Ligand-Dependent Activation of the Chimeric Tumor Necrosis Factor Receptor-Amyloid Precursor Protein (APP) Reveals Increased APP Processing and Suppressed Neuronal Differentiation

Poh-Ling Sim Klaus Heese

Department of Molecular and Cell Biology, School of Biological Sciences, College of Science, Nanyang Technological University, Singapore, Singapore

Key Words

Alzheimer's disease · β -Amyloid precursor protein · Neurodegeneration · Differentiation

Abstract

The β -amyloid precursor protein (APP) is a type I transmembrane protein whose functions and metabolic processing have been implicated in the pathogenesis of Alzheimer's disease (AD). APP's physical resemblance as a glycosylated receptor and the presence of several conserved motifs which are characteristics of a membrane-associated receptor has prompted interest to study and understand the role of downstream signaling events mediated by the activation of APP during both physiological and pathological conditions. Efforts to elucidate the mechanisms of APP signaling have not been conclusive. In order to further characterize the intracellular signaling activities of APP, we have constructed a chimeric APP receptor which is made up of the extracellular domain of human tumor necrosis factor receptor superfamily, member 1B (TNFRSF1B), the membrane-spanning segment of human APP₇₇₀ isoform's β -cleaved carboxyl-terminal fragment (C99), and the green fluorescent protein (GFP) tagged to the carboxyl-terminus of C99. The mammalian rat pheochromocytoma (PC12) cell line is used as the cellular model for transfection of this TNFRSF1B-APP-GFP (TAF) fu-

sion receptor. Ligand-specific stimulation of this TAF receptor using human recombinant TNF reveals the induction of cytoplasmic TAF phosphorylation on Thr743 (numbering for APP₇₇₀ isoform) and an elevated release of APP intracellular domains (AICDs). Time-lapse microscopy shows the trafficking of GFP-tagged AICDs into the nucleus upon TAF receptor activation. The increased presence of AICDs is believed to suppress neuronal differentiation of PC12 cells in response to nerve growth factor treatment, by negatively regulating the levels of p53, cyclin D1 and phosphorylation of the signal transducer and activator of transcription STAT3.

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Introduction

In today's aging society, the most common form of dementia to hit the population is Alzheimer's disease (AD). The numbers of people having AD will double every 5 years beyond the age of 65, and it is estimated according to statistics that nearly half of the population who are aged 85 and above will be susceptible to this incurable disease [1]. The two pathological lesions which are characteristics of the brains of AD patients and used as post-mortem diagnosis are the senile plaques and neurofibrillary tangles [2]. The former are extracellular deposits of

aggregated β -amyloid (A β) peptides permeated by dystrophic neurites, while the latter are composed of intracellular paired helical filaments of the hyperphosphorylated tau, a microtubule-associated protein [3, 4]. These are manifested into clinical symptoms such as a loss of memory and cognitive dysfunctions, due to a loss of cholinergic neurons in the basal forebrain that innervate the hippocampus and the neocortex.

The β -amyloid precursor protein (APP) has been investigated by scientists since its discovery in 1987 and has been widely accepted to play a causative role in the etiology of AD [5]. APP is a type I integral membrane protein that resembles a glycosylated receptor comprising a large extracellular region, a single-pass hydrophobic transmembrane domain and a short cytoplasmic segment (fig. 1). The expression of APP occurs in multiple organs and cell types. There are three different APP isoforms which are derived from a single gene by alternative splicing: APP₆₉₅ (695 amino acids), APP₇₅₁ and APP₇₇₀ in length. The dynamic metabolism of APP can be generally classified into two functional pathways. In the amyloidogenic pathway, APP is cleaved sequentially by β -secretase and γ -secretase to produce the soluble β -ectodomain of APP (sAPP β), A β _{40–42} peptides and γ -cleaved carboxyl-terminal fragments (γ -CTFs) [6]. On the other hand, APP is cleaved by an α -secretase and a γ -secretase to produce sAPP α , p3 peptides and γ -CTFs in the non-amyloidogenic pathway, which therefore precludes the formation of A β peptides by cleaving within the A β sequence [7]. Enhanced proteolytic processing of APP in early-onset or familial AD has resulted in the generation of the highly neurotoxic and fibrillogenic A β peptides. Additional cleavage sites have also been identified, such as the ζ -secretase and ϵ -secretase sites [8, 9].

Presenilins 1 (PS1) and 2 (PS2) are recognized to play important roles in the catalytic functions of γ -, ζ - and ϵ -secretases [10–12]. These secretases work via the common mechanism of regulated intramembrane proteolysis (RIP), which is an important alternative signaling mechanism [13–15]. Like the Notch receptor, APP is cleaved by the RIP mechanism to release the APP intracellular domain (AICD) which can then act as a transcriptional regulator together with Fe65 and Tip60 [16]. Accumulating evidence has also pointed to a possibility of APP's role as a transmembrane receptor mediating the signal transduction of extracellular stimuli upon binding to its cognate ligand. For instance, it has been shown that the cytosolic part of APP can bind to and activate G_o (a major GTP-binding protein in brain) in a ligand-dependent manner [17–19]. Phosphorylation cascades and protein-

protein network interactions have also been implicated in the regulation of APP processing and signaling during both physiological and pathological conditions [20, 21]. At the cellular level, APP has been reported to be involved in the modulation of human neural stem cells differentiation during early embryonic development and in later stages to participate in axonal transport, synaptic plasticity and lipid homeostasis [22–24].

Despite tremendous efforts over the years to investigate the mechanism behind APP signaling, the downstream pathways which are activated upon APP receptor stimulation and its link to APP's proteolytic processing remains unclear. Therefore, in order to characterize APP-mediated intracellular signal transduction, we have established a cellular system based on the analogy of the fusion protein created by Rovelli et al. [25] and Canossa et al. [26]. We constructed a chimeric APP receptor, of which the N-terminus is the ectodomain of human TNFRSF1B (also known as TNFR2), the transmembrane segment is the β -cleaved carboxyl-terminal fragment (C99) of human APP₇₇₀ isoform and the C-terminus is the green fluorescent protein (GFP) (fig. 1). The rat pheochromocytoma PC12 cell line was chosen as the cellular model for neuronal studies [27, 28] and after expressing this fusion protein into PC12 cells, it can be analyzed as a classical ligand-receptor system with human TNF as the specific stimulatory ligand and the TNFRSF1B-APP-GFP (TAF) as the surrogate receptor for APP which mimics the APP receptor activation after binding to its natural ligand. The advantage of this fusion receptor is that it allows a more direct approach of studying APP receptor signaling in an in vitro system. This can then further provide supporting evidence into the relevance of APP's intracellular mechanisms to the development of AD despite that current research on APP has so far focused on mutagenesis, overexpression and knockout studies in mouse models.

