Published in final edited form as:

Neurobiol Dis. 2007 April ; 26(1): 165–173.

Differential regulation of inhibitors of apoptosis proteins in Alzheimer's disease brains

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Abstract

Neuronal degeneration linked to apoptosis can be inhibited by a family of proteins known as inhibitors of apoptosis proteins (IAPs). We examined three members of the IAP family that are implicated in the regulation of neuronal death. We assessed NAIP, XIAP, and cIAP-2 protein levels in the entorhinal cortex of non-demented, cognitively impaired and Alzheimer's disease cases. Levels of paired helical filament-1 (PHF-1), a marker of neurofibrillary tangles, were assessed to determine their relationship to IAP levels. NAIP was decreased in AD cases compared to mildly-impaired and unimpaired cases, and this decrease was associated with increased PHF-1 levels. Low NAIP levels were associated with higher Braak and Braak tangle stage and cognitive dysfunction. XIAP levels were higher in AD cases and cIAP-2 levels did not vary with clinical status. Our data suggest that decreased NAIP may place neurons at risk for the development of tangles and apoptosis.

Keywords

inhibitor of apoptosis; neurodegeneration; Alzheimer's disease; NAIP; XIAP; cIAP-2; caspase activation; tau; neurofibrillary tangles; MMSE

Introduction

Alzheimer's disease (AD) is characterized by a progressive accumulation of extracellular β -amyloid ($A\beta$) in the form of senile plaques and intracellular tau in the form of neuropil threads and neurofibrillary tangles (NFTs). In addition, neuronal and synaptic loss occur during the progression of AD (for reviews, see Cotman, 1998; Mattson, 2004), and this degeneration is linked to the presence of $A\beta$ plaques (Allen et al., 2001; Song et al., 2006; Takuma et al., 2004) and NFTs (Broe et al., 2001, and reviewed in Dickson, 2004; Gomez-Isla et al., 1997; Rohn et al., 2002b).

Recent evidence suggests that neuronal degeneration and loss in AD may result in part from the activation of apoptotic-related pathways or apoptosis itself. In AD and other neurodegenerative disorders, neurons induce a series of proteases including caspases, a class of cysteine proteases, which are key molecules in the execution of apoptosis (reviewed in

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Prunell and Troy, 2004). Caspases act to either initiate (initiator caspases) or advance (executioner caspases) apoptosis and result in the characteristic morphological changes of cell death such as shrinkage, nuclear fragmentation and membrane blebbing. Activated initiator and effector caspases are elevated in the brains of AD patients (Guo et al., 2004; Rohn et al., 2001; Rohn et al., 2002a; Su et al., 2002; Su et al., 2001; Zhao et al., 2003) and exhibit a high degree of co-localization with NFTs, granulovacuolar degeneration and dystrophic neurites (Guo et al., 2004; Newman et al., 2005; Rissman et al., 2004; Rohn et al., 2001). An accumulation of caspase cleavage products including cleaved cytoskeletal proteins (e.g., actin, spectrin, tau), amyloid precursor protein (APP), glial fibrillary acidic protein (GFAP) and poly (ADP-ribose) polymerase (PARP) (for reviews, see Marks and Berg, 1999; Rohn et al., 2002b) are also found in the AD brain.

Caspase activation may provide a mechanistic link between A β and NFT pathologies in AD (Cotman et al., 2005). In the AD brain, A β plaques co-localize with cells showing classical apoptotic morphology and caspase activation (reviewed in Cotman et al., 2005). This is consistent with in vitro studies showing that aggregated A β triggers caspase-dependent apoptosis (Lesne et al., 2005; Loo et al., 1993). Several recent studies report that A β -mediated caspase activation may contribute to the development of tangle pathology and neuronal compromise. Caspase-cleaved tau co-localizes with intracellular A β , activated caspase-3, and paired helical filament-1 (PHF-1), a marker of mature tangle pathology, and is detected in patients with mild cognitive impairment but not within control brains, suggesting that caspase cleavage of tau may occur early in the progression of AD (Rissman et al., 2004). Consistent with this hypothesis, A β exposure leads to the formation of paired helical tau-like filaments in vitro (Ferrari et al., 2003; Gamblin et al., 2003), which occurs via A β 's activation of caspases (Ivins et al., 1999; Loo et al., 1993) that in turn cleave tau (Gamblin et al., 2003; Guo et al., 2004; Rissman et al., 2004). Further, caspase cleavage of tau leads to a conformational change that is recognized by markers of early tangle epitopes, like MC1 (Rissman et al., 2004). The resulting cleavage product, Δ Tau or truncated tau, accelerates the aggregation of full-length tau filaments in vitro, suggesting that it may initiate and/or catalyze NFT formation (Gamblin et al., 2003; Rissman et al., 2004; Rohn et al., 2002a).

