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Regulation of gene expression by the amyloid precursor protein: inhibition of the JNK/c-Jun pathway

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Abstract

The amyloid precursor protein (APP) has been suggested to regulate gene expression. GeneChip[®] analysis and in vitro kinase assays revealed potent APP-dependent repression of *c-Jun*, its target gene SPARC and reduced basal c-Jun Nterminal kinase (JNK) activity in PC12 cells overexpressing APP. UV-induced activation of the JNK signalling pathway and subsequent apoptosis were likewise reduced by APP and this effect could be mimicked by the indirect JNK inhibitor CEP-11004. Treatment with a γ -secretase inhibitor did not affect APP-mediated downmodulation of the JNK signalling pathway, suggesting that the effects might be mediated via α secretase processing of APP. In support of these data, overexpression of the Swedish mutant of APP did not inhibit SPARC expression, UV-induced JNK activation and cell death. Our data suggest an important physiological role of APP and α -secretase activity in the control of JNK/c-Jun signalling, target gene expression and cell death activation in response to cytotoxic stress.

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Abbreviations: AD, Alzheimer's disease; AICD, APP intracellular domain; AP-1, activator protein-1; APP, amyloid precursor protein; ECM, extracellular matrix; JIP-1b, c-Jun N-terminal kinase (JNK)-interacting protein-1b; JNK, c-Jun N-terminal kinase; MLK, mixed-lineage kinase; MnSOD, manganese superoxide dismutase; NF- κ B, nuclear factor-kappa B; SPARC, secreted protein, acidic and rich in cysteine

Introduction

In recent years, significant progress has been achieved in elucidating the molecular mechanisms leading to neurodegeneration in Alzheimer's disease (AD). The β -amyloid precursor protein (APP), a type 1 transmembrane protein, plays a pivotal role in the pathophysiology of AD.^{1,2} The majority of APP is cleaved by the activity of α -secretase at the cell surface^{1,3} leading to secretion of sAPP α , which might act as a neuroprotective cytokine and regulate gene expression.⁴ In an alternative processing pathway, APP is cleaved by β -secretase at the N-terminus of the A β domain. The generated cleavage fragment can be further processed by the activity of γ -secretase to yield A β , the major constituent of Alzheimer plaques.^{1,3} Generation of A β peptides can be prevented by α -secretase activity, which cuts APP within the A β sequence. Therefore, α - and β -secretase are concurring activities, the latter being favored by a number of missense mutations identified to date within three genes associated with familial AD (FAD): APP, presenilin 1 and presenilin 2. Presenilins are believed to be the active components of ysecretase activity.^{5,6} However, at present, little is known about the biological function of the APP C-terminal region from which the APP intracellular domain (AICD) is cleaved off by γ-secretase.^{2,7} Recent findings indicate that AICD may serve as a membrane-to-nucleus messenger;^{8,9} thus, both sAPP α and AICD might link APP to regulation of transcriptional processes. The YENPTY motif of the C-terminal domain of APP has been shown to bind to several interaction partners, among them Fe65 and c-Jun N-terminal kinase (JNK)interacting protein-1b (JIP-1b).8,10-12 JIP-1b has been shown to serve as a scaffold molecule for JNKs and their upstream kinases^{13,14} and thus may couple APP to the JNK mitogenactivated protein (MAP) kinase signal transduction pathway. The JNK signalling pathway is a central stress signalling pathway implicated in neuronal plasticity, regeneration and cell death of both neuronal and non-neuronal cells.¹⁵ It can be activated by a large variety of cytotoxic stresses, such as oxidative stress, genotoxic stress, ischemia, heat shock and proinflammatory cytokines. In the present study, we investigated the effect of APP on basal gene expression using microarray technology, and demonstrate an important function of APP in the regulation of the JNK/c-Jun signalling pathway and JNK-dependent apoptosis.

Results

Gene expression microarray analysis of APP-mediated transcriptional changes

To analyze APP-mediated transcriptome alterations, we used high-density oligonucleotide microarrays for global analysis of gene expression in PC12 cells. Comparison of APP-dependent changes in basal gene expression (i.e. in the absence of cytotoxic stress) was performed with two independent PC12 cell lines, PC12 APP M5 and PC12 APP N10, overexpressing equal amounts of the human brain-specific isoform APP695,^{16–18} which lacks the the Kunitz-type protease inhibitor (KPI) domain.¹ Empty vector-transfected PC12 cells (PC12neo O1) served as control. Tables 1 and 2 provide the lists of differentially expressed genes (for a detailed description of data analysis, see Materials and Methods) with an at least 1.5-fold expression increase or decrease in both APPoverexpressing cell clones. A total of 34 upregulated and 13 downregulated genes were identified in APP-overexpressing cells. APP significantly activated expression of the genes encoding the mitochondrial antioxidant manganese

