Review

Cultured cell and transgenic mouse models for tau pathology linked to β-amyloid

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Abstract

The two histopathological signatures of Alzheimer's disease (AD) are amyloid plaques and neurofibrillary tangles, prompting speculation that a causal relationship exists between the respective building blocks of these abnormal brain structures: the β-amyloid peptides (Aβ) and the neuron-enriched microtubule-associated protein called tau. Transgenic mouse models have provided in vivo evidence for such connections, and cultured cell models have allowed tightly controlled, systematic manipulation of conditions that influence links between Aβ and tau. The emerging evidence supports the view that amyloid pathology lies upstream of tau pathology in a pathway whose details remain largely mysterious. In this communication, we review and discuss published work about the Aβ–tau connection. In addition, we present some of our own previously unpublished data on the effects of exogenous Aβ on primary brain cultures that contain both neurons and glial cells. We report here that continuous exposure to 5 μM non-fibrillar Aβ40 or Aβ42 kills primary brain cells by apoptosis within 2–3 weeks, Aβ42 is more toxic and selective for neurons than Aβ40, and Aβ42, but not Aβ40, induces a transient increase in neurons that are positive for the AD-like PHF1 epitope. These findings demonstrate the greater potency of Aβ42 than Aβ40 at inducing tau pathology and programmed cell death, and corroborate and extend reports that tau-containing cells are more sensitive to Aβ peptides than cells that lack or express low levels of tau.
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1. Background

The neurodegenerative tauopathies, including Alzheimer's disease (AD), are characterized by reduced association of tau with microtubules, hyperphosphorylation of tau at multiple sites that are rarely phosphorylated in normal neurons, and the accumulation of intracellular neurofibrillary tangles, which are bundles of highly insoluble, paired helical (PHF) or straight tau filaments. This multi-faceted tau pathology is invariably joined in AD by the accumu-lation of extracellular amyloid plaques formed primarily from Aβ peptides. These proteolytic fragments of the transmembrane β-amyloid precursor protein (APP) are variable in length, but most commonly comprise 40 (Aβ40) or 42 (Aβ42) amino acids.

The direct involvement of APP in AD pathogenesis is based on indisputable genetic evidence. Point mutations in the genes for APP or presenilins, which form part of the γ-secretase complex that helps to generate Aβ from APP, can cause AD with high, if not complete penetrance [10,11]. Nevertheless, deposition of amyloid plaques alone is not well correlated with loss of cognitive function, and the behavioral symptoms of AD seem to require both amyloid and tau pathologies [10]. It may therefore seem puzzling at first that unlike APP and the presenilins, tau has not been linked genetically to AD. There are, however, numerous tau

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mutations that are highly penetrant for some non-AD tauopathies, most notably the frontotemporal dementia, FTDP-17 [12].

Making sense of this collection of complex observations is a challenge, but one hypothesis worth considering is that many roads can lead to tau pathology with its attendant neurodegeneration and dementia, but not all entry points mark a path through APP. These entry points are equivalent to disease-inducing environmental or genetic insults, which in the case of AD are envisioned to occur at or upstream of APP, and lead downstream to tau. In contrast, the entry points for non-AD tauopathies are thought to originate at or upstream of tau, but downstream of APP. While this low-resolution model may seem somewhat "tau-centric", it emphasizes the need to understand how tau pathology is coupled to amyloid pathology in AD, which is a much more common disease than any of the non-AD tauopathies. Two powerful experimental systems that are being used to unravel connections between amyloid and tau pathologies are cultured cells and transgenic mice.

2. Amyloid–tau connections in cultured neurons and transgenic mice

The first reported evidence for signaling from Aβ to tau came from studies of pure fetal neuronal cultures derived from rat hippocampus or human brain cortex [9]. Aβ peptides were found to be slowly cytotoxic, but also to induce two AD-like phospho-epitopes on tau, pS199/pS202 and pS396/pS404, and to reduce the affinity of tau for microtubules in the cells. All of these effects were observed for 20 μM fibrillar Aβ40, and some were reproduced by fibrillar, but not amorphous Aβ42 at the same concentration.

There are at least three other published reports of Aβ peptides inducing AD-like tau phosphorylation in cultured cells. In one case, human SH-SY5Y neuroblastomas stably transfected to express wild-type tau or the P301L tau mutant that causes FTDP-17 were exposed to 10 μM Aβ42. The Aβ42 treatment induced the AD-like pS422 phospho-epitope and some tau filaments in both cell lines, but tau filaments were not observed in cells expressing P301L/S422A or P301L/S422E double mutants of tau [8]. Because the former double mutant was unable to be phosphorylated at S422 and the latter represented a pseudo-pS422 double mutant, tau filament assembly may be favored by regulated phosphorylation of S422. For example, perhaps filament nucleation or elongation is promoted by phosphorylation of S422, but only after one or more other specific residues on tau becomes phosphory-lated. In that case, filament formation would be less favored if S422 were mutated to an amino acid that resembled either a constitutively non-phosphorylated or a constitutively phosphorylated serine.

