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# Alzheimer-associated presenilin-2 confers increased sensitivity to apoptosis in PC12 cells

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**Abstract Presenilin-2 is a gene of unknown function recently identified based upon linkage with some forms of familial Alzheimer's disease. To investigate potential effects of PS-2 on cell viability, rat pheochromocytoma (PC12) cells were stably transfected with cDNA constructs encoding either full-length human PS-2 or, for comparison, mouse Bcl-$x_L$. Overexpression of PS-2 conferred increased sensitivity to the apoptotic stimuli staurosporine and hydrogen peroxide. In contrast, Bcl-$x_L$ overexpression significantly reduced cell death induced by these stimuli. These results suggest that one function of PS-2 may involve modulation of cell viability.**

*Key words:* Presenilin-2; Apoptosis; Bcl-$x_L$; Alzheimer's disease; Stable transfection; PC12

## 1. Introduction

The presenilin-2 (PS-2/STM2) gene was recently identified as the chromosome 1 locus of missense mutations linked to familial Alzheimer's disease (AD) within specific kindreds [1,2]. Although the etiology of AD is unknown, elucidating the contributions of genes linked to the disease promises to provide insight into relevant pathological pathways. In addition to PS-2, genes implicated in AD pathogenesis include allelic variation of apolipoprotein E on chromosome 19 [3,4] as well as mutations of amyloid precursor protein gene (APP) on chromosome 21 [5–7] and presenilin 1 (PS1/S182) on chromosome 14 [8]. Although cellular roles of apolipoprotein E and APP have been reported, the normal function(s) of the related PS-2 and PS-1 genes has not been determined.

Recently, it has been suggested that presenilins may play a role in protein processing and trafficking since mutations in both PS-1 and PS-2 alter APP processing [9]. Another potential functional role of PS-2 that may be consistent with its AD association is modulation of apoptotic cell death. Apoptosis or programmed cell death is an efficient mechanism for cellular removal that is associated with both the normal development and maintenance of many tissues, including brain [10]. We have proposed that apoptotic pathways also may contribute to pathological cell death in chronic neurodegenerative diseases such as AD [11]. Recently, it was reported that a T cell hybridoma transfected with ALG-3, a partial cDNA that appears to be the mouse homologue of PS-2, exhibits decreased vulnerability to T cell receptor- and Fas-induced apoptosis [12]. Unclear, however, is whether the cell death resistance conferred by the truncated PS-2 homologue represents dominant negative inhibition of full-length PS-2 and or direct anti-apoptotic activity of a putatively translated ALG-3 polypeptide. To examine further the potential actions of PS-2 gene in regulation of cell viability, we stably transfected PC12 cells with a full-length human PS-2 cDNA construct and compared its effects on cell viability relative to wild type PC12 cells and those transfected with either empty vector or the anti-apoptotic gene Bcl-$x_L$.

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*Abbreviations:* AD, Alzheimer's disease; APP, amyloid precursor protein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; pcDNA-Bcl-$x_L$, Bcl-$x_L$ transfected cell line; pcDNA-Neo, empty vector transfected cell line; pcDNA-PS2, presenilin-2 transfected cell line; PS-1, presenilin-1; PS-2, presenilin-2

## 2. Materials and methods

### 2.1. cDNA cloning and stable transfection

To obtain a human PS-2 cDNA, a 1 μl aliquot from the human brain cDNA library (Stratagene) was amplified by polymerase chain reaction (PCR) with the following primers [1]: forward GCCAAGAATTCGTGGTGCTTCCAGAGGCA (containing the *Eco*RI restriction site) and reverse GCAGCTCTAGAAAATTCCCTGCAGCTTGCA (containing the *Xba*I restriction site). To obtain full length Bcl-$x_L$ cDNA [13], first strand cDNA synthesized from mouse brain mRNA using a cDNA cyclic kit (Invitrogen) was amplified by PCR with following primers: forward TGGCTCTAGAGACCTTCCGGGGGTTGTACC (containing *Xba*I restriction site) and reverse TGGCTCTAGACAGTGTCTGGTCACTTCCGA (containing *Xba*I restriction site). PCR was performed under the following conditions: 94°C, 1 min; 60°C, 1 min; and 72°C, 2 min (PS-2) or 1 min (Bcl-$x_L$) for 30 cycles. PCR products were digested by corresponding restriction enzymes and ligated to a pcDNA3 vector (Invitrogene). The orientation and sequences of positive inserts were verified using sequenase 2 on double strands. PC12 cells were transfected by electroporation (Electro Cell Manipulator ECM 100, BTX) with the pcDNA3 plasmid containing either human PS-2 or mouse Bcl-$x_L$ under CMV promoter. As a control, transfections were also performed with the pcDNA3 plasmid without an insert. After G418 (800 μg/ml) selection for 2 weeks, colonies were randomly selected by limited dilution.