Table 1 Genes upregulated in APP-overexpressing cells

Upregulated genes	Accession	M5 <i>N</i> -fold	N10 <i>N</i> -fold
TPO1 ^a	AI012275	2.8	3
Calnexin	Al010725	2.8	2.6
Similiar to XRCC1 ^b	AI010580	2.8	1.7
SNAP-25B	AB003992	2.6	4.6
Similiar to RAB10 ^b	AI230406	2.3	2
MnSoD	Y00497	2.3	1.7
SEL10	AB020504	2.1	2.1
Lipoprotein lipase	L03294	2	2.8
Phosphoserine aminotransferase ^a	AI230228	2	2.6
Tieg	AI071299	2	2.6
Aspartyl-tRNA synthase ^a	AI009682	2	2.1
Inducible carbonyl reductase	D89069	2	1.9
Similiar to NADH dehydrogenase $1\beta^{\text{D}}$	AI171542	2	1.7
Dynactin 4 ^a	AI235707	1.9	2.1
Dnaja2ª	AI170685	1.7	1.5
Cd24 ^a	AI171462	1.7	1.6
Cytochrome c ^a	AI008815	1.7	2
Cdo1 ^a	AA942685	1.6	1.5
Msh2	AA874919	1.6	1.6
Noninducible carbonyl reductase	D89070	1.6	1.6
Ubiquitin conjugating enzyme	M62388	1.6	1.6
Beta-tubulin	AB011679	1.6	1.6
Krüppel-like factor 9	D12769	1.6	1.5
Immunoglobulin binding protein 1°	AI011179	1.6	2.5
RNA helicase	025746	1.6	1.7
Similiar to FK506 binding protein 2°	AI103874	1.5	1.5
Catalase	AA926149	1.5	2.5
Similiar to cytochrome P450°	AA866240	1.5	1./
I ransferrin receptor	M58040	1.5	4.3
MEK5	037464	1.5	1.5
Mt hsp/0	\$75280	1.5	2.6
Syntaxin /	AF031430	1.5	1.9
Primase (DNA POI α subunit III) ZOG	AJU11607 D84336	1.5 1.5	1.6 1.5

^aEST sequence with indicated gene identity on the Affymetrix website. ^bEST sequence without indicated gene identity on the Affymetrix website. Expression profiling by microarray analysis in PC12 APP M5 (M5) and PC12 APP N10 (N10) cells in comparison to the baseline control (PC12 neo O1). cRNA was prepared and hybridized to U34A microarrays after which relative mRNA levels were quantified with the Affymetrix Microarray Suite 5.1 software (see Materials and Methods). To be included, genes had to be at least 1.5-fold upregulated in both APP-overexpressing cell lines (PC12 APP M5 and PC12 APP N10) and the changed P-value had to be below 0.005. Abbreviations: XRCC1, X-ray repair complementing defective repair in Chinese hamster cells 1; TPO1, developmentally regulated protein TPO1; SNAP-25B, synaptosomal-associated protein; MnSoD, manganese superoxide dismutase; RAB10, member RAS oncogene family; SEL10, suppressor and/or enhancer of lin-12 10; Tieg, TGF-beta-inducible early protein; Dnaja2, DnaJ (Hsp40) homolog, subfamily A, member 2; Cd24, CD24 antigen; Cdo1, cytosolic cysteine dioxygenase 1; Msh2, mismatch repair protein; MEK5, mitogen-activated protein kinase kinase 5; ZOG, delta-like homolog (Drosophila); mt hsp70, mitochondrial hsp70; in case genes were found more than once on the chip, the probe set with the highest fold induction values is given

Table 2 Genes downregulated in APP-overexpressing cells

Downregulated genes	Accession	M5 <i>N</i> -fold	N10 <i>N</i> -fold
c-Jun ^a	AA945867	4.6	2.8
Alpha-actin ^a	AA900769	3.0	4.9
S100 A4	X06916	3.0	2.3
SPARC/osteonectin ^a	AA891204	2.8	1.8
SH3-domain kinase binding protein 1 ^a	AA875172	2.3	2.8
Plod2 ^a	AA892897	2.1	2.0
Similiar to filamin 2 ^b	AA799773	2.0	2.5
Aldehyde dehydrogenase	AF001898	1.9	9.2
Pama	AA799575	1.6	2.3
Htr3a	U01227	1.5	1.9
Calponin 3 ^a	AA944422	1.5	1.7
S100 A10	J03627	1.5	1.7
Similiar to MLC, muscle isoform	AA875523	1.5	1.7

