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Aβ1-42 monomers or oligomers have different effects on autophagy and apoptosis

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Abbreviations: Aβ, amyloid β; ACTB, β actin; AD, Alzheimer disease; APP, amyloid precursor proteins; AVs, autophagic vacuoles; BACE1, beta-site APP-cleaving enzyme 1; BAK1, pro-apoptotic protein; BCL2, B-cell lymphoma 2 protein; BECN1, Beclin 1; CASP3, caspase 3; CTSD, cathepsin D; CYCS, cytochrome c; LAMP1, lysosomal-associated membrane protein 1; LC3, microtubule-associated protein 1 light chain 3; LDH, lactate dehydrogenase; MAPK8/JNK1, mitogen-activated protein kinase 8; MAPK9/JNK2, mitogen-activated protein kinase 9; SQSTM1/p62, sequestosome1

The role of autophagy and its relationship with apoptosis in Alzheimer disease (AD) pathogenesis is poorly understood. Disruption of autophagy leads to buildup of incompletely digested substrates, amyloid-β (Aβ) peptide accumulation in vacuoles and cell death. Aβ, in turn, has been found to affect autophagy. Thus, Aβ might be part of a loop in which it is both the substrate of altered autophagy and its cause. Given the relevance of different soluble forms of Aβ1-42 in AD, we have investigated whether monomers and oligomers of the peptide have a differential role in causing altered autophagy and cell death. Using differentiated SK-N-BE neuroblastoma cells, we found that monomers hamper the formation of the autophagic BCL2-BECN1/Beclin 1 complex and activate the MAPK8/JNK1-MAPK9/JNK2 pathway phosphorylating BCL2. Monomers also inhibit apoptosis and allow autophagy with intracellular accumulation of autophagosomes and elevation of levels of BECN1 and LC3-II, resulting in an inhibition of substrate degradation due to an inhibitory action on lysosomal activity. Oligomers, in turn, favor the formation of the BCL2-BECN1 complex favoring apoptosis. In addition, they cause a less profound increase in BECN1 and LC3-II levels than monomers without affecting the autophagic flux. Thus, data presented in this work show a link for autophagy and apoptosis with monomers and oligomers, respectively. These studies are likely to help the design of novel disease modifying therapies.

Introduction

Macroautophagy (hereafter referred to as autophagy) and apoptosis represent the 2 processes through which altered or aged cells and organelles are removed. Autophagy involves sequestration of portions of the cytoplasm and intracellular organelles within autophagic vacuoles (named autophagosomes) that are sent to lysosomes for degradation.[1] Apoptosis is the best-known form of programmed cell death and involves the activation of catabolic pathways that lead to the rapid destruction of cellular organelles.[2] Interestingly, the 2 processes are likely to be connected with each other in Alzheimer disease pathogenesis. Disruption of autophagy leads to amyloid-β (Aβ) peptide accumulation in vacuoles and cell death.[3,4] Aβ, in turn, has been shown to affect autophagy.[5] Thus, it is plausible that Aβ plays a key role in linking the 2 processes with each other during AD.

Aβ is the central peptide in the amyloid hypothesis that has dominated the AD field for many years.[6] Aß derives from amyloid precursor protein through BACE1 and γ-secretase processing. Prior to its deposition in amyloid plaques, Aβ forms soluble oligomers that constitute the major killer form of the peptide.[7] Interestingly, concentrations of Aβ1-42 oligomers are higher in plasma of AD patients that control subjects (642.5 ng/ml vs. 441.8 ng/ml). Furthermore they are negatively correlated with cognition while plasma levels of Aβ1-42 monomers do not differ between AD patients and controls (9.94 pg/ml vs. 8.42, respectively).[8] Monomers, instead, have been proposed to be involved in physiological processes. For instance, we recently identified a physiological function for monomeric Aβ1-42 that was able to trigger *BACE1* transcription without affecting cell survival suggesting that the boundary separating toxicity from a probable physiological signaling of Aβ is very narrow.[9] However, oligomers

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of Aβ1-42 are also likely to play a physiological role in synaptic plasticity and memory.[10] Indeed, the membrane-affinity of oligomers and monomers is very similar,[11,12] with a higher propensity to attach to the membrane by the oligomers,[13] and a lower affinity to bind to the cell membrane by the monomers.[14] There is the need to better differentiate the effects of the 2 Aβ species.

Defective autophagy has been implicated in AD.[15] Autophagy initiation was found to be increased after Aβ stimulation[16] and in an AD mouse model.[3] Extensive autophagic-lysosomal pathology was found in the TgCRND8 mouse model of AD, an animal with an aggressive production of Aβ1-42.[17] On the other hand, strong evidence indicates that Aβ is both produced and degraded during autophagy.[3,4] In the healthy brain, autophagy may play a relatively minor role in basal Aβ production because efficient clearance of autophagic vacuoles (AVs) and lysosomal degradation of Aβ prevent its accumulation.[18] However, in pathological conditions both endocytic and autophagic pathways are sites of APP processing and Aβ production.[4] AVs are numerous in AD brains, particularly, in dystrophic neuritis.[19] Autophagy is also evident in the perikarya of neurons with tangles. The accumulation of immature AV forms in dystrophic neurites suggests that the transport and maturation of AVs to lysosomes may be affected in AD. Of note, AVs are a major reservoir of intracellular Aβ in the brain.[3] Purified AVs contain full-length APP as well as β-secretase and the γ-secretase complex subunits PSEN1 (presenilin 1) and NCSTN (nicastrin).

Additional evidence in favor of an involvement of autophagy in relationship to Aβ in AD derives from studies on the expression of a key regulator of the initiation of the autophagic process, BECN1. BECN1 levels are decreased in AD patients.[20,21] The reduction of BECN1 is supposed to be caused by caspase-mediated cleavage,[22] another phenomenon involved in APP processing and AD pathogenesis. CASP3 (caspase 3) processes GGA3 (golgi-associated, gamma adaptin ear-containing, ARF binding protein 3),[23] an adaptor protein involved in trafficking of BACE1 to the lysosome for degradation. The GGA3-depletion induced by CASP3 affects the degradation of BACE1 and induces BACE1 activity, determining an enhancement of Aβ peptide generation.[23] Moreover, a selective increase of CASP3 has been found in the post-synaptic compartment of brains from early cases of AD.[24] Autophagic markers ATG5, ATG12, and LC3 are also linked with plaques and tangles in AD.[25] Morphological evidence also shows that APP and Aβ peptides are colocalized with LC3-positive autophagosomes in a cell line overexpressing APP and in AD mouse models,[3,26] suggesting that Aβ can be a substrate of autophagy. Finally, it has been found that the accumulation of insoluble Aβ1-42 and SQSTM1, a marker of autophagic flux, precedes the impairment of autophagic clearance and may be a cause of lysosomal failure.[27] Together, these data support the model in which autophagy is crucial for the removal of Aβ peptides.

Collectively, these findings suggest that modulation of autophagy may be a therapeutic target for diseases associated with protein aggregation. While autophagy clears certain aggregated proteins, upregulation of autophagy can also influence Aβ pathology, as autophagic vacuoles may represent a site of Aβ production and neurodegeneration. In this manuscript, given the relevance of soluble monomeric as well as oligomeric preparations of Aβ1-42 in AD pathogenesis, we studied their role in altering autophagy and causing cell death.

Results

Both Aβ1-42 monomers and oligomers impair autophagy

To determine whether Aβ1-42 oligomers and monomers affect autophagy, we measured levels of autophagy-associated proteins, BECN1, and LC3-II in differentiated SK-N-BE neuroblastoma cells treated with 1 μM Aβ1-42 oligomers and monomers. BECN1 participates in autophagosome formation. MAP1LC3/LC3 (microtubule-associated protein 1 light chain 3) is a ubiquitin-like protein that becomes lipidated (LC3-II) and is tightly associated with the phagophore and autophagosomal membranes.[28] The distribution of these 2 proteins using a confocal scanning laser microscope is reported in **Figure 1A**. After 24 h both monomeric and oligomeric Aβ1-42 preparations increased BECN1 immunoreactivity; however a higher number of positive cells were observed after treatment with the monomeric preparation. LC3 A/B immunoreactivity was similar after 24 h of incubation with Aβ1-42 monomers and oligomers. Then, to further study whether Aβ1-42 oligomers and monomers affect autophagy, we measured protein levels of BECN1 and LC3 by western blot (**Fig. 1B**). Both preparations were able to significantly increase BECN1 levels, but the increase was more pronounced with monomers than with oligomers (up to 3-fold with monomers vs. 2.5-fold with oligomers). Moreover, the increase was already present at 1 h of treatment, whereas it took about 8 h to start for oligomers. Examination of LC3-II levels also showed an increase in protein levels for both monomeric and oligomeric preparations. Monomers of Aβ1-42 produced a marked and significant increase in LC3-I-II protein levels that started at 3 h and was still present after 48 h of treatment, whereas oligomers produced a transient increase that was no longer present after 8h to 12 h of incubation.

We also examined the effect of Aβ1-42 species on the autophagic flux by assessing the levels of SQSTM1, one of the selective substrates of autophagy. SQSTM1 is a protein that is degraded by autophagy and starts lysosomal degradation linking ubiquitinated proteins to the autophagic machinery. Since SQSTM1 accumulates when autophagy is inhibited, and decreases when autophagy is induced, SQSTM1 may be considered a marker of autophagic flux.[29] The SQSTM1 protein levels after treatment with Aβ1-42 monomers and oligomers are reported on **Figure 2A**. Cell treatment with monomeric preparations produced a significant (> 2-fold) increase in SQSTM1 levels after 1 h of incubation that lasted up to 24 h, whereas oligomers of Aβ1-42 caused a slight, but not significant decrease in SQSTM1 protein levels.

To determine whether Aβ1-42 monomeric or oligomeric preparations are able to affect the number of early and late autophagosomes, we used a *RFP-GFP LC3* DNA construct. As expected, in **Figure 2B**, both treatments with monomers and oligomers induced a significant increase in the number of late

autophagosomes (GFP- RFP+ LC3-positive dots) suggesting that, at this time point, the autophagic flux is increased. More interestingly, treatment of cells with monomers also strongly increased the number of early autophagosomes (GFP+ RFP+ LC3-positive dots). Taken together these findings suggest that monomers and oligomers affect differently the autophagic flux and that monomers induced a too-strong increase in new autophagosomes that could be only partially degraded by the lysosomes.

Our next goal was to determine whether early autophagosomes are fused (the GFP- RFP+ LC3-positive dots are autophagosomes that have fused with the lysosomes) and digested by lysosomes. First we analyzed lysosomal formation. **Figure 3A** shows LAMP1 (lysosomal-associated membrane protein 1) and Aβ1-42 immunostaining after 24 h of treatment with different Aβ species, suggesting that lysosomal vesicles are formed and also that there is a colocalization with lysosomes and the 2 Aβ1-42 preparations. This finding does not necessarily mean that Aβ forms are transported (and also degraded) to lysosomes by autophagy because this process can also occur through endocytosis, chaperone-mediated autophagy, or direct binding to lysosomal membrane or lysosomal membrane proteins.

Next, we measured lysosomal activity by evaluating CTSD/cathepsin D activity. We found that Aβ1-42 monomers, but not oligomers, impair CTSD activity (~60% - 70%) after 6 h and up to 24 h of treatment (**Fig. 3B**). Pepstatin, a CTSD inhibitor, used as a positive control, induced a decrease (~85%) of the enzymatic activity after 8 h of incubation. Finally, we pretreated cells with the lysosomal inhibitor chloroquine at the concentration of 100 μM for 24 h followed by exposure to the oligomeric preparation. Aβ1-42 was capable of mediating a strong and significant increase in LC3-II protein levels that was present at 3 h and lasted at least for 24 h (**Fig. 3C**), with a trend almost similar to monomers.