### 2.2. RNA extraction and Northern blot analysis

Total RNA was isolated from the different colonies by the guanidine thiocyanate method [14]. 10-μg aliquots of total RNA were denatured in 2.2 M formaldehyde at 65°C for 15 min and electrophoresed on 1.2% agarose/2.2 M formaldehyde gels at 50 V for 4 h. The separated RNA was transferred to nylon membrane by the overnight capillary blotting method in 6×SSC buffer and immobilized by UV irradiation. Hybridization was performed with [$^{32}$P]dCTP-labeled human PS2 and mouse Bcl-$x_L$ cDNA fragment in a solution containing 5×SSPE, 5×Denhardt's, and 1% SDS at 65°C overnight. The blots were washed twice in 2×SSPE/0.5% SDS, 0.5×SSPE/0.5%SDS and 0.2×SSPE/0.5%SDS solution at 65°C, 15 min each and then were exposed to film at −80°C for 3–5 days.

### 2.3. Cytotoxicity assays

To assess cell survival, WT and transfected PC12 cells were plated on poly(L-lysine)-coated multiwell plates at $1\times10^4$ cells/cm$^2$ in Dulbecco's modified Eagle's medium (DMEM) medium containing 10%


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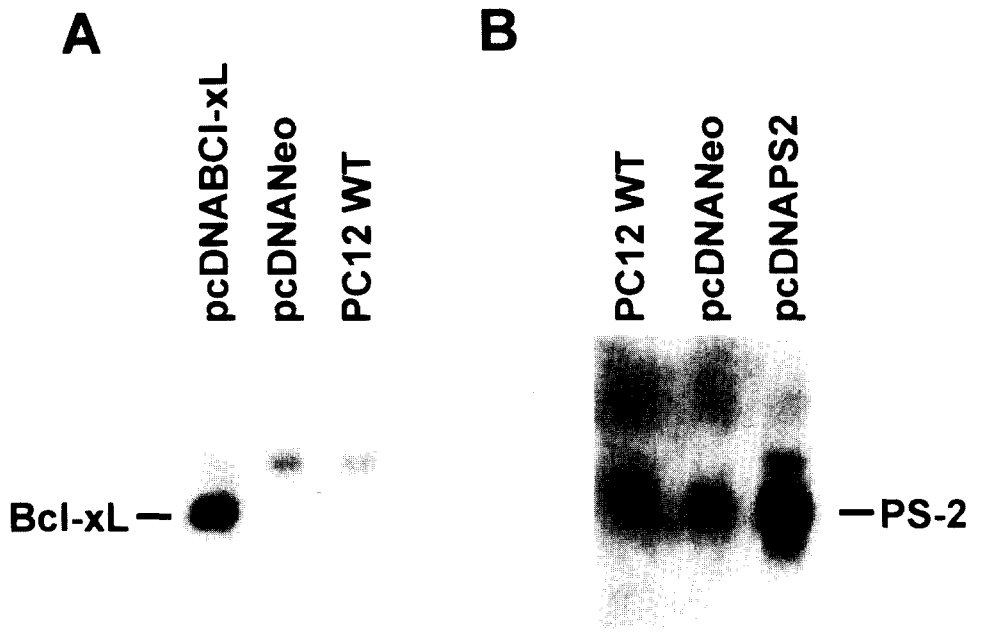


Fig. 1. Expression of Bcl-$x_L$ and PS-2 mRNA in transfected and wild type PC12 cells. 10 μg of total RNA isolated from the indicated clones was separated on 1.2% agarose/formaldehyde gels. Northern blot analysis was carried out with either a 0.9 kb mouse Bcl-$x_L$ or a 1.4 kb human PS-2 probe. (A) Expression of Bcl-$x_L$ mRNA. An approx. 2.4 kb endogenous transcript appeared in PC12 wild type (PC12WT), empty vector (pcDNA-Neo) and Bcl-$x_L$ (pcDNA-Bcl-$x_L$) transfectants. A major Bcl-$x_L$ band was expressed in pcDNA-Bcl-$x_L$. (B) Expression of PS-2 mRNA. An approx. 2.5 kb endogenous transcript was found in all clones. Human PS-2 transfectant (pcDNA-PS2) expressed two more bands derived from pcDNA-PS2 vector.

