The Role of Autophagy in Alzheimer Disease - from Cellular Mechanisms to Systems Proteomics

Dissertation

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By
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Ex annis asperis, nunc propero ad Astra [sic].

This thesis work was performed between July 2006 and January 2011 under the supervision of Prof. Dr. Tony Wyss-Coray at Stanford University, Palo Alto, CA, USA.

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Title	**Contribution**
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Jaeger PA, Wyss-Coray T. *Beclin 1 complex in autophagy and Alzheimer disease.* Arch Neurol. **2010** Oct;67(10):1181-4.	100%
Jaeger PA, Pickford F, Sun CH, Lucin KM, Masliah E, Wyss-Coray T. *Regulation of amyloid precursor protein processing by the Beclin 1 complex.* PLoS One. **2010** Jun 15;5(6):e11102.	80%
Jaeger PA, Wyss-Coray T. *All-you-can-eat: autophagy in neurodegeneration and neuroprotection.* Mol Neurodegener. **2009** Apr 6;4:16.	100%

I confirm that the above stated author contributions are correct and that all my research has been conducted independently, following the ethical and practical standards of good scientific practice.

Philipp Jäger

Palo Alto, the 25th of February 2011

Talks and poster presentations

The author has presented the content of this research thesis at the following conferences.

Talks:

PA. Jaeger, M. Britschgi, CH. Sun, H. Johns, S. Pradhan, RC. Petersen, DS. Knopman, BF. Boeve, AL. Boxer, A. Karydas, BL. Miller, R. Rademakers, DW. Dickson, N. Graff-Radford, T. Wyss-Coray: *The Communicome of a Disease: Application and Potential of Human Plasma Proteomics to Study Frontotemporal Dementia.* 40th Annual Meeting of the Society for Neuroscience 2010, San Diego/CA, USA, Nov 13-17, **2010**.

PA. Jaeger, M. Britschgi, H. Johns, CH. Sun, T. Wyss-Coray: *Exploring the Communicome of Frontotemporal Dementia Patients through Plasma Proteomics.* Consortium for Frontotemporal Dementia Research (CFR): Research in Progress Meeting, The J. David Gladstone Institute, San Francisco/CA, USA, Jun 4, **2010**.

PA. Jaeger, F. Pickford, CH. Sun, KM. Lucin, T. Wyss-Coray: *Beclin 1 Regulates APP Turnover in the Endosomal-lysosomal Pathway.* Cold Spring Harbor Meeting "Neurodegenerative Diseases", Cold Spring Harbor Laboratory, Cold Spring Harbor/NY, USA, Dec 4-7, **2008**.

F. Pickford, **PA. Jaeger**, E. Masliah, T. Wyss-Coray: *Amyloid Precursor Protein Processing, Autophagy, and Alzheimer's Disease.* Herbsttagung der Gesellschaft für Biochemie und Molekularbiologie (GBM), Hamburg, Germany, Sep 16-19, **2007**.

Poster presentations:

PA. Jaeger, M. Britschgi, CH. Sun, S. Pradhan, H. Johns, T. Wyss-Coray: *The Plasma Proteome of Secreted Cellular Communication Factors as a Tool to Study Dementia.* Keystone Symposium "Alzheimer's Disease Beyond Abeta", Copper Mt, CO, Jan 10-15, **2010**.

PA. Jaeger, F. Pickford, CH. Sun, KM. Lucin, AR. LaSpada, T. Wyss-Coray: *Beclin 1 Reduction Causes Accumulation of APP and APP Metabolites in Cells.* Alzheimer's Association Research Symposium, San Francisco/CA, USA, Jun 25, **2008.**

F. Pickford, **PA. Jaeger**, E. Masliah, CH. Sun, M. Britschgi, S. Small, B. Spencer, E. Rockenstein, N. Mizushima, B. Levine, T. Wyss-Coray: *Effects of Autophagy on the Distribution and Processing of APP.* 37th Annual Meeting of the Society for Neuroscience , San Diego/CA, USA, Nov 3-7, **2007**.

F. Pickford, **PA. Jaeger**, E. Masliah, CH. Sun, M. Britschgi, S. Small, E. Rockenstein, N. Mizushima, B. Levine, T. Wyss-Coray: *Beclin 1 Deficiency in Alzheimer's Disease Links Autophagy with Amyloidosis and Neurodegeneration.* Keystone Symposium "Autophagy in Health and Disease", Monterey/CA, USA, Apr 15-20, **2007**.

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For Lora and Anna, with love.

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Zusammenfassung (German)

Während meiner Doktorarbeit lag der Schwerpunkt meines wissenschaftlichen Interesses bei der Erforschung schädlicher Protein-Aggregate und von Entzündungsstoffen, und deren Rolle bei der Entstehung neurodegenerativer Erkrankungen. Drei zentrale Fragen haben mich dabei beschäftigt: Spielt die Autophagie bei der Verstoffwechslung des Amyloid-Vorläufer Proteins (APP) im Gehirn eine Rolle? Hat die Aktivität der Autophagie einen Anteil an der Entstehung der Alzheimer Erkrankung? Und was für ein Zusammenhang besteht zwischen einer fortschreitenden, systemweiten Entzündungs-Reaktion, intrazellulärer Kommunikation und der Alzheimer Erkrankung im Menschen?

Meine Doktoarbeit basiert auf der Entdeckung, dass Beclin 1 (BECN1), ein Protein, das eine wichtige Rolle bei der Initiation von Autophagie spielt, im Cortex von Alzheimer Patienten reduziert zu sein scheint. Unser Labor entwickelte daraufhin ein Alzheimer-Maus-Modell mit reduzierter BECN1 Expression und stellte fest, dass diese Mäuse unter erhöhter Ablagerung von Aβ Plaques, erhöhter Aktivität von Mikroglia und fortgeschrittenem Verlust von Nervenzellen leiden (Pickford et al., 2008).

Um den Zusammenhang zwischen BECN1, Autophagie und Alzheimer Erkrankung besser zu verstehen, habe ich daraufhin eine Reihe verschiedener Zellkultur-Experimente entwickelt. Verringerung und Über-Exprimierung von BECN1 durch siRNA Plasmide und Lenti-Virus Partikel haben mir geholfen aufzuzeigen, dass der zelluläre APP Gehalt tatsächlich durch Autophagie reguliert werden kann. Dementsprechend kann APP gezielt abgebaut und eine Aβ-Ansammlung verringert werden, wenn man Autophagie entweder durch Nährstoffs-Entzug oder durch Pharmaka künstlich aktiviert (Jaeger et al., 2010).

Um der Frage nachzugehen, wie Entzündungsstoffe, Immunsignale und zelluläre Kommunikations-Faktoren während einer neurodegenerativen Erkrankung die Vorgänge (u.U. auch die Autophagie) in Nervenzellen beeinflussen, hat unser Labor einen speziellen antikörper-basierten Biochip entwickelt. Mit diesem Biochip sind wir in der Lage, hunderte verschiedener Kommunikations-Faktoren (z.B. Chemokine, Zytokine, Wachstumsfaktoren, Neurotrophine usw.) in Blut-Plasma-Proben demenz-erkrankter

Patienten oder gesunder Probanden zu messen. Basierend auf diesem Chip-Design habe ich neuartige Methoden zur Daten-Extraktion, Daten-Analyse und Daten-Interpretation entwickelt. Dabei habe ich eine signifikante Deregulation in verschiedenen Signal-Kaskaden entdeckt, wie zum Beispiel in der TNF-α oder der TGF-β Kaskade (Manuskript in Vorbereitung). Unser Labor ist nun damit beschäftigt, diese potenziellen Signal-Kaskaden weiter zu erforschen, und zu überprüfen, welche bei der beobachteten Reduktion der Autophagie beteiligt sein könnten.

Literatur:

Pickford F, Masliah E, Britschgi M, Lucin K, Narasimhan R, **Jaeger PA**, Small S, Spencer B, Rockenstein E, Levine B, Wyss-Coray T. *The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid beta accumulation in mice.* J Clin Invest. **2008** Jun;118(6):2190-9.

Jaeger PA, Pickford F, Sun CH, Lucin KM, Masliah E, Wyss-Coray T. *Regulation of amyloid precursor protein processing by the Beclin 1 complex.* PLoS One. **2010** Jun 15;5(6):e11102.

Jaeger PA, Britschgi M, Rufibach K, Burkholder B, Johns, H, Sun CH, Pradhan S, Petersen R, Knopman DS, Boeve BF, Boxer, AL, Karydas A, Miller BL, Rademakers R, Dickson DW, Yonnkin S, Graff-Radford N, Wyss-Coray T. *Plasma Protein Changes in Sporadic Alzheimer Disease Patients are Linked to Cognitive Decline and Identify Disease-related Pathways* (in Vorbereitung)

Summary

I am interested in inflammation and protein aggregation in neurodegenerative diseases. I pursued three main questions during my PhD project in the Wyss-Coray lab: Does autophagy contribute to the metabolism of Amyloid Precursor Protein (APP) in the brain? Does autophagy activity play a role in Alzheimer Disease (AD) pathology? And how does inflammation and cellular communication influence dementia pathology in humans?

My PhD project is based on the discovery that Beclin 1 (BECN1), a protein involved in autophagy initiation, is selectively reduced in AD patients' cortex. We created an AD mouse model with reduced BECN1 levels and observed significantly enhanced deposition of Aβ plaques, increased microglia activity, and increased neuronal loss (Pickford et al., 2008).

Next, I established various cell culture models of BECN1 deficiency and overexpression (using siRNA and Lentivirus) and demonstrated that BECN1 regulates APP levels through autophagy. Accordingly, I found that enhancing autophagosomal turnover through starvation or pharmacological treatments reduced levels of APP and its metabolites (Jaeger et al., 2010).

To explore how systemic inflammatory, immune signaling, and cellular communication factors modulate neuronal processes (and potentially autophagy) in neurological disorders we developed an antibody-based protein microarray technique to simultaneously measure hundreds of plasma based communication factors (chemokines, cytokines, growth factors, neurotrophins etc.) in blood from human dementia patients and unaffected controls. I adapted existing genomics tools and developed novel data extraction, data handling, and analytical methods to interpret the plasma proteomics data. I discovered a significant de-regulation of a variety of important biological pathways such as TNF-α or TGF-β signaling (Jaeger et al., manuscript in preparation).

Based on my findings, our laboratory will now continue to explore potential candidate pathways that might underlie the observed de-regulation of brain autophagy in AD, both on a cellular and a systemic level.

Summary

References:

Pickford F, Masliah E, Britschgi M, Lucin K, Narasimhan R, **Jaeger PA**, Small S, Spencer B, Rockenstein E, Levine B, Wyss-Coray T. *The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid beta accumulation in mice.* J Clin Invest. **2008** Jun;118(6):2190-9.

Jaeger PA, Pickford F, Sun CH, Lucin KM, Masliah E, Wyss-Coray T. *Regulation of amyloid precursor protein processing by the Beclin 1 complex.* PLoS One. **2010** Jun 15;5(6):e11102.

Jaeger PA, Britschgi M, Rufibach K, Burkholder B, Johns, H, Sun CH, Pradhan S, Petersen R, Knopman DS, Boeve BF, Boxer, AL, Karydas A, Miller BL, Rademakers R, Dickson DW, Yonnkin S, Graff-Radford N, Wyss-Coray T. *Plasma Protein Changes in Sporadic Alzheimer Disease Patients are Linked to Cognitive Decline and Identify Disease-related Pathways*. (in preparation)

Thesis Introduction

Alzheimer Disease

Alzheimer Disease (AD) is an age-dependent neurodegenerative disorder that causes a progressive loss of cognitive function. It is characterized by the accumulation of Aβ into amyloid plaques and cerebrovascular deposits in the extracellular space (Glenner and Wong 1984; Hardy and Selkoe 2002). In addition, abnormal phosphorylation of the microtubule associated protein tau results in the formation of tangles inside neurons (Terry, Masliah et al. 1994). These protein deposits are associated with a dramatic loss of neurons in the cortex and hippocampus, leading to severe memory deficits, speech difficulties, and personality changes. Protein aggregation is also a defining hallmark of other neurodegenerative diseases including Huntington's disease, Parkinson's disease, Prion diseases, and tauopathies. While much research has centered on abnormal folding or proteolytic processing of key components of these protein deposits, less focus has been placed on the possibility that dysfunction or deficiency of intracellular protein degradation pathways may drive the disease process (Kopito 2000; Cataldo, Petanceska et al. 2004; Levine 2005; Williams, Jahreiss et al. 2006). Autophagy (also known as macroautophagy) is the major pathway involved in the degradation of proteins and organelles, cellular remodeling, and survival during nutrient starvation (Klionsky and Emr 2000; Levine and Klionsky 2004; Levine 2005). Autophagy has been implicated in an increasing number of diseases from cancer to viral diseases and more recently in neurodegeneration (Shibata, Lu et al. 2006). Interestingly, autophagy decreases with age (Martinez-Vicente, Sovak et al. 2005; Shibata, Lu et al. 2006) and may contribute to abnormal protein accumulation in different cell types with age and thus increase the risk for neurodegenerative diseases. Autophagy has been described in AD brains and implicated in APP metabolism (Cataldo and Nixon 1990; Nixon, Wegiel et al. 2005; Yu, Cuervo et al. 2005) but it is unclear whether this process exerts beneficial or detrimental effects (or both) in AD pathogenesis.

APP metabolism and trafficking is central to AD

There is strong evidence from human genetics, animal models, and cell culture studies that abnormal production or accumulation of Aβ is a key factor in the pathogenesis of AD (Selkoe 1999; Haass and Selkoe 2007). All known genetic mutations in cases of familial AD (FAD) increase production of Aβ42, increase the Aβ42/40 ratio or increase the amyloidogenicity of Aβ. FAD mutations cause less than 2% of total AD cases, however, they point to the centrality of APP processing in AD, and it is highly likely that other genetic or environmental factors that alter APP metabolism may contribute the other 98% of AD cases (Hardy and Selkoe 2002). Transgenic mouse models overproducing FAD-mutant human APP reproduce important aspects of AD, including amyloid plaques, neurodegeneration, and cognitive deficits.

APP is a transmembrane protein that is translated in the endoplasmic reticulum, modified and glycosylated in the Golgi network and then enters the secretory pathway (Fig. 1). It may be processed by one of two mutually exclusive cleavage pathways: by either α-secretase (non-amyloidogenic processing) or β-secretase (amyloidogenic processing) and subsequently γ-secretase (Fig. 2). Non-amyloidogenic cleavage occurs mainly at the cell surface, whereas amyloidogenic processing takes place in intracellular compartments (including endosomes, lysosomes and possibly autophagosomes [Yu, Cuervo et al. 2005; Vetrivel and Thinakaran 2006]). Amyloidogenic processing releases Aβ that is subsequently secreted from the cells.

Recent advances have shown that subtle alterations in the intracellular distribution of APP can have strong effects on whether it is processed by the non-amyloidogenic or amyloidogenic pathways. This is in part due to different enzymatic compositions and pH of subcellular compartments. For example, the protein SORL1 (SorLA/LR11) is involved in sorting of APP promoting its recycling and protecting it from processing to Aβ and degradation (Fig. 1). As a result, overexpression of SORL1 reduces extracellular Aβ and lowers APP levels while reduced expression leads to increased Aβ production (Andersen, Reiche et al. 2005; Offe, Dodson et al. 2006). Interestingly, SORL1 has been shown to be reduced in AD brains (Scherzer, Offe et al. 2004; Andersen, Reiche et al. 2005) and has been genetically linked to late-onset AD (Rogaeva, Meng et al. 2007).

While the number and distribution of amyloid plaques correlate only weakly with AD symptoms the amount of soluble Aβ and Aβ oligomers is a better indicator of disease (Naslund, Haroutunian et al. 2000). In addition, Aβ can accumulate inside neurons and there is growing evidence that this type of accumulation occurs early in the disease and may be neurotoxic (Laferla, Green et al. 2007). Such Aβ may be generated in late endosomes or autophagosomes (Yu, Kumar et al. 2004) and seems to accumulate in part in multi-vesicular bodies (MVBs) where it has been linked to synaptic pathology (Takahashi, Milner et al. 2002). In Down syndrome patients (Gyure, Durham et al. 2001; Mori, Spooner et al. 2002) and in 3xTg-AD mice (Oddo, Caccamo et al. 2006) Aβ appears first inside neurons and as intraneuronal Aβ levels decrease, extracellular deposits start to form.

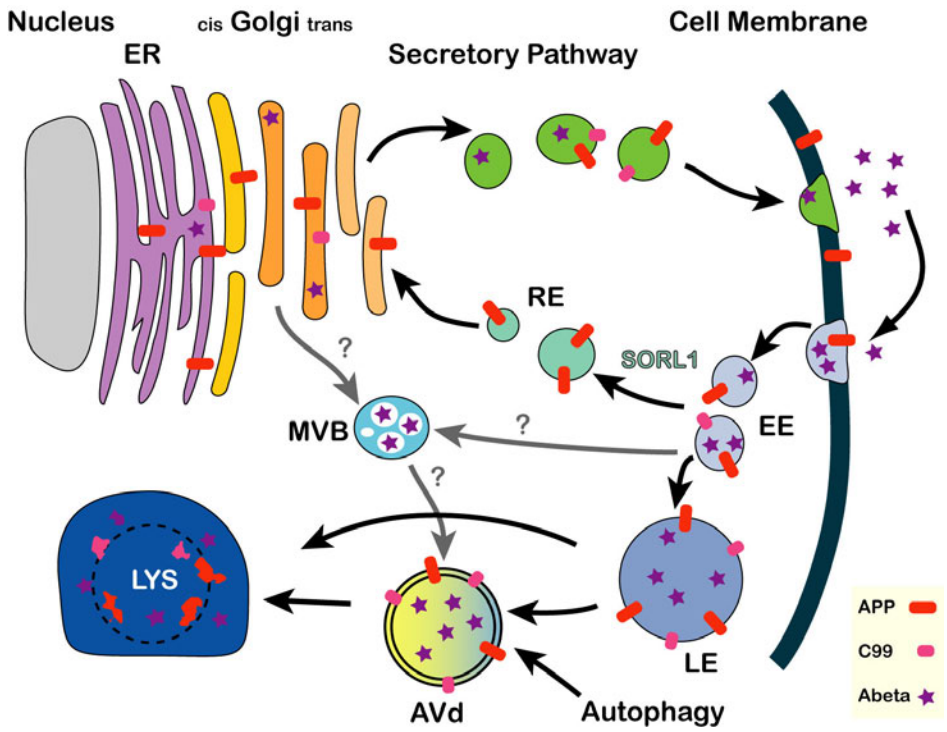

Figure 1: APP trafficking and Aβ production

The Amyloid Precursor Protein (APP) is translated in the endoplasmatic reticulum (ER). After folding and potential post-translational modifications APP is shuttled to the cell membrane via the secretory pathway. Some APP cleavage occurs in this early stages already, as indicated by the presence of Aβ and C99, the C-terminal cleavage product of APP. APP is then integrated into the cell membrane and remains at the cell surface, where most of its cleavage occurs. After some time APP and/or its cleavage products can become endocytosed and are taken up through early endosomes (EE). APP can either re-enter this cycle by passing through recycling endosomes (RE) or it can be targeted for degradation. Then, late endosomes form (LE), which subsequently fuse with degradative autophagic vesicles (AVd) and finally enter lysosomes (LYS) for degradation. Late endosomes might be able to target lysosomes directly and other potential vacuolar compartments might also be involved, as indicated by grey arrows (multivesicular-bodies, MVB).

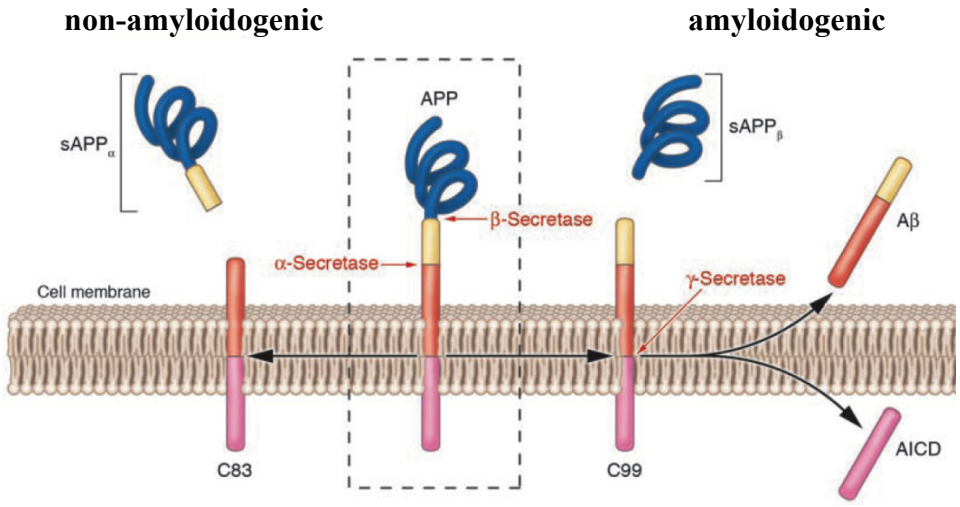

Figure 2: APP processing

Mature APP (center, inside dashed box) is metabolized by two competing pathways, the α-secretase pathway that generates sAPPα and C83 (also known as CTFα; left) and the β-secretase pathway that generates sAPPβ and C99 (right). The carboxyterminal fragments C83 and C99 are substrates for γ-secretase, generating the APP intracellular domain (AICD) and, respectively, the secreted peptides p3 (not shown) and Aβ (right). (Modified from S. Gandy (Gandy 2005))

Mechanism of autophagy

Autophagy is involved in the intracellular turnover of proteins and cell organelles and has a key role in regulating cell fate in response to stress (Klionsky and Emr 2000; Levine 2005). It is a highly conserved process that occurs in all species and cell types studied so far. A cup-shaped isolation membrane forms around cytosolic components, eventually fusing to form a double membrane (two bi-layered membranes) bound vesicle (Wang and Klionsky 2003). This autophagosome undergoes several microtubule-dependent maturation events including fusion with endosomes and multilamellar vesicles before it fuses with lysosomes (Lucocq and Walker 1997; Berg, Fengsrud et al. 1998; Eskelinen 2005) (Fig. 3). Lysosomal enzymes degrade the autophagosome inner membrane and cytosolic contents. Autophagy decreases with age (Martinez-Vicente, Sovak et al. 2005; Shibata, Lu et al. 2006) and age is also the biggest risk factor for AD. The molecular components of autophagy were first discovered in yeast, and mammalian homologues have subsequently been identified (Klionsky, Cregg et al. 2003).

Two ubiquitin-like modification systems are essential for mammalian autophagy; Atg 12 is activated by Atg7, transferred to Atg10, conjugated to Atg5 and subsequently forms a complex with Atg 16L. This step is necessary early in autophagy for the formation of the phagophore or isolation membrane. LC3-l, the cytoplasmic homologue of yeast Atg8, is activated by Atg7, transferred to Atg3, cleaved by Atg4, and conjugated to the phospholipid phosphatidylethanolamine (PE). This form known as LC3-ll, localizes to the autophagosome membrane and is subsequently degraded in the lysosome (Kabeya, Mizushima et al. 2000; Klionsky and Emr 2000). Several different signaling cascades are regulating autophagy. At least three of them work through modification of the mammalian target of rapamycin (mTOR), which inhibits autophagy. These include insulin and growth factors which signal through AKT to inhibit the tuberous sclerosis complex (TSC1/2) and alleviate inhibition of mTOR, energy depletion which activates AMP kinase and downregulates mTOR (Inoki, Zhu et al. 2003; Corradetti, Inoki et al. 2004), or through nutrient depletion and subsequent modulation of TSC1/2 and other regulatory proteins. Other signaling cascades implicated in the regulation of autophagy include Ras/Raf and ERK signaling (mTOR dependent [Furuta, Hidaka et al. 2004] or

independent [Pattingre, Bauvy et al. 2003]), calcium sensing calmodulin-dependent kinase kinase-beta signaling (Hoyer-Hansen, Bastholm et al. 2007) (converging onto the AMPK pathway) and the mTOR independent inositol signaling pathway (Sarkar, Floto et al. 2005; Criollo, Maiuri et al. 2007). Lastly, autophagy may be induced "directly" through the presence of intracellular inclusions (Yamamoto, Cremona et al. 2006; Lunemann, Schmidt et al. 2007; Malicdan, Noguchi et al. 2007).

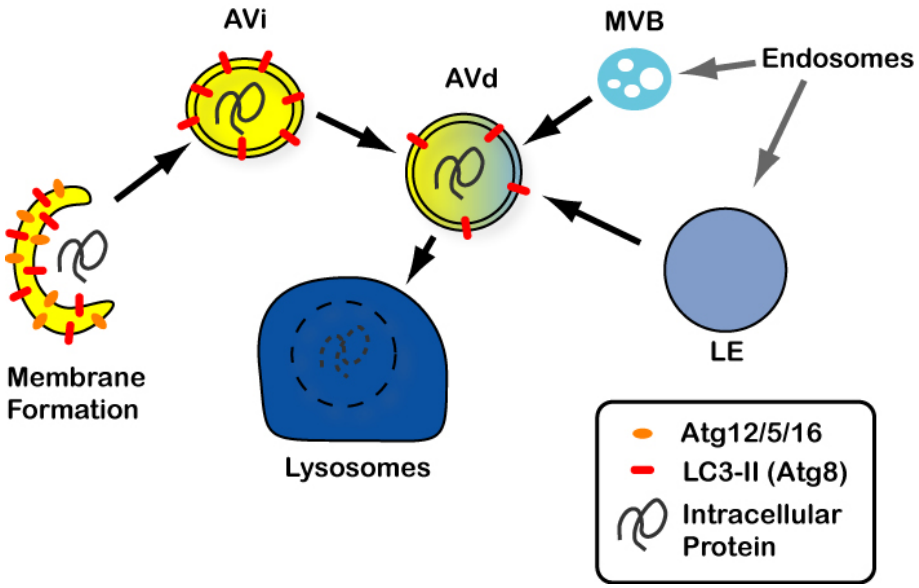

Figure 3: Vesicle trafficking in autophagy

Autophagy degrades cytoplasmic proteins, damaged organelles and membrane proteins eg. APP. (see text page 23-24). AVi; initial autophagic vesicle, AVd; degradative autophagic vesicle, LE; Late endosome, MVB; multivesicular body.

Beclin 1 in autophagy

Beclin 1 is necessary for autophagy (Liang, Jackson et al. 1999; Yue, Horton et al. 2002; Qu, Yu et al. 2003; Yue, Jin et al. 2003) and is thought to be involved in the remodeling of membranes to form autophagosomes (Figure 5). It is a 60 kd membrane protein that was first identified as a Bcl-2 interacting protein (Liang, Kleeman et al. 1998), and is the human homolog of the yeast autophagy protein Atg6 (Kametaka, Okano et al. 1998). Beclin 1 modulates the activity of the class III PI(3) kinase hVps34 (Kihara, Kabeya et al. 2001), to generate PI3P. The majority of the Beclin 1/hVps34 complex resides in the TGN, and is thought to be important in targeting other autophagy proteins to pre-autophagosomal membranes (Kihara, Kabeya et al. 2001). Recent reports have shown that although Vps34 has other intracellular membrane trafficking roles in mammalian cells, Beclin 1 acts only to control its autophagy function (Zeng, Overmeyer et al. 2006). Another binding partner for Beclin 1 is UVRAG (Liang, Feng et al. 2006) which enhances the interaction between Beclin 1 and hVps34/PI3-kinase to promote autophagy.

Separate domains have been identified in Beclin 1 corresponding to its Bcl-2 binding and autophagy functions (Liang, Kleeman et al. 1998; Liang, Yu et al. 2001; Pattingre, Tassa et al. 2005). Beclin 1 mRNA and protein are expressed in neurons and glia in human and mouse brains (Liang, Kleeman et al. 1998) although this analysis was not detailed. Knockout mice lacking beclin 1 (beclin 1–/–) die during embryogenesis (Qu, Yu et al. 2003; Yue, Jin et al. 2003). In contrast, beclin 1+/– mice are viable; they have reduced autophagosome formation in skeletal muscle, bronchial epithelial cells and B lymphocytes (Qu, Yu et al. 2003), but the neuronal phenotype of these mice has not been characterized.

Autophagy in neurodegenerative disease

Autophagy was initially identified in yeast as a physiological response to starvation in order to degrade and recycle non-essential intracellular macromolecules. Identification of mammalian homologues led to the investigation of the role of autophagy in cancer, programmed cell death, and virus replication. Recently, increasing attention has been focused on the role of autophagy in degradation of misfolded proteins and neuronal cell death in neurodegeneration (Martinez-Vicente and Cuervo 2007; Rubinsztein, Gestwicki et al. 2007). The key question as to whether autophagy protects neurons or executes their death in neurodegenerative disease remains unanswered.