A second study examined the effects of 0.2–20 μM fibrillar versus soluble Aβ40 on primary rat hippocampal neurons that had been allowed to differentiate in culture for 30 days and thereby express adult tau isoforms. Here again, the pS199/pS202 and pS396/pS404 phospho-tau epitopes were induced by aggregated, but not by freshly solubilized Aβ [13]. In a third study, 10 μM Aβ40 was found to be cytotoxic to primary embryonic rat cortical neurons within 4 days, and to induce a slight increase in the pS199/PS202 tau epitope. Interestingly, Aβ40 was not cytotoxic to cells whose tau levels were reduced by antisense oligonucleotides [7]. Consistent with the idea that tau expression promotes Aβ cytotoxicity is another report demonstrating that 20 μM fibrillar Aβ40 killed primary hippocampal neurons isolated from wild-type mice, but not from tau knockout mice. Sensitivity to Aβ in the knockout cells was restored by expression of human tau, but no effects of Aβ40 on tau phosphorylation were reported for this study [6].

Together, these studies make a strong case that exposure of neurons to Aβ peptides transforms tau into a phosphorylated state reminiscent of that found in AD and other tauopathies, and may even provoke the assembly of PHF-like tau filaments. They imply, furthermore, that tau expression makes cells more vulnerable to the cytotoxic effects of Aβ. Several in vivo studies using transgenic mice have fortified this view. For instance, increased levels of the pS199/pS202 tau epitope and two other AD-like tau epitopes were observed in brain neurons of mice expressing the APP "Swedish" (K670N/M671L) double mutant [14,15] that leads to overproduction of Aβ peptides and causes AD in humans. This study did not present evidence for further tau pathology [5], however, and mice of a different transgenic strain expressing the APP Swedish mutations apparently do not make tau filaments [16].

Two reports that were published sequentially in a 2001 issue of *Science* provided the first in vivo evidence for an Aβ–tau connection that leads to tau filament formation. Both studies made use of transgenic mouse strains that expresses the P301L mutant of the largest human tau isoform. In one case, P301L mice were cross-bred with mice expressing the human APP Swedish mutations. Prior studies had indicated that the parental P301L mice [17,18], but not the parental APP mutant mice [16], accumulate tau filaments. The double transgenic mice accumulated PHF-like tau filaments much more rapidly than the P301L single transgenics [2], raising the possibility that simply increasing the supply of extracellular Aβ peptides in the brain upregulates polymerization of tau into filaments. It should be noted, however, that overproduction of Aβ peptides also entails altered production of other APP metabolites that potentially impact on tau.

The companion study published in *Science* yielded strikingly similar results using a very different strategy. In this case, Aβ42 was simply injected into the sensory cortex and hippocampus of P301L mice. Tau filament accumu-lation was accelerated in these mice, but not in comparable

P301L mice that had been injected with a 42-amino acid peptide with the reverse sequence of Aβ42 [3]. These results provide compelling evidence that excess extracellular Aβ42 can trigger tau filament assembly, although they do not address further details of the mechanism.

An additional pair of transgenic mouse studies indicating a metabolic link between amyloid and tau filament formation has extended these observations. One strategy entailed the use of triple transgenic animals expressing the AD-causing M146V mutant of presenilin-1 [11], along with P301L tau and the APP Swedish mutations. These mice developed neurofibrillary tangles, which unlike those in other transgenic mice, followed a developmental progression within the brain similar to that seen in AD patients [1]. The final transgenic mouse study to be discussed represents a strategic departure from the others mentioned here, because it did not include mice expressing mutant tau. Instead, double transgenics expressing the Swedish APP mutations plus the M146L mutant of presenilin-1 were generated [4]. This presenilin-1 mutation is also associated with AD [11], and like the single transgenic mice that express the APP Swedish mutations, mice that are singly transgenic for M146L presenilin-1 are not known to produce tau filaments. Unlike the parental strains, the APP-presenilin-1 double transgenics accumulated abundant tau filaments. In addition, immunohistochemical analysis of brain sections indicated the presence of four AD-like phospho-tau epitopes in the double transgenics [4].

3. Aβ effects on mixed brain cell cultures

The studies discussed to this point provide strong evidence that tau-expressing cells are particularly vulnerable to damage by Aβ peptides, and that tau pathology in the form of AD-like hyperphosphorylation and tau filament assembly can be caused by Aβ peptides, and perhaps by other by-products of pathological APP metabolism as well. It is equally evident that many questions about the amyloid–tau connection at both the basic and detailed mechanistic levels still await answers. The remainder of this communication addresses several basic questions that we have investigated by exposing primary mixed brain cell cultures that contain both neurons and glial cells to low (5 μM) concentrations of Aβ peptides for up to 3 weeks. Several novel observations were made about the effects of Aβ peptides on cultured brain cells. Most notably, we found that Aβ42 killed cells by an unusually prolonged apoptotic mechanism, was more toxic to neurons than to glial cells, was more potent and neuron-selective than Aβ40 at exerting these effects, and in contrast to Aβ40, was able to induce an AD-like epitope on tau. These results emphasize the potential cytotoxicity of low levels of soluble β-amyloid peptides (Aβ) in vivo, and raise the possibility that extracellular amyloid plaques represent a source from which such peptides can diffuse through the brain and thereby damage neurons.