Aβ1-42 oligomers, but not monomers, induced necrotic and apoptotic cell death

Our next goal was to examine the necrotic cell death as LDH release in culture medium (**Fig. 4A**). The treatment of differentiated neuroblastoma cells with oligomeric Aβ1-42 preparation induced a stronger (~60% vs. 20%) release of LDH compared with the monomeric form. Measurement of expression of cleaved CASP3, a critical executioner

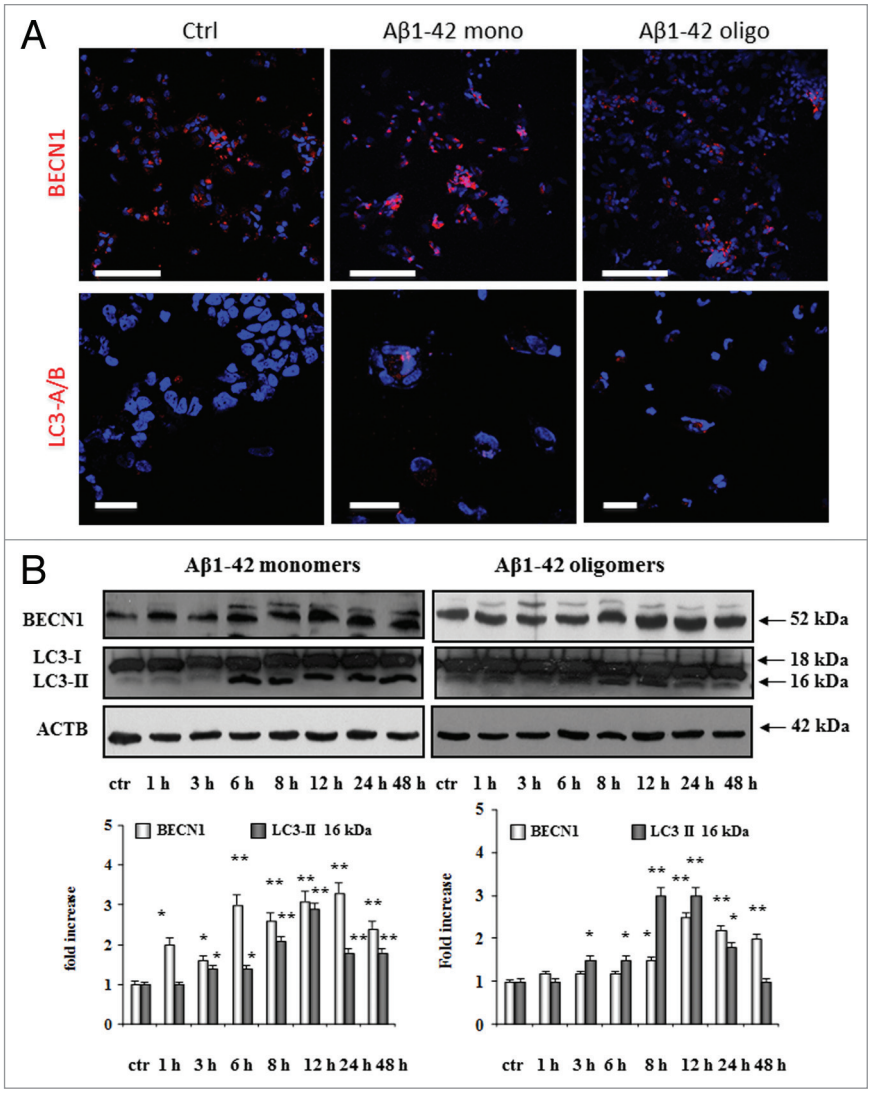

Figure 1. Treatment of differentiated SK-N-BE cells with 1 μM Aβ1-42 monomers and oligomers significantly increase BECN1 and LC3-I and LC3-II fluorescence intensity and protein levels. (**A**) Cells treated with 1 μM Aβ42 monomers and oligomers for 24 h were stained, after fixation and permeabilization, with BECN1 or LC3 antibodies. After washing in PBS, sections were incubated with secondary antibodies raised in different species: 1:200 cyanine 3-conjugated donkey anti-mouse secondary antibody and 1:100 cyanine 2-conjugated anti-rabbit secondary antibody with 1% BSA in PBS. For counterstaining, cells were incubated for 3 min with DAPI diluted 1:50 in methanol 0.1 M and rinsed with PBS. After 24 h both monomeric and oligomeric Aβ1-42 preparations increased BECN1 immunoreactivity; however a higher number of positive cells were observed after treatment with the monomeric preparation. (**B**) BECN1 and LC3-I/-II protein levels in cells treated with 1 μM Aβ42 monomers and oligomers at different time points up to 48 h. Both preparations were able to significantly increase BECN1 levels, but the increase was more pronounced with monomers than with oligomers (up to 3-fold with monomers vs. 2.5-fold with oligomers). Examination of LC3-II levels also showed an increase in protein levels for both monomeric and oligomeric preparations. Monomers of Aβ1-42 led to a marked and significant increase in LC3-II protein levels that started at 3 h and was still present after 48 h of treatment, whereas oligomers produced a transient increase that was no longer present after 8 h to 12 h of incubation. The error bars represent standard deviations. Experiments were conducted in triplicate. *, significantly different from controls ($P < 0.05$); **, significantly different from controls ($P < 0.02$).

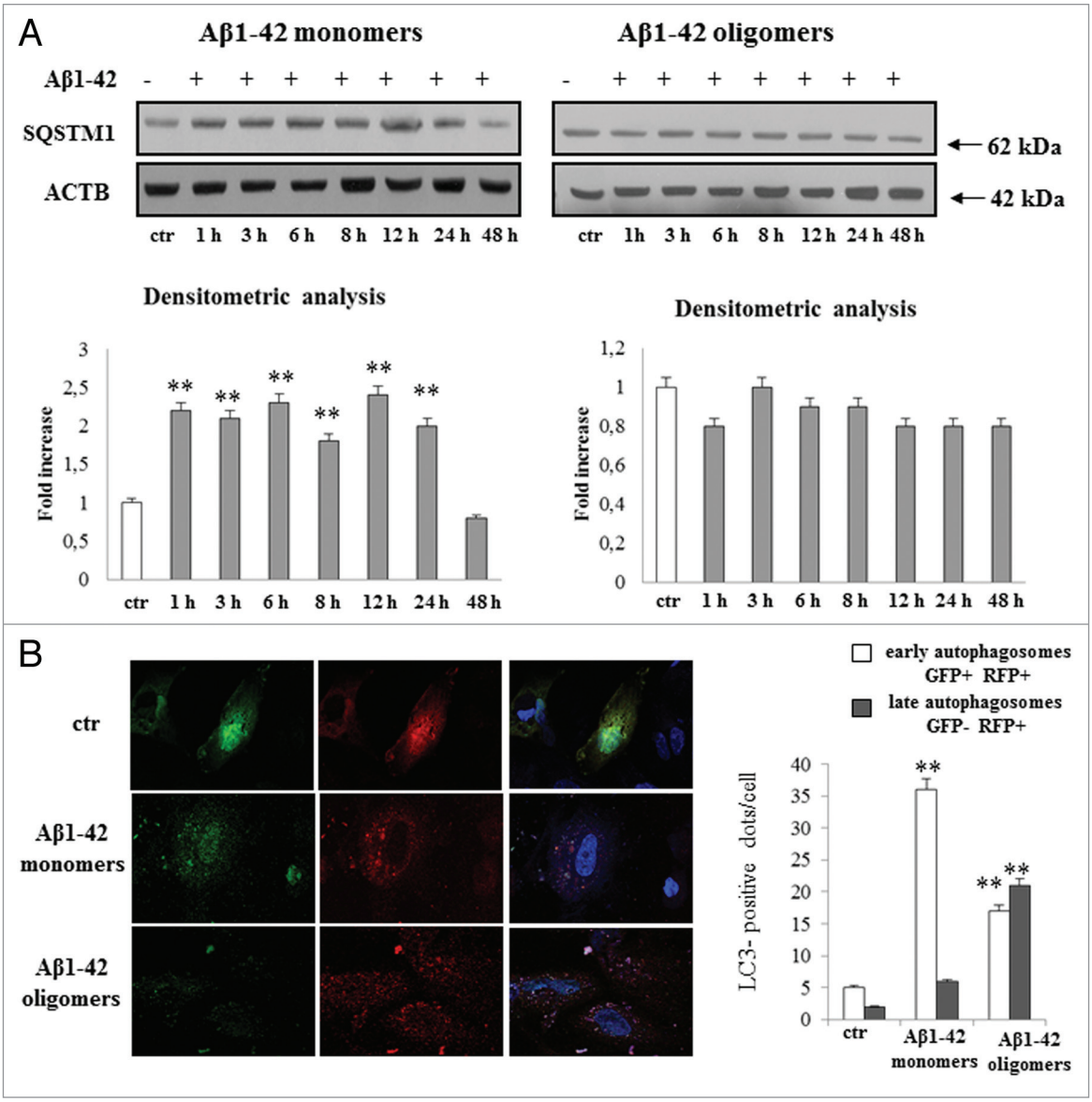

Figure 2. Treatment of differentiated SK-N-BE cells with 1 μM Aβ1-42 monomers, but not oligomers, significantly decreased autophagic flux, through accumulation of early endosomes. (**A**) SQSTM1 protein levels in cells treated with 1 μM Aβ1-42 monomers and oligomers up to 48 h. Treatment of cells with monomeric preparations was followed by a significant (> 2-fold) increase in SQSTM1 levels after 1 h of incubation that lasted up to 24 h, whereas oligomers of Aβ1-42 caused a slight, but not significant decrease in SQSTM1 protein levels. (**B**) On the left, confocal images of representative cultured SK-N-BE neuronal cells transfected with GFP-RFP-LC3 DNA for 24 h following 8 h of treatment with 1 μM Aβ1-42 monomers or oligomers. On the right, quantification of early and late autophagosomes per cells (15 cells) after treatment with 1 μM Aβ1-42 monomers and oligomers. The increase in number of early autophagosomes (GFP^+-RFP^+) was much higher in cells treated with monomeric preparation than in cells treated with oligomers, whereas late endosomes (GFP^- RFP^+) were significantly increased after treatment with both preparation. For counterstaining, cells were incubated for 3 min with DAPI diluted 1:50 in methanol 0.1 M and rinsed with PBS. Experiments were conducted in triplicate. *, significantly different from controls ($P < 0.05$); **, significantly different from controls ($P < 0.02$).

of apoptosis, through western blotting (WB) technique revealed that oligomeric Aβ1-42, but not the monomeric preparation, was capable of significantly increasing (2.5-fold) the cleavage of CASP3 at 3 and 6 h of incubation (**Fig. 4B**). Consistent with these results, immunocytochemistry of cleaved CASP3-positive neurons and terminal deoxynucleotidyltransferase-mediated biotinylated UTP Nick End Labeling and (TUNEL)-staining showed an increase in CASP3 cleavage and apoptotic nuclei at the same time points (**Fig. 4C**). Thus, differently from monomers, oligomers are the only Aβ species that are likely to induce cell death.

The induction of apoptotic cell death mediated by oligomeric preparations of Aβ1-42 was corroborated by analysis of the CYCS release as well as the protein levels of the proapoptotic effector BAK1 in the cytosol (**Fig. 5A**). Treatment with oligomeric Aβ1-42 increased the CYCS release (100 to 120%)

after 3 h and up to 24 h, and BAK1 protein levels (120 to 150%) after 1 h and up to 24 h (**Fig. 5A**). Data reported in **Figure 5A** indicate that the same experimental conditions that induced an increase of BAK1 levels also produced a significant decrease of the antiapoptotic protein BCL2 (B-cell CLL/lymphoma 2) (50%). As expected, treatment of cells with monomeric preparations of Aβ1-42 did not affect these parameters (**Fig. 5A**).