Pharmacological stimulation of autophagy attenuated disease severity in models of Huntington's disease (Ravikumar, Duden et al. 2002; Iwata, Christianson et al. 2005; Berger, Ravikumar et al. 2006) or amyotrophic lateral sclerosis (Kabuta, Suzuki et al. 2006) and promoted clearance of α-synuclein in PC12 cells (Webb, Ravikumar et al. 2003). Intracellular huntingtin inclusions can induce autophagy and sequester Beclin 1 (Shibata, Lu et al. 2006; Yamamoto, Cremona et al. 2006). Mice lacking either Atg5 or Atg7, two key components of the autophagy pathway, developed ubiquitin positive inclusions, behavior abnormalities and neuronal loss (Hara, Nakamura et al. 2006; Komatsu, Waguri et al. 2006). These data suggest that autophagy is protective against neurodegeneration. On the other hand, ultrastructural analyses have identified the presence of double membrane autophagic vesicles in dystrophic neurites in AD brains, and APP/PS1 transgenic mice (Nixon, Wegiel et al. 2005) and more recently, autophagy of mitochondria in AD brains (Moreira, Siedlak et al. 2007). Autophagosomes have been identified as a site for Aβ production (Yu, Cuervo et al. 2005). Continuous autophagy may be responsible for cell death (Mills, Reginato et al. 2004), or required for cell death (Yu, Alva et al. 2004), however the interactions between autophagic cell death and apoptosis remain complex. Finally, autophagy has been implicated in Purkinje cell death in brains of Lurcher mice (Yue, Horton et al. 2002). The Lurcher mutation in these mice causes constitutive activation of the GluRδ2 channel and also the recruitment of a complex of proteins including Beclin 1. This may induce autophagic cell death in the affected Purkinje neurons (Zuo, De Jager et al. 1997; Yue, Horton et al. 2002) although a

recent study from the same group indicates that the early induction of autophagy in dystrophic axons may be a protective response (Wang, Ding et al. 2006).

Together, the data suggest that autophagy plays an important role in the normal function of neurons. It may also be involved in the clearance of aggregated proteins and in the eventual death of neurons in disease. So far, no genetic or causal defects in the autophagy pathway have been linked to any neurodegenerative disease and the role of autophagy in AD remains unclear. What makes autophagy a particularly interesting pathway to study in AD are its postulated role in Aβ production, its general function in intracellular degradation of proteins and protein aggregates, and its role in the cellular stress response. In addition, disruption of axonal transport in AD (Gunawardena and Goldstein 2001; Mandelkow, Stamer et al. 2003; Ravikumar, Acevedo-Arozena et al. 2005) may be linked to the accumulation of autophagosomes in dystrophic neuritis and presenilins have been shown to be involved in the fusion of autophagosomes with lysosomes (Esselens, Oorschot et al. 2004; Wilson, Murphy et al. 2004; Yu, Kumar et al. 2004).

Beclin 1 and autophagy in Alzheimer Disease

Based on the above-mentioned involvement of autophagy in protein degradation, our laboratory became interested in investigating the role of autophagy related proteins in AD (Pickford et al., 2008). A model-guided microarray study on tissue from AD patients' brains had previously discovered reduced levels of Beclin 1 mRNA in their entorhinal cortex (Small et al., 2004). Thus we decided to measure Beclin 1 protein and mRNA levels in AD brain tissue and found both reduced (Fig 4A and data not shown). Existing mouse-models of AD appeared to not have reduced Becn1 levels (data not shown) so our laboratory produced a special, Beclin 1 deficient AD mouse line. When we crossed Becn1 +/- mice with mice overexpressing human APP, we observed increased plaque deposition and Aβ levels in their brain tissue (Fig. 4B and C). This increased plaque formation was accompanied by elevated markers of microglia activation and neurodegeneration (data not shown and Fig. 4D). Interestingly, when we examined the brain tissue by electron microscopy, we found evidence of impaired protein homeostasis, as the neurons frequently contained numerous enlarged vacuoles that were filled with electron dense material (Fig. 4E). Based on these findings I then started to investigate if the role of Beclin 1 in AD is indeed tied to APP metabolism via autophagy or based on an unknown, autophagy independent function of Beclin 1.

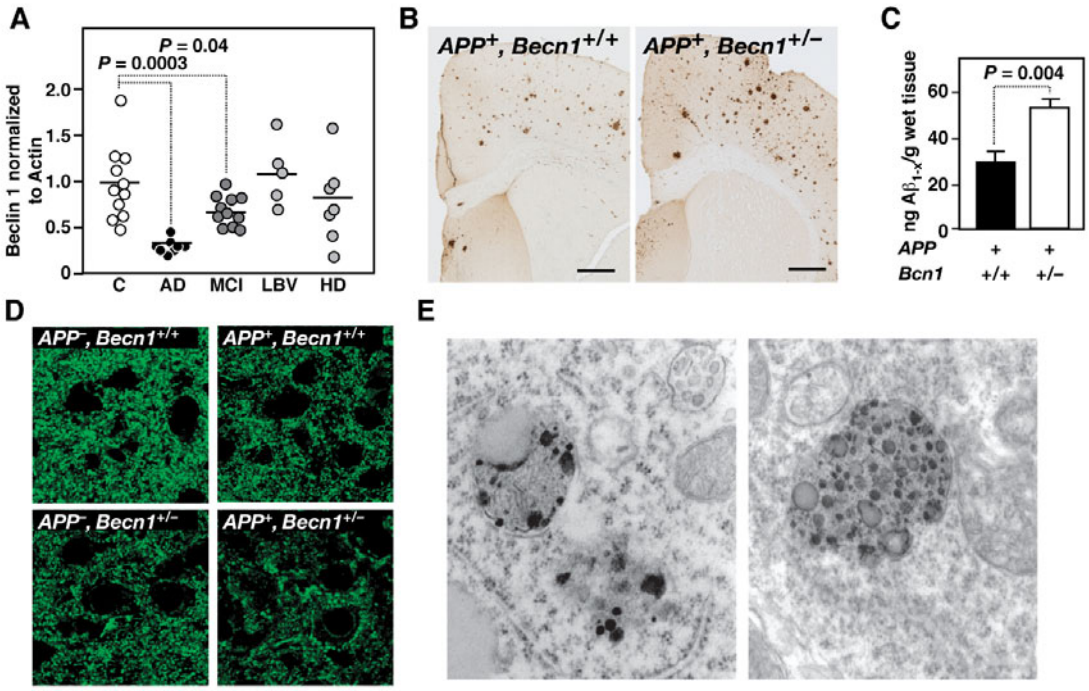

Figure 4: Beclin 1 deficiency in AD and APP transgenic mice

(A) Beclin 1 levels are strongly reduced in AD patient's brain tissue and diminished in tissue from patients with mild cognitive impairment (MCI). However they are unchanged in patients with the Lewy-body variant of AD (LBV) or Huntington Disease patients (HD). **(B)** Human APP transgenic mice with reduced Beclin 1 levels exhibit increased plaque formation. **(C)** Beclin 1 reduction leads to increased levels of secreted Aβ. **(D)** Neurodegeneration in 9-month-old female APP-Becn1+/- mice and age matched littermate controls (synaptophysin stain, magnification 980x). **(E)** Abnormal, electron-dense material-filled vacuoles in neurons from 5-month-old APP-Becn1+/- mice (magnification 25.000 x). [Modified from F. Pickford (Pickford et al., 2008)]

Significance

AD and other neurodegenerative diseases afflict millions of individuals in this country and there are no effective treatments available. Aging is the main risk factor for the sporadic forms of the disease and greatly influences age of onset in FAD. It is therefore likely that changes in gene expression with age determine the susceptibility to disease. Autophagy is a major pathway necessary for the turnover of long-lived proteins and cellular organelles and autophagy decreases with age. Our preliminary data suggest that APP, a molecule central in the development of AD, is degraded by autophagy. We hypothesize that reduction of the autophagic degradation of APP and possibly other proteins promotes AD pathogenesis (Fig. 5). There is extensive literature placing changes in APP metabolism at the heart of AD, however our hypothesis outlines a novel approach and may not only help in a better understanding of the disease process but also identify potential new therapeutic targets. Screens for autophagy inducing compounds for the treatment of Huntington's disease are already underway (Sarkar, Perlstein et al. 2007) and new compounds could possibly be employed in AD as well.

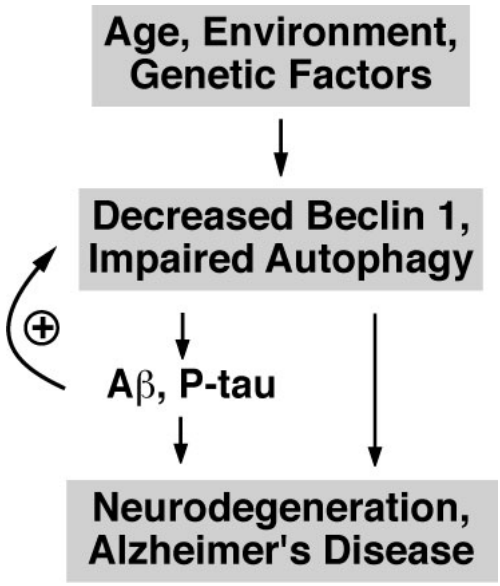

Figure 5: General thesis hypothesis

Age, environmental and genetic factors reduce Beclin 1 levels and autophagy in neurons and possibly other cells. Impaired autophagy will lead to accumulation of intracellular proteins and damaged organelles and disruption of the lysosomal pathway. This will lead to neurodegeneration either directly or via increased APP and it degradation products including Aβ or increased phosphorylation of tau (P-tau). It is also possible that APP and Aβ or, in autosomal dominant forms of AD, mutant APP or presenilins result in a decrease in beclin 1 and/or autophagy. Restoring Beclin 1 to normal levels in aging brains or brains with AD pathology may prevent or reduce neurodegeneration and pathology.

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Chapter 1: Autophagy in Neurodegeneration and Neuroprotection

Summary

Autophagy is the major pathway involved in the degradation of proteins and organelles, cellular remodeling, and survival during nutrient starvation. Autophagosomal dysfunction has been implicated in an increasing number of diseases from cancer to bacterial and viral infections and more recently in neurodegeneration. While a decrease in autophagic activity appears to interfere with protein degradation and possibly organelle turnover, increased autophagy has been shown to facilitate the clearance of aggregation-prone proteins and promote neuronal survival in a number of disease models. On the other hand, too much autophagic activity can be detrimental as well and lead to cell death, suggesting the regulation of autophagy has an important role in cell fate decisions. An increasing number of model systems are now available to study the role of autophagy in the central nervous system and how it might be exploited to treat disease. We will review here the current knowledge of autophagy in the central nervous system and provide an overview of the various models that have been used to study acute and chronic neurodegeneration.

Background

Cells have a constant need for the building blocks of life: amino acids, lipids, carbohydrates, and nucleic acids. To sustain this catabolic and anabolic need, they rely on uptake and recycling. While nutrient uptake is important, different degradation systems are in place to efficiently turnover recyclable intracellular material and provide quality control. The main pathways for protein degradation and recycling are the ubiquitin/proteasome pathway (for degrading short-lived cytosolic and nuclear proteins) [1]*, the lysosomal pathway (for cytosolic proteolysis), and autophagy (for bulk cytosolic degradation and organelle recycling) [2]. Deficits in any of these recycling pathways can

* To preserve space and the original table layout, references for chapters 1-4 are numbered based on the sequence of citations in the original publications and references can be found starting at p. 159.

result in uncontrolled accumulation of cellular debris or severe deficiencies in metabolic productivity, ultimately causing cell death.

The term autophagy, coined from the Greek words of αυτός (‘autos’, self) and φαγειν (‘phagein’), meaning 'eating', was first used in 1963 by Christian de Duve to establish a nomenclature for different cellular pathways and compartments in the endosomal-lysosomal pathway [3]. Early work in autophagy research was done in rat liver cells and autophagy was characterized as a physiological response to starvation in order to degrade and recycle non-essential intracellular macromolecules [4-6]. Later, autophagy [7] and many of the autophagy genes [8] were identified in yeast, which gave the scientific community access to powerful cloning and pathway analysis tools. Subsequent identification of mammalian homologues led to the investigation of the role of autophagy in cancer, programmed cell death, tissue remodeling, heart, liver and muscle diseases, and bacterial and viral infections [9].

In recent years, increasing attention has been focused on the role of autophagy in metabolism of misfolded proteins and neuronal cell death in neurodegeneration (for comprehensive reviews see [10-13]). Abnormal autophagy has been implicated in the pathology of numerous diseases of the central nervous system (CNS), both chronic disorders (such as proteopathies) and many acute injuries. While it is still early in our understanding of this pathway, autophagy seems to have both beneficial and detrimental effects in disease, and it will be key to define the context that determines the outcome.

Types of neuronal autophagy

Autophagy is involved in the intracellular turnover of proteins and cell organelles and has an important role in regulating cell fate in response to stress [14, 15]. It is a highly conserved process that occurs in all species and cell types studied thus far. Two main types of mammalian autophagy have been identified and implicated in CNS injury and disease: macroautophagy and chaperone-mediated autophagy. Other more specialized forms of autophagy exist, such as mitophagy (direct targeting of mitochondria to lysosomes) [16], pexophagy (selective degradation of peroxisomes) [17, 18], xenophagy (degradation of intracellular bacteria and viruses) [14, 19], crinophagy

(lysosomal fusion with re-directed exosomes) [20], microautophagy (direct engulfment of cytosol by lysosomes) [21, 22], and piecemeal microautophagy of the nucleus (partial sequestration and degradation of the nucleus) [23], but most of them have only been observed in yeast or under special conditions and are not reviewed here.

Macroautophagy is a bulk degradation pathway and the only intracellular mechanism potentially capable of degrading large protein aggregates or damaged organelles. It is a well-understood process in yeast, but details about the exact sequence of events and the proteins involved are still uncertain in mammals. A cup-shaped isolation membrane forms around cytosolic components, eventually fusing to form a double membrane bound vesicle [24, 25]. The origin of the membrane material for the formation of the isolation membrane is still under investigation, but recent evidence suggests that it might be derived from the endoplasmatic reticulum (ER) [26]. The protein MAP1LC3 is anchored via conjugated phosphatidylethanolamine (MAP1LC3-II) to the isolation membrane and is a specific marker for the so-called autophagosomes [27]. The autophagosome undergoes several microtubule- [28, 29] and dynein-dependent maturation events [30, 31], including fusions with multivesicular bodies (MVB), early and/or late endosomes [32, 33], before it fuses with lysosomes [34, 35] (Fig. 6, for a more comprehensive overview of autophagosome turnover see [28, 36]).

At least 12 Atg (autophagy-related) and 4 other proteins are known to be involved in mammalian macroautophagy initiation and execution [37, 38] (see Fig. 7). Whether direct autophagosomal-lysosomal fusion is possible, or endosomes first have to deliver essential enzymes to the maturating autophagosomes, is unclear. While the content of the autophagosome initially has the same pH as the surrounding cytosol, it becomes more acidic during its maturation [39, 40]. For successful degradation of the autophagosomal content, autophagosomes need to migrate from their site of formation to lysosome rich peri-nuclear regions [29, 41]. After fusion with the lysosome the outer autophagosome membrane can be reused, while lysosomal enzymes degrade the inner membrane and its cytosolic contents, enabling the recycling of macromolecules [42] (Fig.1). It is unknown which markers, if any, label organelles or cytoplasm for sequestration and inclusion into autophagosomes. One possible marker for protein aggregates is the ubiquitin binding protein sequestosome 1 (SQSTM1, also known as p62) [43]. Almost all protein

aggregates are poly-ubiquitinated and SQSTM1 binds both, MAP1LC3 and ubiquitin [44-46]. Macroautophagy components are expressed in neurons and neuronal cell lines (Tab. 1). While the function of autophagy-related proteins has been described for some, it is still unknown for others (Tab. 2). Macroautophagy has been implicated in chronic neurodegenerative diseases and acute neuronal injuries (Tab. 3 and 4).

Chaperone-mediated autophagy (CMA) is distinctly different from macroautophagy in that no vesicular trafficking is involved (Fig. 6). Instead, a pentapeptide motif in substrate proteins allows their specific translocation to the lysosome membrane (reviewed in [47-49]). Thus, CMA degrades only proteins with the motif KFERQ or a biochemically related sequence, which is present in about 30% of all cytosolic proteins [50]. It has recently been suggested that 80% of aminoacyl-tRNA synthases are also substrates for CMA [48], indicating a possible role of CMA in protein synthesis control under starvation conditions.

To be targeted for CMA, substrate proteins first bind to a cytosolic complex containing the chaperone HSC70 (Fig. 6). This complex then interacts with a lysosomal membrane complex containing LAMP2A and HSP90 [51]. The substrate protein is finally degraded after unfolding and translocation into the lysosomal lumen (with the help of lys-HSC70, a luminal form of HSC70) [51]. The chaperone complex consists of many more proteins but their exact localization and role in CMA is presently unclear [52].

Macroautophagy and CMA are interconnected, although the details of this crosstalk are not well understood. A possible connection is BCL2 associated athanogene (BAG1) which functions as a nucleotide exchange factor for HSC70 [53] and has been reported to bind MAP1LC3 [54]. Impairing macroautophagy, either genetically or pharmacologically, results in a compensatory up-regulation of CMA [55]. CMA components are expressed in neurons and neuronal cell lines (Tab. 1) and CMA has also been implicated in chronic neurodegenerative diseases (Tab. 3).

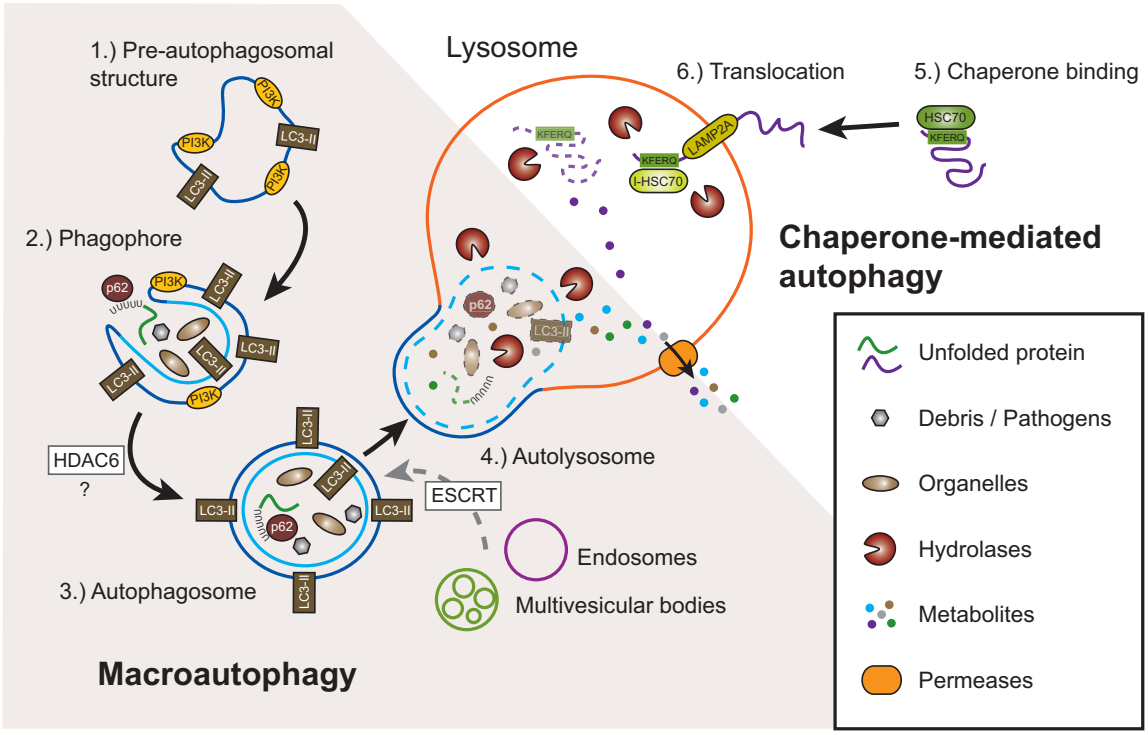

Figure 6: Steps in macroautophagy and chaperone mediated autophagy.

Macroautophagy: **1.) Nucleation.** An unidentified membrane source delivers lipid bi-layers for the formation of the phagophore. In yeast this early structure is termed pre-autophagosomal structure (PAS), its identity in mammalian cells is uncertain. A class III PI3K complex consisting of at least BECN1, PIK3C3, PIK3R4, UVRAG, and AMBRA1 is required for PAS formation and MAP1LC3 is anchored to the membrane via a phosphoethanolamine (PE) anchor (LC3-II). **2.) Expansion.** The PAS or a comparable structure in mammals sequesters cytosolic cargo (either specifically via SQSTM1 [p62] or nonspecifically) by invagination, forming a double-membranous vesicle. This stage is also called "isolation membrane". More membrane and LC3-II is being recruited to the developing vacuole. **3.) Maturation.** The completed autophagosome undergoes multiple maturation steps and fusion events with multi-vesicular bodies (MVB) or endosomes. The exact nature and sequence of this maturation, and whether these steps are always required is currently unknown. The autophagosomal lumen becomes more acidified during this maturation. **4.) Docking and fusion.** During docking and fusion the inner membrane compartment together with its content gets released into the lysosome/autolysosome and is being degraded by lysosomal hydrolases. The components of the outer membrane are available for re-usage. Chaperone-mediated autophagy: **5.) Recognition and binding.** The HSC70 chaperone complex (consisting of HSC70, HSP90 and maybe other proteins) recognizes unfolded proteins with the KFERQ sequence and moves them to the lysosome. **6.) Translocation.** LAMP2A and a lysosomal form of HSC70 (l-HSC70) translocate the substrate protein across the lysosomal membrane into the lumen for degradation. The autophagy delivered substrates get degraded inside the lysosomes and their macromolecular components are made available to the cell's metabolism via permeases that allow their transport back into the cytosol.

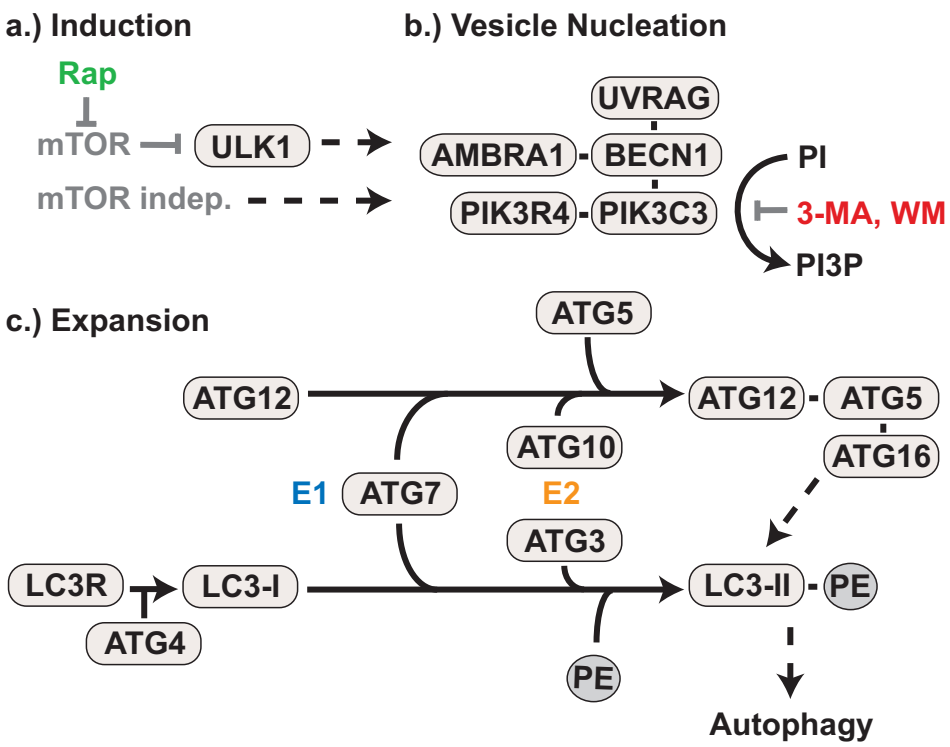

Figure 7: Autophagy pathway in mammals.

The formation of autophagosomes appears to follow a pathway conserved across species and most findings made in yeast or other organisms also apply to mammalian autophagy. a.) Autophagy can be induced via mTOR dependent or independent pathways (for more information, see text and Fig. 3) which stimulate the nucleation and expansion of the phagophore / isolation membrane. b.) A multi-protein complex surrounding BECN1 with PI3K activity (mediated by PIK3C3) is important for the formation of the autophagosomal membrane. c.) Two ubiquitin-like modification systems are essential for mammalian autophagy; ATG12 is activated by ATG7 (E1 step), transferred to ATG10 (E2 step), conjugated to ATG5 and subsequently forms a complex with ATG16. This step is necessary early in autophagy for the formation of the phagophore or isolation membrane. MAP1LC3 (LC3) is cleaved by ATG4, activated by ATG7 (E1 step), transferred to ATG3 (E2 step), and conjugated to the phospholipid phosphoethanolamine (PE). This form known as MAP1LC3-II (LC3-II), localizes to the autophagosome membrane and is subsequently degraded in the lysosome. ATG4 cleaves off a C-terminal arginine (R) to expose a glycine residue that is then being linked to PE. Rapamycin (Rap) inhibits mTOR and activates macroautophagy, while 3-methyladenin (3-MA) and wortmannin (WM) inhibit the PI3K activity and de-activate macroautophagy.

Gene	H. sapiens		M. musculus		R. norvegicus	Gene	D. melanogaster	Gene	C. elegans
	mRNA	Protein		AllenB					
ULK1	[67]		[99,100,107]	Yes	[92]	*Atg1*	[97]	*unc-51*	WoBa [109,110,112,113]
ATG3	[74]		[104]	Yes		*Atg4/Aut1*	[111]	*atg-3*	
ATG4	[68]		[90]	Yes	[105]	*Aut2/Atg4*		*atg-4.1-2*	WoBa
ATG5			[60,91,93,96]	Weak	[87]	*Atg5*		*atg-5/atgr-5*	
BECN1	[59,69]	[59,65,69]	[59,81,96]	Yes	[80,94,95,106]	*Atg6*	[97]	*bec-1*	WoBa [116]
PIK3C3	[66]	[77]		Yes		*Vps34/Pi3K59F*		*vps-34/let-512*	[114]
PIK3R4	[71]			Weak		*Vps15/ird1*		*ZK930.1*	
UVRAG	[72]			Yes					
AMBRA1			[83]	n.a.					
ATG7	[75]		[61,96]	Weak	[75]	*Atg7*		*atg-7/atgr-7*	
MAP1LC3		[58,65]	[56,60,65,79,84,86, 96]	Yes	[87-89,95,103,106]		[97]	*lgg-2*	
GABARAP	[78]			Yes	[89,92]	*Atg8a*		*lgg-1*	WoBa
GABARAPL2	[78]			Yes	[89]				
ATG12			[82,96]	Weak	[87]	*Atg12*		*lgg-3*	
CHMP4B			[85]	n.a.		*shrb/Vps32*	[108,115]	*vps-32.1*	WoBa
HSPA8	[70]	[63,73,76]		Yes	[76,98,101]	*Hsc70-4*		*hsp-1*	
LAMP2		[64]	[102]	Weak	[102,103]				

Table 1: Presence of autophagy related gene expression in neuronal tissue

Examples of autophagy related gene expression in humans and common model organisms (mRNA and/or protein). For human, mouse, and rat genes the approved human gene symbol is used (www.genenames.org), for D. melanogaster and C. elegans their respective gene symbols (if existent) are provided. (*AllenB):* Gene mRNA is detectable by hybridization as published in the Allen Brain Atlas [www.brain-map.org]; (*WoBa):* neuronal expression data available at WormBase [www.wormbase.org]; *(n.a.):* not available.

Autophagy in the healthy nervous system

The brain is well protected against short-term periods of systemic starvation. Selective transport of glucose, amino acids, and hormones across the blood-brain-barrier ensures ample supply of metabolites and local populations of glia cells release trophic factors under normal or energy restricted conditions. High levels of constitutive autophagy in neurons may therefore not be necessary to maintain the cellular energy needs; indeed, forty-eight hours of food deprivation caused no apparent autophagy induction in the mouse brain [56].

Instead, autophagy probably supports local housekeeping functions within the neuron: macroautophagy is the only cellular mechanism capable of degrading expired organelles in neurons that can live for decades. In addition autophagy is a potential clearing mechanism for protein aggregates that occur frequently in aging neurons, but not in young and healthy cells. Consistent with such a role in the normal brain autophagosome numbers [57] and the levels of MAP1LC3-II protein [56, 58, 59] are low when compared with other tissues. Nevertheless, recent findings show that autophagy in neurons is indeed constitutively active [60, 61] and autophagosomes accumulate rapidly when their clearance is blocked [62], indicating fast basal turnover.

A number of autophagy related genes are expressed (measured either by mRNA or protein analysis) in neuronal tissues of humans [58, 59, 63-78], rodents [56, 59-61, 65, 75, 76, 79-107], and insects [97, 108-116] (Tab. 1). Electron microscopy of human and mouse brain tissue shows the presence of lysosomes and autophagosomes in neurons further supporting a basal level of autophagy during normal neuronal homeostasis [57, 58, 117, 118]. Model organisms have been crucial for the identification of genes that regulate autophagy and clarification of their function as detailed in Tab. 2 [27, 40, 59-61, 79, 83, 85, 90, 91, 97, 99, 100, 107, 111-116, 119-146].