Taken together, these findings suggest that activation of apoptotic-related caspase pathways and/or apoptosis itself contributes to the progression of neuropathological changes and cell death in AD. If fully activated, indications of apoptosis in the AD brain should be obvious yet there are contrasting and conflicting reports on the existence of DNA fragmentation, nuclear apoptotic bodies, and other classical apoptotic morphology in the AD brain (for example, Woodhouse et al., 2006). Further, caspase activation can occur within a cell without resulting in classical apoptosis, and prolonged caspase activation sometimes occurs without cell death (reviewed in Cotman et al., 2005). Therefore, there must be counteractive anti-apoptotic mechanism(s) acting to limit cell death whose down-regulation could leave neurons susceptible to apoptotic death and up-regulation could inhibit caspase activation and arrest apoptosis.

A family of structurally related endogenous proteins termed inhibitors of apoptosis (IAPs) are key in acting to restrain the activity of both initiator and effector caspases (reviewed in Liston et al., 2003). Eight mammalian IAPs have been identified to date, and include: XIAP, cIAP-1, cIAP-2, NAIP, Livin, TsIAP, Survivin and Bruce (Prunell and Troy, 2004). Of these, NAIP, XIAP and cIAP-2 are implicated in the regulation of neuronal death in several disease models (reviewed in Prunell and Troy, 2004). NAIP directly inhibits caspase-3, -7 and -9 (Maier et al., 2002; Davoodi et al., 2004) and XIAP directly inhibits caspase-3 and caspase-7 (Deveraux et al., 1997). cIAP-2 may inhibit caspase activation and proteolytic processing of pro-caspase-3 (Roy et al., 1997, but see Eckelman and Salvesen, 2006). Elevated neuronal expression of NAIP reduces ischemic damage in the rat hippocampus (Xu et al., 1997), and is protective in a 6-hydroxydopamine model of Parkinson's disease (Crocker et al., 2001). Consistent with

these findings, *in vitro* studies report that up-regulation of NAIP and decreased interaction of NAIP with its endogenous inhibitor, Smac, by the presence of neurotrophin-3, protects neurons from A β induced death (Lesne et al., 2005). Transgenic XIAP-overexpressing mice show reduced caspase-3 activation, fewer cells with DNA fragmentation, reduction in infarct size and better neurological outcome after transient cerebral ischemia (Trapp et al., 2003). Following axotomy in neonates, overexpression of cIAP-2 is protective (Perrelet et al., 2000), and increases in cIAP-2 are detected following traumatic brain injury (Keane et al., 2001).

Few studies have examined how IAPs are regulated in human neurodegenerative disorders or if their expression in the AD brain is related to the presence of the disease's hallmark pathological features or cognitive decline (Seidl et al., 1999). In the present investigation, we sought to determine the levels of NAIP, XIAP, and cIAP-2 in a region of the brain vulnerable to AD pathology, the medial temporal lobe, of nondemented aged individuals compared to patients with dementia of varying severity. We also assessed levels of a marker of mature hyperphosphorylated tau (PHF-1) in order to determine the association between NAIP, XIAP and cIAP-2 and tangle pathology. Finally, we investigated the ability of IAP level, selectively, to predict global cognition and Braak and Braak tangle and plaque staging. We report that (1) NAIP levels are decreased in AD patients compared to mildly-impaired and control cases, (2) NAIP levels are inversely related to PHF-1 levels, and (3) low NAIP and high XIAP immunoreactivity are associated with impaired cognitive function and increased tangle pathology. Our data suggest that the expression of at least one apoptotic inhibitory protein, NAIP, may be protective against the development of tangle pathology and cognitive decline in AD.