^aEST sequence with indicated gene identity on the Affymetrix website. ^bEST sequence without indicated gene identity on the Affymetrix website. Expression profiling by microarray analysis in PC12 APP M5 (M5) and PC12 APP N10 (N10) cells in comparison to the baseline control (PC12 neo O1). cRNA was prepared and hybridized to U34A microarrays after which relative mRNA levels were quantified with the Affymetrix Microarrays Suite 5.1 software (see Materials and Methods). To be included, genes had to be at least 1.5-fold downregulated in both APP-overexpressing cell lines (PC12 APP M5 and PC12 APP N10) and the changed *P*-value had to be above 0.995. Abbreviations: Plod2, procollagen lysine, 2-oxoglutarate 5-dioxygenase 2; SPARC, secreted protein, acidic and rich in cysteine; Pam, peptidylglycine alpha-amidating monooxygenase; Htr3a, 5-hydroxytryptamine (serotonin) receptor 3a; MLC, myosin light chain; in case genes were found more than once on the chip, the probe set with the highest fold induction values is given

superoxide dismutase (MnSOD, 2.3- and 1.7-fold), cytochrome *c* (2.0- and 1.7-fold), the presenilin-interacting protein Sel-10 (2.1- and 2.1-fold), the developmentally expressed protein TPO1 (2.8- and 3.0-fold) and the synaptosomalassociated protein 25 kDa (SNAP-25B, 2.6- and 4.6-fold). Other targets found to be transcriptionally activated by APP included *calnexin* and the mitochondrial heat shock protein gene *mt-hsp70/mortalin* (Table 1). Increased expression of selective APP-induced genes was confirmed by semiquantitative RT-PCR analysis (Figure 1a).

Inhibition of expression of c-Jun/AP-1 target genes and reduced basal JNK activity in APPoverexpressing cells

Interestingly, among the genes most prominently downregulated in APP-overexpressing cells, we found SPARC/ osteonectin and the transcription factor c-Jun (Table 2). SPARC/osteonectin (SPARC: secreted protein, acidic and rich in cysteine) has been shown to be induced in response to c-Jun overexpression and is a proposed c-Jun/activator protein-1 (AP-1) target gene.¹⁹ It is to be noted that, c-Jun also is a c-Jun/AP-1 target gene, and is believed to amplify c-Jun-dependent transcription.²⁰ Downregulation of both *c-Jun* and SPARC/osteonectin was confirmed by RT-PCR analysis in the APP-overexpressing cells (Figure 1a). To analyze the role of the APP metabolism in APP-dependent transcriptional regulation, we employed two PC12 cell lines stably overexpressing KM670/671NL, the Swedish double mutant of APP (APPsw) (Figure 1b), which is expressed at equal levels as APP wild type (APPwt).¹⁶ KM670/671NL is preferentially



Figure 1 Semiquantitative RT-PCR analysis of transcriptional alterations induced by overexpression of APP. (a) Basal expression of putative APP target genes *c-Jun, SPARC/osteonectin, MnSOD* and *Sel-10* was analyzed in PC12 APPwt M5 and PC12 APPwt N10 cells, with PC12 neo O1 (neo) serving as a negative control. Expression of *GAPDH* served as an internal control. Expression levels of individual genes in PC12 APPwt M5 and PC12 APPwt N10 relative to the PC12 neo O1 control were analyzed with the ImageJ software. (b) Expression of *c-Jun* target gene *SPARC/osteonectin* is not altered by application of a γ -secretase inhibitor. RT-PCR analysis in PC12 APPwt N10, PC12 APPwt M5, PC12 APPwt N10, PC12 APPsw Q8 and PC12 APPsw Q7 cells left untreated (upper panel) or treated with 10 μ M γ -secretase inhibitor (γ -SI, lower panel) for 24 h. Expression of *GAPDH* served as an internal control

processed via the amyloidogenic β -secretase pathway and is known to be associated with enhanced A β levels and abrogation of sAPP α formation. In comparison to APPwtoverexpressing cells, expression of SPARC/osteonectin was significantly enhanced in cells overexpressing APPsw (Figure 1b). A fenchylamine sulfonamide γ -secretase inhibitor²¹ had no discernible effect in any of the cell lines (Figure 1b), indicating that APP processing by α -secretase might be required for the APP-mediated inhibition of SPARC/ osteonectin expression. We subsequently investigated whether APP exerts a regulatory effect on the JNK signalling pathway. To this end, we analyzed basal JNK activity using an in vitro kinase assay (Figure 2a). Incubation with cellular extracts of PC12 neo cells and PC12 APPsw cells led to detectable amounts of phosphorylated recombinant GSTc-Jun, which were greatly diminished after incubation with extracts of PC12 APPwt cells.