Age is a major risk factor for many neurodegenerative diseases and a number of studies suggest a role for autophagy in aging. Interestingly, protein degradation and specifically autophagy (both macroautophagy and CMA) decline with age, although to what extent that reduction occurs within the CNS is not clear [147-150]. An age related decline of Atg genes has been shown in *D. melanogaster*, and Atg8 overexpression

increases the fly's lifespan [151, 152] while RNAi of autophagy genes in *C. elegans* leads to decreased lifespan [136, 153]. If and how decreasing autophagy activity in the aging human CNS contributes to the higher prevalence of neurodegenerative diseases and accumulation of various protein aggregates will have to be clarified in future studies.

Gene (Alias)	Protein function	Knockout/ knockdown	OE/TG	ES/M @ IMSR	Neuronal phenotype after k.o./k.d. (Animal model)	K.o. embryonic lethal
ULK1 (ATG1)	Ser/Thr protein kinase (regulation and vesicle formation)	[107,112,113] * [97,99,100,131,132,135,141,145]	[140] (OE)	ES M (GT)	Impaired endocytosis of nerve growth factor, excessive axon arborization, stunted axon elongation (MM) Paralysis, aberrant axon growth, abnormal vesicles, arrested differentiation (CE)	Yes (DM)
ATG3	Ubiquitin-conjugating-like enzyme (attaches MAP1LC3 to PE)	[111,143]		n.a.	Not reported	Yes (DM)
ATG4	Cystein protease (cleaves C-terminus of MAP1LC3 for conjugation)	[90,144]		ES M (GT/TG)	Not reported	Yes (CE)
ATG5	Unknown (conjugates to ATG12, binds ATG16)	[60] * [91,130,141]		ES M (MUT)	Progressive motor deficits, accumulation of inclusion bodies, neurodegeneration, aberrant vacuoles in Purkinje cells (MM)	No # (DM/MM)
BECN1 (ATG6)	Unknown (part of class III PI3K complex, anchor protein, autophagy initiation)	[59] * [97,116,124,137,146]	[119] (TG)	M (TG)	Neurodegeneration, lysosomal abnormalities (MM)	Yes (MM/CE/DM)
PIK3C3 (VPS34)	Class III PI3K complex (forms complex with BECN1/PIK3R4/AMBRA1/ UVRAG, autophagy initiation)	[123,142] * [114,126]		ES	Abnormal protein aggregation, abnormal locomotion (CE)	Yes (CE)
PIK3R4 (VPS15, P150)	Ser/Thr protein kinase (forms a complex with and activates PIK3C3)	[134]		ES	Not reported	Yes (DM)
AMBRA1	Unknown (component of the class III PI3K complex)	[83] *		ES	Neural tube defects, polyU aggregates, unbalanced cell proliferation, cell death (MM)	Yes (MM)
ATG7	Ubiquitin-activating-like enzyme (activates MAP1LC3 and ATG12 for conjugation)	[61,123,129] * [124,125,127,128, 136,141]		ES	Behavioral deficits, neuronal loss, polyU inclusions, axonal dystrophy, axonal terminal degeneration (MM) PolyU aggregates, neuronal degeneration (DM) Abnormal protein aggregation (CE)	No # (DM/MM)

Table 2: Neuronal phenotype of autophagy related knockout/knockdown animals.

Examples of model organism with knockout, knockdown, or overexpression of autophagy genes and the corresponding neuronal phenotype. Approved human gene names are used [www.genenames.org], in addition commonly used aliases are provided. # While non-neuronal Atg5 and Atg7 k.o. mice survive birth, they die within one day postnatal. (MM): M. musculus; (DM): D. melanogaster; (CE): C. elegans; (OE): overexpression; (ES): embryonic knockout stem cell; (M): mouse line; (TG): transgenic; (GT): gene-trap; (MUT): targeted mutation; (IMSR): knockout ES / mice available through the International Mouse Strain Resource [www.informatics.jax.org/imsr/index.jsp]; (*): neuronal tissue examined; (n.a.): not available.

MAP1LC3 (LC3)	Unknown (similarity with ubiquitin, part of autophagosomal membrane)	[123,145] * [79,97]	[27] (TG)	ES M (TG)	Abnormal protein aggregation (CE)	Yes (CE) No (MM)
ATG12	Unknown (similarity with ubiquitin, conjugated to ATG5)	[123] *		n.a.	Abnormal protein aggregation (CE)	Unknown
CHMP4B (SNF7-2)	Unknown (part of the ESCRT-III complex, involved in surface receptor degradation, formation of MVBs and autophagosomes)	[85,115,138]		ES	Dendritic and axonal branching impaired, dendritic retraction, reduced cell viability, autophagosomes accumulate, increased htt toxicity (DM)	Yes (MM)
HSPA8 (HSC70)	Chaperone (recognizes CMA motif, lysosomal translocation)	[121,139] *	[120] (OE)	ES	Impaired transmitter release, o.e. rescues α-synuclein pathology, Bolwig's nerve projection abnormalities (DM)	Yes (DM)
LAMP2	Unknown (Lysosomal membrane glyco-protein, forms complex with HSPA8)	[40]		ES	Not reported	No (MM)

Table 2 (Continued)

Autophagy as a clearing mechanism for protein degradation

The strongest evidence for an active role of autophagy in maintaining neuronal homeostasis comes from engineered mutant mice lacking autophagy genes. While *Atg5* and *Atg7* knockout mice had been created before [128, 130], their early developmental mortality made the study of the adult CNS impossible. To overcome this limitation, two landmark studies generated conditional knockout mice lacking *Atg5* and *Atg7* only in neurons [60, 61].

The *Atg5*$^{flox/flox}$;nestin-Cre mice showed growth retardation, progressive motor and behavioral deficits, prominent neurodegeneration and axonal swelling in a number of brain regions. Histological examination also revealed abundant ubiquitin-positive inclusions in neurons, indicating a crucial role of autophagy in the turnover of diffuse cytosolic proteins labeled for degradation [60].

In the *Atg7*$^{flox/flox}$;nestin-Cre mice, strikingly similar pathological changes occurred: reduced growth, motor and behavior changes, loss of Purkinje cells, activation of glia cells, and accumulation of ubiquitinated inclusions. Proteasomal function was not impaired by autophagy inhibition, which shows that autophagy has an important role in the basal turnover of poly-ubiquitinated (polyU) proteins together with the proteasome [61]. The ubiquitin-positive aggregates also contain abnormal amounts of SQSTM1 [127].

While polyU proteins themselves are sticky but not highly aggregating, the presence of large amounts of SQSTM1 might enhance their aggregation [43, 154]. SQSTM1 can directly interact with MAP1LC3 [45] and tags ubiquitinated protein-aggregates for autophagic degradation [43, 155]. It appears that impairment of autophagy leads to the accumulation of SQSTM1, which in turn increases the rate of aggregation for diffuse ubiquitinated proteins. Interestingly, the double knockout of *Atg7* and *Sqstm1* prevents the formation of ubiquitinated aggregates in neurons, but has no effect on the other observed neurodegenerative phenotypes [127], indicating that autophagy plays multiple roles in neuronal homeostasis, not just clearance. This crosstalk between autophagy and the ubiquitin-proteasome system (UPS) is supported by *in vitro* induction of autophagy in response to impaired UPS [156]. SQSTM1 is not the only protein

facilitating the degradation of protein aggregates via autophagy, as HDAC6, a microtubule-associated histone deacetylase that interacts with polyU proteins, also provides a link to autophagy (see below [156, 157]).

Additional evidence for a role of autophagy in protein turnover comes from mice lacking *Ambra1*, a recently discovered regulator of autophagy that interacts with Beclin 1 (BECN1) [83] (Fig. 7). *Ambra1* knockout mice show polyU inclusions and severe neural tube deficits, unbalanced cell proliferation, and excessive apoptotic cell death. Autophagy has a complex interplay with apoptosis, where it can serve both as an alternative cell-death and as an anti-apoptotic survival mechanism. More details of this relationship will be discussed at the end of this article and comprehensive reviews have been published on this topic elsewhere [133, 158].

Autophagy in vesicle sorting and organelle turnover

Another set of important findings indicates that endosomal sorting and endosomal-autophagosomal fusion are impaired in certain neurodegenerative diseases. ESCRT-0 to III (endosomal sorting complex required for transport) orchestrate the progression of endosomes along the endosomal-lysosomal pathway. Dysfunction of one of these complexes (ESCRT-III), either by RNAi depletion of its essential subunit *CHMP4B* (also known as *SNF7-2*) or by expression of a mutant CHMP2B protein (another subunit of ESCRT-III and associated with Frontotemporal dementia linked to chromosome 3), caused autophagosome and polyU protein aggregate accumulation, and dendritic retraction followed by neuronal death in cultured mature cortical neurons [85]. It has been suggested that the endosomal and autophagosomal pathways merge upstream of lysosomal fusion [159-161], in particular that intact multivesicular bodies (MVB) are essential for autophagosome maturation [138, 162]. ESCRT-III seems to play an important role during this endosomal-autophagosomal fusion event and its dysfunction leads to impaired processing and accumulation of autophagosomes. In a recent paper, deletion of the *Hrs* (also known as *Hgs*) gene, a component of ESCRT-0, in the neurons of $Hrs^{flox/flox}$;SynI-cre mice caused apoptosis, loss of hippocampal CA3 pyramidal

neurons, and accumulation of polyU proteins and SQSTM1 [163]. Accordingly, locomotor activity and learning ability were severely reduced in these mice.

While no evidence for the autophagosomal degradation of specific neuronal organelles (such as synaptic vesicles) in healthy neurons exists thus far, mitochondria were selectively degraded by macroautophagy in neurons exposed to experimental neurotoxins 1-methyl-4-phenylpyridinium (MPP+) or 6-Hydroxydopamine, which induce mitochondrial damage [164, 165]. Autophagosomes were also observed in dopaminergic neurons treated with methamphetamine [166], supporting the idea that autophagy serves to clear damaged organelles in neurons. Together, these studies underline the critical role of autophagosomal-endosomal-lysosomal trafficking and sorting in neuronal homeostasis.

Autophagosomes as transport vacuoles

Autophagosomes are not only found in the soma but also in the distal parts of the axon and dendrites and can be retrogradely transported to the cell soma for degradation [167]. Autophagy may thus support neurite and growth cone remodeling and clear axons and dendrites of defective larger structures. Efficient bi-directional transport along the axon is necessary for neuronal survival [168, 169] and supports the clearing of protein aggregates by autophagosomes [31].

In addition, autophagosomes are retrogradely transported, making them potential transport vacuoles for the delivery of trophic factors from the synapse to the cell body. Autophagosomes can travel along microtubules, possibly facilitated through an interaction between MAP1LC3 and MAP1A/B [29, 41]. Some evidence exists that signaling endosomes containing nerve growth factor (NGF) might be derived from or be related to autophagosomes, based on the microscopic association of fluorescently labeled LC3 with retrogradely transported NGF and the NGF receptors TrkA and p75 [170]. This finding could indicate that disturbed autophagy (for example, as a result of changes in APP expression or metabolism) might contribute to the reported impairment of NGF transport in neurodegenerative diseases such as Down syndrome. In this condition, an extra copy of chromosome 21, which contains the *APP* gene, results in increased APP

expression and the development of Alzheimer-like dementia. Intriguingly, in a trisomic mouse model of Down syndrome deletion of one copy of *APP* led to a marked improvement in transport of signaling endosomes containing NGF, reduced neurodegeneration, and improved cognitive function [171].

Several studies point towards an important role of ULK1 in this trafficking role of autophagy. For example, knockdown of *ULK1* by RNAi in cultured mouse spinal sensory neurons leads to impaired endocytosis of NGF [107]. Axonal growth appears stunted in *C. elegans* in *unc-51* mutants [110, 112] and after *ULK1* knockdown in mouse neuronal cells [107], while dominant negative *ULK1* mutants expressed in immature murine cerebellar granule cells lead to inhibition of neurite outgrowth and developmental arrest [99]. ULK1 is important for autophagy initiation and has been reported to interact with GABARAP and GABARAPL2 (also known as GATE16), two homologues of MAP1LC3, in mouse pyramidal, mitral, and Purkinje cells. This interaction indicates an involvement of autophagosome transport in some of the *ULK1* knockdown phenotypes [92], although it clearly has functions independent of autophagy [100, 172].

Another interaction between autophagy and neuronal receptors was found in Lurcher mice, which have a mutation in the glutamate receptor GluRδ2 and are a model for ataxia. The mutated receptor GluRδ2^{Lc}, but not the wildtype receptor, binds to BECN1 and may thus trigger autophagy in dying Purkinje cells in Lurcher mice [173, 174]. In this way, autophagy might serve as an early stress response to axonal dystrophy. Autophagosomes appear rapidly in axons in Lurcher mice and this is attributed to the induction and local synthesis of autophagosomes in axon terminals in response to stress [174]. How autophagosomes form so fast in distal cell parts is unclear, but early ultrastructural studies suggest that smooth ER in axons might be a source for quick membrane supply [175, 176].

Regulation of autophagy

Because of its key function in cell homeostasis, multiple signaling cascades have been implicated in the regulation of autophagy (Fig. 8). A large amount of this knowledge has been acquired in yeast and it is unknown how much can be translated to

mammalian cells (for reviews see [177-179]). One of the key regulators of autophagy is the level of amino acids, both extracellular and intracellular. Cells measure intracellular amino acid levels via the protein kinase EIF2AK4 (also known as GCN2), which is activated by unloaded transfer RNAs. Low levels of intracellular amino acids leading to free transfer RNAs thus activate autophagy through phosphorylation of the eukaryotic initiation factor eIFα2 [180]. Extracellular amino acids are sensed via a putative receptor in the cell membrane [181], which seems to signal through mammalian target of rapamycin (mTOR, also known as FRAP1). mTOR is a protein kinase that plays a central role in nutrient sensing, cell proliferation, and metabolism [182-184], integrating many signaling pathways. Activated mTOR promotes protein synthesis and inhibits autophagy via phosphorylation of the ULK1 binding partner ATG13, while deactivated mTOR activates autophagy [185]. Insulin and growth factors signal through AKT, activate mTOR [182, 186] and deactivate autophagy, while energy depletion [187] or elevated intracellular calcium [188] inhibit mTOR through AMP-activated protein kinase (AMPK) and activate autophagy. Other signaling cascades implicated in the regulation of autophagy include Ras/Raf and ERK signaling (mTOR dependent [189] or independent [190]) and the mTOR independent inositol signaling pathway [191, 192]. Lastly, autophagy may be induced "directly" through the presence of intracellular inclusions [193-195]. It is unclear which of these pathways are involved in neurodegenerative conditions.

Even less is known about the transcriptional control of autophagy, especially in neurons. Nevertheless, a number of important transcription factors have been associated with the regulation of autophagy genes in non-neural cell types. Since these processes are likely conserved, they may contribute to the control of autophagy in neurons as well.

In one study, a high-affinity E2F4 transcription factor-binding region in the *BECN1* promoter was identified [196]. A number of autophagy proteins are also controlled by the FOXO3 transcription factor in muscle cells [197, 198] and potentially hepatoma and pheochromocytoma cells [198]. In these cell types, FOXO3 binds directly to the promoters of *MAP1LC3*, *ATG12*, and *GABARAP* genes to increase their expression and induce autophagy [197]. Indeed, FOXO3 increases the expression of ATG4, PIK3C3 and BECN1, but the exact mechanisms are unknown [198].

Members of the p53 family also play important roles in autophagy control: Cytosolic p53 inhibits autophagy [199], whereas nuclear p53 activates it [200]. The localization of p53 appears to be a sensor for genotoxic stress. In addition, p53 acts upstream of mTOR, inhibiting its activity through AMPK, thus stimulating autophagy. Recently, a p53 homologue, p73, has been identified by integrating whole-genome chromatin immunoprecipitation and expression profiling in cell culture that binds to regulatory regions of several autophagy genes (*ATG5, ATG7, UVRAG, GABARAP, AMBRA1, ATG16, PIK3C3*) presumably through its nuclear activity [201, 202]. Further studies that investigate the upstream control of autophagy in neurons will greatly help to improve our understanding of the potential misregulation of autophagy during neurodegeneration.

The above findings suggest three main roles for autophagy in neuronal homeostasis: First, impaired autophagy results in abnormal protein aggregation across species, indicating an involvement of autophagy in the clearance of intracellular protein aggregates, especially when these aggregates are poly-ubiquitinated. Second, changes in vesicular appearance and trafficking point towards a crucial role of autophagy in maintaining the normal turnover and flux of vacuolar compartments and possibly trophic factors through the neuron. And third, disrupted autophagy leads to changes in neuronal morphology and connectivity, such as excessive axon arborization, stunted axon growth, axonal dystrophy, axonal terminal degeneration or impaired axonal projections, implicating autophagy genes and their gene products in neuronal shaping, connectivity, and development. Whether these observations are always directly linked to the gene's role in autophagy or are sometimes a result of non-autophagic functions remains to be determined.

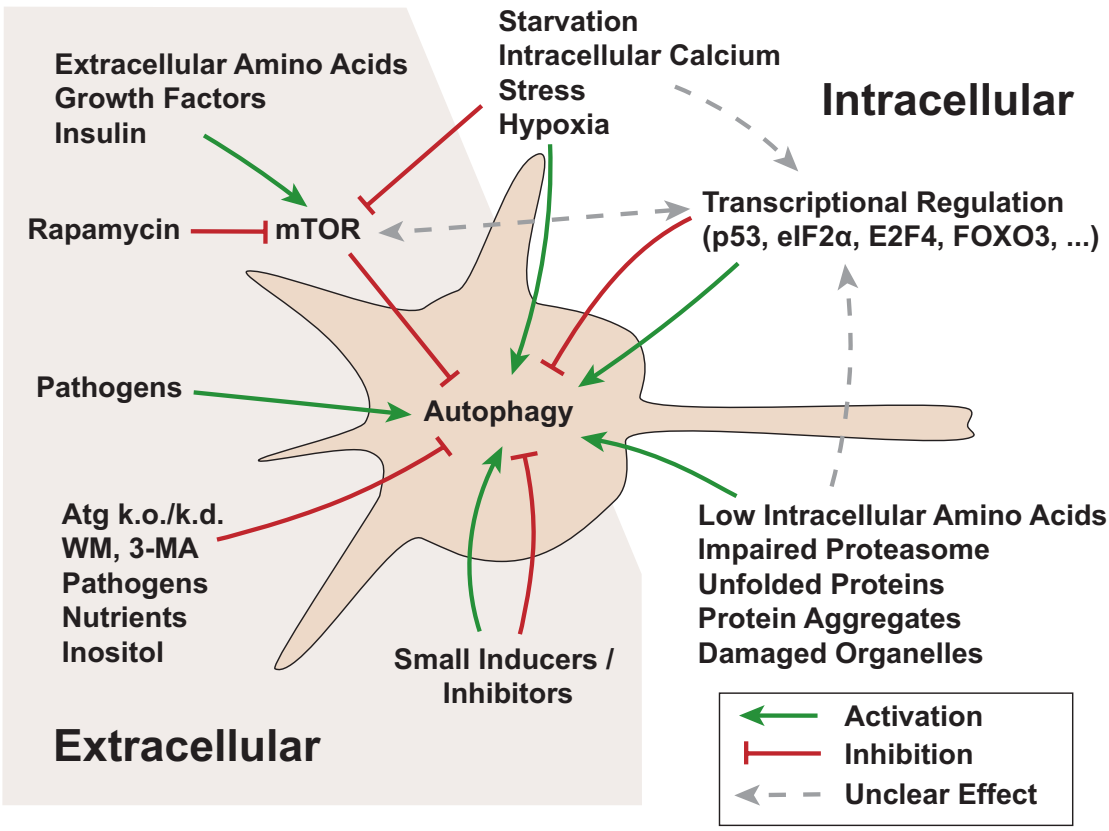

Figure 8: Control of autophagy

Autophagy is a major housekeeping pathway and under the control of many different signaling cascades. Mammalian Target of rapamycin (mTOR) plays a central role in the regulation of autophagic activity as it integrates signaling from different sensors of cellular homeostasis. When mTOR is active in yeast it keeps an important ULK1 binding partner (ATG13) phosphorylated, thus inhibiting the induction of autophagy. While signals indicating abundant nutritional and trophic support activate mTOR (and deactivate autophagy), signals of starvation or other stressors inhibit mTOR (and activate autophagy). Autophagy can be directly stimulated by intracellular debris (such as unfolded proteins and damaged organelles) or by indicators of an overwhelmed ubiquitin-proteasome system (UPS). Also certain pathogens activate autophagy. Autophagy can be directly inhibited by genetic ablation of important Atg genes, inhibitors of the class III PI3K-complex (WM, 3-MA), high nutrient levels, and inositol signaling. More recently screenings of small compound libraries have yielded inducers and inhibitors of autophagy, both mTOR dependent and independent. And last, transcriptional regulators, such as p53, eIF2α, E2F4, or FOXO3 regulate autophagy by controlling the expression levels of many Atg genes. For further details, please refer to the text.

Autophagy in CNS disease and injury

Several excellent reviews have recently covered the emerging relationship between autophagy and various neurodegenerative diseases [10-13] and we provide only a brief overview of the most prevalent diseases associated with histopathological changes in autophagy. Instead, we summarize here which aspects of autophagosomal pathology that have been observed in human disease are now being successfully replicated in model systems (Tab. 3 and 4).

In general, the effect of autophagy in neurons during disease can be broadly divided into two classes: autophagosomal degradation is either impaired or excessively activated, leading to an apparent disruption of the intracellular organelle organization and accumulation of autophagosomes in neurons over long periods of time (chronic conditions, Tab. 3), or autophagy genes are activated in response to temporary injury/stress (acute response, Tab. 4).

Autophagy in chronic CNS diseases

Typical examples of the first class of diseases are Alzheimer (AD) [57-59, 62, 118, 203-209], Parkinson (PD) [102, 117, 192, 210-220], and Huntington disease (HD) [195, 203, 216, 221-231] (Tab. 3). In these diseases, the pathological accumulation of autophagosomes/autophagosome-like structures and abnormalities in the endosomal-lysosomal pathway were documented by electron microscopy (EM) in human postmortem brain tissue [57, 58, 117, 118, 207]. Diseases with a seemingly more endosomal pathology, but an autophagic component, are Amyotrophic lateral sclerosis (ALS) and Frontotemporal dementia (FTD) [85, 86, 162, 232, 233].

In Alzheimer research, expression analysis revealed that *BECN1* mRNA is reduced in AD brain tissue [59, 234], and BECN1 protein levels are significantly lower in the cortex of AD patients compared with age-matched controls [59]. This is despite the fact that an increase in autophagosome numbers in neurons from AD patients is obvious by EM, and AD brains also show increased levels of MAP1LC3-I and MAP1LC3-II [58]. A possible explanation for this apparent contradiction is that reduced BECN1 levels lead

to changes in autophagosomal flux. This in turn could impair endosomal-lysosomal degradation, leading to a built-up of intracellular vesicular compartments over time. Changes in the endosomal-lysosomal pathway are amongst the earliest changes in AD [235] and a possible indicator for disturbed vacuolar trafficking.

While the aforementioned studies were descriptive, one of the first mechanistic insights into the possible role of autophagy in neurodegenerative diseases was provided by a study of primary neurons from a mouse model for HD. The authors observed increased autophagy, increased oxidative stress, and polyU aggregates in cultured striatal neurons from transgenic mice expressing mutant human huntingtin in response to a single exposure of a neurotoxic concentration of dopamine [223]. The results suggest that dopamine triggered free radical-mediated oxidation of macromolecules and stimulated autophagy. Subsequent studies demonstrated that SQSTM1 extensively decorates polyU protein aggregates, co-localizes with MAP1LC3 and becomes sequestered in autophagosomes. This highlights the importance of autophagy as a degradative pathway for polyU aggregates [43]. Another link between autophagy and protein aggregates was provided by a study showing that mTOR accumulates in huntingtin aggregates in cells, mice, and human brains [226]. The authors speculate that mTOR can be sequestered and inactivated in this way, leading to a protective induction of autophagic degradation of protein aggregates. Arguing against this interpretation is the observation that BECN1, a protein necessary for the induction of autophagy, is recruited into pathological huntingtin aggregates in human brain tissue as well [230].

The effect of autophagy on the degradation of protein aggregates was investigated further in cell culture and animal models using pharmacological inducers and inhibitors of autophagy (see Tab. 4). It was discovered that rapamycin, an inducer of autophagy, leads to the clearance of polyQ/polyA aggregates in cell culture, fly, and mouse models of HD [195, 226]. This finding was confirmed for α-synuclein in cell culture [218] and wildtype tau in flies [203]. Together, these results triggered a concerted research effort to find mTOR dependent and independent pharmacological inducers of autophagy and led to the discovery of many small compounds that facilitate the clearing of aggregated proteins [216, 219, 229, 236]. While pharmacological autophagy stimulation reduces the toxicity of many aggregate-prone proteins, experiments in cell culture demonstrate that α-

synuclein can be degraded by both the proteasome and autophagy. Pharmacological inhibition of either pathway leads to increased intracellular α-synuclein levels [218]. Interestingly, pharmacological inhibition of microtubule formation by nocodazole treatment inhibits polyQ aggregate formation and at the same time increases its toxicity in cell culture [237, 238]. This is at least partially due to the inhibition of autophagosome-lysosome fusion [239], demonstrating that intracellular transport is essential for proper aggresome/inclusion body formation and autophagosomal function. Furthermore, activation of autophagy through starvation in primary cortical mouse neurons expressing polyQ proteins protects against cell death [186]. In summary, autophagy might be especially effective in clearing aggregated proteins.

While these pharmacological studies increase our understanding of some aspects of autophagy in neurodegeneration, they mostly employ drugs that are rather nonspecific and they target proteins such as mTOR and AKT, which have broad functions outside autophagy. Genetic or RNAi-based methods overcome some of these limitations.

It has been shown, for example, that cytosolic protein aggregates can be specifically targeted by autophagy and that their aggregation increases after inhibition of autophagy by siRNA knockdown of *MAP1LC3* in cell culture [221]. In *C. elegans*, RNAi mediated deletion of *bec-1*, *atgr-7*, and *Ce-atg18* led to increased accumulation of polyQ aggregates in models for HD, confirming the earlier studies in mammalian cell culture systems [124].

The cytoplasmic histone deacetylase HDAC6, although not directly an autophagy related protein, plays an essential role in the microtubule- and dynein-dependent intracellular movement of polyU protein aggregates [240]. *HDAC6* RNAi impairs retrograde transport of autophagosomes and lysosomes [156]. HDAC6 overexpression, on the other hand, is sufficient to rescue neurodegeneration caused by proteasome mutations or polyQ toxicity in transgenic flies via autophagy, providing a direct link between UPS and autophagy [157]. HDAC6 activates autophagy by an unknown mechanism, leading to accelerated protein turnover. Potential mechanisms include modulation of HSP90 (and maybe CMA), a substrate of HDAC6 [241], accelerated transport of polyU-proteins into aggregates and to autophagosomes [240], and enhanced transport of lysosomes to autophagosomes [156]. The importance of autophagosomal

transport for effective clearance of aggregated proteins has been demonstrated in HD fly and mouse models, where dynein mutations caused increased aggregate formation and decreased autophagosome-lysosome fusion [31].

Recently, autophagy was genetically manipulated in a mouse model of AD by crossing *Becn1* heterozygous knockout mice (*Becn1*$^{+/-}$) with human amyloid precursor protein (APP) transgenic mice. *Becn1* deficiency resulted in neurodegeneration and increased β-amyloid (Aβ) deposition in APP mice [59]. Based on these findings and new cell culture data from our lab (Jaeger *et al.*, manuscript in preparation) we propose that autophagosomes can degrade APP and thus lower Aβ accumulation [59]. On the other hand, autophagosomes contain the enzymes necessary for processing of APP into Aβ and are potential producers of this toxic peptide [58]. A decisive factor that determines whether autophagy reduces or promotes Aβ accumulation might be the speed of autophagosomal turnover and the clearance of autophagic vesicles. Both are impaired under disease conditions [62]. Disturbances in autophagy initiation due to insufficient BECN1 levels could cause expansion of the endosomal-lysosomal system, producing a high load of potentially Aβ generating vacuoles. Interestingly, two APP mouse models for AD have been analyzed for changes in Becn1 levels, but no differences were detected [59]. These findings hint at an autophagy dysfunction upstream of APP pathology in AD.

CMA is also clearly involved in chronic neurodegenerative diseases, most prominently in PD: HSP90 levels are increased in human PD brains and are correlated with the levels of insoluble α-Synuclein [242]. In the same study, immunohistochemistry and EM show that HSP90 co-localizes with α-synuclein in Lewy bodies, Lewy neurites, and glia cell inclusions, both in PD patients and α-synuclein transgenic mice. Furthermore, HSP90 and HSC70 co-immunoprecipitate with α-synuclein in cell culture [242]. While this could indicate increased (protective) CMA in PD, a recent gene expression profiling of substantia nigra tissue from sporadic PD patients revealed reduced expression of UPS proteins and reduced HSC70 [243]. At some point during disease progression, HSP90 may be sequestered into α-synuclein aggregates and deactivated, thus reducing CMA activity.