3.1. The methods

A previously described method for making primary cultures of embryonic rat brain cells [19] was adapted to newborn mouse brain without modification. To allow expression of adult tau isoforms, the cells were cultured for 1 month [13] before being initially exposed to medium containing 5 μM Aβ peptides. Subsequent medium changes in either the presence or absence of Aβ were made twice weekly. Previously described methods were used to synthesize and purify Aβ40 and Aβ42 [20], and for immunofluorescence microscopy [21]. Primary antibodies for immunofluorescence included a rabbit polyclonal antibody to MAP2 [19], and five mouse monoclonal IgG1 antibodies: Tau-1, which reacts with tau that is dephosphorylated at serine 199/202, but does not react well with AD tau [22–24]; tau-5, which recognizes all tau splice variants independently of their phosphorylation state [24]; PHF-1 anti-tau, which reacts with an epitope that includes phosphorylated serine 396/404 and is characteristic of AD tau [25]; DM1A anti-tubulin, which binds to an α-tubulin epitope that is not known to be post-translationally modified, and is highly conserved in amino acid sequence across broad species barriers and among all known isoforms of α-tubulin [26,27]; and anti-glial fibrillary acidic protein (GFAP), a marker for astrocytes. To detect fragmented DNA characteristic of apoptotic cells, cover slips were processed by a TUNEL assay immediately after being fixed and permeabilized. This entailed incubating the cover slips with calf thymus terminal transferase followed by fluorescein-labeled dUTP, according to the vendor's (Boehringer-Mannheim, Indianapolis, IN) instructions. For labeling of both TUNEL-positive cells and neurons on individual cover slips, the cover slips were processed first with the TUNEL reagents, and then with Tau-5 (as a neuron marker) followed by Texas red-labeled goat anti-mouse IgG.

To estimate the relative numbers of live cells that had been present in each well of six-well culture dishes, the cells in each well were dissolved in 200 μl of SDSPAGE sample buffer [28] lacking the bromophenol blue tracking dye. The protein concentration of each sample was then measured using a bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co.). Cell numbers were assumed to be directly proportional to protein concentrations. Each bar on the graph shown in Fig. 3 represents the average protein concentration for three separate samples that were grown in parallel.

3.2. Aβ42 induces accumulation of TUNEL-positive cells and subsequent cell loss

Compared to controls, cultures that were exposed to 5 μM Aβ42 for 14 days or longer appeared to have lost a substantial number of cells when observed at low magnification on a tissue culture microscope. In light of this

apparent cell loss caused by Aβ42 and prior reports that Aβ peptides are capable of inducing programmed cell death, or apoptosis [29], cultures exposed to Aβ42 were analyzed by a fluorescent TUNEL assay commonly used to detect the fragmented DNA which is characteristic of apoptotic cells. As can be seen by fluorescence microscopy in Fig. 1, TUNEL-positive cells were rare in control cultures at all time points. In cultures exposed to soluble Aβ42, however, TUNEL-positive cells were more common, and were especially abundant after 14 or 21 days of exposure to the peptide.

Quantitation of the TUNEL assays is shown in Fig. 2. Approximately 2% of the cells were TUNEL-positive at all time points in control cultures. In contrast, TUNEL-positive cells accounted for ~15%, ~25% and ~30% of the total cells after 7, 14 and 21 days, respectively, of culture in the continuous presence of Aβ42. Paralleling the accumulation of TUNEL-positive cells in cultures exposed to Aβ42 was a decrease of total cells. As illustrated in Fig. 3, Aβ42 caused little, if any cell loss after 7 days. In cultures that were exposed to Aβ42 for 14 or 21 days, however, cell numbers were estimated to be only 60% and 54%, respectively, of