Figure 2 APPwt inhibits both basal and stress-induced JNK activity. (a) PC12 neo O1, PC12 APPwt M5 and PC12 APPsw Q8 cultures were UV-irradiated (UV) or left untreated (Basal). JNK activity was analysed by a GST-c-Jun in vitro kinase assay (upper panel). To confirm equal c-Jun levels in the samples, the residual protein in the gel was stained with Simply Blue Safe Stain (Invitrogen Life Technologies) after blotting (lower panel). (b, c) APPwt inhibits UV-triggered JNK phosphorylation. For analysis of JNK activation, cultures of PC12 neo O1, PC12 APP M5 and PC12 APPsw Q8 (b, left panel) or PC12 neo O3, PC12 APPwt N10 and PC12 APPsw Q7 (c) were subjected to UV irradiation (UV) or left untreated (Basal). At 4 h postirradiation, cultures were lysed and subjected to Western blotting with the anti-ACTIVE[®] JNK antiserum. Effect of a γ -secretase inhibitor (y-SI) on UV-induced JNK activation (b, right panel) is shown. PC12 neo O1, PC12 APP M5 and PC12 APPsw Q8 cells were preincubated with γ -SI for 24 h at 10 μ M, UV-irradiated and continuously incubated in the presence of γ -SI for 4 h postirradiation. (d) APPwt inhibits basal and UV-induced c-Jun expression. For analysis of c-Jun levels, cultures of PC12 neo O1 and PC12 APP M5 were subjected to UV irradiation (UV) or left untreated (Basal). At 4 h postirradiation, cultures were lysed and subjected to Western blotting with the c-jun (Ab-3) antibody. JNK1 served as an internal loading control

APP inhibits UV-induced JNK activation and cell death

We also determined whether APP modulated stress-induced JNK activity. UV irradiation triggers activation of the JNK signalling pathway in a wide array of cell types, including neurons.²² UV induced a marked elevation of the active forms of JNK1/JNK2 in two independent vector-transfected PC12 cell lines (PC12 neo O1 and PC12 neo O3) (Figure 2b, left panel, and Figure 2c) as detected with a phospho-specific antibody binding to the dually phosphorylated Thr/Pro/Tyr region of the JNKs. Remarkably, UV-triggered JNK phosphorylation was potently inhibited in the two APPwt-overexpressing cell lines (PC12 APPwt M5 and PC12 APPwt N10), but not in the APPsw-overexpressing cell lines (PC12 APPsw Q8

and PC12 APPsw Q7) (Figure 2b, left panel, and Figure 2c). APPwt also potently downmodulated UV-induced JNK activity detected with the *in vitro* kinase assay (Figure 2a). The γ -secretase inhibitor had no effect on stress-induced JNK activation in APPwt and APPsw cells (Figure 2b, right panel). In addition to the inhibitory effect of APPwt on *c-Jun* transcription (Figure 1a), we observed significantly lowered *c-Jun* protein levels in PC12 APPwt cells, both under basal conditions and after UV irradiation (Figure 2d).

In light of the strong inhibitory effect of APP on UV-triggered JNK activation, we investigated whether APP overexpression affected cell survival after UV irradiation. The JNK signalling pathway is known to play a fundamental role in UV-triggered, caspase-dependent apoptosis.^{23,24} To elucidate the potential role of APP in modulation of UV-induced apoptosis, we analyzed the activation of the effector caspase caspase 3 by Western blotting. In comparison to neo cells, we found significantly lowered amounts of the active caspase 3 subunit in extracts of UV-treated PC12 APPwt cells, but not in PC12 APPsw cells (Figure 3a). To investigate nuclear alterations during UV-induced apoptosis, we performed Hoechst staining 16h postirradiation. A large number of nuclei were condensed and/or fragmented in UV-treated PC12 neo cells. In contrast, the number of apoptotic nuclei of UV-treated PC12 APP cells was markedly lower (Figure 3b). Analysis of effector caspase activation with an enzymatic assay revealed that APPwt not only suppressed UV-triggered JNK activation but also significantly reduced UV-triggered activation of DEVDases in comparison to PC12 neo controls (Figure 3c). Since APPsw cells showed no reduction of JNK activation after UV irradiation, we also compared the effect of overexpressed APPwt and APPsw on UV-triggered apoptosis. In contrast to APPwt, APPsw did not enhance cellular resistance against apoptosis (Figure 3c). Since $A\beta$ has been implicated in caspase-independent cell death,²⁵ we also performed a quantitative comparison of apoptosis and (primary and secondary) necrosis in UV-treated cultures by simultaneous staining with Hoechst and propidium iodide (PI) (Figure 3d). In concordance with the DEVDase assays, overexpression of APPwt reduced the percentage of apoptotic cells after UV irradiation compared with vector-transfected controls. It is to be noted that overexpression of APPsw significantly increased necrotic cell death in the UV-treated cultures.