In this study, we aim to investigate some of the downstream signaling pathways which are activated by the intracellular domain of APP, the metabolic processing of APP's C-terminus and the migration of AICD in response to stimulus in the absence and presence of α - and β -secretase inhibitors. In addition, murine nerve growth factor (NGF) was used to investigate the potential transsignaling between its receptor, Ntrk1 (also known as TrkA), and APP as studies have suggested that an imbalance of neurotrophin receptor signaling may be involved in AD [29–31].

Fig. 1. Comparison between the APP₇₇₀ isoform and the chimeric APP receptor TAF. **a** APP can be cleaved on several sites on its extracellular and transmembrane domain by a family of enzymes called the secretases. The α - and β -secretases cleave at the juxta-membrane region, while the γ -, ζ - and ϵ -secretases cleave in the hydrophobic environment of the lipid bilayer. The γ -secretase is a multimeric complex consisting of nicastrin, Aph-1, Pen-2 and presenilins. **b** The TNFRSF1B-APP-GFP (TAF) fusion receptor is

constructed of the extracellular part of human TNFRSF1B, the transmembrane and intracellular part of the human APP₇₇₀ isoform and the GFP tagged to the carboxyl-terminus of the β -cleaved fragment (β -CTF or C99) of APP. Human TNF can be used as the specific ligand for stimulation of this classical ligand-receptor system, thereby enabling the study of intracellular APP signaling upon its activation.

Materials and Methods

Unless indicated, all reagents used for biochemical methods were purchased from Sigma-Aldrich (St. Louis, Mo., USA).

Antibodies, Growth Factors and Inhibitors

For Western blotting: anti-hsTNFRSF1B (1:1,000, goat polyclonal; R&D Systems Inc., Minneapolis, Minn., USA), anti-APP's C-terminus A8717 (1:10,000, rabbit polyclonal; Sigma-Aldrich), anti-GFP (1:1,000, rabbit polyclonal; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., USA), anti-Tp53 (1:1,000, mouse monoclonal; Cell Signaling Technology, Inc., Danvers, Mass., USA), anti-cyclin D1 (1:2,000, rabbit polyclonal; Santa Cruz Biotechnology), anti-JNK (c-Jun N-terminal kinase, 1:2,000, rabbit polyclonal; Cell Signaling Technology), anti-STAT3 (1:500, rabbit polyclonal; Santa Cruz Biotechnology), anti-phospho-APP (Thr668, numbering for APP₆₉₅) (1:1,000, rabbit polyclonal; Cell Signaling Technology), anti-phospho-JNK (Thr183/Tyr185) (1:2,000, rabbit polyclonal; BioSource, Inc., Invitrogen, Carlsbad, Calif., USA), anti-phospho-Ntrk1 (Tyr490) (1:500, rabbit polyclonal; Cell Signaling Technology), anti-phospho-STAT3 (Tyr705) (1:500, mouse monoclonal; Santa Cruz Biotechnology), anti-GAPDH (1:10,000, mouse monoclonal; Santa Cruz Biotechnology), anti- α -tubulin (1:2,000, mouse monoclonal; Santa Cruz Biotechnology) and donkey/sheep polyclonal anti-rabbit/mouse IgG (horseradish-peroxidase conjugated, 1:5,000; Amersham, Piscataway, N.J., USA). The human recombinant TNF was from Sig-

ma-Aldrich, murine NGF 2.5S was from Invitrogen, α -secretase inhibitor (*N*-(R)-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl-L-naphthylalanyl-L-alanine, 2-aminoethyl amide; TAPI-1) and β -secretase inhibitor (KTEETSEVN(stat)VAEF) were from Calbiochem (Merck, Darmstadt, Germany).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Subcloning

The RT-PCR method was used for generation of the fusion-protein TAF according to Rovelli et al. [25] and Canossa et al. [26]. Human TNFRSF1B sequence or human APP sequence was subjected to PCR amplification (25 μ l reaction volume) as described previously [32]. [Human APP (Access. No. A31584): from: nt2013–nt2310 = 297nt = 99aa; human TNFRSF1B (Access. No. M32315): from: nt90–nt860 = 771nt = 257aa. Primers were from Invitrogen. First: P1 + P2 (for TNFRSF1B-N-terminus) and P4 + P5 (for APP-C-terminus); second: P1 + P3 and P6 + P5; finally: P1 + P5; (primers: P1: 5'-gccatggcgcgcgcgtcgcgtctggg-3', P2: 5'-gtcgccagt-gctcccttcagctgggggctggggccattgg-3', P3: 5'-ggaattctgcatc/gtcg-ccagtgtcccttcagctgggggct-3', P4: 5'-gcactggcgac/gatgcagaattcc-gacatgactca-3' (') indicates: TNFRSF1B/APP-overlap), P5: 5'-tgttctgcatctgctcaagaactt-3' (without stop-codon for expressing as a GFP-fusion protein), P6: 5'-gccccccagctgaaggagcactggcgacgatg-cagaattccga-3'); the fusion protein contains the extracellular part of human TNFRSF1B and the transmembrane/intracellular part of human APP including the A β (1–42) peptide that starts from: '-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLM-

VGGVVIATVIVITLVMKKKQ...→C-terminus of APP.] The amplification steps involved denaturation at 94°C for 1 min, annealing for 15 s at 65°C (AnnT) with specific primers and extension for 0.5 or 1 min at 68°C (AnnT: 65°C/24 cycles). The PCR reactions were analyzed by DNA electrophoresis in 1.5% agarose gels. Finally, an expression construct was generated by inserting the fusion protein in-frame with the GFP of pcDNA3.1CT-GFP-TOPO® (Invitrogen) at the C-terminus of TAF (fig. 1) and the fusion protein has been confirmed by full-length sequencing analysis.

Cell Culture and Transfection

The rat pheochromocytoma PC12 cells (American Type Culture Collection, Manassas, Va., USA) were propagated in Dulbecco's modified eagle medium (DMEM GlutaMax™) supplemented with 10% fetal bovine serum and 5% horse serum from Gibco™ (Invitrogen). Incubation of the cells was performed in humidified 5% CO₂ and 95% air at 37°C and the culture medium was changed every 2–4 days. The PC12 cells were transfected with the TAF pDNA using the Lipofectamine™ 2000 (Invitrogen) transfection reagent according to the manufacturer's protocol. Cells with positive expression of the TAF fusion protein were assessed 48 h post-transfection by fluorescence microscopy (Nikon TE2000S, Nikon, Singapore) and then purified by FACS (fluorescence-activated cell sorting; FACS Aria Flow Cytometer, BD Biosciences, Singapore). This stable cell line expressed the GFP-tagged protein for at least 3 months before repurification with FACS is needed for culture maintenance.

