*HI restriction enzyme digestion of the $APP_{751}$ cDNA and labeled with [α-$^{32}$P]dCTP (GE Healthcare) using the High

Prime DNA labeling kit (Roche, Alfagene). Purification of the probe through NucTrap Probe purification columns (Stratagene, Alfagene) was performed prior to hybridization. APP hybridizing RNA was detected using Kodak Biomax XAR film (Sigma).

### Monitoring Nuclear Targeting of APP C-Terminal Proteolytic Products

For evaluating nuclear targeting of APP C-terminal proteolytic products and Fe65, cells were fixed with 4% paraformaldehyde, permeabilized with methanol for 2 min, and blocked with 3% bovine serum albumin in phosphate-buffered saline (PBS) for 1 h and further incubated with primary antibody (C-terminal APP antibody and Fe65 antibody) for 3 h. The antibody 4G8 (anti-Abeta 17–24 aa antibody, Chemicon) was used in co-localization studies with the APP C-terminal antibody to rule out nuclear CTFs and confirm the identity of AICD at the nucleus. After washing with PBS, Texas Red-conjugated goat anti-rabbit (Molecular Probes) or fluorescein-conjugated goat anti-mouse (Calbiochem) secondary antibodies were added for 2 h at room temperature. Coverslips were mounted on microscope glass slides using FluoroGuard (BioRad) as an antifading reagent or Vectashield (Vector Laboratories), an antifading reagent containing 4′,6-diamidino-2-phenylindole (DAPI) for nucleic acid labeling. Nuclear targeting and co-localization studies of APP C-terminal fragments and Fe65 was carried out by immunofluorescence analysis. Acquisition of epifluorescence images made use of a LSM 510-Meta confocal microscope (Zeiss) and a 63×/1.4 oil immersion objective. The argon laser lines of 405 nm (DAPI), 488 nm (fluorescein), and a 561-nm DPSS laser (Texas Red) were used. Microphotographs were acquired in a sole section in the *z*-axis (*xy* mode) and represent a mean of 16 scans.

## Results and Discussion

### Aβ Affects APP Expression Levels

Aβ effects on APP metabolism are unclear, with some reports suggesting that Aβ may be affecting *APP* transcription, while others suggest it to have an effect at the APP processing/catabolic levels (Davis-Salinas et al. 1995; Schmitt et al. 1997; Carlson et al. 2000). In our work, primary neuronal cultures were incubated with and without $A\beta_{25–35}$ during 24 h and total RNA extracted for Northern blot analysis (Fig. 1a). Although Aβ induction of *APP* transcription was previously reported in a neuronal hybrid cell line and in cultured astrocytes (Le et al. 1995; Moreno-Flores et al. 1998), under our experimental conditions, Aβ treatment lead to a clear decrease in *APP* expression in primary cortical cultures. Accordingly, APP intracellular protein levels also dropped a concordant 0.4-fold below control levels upon Aβ exposure (Fig. 1b). Similar results were obtained for hippocampal cultures, with Aβ leading to a decrease in APP intracellular levels (as detected using an APP C-terminal antibody, Fig. 2a), again supporting a role for Aβ in modulating *APP* transcriptional levels in primary hippocampal cultures.