fetal bovine serum (FBS). After 1 day in vitro, cultures were shifted to DMEM/1% FBS for 1 day and then to serum-free DMEM containing different levels of staurosporine and hydrogen peroxide for 18–24 h. Over the 18–24 h experimentation period, the absence of serum did not result in significant cell loss. Cell viability was quantitatively assessed by spectrophotometric measurement of MTT reduction [15]. These data were verified by morphological evaluation under bright-field and phase contract optics and by nuclear examination under fluorescent illumination following addition of the nucleic acid stain Syto-11 (Molecular Probes). All conditions were performed in triplicate wells and were repeated in at least 3 independent experiments with similar findings. To permit comparisons between cell lines, raw MTT data were normalized to percentage values of untreated controls and statistically examined using ANOVA followed by Fisher LSD test. For graphical presentation, data from single, representative experiments are shown.

## 3. Results

### 3.1. Transfected PC12 cells express human PS-2 and mouse Bcl-$x_L$ RNAs

PC12 cells were transfected with either empty vector (pcDNA-Neo), human PS-2 gene (pcDNA-PS2) or mouse Bcl-$x_L$ (pcDNA-Bcl-$x_L$) and stable transfectants were selected by G418 exposure. Successful expression of PS-2 and Bcl-$x_L$ constructs was confirmed by Northern blot analysis (Fig. 1). Northern blots revealed that WT and pcDNA-Neo cells have low endogenous levels of PS-2 expression with an approx. 2.5 kb transcript. In addition, pcDNA-PS2 cells exhibit one major transcript corresponding to the coding region ($\approx$1.4 kb) and

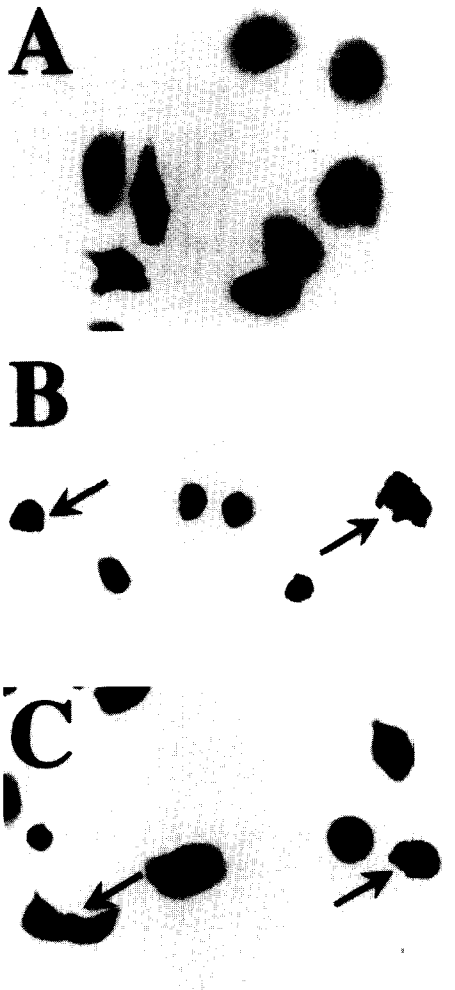


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Fig. 2. Nuclear staining with the dye Syto-11 viewed under fluorescent illumination shows pyknotic nuclei (arrows), a characteristic of apoptosis, in PC12 WT cultures treated with 4 nM staurosporine (B) and 100 μM hydrogen peroxide (C) but not in untreated controls (A). For clarity, figure shows negative images.