Time-Point Inductions and Cell Lysis

For short-term induction, both non-transfected PC12 cells and cells transfected with TAF (PC12-TAF) were deprived of serum for 5 h before stimulating them with TNF (10 ng/ml) or NGF (100 ng/ml) for 5, 15, 30 and 60 min in the absence of α - and β -secretase inhibitors, while in the presence of 20 μ M α -secretase inhibitor and 1 μ M β -secretase inhibitor, the durations of stimulation were 15 and 30 min. For long-term induction, PC12 and PC12-TAF cells were treated with TNF for 7 days in the absence of secretase inhibitors, or NGF for 2–10 days. Cells were then washed twice with ice-cold Ca²⁺/Mg²⁺-free Dulbecco's phosphate-buffered saline (DPBS–/–). Cold lysis buffers [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% (v/v) Triton X-100, 0.1% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS), 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 ng/ml of aprotinin, Complete protease inhibitor (Roche, Mannheim, Germany) and a phosphatase inhibitor cocktail 1 & 2 (Sigma-Aldrich)] were added onto cells followed by 5 min incubation on ice. The cell debris and lysates were collected by disposable cell scrapers (Greiner Bio-One, Frickenhausen, Germany) and centrifuged at 13,000 g at 4°C for 10 min. The supernatant was either immediately used for further analysis or stored at –20°C.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis

20 μ g of proteins were separated on 8–12% resolving SDS-polyacrylamide gel electrophoresis at 120 V constant voltage and transferred to a polyvinylidene difluoride membrane (0.2 μ m; Bio-Rad Laboratories, Singapore) by applying the 'wet transfer' method for 2 h at 80 V in buffer containing 25 mM Tris, 192 mM

glycine and 20% (v/v) methanol. The membrane was blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline (TBS) solution with 0.1% (v/v) Tween-20 (TBST) for 1 h at room temperature, followed by incubation with primary antibody (diluted in 5% milk in TBST) overnight at 4°C. Membrane was then washed with TBST three times at 10 min each and incubated with HRP-conjugated secondary antibody for 50 min at room temperature. After washing the membrane five times at 10 min each, the Western blot was developed using Pierce SuperSignal West Femto chemiluminescent substrate (Thermo Fisher Scientific, Inc., Rockford, Ill., USA) and then exposing the membrane on X-ray films (Konica Minolta, Inc., Tokyo, Japan). For equal sample loading, protein quantification was done via the Bradford assay with at least two independent replicates. Bovine serum albumin (Bio-Rad Laboratories) was used as the standard for protein quantification. Equal sample loading was confirmed by using α -tubulin or GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as a reference protein. Western blot experiments were done at least three times and representative blots are shown. Densitometry was carried out using Adobe Photoshop CS3 and the Student's t test was applied for statistical analysis.

Enzyme-Linked Immunosorbent Assay (ELISA)

To measure the concentration of human soluble TNFRSF1B (hsTNFRSF1B) shed extracellularly, the quantitative sandwich enzyme immunoassay technique was employed. A microplate (Quantikine® DRT200, R&D Systems) which had been precoated with an antibody specific for hsTNFRSF1B was incubated with the culture supernatant of the samples initially seeded with equal number of cells. One set of samples was obtained after treating both PC12 and PC12-TAF cells with 20 μ M of α -secretase inhibitor and 1 μ M of β -secretase inhibitor, another set was obtained after treatment with 30 μ M of α -secretase inhibitor and 2 μ M of β -secretase inhibitor, and the last set was treated with 20 μ M of α -secretase inhibitor alone and 1 μ M of β -secretase inhibitor alone. The control samples were not treated with any secretase inhibitors. After adding the enzyme-conjugated antibody against bound hsTNFRSF1B followed by the substrate, a color reaction was developed. By comparison with the standard curve, the hsTNFRSF1B concentrations in the samples were determined. Absorbance measurements were taken at 450 nm using the Safire™ microplate reader (Tecan Group Ltd, Männedorf, Switzerland) and the Magellan™ (Tecan) software. The correction of wavelength was performed at 540 nm. Statistical evaluation of results was performed by applying the analysis of variance method and the statistical error is indicated as the standard error of the mean (SEM).

Neurite Outgrowth Assay

The PC12 and PC12-TAF cells were each seeded at approximately 1×10^5 onto 60-mm poly-D-lysine-coated dishes (Techno Plastic Products AG, Trasadingen, Switzerland) and cultured in DMEM complete medium supplemented with NGF (100 ng/ml) for 7 days. The culture medium was changed every 2–3 days. Bright-field images of the respective cells at 100 $\times$ magnification were captured using the Nikon TE2000S microscope. 250 cells each from differentiated PC12 and PC12-TAF cells were counted for the number of cell bodies bearing neurites greater than two-body length. Cells bearing at least one neurite greater than two-body length were counted as positive. The experiments were re-

Fig. 2. Characterization of the protein expression and subcellular localization of TAF in transfected PC12 cells. Western blot analysis on PC12-TAF (TAF) and control PC12 (CT) cell lysates was carried out using antibodies against (a) TNFRSF1B's N-terminus and (b) APP's C-terminus. The major expression band of the full-length TAF protein is shown at ~75 kDa and some of the other bands are indicated as shown. Under the fluorescence microscope, (c) naive and (d) differentiated PC12-TAF cells treated with NGF (100 ng/ml) for 7 days show the localization of TAF protein in the cytosolic compartment. Scale bar = 50 μ m.

peated for a total of two independent sets and the results were analyzed using the Student's *t* test. The statistical error is indicated as the standard deviation.

Time-Lapse Microscopy

PC12-TAF cells were grown on 35-mm poly-D-lysine-coated glass-bottom FluoroDish™ (World Precision Instruments, Inc., Berlin, Germany) at about 40% confluency and then differentiated into neuronal-like cells with NGF (100 ng/ml) for 7 days. The DMEM culture medium was replaced with Gibco™ Leibovitz's L-15 medium (Invitrogen) prior to real-time imaging. Upon the addition of TNF (15 ng/ml) for stimulation, visualization of the GFP in cells was done using the fluorescent isothiocyanate filter set on the Zeiss Axiovert 200M Fluorescence/Live cell Imaging Microscope (Carl Zeiss MicroImaging, Inc., Thornwood, N.Y., USA) and images were captured at every interval of 15 min. Image analysis was subsequently performed using the AxioVision 4.6.3 software (Carl Zeiss MicroImaging) for measuring the intensity of GFP migration into the nucleus. PC12-TAF cells which had not been treated with TNF were used as the control. The experiment was repeated for a total of three sets and the Student's *t* test was applied for statistical analysis.