Materials and methods

Subjects

Participants were recruited to enter the Alzheimer's Disease Research Center (ADRC) at the University of California, Irvine and completed a standard neuropsychological test battery. Measures of global cognitive status from the Mini-Mental State Examination (MMSE; Folstein et al., 1975) and recent verbal memory from the CERAD Word List (CWL; Morris et al., 1989) were used to examine the relationship between IAP immunoreactivity and cognitive ability. Scores from the final tests prior to death were used in statistical analyses.

Brain tissue from participants was collected at autopsy from the Institute for Brain Aging and Dementia Tissue Repository for Western blot and immunohistochemical analysis. Entorhinal and hippocampal tissue from 15 cases with short postmortem intervals was examined. Table 1 summarizes demographic information for these cases, which were categorized into three groups based on their clinical diagnostic status at time of death as (1) non-demented individuals (NL, N=4), (2) age-matched individuals who were diagnosed as cognitively impaired but not demented (CIND), mild cognitive impairment (MCI) or mild AD (CIND/MCI/mild AD, N=3), and (3) age-matched AD cases (AD, N=8) (Morris et al., 1989). AD cases were further selected to include a range in severity of cognitive decline and Braak and Braak staging for tangles and plaques (Braak and Braak, 1991).

Antibodies

Details regarding the antibodies used in the present study are listed in Table 2. Briefly, rabbit anti-human NAIP antibody raised against amino acids 473-490 of human NAIP was purchased from R & D Systems (Minneapolis, MN). Monoclonal anti-XIAP generated from human IAP-like protein was purchased from BD Biosciences (San Diego, CA) and rabbit polyclonal anti-c-IAP2 raised against amino acids 94-178 of human cIAP-2 was purchased from Santa Cruz

Biotechnology (Santa Cruz, CA). A well-characterized monoclonal antibody, anti-PHF-1 (a generous gift from Peter Davies, Yeshiva University, Bronx, NY), was used to detect the presence of hyperphosphorylated tau. Monoclonal anti- β -Actin (mouse IgG2a isotype, Sigma-Aldrich, Saint Louis, MO) was used as a protein loading control for Western blots.

Western blot analysis

Samples of entorhinal cortex (~ 100mg) prepared from fresh frozen brain tissue were homogenized in 1:10 volume extraction buffer (10mM Tris, 10mM EDTA, 150mM NaCl, 0.1% mSDS, 1% Triton, 0.5% NP40, 0.2mM Na_3VO_4 , 0.2mM PMSF, 50mM NaF, and a Protease Inhibitor cocktail from Sigma P-8340). The homogenates were centrifuged for 20 min at 1500 g to obtain the supernatants. Protein concentrations were determined by BCA assay (Pierce Biotechnology, Rockford, IL) and all samples were adjusted for total protein to ensure equal loading of the gels. Samples from the tissue homogenates were run on a 10% SDS-PAGE gel (Bio-rad Laboratories, Hercules, CA) and then transferred to a nitrocellulose membrane (0.2 μm ; Whatman Schleicher & Schuell BioScience, Keene, NH). In order to block nonspecific binding, the membranes were incubated in Tris-buffered Saline with Triton X-100 (TTBS) with 5% skim milk. Membranes were incubated overnight in antibodies against NAIP, XIAP, c-IAP2 or for two hours in PHF-1 (dilutions listed in Table 1), and then washed and incubated in anti-rabbit-HRP secondary antibody or in anti-mouse-HRP (Vector Laboratories, Burlingame, CA). Membranes were later re-probed using monoclonal anti- β -actin. Proteins were detected by chemiluminescence (SuperSignal West Pico; Pierce Biotechnology, Rockford, IL) and quantified by densitometric analysis using public domain software (Scion Image for Windows, v. 4.0.2; Scion Co., Frederick, MD).

Immunohistochemistry and confocal microscopy

Fresh brain tissue was fixed in 10% formalin or 4% paraformaldehyde in 0.1 M Sorensen's buffer (pH 7.3) for 48-72 h and then stored in 0.1 M PBS with 0.02% sodium azide at 4°C. Samples from the hippocampal formation were sectioned using a vibratome (50 μm) and collected in PBS. Tissue sections were treated for 20 min with 1% H_2O_2 to inactivate endogenous peroxidases. Sections were boiled with antigen unmasking solution (Vector Laboratories, Burlingame, CA) for five minutes and incubated overnight at room temperature in primary antibody (dilutions listed in Table 1), rinsed, and incubated in biotinylated secondary antibody and avidin-biotin complex for 1 h, respectively (ABC Kit, Vector Laboratories). IAPs were detected using a DAB kit (brown reaction product) and an SG kit was used to detect PHF-1 bearing neurons (blue-gray reaction product) (Vector Laboratories). Sections incubated in parallel without primary antibody failed to develop specific staining (data not shown).