To define the molecular mechanisms behind apoptotic cell death, we analyzed the interaction between BCL2 and BECN1. It has been reported that BCL2 interacts with BECN1[30] promoting apoptosis,[2] whereas its phosphorylation is likely to impede formation of the BCL2-BECN1 complex permitting autophagy. Lysates from cells treated with both Aβ1-42 preparations, were immunoprecipitated with a polyclonal anti-BECN1 antibody and blotted with BCL2 antibody. As shown in **Figure 5B**, we observed that only the treatment with Aβ1-42 oligomers allowed the interaction between BCL2 and BECN1, as demonstrated by the increase in BCL2 in samples immunoprecipitated with BECN1 after 3 and 6 h of incubation. Thus, the formation of BCL2-BECN1 complex may represent the mechanism through which oligomers promote apoptotic cell death.

Next, we studied levels of cleaved CASP3 in SK-N-BE differentiated cells that overexpress BCL2 to investigate whether the BCL2-BECN1 complex formation mediates apoptosis. As shown in **Figure 5C**,

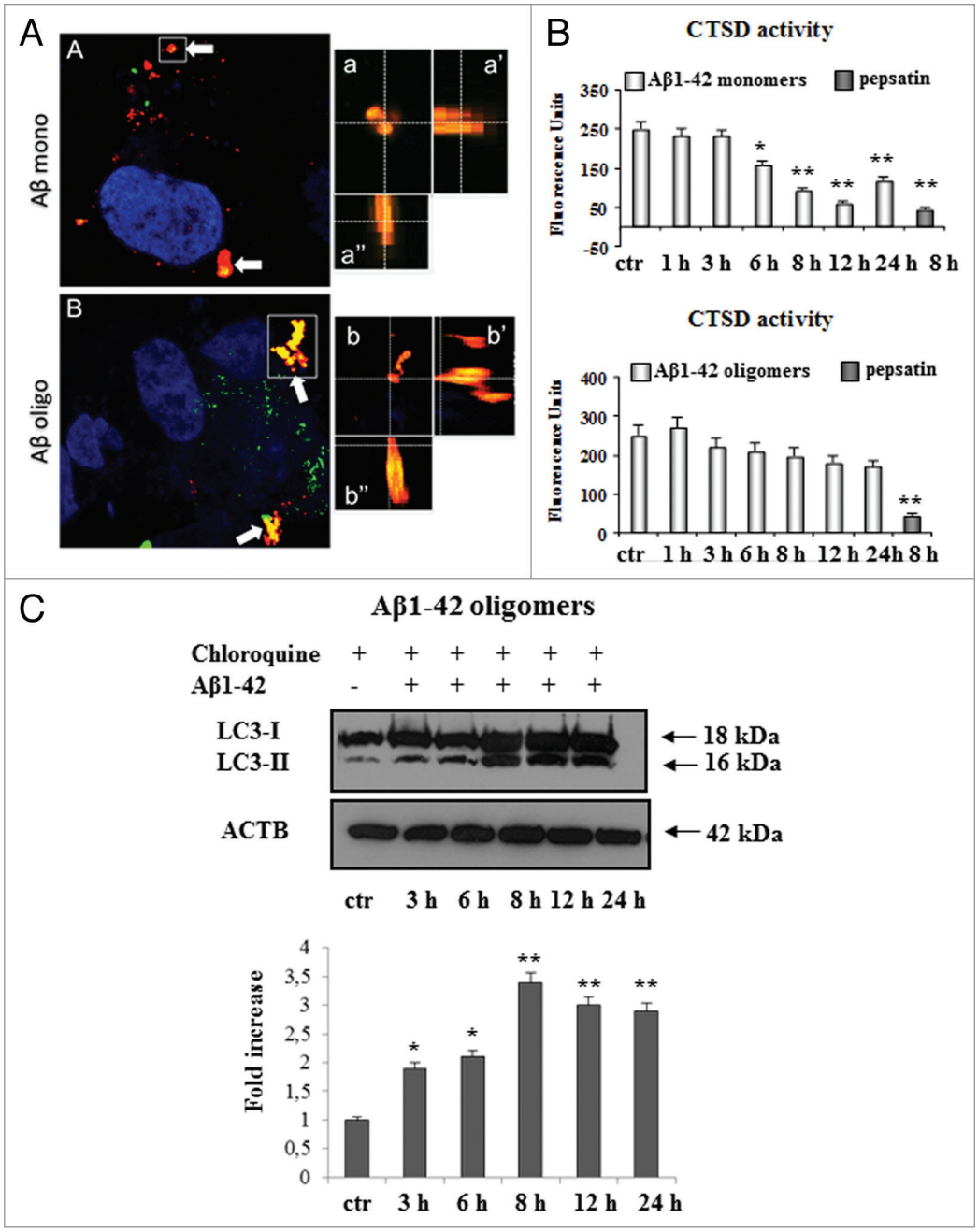

Figure 3. Treatment of differentiated SK-N-BE cells with 1 μM Aβ1-42 monomers affects autophagic flux through impairment of lysosomal activity. (**A**) Lysosomal-associated membrane protein 1 (LAMP1) immunostaining after 24 h treatment with the Aβ species. Cells treated with 1 μM Aβ42 monomers and oligomers for 24 h were stained, after fixation and permeabilization, with LAMP1 monoclonal and Aβ42 polyclonal antibodies. After washing in PBS, sections were incubated with secondary antibodies raised in different species: 1:200 cyanine 3-conjugated donkey anti-mouse secondary antibody and 1:100 cyanine 2-conjugated anti-rabbit secondary antibody) with 1% BSA in PBS. For counterstaining, cells were incubated for 3 min with DAPI diluted 1:50 in methanol 0.1 M and rinsed with PBS. As shown, lysosomal vesicles are formed and also there is a colocalization with lysosomes and the 2 Aβ1-42 preparations. (**B**) CTSD activity evaluation in SK-N-BE differentiated cells after treatments with the 2 Aβ1-42 preparations. Only treatment with Aβ1-42 monomers was followed by a significant decrease in the CTSD activity after 6 h and up to 24 h. Pepsatin, used as a positive control induced ~80% inhibition of the enzyme activity after 8 h of incubation. (**C**) LC3-II protein levels in SK-N-BE cells pretreated with 100 μM chloroquine for 24 h and then treated with Aβ1-42 oligomeric preparation for 8 h. Pretreatment of cells with the lysosomal inhibitor was able of mediating a strong and significant increase in LC3-II protein levels, with a trend almost similar to monomers. The error bars represent standard deviations. Experiments were conducted in triplicate. *, significantly different from controls ($P < 0.05$); **, significantly different from controls ($P < 0.02$).

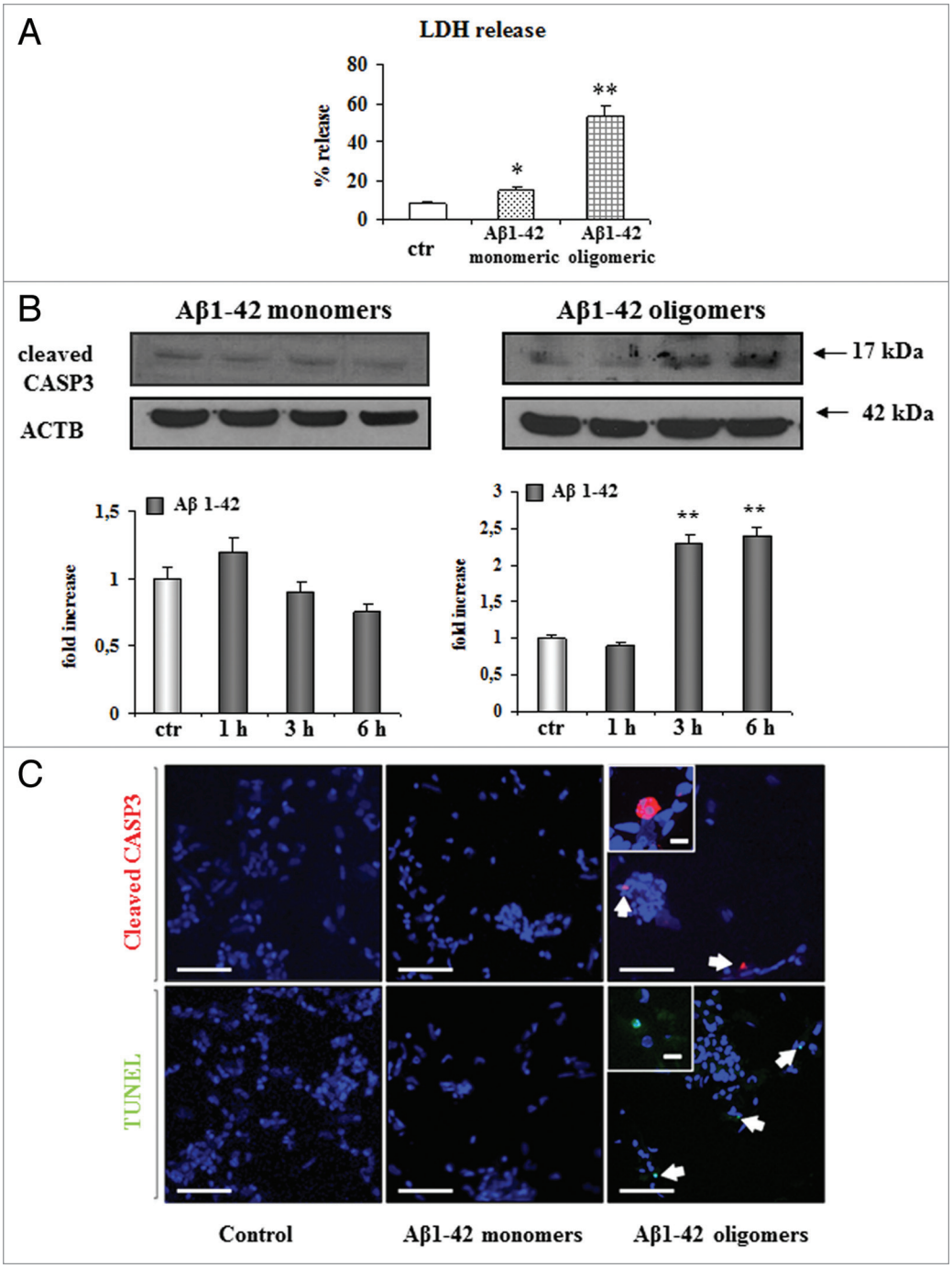

Figure 4. Treatment of differentiated SK-N-BE cells with 1 μM Aβ1-42 oligomers, but not monomers, significantly increases LDH release in the medium as well as cleavage of CASP3 and DNA fragmentation. (**A**) LDH release in differentiated SK-N-BE treated with Aβ1-42 oligomers as well as monomers for 24 h. The treatment of differentiated neuroblastoma cells with oligomeric Aβ1-42 preparation induced a more severe (~60% vs. 20%) release of LDH compared with the monomeric form. (**B**) Cleaved-CASP3 protein levels in cells treated with different Aβ species. We found that only oligomeric Aβ1-42, significantly increased (2.5-fold) the cleavage of CASP3 at 3 h and 6 h of incubation. (**C**) Cells treated with both oligomers and monomers of Aβ1-42 for 6 h were fixed, permeabilized, and stained with polyclonal antibodies against cleaved CASP3 (red) or with the DNA fragmentation stain TUNEL (green), and then analyzed by confocal microscopy. Monomers did not change cell number compared with controls, whereas oligomers strongly decreased it (oligomers: 61% increase in apoptotic nuclei vs. control; monomers: 18% increase in apoptotic nuclei vs. controls). Arrows indicate puncta of either cleaved CASP3 or TUNEL staining. Higher magnification views of given areas are encapsulated in squares. The nuclear stain DAPI (4,6 diamidino-2phenylindole, blue) was used to stain cells. Scale bars: 100 μm, or 15 μm for higher magnification views. The error bars represent standard deviations. Experiments were conducted in triplicate. *, significantly different from controls ($P < 0.05$); **, significantly different from controls ($P < 0.02$).