Results

Characterization of the Chimeric TAF Receptor

After transfecting PC12 cells with the TAF fusion protein construct, expression of the TAF protein in the cells was confirmed through Western blot analysis. The TAF protein has an approximate molecular weight of 75 kDa, as shown in figure 2a and b. Despite the presence of some possible minor cleaved products of TAF, the majority of the basal expression of TAF protein is left as the intact full-length form. Figure 2b also shows that there are two major isoforms of APP in rat PC12 cells. Although the non-transfected control (CT) PC12 cells have low expression levels of rat TNFRSF1B (fig. 2a), this is much lesser in comparison with the overexpressed human TAF and thus, due to species specificity, will not pose as a potential signaling competitor with TAF's human TNFRSF1B ectodomain to be stimulated by human TNF. In addition, we had performed RT-PCR and found that the mRNA levels of TNFRSF1B in PC12 cells to be negligible in comparison with TAF-transfected cells (data not shown). Flu-

Fig. 3. Inhibition of the proteolytic processing of TAF by α - and β -secretase inhibitors. Control (CT) PC12 and PC12-TAF (TAF) cells were treated with the indicated concentrations of the respective secretase inhibitors for 1 and 3 days. Subsequently, the amount of hsTNFRSF1B which was cleaved and shed into the culture medium was measured by ELISA. Experiments were performed in duplicates and concentrations are as shown $\pm$ SEM. * $p < 0.05$.

orescence microscopy showed that the TAF protein may be localized in the endoplasmic reticulum and Golgi compartments, which is in a similar subcellular distribution as with endogenous APP in both naive and differentiated PC12-TAF cells (fig. 2c, d) [33, 34].

The TAF protein is constructed of the N-terminus of human TNFRSF1B at the ectodomain, β -cleaved membrane-spanning APP (C99) fragment and GFP at the C-terminus. Due to the presence of the α - and β -secretase cleavage sites on the C99 fragment, the TAF protein may also be prone to hydrolysis events mediated by these enzymes. In order to find out if the ectodomain cleavage of the TAF fusion receptor is functional at the cellular level and subjected to inhibition like APP, α -secretase and β -secretase inhibitors were added at varying concentrations for 1–3 days, after which ELISA was performed to determine the amount of hsTNFRSF1B shed into the culture medium (fig. 3). These inhibitors were chosen to study APP metabolism because the α - and β -secretases are the major cleavage sites occurring at the juxtamembrane region of APP during physiological conditions and they regulate the downstream intramembrane cleavage by γ -secretase [35]. As shown in figure 3, the extracellular cleavage and release of hsTNFRSF1B was inhibited in the presence of both secretase inhibitors, with a much greater effect exerted by the α -secretase inhibitor alone than the β -secretases inhibitor alone, illustrating that the proteolytic mechanism of TAF works similarly to that as APP. The lack of β -secretase activity due to the presence of just the amino acids sequence which is C-terminal to the β -secretase cleavage site could explain the lack of in-

hibition of this aspartic proteinase. In addition, we had performed an ELISA on secreted A β in the extracellular medium; however, no significant differences were found between the controls and PC12-TAF cells (data not shown). In the absence of secretase inhibitors, there was an accumulation of hsTNFRSF1B secreted into the extracellular medium from PC12-TAF cells. The control PC12 cells have negligible amount of hsTNFRSF1B due to species specificity of the detection epitope.

Transactivation of TAF's APP Cytoplasmic Domain Using TNF as Ligand

After establishing the functional similarity of TAF and APP with respect to proteolytic processing at the ex-

Fig. 4. Effects of TAF receptor activation by TNF on its phosphorylation, C-terminus's proteolytic processing and JNK signaling in the absence and presence of secretase inhibitors. Control PC12 (C) and PC12-TAF (T) cells were treated with TNF (10 ng/ml) for the indicated durations either in the absence (a, c, g) or presence (b, d, h) of 20 μ M α -secretase inhibitor and 1 μ M β -secretase inhibitor. Western blot analysis was then carried out on the cell lysates using specific antibodies against phosphorylated APP on Thr743 (numbering for APP₇₇₀), APP's C-terminus, JNK and phosphorylated JNK on Thr183 and Tyr185. **e** Quantitative analysis of the Western blots shown in a and c. **f** Quantitative analysis of the Western blots shown in b and d. The relative protein expression of phosphorylated TAF and CTFs in PC12-TAF cells at different durations of TNF treatment were compared respectively to the time-point at 0 min which was designated with a ratio of 1. Error bars represent the standard deviation. * $p < 0.05$.

Fig. 5. Downregulation of Tp53 and cyclin D1 expression levels after long-term constitutive activation of the TAF receptor by TNF. Control PC12 (C) and PC12-TAF (T) cells were treated with TNF (10 ng/ml) in the absence of secretase inhibitors for 7 days, after which the (a) Tp53 and (b) cyclin D1 expression levels were probed using their respective specific antibodies.

tracellular domain, control PC12 and PC12-TAF cells were stimulated with TNF in the absence and presence of secretase inhibitors in order to investigate the downstream signaling events mediated by TAF receptor's activation and to compare their effects under the influence of metabolic processing (fig. 4). Upon TNF stimulation, there was initially an elevation of intracellular TAF phosphorylation on Thr743 (numbering for APP₇₇₀) residue of the APP domain which subsequently decreased with time both in the absence and presence of secretase inhibitors (fig. 4a, b, e, f). This increase in its phosphorylation was even more significant in the presence of secretase inhibitors (fig. 4b, f). The increase in TAF phosphorylation in the absence of secretase inhibitors corresponded with an increased proteolytic processing of TAF's C-terminus, resulting in more intracellular release of the carboxyl-terminal fragments (CTFs) of TAF (fig. 4c, e). However, in the presence of secretase inhibitors, the level of CTFs remained fairly constant despite TAF's increased phosphorylation (fig. 4d, f). It is important to note that the CTFs of endogenous APP in control PC12 cells were not observed due to their low basal expression compared to the overexpressed TAF and its CTFs which are conjugated to GFP.

Besides an increase in phosphorylation of the APP domain of TAF, we have also found the c-Jun N-terminal kinase (JNK) signaling molecule to be regulated by the metabolism and activation of the TAF receptor. In the presence of secretase inhibitors whereby the TAF receptor is mostly intact and processing of the CTFs is negligible, there was significantly more phosphorylated JNK in PC12-TAF cells than in control PC12 cells (fig. 4h). Furthermore, with increasing duration of TAF receptor stimulation by TNF, the phosphorylation of JNK in PC12-TAF cells was further upregulated. On the contrary, there were no changes in JNK phosphorylation in the absence of secretase inhibitors (fig. 4g).

In order to study the effects of long-term constitutive APP activation on physiological changes in the cell, control PC12 and PC12-TAF cells were treated with TNF for 7 days. This was done in the absence of secretase inhibitors so as to observe downstream AICD signaling. As shown in figure 5, there were decreased expression levels of Tp53, a pivotal tumor suppressor protein [36], and cyclin D1, a cell cycle regulator [37], after 7 days of stimulation in PC12-TAF cells. It is interesting to note that both Tp53 and cyclin D1 are involved in the control of the cell cycle checkpoint [38].

As the AICD has been implicated as a transcriptional regulator and we have shown that stimulation of the TAF receptor, in analogy to the APP receptor, resulted in an increased phosphorylation and release of the AICDs, we next investigated whether these AICDs translocate into the nucleus where they get involved with gene transactivation or repression. Terminally differentiated PC12-TAF cells were used for TNF induction and time-lapse imaging showed that the GFP-tagged AICDs indeed migrated into the nucleus in a time-dependent fashion after the addition of TNF (fig. 6). In another control experiment where no TNF was added to differentiated PC12-TAF cells, there was no migration of the GFP signal into the nucleus (data not shown).