For confocal microscopy, IAPs were detected using Alexa Fluor 488 conjugated IgG (green fluorescence, Molecular Probes Inc., Eugene, OR) and PHF-1 was detected with Cy3 (Jackson ImmunoResearch, Westgrove, PA) or Alexa Fluor 568 conjugated IgG (red fluorescence) (Molecular Probes Inc.). For double immunofluorescence staining using primary antibodies from the same species, after localizing the first antigen, sections were immersed in 37% formaldehyde solution for 2 h at 37°C to minimize crossreactivity. Fluorescently stained specimens were viewed with a 60x or 40x objective lens on an Olympus IX70 inverted microscope and confocal images were captured on an Olympus FluoView system or MRC 1024 confocal system (Bio-Rad, Hercules, California). For each observation, consecutively scanned 2.0 μm thick images were obtained via two channels. Channel 1 was used to collect green fluorescence images and channel 2 was used to acquire red fluorescence images. These images were displayed on the monitor and merged using Adobe Photoshop (version 7.0, © Adobe Systems Inc.) graphics software.

Statistical Analyses

All statistical analyses were performed using SPSS (version 11.0 for Mac OS X; SPSS, Chicago, IL). As additional confirmation of our clinical status groupings, the relationship between Braak and Braak tangle and plaque staging and performance on the MMSE (i.e. total score out of 30) was examined by coding Braak stage (i.e. for tangles, 0 was coded as 0, I and 1, II as 2, etc., and for plaques, A was coded as 1, B as 2, etc.) and performing partial correlation analyses controlling for test interval (i.e. time elapsed between final clinical evaluation and death in months). For each comparison, a Bonferroni-adjusted two-tailed p-value less than or equal to 0.025 (i.e. 0.05/2) was considered statistically significant.

A non-parametric Kruskal-Wallis test was conducted in order to determine if IAP immunoreactivity differed between the AD, CIND/MCI/Mild AD and control groups. Post hoc Mann-Whitney U tests were conducted when significant overall group effects were found.

Partial correlation analyses, controlling for postmortem interval, were performed in order to examine the levels of the IAP markers (i.e. NAIP, XIAP and cIAP-2) in relation to PHF-1 levels. For all markers, densitometric measures for each case were standardized using their β -actin loading control values. A Bonferroni-adjusted two-tailed p-value less than or equal to 0.017 was considered statistically significant.

In order to determine if IAP levels would predict disease severity, stepwise linear regression analyses were performed using NAIP, XIAP and cIAP-2 optical density measurements as the predictor variables. Age, post mortem interval and test interval were included as variables in the analyses in order to rule them out as potential predictors of IAP level. Four separate analyses were performed for four disease severity criteria; MMSE score, overall learning and retention on the CERAD Word List (CWLTL), Braak and Braak tangle stage, and Braak and Braak plaque stage. For the MMSE, the total score out of 30 was used, and for the CWLTL, the pooled score from trials 1, 2 and 3 and 5- and 30-minute recall (out of a total of 50) was used as a measure of overall learning and retention. A p-value of 0.05 was used for entry of the predictor variables into the model.

Results

Subject Demographics

As expected, Braak and Braak staging was related to level of performance on the MMSE in our sample (Cummings et al., 1996; Tiraboschi et al., 2004). Both tangle ($r = -0.875$, $p < 0.001$) and plaque ($r = -0.797$, $p = 0.002$) stages showed significant inverse relationships with MMSE score, controlling for test interval; lower MMSE scores were associated with higher (i.e. more advanced) Braak and Braak plaque and tangle stages (data not shown).