The death-inhibiting function of APP can be mimicked by an MLK inhibitor

Further proof for the critical involvement of the JNK signalling pathway and its regulation by APP in UV-triggered apoptosis was obtained from experiments with the synthetic and specific mixed-lineage kinase (MLK) inhibitor CEP-11004^{26,27} (Figure 4). MLKs have been shown to bind to JIP-1b and are known upstream activators of JNKs. CEP-1004 almost completely abolished UV-induced Ser63 phosphorylation of c-Jun after UV irradiation in PC12 neo cells (Figure 4a). Application of CEP-11004 downmodulated apoptosis in PC12 neo cells to a level comparable to that observed in PC12 APPwt cells, while it had only a very moderate



Figure 3 Overexpression of APP protects PC12 cells from UV-triggered apoptosis. (a) Analysis of UV-induced procaspase 3 cleavage in PC12 neo O1 (neo) and PC12 APP M5 (APP) cells treated with UV. Active caspase 3 p17 subunits were detected by Western blotting. (b) Nuclear apoptosis visualized by Hoechst staining. Cultures of PC12 neo O1 (neo) and PC12 APP M5 cells (APP) were UV-irradiated or left untreated. Images were taken 20 h postirradiation. Scale bar: 25 µm. (c) PC12 neo O1 (neo), PC12 APPwt M5 (M5), PC12 APPwt N10 (N10), PC12 APPsw Q8 (Q8) and PC12 APPsw Q7 (Q7) cells were UVirradiated (UV). Control cultures were left untreated (Con). At 20 h postirradiation, the cultures were lysed and caspase 3-like activity was monitored over 1 h. Data are means \pm S.E.M. from n = 4 cultures per treatment. Experiments were repeated two times with similar results. AU = arbitrary fluorescence units. *P<0.05 difference from vehicle-treated controls. . P<0.05: difference from respective control cell line PC12 neo. (d) Parallel quantification of apoptosis and (primary and secondary) necrosis after UV irradiation. PC12 neo O1 (neo), PC12 APPwt M5 (APP) and PC12 APPsw Q8 (sw) cells were UV-irradiated (UV) or left untreated (Con). At 20 h postirradiation, the percentage of apoptotic and necrotic cells in the different cultures was analyzed as described in Materials and Methods. Data are means \pm S.E.M. from n = 4 cultures per treatment. Experiments were repeated two times with similar results. *P<0.05: difference from vehicle-treated controls. , P<0.05: difference from respective control cell line PC12 neo

protective effect in the presence of overexpressed APP (Figure 3b).

Discussion

Here, we analyzed the effect of APP on global gene expression in PC12 cells. Our data demonstrate that APP induces a specific subset of neuroprotective and antioxidative genes, genes regulating mitochondrial function, as well as developmental genes. GeneChip analysis also demonstrated that APP potently downmodulated basal expression of *c-Jun* and the c-Jun/AP-1 target gene *SPARC/osteonectin*. Furthermore, we established an inhibitory role of APP in the regulation of the stress-induced JNK signalling cascade.



Figure 4 (a) CEP-11004 significantly inhibits c-Jun phosphorylation. PC12 neo O1 cells were UV-irradiated (UV) or left untreated (Con). Where indicated, CEP-11004 was added at a final concentration of 200 nM 2 h prior to the irradiation time point. c-Jun phosphorylation was analyzed by Western blotting with the polyclonal phospho-c-Jun (Ser63) II antibody with JNK1 serving as a loading control. (b) The synthetic MLK inhibitor CEP-11004 can mimic the neuroprotective function of APP. PC12 neo O1 (neo) and PC12 APPwt M5 (APP) cells were UV-irradiated (UV) or left untreated (Con). Where indicated, CEP-11004 was added at a final concentration of 200 nM 2 h prior to the irradiation time point. At 20 h postirradiation, the cultures were lysed and caspase 3-like activity was monitored over one hour. Data are means \pm S.E.M. from n = 4 cultures per treatment. Experiments were repeated two times with similar results. AU = arbitrary fluorescence units. *P < 0.05: difference from vehicle-treated controls

At least two proteolytic cleavage products of APP, sAPP α and AICD, which are released by y-secretase cleavage, have been previously implicated in the regulation of transcriptional processes by APP.^{4,8} It has been reported that sAPP α may exert its neuroprotective effect by activation of the nuclear factor-kappa B (NF- κ B) signalling pathway.⁴ Our microarray analysis of global changes in gene expression revealed APPmediated induction of MnSOD, which has been described as an NF- κ B target gene.²⁸ In addition, APP activated expression of TPO1, genes regulating protein folding (mt-hsp70/mortalin) and ATP production (cytochrome-C) in mitochondria, as well as the ER chaperone calnexin. Interestingly, TPO1 was suggested to be a key player in myelin biogenesis,²⁹ linking APP to protection from white matter degeneration known to occur in AD.³⁰ Disturbance of the oxidative stress response (MnSOD) and mitochondrial function (mt-hsp70/mortalin) has been suggested to play an important role in the pathogenesis of AD.³¹ Calnexin serves both as a molecular chaperone and a calcium ion storage protein.³² In light of the fact that ER Ca²⁺ store depletion has been implicated in UV-triggered apoptosis³³ and the pathophysiology of AD,³⁴ induction of proteins regulating ER Ca²⁺ homeostasis might be an underlying mechanism of the observed neuroprotective effects of APP.