BCL2 protein levels were drastically increased in BCL2 overexpressing cells compared with normal cells (+300%). Moreover, treatment with 1 μM Aβ1-42 oligomers caused a significant decrease in BCL2 levels in normal cells but not in overexpressing cells. Most importantly, BCL2 overexpression was able to attenuate the cleavage of CASP3 mediated by oligomers, supporting the hypothesis that BCL2-BECN1 complex formation mediates apoptosis.

Monomeric Aβ1-42, but not the oligomeric species, causes activation of the MAPK8/JNK1-MAPK9/JNK2 pathway with an increase in BCL2 phosphorylation, as well as BACE1 expression and activity

Given that in previous experiments we have demonstrated that Aβ1-42 monomers are able to activate the MAPK8/JNK1-MAPK9/JNK2 pathway,[31] the kinase that phosphorylates BCL2[32] suppressing its complex with BECN1, we evaluated levels of nuclear phospho-MAPK8/JNK1-MAPK9/JNK2. Incubation of cells with Aβ1-42 monomeric preparation was followed by a robust activation of the MAPK8/JNK1-MAPK9/JNK2 pathway, as shown by the significant increase (~3 to 2.5-fold) in levels of phospho-MAPK8/JNK1-MAPK9/JNK2 in SK-N-BE nuclear fractions (**Fig. 6A**). In addition, measurement of BCL2 phosphorylation by WB in cytosolic fractions of cells treated with Aβ1-42 monomers revealed a significant increase (~2.5-fold) in BCL2 phosphorylation, as expected with the activation of the MAPK8/JNK1-MAPK9/JNK2 pathway (**Fig. 6B**).

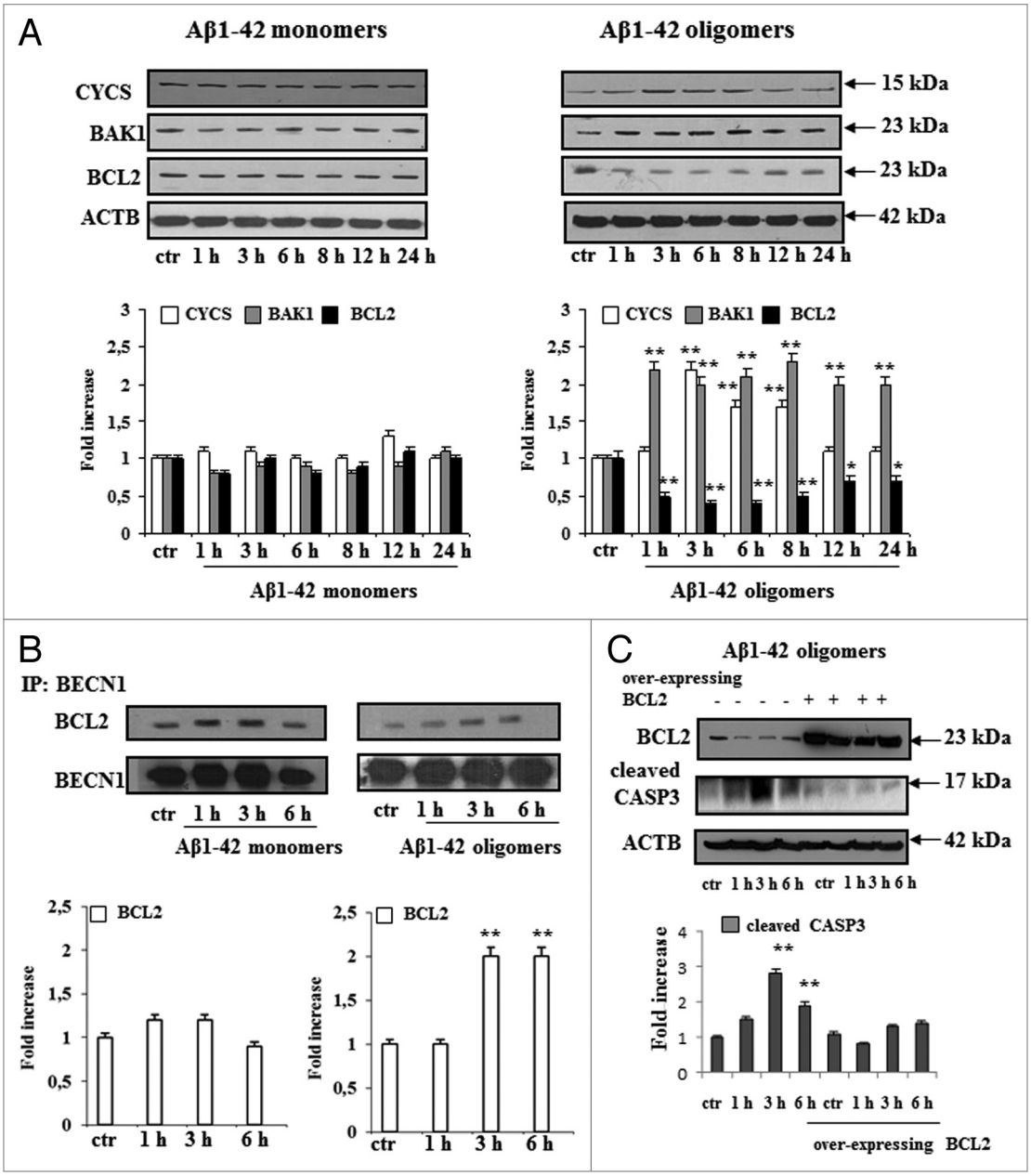

Figure 5. Treatment of differentiated SK-N-BE cells with 1 μM Aβ1-42 oligomers significantly increases CYCS and BAK1 and decreases BCL2 levels and allows the interaction between BCL2 with BECN1. (**A**) CYCS, BAK1 and BCL2 protein levels in differentiated SK-N-BE cells treated with Aβ1-42 oligomers as well as monomers up to 24 h. Treatment with oligomeric Aβ1-42 increased the CYCS release (100 - 120%) after 3 h and up to 24 h, and BAK1 protein levels (120–150%) after 1 h and up to 24 h, whereas BCL2 levels resulted significantly decreased (50%). Treatment of cells with monomeric preparations of Aβ1-42 did not affect these parameters. (**B**) Cells treated with Aβ preparations were immunoprecipitated with polyclonal BECN1 antibody and revealed with monoclonal BCL2 antibody. We found that BCL2 is linked to BECN1 only after 3 h to 6 h of treatment with Aβ1-42 oligomers. (**C**) BCL2 and cleaved CASP3 protein levels in differentiated SK-N-BE control and overexpressing BCL2 cells treated with 1 μM Aβ1-42 oligomers. BCL2 overexpression was able to attenuate the cleavage of CASP3 mediated by oligomers. The error bars represent standard deviations. Experiments were conducted in triplicate. **, significantly different from controls ($P < 0.02$).

Thus, destruction of the BCL2-BECN1 complex mediated by activation of the MAPK8/JNK1-MAPK9/JNK2 pathway by Aβ1-42 monomers may represent the mechanism that cells use to promote autophagy instead of apoptosis under treatment with Aβ1-42 oligomers.

We previously demonstrated that monomers of Aβ1-42 augment BACE1 gene transcription through this MAPK8/JNK1-MAPK9/JNK2 signaling pathway activation.[31] We therefore confirmed that MAPK8/JNK1-MAPK9/JNK2 activation precedes a significant increase in *BACE1* mRNA, as well as its protein levels and activity. As shown in **Figure 6**, Aβ1-42 monomers caused a 1.5- to 2.5-fold increase in *BACE1* mRNA at 1 h to 6 h of incubation (**Fig. 6C**), BACE1 protein levels (~2 to 3-fold) starting at 3 h and still present at 48 h (**Fig. 6D**), and

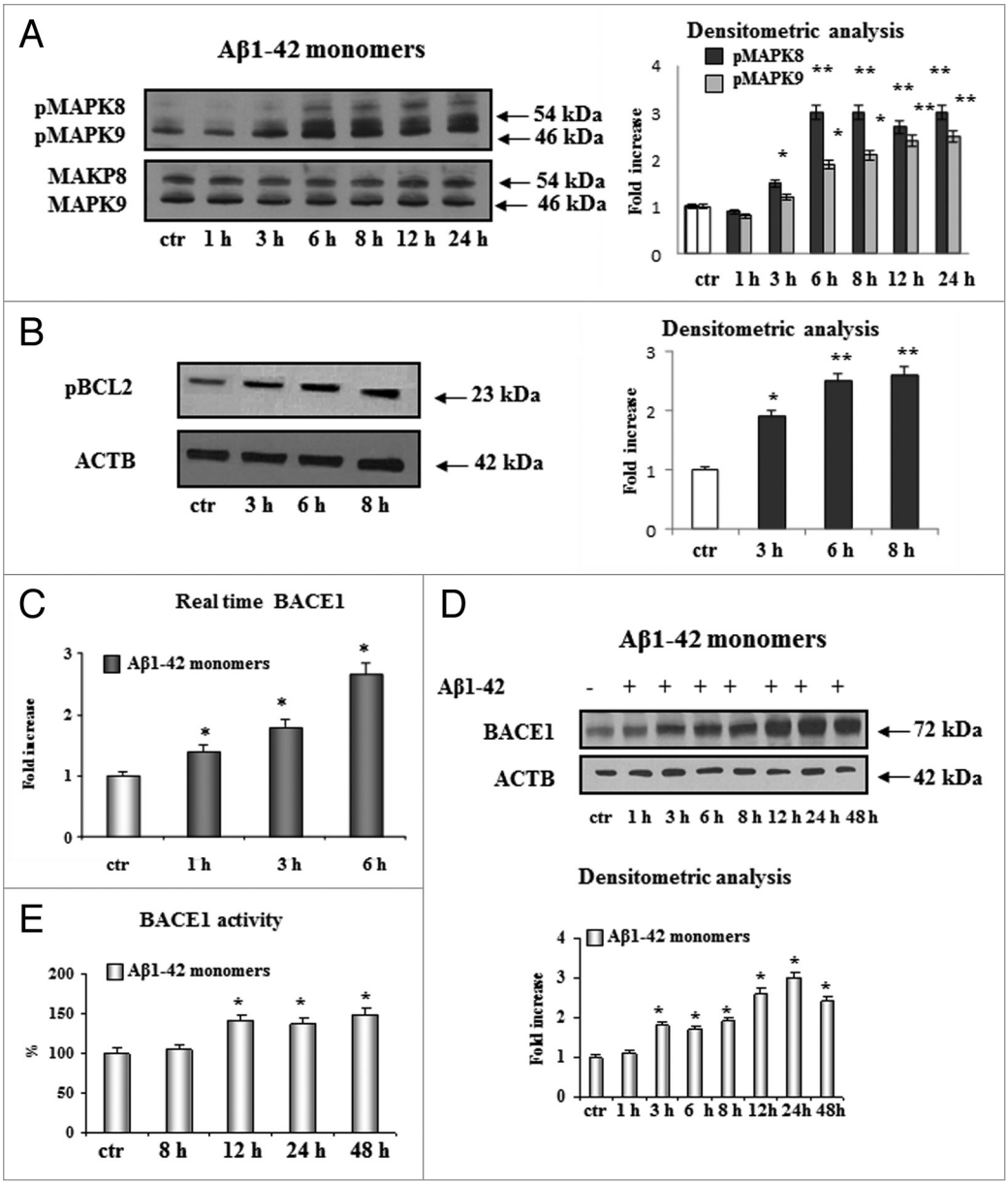

Figure 6. Treatment of differentiated SK-N-BE cells with 1 μM Aβ1-42 monomers increases BACE1 expression and activity through the activation of the MAPK/JNK pathway. (**A**) Nuclear pMAPK/JNK protein levels in differentiated SK-N-BE treated with Aβ1-42 monomers up to 24 h. The treatment was followed by a robust activation of the MAPK/JNK pathway, as shown by the significant increase (~3 to 2.5-fold) in levels of phospho- MAPK/JNK in nuclear fractions. (**B**) Phosphorylated BCL2 protein levels of in cytosolic fractions of cells treated with monomers up to 8 h. As expected with the activation of the MAPK/JNK pathway, cells treated with Aβ1-42 monomers revealed a significant increase (~2.5-fold) in BCL2 phosphorylation (**C–E**) BACE1 expression, protein levels, and activity in cells treated with Aβ1-42 monomeric preparation. Treatment of cells with Aβ1-42 monomers induced a 1.5- to 2.5-fold increase in *BACE1* mRNA, as well as a 100% to 200% increase in BACE1 protein levels and a 35% increase in BACE1 activity. The error bars represent standard deviations. Experiments were conducted in triplicate. *, significantly different from controls ($P < 0.05$); **, significantly different from controls ($P < 0.02$).