A landmark study identified α-synuclein as a target for CMA and demonstrated that the PD associated mutations A53T and A30P cause α-synuclein to bind to the CMA

receptor and inhibit both the degradation of the receptor itself and that of other CMA substrates [210]. While these α-synuclein mutations are relatively rare, recent findings demonstrate that post-translational modifications of wildtype α-synuclein through dopamine can cause a similar toxic gain-of-function behavior [213]. Furthermore, inhibition of CMA by lentiviral RNAi against *LAMP2* increases the level of endogenous α-synuclein in rat cortical neurons [102]. Additionally, a link has been suggested between the PD associated mutant ubiquitin carboxyl-terminal esterase L1 (UCH-L1) and the lysosomal receptor for chaperone-mediated autophagy. This mutant UCH-L1 interacts aberrantly with LAMP2, HSC70, and HSP90, inhibits CMA and causes an increase in α-synuclein in cell culture [212].

While the role of autophagy in neurodegenerative diseases is far from being understood, the available data indicate it plays an integral role in the cellular response to intracellular protein aggregation common to these diseases. Autophagy appears impaired in the final stages of neurodegenerative diseases, whereas alterations in vacuolar trafficking are apparent in early stages, often before other histopathological changes manifest themselves. It is therefore likely that autophagy, UPS, the endosomal-lysosomal pathway, and the escalating accumulation of toxic proteins are tightly connected. Whether mutant or misfolded proteins are causing the changes in vacuolar trafficking and later autophagy or whether abnormalities in these protein degradation pathways precede protein aggregation remains to be shown.

Disease	Autophagosomal phenotype	Ref.
Alzheimer disease	Autophagy appears impaired, autophagosomes accumulate, endosomal-lysosomal abnormalities, increased mitophagy, reduction of macroautophagy enhances pathology, pharmacological activation of macroautophagy can promote the clearance of Aβ/APP and reduces tau pathology, autophagosomes contain APP/Aβ/secretases.	[206,208,59,62,204,207,203,209,205,57,58,118]
Parkinson disease	Autophagy/mitophagy appears impaired, autophagosome-like structures accumulate, pharmacological activation of macroautophagy enhances α-synuclein clearance and is neuroprotective, α-synuclein is a target of CMA and macroautophagy and the proteasome, dopamine-modified/mutated α-synuclein blocks CMA and dopamine induces autophagic cell death and α-synuclein accumulation, mutant UCH-L1 binds to LAMP2A and inhibits CMA.	[220,214,215,213,219,212,102,216,192,210,211,218,217,117]
Huntington diseases	Impaired sorting/degradation of autophagosomes, autophagosomes accumulate, BECN1 is recruited to htt inclusions and BECN1 reduction causes enhanced htt accumulation, pharmacological or signaling mediated activation of macroautophagy reduces htt toxicity, mTOR is sequestered into htt inclusions, which causes macroautophagy activation.	[225,227,228,216,229-231,203,221,226,224,195,223,222]
Frontotemporal dementia	Impaired endosome maturation, enlarged autophagosome accumulation, mutant CHMP2B disturbs the ESCRT-III complex for endosomal sorting which results in polyU/SQSTM1 aggregates.	[162,85]
Amyotrophic lateral sclerosis	Impaired early endosomes, impaired sorting/degradation of autophagosomes, CHMP2B disturbs the ESCRT-III complex for endosomal/MVB sorting which results in polyU/SQSTM1 aggregates, MVBs are required for TDP-43 clearance, Lithium activates protective autophagy.	[232,86,162,233]

Table 3: Autophagy in common chronic neurodegenerative diseases

Autophagy in acute CNS diseases and injuries

The second class of brain insults that present with an autophagy phenotype are acute injuries or stressors which activate competing cellular death and pro-survival pathways (Tab. 4). Examples include hypoxia/ischemia [80, 94, 104, 244-247], brain trauma [65, 81, 84, 87, 95, 106, 248-250], experimental pharmacological injury models (kainate, methamphetamine, oxidative stress and others) [96, 103, 166, 251-254], and trophic factor deprivation [255-259]. Similar to chronic neurodegenerative conditions, many observational studies find increased levels of autophagy proteins and/or numbers of autophagosomes after acute CNS injury such as hypoxia/ischemia or trauma [81, 87, 94, 95, 104, 106, 244, 246, 248, 250].

As described in the previous chapter above, autophagy has beneficial functions in neurons that seem to be relevant for acute injury as well. For example, the autophagy inducing drug rapamycin reduced brain injury and protected neurons in a rat model of neonatal hypoxia/ischemia [80, 249] or traumatic brain injury in mice [80, 249]. Consistent with these findings, RNAi mediated knockdown of *bec-1*, *lgg-1*, and *lgg-2*, or mutation of *unc-51* reduced survival after hypoxia in *C. elegans* [247].

However, in contrast to most studies in chronic degenerative models, acute pharmacologically induced injury or withdrawal of trophic support triggered cell death that involved autophagy and signs of apoptosis (Tab. 4). In support for a role in promoting cell death, inhibition of autophagy by 3-methyladenine (3-MA) treatment, decreased the toxic effects or delayed neuronal loss after noxious treatments [103, 253, 254, 260]. Likewise, knockdown of *ATG5* or *BECN1* by RNAi reduced cell death in photoreceptor cells that were exposed to oxidative stress [253]. Maybe most convincingly, $Atg7^{flox/flox}$;nestin-Cre mice lacking *Atg7* in the neuronal lineage are almost completely protected against stroke-induced neurodegeneration [245].

Why seemingly similar studies come to these opposing conclusions is not clear at this point but differences in the models, the tools used to analyze autophagy, or the time of analysis after injury could be responsible. In support of the last point, autophagy was still increased in surviving cells at the injury site one month after traumatic brain injury [106] while cells undergoing necrotic or apoptotic death (and possibly involving

autophagy in its detrimental role) would likely have disappeared. It will therefore be interesting to explore whether inhibiting autophagy early or late after a traumatic brain injury may have different outcomes. In addition, a better understanding of how exactly autophagy contributes to cell death and how it interacts with necrotic and apoptotic death programs is necessary.

Injury	Autophagy related changes	Ref.
Hypoxia/Ischemia	Mixed results after hypoxic treatments: Knockout of Atg genes in C. elegans decreases survival after hypoxia and autophagy activation by rapamycin treatment leads to injury reduction in rat and rat tissue. On the contrary, *Atg7*$^{-/-}$ mice lacking functional autophagy in the CNS are largely protected from neurodegeneration.	[247,80,104,94,244,246,245]
Trauma	Macroautophagy appears to be beneficial: Autophagy can be activated for more than a month following brain trauma (elevated BECN1, MAP1LC3-II, ATG5-12 levels, increased AV numbers) in rodents, autophagy appears activated in human tissue samples. Rapamycin treatment is neuroprotective in mice.	[106,87,249,248,65,95,84,81,250]
Pharmacological injury	Autophagy appears to be deleterious: Transient activation of autophagy after injury (elevated MAP1LC3-II, p-mTOR, LAMP2, increased AV numbers) and activation of apoptosis in rodents and primary neuronal culture. 3-MA treatment or RNAi against *ATG5* or *BECN1* blocks cell death.	[96,252,166,103,253,251,254]
Trophic deprivation	Autophagy appears to be deleterious: Growth factor withdrawal leads to autophagic cell death in rodents or chicken, 3-MA blocks cytochrome C release and delays apoptosis.	[257,255,256,259,258]

Table 4: Autophagy in acute neuronal injury

Autophagy and apoptosis

As described in the previous part of this chapter, autophagy in the CNS can be protective under some circumstances, while it leads to cell death in others. Furthermore the resulting cell death can be either apoptotic (type I cell death) or autophagic (type II cell death), depending on the cellular setting and inducing stressor (see also reviews [133, 158]). This dichotomous role of autophagy is the result of a complex relationship between the autophagy and apoptosis pathways (Fig. 9). While some mixed phenotypes have been reported [261-263], autophagy and apoptosis ultimately develop in a mutually exclusive way and appear to inhibit each other [264-267].

Strong evidence for a role of autophagy as an alternative cell death mechanism comes from mice deficient in apoptosis. One of the key features of apoptotic cell death is the mitochondrial outer membrane permeabilization (MOMP), which requires the two BCL2 family proteins BAX and BAK1. Cells from *Bax*$^{-/-}$ *Bak*$^{-/-}$ knockout mice are resistant to various apoptotic stimuli, but can die through a delayed autophagic cell death in response to DNA damage [268]. Autophagic cell death can also be observed after caspase inhibition, a treatment that disrupts normal apoptosis [266]. Conversely, inhibition of autophagy via RNAi targeting various autophagy genes (*ATG5*, *ATG7*, *BECN1*) can reduce autophagic cell death in certain situations [268-270].

In contrast to its function as a cell death mechanism, autophagy is induced under starvation conditions to supply the cell's metabolic needs. Under these conditions, inhibition of autophagy results in cell death [8]. Even without starvation, loss of autophagy itself (as in the *Atg5*$^{-/-}$ or *Atg7*$^{-/-}$ knockout mice) is sufficient to cause neuronal apoptosis [60, 61], and it has been suggested that autophagy is primarily a pro-survival pathway [271].

It has been shown that autophagy and apoptosis share common inducers such as reactive oxidative species (ROS), ceramide, and intracellular calcium [188, 272-275]. The two pathways are further linked through ATG5 proteolysis [275], the transcription factor p53 [276], and the BCL2 protein family (via BECN1) [277] (Fig. 9). How the balance between autophagy and apoptosis is maintained in neurons requires further investigation.

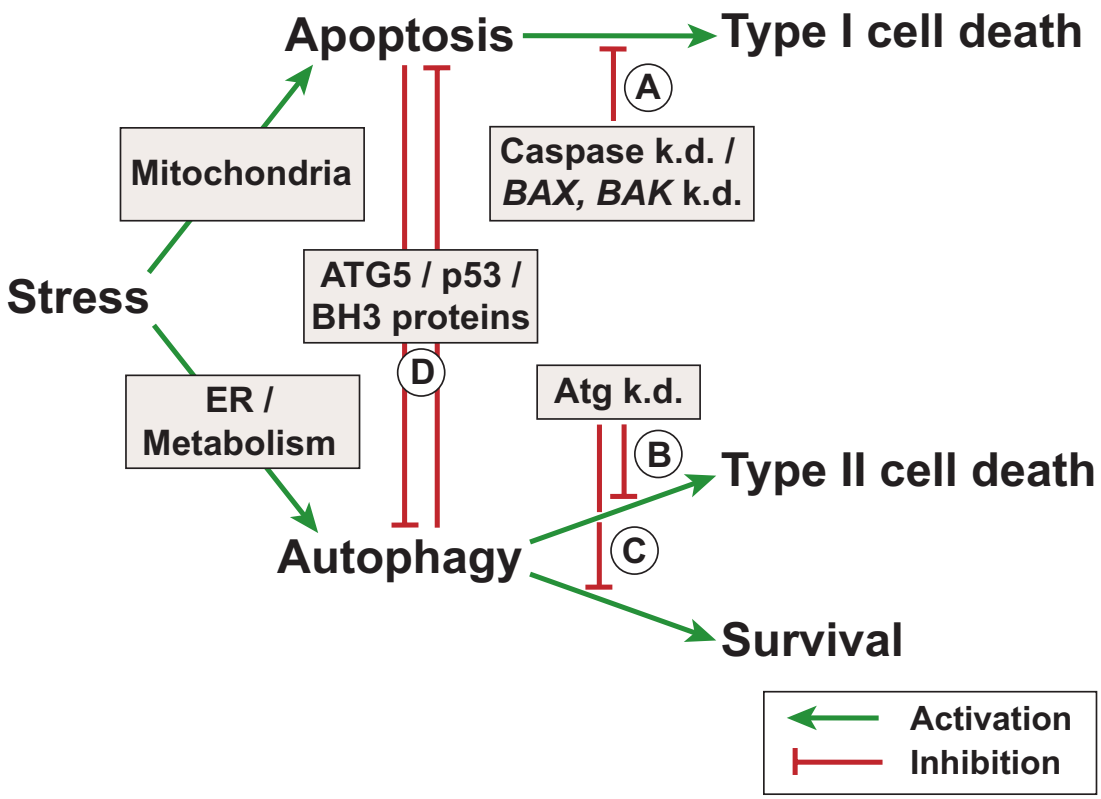

Figure 9: Interactions between autophagy and apoptosis

Cellular stressors can lead to mitochondria outer membrane permeabilization (MOMP) and subsequent cytochrome c release and apoptosis, while nutrient deficiency or ER stress can cause autophagy activation. Under physiological conditions autophagy and apoptosis keep each other inactive through mutual inhibition. A strong apoptotic stimulus (for example DNA damage, death-receptor stimulation, or cytokine deprivation) can drive a cell into apoptotic 'type I' cell death. If apoptosis is inhibited under such conditions (by caspase knockout or *Bax/Bak* knockout, [A]), autophagy can become activated and result in a delayed 'type II' cell death through degradation of most cytoplasmic cell components and organelles. Under these circumstances the knockdown of autophagy related genes [B] reduces cell death. Autophagy can become activated through ER stress (for example accumulation of misfolded proteins in the ER, intracellular calcium release from the ER) or nutrient deficiency. The cell then ensures survival by enhancing metabolic recycling through autophagy and adapting to the new nutrient conditions. Knockdown of autophagy genes in such a situation leads to an increase in apoptotic 'type I' cell death [C]. The crosstalk between autophagy and apoptosis [D] is mediated via proteolytic processing of ATG5, the transcription factor p53, and the binding and subcellular localization of BCL2 family proteins with BH3 domains. For further details, please refer to the references in the text.

Concluding remarks

Unknown to most neuroscientists just a few years ago, autophagy has gained increasing attention not only from translational researchers but also from basic neuroscientists interested in neuronal cell biology. Consequently, there are few answers as to the role and relevance of autophagy in neurons, let alone in glia cells, and very few genetic *in vivo* studies have been conducted to investigate its role in neurological disease. Nevertheless, it seems clear that neurons require autophagy for normal function and that neuronal stress will rapidly trigger this pathway. There is growing consent that intraneuronal protein aggregates trigger autophagy and that this response is beneficial – at least in its intent. This notion is supported by a limited number of pharmacological and genetic studies in animal models, which demonstrate that reduced autophagy promotes neurodegenerative disease while increased autophagy is beneficial. In contrast, work from stroke models and other acute forms of neural injury indicate that autophagy can be detrimental in such circumstances and promotes cell death. It will be necessary to employ state of the art genetic and molecular tools to dissect the role of autophagy in normal and pathological conditions in cell culture and in mammalian disease models. Conditional knockout mice are being developed or are already available to target autophagy not only in neurons but also in astrocytes, oligodendrocytes and microglia. Such studies are likely to add additional complexity to our understanding of autophagy but they may also uncover new therapeutic opportunities. Self-eating, after all, does not equate with self-destruction but may in fact be a powerful survival pathway for the cell, and as such, of key importance to neurodegeneration or neuroprotection.

Abbreviations

3-MA: 3-Methyladenine; Atg: Autophagy related genes; AD/PD/HD: Alzheimer/Parkinson/Huntington disease; APP: Amyloid precursor protein; AV: Autophagic vesicles; CMA: Chaperone-mediated autophagy; CNS: Central nervous system; EM: Electron microscopy; ER: Endoplasmatic reticulum; htt: Huntingtin; MOMP: Mitochondrial outer membrane permeabilization: MVB: Multivesicular body;

NGF: Nerve growth factor; PE: Phosphoethanolamine; PI3K: Phosphoinositide 3-kinase; polyQ/polyA/polyU: Proteins with long sequences of Glu/Ala or that are ubiquitin decorated; Rap: Rapamycin: ROS: Reactive oxidative species: UPS: Ubiquitin-proteasome system; WM: Wortmannin;

Chapter 2: Regulation of Amyloid Precursor Protein Processing by the Beclin 1 Complex

Summary

Autophagy is an intracellular degradation pathway that functions in protein and organelle turnover in response to starvation and cellular stress. Autophagy is initiated by the formation of a complex containing Beclin 1 (BECN1) and its binding partner Phosphoinositide-3-kinase, class 3 (PIK3C3). Recently, BECN1 deficiency was shown to enhance the pathology of a mouse model of Alzheimer Disease (AD). However, the mechanism by which BECN1 or autophagy mediate these effects are unknown. Here we report that the levels of Amyloid precursor protein (APP) and its metabolites can be reduced through autophagy activation, indicating that they are a substrate for autophagy. Furthermore, we find that knockdown of Becn1 in cell culture increases the levels of APP and its metabolites. Accumulation of APP and APP C-terminal fragments (APP-CTF) are accompanied by impaired autophagosomal clearance. Pharmacological inhibition of autophagosomal-lysosomal degradation causes a comparable accumulation of APP and APP-metabolites in autophagosomes. Becn1 reduction in cell culture leads to lower levels of its binding partner Pik3c3 and increased presence of Microtubule-associated protein 1, light chain 3 (LC3). Overexpression of Becn1 on the other hand reduces cellular APP levels. In line with these observations, we detected less BECN1 and PIK3C3 but more LC3 protein in brains of AD patients. We conclude that BECN1 regulates APP processing and turnover. BECN1 is involved in autophagy initiation and autophagosome clearance. Accordingly, BECN1 deficiency disrupts cellular autophagy and autophagosomal-lysosomal degradation and alters APP metabolism. Together, our findings suggest that autophagy and the BECN1-PIK3C3 complex regulate APP processing and play an important role in AD pathology.

Background

Alzheimer Disease (AD) affects a growing number of the elderly and results in dramatic loss of cognitive function. It is characterized pathologically by the presence of extracellular beta amyloid (Aβ) assemblies called plaques [278, 279], and intracellular accumulation of Aβ [280] and tau [281]. These lesions are hallmarks of the disease and are associated with neurodegeneration and inflammation [282]. Currently it is unclear how these lesions form, and how protein aggregation and neuronal loss are connected [283]. While much research has centered on abnormal proteolytic processing of Amyloid precursor protein (APP) and tau, less focus has been placed on the possibility that slow, progressive dysfunction of intracellular protein sorting and degradation pathways, such as macroautophagy, may drive pathogenesis steadily over time, especially in cases of sporadic AD [284, 285].

APP is a type I transmembrane protein that can be processed by one of two mutually exclusive cleavage pathways: α-secretase (non-amyloidogenic processing) or β-secretase (amyloidogenic processing) followed by γ-secretase cleavage. Non-amyloidogenic cleavage occurs mainly at the cell surface, whereas amyloidogenic processing takes place in intracellular compartments, including endosomes, lysosomes, and autophagosomes [286, 287]. Amyloidogenic processing releases Aβ which can subsequently be secreted from cells. In addition, APP C-terminal fragments (APP-CTF) of both cleavage pathways can translocate to the nucleus and induce nuclear signaling [288-291]. Both, Aβ and APP-CTF potentially contribute to AD pathology and can exhibit neurotoxic properties through multiple pathways [292, 293].

APP levels, Aβ levels, and neurodegeneration are tightly coupled. Less than 1% of all AD cases are autosomal dominant early-onset familial AD (FAD) and are caused by mutations in one of three major genes APP, Presenilin-1 (PSEN1), or Presenilin-2 (PSEN2) [294]. These mutations lead to the predominant amyloidogenic cleavage of APP. Additionally, FAD can be caused by APP locus duplication [295] and polymorphisms in the APP promoter region that increase APP levels have been linked to an increased risk for AD [296]. In Down Syndrome an additional copy of chromosome 21, which harbors the APP gene, leads to overexpression of APP protein, elevated Aβ

levels, plaque deposition and AD-like disease in all older Down patients [297-299]. While this illustrates the importance of APP gene regulation and APP protein levels in AD, little is known about the regulation of APP metabolism in sporadic AD cases. The levels of APP protein and APP mRNA in AD cases versus control has been reported in the past with conflicting results, but more recent research indicates increased levels of APP and APP-CTFs in sporadic AD brains [300-303].

Macroautophagy (in this paper referred to as 'autophagy') is a major pathway involved in the degradation of long-lived proteins, protein aggregates, and organelles, cellular remodeling, and survival during starvation [304, 305]. Autophagy is characterized by the formation of a cup-shaped isolation membrane that develops around cytosolic components and eventually fuses to form a double membrane bound vesicle [306-309]. The protein Microtubule-associated protein 1, light chain 3 (LC3) is anchored via conjugated phosphatidylethanolamine to the vesicle's membrane. While the un-conjugated LC3 is called LC3-I, the phosphatidylethanolamine conjugated LC3 is referred to as LC3-II and is a specific marker for these so-called autophagosomes [310]. Autophagosomes then undergo several microtubule- [311] and dynein-dependent maturation events [312, 313], including fusions with multivesicular bodies, early and/or late endosomes [314], before eventually fusing with lysosomes [315, 316].

Autophagy has recently been implicated in a number of diseases including neurodegenerative conditions and it appears that autophagy can exert both a pathological or protective role, depending on the setting [317]. While it is still largely unknown how dysfunction of the autophagy pathway might contribute to neurodegeneration and AD, recent papers suggest a role for Beclin 1 (BECN1) in AD and mild cognitive impairment [318-320]. Haploinsufficiency of Becn1 in mice decreases neuronal autophagy and promotes neuronal degeneration [318]. Moreover, in a mouse model for AD genetic reduction of Becn1 expression results in increased accumulation of APP fragments and Aβ, increased neurodegeneration and increased inflammation [318]. In addition, Autophagy has been shown to protect neurons from Aβ induced cytotoxicity [321-323].

BECN1 plays an important role in autophagy [324-328] and is the human homolog of the yeast autophagy protein Atg6/Apg6 [329]. BECN1 forms a core complex with the class 3 phosphoinositide-3-kinase PIK3C3 (also known as Vps34) [328, 330,

331]. Other proteins such as UVRAG, Atg14L, PIK3R4/Vps15, Ambra1, Rubicon, or Bif-1, join this complex depending on its physiological function in autophagy or endosomal trafficking [332-334]. Becn1 and Pik3c3 mRNA and protein are expressed in human and mouse brains [317] (Fig. 10, from the Allen Mouse Brain Atlas. Seattle (WA): Allen Institute for Brain Science. Available from: http://mouse.brain-map.org [335]). Knockout mice lacking Becn1 (Becn1-/-) die during embryogenesis [325, 327].

To date, the mechanism describing how deficiency in BECN1 can cause changes in APP processing and amyloid accumulation is unknown. Here we characterize the relationship between BECN1 levels, autophagy, and APP processing in cell culture and in human brain tissue. We show that intracellular APP, APP-CTFs, and Aβ can be reduced by autophagy activation and that the BECN1-PIK3C3 complex regulates APP processing and accumulation.

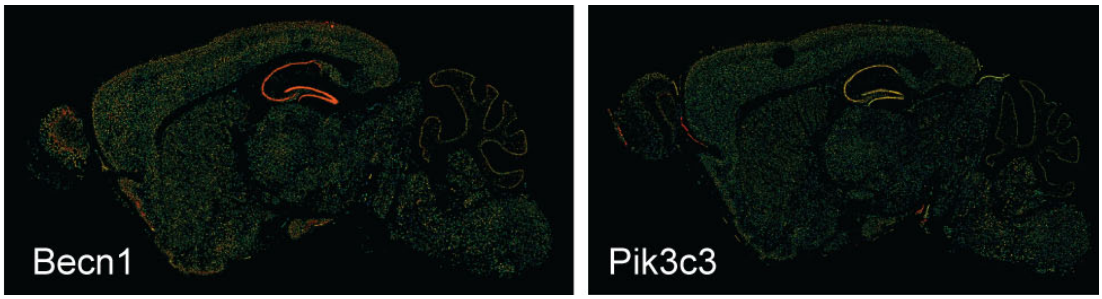

Figure 10: Expression of Becn1 and Pik3c3 in the mouse brain

Expression of Becn1 and Pik3c3 in the mouse brain, especially in the hippocampus, indicates an important function of autophagy in neuronal homeostasis (from the Allen Mouse Brain Atlas. Seattle (WA): Allen Institute for Brain Science. Available from: http://mouse.brain-map.org).

Results

Activation of autophagy promotes APP, APP-CTF, and Aβ degradation

Activation of autophagy can lead to degradation of α-synuclein, huntingtin, and poly-ubiquitinated proteins [336-339]. To test whether APP and APP-CTFs can also be reduced through this mechanism, we induced autophagy in B103 rat neuroblastoma cells which lack endogenous rat APP and are stably transfected with wildtype human APP695 (B103/hAPP) [340]. To induce autophagy we used either starvation [341] or rapamycin treatment which inhibits mTOR and activates autophagy [342] (Fig. 11A). APP and APP-CTF levels were significantly reduced in starved B103/hAPP cells and further reduced in rapamycin treated B103/hAPP cells (Figure 6B-C). Rapamycin treatment did not affect APP mRNA levels analyzed by qRT-PCR (data not shown). Furthermore, inhibition of autophagy through lenti-viral Atg5 siRNA significantly impaired starvation-induced autophagosomal clearance of APP (Fig. 12). Similar to the findings in neuronal cells, Chinese hamster ovary (CHO) cells stably transfected with human APP695 (CHO/hAPP) [289] and treated with the autophagy inducer thapsigargin [343] showed a more than 50% reduction in APP and APP-CTF levels (Fig. 11D-F) and significantly reduced levels of Aβ in the cell supernatant (Fig. 11G). Consistent with these biochemical findings, microscopy (Fig. 1H) revealed reductions both in intracellular APP (detected with CT20 antibody) and in cell surface APP (detected on non-permeabilized cells with 8E5 antibody). These findings indicate that autophagy activation can reduce levels of APP and APP metabolites.

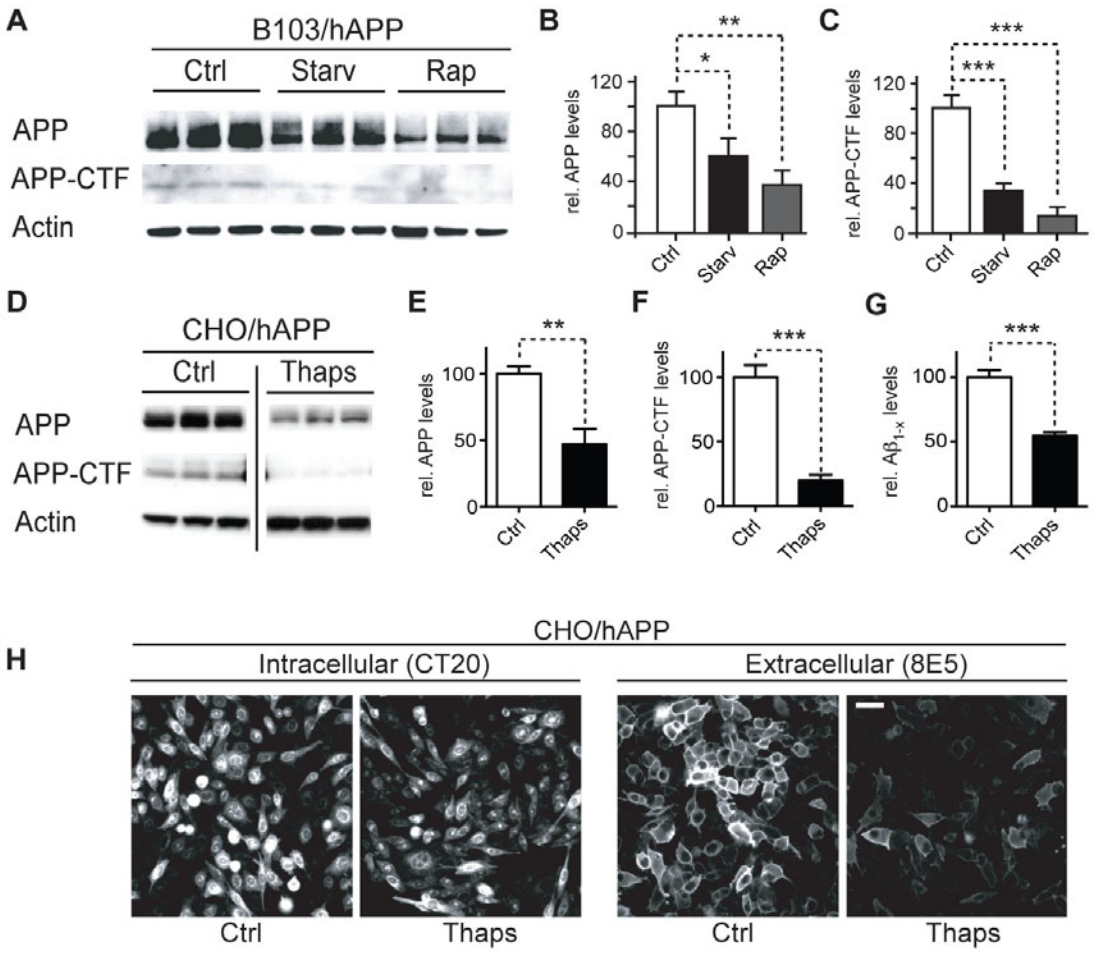

Figure 11: Activation of autophagy promotes APP, APP-CTF, and Aβ degradation

A-C. B103/hAPP cells were left untreated (Ctrl), starved for 90min in HANKS solution (Starv), or treated with 100nM rapamycin in DMEM (Rap) for 90min. Western blots (A) and quantification (B, C) of RIPA cell lysates probed with the CT15 antibody recognizing full-length APP and APP-CTFs and with an actin antibody as a control for loading. **D-F.** CHO/hAPP cells were left untreated (Ctrl) or treated for 12 hrs with 3μM thapsigargin (Thaps) in DMEM/10%FBS. Western blots (D) and quantification (E, F) of RIPA cell lysates probed with antibodies as in A. (Data from the same blot. The vertical line indicates removal of three lanes not part of this experiment.) **G.** Secretion of Aβ into the cell supernatant was measured by ELISA (12 hrs/1μM Thaps) **H**. Epifluorescence microscopy images of CHO/hAPP cells treated as in D, permeabilized with Tween and stained with antibody CT20 to label all cellular APP, or not permeabilized and stained with antibody 8E5 which recognizes the ectodomain of APP at the cell surface (scale bar represents 25μm). Bars are mean ± SEM from triplicate cultures of at least two independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by unpaired Student's t test.