It is to be noted that our microarray analysis suggested that overexpression of APP provoked a pronounced inhibition of c-Jun/AP-1 target gene expression. This downregulation was associated with a potent reduction in JNK activity. Interestingly, it was recently demonstrated that inhibition of c-Jun by a dominant-negative c-Jun peptide also leads to activation of MnSOD expression.³⁵ This suggests that in addition to its implication in the NF-*k*B pathway (see above), APP-mediated activation of MnSOD might be causally linked to the observed inhibition of the JNK signalling pathway. The proposed c-Jun/ AP-1 target gene SPARC/osteonectin belongs to the group of matricellular proteins. Components of the extracellular matrix (ECM) mediate neuron-glia interactions, thus stabilizing the neuronal-astroglial network. It is to be noted that expression of SPARC/osteonectin has been shown to weaken cellular adhesion to the ECM by counteracting integrin-ECM interactions.³⁶ By controlling the level of SPARC/osteonectin expression, APP may counteract neurodegeneration and neuronal cell death induced by matrix detachment (anoikis) and loss of cell/cell contacts. It is tempting to speculate that downmodulation of SPARC/osteonectin may be linked to ADassociated neuronal atrophy and progressive loss of neurons during the disease.

Disruption of the JNK/c-Jun signalling pathway has been shown to alleviate apoptosis triggered by UV, a classical stimulus of the JNK signalling pathway.23,24 Following UV irradiation, we observed a potent APP-mediated inhibition of JNK activation, which was accompanied with repression of c-Jun and significant protection from UV-induced cell death. In another study, APP was shown to inhibit neuronal cell death induced by nerve growth factor (NGF)- deprivation,³⁷ which is known to require activation of the JNK pathway.³⁸ Collectively, these data suggest that following neuronal insults, APP may exert neuroprotective effects by blocking activation of the proapoptotic JNK signalling cascade. MLKs are known to be essential upstream components of UV-induced JNK activation.³⁹ Indeed, application of the MLK inhibitor CEP-11004 almost completely abolished UV-induced c-Jun phosphorylation in PC12 neo cells. CEP-11004 also significantly reduced UV-induced apoptosis in PC12 neo cells to a level comparable to that observed in PC12 APPwt cells. Thus, our data suggest that APP-mediated protection from UV-triggered cell death may to a large extent be attributable to the observed inhibition of JNK activation. It is to be noted that JNKs can also activate apoptosis by transcription-independent mechanisms. For example, it has been shown that JNKs activate translocation of Bax and Bim to the mitochondria. 40,41

Increasing evidence points to a pivotal role of the JNK signalling pathway in the development of neurodegenerative diseases, such as AD.^{42,43} *In vitro* exposure of neurons to A β peptides has been shown to induce cell death via activation of the JNK signalling cascade.^{44,45} Enhanced JNK activation has also been demonstrated in degenerating neurons of postmortem brain sections from AD patients.^{45,46} It was recently demonstrated that the C-terminal domain of APP is physically bound to the adapter protein JIP-1b,^{10–12} which serves as a

scaffolding protein for JNKs and upstream kinases such as MKK4, MKK7 and MLKs. Interestingly, our experiments revealed that the Swedish APP mutant did not inhibit stressinduced JNK signalling. Full-length APPsw was previously shown to bind JIP1b with a similar efficiency as APPwt, suggesting that APP-mediated JNK inhibition is not due to sequestration of JIP-1b by APP and prevention of scaffolding of JNKs and their upstream kinases. Furthermore, the inhibitory effect on JNK signalling could not be restored by application of a γ -secretase inhibitor in PC12 APPsw cells, indicating that extracellular sAPPa rather than AICD might be the proteolytic cleavage product responsible for antagonizing JNK signalling. Although sAPP α has been described as an injury-induced neurotrophic factor,47 the receptor that is required for transducing its neuroprotective effect has not been identified so far. However, it has been shown that APPwt, but not mutant APP, can activate the antiapoptotic PI3K/Akt pathway,⁴⁸ which is known to block the JNK pathway via phosphorylation of MLK3.49 Furthermore, JNK activation increases non-amyloidogenic α-secretase cleavage of APP,⁵⁰ indicating the possible existence of a negative feedback loop between APP and the JNK pathway after neuron damage. In light of the observed inhibitory effect of APPwt on JNKs, overactivation of the JNK signalling pathway in AD⁴⁴⁻⁴⁶ might reflect a potential loss of the physiological function of APP. Parallel to this loss of function, enhanced A β production might further enhance JNK activation, oxidative stress and mitochondrial dysfunction in AD.

Materials and Methods

Materials

Acetyl-DEVD-7-amido-4-methylcoumarin (Ac-DEVD-AMC) was purchased from Bachem (Heidelberg, Germany). The MLK inhibitor CEP-11004 was a generous gift from Cephalon Inc. (West Chester, USA).²⁶ The γ -secretase inhibitor 1-(S)-*endo-N*-(1,3,3)-trimethylbicyclo[2.2.1]hept-2-yl)-4-fluorophenyl sulfonamide²¹ was from Merck Biosciences (Schwalbach, Germany). All other biochemicals and chemicals came in analytical grade purity from Roche Diagnostics (Mannheim, Germany) or Roth (Karlsruhe, Germany).