NAIP is decreased in AD cases and inversely related to paired helical filament-1 levels

Western blots showed a predominant band labeled with NAIP at ~ 120 kDa in all four control cases examined (Fig. 1). A similar band was detected in the MCI and CIND cases but not in the mild AD case, and a lighter band was observed in three of eight AD cases. The calculated molecular weight of full-length NAIP is ~ 160 kDa but our observation of a ~ 120 kDa band is consistent with a previous report (Lesne et al., 2005). We confirmed these results with an additional Western blot experiment using a subset of the same AD, CIND/MCI/Mild AD and control cases with two other antibodies (Table 2). We found the same 120-kDa bands using all three antibodies (data not shown), demonstrating that we were in fact detecting NAIP. Further, we concurrently ran human NAIP-expressing HeLa cells (generously provided by Dr. A.E. MacKenzie, Children's Hospital of Eastern Ontario, Ottawa, Canada) in the same Western blots

and observed immunoreactive bands at ~120 kDa (data not shown) using all three commercial antibodies.

A significant overall effect of diagnostic group on NAIP immunoreactivity levels was found ($\chi^2=9.019$; $p=0.011$). Post hoc tests revealed that NAIP immunoreactivity levels were significantly lower in AD cases compared to age-matched control cases (Fig. 2; $p=0.004$). AD levels tended to be lower compared to CIND/MCI/Mild AD levels, although this trend did not reach statistical significance ($p=0.085$).

NAIP levels were inversely related to PHF-1 (Fig. 3A; $r=-0.856$, $p<0.001$) level. In the control cases, which clearly showed NAIP, PHF-1 was not detected. The MCI and CIND cases with NAIP bands showed no PHF-1, whereas the mild AD case showing no detectable NAIP did show PHF-1. Further, AD cases with high PHF-1 levels showed faint or negative NAIP bands, whereas AD cases with low PHF-1 levels showed light NAIP bands (Fig. 1).

A dissociation in NAIP and PHF-1 was also evident from observation of the double-labeled hippocampal sections (Fig. 3B, 3C). The majority of NAIP-positive neurons did not contain PHF-1. Weak NAIP immunoreactivity was detected within some tangle-bearing neurons, but the morphological characteristics of these neurons suggests that they are pre-tangle neurons based upon punctate rather than fibrillar intracellular labeling. By contrast, weak or no NAIP immunoreactivity was seen in neurons containing intracellular tangles or in association with extracellular tangles. Figures 3B and C depict a representative AD case demonstrating this dissociation in the hippocampus. Similarly, double immunofluorescence confocal images show a similar dissociation in NAIP versus PHF-1 labeled cells (Fig. 4A, B and C).

XIAP and cIAP-2 are not related to paired helical filament-1 levels

XIAP protein levels were low in all cases examined however, after a prolonged exposure time a band was detected at approximately 57 kDa in four AD cases (Fig. 1). A significant overall effect of diagnostic group on XIAP immunoreactivity levels was found ($\chi^2=6.057$; $p=0.049$). As shown in Figure 2, XIAP levels were significantly higher in AD cases compared to controls ($p=0.028$). This effect is opposite to the decrease in NAIP levels seen in AD and CIND/MCI/Mild AD cases. Also unlike NAIP, XIAP was not correlated with PHF-1 immunoreactivity ($p=0.776$).

cIAP-2-positive bands at approximately 68 kDa were detected in all cases and did not vary in intensity in relation to case diagnosis (Fig. 1). cIAP-2 levels did not differ significantly between AD, CIND/MCI/Mild AD and control cases (Fig. 2; $p=0.330$). Further, cIAP-2 levels were not significantly correlated with PHF-1 levels ($p=0.908$).

NAIP and XIAP levels predict disease severity

We next hypothesized that NAIP and XIAP protein levels may predict Braak and Braak stage and cognitive function. Stepwise linear regression analyses revealed that, of the three IAPs, NAIP and XIAP were significant predictors of both MMSE score (Fig. 5A and B; $F(2, 13)=7.619$; $p=0.008$; $R^2=0.581$) and Braak and Braak tangle stage (Fig. 5C and D; $F(2, 14)=15.142$; $p=0.001$; $R^2=0.716$). NAIP was also a significant predictor of overall learning and retention on the CWLT task (Fig. 5E; $F(1, 13)=14.120$; $p=0.003$; $R^2=0.541$). None of the IAPs were significant predictors of Braak and Braak plaque stage. Neither age, post mortem interval nor test interval were significant predictors of disease severity.