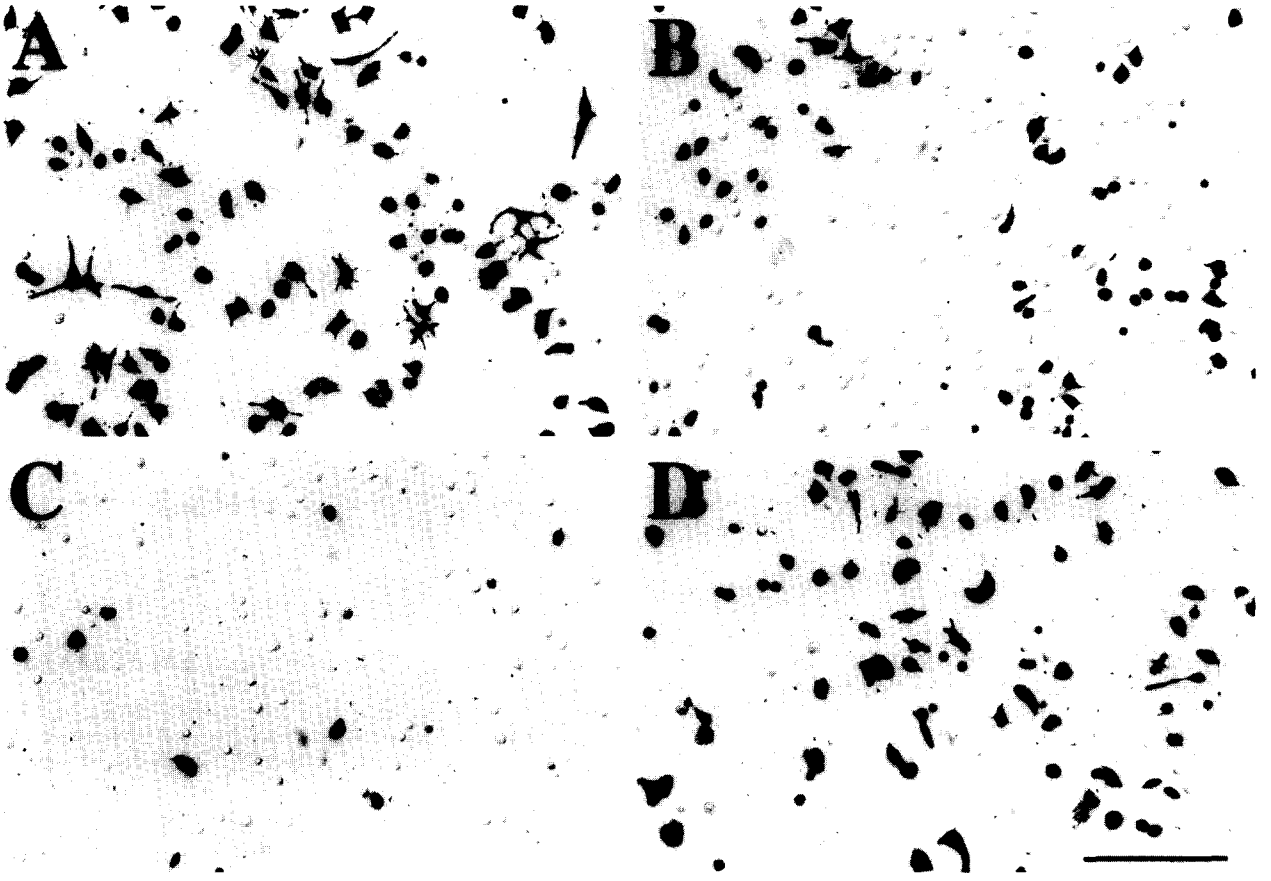


Fig. 3. Microscopic inspection of cultures shows that staurosporine toxicity is potentiated in PS-2 transfected cells but attenuated in Bcl-$x_L$ transfected cells: (A) untreated pcDNA-Neo cells, (B) pcDNA-Neo cells treated with 4 nM staurosporine, (C) pcDNA-PS2 cells treated with 4 nM staurosporine, (D) pcDNA-Bcl-$x_L$ cells treated with 4 nM staurosporine. Viable cells are darkly stained with blue formazan product following 1 h exposure to MTT reagent [15], as viewed under bright-field microscopy. Scale bar, 100 μm.

one minor transcript probably corresponding to vector (Fig. 1A). In pcDNA-Bcl-$x_L$ cells, high levels of a major Bcl-$x_L$ transcript (≈1 kb) were detected as well as an endogenous transcript (≈2.5kb) (Fig. 1B).