BACE1 activity (~50%) starting at 12 h and still present at 48 h (**Fig. 6E**).

Next, we asked whether Aβ1-42 oligomers are capable of activating the MAPK8/JNK1-MAPK9/JNK2 pathway. Exposure of differentiated neuroblastoma cells to oligomeric preparations did not modify the nuclear translocation of phospho-MAPK8/JNK1-MAPK9/JNK2 (**Fig. 7A**). Furthermore, oligomers did not induce a significant increase in *BACE1* mRNA levels (**Fig. 7B**), as well as protein levels (**Fig. 7C**) and activity (**Fig. 7D**). Taken all together, these findings demonstrate that monomeric Aβ1-42, but not the oligomeric species, causes MAPK8/JNK1-MAPK9/JNK2 pathway activation.

In order to exclude that differences in mass between monomers and oligomers or in rate and/or proportion of monomers and oligomers entering cells, produce apparent differences between the 2 Aβ species, we treated cells with both oligomeric and monomeric Aβ1-42 at different concentrations (100 nM, 200 nM, 1 μM, 5 μM) for 6 h. As shown in **Figure 8**, we observed that monomers were not capable of increasing CASP3 levels even at higher concentrations, whereas they increased BACE1 protein levels (2.5–3.5-fold increase) at any concentration. Oligomers, in turn, failed to increase BACE1 levels at any concentration, but significantly increased cleaved CASP3 protein levels at all concentrations. Taken together, these findings suggest that our results were not due to differences in the amount of oligomers and monomers reaching the cells as a consequence of differences in mass between monomers and oligomers, as well as rate or proportion of monomers and oligomers entering cells.

The presence of BECN1 is crucial for the toxicity mediated by Aβ1-42 oligomers

To confirm that the mechanism underlying the toxicity exerted by Aβ1-42 oligomers may be mediated by BECN1-BCL2 interaction, we studied some parameters of toxicity in SK-N-BE differentiated cells stably silenced for *BECN1*. As shown in

Figure 9A, silenced cells did not display a band for BECN1 protein. Furthermore, silencing *BECN1* completely protected the decrease of BCL2 and the cleavage of CASP3 (**Fig. 9A**), demonstrating that BECN1 is necessary for oligomeric Aβ1-42-induced toxicity.

Evaluation of the LDH release in culture medium of control and *BECN1* silenced neuroblastoma cells treated with Aβ1-42 oligomers showed no LDH release after 12 h and 24 h of incubation following silencing of BECN1 compared with a 50% release in control cells (**Fig. 9B**).

Finally, we studied if *BECN1* silencing changes autophagic flux. As shown in **Figure 9C**, the absence of BECN1 lowered the basal levels of both LC3 and SQSTM1 and treatment of cells with the oligomeric preparation of Aβ1-42 did not modify this trend.

Primary cortical neuronal cultures treated with Aβ1-42 monomers and oligomers confirm findings from SK-N-BE neuroblastoma cell lines on autophagy and apoptosis

To enhance the significance of our findings obtained on a neuroblastoma cell line, we investigated the effects of Aβ1-42 monomers and oligomers on autophagy and apoptosis, using mouse primary cortical neuronal cultures. We treated cells with 1 μM Aβ1-42 monomers or oligomers for 6, 8, 12, 24, and 48 h. As shown in **Figure 10A**, monomers induced a significant increase in LC3-II protein levels that was present at 6 h and at the following time point including 48 h. Treatment of neuronal cells with oligomers produced, in turn, a transient increase of LC3-I-II protein levels including the 6 h and the 12 h time points, but not the 24 h and 48 h time points. When we assessed CTSD activity we observed that Aβ1-42 monomers decreased CTSD activity by about 40% to 60%, whereas oligomeric Aβ1-42 did not (**Fig. 10B**). Finally, we found that monomers, but not oligomers, increased BACE1 activity of approximately 30% after 12 to 48 h of treatment (**Fig. 10C**). On the other hand, oligomers, but not monomers, induced apoptotic cell death as confirmed by the significant decrease (-50%) of BCL2 levels after 3 and 6 h of treatment and the increase (+100 to 150%) of cleaved CASP3 protein levels (**Fig. 10D**). Taken all together, these findings extend to the primary culture preparation findings from the neuroblastoma cell line on the involvement of Aβ1-42 monomers and oligomers in autophagy and apoptosis, respectively.

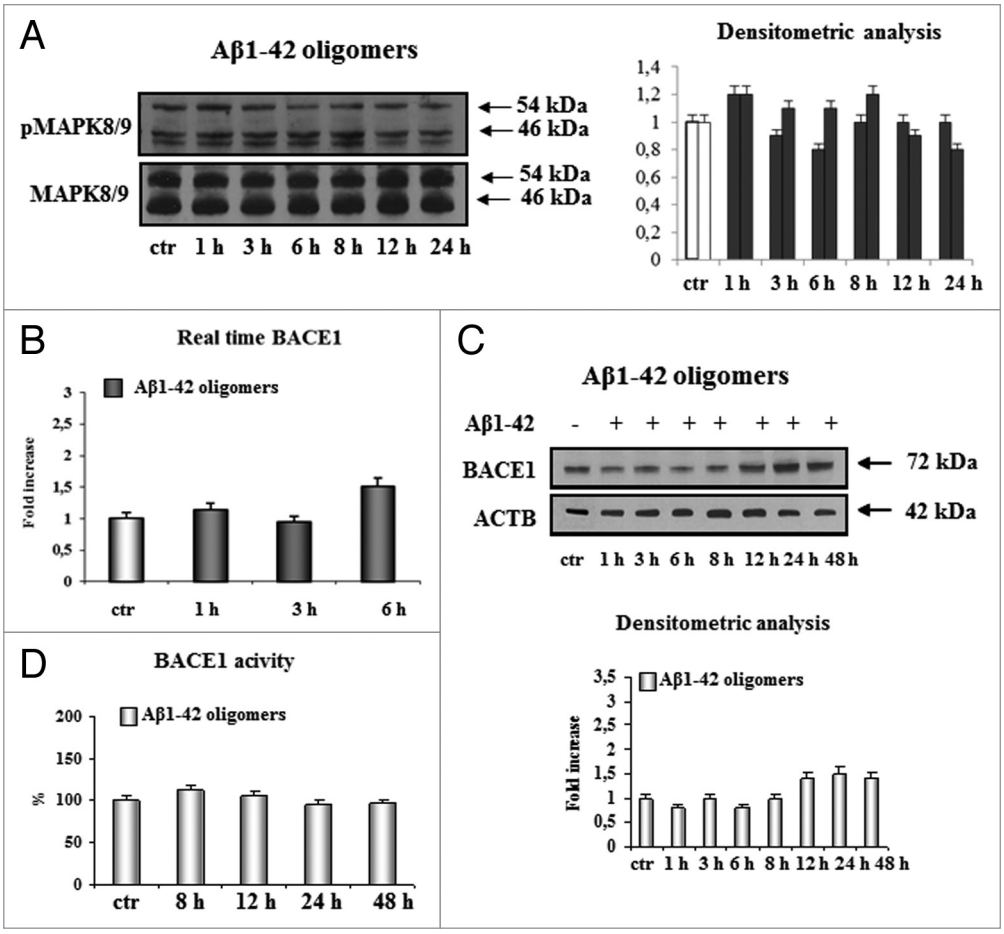

Figure 7. Treatment of differentiated SK-N-BE cells with 1 μM Aβ1-42 oligomers does not affect BACE1 expression and the JNK pathway. (**A**) Nuclear pJNK protein levels in differentiated SK-N-BE treated with Aβ1-42 oligomers up to 24 h. Aβ1-42 oligomers do not activate JNK phosphorylation in nuclear fraction of differentiated SK-N-BE cells. (**B–D**) BACE1 expression, protein levels, and activity in cells treated with Aβ1-42 oligomeric preparation. Treatment of cells with Aβ1-42 oligomers was not followed by significant induction of BACE1 mRNA, protein levels, and activity. The error bars represent standard deviations. Experiments were conducted in triplicate.

Discussion

We have found that Aβ1-42 monomers and oligomers have different effects on autophagy and cytototoxicity. Monomers hamper the formation of the BCL2-BECN1 complex activating the JNK pathway that phosphorylates BCL2, as well as inhibiting apoptosis and allowing autophagy. Monomers also cause intracellular accumulation of autophagosomes with elevation of levels of BECN1 and LC3-II, resulting in inhibition of substrate degradation due to reduction of lysosomal activity. Oligomers, in turn, facilitate the production of the BCL2-BECN1 complex favoring apoptosis. In addition, they cause a less profound

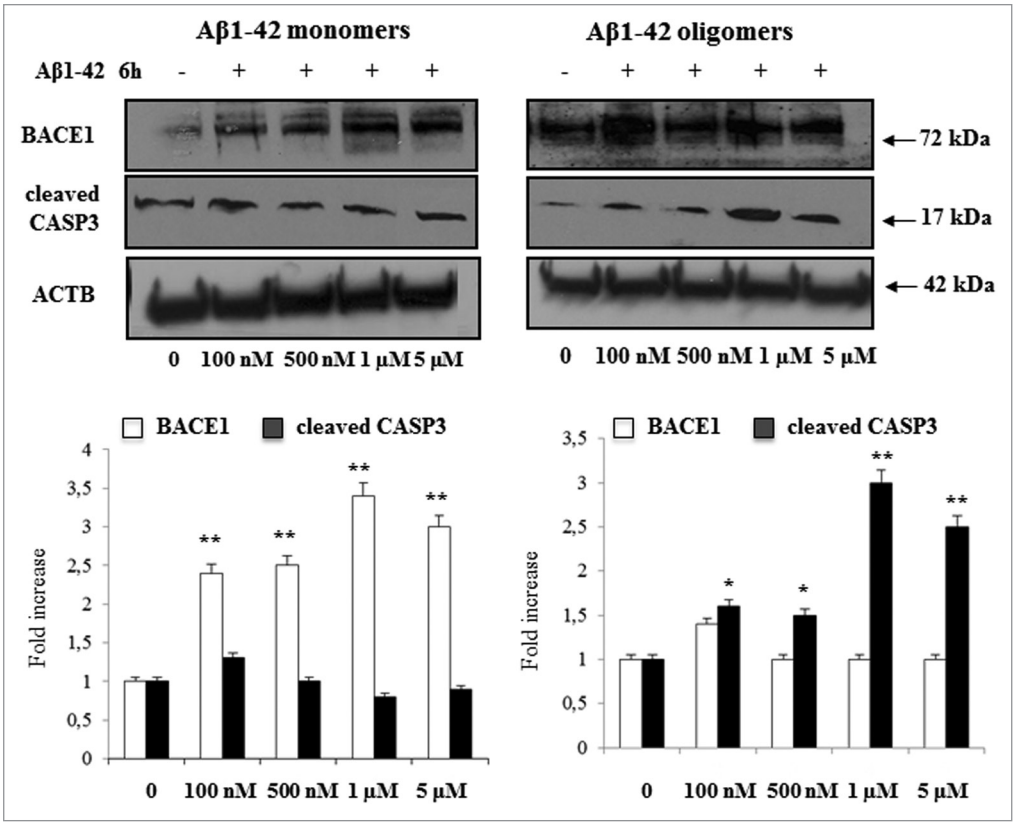

Figure 8. Treatment of differentiated SK-N-BE cells with different concentrations of Aβ1-42 monomers or oligomers does not modify the effects observed with the 1 μM concentratiob. BACE1 and CASP3 protein levels in differentiated SK-N-BE cells treated with a range (100 nM–5 μM) of Aβ1-42 monomers or oligomers for 6 h. We observed that monomers are not capable of increasing CASP3 levels even at higher concentrations, whereas they increase BACE1 protein levels at any concentration. Oligomers, in turn, fail to increase BACE1 levels at any concentration, but significantly increase cleaved CASP3 protein levels at all concentrations. The error bars represent standard deviations. Experiments were conducted in triplicate. *, significantly different from controls ($P < 0.05$); **, significantly different from controls ($P < 0.02$).

increase in BECN1 and LC3-II levels with no effect on the autophagic flux (**Fig. 11**).