Effects of NGF-Induced Ntrk1 Activation on the Trans-Signaling with TAF's Cytosolic Domain

The high-affinity NGF receptor Ntrk1 had been previously reported to influence the tyrosine phosphorylation and processing of AICD [39]. Therefore, we proceeded to study the effect of trans-signaling between the two receptors Ntrk1 and APP by using the TAF receptor system in the presence of NGF. Figure 7a shows that upon activation of the Ntrk1 receptor by NGF, there was spontaneously a significant increase in tyrosine phosphorylation of the Ntrk1 receptor in both control PC12 and PC12-

Color version available online

Fig. 6. Intracellular trafficking of the AICD-GFP into the nucleus after stimulation of the TAF receptor by TNF. The PC12-TAF cells were differentiated with NGF (100 ng/ml) for 7 days and time-lapse images were captured at an interval of every 15 min after the addition of TNF (15 ng/ml). The durations of TAF receptor stimulation are as shown in (a) 0 h, (b) 3 h 15 min, (c) 7 h 15 min and (d) 10 h 15 min. Scale bar = 50 μ m. **e** Quantitative analysis of the time-lapse imaging in a–d. The experiment was repeated for a total of three sets and error bars represent the standard deviation. * $p < 0.05$.

TAF cells. However, the kinetics of Ntrk1 dephosphorylation differed as the level of Ntrk1 phosphorylation retreated to its basal level faster in PC12-TAF cells while in control PC12 cells, the peak phosphorylation of Ntrk1 was sustained longer. At the same time, short-term stimulation with NGF caused an elevation in the phosphorylation of Thr743 (numbering for APP₇₇₀) on the TAF receptor (fig. 7b, d) which is nonetheless lower in comparison to that when TNF was used for stimulation (fig. 4e). This observable increase in TAF phosphorylation on the APP domain unlike endogenous APP may be explained by the much higher level of overexpressed TAF receptor and that the phosphorylation of endogenous APP on Thr743 will have required between 48 and 72 h of NGF induction under normal conditions, which coincides

with the time when neurite extension first occurs [34]. Coupled with the elevated TAF phosphorylation, there was also an increase in the processing of APP's C-terminus in TAF (fig. 7c, d). The cleavage and release of CTFs followed the pattern of TAF phosphorylation which decreased after peaking at 30 min. The increase in the level of intracellularly released CTFs was also not as great and sustained in comparison with that of stimulation using TNF (fig. 4e).

Long-term induction with NGF for 7 days on the control PC12 and PC12-TAF cells showed differences in their morphology and ability for neurite extension. As shown in figures 8a and b, PC12-TAF cells exhibited a significant reduction of about sixfold in neurite outgrowth compared to control PC12 cells. As mentioned

Fig. 7. Effects of Ntrk1 receptor activation on the trans-signaling involving TAF phosphorylation and the proteolytic processing of its C-terminus. Naive control PC12 (C) and PC12-TAF (T) cells were stimulated with NGF (100 ng/ml) for the indicated durations after which Western blot analysis was carried out on the cell lysates using specific antibodies against (a) phosphorylated TrkA on Tyr490, (b) phosphorylated APP on Thr743 (numbering for

APP₇₇₀) and (c) APP's C-terminus. d Quantitative analysis of the Western blots shown in b and c. The relative protein expression of phosphorylated TAF and CTFs in PC12-TAF cells at different durations of NGF treatment were compared respectively to the time-point at 0 min which was designated with a ratio of 1. Error bars represent the standard deviation. * $p < 0.05$.

previously, it was reported by Ando et al. [34] that the phosphorylation of APP coincides with the beginning of neurite extension. Therefore, we treated control PC12 and PC12-TAF cells with NGF for 2 and 4 days and found that both endogenous APP and TAF showed a normal increase in phosphorylation on the fourth day (fig. 8c). However, long-term treatment with NGF for up to 10 days showed an elevated Tp53 expression and phosphorylated STAT3 level in control PC12 cells (fig. 8d, e), both of which are required for mediating NGF-induced neuronal differentiation [40–42]. The Tp53 expression and especially the STAT3 phosphorylation level were clearly suppressed in PC12-TAF cells, which in turn corresponded with the observed reduction in the extent of neurite outgrowth after differentiation induced by NGF treatment.

Discussion

AD is an age-related progressive neurodegenerative disorder characterized by the presence of senile plaques and neurofibrillary tangles in the brain, of which particular focus has been emphasized in the past decade on the etiology of APP [43]. The understanding of the role of APP in the central pathogenesis of AD has been compounded by the lack of characterization of its ligand, until recently when a group of scientists proposed a novel ligand called CNTN2 (also known as TAG1) for APP [44]. Another candidate ligand for APP is the F-spondin which binds to the extracellular domain of APP and inhibits β -secretase cleavage activity [45]. In our present study, the genetically engineered ligand (TNF)-receptor (TAF) sys-

Fig. 8. Effects of long-term NGF stimulation on TAF phosphorylation, Tp53 expression, phosphorylated STAT3 level and neurite outgrowth. **a** Control PC12 and PC12-TAF cells were differentiated with NGF (100 ng/ml) for 7 days and their morphologies were compared under the bright-field microscope. Images of their representative fields are shown. Scale bar = 100 μ m. **b** 250 cells each from differentiated PC12 and PC12-TAF cells in **a** were counted for the number of cells with neurites greater than two-body length

and the percentages are illustrated in a histogram. Experiments were performed in two independent sets and the error bars show the standard deviation. * $p < 0.05$. Control PC12 (C) and PC12-TAF (T) cells were also treated with NGF (100 ng/ml) for the indicated number of days followed by Western blot analysis on the cell lysates using specific antibodies against (c) phosphorylated APP on Thr743 (numbering for APP₇₇₀), (d) Tp53 and (e) phosphorylated STAT3 on Tyr705.

tem mimics the APP receptor activation after binding by its cognate ligand, thus facilitating the study of its downstream signaling events. The TAF receptor shares several similar properties with APP in terms of its subcellular localization and its function as an integral membrane protein. In addition, the TAF receptor is also subjected to ectodomain cleavage by α - and β -secretases which can be prevented through the action of their respective inhibitors.