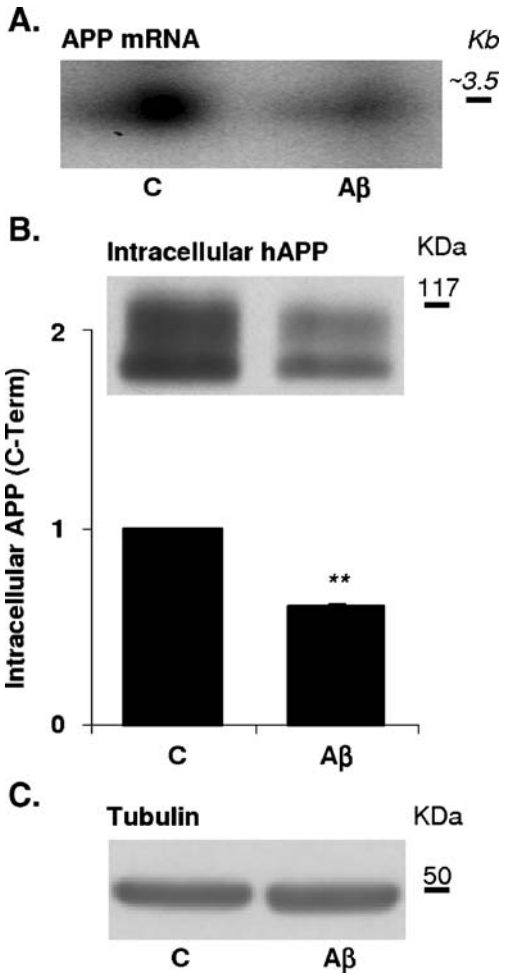


**Figure 1** Aβ decreases *APP* expression in primary neuronal cultures. Primary cortical neuronal cultures were treated with 20 μM $A\beta_{25–35}$ for 24 h as described in "Materials and Methods". **a** Total mRNA was isolated and APP mRNA expression in primary cultures was monitored in response to Aβ exposure. **b** Intracellular holoAPP (*hAPP*) protein detection was carried out using an antibody to the APP C terminus. **c** Tubulin was used as a control. *C* control, *Aβ* Aβ treatment for 24 h. $^{**}P<0.01$, significantly different from control using Dunnett post hoc test

### Aβ Induces Accumulation of APP C-Terminal Fragments

Our data show that $A\beta_{25–35}$ provokes a decrease in intracellular holoAPP (hAPP, Fig. 2a), detected using the APP C-terminal antibody in primary neuronal cultures. Concomitantly, using the APP N-terminal antibody 22C11,