Little is known on the relationship between autophagy and apoptosis. This relationship is multifaceted in the sense that autophagy might represent a stress adaptation that suppresses apoptosis, avoiding cell death, whereas in other cellular settings, autophagy represents an alternative mechanism to cellular damage that is called autophagic cell death (or type II cell death). Autophagy induction may have a general inhibitory effect beyond protein aggregate removal. For instance, pretreatment of cells with rapamycin (an autophagy inducer) decreases the mitochondrial mass by ~50% and reduces the susceptibility of cells to mitochondrial outer membrane permeabilization, one of the crucial steps that mediate apoptotic cell death.[33] In this system, inhibition of autophagy mediated by depletion of ATG7 suppressed the protective effect of rapamycin in vitro, in human cells.[33] On the other hand, mouse embryonic fibroblasts (MEFs) derived from double-knockout *bax*$^{-/-}$ *bak1*$^{-/-}$ mice when treated with the topoisomerase-2 inhibitor, etoposide, an agent capable of damaging DNA, do not undergo apoptosis and show massive autophagy. BAX and BAK1 are 2 members of the BCL2 family, that are required for mitochondrial outer membrane permeabilization and thus for apoptotic cell death.[34] In other cellular settings, other types of damages in the apoptotic machinery, such as caspase inhibition, induce cell death mediated by autophagy. Treatment of selected cell types (such as the L929 mouse fibrosarcoma and the human Jurkat T cell lymphoma) with Z-VAD-FMK, an inhibitor of cysteine proteases, can induce autophagic cell death.[35]

Through our work, we gathered a more complete understanding of the interplay between the 2 processes in AD pathogenesis in relationship to the elevation of different soluble Aβ species. We have demonstrated that oligomers lead to apoptosis favoring the formation of the BCL2-BECN1 complex. Consistent with this finding, compelling evidence demonstrates that the regulation of BCL2-BECN1 interaction represents a central mechanism by which autophagy is turned off in response to different cellular stimuli.[36] BECN1 interaction with BCL2 and BCL2L1 would lead to protection against autophagy. On the contrary, the disruption of the BCL2-BECN1 complex may be a mechanism through which cells promote autophagy during different stress conditions.[37,38] Thus, in some circumstances, such as nutrient deprivation or hypoxia, it has been reported that inhibition of autophagy is followed by an accelerated cell death with the presence of hallmarks of apoptosis, such as chromatin condensation and caspase-activation.

The mechanisms through which autophagy inhibition may facilitate cell death are not completely clear. It is possible that autophagy inhibition results in a bioenergetic deficiency that mediates apoptosis.[39] Thus, the deficiency in redox equivalents favors oxidative reactions, probably through a direct effect on mitochondria that triggers apoptosis.[40] This is in agreement with our previous results showing that Aβ1-42 oligomers, but not monomers, increase oxidative stress.[41]

We found that suppression of BECN1 leads to a protection of apoptotic cell death mediated by Aβ1-42 oligomers. This finding is in apparent contrast with the demonstration that activation of autophagy or overexpression of BECN1 can prevent neuronal cell death and promote clearance of toxic protein aggregates.[16] Furthermore, it has been reported that reduced

expression of BECN1 in AD brains is associated with an increase in Aβ levels and with a derangement of autophagosome degradation.[42] One hypothesis is that in absence of BECN1 the apoptosis is turned off with activation of a BECN independent autophagic machinery and consequent overproduction of Aβ mediated by BACE1 overexpression. These apparently conflicting findings would require further investigation.

On the other hand, Aβ1-42 monomers lead to autophagy and hamper the formation of the BCL2-BECN1 complex by activating the MAPK8/JNK1-MAPK9/JNK2 pathway that phosphorylates BCL2.[32] This pathway is connected with all the pathological hallmarks of AD. MAPK8/JNK1-MAPK9/JNK2 activation modulates the phosphorylation of APP, leading to regulation of Aβ levels,[43] as well as to MAPT/tau phosphorylation in vitro.[44] Furthermore, the MAPK8/JNK1-MAPK9/JNK2 pathway is activated in preclinical models of AD, including Tg2576 and Tg2576/PS1P264 L transgenic mice,[45,46] as well as in brains of AD patients.[47] Indeed, we have previously detected a significant activation of MAPK8/JNK1-MAPK9/JNK2-AP1 in oxidative stress in vitro as well as in vivo models, as a pathway that mediates the upregulation of BACE1.[48]

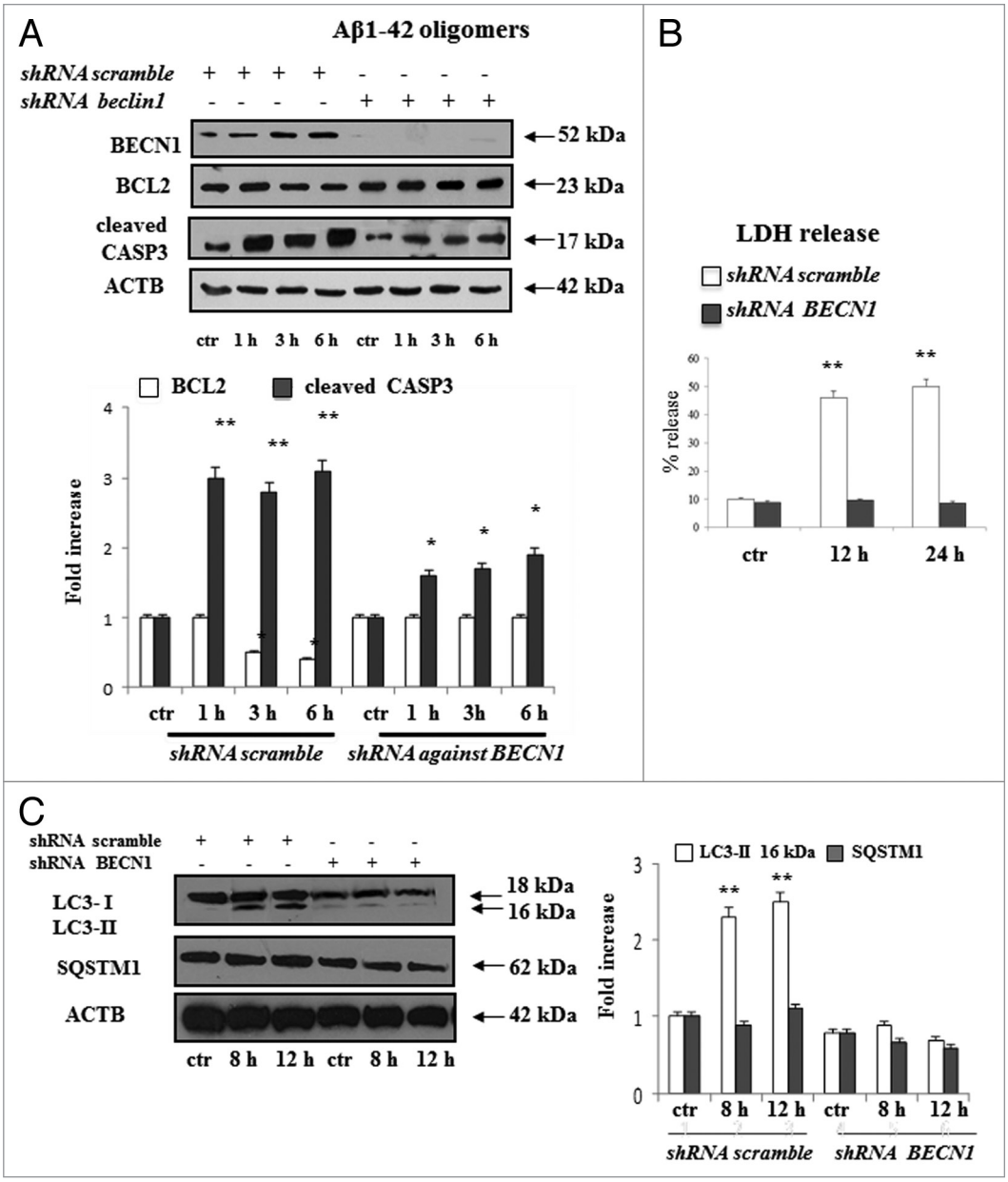

Figure 9. BECN1 is needed for the toxicity mediated by Aβ1-42 oligomers. (**A**) BECN1, BCL2, and cleaved CASP3 protein levels in SK-N-BE cell lines stably expressing a specific shRNA against *BECN1* or scrambled shRNA treated with Aβ1-42 oligomers up to 6 h. BECN1 is observed only in cells expressing scrambled shRNA while a band corresponding to the protein is not observed in cell expressing shRNA against *BECN1*. BCL2 protein levels remain unchanged in cells silenced for BECN1 treated with Aβ1-42 oligomers up to 6 h, whereas cleaved CASP3 are protected but slightly significant with respect to untreated cells. (**B**) LDH release in culture medium in control as well as in silenced neuroblastoma cells treated with Aβ1-42 oligomers for 24 h. In silenced cells oligomers failed to induce necrotic cell death after 12 h and 24 h of treatment. (**C**) LC3-II and SQSTM1 protein levels in cells with and without silencing for BECN1 and treated with oligomers of Aβ1-42 for 8 h and 12 h. The absence of BECN1 lowered the basal levels of both LC3 and SQSTM1 and treatment of cells with the oligomeric preparation of Aβ1-42 did not modify this trend. The error bars represent standard deviations. Experiments were conducted in triplicate. *, significantly different from controls ($P < 0.05$); **, significantly different from controls ($P < 0.02$).