NAIP and XIAP showed opposite relationships to measures of disease severity; higher NAIP levels were associated with better performance on the Mini-Mental State Examination (MMSE) and CERAD Word List task (CWLT) and less tangle pathology while increased XIAP predicted lower MMSE scores and greater tangle pathology.

Discussion

In the present study, we report that NAIP immunoreactivity in the entorhinal cortex is decreased in AD patients compared to age-matched normal and mildly-impaired individuals. This is consistent with a previous report in which individuals with AD showed decreased NAIP immunoreactivity compared to control subjects in the temporal cortex, along with the frontal and occipital cortices (Seidl et al., 1999). We have extended these previous results to the entorhinal cortex, and demonstrated that early AD may be intermediate in levels between normal and moderate to severe AD cases.

We also report that decreased NAIP is associated with increased paired helical filament-1 (PHF-1) levels. Individual cells positive for NAIP in the hippocampal formation do not co-localize with cells expressing PHF-1. Further, low NAIP levels are associated with high Braak and Braak tangle stage, low global cognitive performance on the Mini-Mental State Examination (MMSE), and impaired verbal learning and retention on the CERAD Word List task (CWLTT) task. All of these findings together suggest that compromised inhibition of apoptosis may be related to the emergence of tangle pathology and cognitive decline in AD.

Decreased NAIP levels will result in decreased inhibition of caspase-3, -7, and -9 (Davoodi et al., 2004; Maier et al., 2002). Tau protein is cleaved by caspase-3 and -7 (Gamblin et al., 2003; Rissman et al., 2004), and therefore, lower levels of NAIP could result in increased cleavage of tau, which could in turn lead to accelerated assembly of neurofibrillary tangles (NFTs) (Gamblin et al., 2003; Rissman et al., 2004; Rohn et al., 2002a; Rohn et al., 2002b) and activation of apoptotic pathways, since caspase-cleaved tau is itself pro-apoptotic (Guo et al., 2004). Therefore, if NAIP levels are decreased in the AD brain, caspase activation in response to any pro-apoptotic stimulus (for example, A β plaques (Ivins et al., 1999; Loo et al., 1993) will evolve uninhibited and might promote neuronal degeneration via apoptosis. If NAIP expression is uncompromised, however, caspase activation will be inhibited (Davoodi et al., 2004; Maier et al., 2002), and therefore caspase cleavage of tau, NFT formation and cell death would be expected to decrease. The results of the present study can be strengthened in future studies by determining whether decreased NAIP levels are directly related to increased caspase-related protein cleavage in the AD brain.

Future studies will determine the precise mechanism(s) by which NAIP acts to regulate NFT formation and cell death in the AD brain and if neurotrophic factors might mediate these effects. In the case of A β -induced death, neurotrophin-3 protects neurons by up-regulating NAIP and decreasing NAIP's interaction with its endogenous inhibitor, Smac, demonstrating that NAIP plays a central role in the regulation of A β by directly inhibiting effector caspases (Lesne et al., 2005). Although there is controversy over whether neurotrophin-3 levels increase or decrease in the AD brain, it is possible that, along with other regulatory elements, neurotrophin signaling may be insufficient to upregulate NAIP levels in AD (Tong et al., 2004).

The observed reduction in NAIP immunoreactivity in the present study could be the result of decreased neuronal density in the entorhinal cortex given that progressive neuronal loss in this region is a pathological hallmark in AD (Braak and Braak, 1991; Gomez-Isla et al., 1996; West and Gundersen, 1990). However, we detected equivalent levels of a cytoskeletal protein, β -actin, across cases suggesting that decreased neuronal density alone cannot account for the reduction in NAIP levels (Fig. 1). In the current set of samples, a Kruskal-Wallis test using β -actin optical density values revealed that they did not vary significantly between the diagnostic groups ($p>0.05$). Further, our findings that AD patients show increased XIAP immunoreactivity compared with unimpaired controls and cIAP-2 levels do not change with clinical status argue against this point.