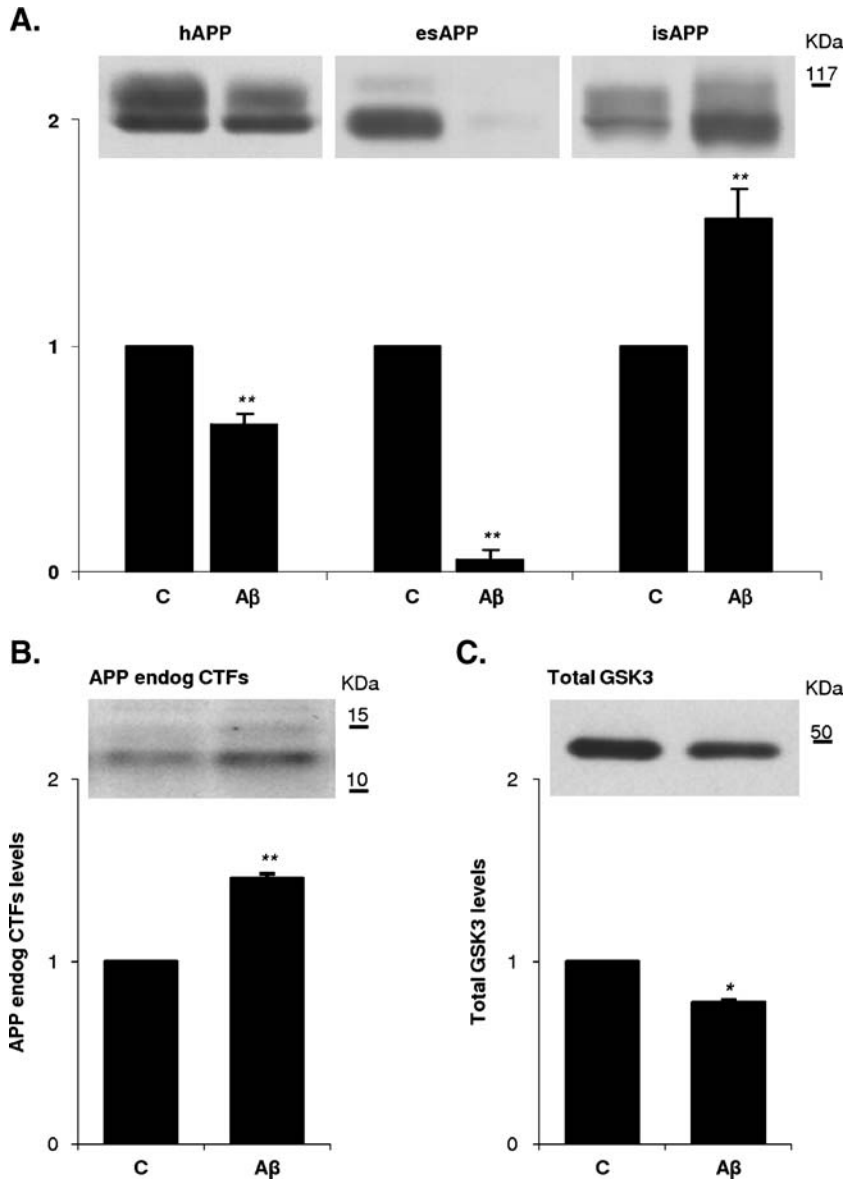


**Figure 2** Effect of Aβ on CTF production. Following incubation of hippocampal neurons with Aβ peptide, cell lysates and conditioned medium were collected and analyzed. **a** Endogenous intracellular holoAPP (*hAPP*), extracellular secreted sAPP (*esAPP*), and intracellular sAPP (*isAPP*). APP and sAPP were distinguished using the APP C-terminal antibody and the APP N-terminal antibody. **b** APP C-terminal fragments (*APP CTFs*) were detected with an APP C-terminal antibody. **c** Total GSK3 expression levels. **P*<0.05 and ***P*<0.01, significantly different from control using Dunnett post hoc test. Values are expressed as mean±SEM from three independent experiments. *C* control, *Aβ* Aβ treatment for 24 h

we could observe a decrease in extracellular sAPP secretion (esAPP, Fig. 2a) and an increase in APP intracellular levels. The latter represents intracellularly accumulated sAPP (isAPP, Fig. 2a) since hAPP levels decreased. This isAPP retention was previously reported by us (Henriques et al. 2009) in various cell types, and $A\beta_{1-42}$ elicits a similar response. Retention of isAPP was also observed with the physiological $A\beta_{1-40}$ peptide (Carlson et al. 2000). In this case, increases in medium secreted proteins, such as IL-8, concomitant with decreases in sAPP secretion, by 10–30%, were reported. Given that Aβ was clearly altering APP processing (Fig. 2), we monitored the levels of other APP proteolytic fragments and observed that these too were affected. The levels of endogenous CTFs produced by APP proteolytic processing increased with Aβ treatment (Fig. 2b). This suggests that γ-secretase activity was inhibited by Aβ and/or that CTFs were not accessible for γ-secretase cleavage. However, direct measurement of γ-secretase