Data obtained in the present work have confirmed our previous results showing that Aβ1-42 monomers are capable of upregulating BACE1 expression by interfering with its lysosomal degradation leading to an amyloid vicious cycle.[49] We recently found that Aβ1-42 monomers, but not oligomers (data not shown), downregulate the activity of UCHL1 (ubiquitin carboxyl-terminal esterase L1 [ubiquitin thiolesterase]), through the activation of total NFKB pathway. The decrease in UCHL1 activity interferes with the lysosomal machinery as demonstrated by the decrease in CTSD activity and the accumulation of BACE1

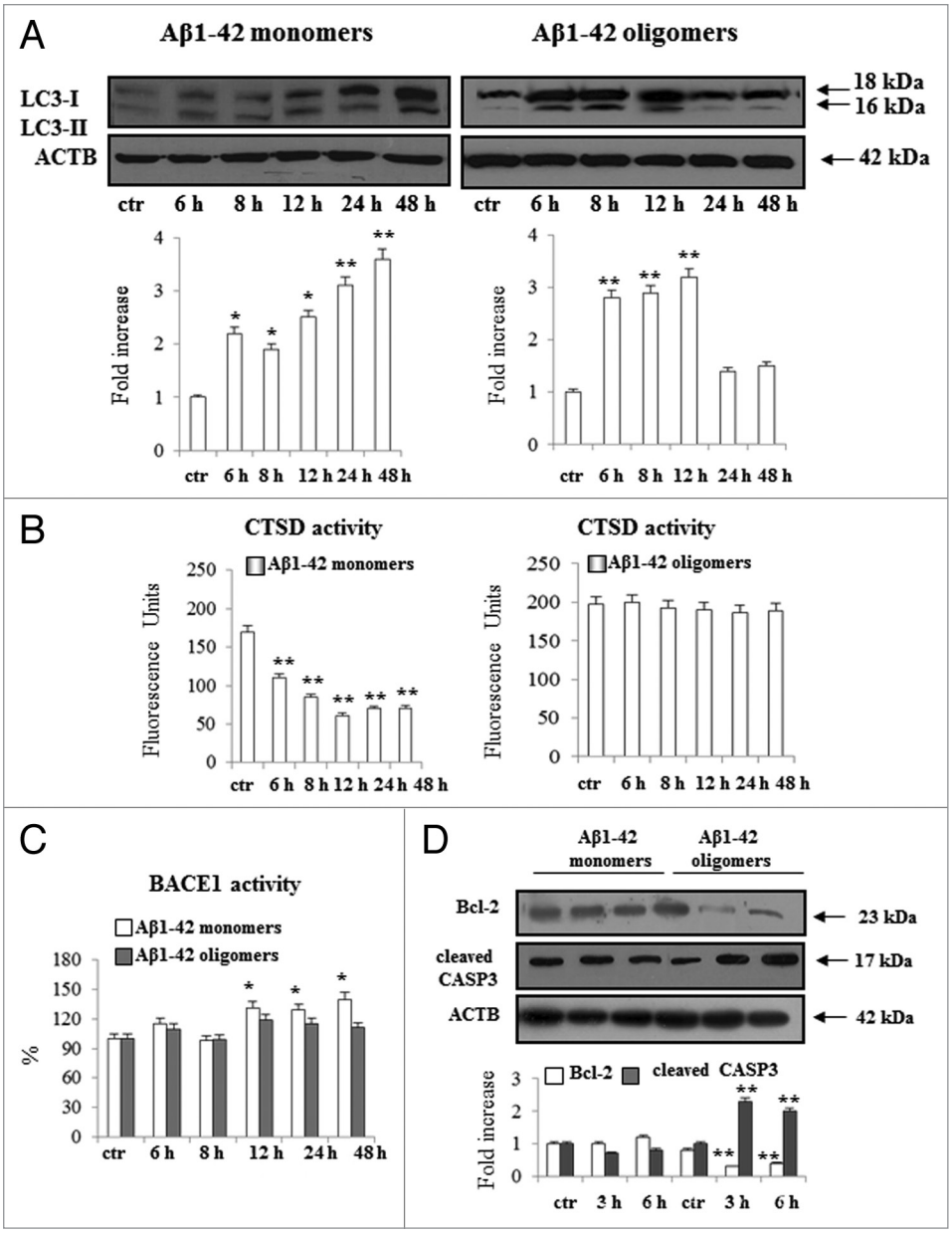

Figure 10. Primary cortical neuronal cultures treated with 1 μM Aβ1-42 monomers and oligomers confirm findings from SK-N-BE neuroblastoma cell lines on autophagy and apoptosis. (**A**) LC3 I-II protein levels in cells treated with Aβ1-42 monomers and oligomers at different time points. Monomeric preparations induce a significant increase in LC3-II protein levels that started at 1 h and was still observed after 48 h of incubation. Treatment of neuronal cells with oligomeric preparation produced a transient increase of LC3-II protein levels that was present after 6 h and 12 h, but not 24 h and 48 h. (**B**) CTSD activity in cortical cultures treated with Aβ1-42 monomers and oligomers up to 48 h. Only Aβ1-42 monomers significantly decreased CTSD activity about 40% to 60% between 6 and 48 h. (**C**) BACE1 activity in cortical cultures treated with Aβ1-42 monomers and oligomers. Monomers, but not oligomers, increase BACE1 activity of approximately 40% after 12 h to 48 h of treatment. (**D**) BCL2 and cleaved CASP3 protein levels in primary cortical cultures treated with Aβ1-42 monomers and oligomers. Oligomers, but not monomers, induce apoptotic cell death as confirmed by the significant decrease (-50%) of BCL2 and increase (+100 to 150%) of cleaved CASP3 protein levels. The error bars represent standard deviations. Experiments were conducted in triplicate. *, significantly different from controls ($P < 0.05$); **, significantly different from controls ($P < 0.02$).

in lysosomes after treatment with Aβ1-42 monomers as well as with an UCHL1 inhibitor. The mechanism through which this enzyme impairs lysosomes needs further investigations.

Alterations in the endosome-lysosome machinery have also been previously reported in AD.[50] BACE1 and Aβ are enriched in autophagic vacuoles in APP transgenic mouse models.[17] Furthermore, autophagic vacuoles accrue in dystrophic neurites in AD brains.[19]

Our manuscript shows a link for autophagy and apoptosis with monomers and oligomers, respectively. This is important in light of the fact that the exact role of monomers and oligomers in AD remains to be clearly defined. The higher neurotoxicity exerted by oligomers with respect to monomers suggests that this event can be mediated by production of reactive oxygen species[41] or other mediators and is able to kill neurons to induce apoptosis. On the other hand, monomers, being less cytotoxic, preferentially induce toxicity by modulating APP processing, increasing BACE1 expression and activity and thus resulting in a further accumulation of Aβ. Thus, our data suggest that both Aβ species are relevant to AD pathogenesis. Most importantly, these studies will help designing novel therapies that will cure the disease or at least alleviate its symptoms.

Materials and Methods

Cell culture differentiation and treatments

SK-N-BE neuroblastoma cells were maintained in RPMI (Roswell Park Memorial Institute) 1640 medium (Sigma-Aldrich, R8758) containing 2 mM glutamine (Sigma-Aldrich, G7513) and supplemented with 10% fetal bovine serum (Sigma-Aldrich, F7425), 1% nonessential amino acids (Sigma-Aldrich, R7131), and 1% antibiotic mixture (penicillin-streptomycin-amphotericin (Sigma Aldrich A5955) in a humidified incubator at 37 °C with 5% CO_2. For differentiation, 2 × 10^6 cells were plated in 75-cm^2 culture flasks (Costar, 430651) and treated with 10 μM

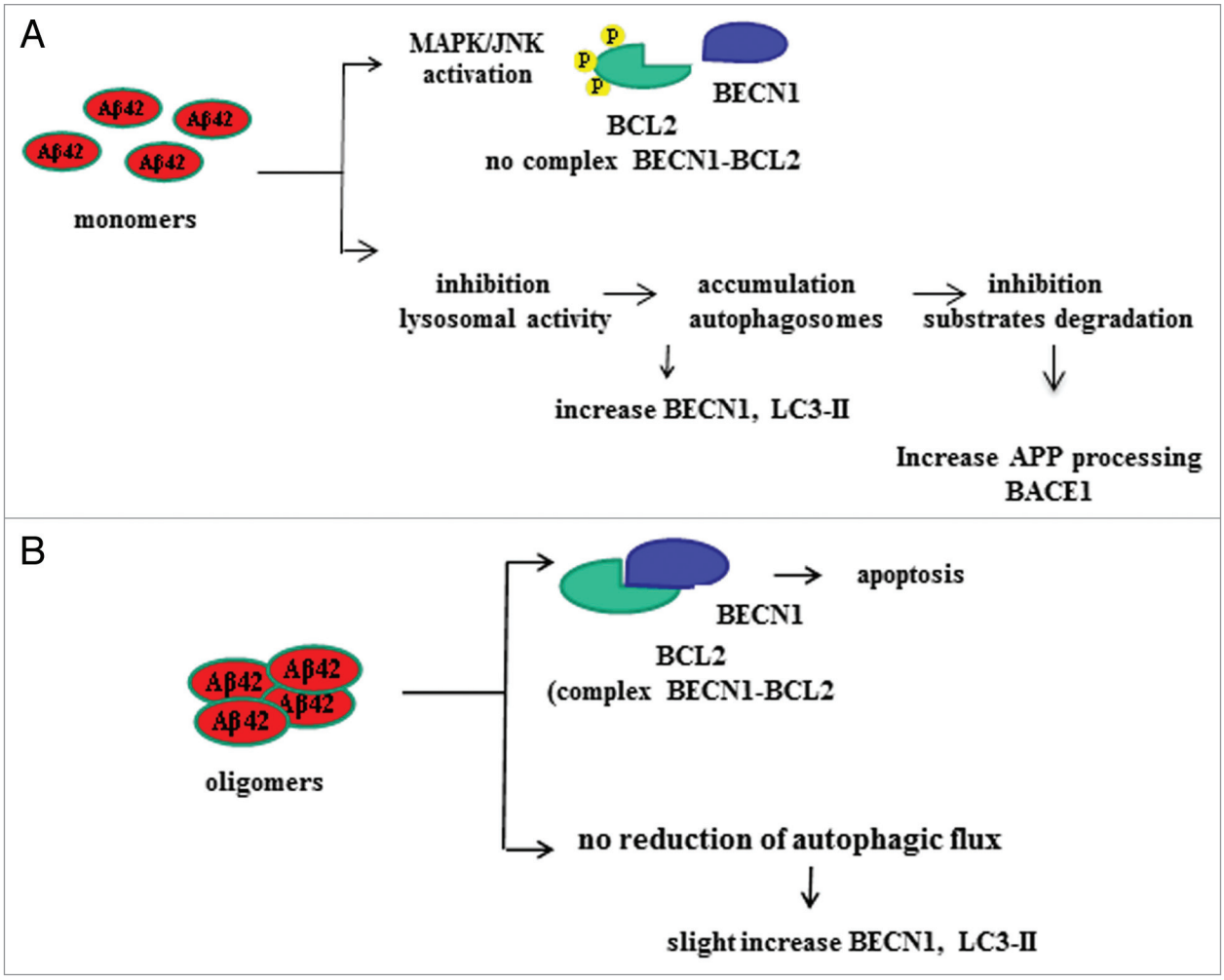

Figure 11. Proposed effects on autophagy and apoptosis elicited by Aβ1-42 monomers and oligomers. (**A**) Monomers impede formation of the BCL2-BECN1 complex by activation of the MAPK/JNK pathway that phosphorylates BCL2. Monomers also cause early endosome accumulation due to an inhibitory action on lysosomal activity, resulting in intracellular accumulation of autophagosomes with elevation of levels of BECN1 and LC3-II, and inhibition of substrate degradation. (**B**) Oligomers favor the formation of the BCL2-BECN1 complex leading to apoptosis. They also cause a less profound increase in BECN1 and LC3-II levels and do not affect the autophagic flux.

retinoic acid (Sigma-Aldrich, R2625) for 10 d. Cells were incubated with 0.1–5 μM Aβ1-42 peptides (Anaspec, 24224) up to 48 h. The lyophilized synthetic peptides were dissolved as a stock solution in 1,1,1,3,3,3-hexafluoro-2-propanol (Sigma-Aldrich, 105228) and stored at −80 °C in aliquots. Monomeric preparations, after evaporation of propanol, were brought to 1 μM (final concentration) with sterile double distilled water, centrifuged at 10000 g for 10 min to avoid aggregation and immediately added to the cell culture.[49] Oligomeric preparations were maintained at 4 °C for 24 h and then added to the cells.[41,51,52] The quality of these Aß preparations was routinely controlled using WB and by transmission electron microscopy.

The lysosomal inhibitor chloroquine (Sigma-Aldrich, C6628) was used at the concentration of 100 μM for 24 h.