Unlike the observed decrease in NAIP levels, XIAP levels were significantly higher in our AD cases compared to the unimpaired control cases. Further, higher XIAP levels predicted higher Braak and Braak tangle stage, which indicates that increased XIAP is insufficient to arrest tangle formation and favors continued decline, as suggested by its relationship with disrupted MMSE performance. It is possible that increased XIAP represents a compensatory mechanism in AD without which decline would be greater. For instance, in the AD brain, XIAP may be upregulated in order to suppress caspase activation. Although XIAP levels were not significantly correlated with PHF-1, the marker of tau used in the present study, it is possible that increased XIAP acts as a compensatory mechanism against caspase activation and the resultant cleavage of various proteins leading to cell compromise and death in AD. Based on the present results, we also cannot rule out the possibility that XIAP-expressing cells were selectively preserved and NAIP-expressing cells eliminated in our AD cases. Thus, these results may provide support for NAIP-related selective neuron loss during AD progression.

In line with the inability of cIAP-2 to discriminate the diagnostic groups, protein levels of this member of the IAP family were unrelated to disease severity in the present sample. cIAP-2 was previously thought to inhibit the effector caspases (Deveraux et al., 1998; Roy et al., 1997), however a more recent study reports that although cIAP-2 (and cIAP-1) is able to bind caspase-3 and -7, it does not act to inhibit their activity (Eckelman and Salvesen, 2006). Taken together, the results suggest that regulation of the various IAPs is differentially affected in AD.

NAIP is unique among the IAPs in that it contains both a nucleotide binding oligomerization domain (NOD) and a leucine-rich repeat (LRR) domain, which suggests that it is regulated in a manner distinct from the other IAPs (Davoodi et al., 2004) and thus may be involved in additional apoptosis-related pathways. Davoodi and colleagues (2004) report that NAIP acts to inhibit caspase-9 in an interaction that, unlike XIAP, requires structural change of its baculoviral IAP repeat-3 (BIR3) region mediated by ATP binding to its NOD domain. Based on this finding and the shared structural properties of NAIP with other members of the NOD family, the authors suggest that NAIP may be activated by pathogen-associated molecular patterns related to proinflammatory caspases (Davoodi et al., 2004). NAIP might serve at least two roles in mediating programmed cell death, acting to suppress unintentional activation of caspase-3, -7 and -9, along with XIAP, and allowing caspase-1 activation, unlike XIAP. The observed relationship between NAIP and PHF-1 suggests that changes in interleukin processing and increased inflammation may be related to the presence of NFTs in the entorhinal cortex, a relationship suggested in the pathogenesis of AD by several previous studies (reviewed in Griffin et al., 2000; Kitazawa et al., 2004). Thus, reduced NAIP may permit several mechanisms of tangle formation to proceed.

In conclusion, the present study demonstrates that levels of IAP family members are differentially affected in AD. NAIP is decreased in the AD entorhinal cortex compared to unimpaired and mildly-impaired cases, XIAP is increased and cIAP-2 appears to be unrelated to clinical status.

NAIP levels are inversely related to PHF-1 levels, a marker of mature tangle pathology, suggesting that compromised ability to inhibit apoptosis is related to the formation of NFTs in AD. The levels of both NAIP and XIAP are predictive of disease severity, but in opposing directions; low NAIP and high XIAP are associated with higher Braak and Braak tangle stage and diminished cognitive performance. These findings suggest that the presence of NAIP provides one mechanism for resistance to tangle formation, and point to NAIP as a potential intervention target for the prevention of tangle formation, global cognitive decline and verbal learning and retention deficits in AD.

Acknowledgements

The authors gratefully acknowledge Dr. E. Head for critical reading of the manuscript and for helpful discussion. We also thank S. Balabanian and Dr. A. MacKenzie for provision of NAIP expressing HeLa cells and Dr. P. Davies for provision of the PHF-1 antibody. This work was supported by NIA grants P50 AG16573 and P01 AG00538 (C.W.C.)

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Figure 1. Western blot analysis of NAIP, XIAP, cIAP-2 and PHF-1 in the human entorhinal cortex NAIP expression is high in control cases (NL-1 to -4) and decreased in AD cases (AD-1 to -8). By contrast, XIAP expression is absent in control and CIND/MCI/Mild AD cases and is detected in four of eight AD cases. PHF-1 expression is higher in CIND/MCI/Mild AD and AD cases and absent in control cases. cIAP-2 and β -actin levels do not consistently vary in relation to diagnostic status.