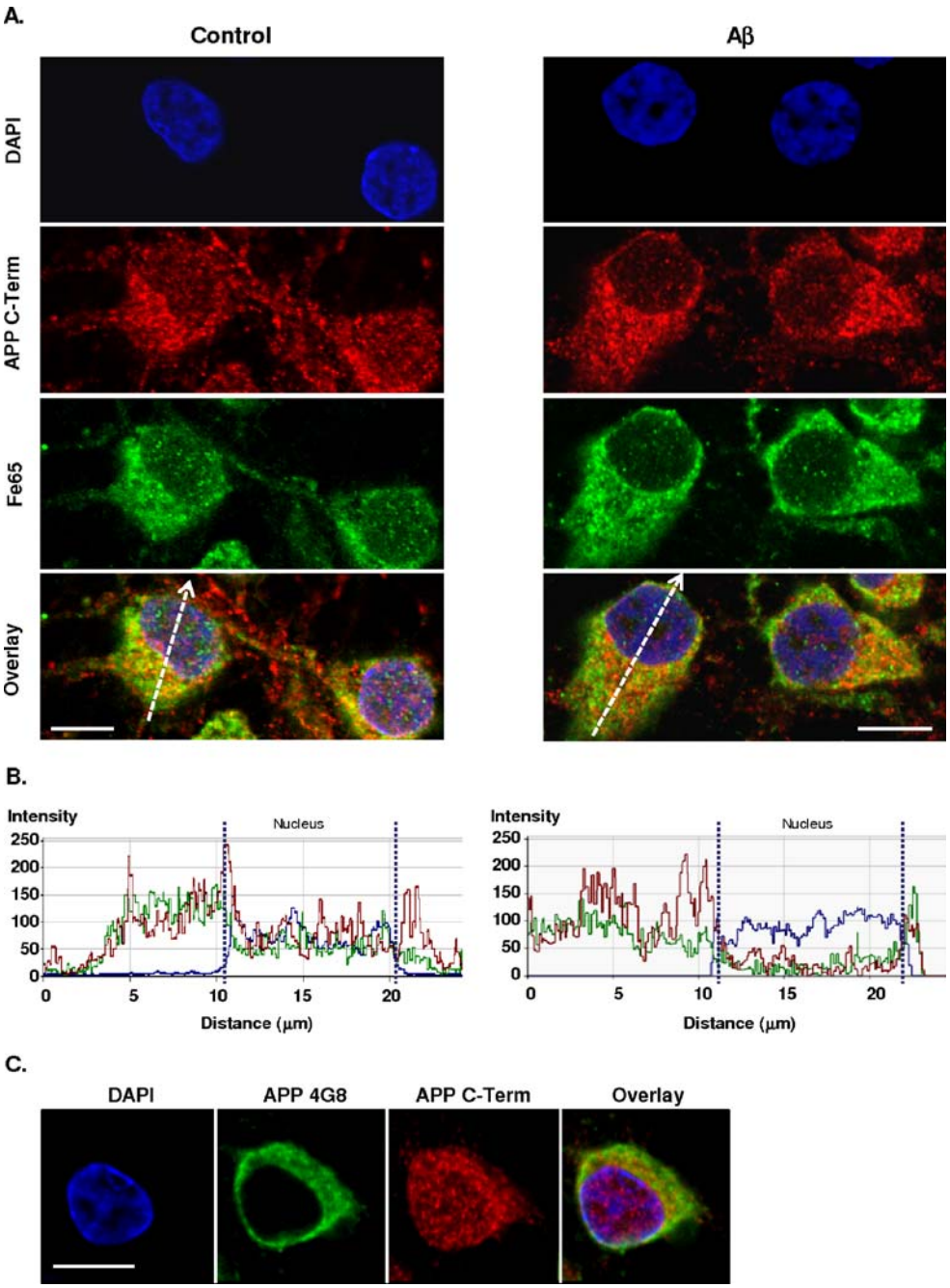


**Figure 3** Aβ induces alterations in AICD and Fe65 nuclear targeting. Hippocampal neuronal cultures were incubated with $A\beta_{25–35}$ for 24 h and subsequently fixed with paraformaldehyde. Immunofluorescence analyses were carried out using anti-β-APP C-terminal and anti-Fe65 antibodies, labeled with a Texas Red-conjugated (*red staining*) or with a fluorescein-conjugated (*green staining*) antibody, respectively. Cells were mounted with an antifading reagent containing DAPI for nuclei acids staining. **a** AICD and Fe65 immunoreactivity was analyzed by confocal microscopy. **b** Representative profiles. Fluorescence intensity profiles represent the voxels through the *white lines* indicated in the merged image shown in **a**. **c** The identity of AICD fragments at the nucleus was shown by the positive immunoreactivity for the APP C-terminal antibody (Texas Red-conjugated, *red staining*) and the negative immunoreactivity for the 4G8 antibody (fluorescein-conjugated, *green staining*). *C* control, *Aβ* Aβ treatment for 24 h

activity did not yield any significant alteration (data not shown), suggesting that CTF cleavage by γ-secretase activity per se was not hindered. An accumulation of amyloidogenic APP CTFs in response to $A\beta_{1–42}$ exposure was previously observed by Yang et al. (1995) in APP transfected HEK293 cells. In neuronally derived cells, γ-secretase cleavage was described to occur at the plasma membrane and/or early endosomes (Kaether et al. 2006). Thus, the neuronal CTF increase observed (Fig. 2b) probably reflects a block in the transport to plasma membrane and a subsequent decrease in proteolytic cleavage of CTFs. This correlates well with our findings of isAPP retention within cytoskeleton-associated vesicular-like structures (Henriques et al. 2009). Hence, increased accumulation of CTFs also correlates with decreased AICD production.