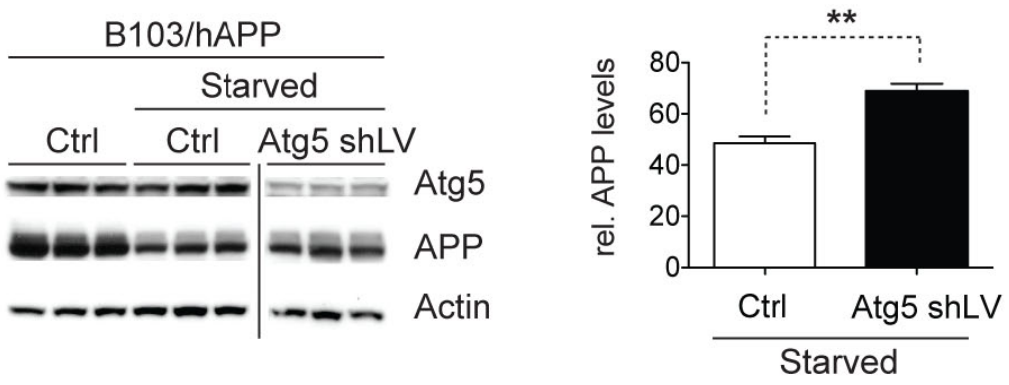

Figure 12: Effects of Atg5 knockdown on APP

Control or Atg5 shLV treated B103/hAPP cells were starved in DPBS for 4 hrs. Atg5 and APP levels were measured by Western-blotting and quantified. Atg5 reduction significantly impairs starvation induced autophagosomal APP degradation (Data from the same blot. The vertical line indicates removal of three lanes not part of this experiment.). Bars are mean ± SEM from triplicate cultures. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by unpaired Student's t test.

Becn1 knockdown increases APP, APP-like proteins, APP-CTFs, and Aβ

The reported reduction in BECN1 in AD brains [318, 319] and the increased plaque formation and neurodegeneration in Becn1+/-APP mice [318] led us to investigate whether Becn1 deficiency affected APP production, processing, or degradation in vitro. Reduction of Becn1 by siRNA in B103/hAPP cells more than doubled the levels of cellular APP and APP-CTFs (Fig. 13A and 14). Moreover, the reduced levels of Becn1 also increased the amount of secreted Aβ in the cell culture supernatant when compared to cells treated with a scrambled control siRNA (Fig. 13B). Similar results were obtained with two different siRNA sequences (data not shown). CHO/hAPP cells treated with Becn1 siRNA also showed twofold increases in APP and APP-CTFs (Fig. 13C and D). This prominent increase in APP protein in Becn1 siRNA treated cells could also be visualized and quantified with fluorescent microscopy showing increased immunoreactivity for both, C-terminal (CT20) and N-terminal (8E5) APP antibody stains (Fig. 15).

Reduced autophagic activity could be specific for APP degradation or it could also affect the processing of amyloid precursor-like proteins. Both, Amyloid precursor like protein-1 (APLP1) and Amyloid precursor like protein-2 (APLP2) are substrates of α-, γ-, and ε-secretase cleavage in a similar manner as APP, while APLP2 can also be cleaved by β-secretase [344]. APP, APLP1, and APLP2 can form homo- and heterodimers [345], making it possible that they are affected similarly by processing alterations. To test if autophagy plays a role in APLP1 and APLP2 degradation, we applied Becn1 siRNA to cell lines stably expressing human APLP1 or APLP2 [289]. Reducing Becn1 in CHO/hAPLP1 and CHO/hAPLP2 cells resulted in significant increases in APLP1 (Fig. 13E-F) and APLP2 levels, respectively (Fig. 13G-H).

To exclude the possibility that the observed cellular changes in APP, APP-CTF, and Aβ levels in response to Becn1 siRNA could be accounted for by transcriptional up-regulation of APP mRNA levels, we performed qRT-PCR on Becn1 or control siRNA treated B103/hAPP cells. APP mRNA levels decreased slightly in Becn1 siRNA treated B103/APP cells (Fig. 13J), therefore increases in APP, APP-CTFs, and Aβ cannot be attributed to increased transcription of the precursor.

To reduce potential transfection related effects, we transduced CHO/hAPP cells with a lentivirus (LV) containing either a control plasmid encoding only GFP (GFP LV) or a GFP plasmid encoding a Becn1 shRNA sequence (Becn1 shLV; different sequence from the siRNA's used above). The Becn1 shRNA LV treated cells exhibited a significant increase in APP immunofluorescence when compared to GFP LV treated control cells (Fig. 13K-L).

In the Becn1 siRNA treated cells there was a significant correlation between Aβ and APP, and between Aβ and APP-CTFs ($R=0.619$, $p=0.03$ and $R=0.698$, $p=0.01$, respectively, data not shown), suggesting that the increase in secreted Aβ was due to increased levels of its precursor, APP. The Aβ/APP ratio was similar in control and Becn1 siRNA treated B103/APP cells (data not shown), suggesting unchanged γ-secretase activity. To further test the role of γ-secretase in the observed effects, we treated control or Becn1 shLV transduced B103/hAPP cells with DAPT, a γ-secretase inhibitor. This treatment had no significant effect on the accumulation of full-length APP in control cells (Fig. 13M and 16) and did not significantly enhance the levels of full-length APP in Becn1 shLV treated cells any further. The APP-CTF levels on the other hand were significantly increased after DAPT treatment (indicating successful γ-secretase inhibition) and this effect was additive when DAPT was applied together with Becn1 shLV. These results indicate that the accumulation of APP and APP-CTFs in the Becn1 deficient cells are unlikely the result of substantial changes in γ-secretase activity.

In summary, these findings show that reduced Becn1 levels can cause intracellular accumulation of APP and its metabolites and increased secretion of Aβ. This accumulation appears not to be restricted to APP but also affects other APP-family members, suggesting that the observed accumulations are due to changes in shared processing and trafficking pathways. Finally, the buildup of APP and APP-CTFs mediated by Becn1 deficiency appears to be independent of γ-secretase activity.

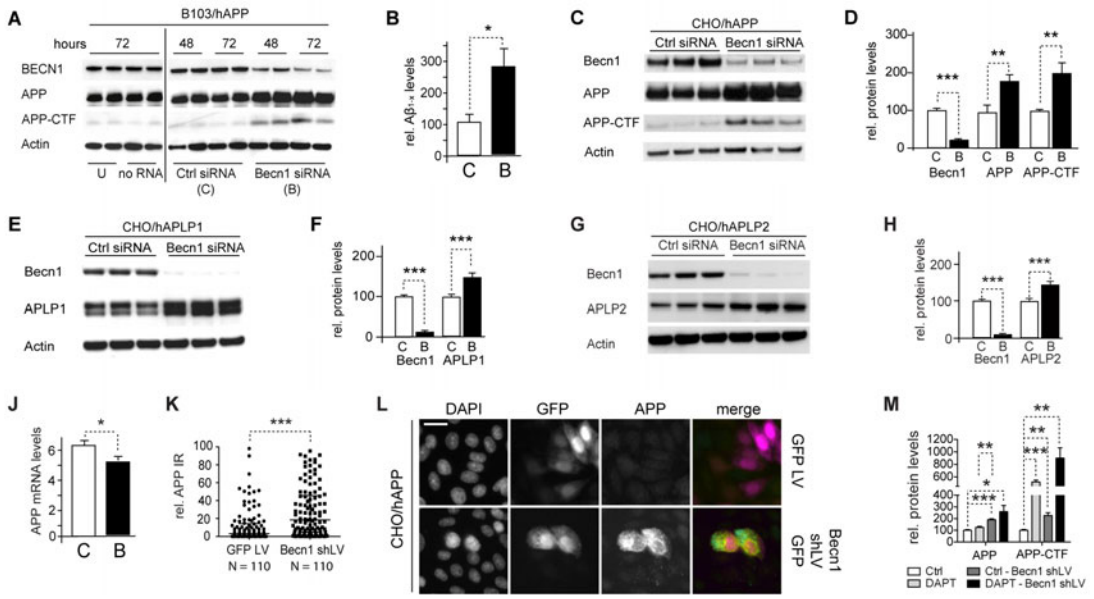

Figure 13: Becn1 knockdown increases APP, APP-like proteins, APP-CTFs, and Aβ

A-B. B103/hAPP cells were treated with Becn1 siRNA for 48-72hrs. Cells were left untreated (U), treated with transfection reagent alone (no RNA), treated with scrambled siRNA (Ctrl siRNA [C]), or treated with Becn1 siRNA (Becn1 siRNA [B]). Western blots (A) of RIPA cell lysates were probed with a Becn1 antibody, the CT15 APP antibody, and with an actin antibody as a control for loading. For quantification see Fig, S2. (Data from two blots with identical exposure times. Blot border indicated by vertical black line.) Total Aβ1-x concentrations measured by ELISA in cell culture supernatant from the same cells at 72 hrs (B). **C-D.** CHO/hAPP cells were treated with Becn1 siRNA for 48hrs. Western blots (C) and quantification (D) of RIPA cell lysates that were probed with a Becn1 antibody, the CT15 APP antibody, and with an actin antibody as a control for loading. **E-F.** CHO/APLP1 cells were treated with Becn1 siRNA for 48hrs. Western blots (E) and quantification (F) of RIPA cell lysates that were probed with a Becn1 antibody, an APLP1 antibody, and with an actin antibody as a control for loading. **G-H.** CHO/APLP2 cells were treated with Becn1 siRNA for 48hrs. Western blots (G) and quantification (H) of RIPA cell lysates that were probed with a Becn1 antibody, an APLP2 antibody, and with an actin antibody as a control for loading. **J.** Levels of APP mRNA were compared by qRT-PCR in scrambled [C] or Becn1 [B] siRNA treated B103/hAPP cells. **K-L.** CHO/hAPP cells were treated with either GFP lentivirus or Becn1 shRNA-GFP lentivirus. Quantification of the relative APP immunofluorescence (K) and epifluorescence microscopy (L) of GFP lentivirus or Becn1 shRNA-GFP lentivirus treated permeabilized CHO/hAPP cells, probed with DAPI and CT20 APP antibody (scale bar represents 10μm). **M.** Inhibition of γ-secretase activity through 100nM DAPT treatment had no significant effect on APP levels and an additive effect on APP-CTF accumulation with Becn1 shLV treatment. Bars are mean ± SEM from duplicate/triplicate cultures of at least two independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by unpaired Student's t test.

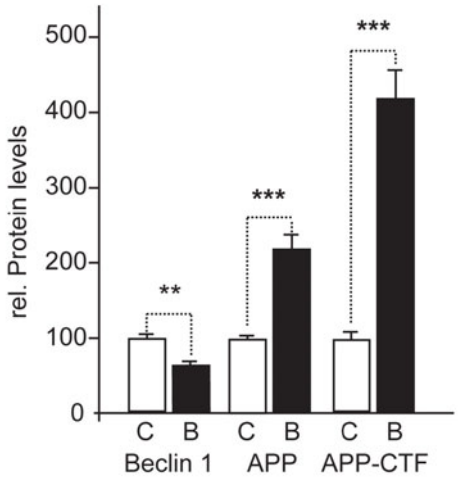

Figure 14: Quantification of B103/hAPP cells

Quantification of B103/hAPP RIPA cell lysates, 72 hrs after siRNA knockdown. All bars are mean ± SEM. Means from at least two independent experiments were compared by unpaired Student's t test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

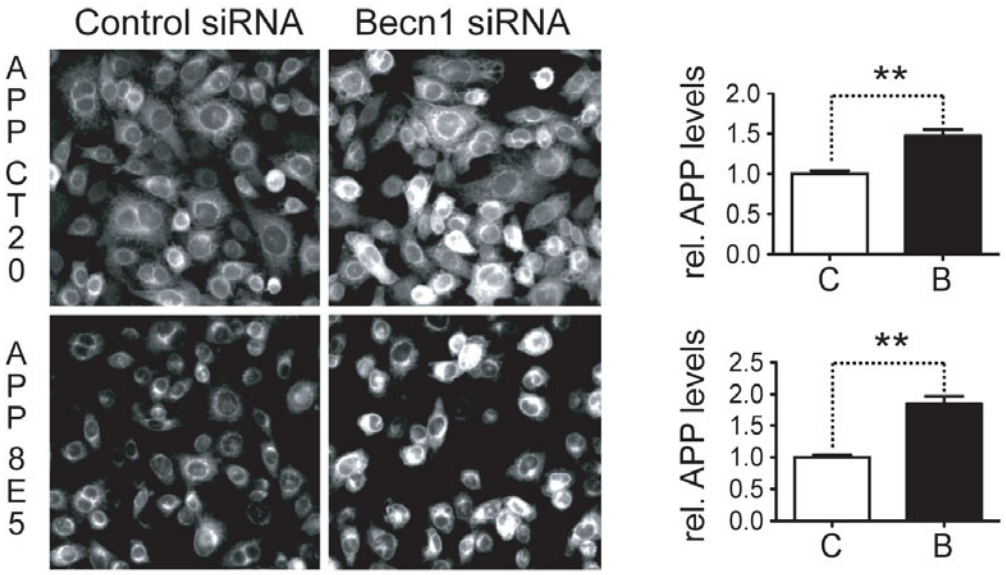

Figure 15: APP accumulation in CHO/hAPP cells after Becn1 siRNA

Epifluorescence microscopy of CHO/hAPP cells treated with Becn1 siRNA for 48 hrs. All bars are mean ± SEM. Means from at least two independent experiments were compared by unpaired Student's t test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

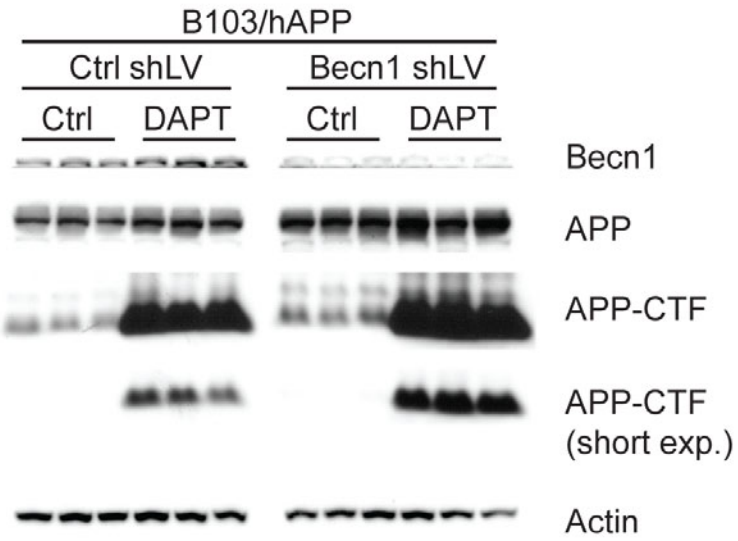

Figure 16: Effects of γ-secretase inhibitors on Becn1 shRNA

Western-blot of control or Becn1 shLV transduced B103/hAPP cells that were treated with vehicle or 100 nM DAPT for 24 hrs. An anti-luciferase shLV was used as control.

Overexpression of APP does not change Becn1 or Pik3c3 protein levels

Brains from AD patients contain less BECN1 protein and mRNA than non-demented controls [318-320]. This reduction could be caused by a disease-related (BECN1-independent) increase in APP levels. To measure the effects of APP expression on Becn1 and Pik3c3 levels, we compared B103 cells that were stably transfected with a mock vector and express no endogenous APP (B103/mock) with cells that were stably transfected with human APP (B103/hAPP; these cells express close to endogenous levels of APP [346]) (Fig. 17A). While APP and APP-CTF levels were strongly increased, Becn1 and Pik3c3 levels were unchanged in B103/hAPP cell compared to B103/mock cells (Fig. 17B).

Expression levels of APP that are chronically much higher than normal could have an effect on Becn1 and Pik3c3 levels. To measure the effects of higher than endogenous levels of APP expression on Becn1 and Pik3c3 levels, we compared CHO cells that were stably transfected with a mock vector and express only endogenous hamster APP (CHO/mock) with cells that were stably transfected with a hAPP vector and express high hAPP levels (CHO/hAPP) (Fig. 17C). Becn1 and Pik3c3 levels remained unchanged despite a strong elevation in APP and APP-CTF levels in these cells (Fig. 17D). These findings indicate that the levels of cellular APP or APP-CTF do not directly influence Becn1 and Pik3c3 levels.

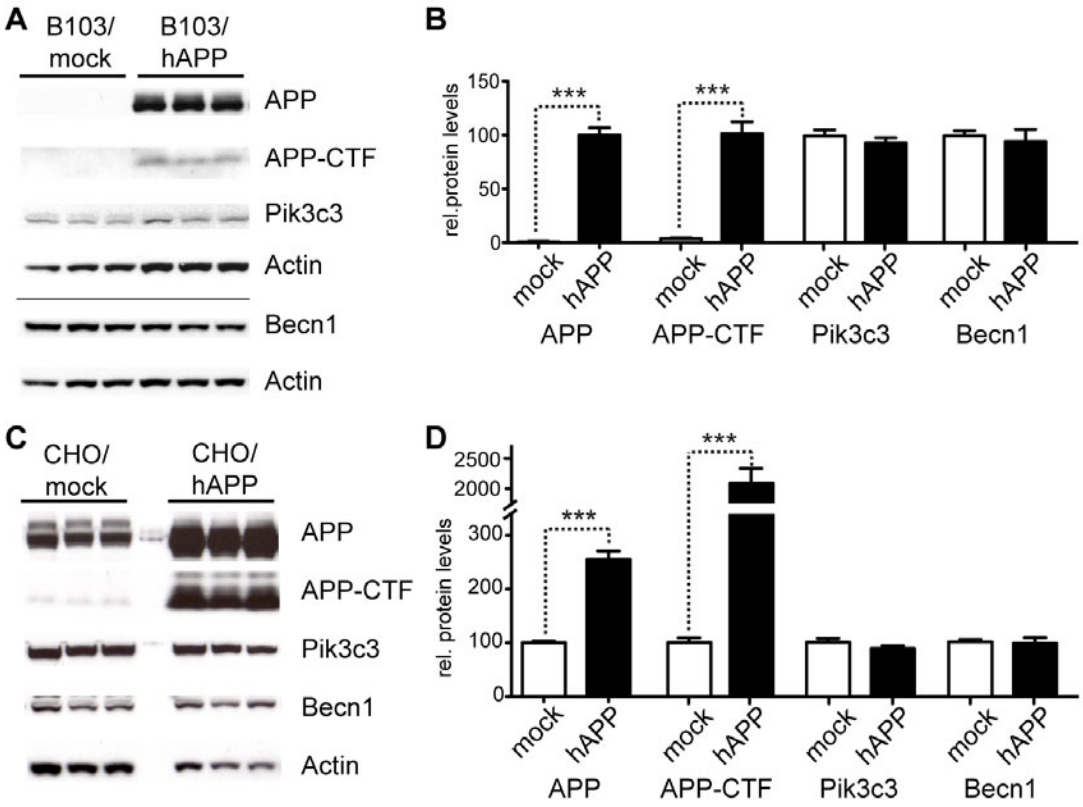

Figure 17: Overexpression of APP does not change Becn1 or Pik3c3 protein levels

A-B. B103 cells were stably transfected with an empty plasmid (mock) or a hAPP encoding plasmid. Western blots (A) and quantification (B) of RIPA cell lysates that were probed with the CT15 APP, a Becn1, and a Pik3c3 antibody. An actin antibody was used as a loading control. **C-D.** CHO cells were stably transfected with an empty plasmid (mock) or a hAPP encoding plasmid. Western blots (C) and quantification (D) of RIPA cell lysates that were probed with the CT15 APP, a Becn1, and a Pik3c3 antibody. Actin antibody was used as a loading control. Bars are mean ± SEM from triplicate cultures of at least two independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by unpaired Student's t test.

Reduction of Becn1 impairs degradation of autophagosomes and reduces Pik3c3 levels

To investigate how the observed effects of Becn1 reduction on APP-family protein processing can be linked to autophagy, we measured the levels of the autophagosomal marker LC3-II in Becn1 siRNA treated CHO/hAPP, CHO/hAPLP1, and CHO/hAPLP2 cells (Fig. 18A and data not shown). A 75% knockdown of Becn1 (Fig. 18B) caused a significant shift in the LC3-II/LC3-I ratio indicating an accumulation of autophagosomes in all three cell lines (Fig. 18C and data not shown).

Becn1 is a core component of the class 3 PI3 kinase complex [347]. Reduction of Becn1 levels could affect the stability of this complex and influence the levels of other proteins in the complex. To address this possibility we measured the levels of Pik3c3 in response to Becn1 siRNA treatment, and the levels of Becn1 in response to Pik3c3 siRNA (Fig. 18D). The cellular levels of both proteins, Becn1 and Pik3c3, appear to be linked, with the reduction of one leading to a comparable reduction of the other (Fig. 18E).

These findings led us to investigate if Pik3c3 reduction by itself can cause a change in APP processing, similar to Becn1 siRNA (Fig. 18A). While we observed a trend towards increased APP-CTF in Pik3c3 siRNA treated cells, we found no significant differences (Fig. 18F-G). These data support a central role for Becn1 in modulating APP levels.

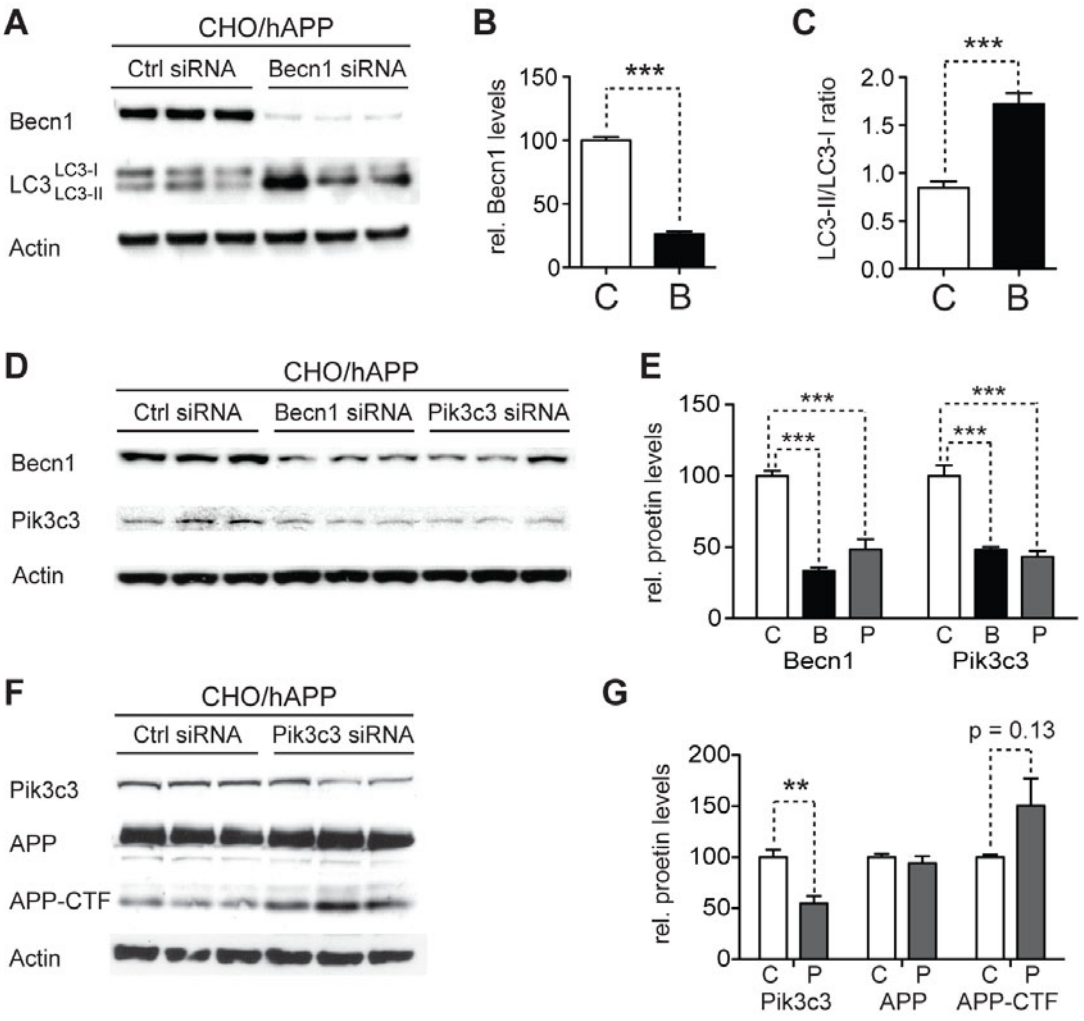

Figure 18: Reduction of Becn1 implairs degradation of autophagosomes and reduced Pik3c3 levels

A-C. CHO/hAPP cells were treated with Becn1 siRNA for 48h. Western blots (A) of RIPA cell lysates were probed with a Becn1 and LC3 antibody. An actin antibody was used as a loading control. Quantification (B) of the Becn1 band intensity and the ratio of LC3-II to LC3-I (C). **D-E.** CHO/hAPP cells were treated with Becn1 and Pik3c3 siRNA for 48h. Western blots (D) and quantification (E) of RIPA cell lysates that were probed with a Becn1 and Pik3c3 antibody. An actin antibody was used as a loading control. **F-G.** CHO/hAPP cells were treated with Pik3c3 siRNA for 48h. Western blots (F) and quantification (G) of RIPA cell lysates that were probed with the CT15 APP antibody and with an actin antibody as a control for loading. Bars are mean ± SEM from triplicate cultures of at least two independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by unpaired Student's t test.

Inhibition of autophagosome turnover leads to a reduction in Becn1 and Pik3c3 levels

BECN1 is reduced in AD brains [318-320], however the mechanism behind this reduction is unknown. One hypothesis is that impaired autophagosomal-lysosomal function may activate a negative feedback loop that subsequently reduces BECN1 levels. It is conceivable that this homeostatic loop could become activated after autophagy is impaired in order to prevent apoptosis or autophagic cell death [348]. An accumulation of autophagosomes in AD brain tissue (indicating impaired autophagosomal degradation) has been reported previously [284, 287, 349, 350]. To test this hypothesis we inhibited autophagosomal-lysosomal fusion using bafilomycin A1 (BafA) [351, 352]. BafA treatment has been shown to lead to accumulation of APP and APP-CTFs in late endosomes and multivesicular bodies (MVB) [353]. We tested if BafA treatment can also lead to APP and APP-CTF accumulation in autophagosomes and if the accumulation of these autophagosomes has any effects on Becn1 or Pik3c3 levels.

In B103/hAPP cells BafA treatment led to a strong increase in APP and APP-CTFs compared to vehicle treated cells (Fig. 19A-C). It also led to a significant accumulation of LC3-I and LC3-II (Fig. 19A), indicating a successful inhibition of autophagosomal degradation through BafA treatment. This impairment of autophagy caused a significant decrease in Becn1 (Fig. 19D, $p=0.025$) and reduced, but not significantly changed, Pik3c3 levels (Fig. 19E, $p=0.063$). Microscopy revealed that APP accumulates primarily in large vacuoles in the perinuclear space (Fig. 19F). Some APP containing vesicles stained positive for LC3 (Fig. 19F, arrowheads) but APP also accumulated in large non-LC3 positive vesicles (Fig. 19F, arrow). In vehicle treated cells only very little APP was found in LC3 positive compartments and these compartments were small in size (Fig. 19F).

Similar results were obtained for CHO/hAPP cells, where treatment with BafA also led to a reduction in Becn1 and Pik3c3 protein levels respectively (Fig. 19G, 14K-L). While CT20 full length APP immunoreactivity slightly decreased (Fig. 19H), a strong increase in APP-CTFs (Fig. 19J) and in sAPP (Fig. 19M-N) were observed. The reduction of full-length APP in CHO/hAPP cells (Fig. 19G-H) can be attributed to

elevated intracellular and extracellular cleavage of APP. The antibody used in Fig. 19G (CT20) does not recognize the N-terminal cleavage product (Fig. 19P) and enhanced APP processing will lead to an apparent reduction in intracellular (full-length) APP (CT20) levels. Accordingly, the N-terminal sAPP cleavage product accumulates both in the cell supernatant (Fig. 19M-N) and in intracellular, LysoTracker-positive vesicles (Fig. 19O) when probed with the N-terminal antibody 8E5. Total APP and its metabolites accumulate in CHO/hAPP cells, consistent with a disruption in autophagosomal degradation.