Cell culture

PC12 cell lines expressing equal amounts of the human wild-type APP695 isoform (PC12 APPwt, clones M5 and N10), PC12 cell lines expressing the Swedish mutant of APP, KM670/671NL (PC12 APPsw, clones Q8 and Q7), as well as vector-transfected control cells (PC12 neo, clones O1 and O3) have been described previously.^{16–18} Cell lines were cultivated in DMEM supplemented with 5% heat-inactivated fetal calf serum, 10% horse serum, 100 U/ml penicillin and 100 μ g/ml streptomycin.

UV irradiation of cells

For UV irradiation of cells, the culture medium was aspirated, and cells were exposed to a dose of $100 \text{ J/m}^2 \text{ UV}$ in a Stratalinker[®] UV Crosslinker (Stratagene, Amsterdam, The Netherlands). After UV irradiation, the culture medium was immediately returned to the respective cultures.

Measurement of caspase 3-like protease activity

Cells were lysed in 200 μ l lysis buffer (10 mM HEPES, pH 7.4, 42 mM KCl, 5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 1 μ g/ml pepstatin A, 1 μ g/ml leupeptin, 5 μ g/ml aprotinin, 0.5% 3-(3-cholamidopropyldimethylammonio)-1-propane sulfonate (CHAPS)). A 50 μ l portion of this lysate was added to 150 μ l reaction buffer (25 mM HEPES, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 3 mM DTT, pH 7.5) and 10 μ M of the fluorigenic substrate Ac-DEVD-AMC. This substrate is preferentially cleaved by caspase 3, and to a lesser extent by caspases 6, 7, 8, 9, and 10.⁵¹ Accumulation of AMC fluorescence was monitored over 1 h using an HTS fluorescent plate reader (excitation 380 nm, emission 465 nm). Fluorescence of blanks containing no cell lysate was subtracted from the values. Protein content was determined using the Pierce Coomassie Plus Protein Assay reagent (KMF, Cologne, Germany). Caspase activity is expressed as change in fluorescence units per microgram protein per hour.

Quantification of apoptosis

Chromatin condensation and fragmentation, which are nuclear hallmarks of apoptosis, were visualized using the DNA-binding fluorescent dye Hoechst 33258 (Sigma, Deisenhofen, Germany). Cells cultured on 24-well plates were UV-irradiated and stained live with Hoechst 33258 at a final concentration of 1 μ g/ml. After incubation for 10 min, nuclear morphology was observed using an Eclipse TE 300 inverted microscope (Nikon, Düsseldorf, Germany) and a \times 20 dry objective with the following optics: excitation, 340–380 nm; dichroic mirror, 400 nm; emission, 435–485. Digital images were acquired with a SPOT-2 camera using SPOT software version 2.2.1 (Diagnostic Instruments, Sterling Heights, MI, USA). For each time point and treatment, a total number of 300 cells were analyzed for apoptotic morphology in three subfields of each culture. All experiments were performed at least three times with similar results.

Western blot

A 30 μ g portion of whole cell lysate (lysis buffer: 68.5 mM Tris/HCl pH 6.8, 2% SDS, 10% glycerol, and protease inhibitors) was loaded onto 12 or 15% SDS–polyacrylamide gels. Proteins were separated at 120 V and then blotted to nitrocellulose membranes (Protean BA 83; 2 μ m; Schleicher & Schuell, Dassel, Germany) in Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol (v/v) and 0.01% SDS) at 18 V for 50 min. The blots were blocked in blocking buffer (5% non-fat milk, 15 mM Tris-HCl pH 7.5, 200 mM NaCl and 0.1% Tween 20) at room temperature for 2 h. Major isoforms of JNKs are p46 JNK1 and p54 JNK2.

For detection of activated JNK1 and JNK2, we diluted the rabbit polyclonal anti-ACTIVE[®] JNK antiserum (Promega, Heidelberg, Germany) 1:5000 in Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA) and 0.1% Tween 20. Primary antibodies were detected by subsequent incubation with a biotinylated anti-rabbit antibody (1:2000; Pharmingen Becton Dickinson, Hamburg, Germany) in blocking solution (TBS containing 3% non-fat milk powder and 0.1% Tween 20) and a horseradish peroxidase (HRP)-conjugated anti-biotin antibody (1:2000; New England Biolabs, Frankfurt, Germany) in blocking solution. Activation of c-Jun and effector caspases was detected with a polyclonal phospho-c-Jun (Ser63) II antibody (New England Biolabs, Frankfurt, Germany), and a rabbit polyclonal anti-active p20 caspase 3 antiserum (Merck-Frosst, Kirkland, Canada; 1:1000 dilution). c-Jun protein levels were detected with the monoclonal c-jun (ab-3) antibody (Merck Biosciences). To ensure equal loading of the samples, membranes were incubated with a mouse monoclonal anti-JNK1 antibody (clone 333, 1:1000; BD Biosciences,

Hamburg, Germany). All antibodies were diluted in blocking solution and blots were incubated overnight at 4°C. Primary antibodies were detected using HRP-conjugated goat anti-mouse or anti-rabbit IgG (Promega, Heidelberg, Germany) used at 1:5000 in blocking solution. Blots were developed using the Pierce SuperSignal substrate chemiluminescence reagent.