AICD has been described as an APP nuclear signal peptide that can form a transcriptional active complex with Fe65 (Cao and Sudhof 2001). AICD-containing complexes were reported to induce transcriptional activation of several genes, including *APP* itself and *GSK3β* (Kim et al. 2003; von Rotz et al. 2004; Ryan and Pimplikar 2005). Thus, increased CTFs and concomitant decreased AICD levels would predict decreases in the AICD nuclear pool and signaling. This is consistent with the observed decrease in

**Table 1** AICD and Fe65 nuclear targeting and co-localization in response to Aβ

| | Nuclear targeting as a % of total population | | | % of each protein co-localizing to the complex in the nucleus | | |
|---|---|---|---|---|---|---|
| | C | Aβ | % Dec + Aβ | C | Aβ | % Dec + Aβ |
| AICD | 46±3.0 | 27±1.7 | 42 | 33±2.0 | 21±1.9 | 36 |
| Fe65 | 36±2.0 | 16±1.4 | 56 | 22±1.1 | 20±1.6 | 10 |

Analysis was carried out using a Zeiss confocal microscope co-localization software. The percentage of nuclear AICD and Fe65 immunopositive pixels were determined relative to the total neuronal cell populations, excluding dendrites and axons (nuclear targeting as a percentage of the total population). The "% of each protein co-localizing to the complex in the nucleus" represents the percentage of each protein (AICD or Fe65) co-localizing to the other and relative to its total nuclear population. "% Dec + Aβ" is the percentage decrease upon Aβ addition. Data are presented as mean±SEM of 40 analyzed cells

APP expression levels (Fig. 1a) and a significant decrease in total GSK3 levels (Fig. 2c). Direct measurements of AICD were not possible given that the endogenous levels in primary cultures are difficult to detect.

## Aβ Decreases Nuclear Targeting of APP C-Terminal Proteolytic Products and Fe65

Our observation that Aβ increased APP CTF levels and decreased *APP* transcriptional activation and GSK3 expression levels (Figs. 1 and 2) is consistent with a decrease in AICD production and subsequent decreased transactivation of AICD downstream genes. Hence, we focused on AICD/Fe65 nuclear targeting and complex formation. The nuclear targeting of APP C-terminal proteolytic products was clearly hindered in the presence of Aβ (Fig. 3a, Texas Red staining). The APP C-terminal peptides detected in the nucleus and positive for the APP C-terminal antibody were negative for the 4G8 antibody, reinforcing the identity of the nuclear targeted APP C-terminal peptides as AICD proteolytic fragments (Fig. 3c). Under our experimental conditions, Aβ exposure decreased not only the intensity of nuclear APP C-terminal punctuate immunoreactivity (Fig. 3a, Texas Red staining) but also Fe65 nuclear intensity and targeting (Fig. 3a, green fluorescein staining). As a consequence, the co-localization of nuclear AICD-Fe65 was also compromised. Confocal profiling demonstrated that both proteins dramatically decreased in the nucleus (DAPI-positive organelle) following Aβ treatment (Fig. 3b). Hence, the blue trace (denotes the nucleus) is sustained with Aβ exposure, whereas the red and green traces for AICD and Fe65, respectively, drop similarly. Of note, this analysis was carried out in non-apoptotic cells, as denoted by nucleus morphology (DAPI staining).

Detailed co-localization studies of AICD and Fe65 immunoreactivity in the nucleus using Zeiss confocal co-localization software (Table 1) also confirmed Aβ-induced alterations in the nuclear targeting of both proteins. The