### 3.2. *Overexpression of human PS-2 gene increases vulnerability to staurosporine toxicity*

To investigate whether PS-2 may modulate cellular sensitivity to degeneration, cultures were examined following exposure to the apoptotic insults staurosporine [16,17] and hydrogen peroxide [18]. In the absence of cellular insults, neither pcDNA-PS2 nor the other PC12 lines exhibited significant spontaneous degeneration over the course of experimentation (data not shown). However, in all PC12 lines 18–24 h exposure to 1–10 nM staurosporine induced cell death with morphological features indicative of apoptosis (Fig. 2B), a finding consistent with reports of staurosporine toxicity in other cell systems [16,17]. Significantly, the four cell lines showed different responses to staurosporine. Specifically, the pcDNA-PS2 line exhibited increased sensitivity to staurosporine toxicity relative to the WT and pcDNA-Neo lines, as assessed by visual inspection (Fig. 3); this effect did not appear to be time-dependent (data not shown). The observed potentiation of staurosporine toxicity in PS-2 transfectants was quantified using the MTT reduction assay. For example, as shown in Fig, 4A, following 4 nM staurosporine treatment WT and pcDNA-Neo lines exhibited 46.5 ± 1.1 and 44.6 ± 0.1% viability, respectively, whereas the pcDNA-PS2 line retained only 17.8 ± 1.4% viability. In contrast to the significant potentiation of cell death observed in the pcDNA-PS2 cells, the pcDNA-Bcl-$x_L$ line exhibited robust protection against staurosporine-induced toxicity (Figs. 3D and 4A).

### 3.3. *Overexpression of human PS-2 gene increases vulnerability to hydrogen peroxide*

To determine if the enhanced vulnerability of pcDNA-PS2 cells to staurosporine toxicity extrapolates to other types of insults, PC12 cell lines were exposed to the pro-oxidant hydrogen peroxide. Exposure of PC12 cells to hydrogen peroxide for 18–24 h caused a dose-dependent reduction in cell viability that exhibited classic features of apoptosis, including apoptotic bodies and pyknotic nuclei (Fig. 2C). In close agreement with the staurosporine data, we found that PS-2 overexpression potentiated hydrogen peroxide toxicity, whereas Bcl-$x_L$ overexpression provided partial protection to PC12 cells (Figs. 3 and 4B).

## 4. Discussion

Identification of PS-2 as a locus for mutations linked to specific forms of early-onset familial AD has generated significant interest in determination of the gene's normal functional role(s) and, subsequently, how their alteration by missense mutations contributes to the disease process. In this