In vitro kinase assays

For determination of JNK activity, a SAPK/JNK Assay Kit was employed (New England Biolabs, Frankfurt, Germany). Briefly, PC12 clones were seeded in six-well plates and UV-irradiated as described. Lysates of UV-treated cultures and control cultures were prepared and incubated with c-Jun fusion beads as described by the manufacturer. After incubation of the obtained pellets in kinase buffer supplemented with 100 μ M ATP for 30 min, kinase reactions were terminated by addition of SDS sample buffer. Reactions were subjected to SDS–PAGE and Western blotting with a Phospho-Ser63 c-Jun antibody and an HRP-conjugated anti-rabbit secondary antibody. Blots were developed using the Pierce SuperSignal substrate chemiluminescence reagent.

Microarray analysis

Total RNA of cell lines PC12 neo O1, PC12 APP M5 and PC12 APP N10 was extracted using the RNeasy Midi Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 20-40 μ g of the total RNA by using the Superscript Choice Kit (Invitrogen Life Technologies, Karlsruhe, Germany). The cRNA was prepared and biotin-labelled by in vitro transcription (Enzo Biochemical, Farmingdale, NY, USA). Labelled cRNA was fragmented by incubation at 94°C for 35 min in the presence of fragmentation buffer (40 mM Tris acetate, pH 8.1, 100 mM potassium acetate and 30 mM magnesium acetate). A 15 μ g portion of fragmented cRNA was hybridized for 16 h at 45°C to a rat U34A array (Affymetrix, Santa Clara, CA, USA). After hybridization, the gene chips were automatically washed and stained with streptavidin-phycoerythrin by using a fluidics station. Finally, probe arrays were scanned at $3 \,\mu$ m resolution using the Genechip System confocal scanner (Aligent Technologies, Palo Alto, CA, USA). The Affymetrix Microarray Suite 5.0 was used to scan and analyze the relative abundance of each gene from the average difference of intensities. For each individual gene, the signal ratio between perfect match and mismatch probe cells was used to determine its 'Absolute Call', indicating whether the corresponding transcript was present (P), absent (A) or marginal (M). For analysis of differential target gene expression, the 'Difference Call Decision Matrix' was employed. Individual transcript levels were ranked as either increased (I), marginally increased (MI), decreased (D), marginally decreased (MD) or not changed (NC). To be considered as differentially regulated, the Absolute Call of individual transcripts had to be present or marginal, and the Difference Call increased/decreased or marginally increased/marginally decreased. The gene identity of the obtained EST sequences represented on the Chips is referring to the assignment given on the Affymetrix Homepage, NetAffx Analysis Center (www.affymetrix.com). EST sequences without given gene identities were analyzed by PubMed database searches (standard nucleotide-nucleotide BLAST (blastn), www. ncbi.nlm.nih.gov).52

Semiquantitative RT-PCR

A 1 μ g portion of total RNA was used in an RT reaction together with 2.5 μ g of random hexamers and dNTPs at a final concentration of 0.5 mM.

After heating to 65°C for 5 min, the mixtures were chilled on ice and supplemented with reaction buffer, DTT (final concentration 10 mM) and 200 U Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). First-strand cDNA synthesis was carried out at 42°C for 50 min. Reactions were stopped by incubation at 70°C for 15 min. cDNAs were subsequently analyzed for expression of putative APP target genes with GAPDH serving as internal control. PCR reactions were performed with Taq polymerase (Eppendorf, Hamburg, Germany) according to the instructions of the manufacturer. Cycle numbers were 25 for c-Jun, SPARC/osteonectin and GAPDH, 30 for Sel-10 and 35 for MnSOD (the sequences of the employed primers can be obtained upon request from the authors). PCR products were separated by agarose gel electrophoresis (1.5%) and visualized with 0.1% ethidium bromide under UV transillumination using a CCD camera-based gel documentation system (MWG, Ebersberg, Germany). Levels of expression in PC12 APPwt clones relative to PC12 neo O1 controls were quantified densitometrically using the public domain Java image processing program ImageJ.

Statistics

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Data are given as means \pm S.E.M. For statistical comparison, *t*-test or one-way ANOVA followed by Tukey's test was employed. *P*-values smaller than 0.05 were considered to be statistically significant.

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