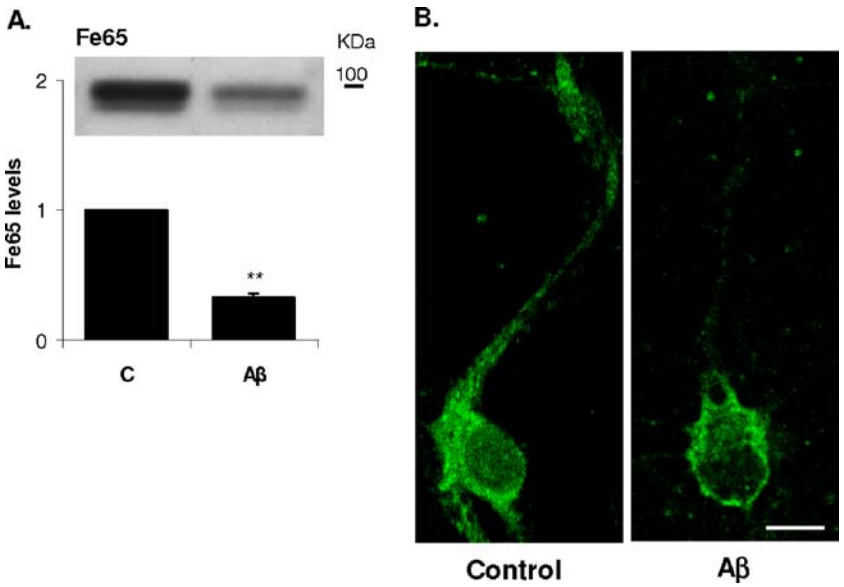


**Figure 4** Aβ effect on Fe65. Fe65 intracellular levels were evaluated using both immunoblotting (**a**) and immunofluorescence (**b**). Fe65 immunoreactivity was analyzed by confocal microscopy at a focus plane above the nucleus and just below the plasma membrane. $^{**}P<0.01$, significantly different from control using Dunnett post hoc test. *C* control, *Aβ* Aβ treatment for 24 h

percentage of both AICD and Fe65 positive pixels present in the nucleus, relative to the total neuronal soma population, showed a decrease of 42% and 56%, respectively. Focusing on the nuclear population alone, we were able to determine that the AICD nuclear population co-localizing with Fe65 dropped from 33% to 21% upon addition of Aβ, which represents a 36% decrease. There was no significant difference in the percentage of the Fe65 nuclear population that co-localized with AICD (22% and 20%). Additionally we determined that the AICD population, as a percentage of the total APP C-terminal immunoreactivity, co-localizing to Fe65 in the nucleus decreased from ~15% (33% of the 46% of the nuclear targeted population, see Table 1) to ~5% (21% of 27%) upon exposure to Aβ. Likewise, we determined the values for Fe65, and the decrease was from ~7% (22% of 36%) to ~4% (20% of 16%).

Given that the nuclear abundance of both AICD and Fe65 were affected, we also tested the latter directly by immunoblotting and immunofluorescence. Our data showed a clear decrease in Fe65 intracellular levels in response to Aβ (Fig. 4a). This was also evident by confocal microscopy at a focus plane above the nucleus and just below the plasma membrane where Fe65 immunoreactivity decreased overall, being particularly visible along neurites (Fig. 4b).

Taken together, our results suggest that Aβ is affecting AICD production, its nuclear translocation, and nuclear complex formation with Fe65 whose nuclear targeting is itself decreased. A decrease in AICD production and in the formation of AICD/Fe65 transactivation complexes potentially leads to altered APP nuclear signaling in the presence of Aβ, leading to impaired gene transcriptional activation.

In summary, we propose that Aβ leads to reduced *APP* expression and consequentially diminished Aβ production, which is important for cells exposed to an Aβ-saturated environment. It is attractive to postulate that the aforementioned mechanisms congregate to reduce intracellular accumulation of Aβ and that exogenous Aβ appears to induce a set of concerted cellular responses to prevent its own production, including reduced AICD/Fe65 nuclear targeting. We hypothesize that a physiologically relevant negative feedback mechanism may be operating, tightly coordinating the levels of APP expression and AICD and Aβ production. Further, as APP CTF levels progressively decrease in AD (Sergeant et al. 2002), this feedback mechanism may be lost with the progression of the disease. This would be a physiologically relevant process given that neurons exhibit higher levels of Aβ production. Nonetheless, we cannot exclude that non-physiological Aβ concentrations may trigger neuronal stress mechanisms which may in turn affect APP metabolism and Aβ production; future research will address this question.

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