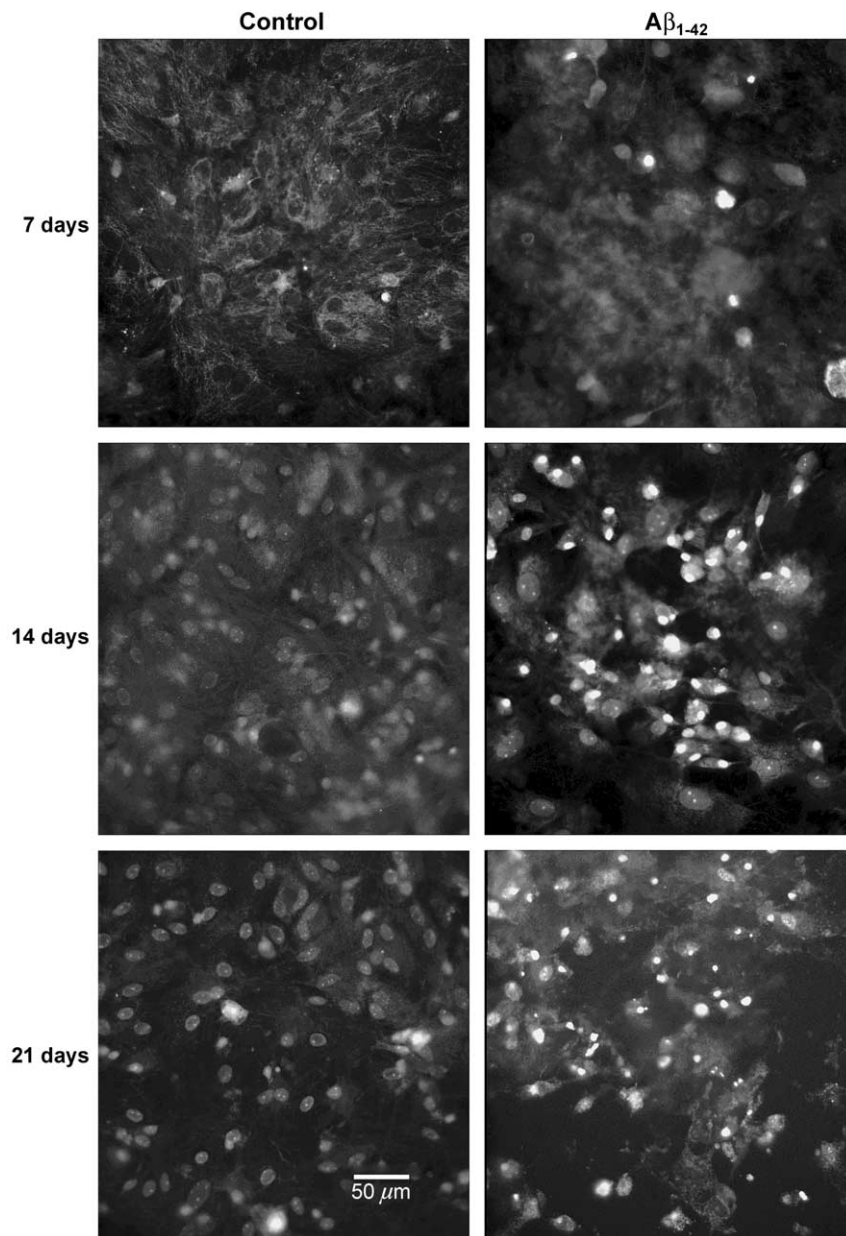Fig. 1. Aβ42 induces apoptosis in cultured mouse brain cells. Primary cultures derived from newborn mouse brains were grown in N2 medium supplemented with 2% calf serum. The cultures comprised ~15% neurons and ~85% glial cells. After 1 month in culture, cells were treated for 7, 14 or 21 days with 5 μM soluble Aβ42 dissolved in DMSO, or with DMSO alone (Control). At each time point, cells were fixed and then stained using fluorescent TUNEL reagents, which labeled nuclei in cells that had entered the apoptotic pathway. As shown here, Aβ42 potently induced TUNEL-positive staining within 14 days.

controls. Thus, Aβ42 was a potent inducer of both DNA fragmentation and cell loss, as occurs in apoptosis.

3.3. Neurons are preferentially sensitive to β-amyloid, and Aβ42 is more cytotoxic than Aβ40

Although the cultures used for this study were obtained from mouse brains, prior studies of comparable rat brain cultures revealed the presence of a rich variety of cell types, including neurons, astrocytes and oligodendrocytes [30]. To determine whether neurons or glial cells were being preferentially targeted for destruction by Aβ42, control and treated cells were analyzed by double fluorescence microscopy. The TUNEL reagent, fluorescein-labeled dUTP, was used to detect putative apoptotic cells, and the Tau-5 monoclonal anti-tau antibody, which was counterstained with Texas red-labeled goat anti-mouse IgG, was used as a marker for neurons. Tau-5 recognizes all tau isoforms independently of their phosphorylation state [24], and yields much brighter staining of neurons than non-neuronal cells.

As illustrated in Fig. 4, a high proportion of the neurons (strongly Tau-5-positive cells with axon-like, but not dendrite-like, immunoreactive processes) were TUNEL-positive after 7, 14 and 21 days of culture in the presence of soluble Aβ42. It must be noted, however, that the absolute number of neurons declined dramatically by 21 days of exposure to Aβ42 (see Fig. 5). In contrast, control cultures at all time points contained very low numbers of TUNEL-positive cells, and far fewer cells that were positive for both the TUNEL assay and Tau-5.