To explore alternative inhibitors of autophagosomal-lysosomal degradation and rule out unspecific BafA effects, we compared control, BafA, chloroquine (CQ), and ammonium-chloride/leupeptin (NL) treated CHO/hAPP and B103/hAPP cells (Fig. 20). We found that both CQ and NL cause an accumulation of APP and APP-CTFs, very similar to BafA. This strongly supports the hypothesis that APP is indeed processed through the autophagosomal-lysosomal pathway. Interestingly, we observe the highest reduction in Becn1 and Pik3c3 levels after BafA treatment. CQ treatment causes a slight ($p=0.06$) reduction in Becn1 and a significant reduction in Pik3c3, while NL has no significant effect on Becn1 or Pik3c3. BafA inhibits autophagosomal-lysosomal fusion, while the two other treatments primarily inhibit autolysosomal degradation. This supports the hypothesis that the accumulation of autophagosomes, rather then the inhibition of lysosomal degradation, affects Becn1 and Pik3c3 levels in a negative feedback-loop.

We conclude that inhibition of autophagosomal-lysosomal fusion through pharmacological treatments leads to accumulation of APP, APP-CTF, sAPP, and autophagosomes. This accumulation results in a reduction of Becn1 and Pik3c3 levels, possibly through a negative feedback mechanism.

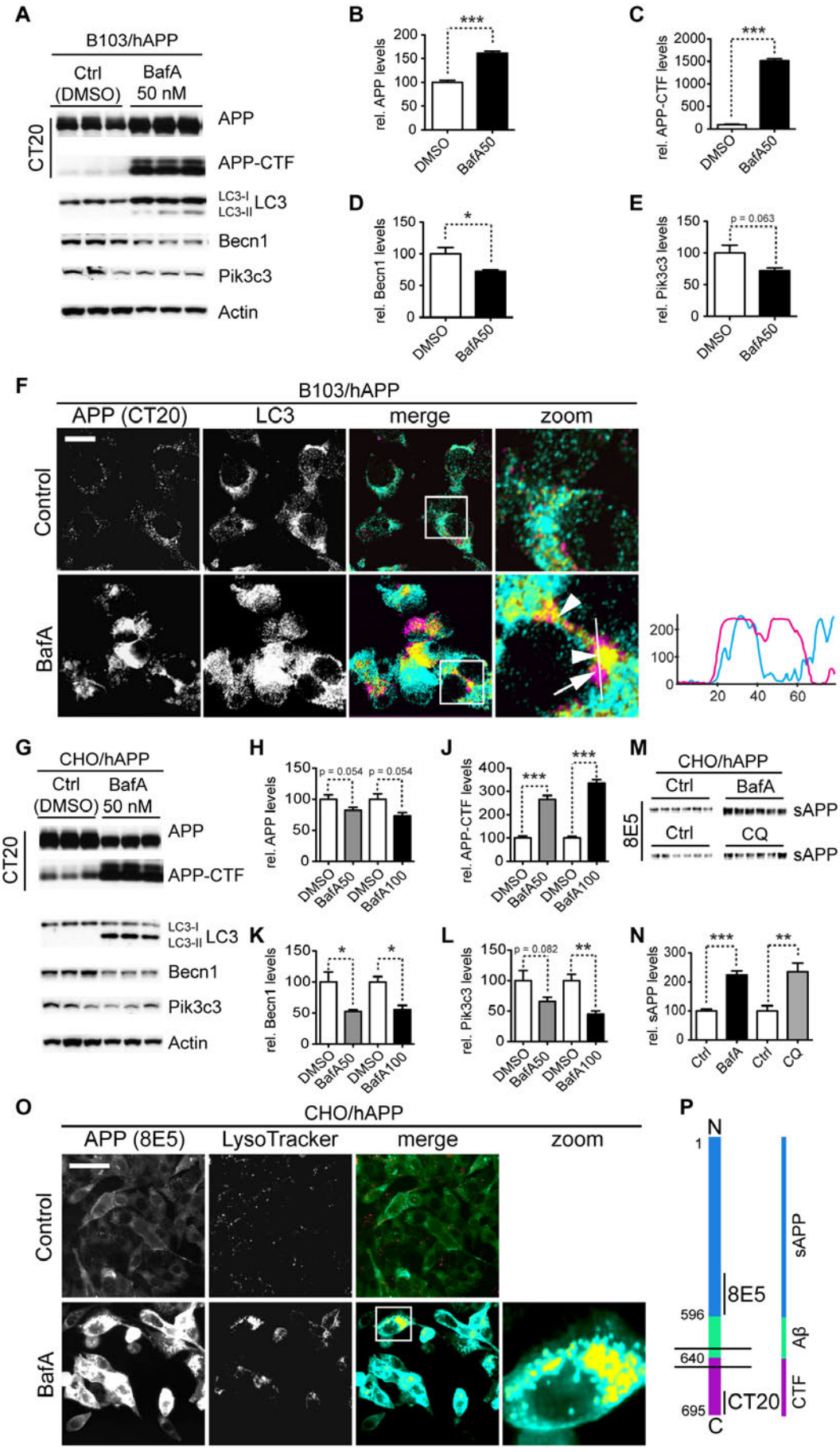
A
B103/hAPP
Ctrl (DMSO)
BafA 50 nM
CT20
APP
APP-CTF
LC3-I
LC3-II
LC3
Becn1
Pik3c3
Actin
B
rel. APP levels
DMSO
BafA50
C
rel. APP-CTF levels
D
rel. Becn1 levels
E
rel. Pik3c3 levels
p = 0.063
F
B103/hAPP
APP (CT20)
LC3
merge
zoom
Control
BafA
G
CHO/hAPP
H
p = 0.054
DMSO
BafA50
BafA100
J
rel. APP-CTF levels
K
rel. Becn1 levels
L
rel. Pik3c3 levels
p = 0.082
M
CHO/hAPP
Ctrl
BafA
CQ
8E5
sAPP
N
rel. sAPP levels
O
CHO/hAPP
APP (8E5)
LysoTracker
merge
zoom
P
N
C
8E5
CT20
sAPP
Aβ
CTF

Figure 19: Inhibition of autophagosomal turnover leads to a reduction in Becn1 and Pik3c3 levels

A-E. B103/hAPP cells were treated with vehicle (DMSO) or 50nM BafA for 24 hrs to inhibit autophagosomal degradation. Western blots (A) and quantification (B-E) of RIPA cell lysates that were probed with CT15 APP, LC3, Becn1, and Pik3c3 antibody. An actin antibody was used as a loading control. **F.** Confocal microscopy of B103/hAPP cells treated with vehicle (DMSO) or 100nM BafA for 24 hrs. Cells were stained with CT20 APP antibody (magenta) and LC3 antibody (cyan). Co-localization is indicated in yellow. Arrowheads indicate LC3 positive APP containing vesicles. The arrow indicates an APP containing LC3 negative vesicle (scale bar represents 10μm). The line indicates cross-section. Cyan line in the cross-section represents APP intensity, magenta line represents LC3 intensity (AU). **G-L.** CHO/hAPP cells were treated with vehicle (DMSO), 50nM, or 100nM BafA (WB data not shown) for 24 hrs. Western blots (G) and quantification (H-L) of RIPA cell lysates that were probed with the CT15 APP, LC3, Becn1, and Pik3c3 antibody. An actin antibody was used as a loading control. **M-N.** BafA and CQ treatment cause increased APP processing which in turn leads to elevated levels of secreted APP (sAPP) in the cell supernatant (M). This is quantified in (N). **O.** Epifluorescence microscopy of CHO/hAPP cells treated with vehicle (DMSO) or 100nM BafA for 12 hrs. Cells were stained with the 8E5 APP antibody (magenta) and LysoTracker (cyan). Co-localization is indicated in yellow (scale bar represents 25μm). **P.** Schematic representation of the APP antibody epitopes. Bars are mean ± SEM from triplicate cultures of at least two independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by unpaired Student's t test.

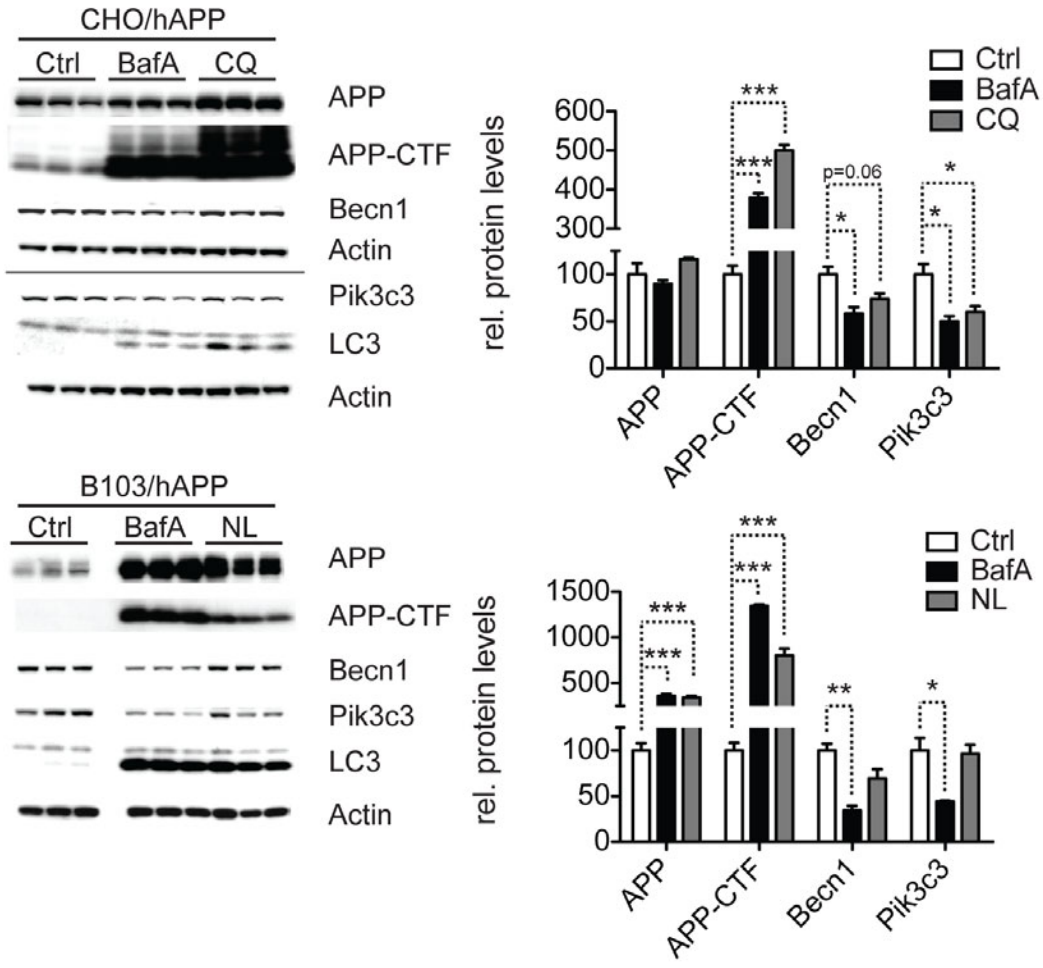

Figure 20: Pharmaceutical inhibition of autophagy in CHO/hAPP and B103/hAPP cells

Western-blots and quantification of CHO/hAPP and B103/hAPP cells treated with chloroquine (CQ) or ammoniumchloride/leupeptin (NL). Means from three independent experiments were compared by unpaired Student's t test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Becn1 overexpression reduces APP immunoreactivity

To determine if Becn1 overexpression alone can reduce APP baseline levels we transduced CHO/hAPP cells with either a Becn1 LV or a control GFP LV (Fig. 21A). While baseline Becn1 levels give only very faint immunoreactivity in fluorescent microscopy, the Becn1 LV treated cells exhibited a wide range of Becn1 expression levels (from baseline to strong overexpression, Fig. 21A). We randomly selected N=214 Becn1 LV treated cells covering the whole spectrum of Becn1 expression from both, the Becn1 (red outline) and APP channel (yellow outline), and measured their relative Becn1 and APP immunofluorescence (Fig. 21B). Next, we grouped these cells into low (<20th percentile), medium (20-80th percentile), and high (>80th percentile) Becn1 expressing cells and compared the median APP immunofluorescence in these groups (Fig. 21C). While no or low overexpression of Becn1 has no effect on APP immunoreactivity (Fig. 21C, 0-20), medium overexpression significantly reduces baseline APP levels (Fig. 21C, 20-80). Very strong, and likely non-physiological overexpression of Becn1 (Fig. 21C, 80-100) had no lowering effect on APP immunoreactivity, but led to either abnormally decreased or increased cell size, indicating that these very high levels of Becn1 expression might impair cellular homeostasis (Fig. 22A-B). This last finding is similar to very high overexpression of GFP protein and probably an artifact. For more details on the effects of GFP overexpression in the control cells, see supplemental Fig. 22B. These results suggest that moderate increases in Becn1 levels alone can have an APP-lowering effect in CHO/hAPP cells, as long as Becn1 is not expressed at extremely high and probably non-physiological levels.

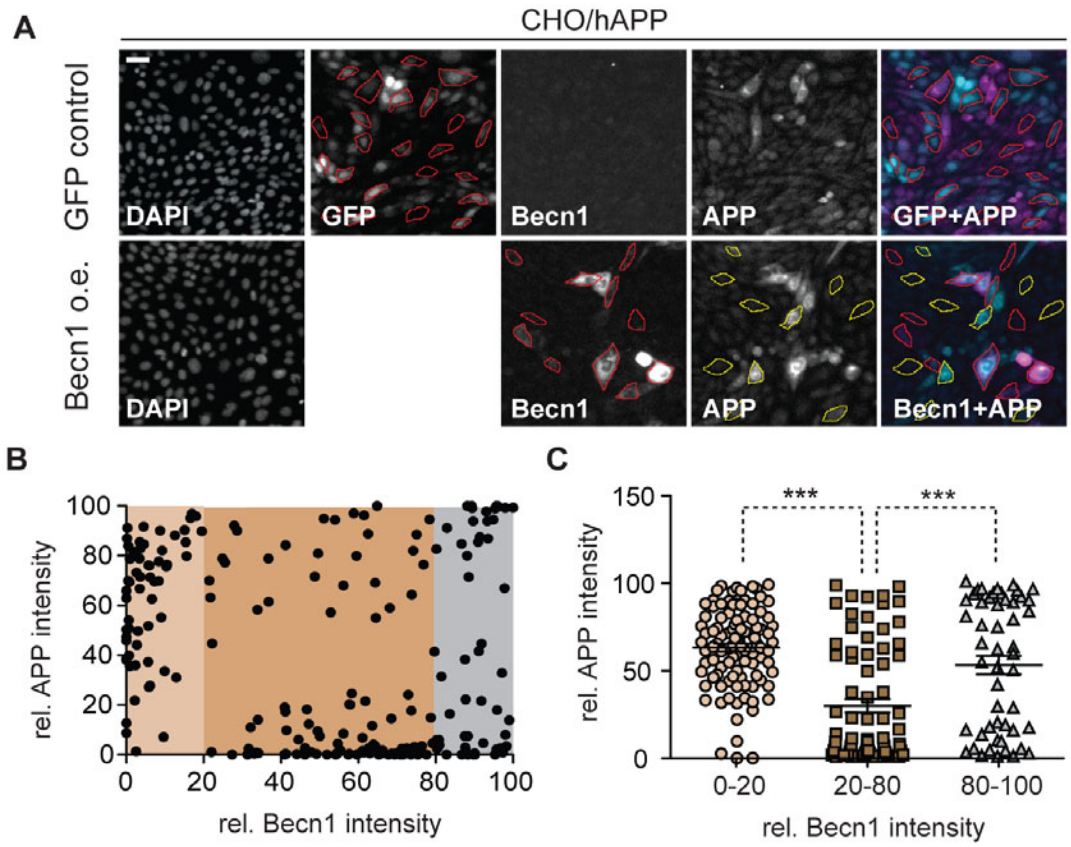

Figure 21: Becn1 overexpression reduces APP immunoreactivity

A. CHO/hAPP cells were transduced with either a GFP LV (GFP control) or a mBecn1 LV (Becn1 o.e.). Epifluorescence microscopy was performed after staining with Becn1 and APP CT15 antibodies (Scale bar represents 25µm). GFP LV transduced cells show very faint Becn1 immunoreactivity, while Becn1 LV transduced cells exhibit a range of Becn1 signal intensity. No GFP signal is present in the Becn1 LV cells. A random selection of cells (N=214) was picked from the GFP LV cells and the Becn1 LV cells. The Becn1 LV cells were randomly selected in both, the APP (yellow outline) and the Becn1 (red outline) channel. **B.** Relative immunofluorescence of the selected cells (AU). They can be divided in low, medium, and high Becn1 expressing cells. **C.** Quantification of the relative APP immunofluorescence in the three cohorts. Medium Becn1 overexpression leads to a significant reduction in APP levels. Medians were compared by Man-Whitney U test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

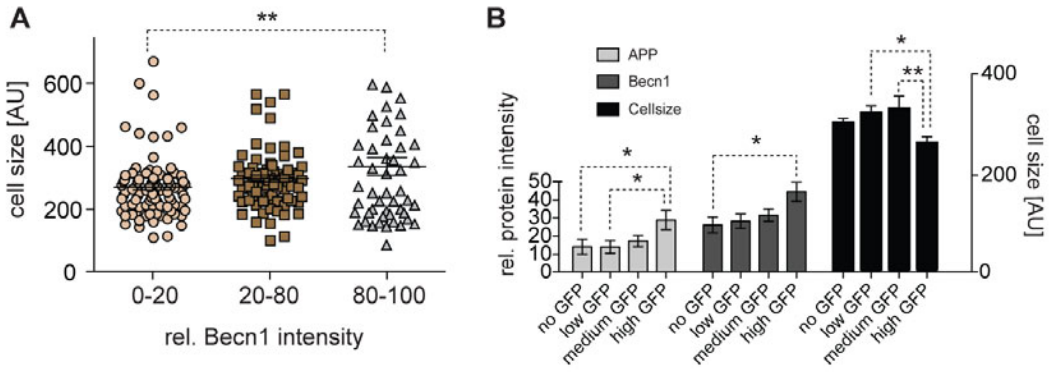

Figure 22: Control experiments for Becn1 lentiviral overexpression

A-B. Control for cell size as a measure of physilogical cell health (A). High Becn1 overexpressors exhibit either swollen or shrunken cell bodies, indicating non-physiological stress. Quantification (B) of APP, Becn1 immunofluorescence, and cell size in GFP LV control cells (N=100) show no difference in APP or Becn1 levels for low and medium overexpression of GFP. High GFP expression induces non-physiolgical conditions leading to an unspecific accumulation of Becn1 and APP and cell shrinkage. This led us to not further explore the effects of the highest Becn1 or GFP expressing cells.

AD brains have less BECN1 and PIK3C3 and more LC3

BECN1 and PIK3C3 form a complex with PI3 kinase (PI3K) activity that is necessary for the classical autophagy-activating pathway through mTOR. We and others have previously shown that BECN1 is strongly and specifically reduced in affected regions of Alzheimer's disease (AD) brains [318-320]. Heterozygous deletion of Becn1 in an AD mouse model caused increased neurodegeneration, decreased autophagy, and disruption of the lysosomal system [318]. Our cell culture findings presented above indicate that BECN1 plays an important role in APP processing and trafficking and that BECN1 reduction has effects on the PI3K complex stability and autophagosomal degradation. To understand if the observed reduction of BECN1 in AD patients is an isolated finding or if it could cause a more general disturbance of the autophagosomal system (similar to our in vitro findings) we measured multiple key proteins involved in autophagy (Fig. 23A) in human brain samples. Protein was extracted from cortical gray matter of confirmed Alzheimer disease patients (N=7, age 81±12.6 years, MMSE 4.3±6.1) and non-demented control subjects (N=10, age 77.7±8.1 years, MMSE 28.3±3.0), using a detergent containing extraction buffer (RIPA). We found PIK3C3 and, consistent with our previously published findings [318], BECN1 to be strongly reduced in AD brains when compared to non-demented age-matched controls (Fig. 23B-C). There was a highly significant correlation between the amount of BECN1 and PIK3C3 (Fig. 23D, $R=0.86$, $p<0.0001$) in agreement with their combined role in forming the autophagy inducing PI3K complex. In support of previous findings by others [287], we measured elevated levels of LC3-I and LC3-II in AD patient brains (Fig. 23E) and we observed a trend towards higher LC3-II/LC3-I ratios (Fig. 23F). In contrast, expression levels of another autophagy protein, ATG5 were unchanged in AD brains, indicating that only portions of the autophagy pathway are de-regulated in AD (Fig. 23A&E). To ensure that the observed reduction in BECN1 and PIK3C3 levels cannot be attributed to a gross decrease in neuronal mass, we measured the levels of the marker neuron-specific enolase (NSE) in lysates from AD and non-demented control brains and found no significant difference (Fig. 23G-H).

Tissue protein measurements are very sensitive to the extraction method used. To rule out extraction artifacts, we extracted a different set of human gray matter tissue (AD N=10, age 77.9±7.7 years, MMSE 4.9±5.4 / Ctrl N=10, age 77.0±8.2 years, MMSE 29.3±1.0) with sequential extraction buffers yielding a cytosolic fraction (RAB buffer) and a membrane bound fraction (RIPA buffer). BECN1, PIK3C3, and ATG5 were predominantly found in the membranous protein fraction with BECN1 and PIK3C3 again significantly reduced in AD brain tissue and ATG5 levels unchanged (Table 5, p=0.003 and p=0.019).

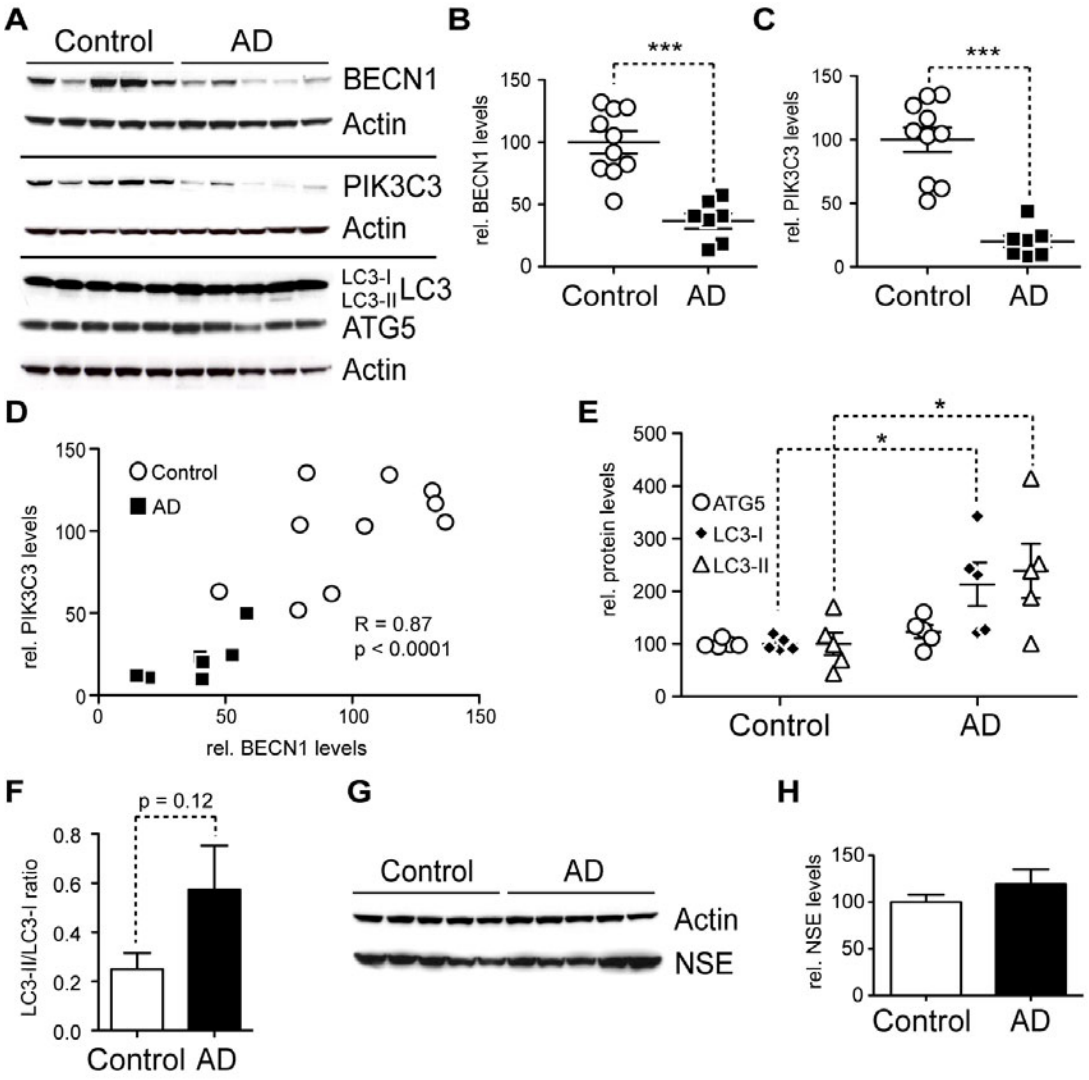

Figure 23: AD brains have less BECN1 and PIK3C3 and more LC3

A-H. Comparison of protein levels in frontal cortex (gray matter) from AD brains and age matched, non-demented, non-pathological controls. Western blots (A) and quantification (B-F) of RIPA lysates that were probed with the CT20 APP, LC3, BECN1, PIK3C3, and ATG5 antibody. An actin antibody was used as a loading control. 7 AD and 10 control cases were used. BECN1 and PIK3C3 levels were significantly reduced in AD cases (B-C). A significant linear correlation exists between BECN1 and PIK3C3 levels ($R=0.86$, $p<0.0001$), consistent with them functioning in a complex (D). While ATG5 levels appear unchanged, LC3-I and LC3-II levels are significantly elevated (E). A slight trend was detected in LC3-II/LC3-I ratio change (F). No significant difference could be detected in the levels of a neuronal marker NSE between the control and AD brains, indicating that the observed changes are not due to gross neuronal loss (G and H). All scattergrams show mean ± SEM. Means were compared by unpaired Student's t test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

		Cytosolic			Membranous		
Protein	**Disease**	**Mean**	**Std Error**	**p value**	**Mean**	**Std Error**	***p* value**
Beclin 1	Control AD	N.D.	N.D.		0.51 0.30	0.05 0.03	*****p=0.003***
ATG5	Control AD	0.37 0.19	0.11 0.03	*p=0.168*	1.79 2.10	0.14 0.19	*p=0.200*
VPS34	Control AD	N.D.	N.D.		0.76 0.41	0.10 0.07	****p=0.019***

Table 5: Detection of autophagy proteins in human AD brain tissue

Human cortical gray matter tissue was subject to sequential RAB / RIPA buffer extraction and Western blotting. Control (N=10) and AD (N=10) cases were compared regarding their relative BECN1, PIK3C3, and ATG5 levels. While BECN1 and PIK3C3 levels were significantly reduced in AD brains when compared to controls, no difference was detectable in ATG5 levels.

Discussion

Recent advances in our understanding of intracellular protein trafficking have shown that subtle alterations in the intracellular distribution of APP can have strong effects on whether it is processed by the non-amyloidogenic or amyloidogenic pathways [354]. In the current study we present data showing that autophagy is a degradative pathway that has the capacity to reduce cellular levels of APP and its metabolites when activated either physiologically (starvation), through pharmacological treatment (rapamycin or thapsigargin), or by lentiviral overproduction of Becn1. Conversely, reduced expression of Becn1 or pharmacological inhibition of autophagosomal degradation (bafilomycin A1, chloroquine, ammonium-chloride/leupeptin) led to an increase in APP and its metabolites. We conclude that Becn1 is a key regulator of cellular APP turnover.

Autophagy is a physiological mechanism that can have both beneficial and detrimental effects on neurons, depending on the circumstances [317]. Whether or not autophagy is increased in AD and whether such an increase reflects a protective attempt by cells to possibly degrade APP and Aβ, or a neurotoxic process promoting autophagic cell death has been debated. However, recent publications indicate that pharmacological stimulation of autophagy can be beneficial and reduce Aβ mediated toxicity [321-323]. In human brains and AD mouse models autophagosomes can be readily detected by electron microscopy and they appear to accumulate in swollen dystrophic neurites [284, 287, 349, 350]. This is most commonly interpreted as a sign of impaired autophagosomal degradation [284]. Furthermore APP-cleaving secretases and Aβ have been localized to autophagosomes and the accumulation of autophagosomes in AD brains and APP/PS1 mice has been interpreted as evidence that autophagy could promote AD pathology [287]. In agreement with these neuropathological findings, we observed that APP transgenic mice accumulate lysosomal and autophagosomal vesicles and that Becn1 deficiency in APP mice further promotes this pathology [318]. In addition, we confirm here that autophagy is activated in AD by detecting increased levels of LC3-II in AD brains (Fig. 23E).