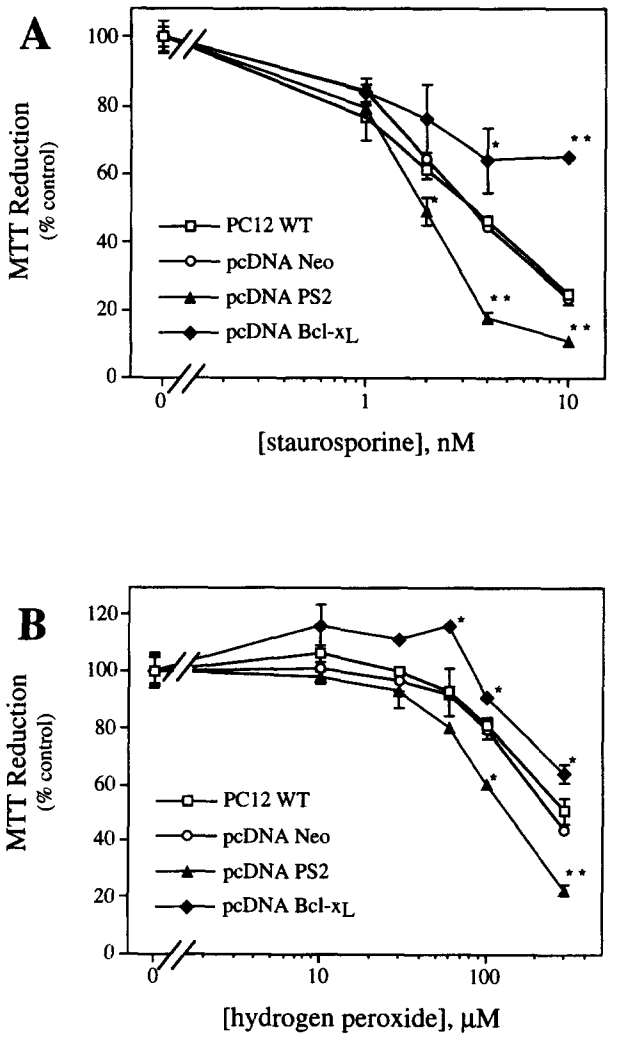


Fig. 4. Quantitative analysis of cell viability regulation by PS-2 and Bcl-$x_L$ overexpression following exposure to various doses of staurosporine (A) and hydrogen peroxide (B). Cell lines are represented as follows: (□) PC12 WT; (○) pcDNA-Neo; (▲) pcDNA-PS-2; (♦) pcDNA-Bcl-$x_L$. Data show mean values (±S.E.M.) normalized to MTT values from respective untreated controls in single, representative experiments. $^*P < 0.05$, and $^{**}P < 0.01$ relative to matched values from WT condition.

report, we have demonstrated that PC12 cells stably transfected with full-length human PS-2 exhibit significantly increased sensitivity to cell death induced by the apoptotic insults staurosporine and hydrogen peroxide, suggesting that one role of PS-2 involves regulation of cell viability.

The proposed role of PS-2 in the cellular regulation of apoptosis is consistent with findings mentioned above that a truncated murine homologue of PS-2 called ALG-3 can rescue a T cell hybridoma from apoptotic insults [12]. One possible mechanism of ALG-3 protection that is consistent with our current results is that ALG-3 may function as an inhibitor of PS-2. If this hypothesis is correct, the pro-apoptotic function of PS-2 may be opposed by ALG-3. Thus, a proper balance between PS2 and its putative truncated protein may be crucial to regulation of cell viability. Although the functional relationship of PS-2 to familial AD is unknown, one might anticipate disruption of this balance in favor of increased apoptosis as a result of mutation-mediated alterations in the processing or activity of PS-2.

The theory that PS-2 function is characterized by an increased sensitivity to apoptotic insults is consistent with pathological mechanisms thought to underlie neuronal loss in AD. Both in vitro [18–21] and in vivo [22,23] data show that cellular insults (e.g. fibrillar β-amyloid, oxidative stress) implicated as causal agents in AD neurodegeneration induce apoptosis. Recent histochemical evidence in AD brain tissues demonstrates several morphological features consistent with apoptosis, including chromatin condensation and nuclear fragmentation as well as the induction of genes related to apoptosis and cell death [24–27]. It appears, however, that the AD brain displays a complex expression pattern of gene products regulating cell death, including those in the Bcl family [27], suggesting competing pro- and anti-apoptotic mechanisms. For example, many neurons that show DNA damage also exhibit increased levels of Bcl-2 [27]. Another member of the Bcl family likely to be important in regulating cell viability in brain is Bcl-$x_L$, which maintains high levels in neurons throughout life. Our culture findings suggest that Bcl-$x_L$ enhances cell survival whereas PS-2 may be involved in promoting cell death.

In addition to the present data suggesting a modulatory role of PS-2 in cell viability, other evidence suggests possible roles in protein trafficking and signal transduction. For example, the amino acid sequence of PS-2 predicts an integral membrane protein with seven transmembrane domains. Partial amino acid sequence homology between PS-2 and the *C. elegans* genes SPE-4 [28] and SEL-12 [29] supports the hypothesis that PS-2 may have a role in protein trafficking or signal transduction. Finally, it has been reported recently that AD-linked mutations in PS-2 cause altered processing of the APP protein yielding increased production of the particularly amyloidogenic X-42/43 forms of β-amyloid [9].

The hypothesized involvement of PS-2 in both protein trafficking and cell death regulation need not be mutually exclusive roles. For example, activation of a single PS-2 intracellular pathway potentially may produce multiple cellular consequences, including those contributing to modulation of cell viability (e.g. calcium regulation) and protein trafficking or processing. One could speculate that AD-linked PS-2 mutations may cause disruption of such a pathway, altering functionality of relevant PS-2 cellular roles. Thus, PS-2 mutations may result in not only increased production of deleterious X-42/43 β-amyloid but perhaps also enhanced sensitivity to apoptotic stimuli (e.g. β-amyloid). The co-occurrence of both pathological pathways would be predicted to interact synergistically, perhaps explaining why familial AD associated with presenilin mutations exhibit a particularly early onset and a relatively high prevalence. Examination of these issues will require study of how AD-linked presenilin mutations affect normal functions in vitro and in vivo.

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