A quantitative summary of these data, along with comparative data for Aβ40, is presented in Fig. 5. Control cultures at all time points contained ~15% Tau-5-positive neurons and ~3% apoptotic cells, and total cell numbers remained relatively constant with time. After 7 or 14 days of exposure to Aβ42, the percent of total cells that were neurons also remained nearly unchanged, but the majority

Fig. 3. Time-dependent cell loss from cultures treated with Aβ42. Cells were fully dissolved in SDS-PAGE sample buffer after having been treated for 7, 14 or 21 days with either 5 μM soluble Aβ42 or DMSO (Control). Each individual sample was obtained from a culture grown in a 35 mm Petri dish, and was dissolved in 200 μl of buffer. To estimate the relative numbers of cells that had been present in each Petri dish, the protein concentration of each sample was then measured. Each bar on the graph shown here represents the average protein concentration for three separate samples that were grown in parallel. Note that in cultures that had been exposed to Aβ42, massive cell death occurred between days 7 and 14, but only a slight, additional loss of cells was induced between days 14 and 21.

of the neurons were TUNEL-positive at both time points and thus were undergoing programmed cell death. It is therefore not surprising that after 21 days of Aβ42 treatment, neurons accounted for only a few percent of the total cells and frequently were apoptotic. Taking into account the fact that Aβ42 had little effect on total cell numbers after 7 days, but caused a 40–50% reduction after 14 and 21 days (Fig. 3), the data summarized in Fig. 5 indicate that compared to controls, cultures exposed to Aβ42 for 21 days had lost nearly all of their neurons and about half of their glial cells. Furthermore, because a high percentage of the glial cells (stained weakly or not at all by Tau-5) were TUNEL-positive after 14 and 21 days in the presence of Aβ42, it is likely that further exposure to the peptide would have eventually killed most of the glial cells. Fig. 5 also demonstrates induction of apoptosis by Aβ40, although in contrast to Aβ42 this did not appear to occur preferentially for neurons. Finally, 21 days of Aβ40 exposure caused only a minor reduction in total cell number (data not shown), but the abundance of TUNEL-positive cells present at that time point (Fig. 5) suggests that longer treatment of cultures with Aβ40 would have resulted in substantial cell death.

3.4. Effects of 5 μm Aβ peptides on tau phosphorylation

Exogenous Aβ peptides have been shown to induce AD-like phosphorylation of tau in serum-free cultures of pure neurons, but only when the peptides were in the fibrillar form [9,13]. In contrast to these prior studies, our cultures contained both neurons and glial cells, were maintained in low (2%) serum, and were exposed to 5 μM freshly solubilized, non-fibrillar, Aβ peptides. In light of these methodological differences, we sought to determine whether
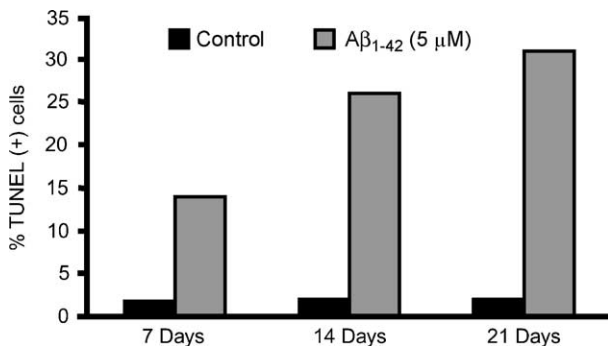

Fig. 2. Quantitation of apoptosis induction by Aβ42. Primary newborn mouse brain cells grown as described for Fig. 1 were scored for percentage of total cells that were TUNEL-positive after having been treated for 7, 14 or 21 days with either 5 μM soluble Aβ42 or DMSO (Control). Illustrated here are the results of one representative experiment. A total of 500 cells were counted for each bar on the graph shown here.
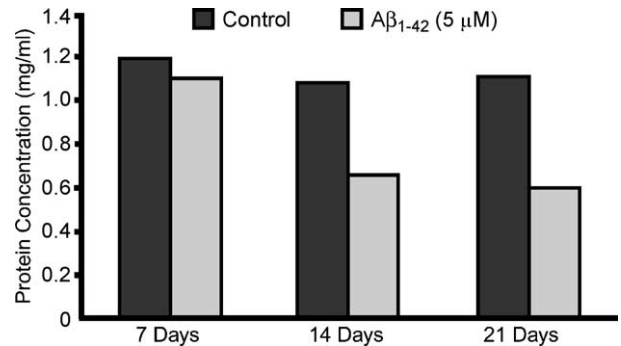
Aβ42 or Aβ40 could influence the phosphorylation state of tau in our mixed brain cell cultures.

Immunofluorescence microscopy with three monoclonal anti-tau antibodies was employed to monitor effects of the Aβ peptides on tau phosphorylation. As described earlier, Tau-5, was used as a marker for total tau because it is insensitive to alternative splicing or phosphorylation of tau [24]. Tau-1, which reacts with tau that is dephosphorylated at serine 199/202, but does not react well with AD tau [22–24], was used as a marker for normal tau. Finally, PHF-1, which reacts with an epitope that includes phosphorylated

serine 396/404 and is characteristic of PHF tau [25], was used as an indicator of AD-like tau.