Using TNF as an extracellular stimulus for the TAF receptor, we have found that a transactivation and phosphorylation of the TAF's APP domain on the Thr743 (numbering for APP₇₇₀) residue was induced. This elevation of TAF phosphorylation was even more significant when the PC12-TAF cells were pretreated with α - and β -secretase inhibitors due to the suppressed release of CTFs resulting from an interference to sequential cleavage. The stabilization of the full-length TAF receptor therefore

leads to a greater observation of the increase in TAF phosphorylation. On the other hand, in the absence of secretase inhibitors, TAF receptor stimulation leads to an increased phosphorylation of its APP's C-terminus which in turn activates RIP to release more AICDs into the cytosol. This is in an analogous manner to Notch receptor signaling. In the in vivo mouse model study conducted by Ma et al. [44] which used the CNTN2 ligand to bind to APP, an increased intracellular release of AICDs was also triggered by a γ -secretase-dependent cleavage activity, eventually leading to suppressed neurogenesis. The phosphorylation of Thr743 on the APP domain after TAF receptor activation could induce a conformational change in the APP cytoplasmic domain, including the 757YENPTY762 motif which is the binding site on APP for Fe65, thus suppressing their interaction [46]. As the Fe65 helps to stabilize the cytoplasmic domain of APP, decreased interaction would promote the cleavage by γ -, ζ -

Fig. 9. A hypothesized signaling scheme of APP receptor activation and its influence on cellular fate during the early stages of nervous system development. When APP binds to its ligand, phosphorylation of the Thr668 residue (numbering for APP₆₉₅ isoform, which is the most abundant in brain) on APP occurs, thus triggering the intramembrane proteolysis of APP and the release of AICDs by γ -, ζ - and ε -secretases. The activation of Ntrk1 can mediate the transactivation and phosphorylation of APP either on Thr668 (numbering for APP₆₉₅), resulting in a transient rise in intracellular AICD levels, or on Tyr682 (numbering for APP₆₉₅). The AICDs, which are released by APP receptor activation, are translocated into the nucleus where they can down-regulate the expression of Tp53 and cyclin D1 proteins, both of which participate in the induction of neuronal differentiation. In addition, the AICD can suppress STAT3 phosphorylation which is a downstream mediator of Ntrk1 receptor signaling. The determination of cell fate on whether to commit to the neuronal lineage depends on a combination of extrinsic and intrinsic factors, the balance between Ntrk1 and APP receptor signaling and the level of APP (or AICD) expression in the cells. An excessive amount of AICD signaling, triggered by the binding of ligand to APP, potentially suppresses neuronal differentiation and in the case of AD during aging, it results in a reduction of the ability for neuronal regeneration.

or ε -secretases, thus causing a greater proteolytic processing of the respective CTFs [47, 48].

The comparison between TAF receptor stimulation in the absence and presence of the secretase inhibitors also revealed differences in JNK signaling. In the presence of secretase inhibitors whereby more of the APP cytoplasmic domains of the TAF receptor remain membrane-anchored, a greater activation of the JNK signaling was observed in PC12-TAF cells than control PC12 cells. It is widely known that the activation of JNK signaling is involved in the response to cellular stress conditions [49, 50]. The increased observation of JNK activation with more membrane-anchored APP could be due to JIP-1 (JNK interacting protein-1) which is an adaptor protein that binds to the ⁷⁵⁷YENPTY⁷⁶² motif of APP [51]. Membrane-tethered APP could recruit the docking of JIP-1 better than free AICDs and thus enhance the downstream phosphorylation and activation of JNK1 and JNK2 through a series of a specific set of kinases such as MLK3 and JNKK2, both of which bind to the scaffolding complex of JIP-1 [52]. In addition, a further increase of JNK phosphorylation was also observed with increasing duration of TAF receptor stimulation. On the other hand, any changes in JNK phosphorylation were prevented during the absence of secretase inhibitors through the release of AICDs.

After the release of AICDs into the cytosol, these AICDs can then translocate into the nucleus where they are involved in the regulation of gene expression [53]. It was found that after 7 days of constitutive TAF receptor activation, the expressions of Tp53 and cyclin D1 were downregulated. One of the functions of Tp53 is to promote cell cycle arrest during NGF-induced differentiation and to enhance neurite outgrowth by transactivating the genes for coronin 1b and GTPase Rab13, both of which associate with the cytoskeleton [40, 54]. Cyclin D1, which is found in the late G₁ phase, is also required for mediating growth arrest prior to the commitment for neuronal differentiation [55]. Therefore, reduced Tp53 and cyclin D1 levels mediated by the increase of APP signal transduction and release of AICDs may enhance cellular proliferation [56] and inhibit neuronal differentiation [44]. In order to investigate the former, we had performed an ATP bioluminescence assay for measuring cell viability and proliferation. Although the PC12-TAF cells showed slightly higher viability than the controls after 7 days of stimulation with TNF, the result was not statistically significant (data not shown).

The short-term induction of PC12-TAF cells with NGF also resulted in an increase in TAF phosphorylation and the proteolytic processing of its CTFs, a similar albeit lesser effect compared to the direct activation of TAF receptor using TNF. This trans-signaling effect of Ntrk1

receptor activation on the intracellular domain of APP could be a regulatory mechanism for the AICD to provide a negative feedback to Ntrk1 phosphorylation. On the contrary, phosphorylation of the tyrosine residue (Tyr757, numbering for APP₇₇₀ isoform) of APP which also occurs during NGF stimulation may work in opposite effects by promoting the docking of ShcA and Grb2 adaptor proteins to the ⁷⁵⁷YENPTY⁷⁶² motif of APP, thus acting in concert with Ntrk1 in the modulation of growth factor signaling to the ERK and PI3K/AKT pathways [57]. In addition, during the long-term NGF-induced differentiation of PC12 and PC12-TAF cells, the Tp53 and phosphorylated STAT3 levels were significantly suppressed in the latter. As noted previously, Tp53 plays an important role during the induction of differentiation and the phosphorylation of STAT3 on Tyr705 occurs in the later stages of neuronal differentiation as one of the downstream mediators of Ntrk1 signaling [42]. The presence of an elevated level of AICDs, both from endogenous APP and TAF, could interfere with Ntrk1 receptor signaling, thus reducing neurite outgrowth and suppressing the neuronal differentiation of PC12-TAF cells. Therefore, the intracellular domain of APP can influence the downstream mechanisms of Ntrk1 receptor signaling differently depending on the site of APP phosphorylation and APP (or AICD) concentrations [58].

We proposed a hypothesis that upon activation of the APP receptor by binding to its natural ligand, a signal transduction is induced in which phosphorylation of the Thr743 residue on the ⁷⁴²VTPEER⁷⁴⁷ motif (numbering for APP₇₇₀) of APP is an important first step (fig. 9). This triggers RIP by γ -, ζ - or ϵ -secretases, therefore releasing more AICDs which can translocate into the nucleus to regulate gene expression. This is very important for the determination of cell fate and works similarly as that for

Notch receptor signaling after it binds to its ligand, for instance Delta-like or Jagged in mammalian systems [59]. Elevated AICD signaling to the nucleus could downregulate the expression of genes such as *p53* and *cyclin D1*, thereby suppressing neuronal differentiation. The observation of erratic APP phosphorylation found in the brains of AD patients [60] lends further support that constitutively activated APP could increase the generation of A β peptides and AICDs, thus impairing neuronal regeneration and losing synaptic contacts. It will be tempting to postulate that during the early stages of nervous system development, AICDs may promote the uncommitted and pluripotency state of cells. However, prolonged AICD activity in cells may cause a switch in its function and turn to mediating apoptosis [61] of those aberrant cells which have remained in the uncommitted state for too long. This molecular switch in function could be attributed by the type of complex formation of AICD with its interacting partners.