Figure 2. IAP immunoreactivity varies between diagnostic groups

NAIP immunoreactivity is significantly reduced in AD (dark gray bars) versus control cases (NL; black bars) (** $p=0.004$) and show a trend towards lower levels in transitional (CIND; light gray bars, indicates CIND/MCI/Mild AD cases) cases. XIAP immunoreactivity is significantly increased in AD versus control cases (* $p=0.028$). cIAP-2 levels do not differ between groups. Error bars represent +1 S.E.M.

Figure 3. Relationship between NAIP expression and tangle pathology in the human entorhinal cortex and hippocampus

A. NAIP and PHF-1 are inversely related ($r = -0.856$; $p < 0.001$) in the human entorhinal cortex. B. Many tangle-bearing neurons (blue) do not co-localize with NAIP (brown and arrows), although NAIP immunoreactivity is detected within some pre-tangle neurons (asterisks) (500X). C. High magnification (1500X) image showing a NAIP-positive neuron (brown) that is not labeled with PHF-1 and a mature tangle-bearing neuron (blue) that is not co-localized with NAIP.

Figure 4. Double-immunofluorescence confocal images show that NAIP-positive neurons do not co-localize with tangle-bearing neurons

A. Neuron containing PHF-1 labeling; B. a strong NAIP-positive neuron; C. The combined image shows only weak NAIP immunoreactivity within the mature, tangle-bearing neuron (arrow). Magnification = 2000X.

Figure 5. NAIP and XIAP expression predict disease severity

NAIP and XIAP expression are significant predictors of Mini-Mental State Examination (MMSE) score (A, B), Braak and Braak tangle stage (C, D), and NAIP is a significant predictor of overall learning and retention on the CERAD Word List (CWLT) task (E). NAIP and XIAP proteins show opposite relationships with disease severity; higher NAIP levels are associated with better MMSE and CWLT scores and decreased tangle pathology, while higher XIAP predicts lower MMSE scores and higher tangle stage.

Table 1

Case demographic data

Case	Age	Sex	Clinical Diagnosis	Pathological Diagnosis	B&B Tangle Stage	B&B Plaque Stage	MMSE	Interval (mos)	PMI (hrs)
NL-1	83	M	NL	Control	II	0	28	18.4	1.7
NL-2	74	F	NL	Control	II	n/a	30	12.0	2.8
NL-3	70	M	NL	Control	0	0	n/a	n/a	5.2
NL-4	74	F	NL	Control	II	B	30	6.8	2.3
MCI	96	M	MCI	SDC	II	0	29	10.0	3.4
CIND	84	M	CIND	SDC	III	0	22	4.3	6.3
Mild AD	81	F	Mild AD	SDC	V	C	23	11.6	9.0
AD-1	88	M	Possible AD	AD	IV	0	18	14.5	3.5
AD-2	81	M	Possible AD	AD	VI	C	0	18.0	2.5
AD-3	83	M	Probable AD	AD	VI	C	0	10.0	3.5
AD-4	75	F	Probable AD	AD	VI	C	0	2.2	4.5
AD-5	83	F	Probable AD/V/D	AD	VI	C	3	82.8	2.3
AD-6	80	F	Probable AD	AD	V	B	3	32.2	4.4
AD-7	82	F	Probable AD	AD	VI	B	17	65.7	6.3
AD-8	71	M	Possible AD	AD	VI	C	3	2.6	5.2

Abbreviations: AD, Alzheimer's Disease; B & B Stage, Braak & Braak stage; CIND, Cognitively-Impaired-Not Demented; Interval, time (months) between final clinical assessment and death; MCI, mild cognitive impairment; MMSE, Mini Mental State Examination (maximum score = 30); n/a, data not available; PMI, postmortem interval; SDC, Senile Degenerative Changes; VD, Vascular Dementia

Table 2

Antibodies used for Western blots and immunostaining.

Antibody	Source	Catalogue #	Host Species	Western Dilution	Immunostaining Dilution
NAIP	R & D Systems Sigma-Aldrich Chemicon BD Biosciences Santa Cruz Biotechnology Gift from Peter Davies Sigma-Aldrich	AF829	rabbit	1:2,000	1:1,000
NAIP		N 6648	rabbit	1:2,000	n/a
NAIP		AB3617	rabbit	1:400	n/a
XIAP		610716	mouse	1:400	1:150
cIAP-2		Sc-7944	rabbit	1:2,000	1:2,000
PHF-1			mouse	1:20,000	1:10,000
β-Actin		A2228	mouse	1:5,000	n/a