Fig. 6 summarizes the results of a typical experiment in which immunofluorescence was used to determine the proportion of total cells that was positive for each of the three anti-tau antibodies. Consistent with the results shown in Fig. 5, at all time points in control medium, 15–18% of the cells were judged to be neurons, based on their strong immunoreactivity with Tau-5 (Fig. 6) and anti-MAP2 (not shown). A similar proportion of the cells were consistently Tau-1-positive, and ~5% of the cells were routinely stained
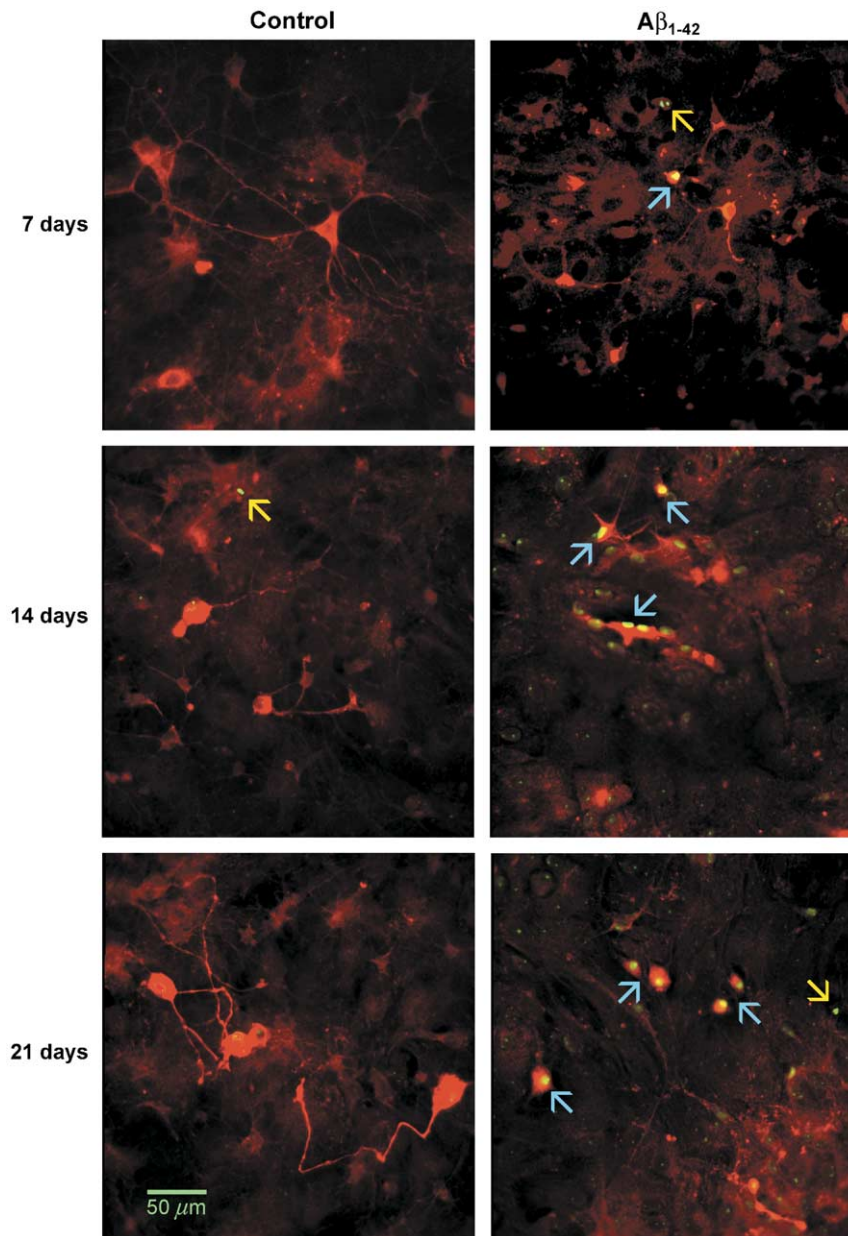