In conclusion, stimulating the 'classical' ligand-receptor (TNF/TAF) system, in analogy to the activation of the type I transmembrane APP receptor, provides an insight into the intracellular signal transduction and metabolic processing triggered by the binding of its natural ligand. This novel cellular system provides a better understanding into the mechanisms of APP receptor signaling and its role in the pathogenesis of AD, and may provide an opportunity in the future for the screening of new drug therapeutics against this irretractable disease.

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References

- 1 Alzheimer's Disease Education & Referral (ADEAR) Center. A Service of the National Institute on Aging. National Institutes of Health. US Department of Health and Human Services: Alzheimer's Disease Fact Sheet. NIH Publ 2008;08-6423:1-6.
- 2 Selkoe DJ: Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev* 2001;81:741-766.
- 3 Mandelkow EM, Mandelkow E: Tau in Alzheimer's disease. *Trends Cell Biol* 1998;8:425-427.
- 4 Gómez-Ramos A, Smith MA, Perry G, Avila J: Tau phosphorylation and assembly. *Acta Neurobiol Exp* 2004;64:33-39.
- 5 Kang J, Lemaire HG, Unterbeck A, Salbaum JM, Masters CL, Grzeschik KH, Multhaup G, Beyreuther K, Müller-Hill B: The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* 1987;325:733-736.
- 6 Dominguez DI, Hartmann D, De Strooper B: BACE1 and presenilin: two unusual aspartyl proteases involved in Alzheimer's disease. *Neurodegener Dis* 2004;1:168-174.
- 7 De Strooper B, Annaert W: Proteolytic processing and cell biological functions of the amyloid precursor protein. *J Cell Sci* 2000;113:1857-1870.
- 8 Weidemann A, Eggert S, Reinhard FB, Vogel M, Paliga K, Baier G, Masters CL, Beyreuther K, Evin G: A novel epsilon-cleavage within the transmembrane domain of the Alzheimer amyloid precursor protein demonstrates homology with Notch processing. *Biochemistry* 2002;41:2825-2835.

- 9 Zhao G, Mao G, Tan J, Dong Y, Cui MZ, Kim SH, Xu X: Identification of a new presenilin-dependent ζ -cleavage site within the transmembrane domain of amyloid precursor protein. *J Biol Chem* 2004;279:50647–50650.
- 10 De Strooper B, Saftig P, Craessaerts K, Vanderstichele H, Guhde G, Annaert W, Von Figura K, Van Leuven F: Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature* 1998;391:387–390.
- 11 Wolfe MS, Xia W, Ostaszewski BL, Diehl TS, Kimberly WT, Selkoe DJ: Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and γ -secretase activity. *Nature* 1999;398:513–517.
- 12 Wilson CA, Doms RW, Lee VM: Distinct presenilin-dependent and presenilin-independent γ -secretases are responsible for total cellular A β production. *J Neurosci Res* 2003;74:361–369.
- 13 Brown MS, Ye J, Rawson RB, Goldstein JL: Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. *Cell* 2000;100:391–398.
- 14 Ebinu JO, Yankner BA: A RIP tide in neuronal signal transduction. *Neuron* 2002;34:499–502.
- 15 Selkoe D, Kopan R: Notch and presenilin: regulated intramembrane proteolysis links development and degeneration. *Annu Rev Neurosci* 2003;26:565–597.
- 16 Von Rotz RC, Kohli BM, Bosset J, Meier M, Suzuki T, Nitsch RM, Konietzko U: The APP intracellular domain forms nuclear multi-protein complexes and regulates the transcription of its own precursor. *J Cell Sci* 2004;117:4435–4448.
- 17 Nishimoto I, Okamoto T, Matsuura Y, Takahashi S, Okamoto T, Murayama Y, Ogata E: Alzheimer amyloid protein precursor complexes with brain GTP-binding protein G $_o$. *Nature* 1993;362:75–79.
- 18 Okamoto T, Takeda S, Murayama Y, Ogata E, Nishimoto I: Ligand-dependent G protein coupling function of amyloid transmembrane precursor. *J Biol Chem* 1995;270:4205–4208.
- 19 Okamoto T, Takeda S, Giambarella U, Murayama Y, Matsui T, Katada T, Matsuura Y, Nishimoto I: Intrinsic signaling function of APP as a novel target of three V642 mutations linked to familial Alzheimer's disease. *EMBO J* 1996;15:3769–3777.
- 20 Da Cruz e Silva EF, da Cruz e Silva OA: Protein phosphorylation and APP metabolism. *Neurochem Res* 2003;28:1553–1561.
- 21 Russo C, Venezia V, Repetto E, Nizzari M, Violani E, Carlo P, Schettini G: The amyloid precursor protein and its network of interacting proteins: physiological and pathological implications. *Brain Res Brain Res Rev* 2005;48:257–264.
- 22 Furukawa K, Barger SW, Blalock EM, Mattson MP: Activation of K $^+$ channels and suppression of neuronal activity by secreted β -amyloid-precursor protein. *Nature* 1996;379:74–78.
- 23 Kwak YD, Brannen CL, Qu T, Kim HM, Dong X, Soba P, Majumdar A, Kaplan A, Beyreuther K, Sugaya K: Amyloid precursor protein regulates differentiation of human neural stem cells. *Stem Cells Dev* 2006;15:381–389.
- 24 Heese K, Akatsu H: Alzheimer's disease – an interactive perspective. *Curr Alzheimer Res* 2006;3:109–121.
- 25 Rovelli G, Heller RA, Canossa M, Shooter EM: Chimeric tumor necrosis factor-TrkA receptors reveal that ligand-dependent activation of the TrkA tyrosine kinase is sufficient for differentiation and survival of PC12 cells. *Proc Natl Acad Sci USA* 1993;90:8717–8721.
- 26 Canossa M, Rovelli G, Shooter EM: Transphosphorylation of the neurotrophin Trk receptors. *J Biol Chem* 1996;271:5812–5818.
- 27 Greene LA, Tischler AS: Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci USA* 1976;73:2424–2428.
- 28 Götz R: Regulation of neuronal cell death and differentiation by NGF and IAP family members. *J Neural Transm Suppl* 2000;60:247–259.
- 29 Hock C, Heese K, Müller-Spahn F, Hulette C, Rosenberg C, Otten U: Decreased trkA neurotrophin receptor expression in the parietal cortex of patients with Alzheimer's disease. *Neurosci Lett* 1998;241:151–154.
- 30 Hock C, Heese K, Hulette C, Rosenberg C, Otten U: Region-specific neurotrophin imbalances in Alzheimer disease: decreased levels of brain-derived neurotrophic factor and increased levels of nerve growth factor in hippocampus and cortical areas. *Arch Neurol* 2000;57:846–851.
- 31 Ginsberg SD, Che S, Wu J, Counts SE, Mufson EJ: Downregulation of trk but not p75NTR gene expression in single cholinergic basal forebrain neurons mark the progression of Alzheimer's disease. *J Neurochem* 2006;97:475–487.
- 32 Heese K, Yamada T, Akatsu H, Yamamoto T, Kosaka K, Nagai Y, Sawada T: Characterizing the new transcription regulator protein p60TRP. *J Cell Biochem* 2004;91:1030–1042.
- 33 Selkoe DJ: The cell biology of β -amyloid precursor protein and presenilin in Alzheimer's disease. *Trends Cell Biol* 1998;8:447–453.
- 34 Ando K, Oishi M, Takeda S, Iijima K, Isohara T, Nairn AC, Kirino Y, Greengard P, Suzuki T: Role of phosphorylation of Alzheimer's amyloid precursor protein during neuronal differentiation. *J Neurosci* 1999;19:4421–4427.
- 35 Ren Z, Schenk D, Basi GS, Shapiro IP: Amyloid β -protein precursor juxtamembrane domain regulates specificity of γ -secretase-dependent cleavages. *J Biol Chem* 2007;282:35350–35360.
- 36 Levine AJ, Momand J, Finlay CA: The p53 tumour suppressor gene. *Nature* 1991;351:453–456.
- 37 King KL, Cidlowski JA: Cell cycle regulation and apoptosis. *Annu Rev Physiol* 1998;60:601–617.
- 38 Galderisi U, Jori FP, Giordano A: Cell cycle regulation and neural differentiation. *Oncogene* 2003;22:5208–5219.
- 39 Tarr PE, Contursi C, Roncarati R, Novielle C, Ghersi E, Scheinfeld MH, Zambrano N, Russo T, D'Adamo L: Evidence for a role of the nerve growth factor receptor TrkA in tyrosine phosphorylation and processing of β -APP. *Biochem Biophys Res Commun* 2002;295:324–329.
- 40 Di Giovanni S, Knights CD, Rao M, Yakovlev A, Beers J, Catania J, Avantiaggiati ML, Faden AI: The tumor suppressor protein p53 is required for neurite outgrowth and axon regeneration. *EMBO J* 2006;25:4084–4096.
- 41 Zhang J, Yan W, Chen X: p53 is required for nerve growth factor-mediated differentiation of PC12 cells via regulation of TrkA levels. *Cell Death Differ* 2006;13:2118–2128.
- 42 Ng YP, Cheung ZH, Ip NY: STAT3 as a downstream mediator of Trk signaling and functions. *J Biol Chem* 2006;281:15636–15644.
- 43 Nathalie P, Jean-Noël O: Processing of amyloid precursor protein and amyloid peptide neurotoxicity. *Curr Alzheimer Res* 2008;5:92–99.
- 44 Ma QH, Futagawa T, Yang WL, Jiang XD, Zeng L, Takeda Y, Xu RX, Bagnard D, Schachner M, Furley AJ, Karagogeos D, Watanabe K, Dawe GS, Xiao ZC: A TAG1-APP signalling pathway through Fe65 negatively modulates neurogenesis. *Nat Cell Biol* 2008;10:283–294.
- 45 Ho A, Südhof TC: Binding of F-spondin to amyloid- β precursor protein: a candidate amyloid- β precursor protein ligand that modulates amyloid- β precursor protein cleavage. *Proc Natl Acad Sci USA* 2004;101:2548–2553.
- 46 Ramelot TA, Nicholson LK: Phosphorylation-induced structural changes in the amyloid precursor protein cytoplasmic tail detected by NMR. *J Mol Biol* 2001;307:871–884.
- 47 Ando K, Iijima KI, Elliott JI, Kirino Y, Suzuki T: Phosphorylation-dependent regulation of the interaction of amyloid precursor protein with Fe65 affects the production of β -amyloid. *J Biol Chem* 2001;276:40353–40361.
- 48 Kimberly WT, Zheng JB, Guénette SY, Selkoe DJ: The intracellular domain of the β -amyloid precursor protein is stabilized by Fe65 and translocates to the nucleus in a notch-like manner. *J Biol Chem* 2001;276:40288–40292.