However, at the same time, we and others found BECN1 [318-320] and in the current study PIK3C3, reduced in AD tissue (Fig. 23B-C and Table 5), suggesting an impairment in the initiation of autophagy. To reconcile these apparently contradictory findings we postulate a dual role for BECN1: one in autophagy initiation, in a complex with PIK3C3, and another in autophagosomal flux and degradation, potentially in a complex with other proteins (Fig. 24). BECN1 has been shown to execute various functions depending on its binding partners and siRNA mediated knockdown of Becn1 has been demonstrated to impair autophagosomal degradation and cause LC3-II accumulation at the same time [334], similar to our findings (Fig. 18A&C). Different experimental models therefore appear to reflect different aspects of this dual role. On one hand Becn1 heterozygous knockout mice have reduced autophagosomes and reduced LC3-II [318], reflecting impaired autophagosomal initiation. On the other hand, Becn1 siRNA treated CHO cells have increased LC3-II levels (Fig. 18A&C). This reflects impaired autophagosomal degradation similar to the pathology observed in AD brains. In either role, reducing BECN1 leads to pathological accumulations of APP and its metabolites through impaired autophagy (Fig. 24).

Our observations regarding the effect of Becn1 siRNA on autophagy confirm that the BECN1-PI3K complex has a crucial role during the initiation stages of autophagy, but they also show that reduction of Becn1 protein levels can have effects on the availability of PIK3C3 and vice versa (Fig. 18D-G). This is supported by recent findings of similar Becn1 reduction after Pik3c3 knockdown [355], although a reduction of Pik3c3 after Becn1 knockdown had not been reported. It will be important do determine if other proteins that are part of the BECN1 complex (Atg14L, PIK3R4, UVRAG, Ambra1, Vps15, Bif-1, or Rubicon) are also reduced in AD or in response to BECN1 reduction, respectively, as this could help explain the (possibly indirect) effects of BECN1 reduction on autophagosomal degradation (Fig. 24). Atg14L and UVRAG are especially interesting candidates for this since both proteins have been shown to determine the stability of Becn1 [355] and Atg14L knockdown causes LC3-II accumulation similar to Becn1 siRNA [334]. Further studies will be needed to precisely determine the role of Becn1 and its binding partners in the modulation of autophagic flux and autophagosomal maturation. Nevertheless, with respect to APP metabolism, Becn1 seems to play central role, since

Pik3c3 siRNA does not cause a comparable effect on APP accumulation in our in vitro system (Fig. 18F-G).

Aiming to validate our cell culture findings in AD brain tissue, we measured the levels of PIK3C3, LC3, and ATG5. We found a reduction not only of BECN1, but also of its binding partner PIK3C3, similar to our cell culture model using Becn1 siRNA (Fig. 23C). Importantly, we observed a linear relationship between the levels of these two proteins (Fig. 23D) similar to the cell culture studies, supporting the idea that reduction in one of the proteins can cause instability of the PI3K complex and increased degradation or reduced production of the respective binding partner. The levels of ATG5 on the other hand were not significantly changed, arguing for a specific disruption of the PI3K complex in AD rather than a general deficiency in the autophagy pathway and signaling cascade. The reduction in PI3K complex components appears to have an inhibiting effect on the degradation rate of autophagosomes, which may lead to the build-up of LC3 protein in brain tissue and a subsequent accumulation of APP and its metabolites.

Which comes first, BECN1/PIK3C3 deficiency or APP accumulation? While the data from the transgenic mice suggested an important role of Becn1 levels on AD pathology [318], it was unclear if this effect is upstream of APP pathology or partially a consequence of disrupted intracellular trafficking due to APP overexpression. Our cell culture data from wildtype human APP overexpressing cell lines demonstrate now that APP overexpression alone does not lead to reduced Becn1 and Pik3c3 levels, leaving the possibility that autophagy disturbance could precede APP/Aβ pathology in vivo, and that the observed reduction of BECN1 in human AD brain tissue is unlikely due to elevated levels of APP or its metabolites alone. Instead, it suggests that an escalating disturbance in autophagosomal flux and degradation could have a negative impact on BECN1 and/or PIK3C3 levels, presumably via a negative feedback loop, downregulating autophagy induction in response to abundant autophagosome numbers (Fig. 24). Such a loop could be in place to prevent an uncontrolled run-off activation of autophagy with potentially disastrous consequences for the cell. In support of such a model, pharmacological inhibition of autophagosomal-lysosomal fusion using BafA causes a strong accumulation of autophagosomes, accompanied with APP and APP-CTF accumulation in those autophagosomes and other intracellular vesicles. This in turn leads to decreased levels of

Becn1 and, at least under some treatment conditions, of Pik3c3 (Fig. 19L). These findings suggest that disturbances in autophagosome turnover can further inhibit proper induction and execution of autophagy, potentially worsening the cellular capacity to degrade APP and its metabolites.

The initial factor that impairs autophagy in AD and reduces BECN1/PIK3C3 still has to be determined. This study however identifies autophagy as an important degradative pathway for APP and suggests that once autophagosomal flux and turnover is impaired an escalating cycle of APP/APP-CTF/Aβ accumulation and further reduced initiation of autophagy occurs (Fig. 24). Future studies of conditional knockout mice for proteins that are part of the BECN1-PI3K complex will help to deepen our understanding of the sequence of events that lead to the disruption of autophagy and how this contributes to the development of AD pathology.

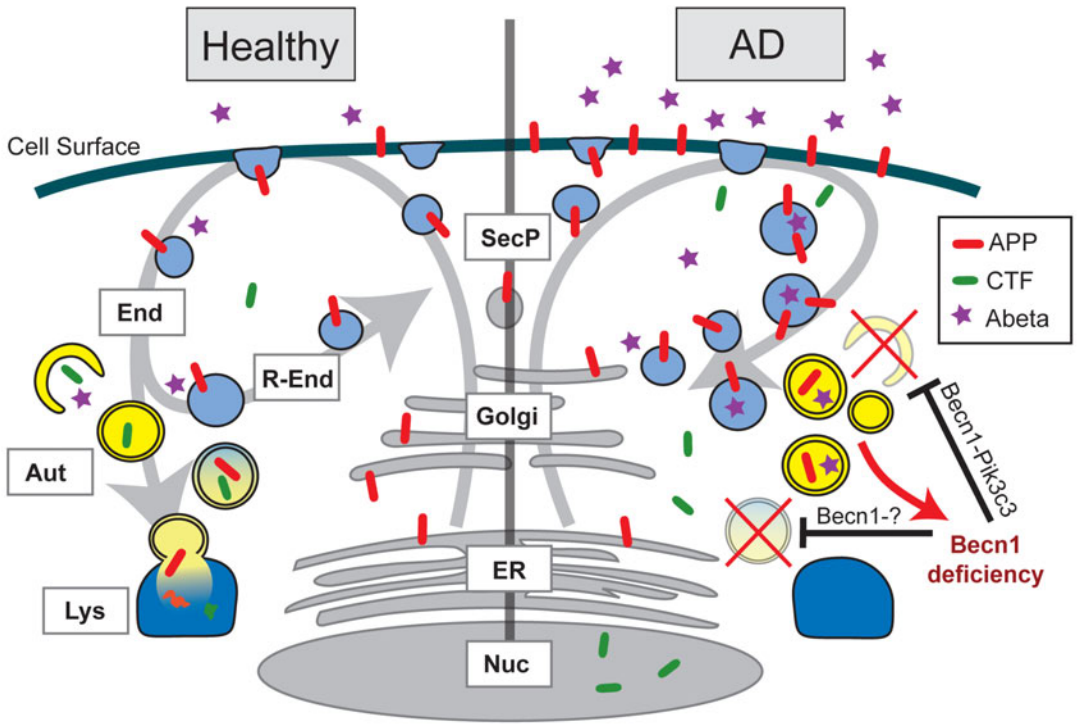

Figure 24: Effects of BECN1 deficiency in AD

In healthy individuals, APP is transcribed in the endoplasmatic reticulum (ER, grey), modified in the golgi network (Golgi, grey) and then shuttled to the cell surface through the secretory pathway (SecP, grey). The cell takes up APP through endocytosis (End, light blue). From here, APP can either be degraded via autophagy (Aut, yellow) and the lysosomes (Lys, dark blue) or APP can be recycled via the recycling endosomes (R-End, light blue) and enter the cycle again. In AD brains and Becn1 deficient cells BECN1 deficiency impairs both induction of autophagy (through the complex with PIK3C3) and autophagosomal degradation (potentially through a complex with an unknown binding partner). APP containing vesicles (endosomes, autophagosomes, and others) build up inside the cell. APP is increasingly cleaved by secretases and large amounts of APP-CTF and Aβ are being released, causing neurotoxic events. The disruption of autophagosomal degradation includes an increasing accumulation of autophagosomes. This accumulation can further inhibit autophagy and BECN1 expression (red arrow), worsening the reduction in APP turnover and degradation.

Material and methods

Cell culture. B103/hAPPwt rat neuroblastoma cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad/CA, USA) containing 10% (v/v) fetal bovine serum and 5% (v/v) horse serum at 37°C with 5% CO2. Selection was maintained with 400µg/ml geneticin/G418 (Invitrogen). CHO/hAPPwt, APLP1 and APLP2 hamster ovary cells were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum and selection maintained using 500µg/ml hygromycin (Invitrogen).

Drug treatments/Starvation. Cells were washed once in warm PBS and covered with fresh medium containing drugs at the indicated concentrations / for the indicated periods: 100nM rapamycin for 90min (Calbiochem, San Diego/CA, USA); 3µM/1µM thapsigargin for 12hrs (Calbiochem, San Diego/CA, USA); 50nM/100nM bafilomycin A1 for 24hrs (LC Laboratories, Woburn/MA, USA); 20mM ammoniumchloride and 10µg/ml leupeptin (Sigma-Aldrich, St. Louis/MO, USA) for 24 hrs; 30µg/ml chloroquine (Sigma-Aldrich, St. Louis/MO, USA) for 16 hrs; 100nM DAPT (Sigma-Aldrich, St. Louis/MO, USA) for 24 hrs. Control cells were treated with the corresponding amount of vehicle. At the end of the incubation period the cells were harvested or imaged as described below. For starvation experiments, the cells were washed twice in warm PBS and then incubated for 90min in HANKS or 4 hrs in DPBS (Invitrogen, Carlsbad/CA, USA) solution.

Antibodies. The following primary antibodies were used: BECN1 antibody #612112 1:500 (BD Biosciences, San Jose/CA, USA); LC3 antibody #PD014 1:500 WB / 1:200 IHC (MBL International, Woburn/MA, USA); PIK3C3 antibody #38-2100 1:500 (Zymed, San Francisco/CA, USA); Actin antibody #A-5060 1:10000 (Sigma-Aldrich, St. Louis/MO, USA); Atg5 antibody 1:2000 (gift from Dr. Noburo Mizushima, Tokyo Metropolitan Institute of Medical Science, Japan); N-terminal APP 8E5 antibody 1:5000(WB)/1:200(IHC) (gift from Elan, South San Francisco/CA, USA); C-terminal APP CT15/CT20 antibody 1:1000(WB)/1:200(IHC) (gift from Dr. Todd Golde, Mayo Clinic, Jacksonville/FL, USA); APLP1 antibody #171615 1:5000 (Calbiochem,

SanDiego/CA, USA); APLP2 antibody #171616 1:5000 (Calbiochem, SanDiego/CA, USA); NSE antibody # MS-171-P1 1:1000 (LabVision, Fremont/C, USA)

RNAi and LV particles. B103/hAPPwt, CHO/hAPP, CHO/hAPLP1 or CHO/hAPLP2 cells were transfected with 40nM synthetic Stealth siRNA (Invitrogen) using Lipofectamine 2000 (Invitrogen, Carlsbad/CA, USA) following manufacturers instructions. The siRNA sequences used were as follows:

BECN1: CCCAGCCAGGAUGAUGUCUACAGAA and GCUAACUCAGGAGAGGAGCCAUUUA.

PIK3C3: CAUUGCCGUUAGAGCCACAGGUGAA and GGAGCCUACCAAGAAGGAUAGUCAA.

Control: GCUACUCGAGGAGGAACCGUAAUUA.

For LV experiments the cells were transduced with virus containing a shRNA plasmid against mBecn1 targeting the nucleotides 405-423 (or against mAtg5) and a GFP-marker. The control LV contained the empty plasmid with only the GFP-marker. For the Becn1 overexpression experiments, the LV particles contained a plasmid encoding mBecn1 alone. Cells were transduced in 96 well plates at 50 MOI in the presence of polybrenen (8μg/ml). Successful transduction was monitored by GFP expression. Following the transduction and expansion the cells were stained or lysed after 36-96 hrs. All LV particles were provided by Dr. E. Masliah, University of California San Diego/CA, USA.

Protein extraction. Samples from human brain tissue were homogenized in extraction buffer (see below) by pulsed ultrasonification at 4ºC, followed by centrifugation at 10000xg at 4ºC for 30min. The resulting supernatant was used for protein analysis. For cell culture samples, cells were washed once with PBS (Invitrogen, Carlsbad/CA, USA) and scraped off the plate. After a brief centrifugation at 4500xg at 4ºC for 5min, the cell pellets were re-suspended in extraction buffer and homogenized by pipetting, three freeze-thaw cycles on dry ice, and 30min incubation on ice. Insoluble particles were pelleted by centrifugation with 10000xg at 4ºC and the resulting supernatant was used for analysis. Proteins were extracted using RIPA buffer (50mM HCl, 150 mM NaCl, 5mM EDTA, 1mM EGTA, 20mM NaF, 1mM Na2VO4, 1% NP40, 0.5% Sodium deoxycholate, 1mM PMSF, 0.1% SDS, pH 7.4) containing 2x Complete proteinase inhibitor (Roche Diagnostics, Mannheim, Germany). When sequential extraction was performed the samples were first extracted with detergent free RAB buffer (MES 100mM, EGTA 1mM, MgSO4 0.5mM, NaCl 750mM, NaF 20mM, EDTA

100mM, Na2VO4 1mM, PMSF 1mM, pH 6.5) containing 2x Complete proteinase inhibitor (Roche Diagnostics, Mannheim, Germany) followed by a RIPA extraction of the pellet.

Western blotting. A pre-cast bis-tris gel (Invitrogen, Carlsbad/CA, USA) and a MOPS buffer system were used and standard Western blotting protocols were followed. 10-20µg of total protein were loaded. Gels were transferred onto 0.4µm nitro-cellulose membranes (BioRad, Hercules/CA, USA) and pre-incubated with MISER antibody extender solution (Pierce, Rockford/IL, USA). Total protein was measured with the BCA Protein Assay Kit (Thermo Scientific, Rockford/IL, USA) against a BSA standard. Antigen specific primary antibodies were incubated 1hr at room temperature or overnight at 4ºC and detected with species-specific horseradish-peroxidase coupled secondary antibodies. The ECL Western Blotting Detection kit (GE Healthcare, Buckinghamshire, UK) was used to obtain a chemiluminescence signal, which was then detected using Amersham Hyperfilm ECL (GE Healthcare, Buckinghamshire, UK) at varying exposure times to obtain images with optimal density within the dynamic range of the film (30s-30min). The films were digitalized at 300dpi and arranged in Photoshop CS4 (Adobe, San Jose/CA, USA) as TIFF files. Band quantification was performed using ImageJ software (NIH, Bethesda/MD, USA). Bands of interest were normalized to a loading control using Microsoft Excel 2008 (Microsoft Corporation, Seattle/WA, USA) and statistical analysis and graph production was performed in Prism5 (GraphPad Software, La Jolla/CA, USA).

Aβ ELISA. ELISAs were performed as described [318] using antibody 266 (Aβ13-28, Elan) as the capture antibody for total Aβ, or antibody 21F12 (Aβ37-42, Elan, South San Francisco/CA, USA) as the capture antibody for Aβx-42 and biotinylated 3D6 (Aβ1-5, Elan, South San Francisco/CA, USA) as the detection antibody. After incubation with the secondary antibody, samples were incubated with avidin-HRP and the signal developed using "1-step slow TMB ELISA solution" (Thermo Scientific, Rockford/IL, USA). For the thapsigargin-treatment experiments, we used a MesoScale detection system (MesoScale, Gaithersburg/MD, USA) and followed the standard protocol with the above antibodies.

Fluorescence Microscopy. For epifluorescence microscopy cells were grown in 12 well tissue culture plates (Becton Dickinson Labware, Franklin Lakes/NJ, USA). They were washed with ice-cold PBS and then fixed in cold 4% PFA in phosphate buffer for 5min at 4ºC followed by 10min at RT. Cells were then washed three times with ice-cold PBS and PFA fluorescence was quenched with ice-cold 100mM tris-HCl pH 8.0 for 3min. The cells were then either washed three times in ice-cold PBS and stained (for cell surface APP) or permeabilized with ice-cold methanol for 6 min at -20ºC, followed by three washes of ice-cold PBS and staining (for intracellular proteins). Staining was performed by blocking cells in blocking buffer (4% donkey serum, 2% bovine serum albumin, 2% fetal calf serum, 0.2% fish gelatin in PBS) for 1hr at RT. Primary antibodies in blocking buffer were applied to the cells for 1hr at RT, followed by three 5min washes in PBS. Fluorescent secondary antibodies in blocking buffer were added and incubated for 1hr at RT, followed by three washes in PBS for 5min. Cells were visualized with a Olympus IX71 (Olympus, Center Valley/PA, USA) microscope with a CoolSnapHQ camera (Roper Scientific, Tucson/AZ, USA). Image analysis was done with MetaMorph 6.1r6 (Molecular Devices, Sunnyvale/CA, USA). For confocal microscopy cells were grown on glass cover slips (Fisher Scientific, Hampton/NH, USA) in 12 well plates, and fixed and stained similar to the epifluorescence protocol above. The glass coverslips were mounted in MoWiol and visualized on a Zeiss LSM 510 confocal microscope (Zeiss, Oberkochen, Germany). Image analysis was done with the Zeiss LSM software package.

RT-PCR. RNA was extracted from B103/hAPP cells (n = 5 wells per treatment group) using Trizol and cleaned using RNAeasy mini kit (Qiagen, Valencia/CA, USA). cDNA was synthesized using TaqMan reverse transcriptase (Applied Biosystems, Branchburg/NJ, USA). cDNA was amplified in triplicate on a MyiQ single color real time PCR detection system using primers specific to human APP (F 5'CACCAATGTGGTAGAAGCCAACC3', R 5'GGGCAACACACAAACTCTACCCC3'), and GAPDH (F 5'TGCGACTTCAACAGCAACTC3', R 5'ATGTAGGCCATGAGGTCCAC3'). The PCR cycle was as follows: 10 min at 95°C, 45 x (30 s at 95°C, 2 min at 60°C, 30 s at 72°C). Cycle numbers for amplification to exceed a pre-set threshold were used to determine the APP mRNA copy number. cDNA prepared without reverse transcriptase was amplified to ensure no genomic DNA contamination of the samples.

Human brain tissue. Brain tissues from confirmed AD and age-matched, non-demented, non-pathological controls were obtained from ADRC at the University of California - San Diego, The Institute for Brain Aging and Dementia Tissue Repository at the University of California - Irvine, and Stanford Brain Bank at Stanford University in strict accordance with all ethical and institutional guidelines. Cortical mid-frontal gray matter tissue was cut out of frozen tissue blocks and subject to protein extraction as described above.

Statistics. Human brain tissue protein data consists of one-sample measurements for each case. The data was normalized against actin and differences calculated using Student's unpaired t-test. Cell culture western blots experiments were conducted in two to three independent experiments consisting of duplicates or triplicates. All measurements were normalized by actin intensities and then calculated as levels relative to control conditions. Differences between treatment conditions were established using Student's unpaired t-test (with two conditions) or one-way ANOVA followed by Dunnett's test for multiple comparisons (for more than two conditions). For fluorescence microscopy, stains were done in independent duplicates and representative images chosen.

Acknowledgements

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Chapter 3: The Beclin 1 Complex in Autophagy and Alzheimer Disease

The article "***Beclin 1 Complex in Autophagy and Alzheimer Disease***" by Philipp Jaeger and Tony Wyss-Coray can be found at the publisher's website following the link below. The original article had to be removed from the online version of this thesis to comply with the publisher's copyright guidelines.

Arch Neurol. 2010;67(10):1181-1184.
http://dx.doi.org/10.1001/archneurol.2010.258

Chapter 4: Plasma Protein Changes in Sporadic Alzheimer Disease Patients are Linked to Cognitive Decline and Identify Disease-related Pathways

Summary

Cells, tissues, and organs communicate through patterns of secreted signaling proteins that circulate in the bloodstream of an organism. Systemic changes, for example during disease, alter this cellular communication, and monitoring these changes could identify pathological pathway alterations. To access this valuable information, we measured more than 700 secreted signaling proteins using an antibody-base microarray with plasma samples from sporadic Alzheimer Disease patients and non-demented controls. This is, to our knowledge, the first large-scale analysis of systemically secreted signaling proteins from individual patients with any neurodegenerative disease. We used a combination of different statistical methods such as relative protein levels, regression analysis, protein-protein connectivity analysis, publication record data mining, and gene-ontology analysis to identify disease relevant pathways. We found significant changes in the levels and patterns of systemic signaling proteins during the disease. We identified changes in pathways involving known players of Alzheimer Disease (such as APP, TNF, IDE, ADAM17, CLU, and APOE) and found strong support for previously implicated pathways (such as TGFβ and TNFα signaling). Confirming their potential role in Alzheimer Disease pathology, our unbiased approach identified factors that are highly enriched for correlation with an independent clinical measure of cognitive decline. Finally, our functional and statistical analysis of the secreted signaling proteins identified a number of novel disease related pathways and proteins, and provides a unique insight in the regulatory pathways potentially involved in the pathology of sporadic Alzheimer Disease.

Background

Measuring changes in the qualitative or quantitative levels of proteins in cells or tissue has been the backbone of molecular and clinical research for many decades. While changes in single proteins are important discoveries, researchers now routinely measure upstream or downstream signaling cascades influencing their protein-of-interest, or proteins that physically interact with it in a biological complex. However, the overall number of proteins investigated for any given scientific question is usually small (<50), compared to the whole proteome (>50'000). This is in strong contrast to DNA or RNA based array technologies, where now 10'000s of sequences are probed routinely. To understand complex diseases however, it will be important to integrate genomics and proteomics data to obtain better understanding of disease systems biology [409, 410]. Here, we present a novel array analysis and data integration method based on hundreds of signaling proteins, measured across over 100 human samples, and linked to a large set of biological databases.

New technologies have been developed in the past two decades to qualitatively and quantitatively explore the proteome on cell, tissue, and organism levels [411]. Yet the vast number of proteins and protein-modifications, the dynamic nature of the proteome, and the huge dynamic range of protein concentrations makes attempts for a complete assessment of the proteome a very challenging task with only limited success [412]. Un-biased mass-spectroscopy techniques on tissue and pooled blood samples have been applied in the cancer field, but sensitivity and throughput limitations remain [413]. Antibody microarrays are an alternative to mass-spectroscopy based proteomics [414]. There, hundreds of different antibodies with known specificities are printed in micrometer-sized spots at known locations onto an absorptive surface (Fig. 27). Antibody microarrays allow for relatively simple and fast screening of biological samples for a large number of proteins simultaneously, but rely on the ability of the antibodies used to detect their respective epitopes in the sample solution. Antibody specificity, availability, and price thus currently limit the number of proteins that can be measured to less then 1'000.

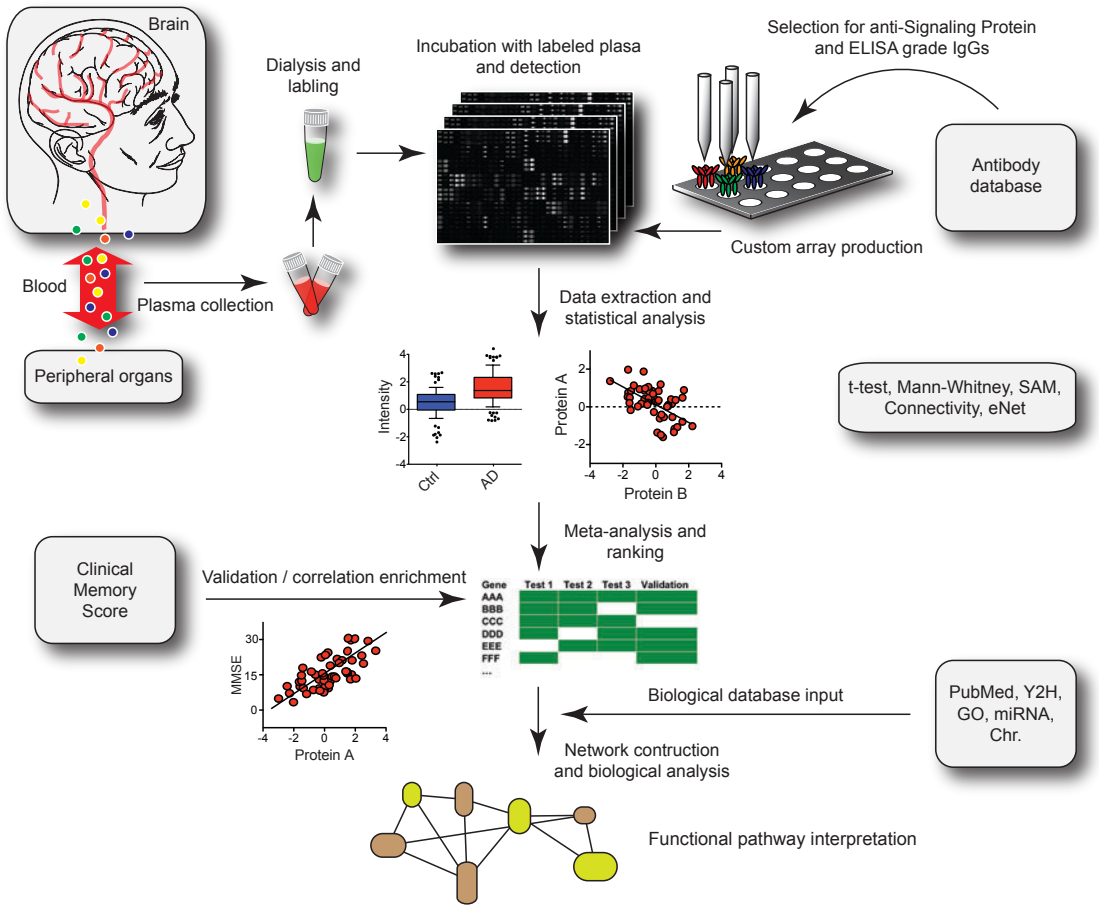

Figure 27: Experimental Design of the microarray production and analysis

Blood carries soluble proteins throughout the body. These proteins contain information about the status of different body-tissues such as the brain. Blood samples were prepared and all plasma proteins were labeled with a biotin-tag. Custom microarrays were printed with selected antibodies against signaling proteins and incubated with the labeled plasma samples. A variety of statistical tools were applied to extract altered proteins, both for differential levels and changes in connectivity patterns. These hits were then validated through enrichment with memory score correlations. Finally, biological databases were interrogated to interpret biological relationships and functions of the identified proteins.

To maximize the amount of biological information that can be extracted through antibody microarrays we applied a focused, but un-biased approach: Instead of measuring every available protein in our samples we focused our antibody selection on a few hundred proteins with known roles in cellular communication such as chemokines, cytokines, growth factors, secreted receptors, etc. (Tab. 7). These secreted signaling proteins represent a natural bottleneck for biological information: Changes, such as during a disease, in complex intracellular processes lead to changes in the secretion patterns of the secreted signaling proteins, which in turn lead to wide-ranging changes in pathways throughout the organism. While current technology does not allow us to measure the proteome as a whole, we can measure the hundreds of secreted signaling factors and how their levels change during disease. By integrating these measurements with data from protein-protein interaction studies, gene ontology, and known biological pathways it is possible to then determine both, pathways that are implied in the production of the secreted signaling factors and pathways that might be affected by them.

For many decades scientists have explored the levels of a wide range of biologically active molecules such as DNA, RNA, lipids, antibodies, or proteins in body fluids or tissues from patients with specific diseases. The major benefit of body fluids over tissue samples is that they are relatively accessible, often without the need for surgical intervention, and thus can be sampled more frequently, allowing higher statistical power and simplified study design. Urine, whole blood, blood serum, blood plasma, and cerebrospinal fluid (CSF) are the most widely used body fluids for such studies. Considerable work has been done across all medical disciplines to discover markers that enable disease classification and to monitor therapy progress, with outstanding success in many cases [415-426].