Fig. 4. Neurons are preferentially sensitive to Aβ42. Cells were stained for double fluorescence microscopy after having been treated for 7, 14 or 21 days with either 5 μM soluble Aβ42 or DMSO (Control). The Tau-5 antibody was used to identify neurons (red) and TUNEL reagents were used to identify apoptotic nuclei (green). Yellow arrows point to apoptotic non-neuronal cells, and blue arrows point to apoptotic neurons. Note that at all time points, neurons accounted for a high proportion of the apoptotic cells in cultures exposed to Aβ42.
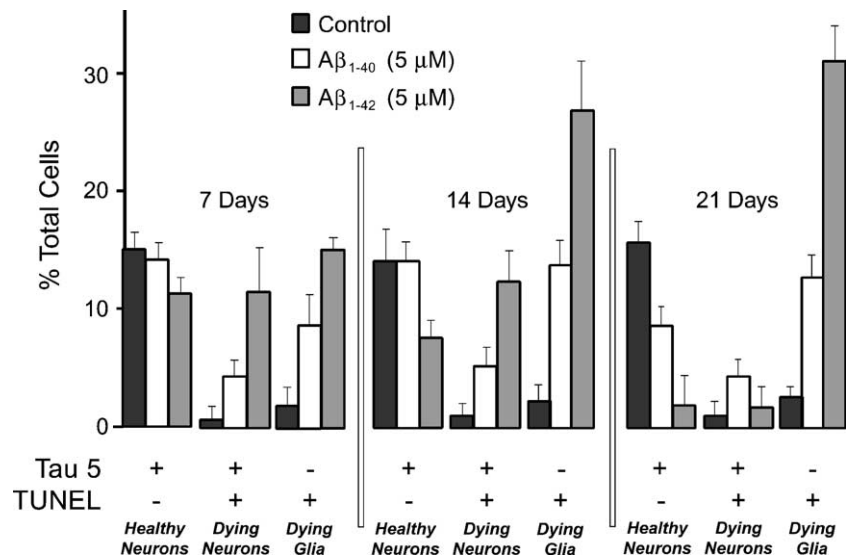
Fig. 5. Aβ42 is more cytotoxic than Aβ40. Cells were stained for double fluorescence microscopy after having been treated for 7, 14 or 21 days with 5 μM soluble Aβ40 or Aβ42, or DMSO (Control). The Tau-5 antibody was used to identify neurons and TUNEL reagents were used to identify apoptotic nuclei, exactly as described for Fig. 4. Five hundred cells from each culture condition were then scored for reactivity with Tau-5 and for TUNEL-positive nuclei. Shown here are the results of one representative experiment. The following conclusions are noteworthy. (1) Comparison of apoptotic neurons (Tau 5+/TUNEL+) to apoptotic glial cells (Tau 5−/TUNEL+) demonstrated that Aβ peptides preferentially induced neurons to become apoptotic. (2) At most time points, Aβ42 induced approximately twice as many TUNEL-positive cells as Aβ40, and is therefore more cytotoxic than Aβ40. (3) Aβ42 killed most neurons (Tau 5+) within 21 days.

by PHF-1. An anti-GFAP, which according to the vendor (ICN Biomedicals, Inc.) recognizes astrocytes and Bergmann glia in vivo, stained the majority of control cells at all time points (not shown), but no effort was made to quantify the GFAP-positive cells more accurately.

As shown in Fig. 6, cultures suffered only modest losses of immunoreactivity with tau antibodies after 21 days of exposure to 5 μM soluble Aβ40. By comparison, 5 μM soluble Aβ42 appeared to have a dramatic effect on tau-positive cells. After 7–14 days in the presence of soluble Aβ42, the percentage of cells stained by Tau-5 or Tau-1 were nearly equal, and almost undistinguishable from controls. By 21 days, however, almost no Tau-5-positive or tau-1-positive cells remained, confirming the near complete loss of neurons documented in Fig. 5 for such conditions. PHF-1-positive cells were ~2.5-fold more abundant after 7 days in the presence of Aβ42 than in controls, but decreased to control levels after 14 days, and were virtually undetectable by 21 days. Thus, Aβ42, but not Aβ40, induced a transient increase in neurons expressing the AD-like PHF-1 epitope, and caused a precipitous decline in the number of total neurons (Tau-5-positive cells) within 3 weeks of continuous exposure to the peptide.

3.5. How do Aβ peptides induce tau pathology and apoptosis in vivo?

Previously published studies of cultured neurons and neuroblastoma cells, and transgenic mice have indicated that Aβ peptides, and perhaps other by-products of APP metabolism as well, can induce tau pathology and act as

pro-apoptotic factors. The new data described here for mixed brain cell cultures are largely consistent with those earlier studies, and extend them in three novel directions. They demonstrate that compared to Aβ40, Aβ42 is more cytotoxic, more selective for neurons, and more capable of inducing the AD-like, PHF-1 epitope on tau. This is consistent with previously reported results that show that Aβ42 aggregates to form cytotoxic oligomers more rapidly than Aβ40, and it suggests that the enhanced cytotoxicity of Aβ42 is due to its enhanced aggregation [31]. What does this collection of old and new data imply about the pathways for Aβ signaling to tau and the apoptotic machinery in vivo?