- 49 Ip YT, Davis RJ: Signal transduction by the c-Jun N-terminal kinase (JNK) – from inflammation to development. *Curr Opin Cell Biol* 1998;10:205–219.
- 50 Mielke K, Herdegen T: JNK and p38 stress kinases – degenerative effectors of signal-transduction cascades in the nervous system. *Prog Neurobiol* 2000;61:45–60.
- 51 Scheinfeld MH, Roncarati R, Vito P, Lopez PA, Abdallah M, D'Adamio L: Jun NH₂-terminal kinase (JNK) interacting protein 1 (JIP1) binds the cytoplasmic domain of the Alzheimer's β -amyloid precursor protein (APP). *J Biol Chem* 2002;277:3767–3775.
- 52 Whitmarsh AJ, Cavanagh J, Tournier C, Yasuda J, Davis RJ: A mammalian scaffold complex that selectively mediates MAP kinase activation. *Science* 1998;281:1671–1674.
- 53 Cao X, Südhof TC: A transcriptionally active complex of APP with Fe65 and histone acetyltransferase Tip60. *Science* 2001;293:115–120.
- 54 Hughes AL, Gollapudi L, Sladek TL, Neet KE: Mediation of nerve growth factor-driven cell cycle arrest in PC12 cells by p53. Simultaneous differentiation and proliferation subsequent to p53 functional inactivation. *J Biol Chem* 2000;275:37829–37837.
- 55 Yan GZ, Ziff EB: NGF regulates the PC12 cell cycle machinery through specific inhibition of the Cdk kinases and induction of cyclin D1. *J Neurosci* 1995;15:6200–6212.
- 56 Conti L, Cattaneo E: Controlling neural stem cell division within the adult subventricular zone: an APpealing job. *Trends Neurosci* 2005;28:57–59.
- 57 Tarr PE, Roncarati R, Pelicci G, Pelicci PG, D'Adamio L: Tyrosine phosphorylation of the β -amyloid precursor protein cytoplasmic tail promotes interaction with Shc. *J Biol Chem* 2002;277:16798–16804.
- 58 Milward EA, Papadopoulos R, Fuller SJ, Moir RD, Small D, Beyreuther K, Masters CL: The amyloid protein precursor of Alzheimer's disease is a mediator of the effects of nerve growth factor on neurite outgrowth. *Neuron* 1992;9:129–137.
- 59 Lai EC: Notch signaling: control of cell communication and cell fate. *Development* 2004;131:965–973.
- 60 Jin LW, Saitoh T: Changes in protein kinases in brain aging and Alzheimer's disease. Implications for drug therapy. *Drugs Aging* 1995;6:136–149.
- 61 Ozaki T, Li Y, Kikuchi H, Tomita T, Iwatsubo T, Nakagawara A: The intracellular domain of the amyloid precursor protein (AICD) enhances the p53-mediated apoptosis. *Biochem Biophys Res Commun* 2006;351:57–63.