Cell extracts

Whole-cell extracts were prepared in ice-cold lysing buffer [1 ml of phosphate-buffered saline (PBS; Sigma-Aldrich, P4417) was mixed with 1% Triton X-100 (Fluka, 93418); 10 μl 10% SDS (Sigma-Aldrich, L4509); 5 μl 1 M dithiothreitol (Sigma-Aldrich, D9163); 6 μl 0.1% phenylmethylsulfonylfluoride (Sigma-Aldrich, P7626), 1% aprotinin (Sigma-Aldrich, A6279)] for 120 min. The lysates were cleared by centrifugation at 14000 g for 25 min. Cytosolic and nuclear fractions were obtained as previously described.[53]

Primary cortical neuronal cultures

Primary neuronal cultures were prepared from the cortices of P0 pups of C57 mice. Culture plates were coated with 15 μg/mL poly-L-ornithine (Sigma-Aldrich, 3655) for 45 min at room temperature. Poly-L-ornithine was then removed and wells were soaked with 4 μg/mL mouse laminin (Gibco, 23017-015) for 12h to 16 h in a cell culture incubator at 37 °C, 95% humidity and 5% CO_2. Forebrains were dissected in ice cold HBSS (Gibco, 14175-053) with 0.5% w/v D-glucose (Sigma-Aldrich, G8270) and 25 mM HEPES (Gibco, 15360-056), under a dissection microscope (Leica, Zoom 2000, MOF-1045). Dissociation was performed in ice-cold dissection medium plus 0.01% w/v Papain

(Worthington Biochemical Corporation, LS003126), 0.1% w/v dispase (Roche, 04 942 078 001) and 0.01% w/v DNase (Worthington Biochemical Corporation, DR1), first by means of sterile razor blades, then by serial pipetting with flamed sterile glass Pasteur pipettes, and incubation at 37 °C twice for 15 min. Cells were then spun down at 220 g for 5 min at 4 °C, resuspended in Neurobasal Medium (Gibco, 21103-049) with 2% B27 (Gibco, 17504-044), 1 mM Na Pyruvate (Gibco, 11360-088), 100 units/ml penicillin-streptomycin solution (Gibco, 15070-063), 2 mM Glutamax (Gibco, 35050-038), filtered through a 40-μm cell strainer, counted and plated on coated 6-well plates at a density of about 400,000 cells/well. Culture medium was completely replaced after 16 h to 20 h, and new medium (30% of starting volume) was added every 3 d until needed. After 15 d cells were treated with 1 μM Aβ 1-42 monomers or oligomers.

Antibodies and immunoblot analysis

The following antibodies were used: polyclonal anti-BECN1 (dilution 1:800; Abcam, ab62472); polyclonal anti LC3 (dilution 1:800; Sigma-Aldrich, L8918) and monoclonal anti-ACTB/β actin (dilution 1:1000; Sigma-Aldrich, A3854); polyclonal anti-CASP3 (dilution 1:1000; Cell Signaling Technology, 9665S) and polyclonal anti-pMAPK8/9 (JNK1/2, dilution 1:1000; Cell Signaling Technology, 4668S); polyclonal anti-MAPK/JNK (dilution 1:200; BD PharMingen Sparks, 610628), monoclonal anti CYCS (dilution 1:250; BD PharMingen Sparks, 556432); polyclonal anti-BAK1 (dilution 1:200; Millipore, 04-433); monoclonal anti-BCL2 (dilution 1:200; Millipore, 05-729); polyclonal anti-pBCL2 (dilution 1:250; Millipore, 05-843R); polyclonal anti-BACE1 (dilution 1:400; Millipore, 195111); anti-SQSTM1 (dilution 1:2000, Sigma-Aldrich, P0067). Cell lysates and nuclear fraction were separated on 9.3% sodium dodecyl sulfate-polyacrylamide gels (Sigma-Aldrich, 01708), using a mini-PROTEAN II electrophoresis cell (Bio-Rad, 165-3301).

Proteins were transferred onto nitrocellulose membranes (GE-Healthcare, RPN203D). Nonspecific binding was blocked with 50 g/l nonfat dry milk in 50 mM TRIS-HCl, pH 7.4, containing 200 mM NaCl and 0.5 mM Tween-20. The blots were incubated with different primary antibodies, followed by incubation with peroxidase-conjugated anti-mouse (Bio-Rad, 170-6516) or anti-rabbit (Bio-Rad, 170-6515) immunoglobulins in Tris-buffered saline Tween [150 mM sodium chloride solution (Sigma-Aldrich, S6546) 10 mM TRIS-HCl pH 7.4 (Sigma Aldrich, T5941) 0.05% Tween 20 (Sigma-Aldrich, P1379)] containing 20 g/l nonfat dry milk. Reactions were developed with an enhanced chemiluminescence system according to the manufacturer's protocol (Bio-Rad, 170-5061). In some experiments, lysates were immunoprecipitated with the polyclonal antibody to BECN1 (Abcam, 62472) and conjugated with protein A Sepharose beads (Sigma-Aldrich, P6649) for 12 h at 4 °C. Immunoprecipitated proteins were separated in 9.3% gels and detected with BCL2 antibody.

Confocal immunofluorescence

For immunofluorescence experiments, cells were plated in 12-well plates (BD Biosciences, 351143), at a concentration of 40,000 cells/cm^2. Cultured cells were fixed with 4% buffered paraformaldehyde for 15 min at room temperature. Samples were washed 3 times with PBS. In order to permeabilize cells, samples were incubated for 10 min in PBS containing 0.3% Triton X-100 (PBST) followed by a washout with PBS. After blocking nonspecific binding sites with 1% BSA Sigma-Aldrich, A7906) in PBST, cells were incubated at 4 °C overnight with the following primary polyclonal antibodies: i) polyclonal anti-BECN1 antibody (1:200; Abcam, 62472), ii) polyclonal anti-LC3A/B antibody (anti-microtubule-associated protein 1 light chain 3, isoforms α and β, 1:200; Novus Biologicals Inc., NB100-2331), iii) monoclonal anti-LAMP1 antibody 1:200; Calbiochem, AB2971), iv) polyclonal Aβ1-42 (1:500; Sigma-Aldrich, A2101), v) poyclonal anti-cleaved-CASP3 antibody (1:400; Cell Signaling Technology Inc., 9665S). After washing in PBS, sections were incubated with secondary antibodies raised in different species: 1:200 cyanine 3-conjugated donkey anti-mouse secondary antibody (1:200; Jackson Research Laboratories, 715-165-151) and cyanine 2-conjugated anti-rabbit secondary antibody (1:100; Jackson ImmunoResearch Laboratories, 711-5461-52) with 1% BSA in PBS. For counterstaining, cells were incubated for 3 min with 4′,6-diamidino-2-phenylindole (DAPI; Sigma, D9564) diluted 1:50 in methanol 0.1 M and rinsed with PBS. Finally, coverslips were mounted with a drop of 1:1 phosphate buffer-glycerol solution. The samples were examined with a Leica TCS SP5 confocal laser scanning microscope (Leica, CLSM).

TUNEL staining

Cell apoptosis was assessed through The DeadEndTM Fluorometric TUNEL System (Promega, Madison, WI, USA) following the manufacturer's instructions. DAPI was employed to stain nuclei.

LDH release

The activity of the lactate dehydrogenase was determined using a commercially available kit (Sigma-Aldrich, TOX7) according to the manufacturer's protocol. The assay is based on the reduction of NAD by LDH. The resulting reduced NAD (NADH) is utilized in the stoichiometric conversion of a tetrazolium dye. The resulting colored compound was measured spectrophotometrically. The amount of LDH activity in cell-free aliquots of culture medium was used as an indicator of relative cell viability as well as a function of membrane integrity. Values for control and treated cells were expressed as a percentage of the LDH released by cells lysed with Triton X-100.

Gene expression analysis

Primer sequences, designed with PRIMER 3 software (Applied Biosystems, 4361938) were:

Human *BACE1*:

5′-CATTGGAGGT ATCGACCACT CGCT-3′

5′CCACAGTCTT CCATGTCCAA GGTG-3′.

Human *ACTB*:

5′-GGCACTCTTC CAGCCTTCCT TC-3′

5′-GCGGATGTCC ACGTCACACT TCA-3′

Quantitative PCR was performed on a real-time iCycler sequence detector instrument (Bio-Rad, iCycler iQ™ Real-Time PCR Detection System). After 3 min of initial denaturation, the amplification profile included 30 s denaturation at 95 °C and extension at 72 °C. Primer annealing was performed for 30 s at 60 °C. The results were obtained with the comparative Ct

method using the arithmetic Equation $2^{-\Delta\Delta Ct}$. Samples obtained from at least 3 independent experiments were used to calculate the mean and SD.

For the quantitative SYBR Green (2× iQ YBR Green PCR Super Mix; Bio-Rad, 170-8882) real-time PCR, 40 ng of cDNA was used per reaction. RNA extraction was performed with the RNeasy kit, according to the manufacturer's protocol, for cell lines (Qiagen, 956034), cDNA was obtained with the SuperScript® III Cells Direct cDNA Synthesis Kit (Invitrogen, 18080200).

BACE1 activity

The activity of BACE1 was determined using a commercially available secretase kit from Calbiochem (565785) according to the manufacturer's protocol. Cells were lysed in cold 1× Extraction Buffer (ready for use in the kit) to yield a final protein concentration of 1 mg/ml. The method is based on the secretase-dependent cleavage of a secretase-specific peptide conjugated to the fluorescent reporter molecules EDANS and DABCYL, which results in the release of a fluorescent signal that can be detected on a fluorescence microplate reader (VICTOR Multilabeled Counter, Perkin Elmer, 1420-018) (excitation wavelength of 355 nm and emission 510 nm). The level of secretase enzymatic activity is proportional to the fluorimetric reaction. Data were expressed as percentage change over activity level of control cells.

CTSD activity assay

The CTSD activity assay kit (Abcam, ab65302) is a fluorescence-based assay that utilizes the preferred CTSD substrate sequence GKPILFFRLK(Dnp)-DR-NH2 labeled with MCA. Cell lysates that contain CTSD cleave the synthetic substrate to release fluorescence, which can be easily quantified using a fluorescence plate reader (VICTOR Multilabeled Counter, Perkin Elmer, 1420- 018) at Ex/Em = 328/460.

mRFP-GFP-LC3 plasmid transfection

Cells were transfected with the tandem mRFP-GFP-LC3-expressing plasmid ptfLC3 (Addgene, 21074) using Lipofectamine 2000 (Invitrogen, 11688-019) according to the manufacturer's instructions.

Lentiviral vectors and virus production

Recombinant lentiviruses delivering anti-*BECN1* shRNA specific for human *BECN1* (TRCN0000033552 for BECN1, GenBankTM NM_003766) from the TRC library (the RNAi consortium) in pLKO lentiviral vectors were used (Openbiosystems, Thermo Scientific). The generation of *BCL2*-encoding lentivirus was achieved by using a SIN-PGK-hBCL2-WHV vector as described previously.[54] A pLKO vector containing scrambled shRNA (Openbiosystems, Thermo Scientific) in BECN1 knockdown experiments or a SIN-PGK-WHV empty vector in BCL2 upregulation experiments were used as control vectors. Self-inactivating lentiviral vectors were produced by cotransfecting 293T cells with the lentivirus expression plasmid and packaging plasmids using the calcium phosphate method. Infectious lentiviruses were harvested at 48 h post-transfection, the supernatant was collected, filtered, concentrated by ultracentrifugation, resuspended in 1% BSA in PBS, and then stored at −80 °C until use as previously described.[55] Viral particle content was assayed for the p24 core antigen by using the p24 antigen enzyme-linked immunosorbent assay (RETROtek, 22-157-319) according to the manufacturer's instructions.

Generation of stable knockdown cell lines

To establish SK-N-BE cell lines stably expressing a specific shRNA against *BECN1* or overexpressing BCL2, 2–5 × 10^6 SK-N-BE cells were seeded in T25 cell culture flasks and transduced with 50 ng of p24/ml culture medium. In parallel, SK-N-BE cells were transduced with a lentiviral vector encoding a scrambled shRNA (shRNAsc Openbiosystems, Thermo Scientific) or a SIN-PGK-WHV empty vector) as an infection control. After 48 h, the transduced cells were trypsinized, and selection was initiated by diluting the cells 10-fold and adding 10 μg/ml puromycin (Sigma-Aldrich, P9620).

Statistical analysis

Data in bar graphs represent mean ± SD, and were obtained from average data of at least 3 independent experiments. Morphological images are representative of at least 3 experiments with similar results. Statistical analysis was performed by the Student *t* test or ANOVA, followed by the Bonferroni post-hoc test, when appropriate ($P < 0.05$ was considered significant).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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