One possible signaling network involves cyclin-dependent kinase 5 (Cdk5), which can be activated by proteolytic conversion of its p35 regulatory subunit to p25. Treatment of cultured rat cortical neurons with Aβ42 has been reported to induce p25 [32], and transgenic mice that express p25 suffer neuronal loss in the hippocampus, and accumulate the pro-apoptotic caspase-3 protease, the AD-like AT8 and PHF-1 tau epitopes, and tau filaments [33]. Interestingly, double transgenic mice for p25 and P301L tau develop AD-like, hyperphosphorylated tau and accumulate tau filaments more rapidly than either singly transgenic parental strain [34], and caspase-3 is one of several caspases that can cleave tau at D421, thereby yielding a truncated tau with enhanced ability to polymerize into filaments in vitro [35]. It is therefore especially significant that this truncated form of tau can be induced in primary rat cortical neurons by exposure of the cells to Aβ42 [35]. Taken together, these findings trace paths from
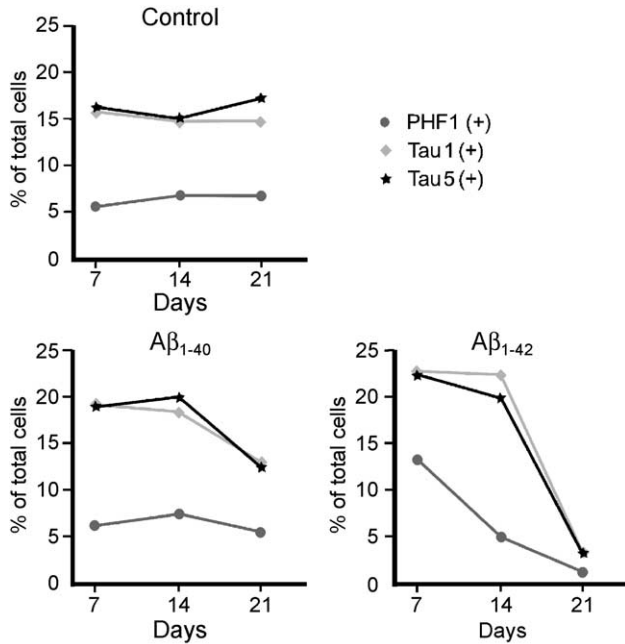
Fig. 6. Aβ peptides do not dramatically affect the phosphorylation states of the tau-1 or PHF-1 epitopes in neurons. To determine whether Aβ peptides influence the phosphorylation states of the Tau-1 or PHF-1 epitopes, cells were stained for immunofluorescence microscopy with Tau-1, Tau-5 or PHF-1 after having been treated for 7, 14 or 21 days with 5 μM soluble Aβ40 or Aβ42, or DMSO (Control). Tau-1 reacts with tau which is dephosphorylated at serine 199/202, Tau-5 recognizes a phosphorylation-independent epitope on tau, and PHF-1 recognizes tau which is phosphorylated at serine 396/404. For each condition the percentage of immuno-positive cells out of 400 total cells were determined for each antibody. Note that Aβ40 and Aβ42 had similar effects on immuno-reactivity of the cultures with Tau-1, Tau-5 and PHF-1, although PHF-1-positive cells nearly disappeared from the cultures within 14 days of exposure to Aβ42.

Aβ42 through Cdk5, and then through pro-apoptotic caspases to tau phosphorylation and filament assembly, and programmed cell death.

As appealing as that signaling network may seem, it is unlikely that it represents the sole route to tau pathology or apoptosis induced by Aβ peptides. For instance, Aβ42 that accumulated intracellularly as a result of direct micro-injection or transient transfection has been reported to be cytotoxic through its effects on p53 and Bax [36], and Aβ42 has been shown in vitro to associate directly with tau, and thereby stimulate tau self-aggregation and the ability of tau to be phosphorylated by tau protein kinase II [37]. Many other protein kinases that phosphorylate tau at sites characteristic of AD tau are known, although we are not aware of any that have been connected yet to Aβ peptides. Nevertheless, it is worth considering the possibility that Aβ peptides signal to tau through kinases like glycogen synthase kinase 3β (GSK3β), which has been implicated in neurodegeneration, and tau hyperphosphorylation and filament assembly in transgenic *Drosophila* [38], and MARK, which phosphorylates tau at S262 and thus impairs its affinity for microtubules [39].

A final issue about which we would like to speculate is the immediate source of the Aβ responsible in vivo for inducing tau pathology and apoptosis. The cultured cell data presented here and elsewhere indicate that extracellular Aβ peptides, in spite of reported differences between Aβ40 and Aβ42, and aggregated versus monomeric forms of the peptides, can kill brain cells quickly at concentrations in the low micromolar range, and perhaps much lower. If such levels of Aβ peptides were steadily being produced and then immediately diffused through brain tissue, one would expect rapid and massive tau pathology and cell death to ensue. Although it is difficult to extrapolate from tissue culture to the human brain, the slow rate at which AD progresses suggests that a different, less swift mechanism applies in vivo. Intracellular Aβ, which only recently has begun to attract attention, could be a culprit. Although there is ample reason to believe that extracellular amyloid deposits are related to AD pathogenesis, perhaps it is not because they are directly toxic, but rather because they represent reservoirs from which Aβ peptides diffuse away at a rate slowly enough to induce tau pathology and apoptosis gradually over the course of many years. In this scenario, extracellular amyloid deposits might be inert themselves, but a potential source of destructive, oligomeric Aβ peptides. Furthermore, perhaps Aβ peptides diffuse away from extracellular amyloid deposits or are degraded quickly after diffusion at variable rates, with high rates causing full blown AD, and low rates being associated with heavy amyloid loads in the absence of tau pathology, dementia or neurodegeneration.

Acknowledgements

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