In this study, we measured the secreted signaling factors in plasma from sporadic Alzheimer Disease (AD) patients and compared it to that from healthy controls (Fig. 6). The known disease pathology in AD is confined to the central nervous system. This pathology consists of neuronal loss, brain atrophy, increased local inflammation, and extracellular Tau and intracellular Abeta aggregates [427-430]. While there are reliable markers for AD in CSF (hyperphosphorylated Tau and Abeta) and other clinical markers [431, 432], there is still a considerable and ongoing debate on whether or not there is a

clinically useful set of blood based AD biomarkers available today. Changes in relatively small numbers of plasma proteins have been reported in the past and used as diagnostic biomarkers by us and others [433-438]. In contrast to these previous reports, this current study uses the much large number of secreted signaling proteins not to detect biomarkers or to classify samples into control or disease, but to instead shed light on functional and pathological pathway changes associated with sporadic AD pathology. This study aims to exemplify the integration of a large variety of statistical and *in silico* tools that cover not only significant one-on-one relationships between proteins A and B, but also the numerous other interactions between proteins, between proteins and clinical scores, and between biological pathways as a whole. By doing this, we attempt to move away from the discovery of pure markers for a disease and towards a better understanding of the complex changes that occur in the physiological networks under disease conditions. The authors hope to demonstrate the large amount of information that can be gathered, processed, and analyzed by today's array technologies and the insights that one can gain from doing so. Focusing on soluble secreted signaling proteins allows to potentially measure changes in the CNS tissue without the need for tissue or CSF collection and could contribute to a much-enhanced understanding of the pathology in neuro-degenerative diseases such as sporadic AD.

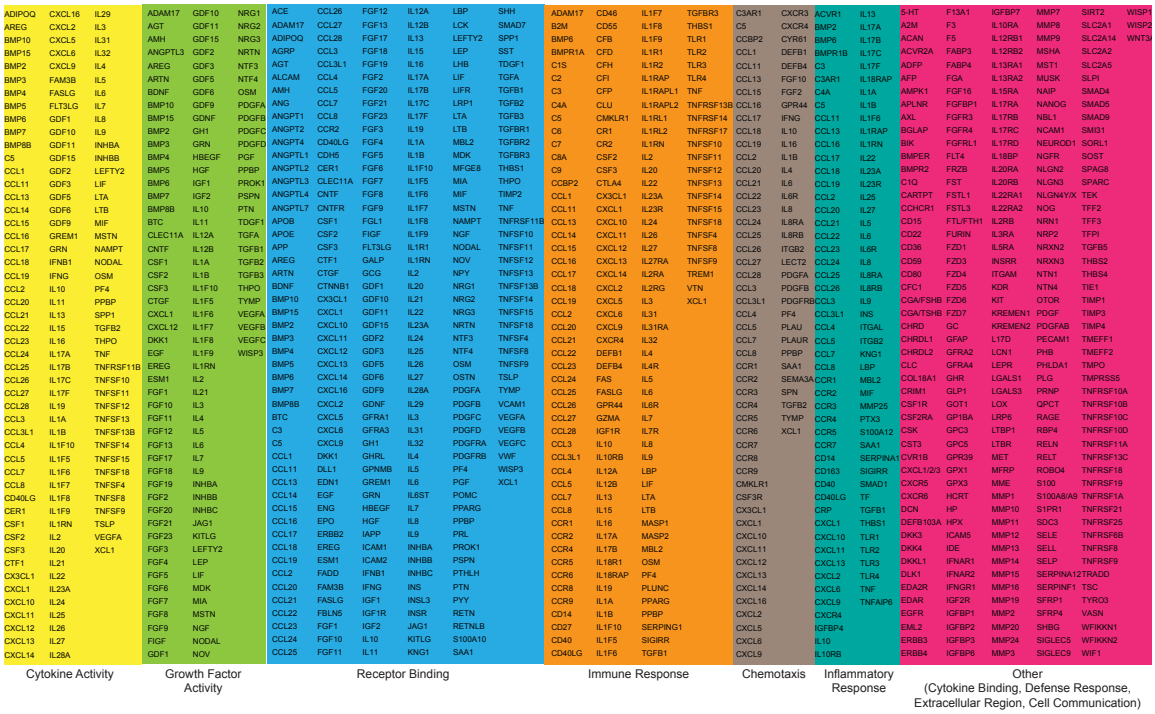

Table 7: Functional grouping of the secreted plasma proteins

The 702 measured plasma proteins, arranged by the most unifying functional gene-ontology terms.

Antibody microarrays can reliably measure relative protein levels in plasma

To measure systemic pathological changes in an intact organism we set out to measure hundreds of secreted signaling proteins and related factors in human plasma. For this we printed custom antibody microarrays, each with more than 2'000 micrometer-sized spots containing a specific antibody at a defined location (Fig. 27). To optimize the signal-to-noise ratio, we performed extensive dilution and dialysis during the preparation of the plasma samples to remove many of the highly variable factors, such as lipids, free carbohydrates, salts, and small protein fragments. To test, if fluorescent signal intensity per spot is indeed a function of antibody-protein complexe number per array surface, we spiked recombinant Green Fluorescent Protein (GFP) at a constant concentration into our processed samples and detected it with four different concentrations of anti-GFP antibody on the arrays (0.125 mg/ml to 1 mg/ml). We were able to extract signals from the anti-GFP antibody following a one-phase saturation curve (Fig. 28a). As part of our quality-control process and to ensure data reliability we ran plasma samples on two different arrays at two different days. The data measurements showed a highly significant correlation (r^2=0.97), indicating good experimental reproducibility (Fig. 28b). This data shows that the fluorescent signal can be used to reliably determine the relative abundance of antibody-protein complexes per spot and thus the relative concentration of protein in the sample solution.

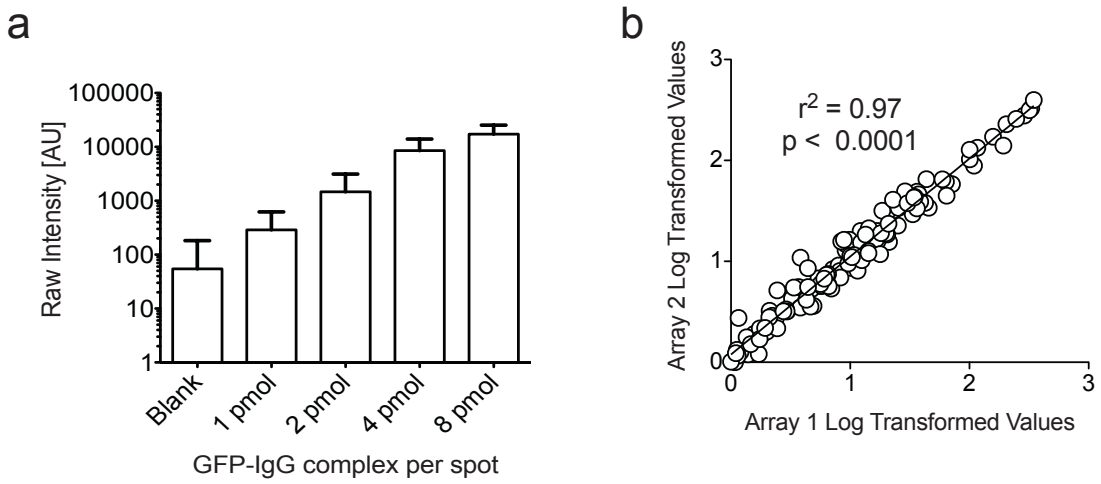

Figure 28: Antibody microarray performance

(a) Titration of different amounts of anti-GFP antibody on our microarrays indicates that the fluorescent signal is a function of antibody-protein complex on the array surface. **(b)** Repeated measurements of the same plasma samples on different arrays and on different days yielded very similar results, indicating good reproducibility.

Secreted signaling protein levels in AD patients differ from non-demented control patients and from patients with non-AD dementia

We selected a cohort of 52 patients with AD and 47 non-demented control patients to measure the secreted signaling factors in plasma. The samples came from two different centers (University of California San Francisco, CA and The Mayo Clinics) and were matched for age, sex, and center (Tab. 8). We measured a total of 776 proteins using our custom printed arrays. After cleaning up the extracted raw data (see methods), measurements for 702 signaling proteins remained. 42 proteins were significantly changed between AD and control samples based on Student's t-test p-values ($p=0.012$) and significance analysis of microarrays (SAM, $q<0.052$). To rank these 42 proteins we computed median-fold change values and Mann-Whitney-U test p-values on the raw data, and Student's t-test values on normalized data (Tab. 9). We then performed an unsupervised clustering of the 42 significantly changed proteins (Fig. 29a). AD patients clustered mostly to the left of the heat map and control patients to the right, indicating that these 42 proteins are a useful representation of disease status. Out of the 42 significantly changed proteins, 12 were elevated in AD and 30 were decreased (Fig. 29a). This pattern is different from a pattern created by matched plasma samples from Fronto-temporal dementia patients of the semantic dementia type (N=50, data not shown), which suggests that it mostly represents specific AD related changes (Fig. 29b). Furthermore, when we extracted measurements for these 42 proteins from a smaller, independent set of AD and control patients (N=6 each) we found a significant overlap in the direction of the observed changes in 31 out of 42 proteins ($p=0.0012$ by binomial test, Fig. 30).

These findings demonstrate that plasma signaling protein levels in AD patients differ significantly from control patients. While this is consistent with previous studies performed by us and others [433-438], we here advanced beyond describing relative fold-changes by integrating three independent analytical modules. To determine which of the 702 signaling proteins and the underlying signaling pathways are altered in AD we combined basic statistics (fold-change, t-test, Mann-Whitney-U test, as described above), linear regression modeling (elastic net, eNet), and correlative network analysis (connectivity), which are described below.

Condition	Ctrl		AD		Total
Gender	Male	Female	Male	Female	Both
N Subjects	28	24	26	21	99
	52		47		
Mean age	70.4 ± 9.2	67.3 ± 9.6	70.6 ± 9.3	67.5 ± 10.4	69.1 ± 9.6
N Mayo	17	14	15	12	58
N UCSF	12	10	11	9	42
Mean MMSE	29.7 ± 0.48	29.7 ± 0.75	18.9 ± 6.7	17.3 ± 7.3	29.7 (Ctrl) 18.3 (AD)
N Pathology confirmed	0	0	15	12	27

Table 8: Human sample demographics

Comparison of control (Ctrl) and Alzheimer Disease (AD) patients. Plasma samples were acquired from two centers (The Mayo Clinics; UCSF, San Francisco, CA). The average minimal mental state exam score is given for each cohort, as well as the number of pathology confirmed subjects (Note: MMSE scores were not available for all subjects).

Meta Rank	Gene	Swiss Prot ID	Ab Name	p-value (TT)	q-value (SAM)	Median f. chg.	p-value (MWU)	Up / Down in AD	AD & FTLD	AD Ref.
1	NTN1	O95631	Netrin1 pAb	0.0010	0.0000	0.4809	0.0002	DOWN		Yes
2	CSF1	P09603	M-CSF	0.0013	0.0000	0.4745	0.0000	DOWN		Yes
3	FURIN	P09958	Furin mAb	0.0002	0.0000	0.5835	0.0004	DOWN	Yes	Yes
4	F5	P12259	Factor V pAb	0.0001	0.0000	0.6096	0.0004	DOWN		
5	FGF1	P05230	FGF acidic mAb	0.0012	0.0000	0.5460	0.0004	DOWN	Yes	Yes
6	KDR	P35968	VEGF R2 (KDR)	0.0002	0.0000	1.7996	0.0029	UP	Yes	
7	FLT4	P35916	VEGF R3	0.0000	0.0000	1.6899	0.0032	UP	Yes	
7	INHBA	P08476	Inhibin A	0.0011	0.0000	0.5880	0.0004	DOWN		
9	MSTN	O14793	GDF8	0.0001	0.0000	0.6163	0.0022	DOWN		
10	IL31	Q6EBC2	IL-31	0.0003	0.0000	0.6055	0.0014	DOWN	Yes	
11	CLEC11A	Q9Y240	SCGF/CLEC11a mAb	0.0002	0.0000	0.7031	0.0003	DOWN		
12	GDF3	Q9NR23	GDF3	0.0009	0.0000	0.6530	0.0006	DOWN		
13	GDF5	P43026	GDF5	0.0006	0.0000	0.6590	0.0014	DOWN		
14	IL29	Q8IU54	IL-29	0.0119	0.0524	0.3222	0.0002	DOWN		
15	GDF6	Q6KF10	BMP-13 pAb	0.0025	0.0349	0.5828	0.0033	DOWN		
15	JAG1	P78504	Jagged 1 mAb	0.0045	0.0524	0.4239	0.0024	DOWN	Yes	
17	NRG1	Q02297	SMDF / NRG1Isoform	0.0012	0.0000	0.6629	0.0013	DOWN	Yes	Yes
18	SMAD5	Q99717	Smad 5	0.0061	0.0465	0.5745	0.0006	DOWN		
19	INHBB	P09529	Inhibin B	0.0035	0.0349	0.3874	0.0187	DOWN	Yes	
19	TNFRSF1A	P19438	TNF RI / TNFRSF1A	0.0010	0.0268	1.5961	0.0161	UP		
21	APP	P05067	APP 444-592 mAb (Clone 1)	0.0102	0.0524	0.6440	0.0001	DOWN		Yes
21	PECAM1	P16284	PECAM-1 /CD31	0.0032	0.0349	0.5741	0.0152	DOWN	Yes	Yes
21	TNFRSF10D	Q9UBN6	TRAIL R4 / TNFRSF10D	0.0007	0.0268	1.3681	0.0063	UP		
24	TNFRSF10A	O00220	TRAIL R1 / DR4 / TNFRSF10A	0.0021	0.0388	1.6065	0.0234	UP		
25	SPARC	P09486	SPARC mAb	0.0112	0.0524	0.5811	0.0045	DOWN		Yes
26	GDF1	P27539	GDF1	0.0024	0.0000	0.7275	0.0068	DOWN		
27	A2M	P01023	alpha-macroglobulin mAb	0.0055	0.0349	0.6509	0.0033	DOWN		Yes
28	GCG	P01275	GLP-1	0.0017	0.0000	0.7467	0.0104	DOWN		Yes
29	FZD5	Q13467	Frizzled-5	0.0081	0.0465	0.6296	0.0143	DOWN		
30	TNFSF15_T	O95150	TL1A / TNFSF15	0.0009	0.0268	1.2138	0.0518	UP	Yes	
31	C5	P01031	rC5a	0.0064	0.0465	0.6841	0.0096	DOWN		Yes
32	FGF16	O43320	FGF-16	0.0036	0.0349	0.7784	0.0072	DOWN		
33	TNFSF15_V	O95150	VEGI / TNFSF15	0.0036	0.0388	1.3771	0.0340	UP		
34	MET	P08581	HGFR	0.0045	0.0349	0.7087	0.0317	DOWN		
35	CSF3	P09919	GCSF	0.0021	0.0000	0.8301	0.0454	DOWN		
35	GREM1	O60565	GREMLIN	0.0095	0.0524	0.7687	0.0068	DOWN		
37	BMP8B	P34820	BMP8b mAb	0.0027	0.0388	1.3618	0.5727	UP		
38	VEGFB	P49765	VEGF-B	0.0042	0.0524	1.3740	0.0527	UP	Yes	Yes
39	TGFB1	P01137	LAP (TGF-B1)	0.0025	0.0388	1.2560	0.3318	UP		Yes
40	C4A	P0C0L4	C4 Binding Protein	0.0032	0.0388	0.8061	0.1165	UP		Yes
41	LGALS3	P17931	Galectin-3	0.0062	0.0349	0.7877	0.0418	DOWN		
42	AFP	P02771	alpha-fetoprotein mAb	0.0028	0.0388	0.9140	0.9804	UP		

Table 9: Plasma proteins with differential levels

The 'Meta Rank' is computed by ranking the four statistical measures for each factor: p-value by Student's T-test (TT) and q-value by Significance Analysis of Miroarrays (SAM) on the normalized Z0-scored data; median fold change and p-value by Man-Whitney-U-test (MWU) on the raw data. To ease identification we provide gene and SwissProt ID as well as the antibody name. The direction of the change in the AD cases is

indicated by ‘Up / Down’. Factors that are also changed in FTLD samples are labled ‘Yes’ in the ‘AD & FTLD’ column. A ‘Yes’ in the ‘AD Ref.’ column indicates that the factor has been implicated in AD. Only factors with a q-value < 0.0524 are shown in this table.

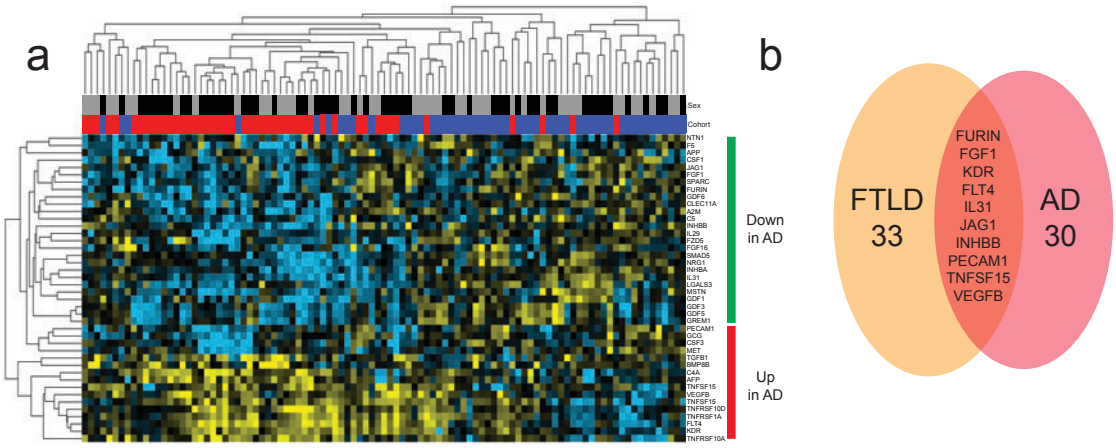

Figure 29: Plasma proteins with differential levels

42 plasma proteins were identified and ranked through a combination of parametric and non-parametric statistical tools and filtered for false-positive discovery rates. **(a)** Unsupervised clustering of the 42 proteins led to a separation between the AD samples (red, more to the left) and the control samples (blue, more to the right). This clustering was sex independent (gray and black indicators on top). **(b)** 30 of the factors were specific for AD when compared to an independent dataset from patients with Front-temporal dementia (FTLD), while 11 factors overlapped in both data sets. These factors might represent a common dementia pathology.

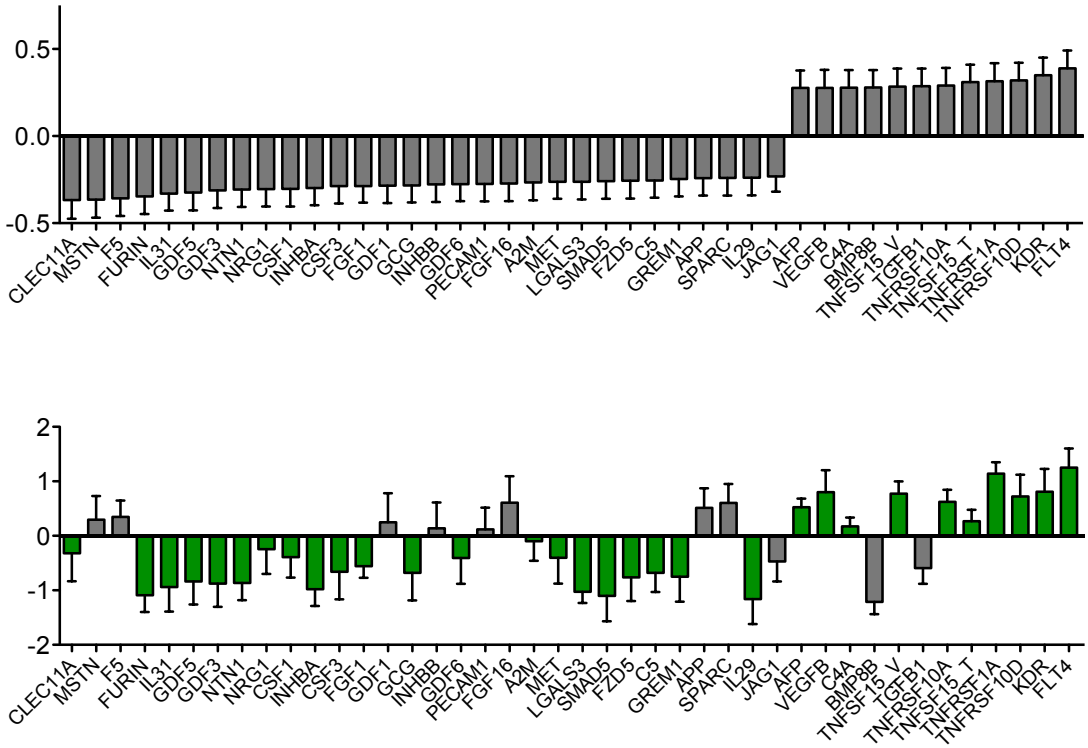

Figure 30: Independent cohort confirmation

Bar graph representation of the differentially secreted proteins in the large 99 sample experiments **(above)**. A similar representation of the same factors, measured in a smaller set of independent patient and control cohorts **(below)**. The directional overlap between the two experiments is highly significant (p=0.0012).

Penalized linear regression modeling confirms and expands the pool of proteins-of-interest

To extract important proteins that predict the identity of the two groups we performed penalized linear regression modeling using the elastic net algorithm (eNet). We used three different eNet approaches (see methods for details). The approaches yielded largely overlapping results and we combined them to compute a rank-value for the significant factors (Tab. 10). While SAM and eNET reached similar results for most top factors, they produced an independent selection of significant markers for the less prominent factors and thus complemented each other to expand the pool of proteins-of-interest for the biological function and pathway level of the analysis.

Antibody	Gene	Rank A α=0.8	Rank B α=0.5	Rank C α = 0.75	Avg Rank	Meta Rank
FGF-8	FGF8		1	1	1.00	1
MMP-9	MMP9		5	6	5.50	2
ADFP	ADFP	6	3	10	6.33	3
C2	C2	3	6	14	7.67	4
VEGF R2 (KDR)	KDR	8	8	13	9.67	5
IL-29	IL29	12	9	8	9.67	5
EGF R / ErbB1	EGFR	15	4	11	10.00	7
CCR9	CCR9	18	11	3	10.67	8
TACE	ADAM17	29	2	4	11.67	9
GDF5	GDF5	7		17	12.00	10
Clusterin	CLU	2	15	22	13.00	11
NRG1 Isoform GGF2	NRG1	21	13	7	13.67	12
Vitamin D binding protein mAb	GC		18	12	15.00	13
BAFF R / TNFRSF13C	TNFRSF13C		17	15	16.00	14
BIK	BIK	32	12	5	16.33	15
MIS/AMH Propeptide mAb	AMH	9	22	19	16.67	16
TRAIL R4 / TNFRSF10D	TNFRSF10D		19	16	17.50	17
GDF1	GDF1	38	7	9	18.00	18
GCSF	CSF3		16	21	18.50	19
Inhibin A	INHBA		36	2	19.00	20
Angiopoietin-2	ANGPT2	11	28		19.50	21
CCR8	CCR8		24	18	21.00	22
TL1A / TNFSF15	TNFSF15	26	10	31	22.33	23
M-CSF	CSF1	1		49	25.00	24
IL-31	IL31	37	14		25.50	25
GM-CSF	CSF2		20	32	26.00	26
RBP4	RBP4	30		23	26.50	27
beta-Catenin	CTNNB1	19		34	26.50	27
CXCL14 / BRAK	CXCL14	16	37		26.50	27
Glucagon	GCG	24	21	36	27.00	30
APP 444-592 mAb (Clone 1)	APP		29	30	29.50	31
beta 2 microglobulin mAb	B2M		35	27	31.00	32
CRP	CRP		41	25	33.00	33
IL-12 p70	IL12A		40	26	33.00	33
BMP-3b / GDF-10	GDF10	36	31		33.50	35
CXCR2 / IL-8 RB	IL8RB		34	38	36.00	36
TLR1	TLR1	40		33	36.50	37
IGFBP-6	IGFBP6	25		48	36.50	37
TRAIL R2 / DR5 / TNFRSF10B	TNFRSF10B	34	39		36.50	37
sFRP-4	SFRP4		23	51	37.00	40
PDGF-C	PDGFC	28		46	37.00	40
GDF-15	GDF15		32	45	38.50	42
FGF-BP	FGFBP1	41	38		39.50	43

Table 10: eNet comparison and ranking

Table 10: eNet comparison and ranking (continued)

Three different eNet linear regression models were used to compute a meta-rank (see methods for details). 'Rank A' is based on data not normalized for antibody intensities, 'Rank B' is based on a single round of antibody and array normalization, while 'Rank C' is based on 5 iterative steps of array and antibody normalizations.

The connectivity between secreted signaling proteins increases significantly in AD patients

Whereas significant changes in the levels of proteins are important and frequently measured, we were also interested in changes in the connectivity between the secreted signaling proteins. To establish this network structure, we calculated the correlation coefficients between all 702 measured signaling proteins and created an un-weighted cross-correlation or adjacency matrix for AD and control samples (Fig. 31a). The sequence of proteins in these adjacency matrices had been established through unsupervised clustering of the control samples alone (data not shown). A black dot in these matrices indicates a significant connection (arbitrarily defined at $|rho|>0.4$, $p<0.006$) between two proteins. Based on these matrices we calculated the unweighted connectivity k_i for each protein (Fig. 31b). The unweighted connectivity k_i represents the number of significant connections that each protein has with all the other proteins. These protein-protein connections can be classified into three groups: They either exist in control samples only, in AD samples only, or in both samples (Fig. 31c). To assess changes in connectivity between AD and control samples we computed a differential connectivity matrix (Fig. 31d). In total we found 16'154 significant control connections, 33'940 significant AD connections, and 10'063 connections that exist in both conditions. Five protein connections became inverted from positively to negatively correlated or vice versa. The average connectivity k_i per protein is significantly higher in the AD samples (61.7 ± 1.5) compared to control samples (36.4 ± 0.9, $p<0.0001$, Fig. 31e). Further analysis of the frequency distribution of the unweighted connectivity k_i in AD and control samples revealed a significant shift from abundant low-connectivity proteins in the control samples towards high-connectivity proteins in the AD samples (Fig. 31f). Finally, we ranked the secreted signaling factors by their connectivity in control, both, and AD samples (Fig. 31g and data not shown). The connected proteins are shown as an example for TNFRSF21 (DR6, Fig. 31h). TNFRSF21 is connected through significant correlation to 71 proteins only in the control samples (blue box; green indicates positive correlation, red negative), is connected to 17 proteins under both conditions (yellow box), and is connected to 25 proteins only in AD samples (red box).

These findings suggest that numerous biological pathways become activated and start communicating with each other during AD. Our data indicates that brain tissue driven AD pathology causes systemic changes in the connectivity of plasma signaling proteins.

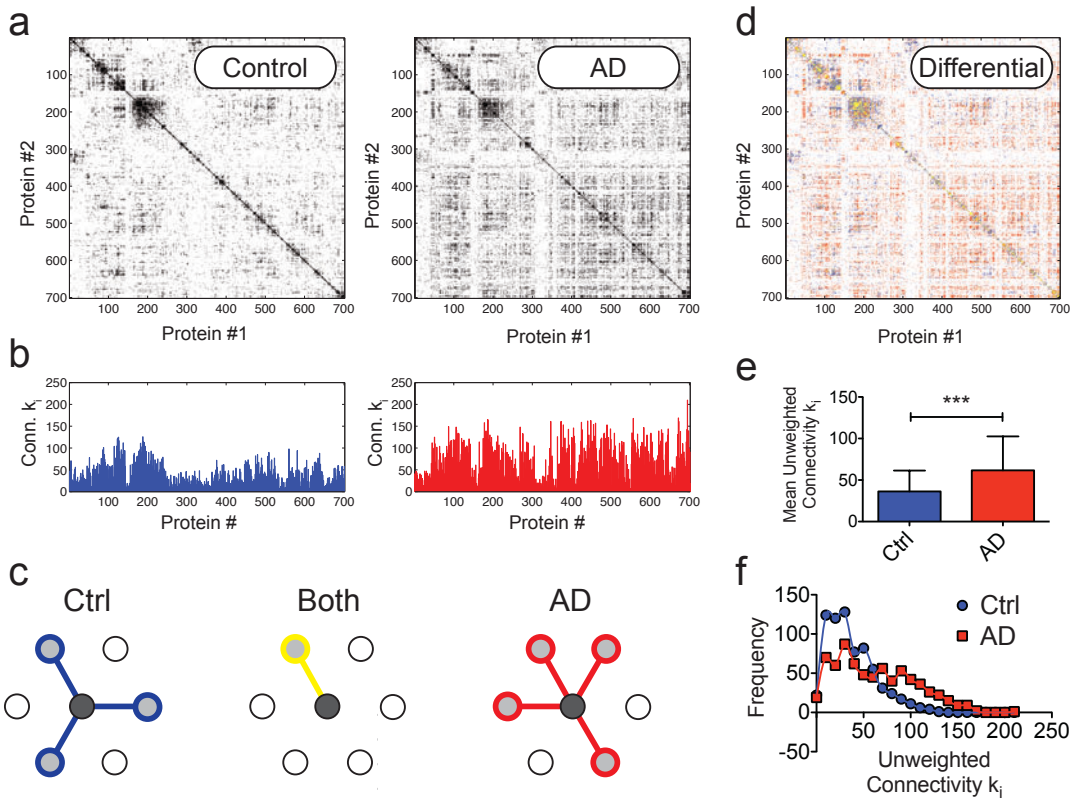

Figure 31: Plasma proteins with differential connectivity

Correlation analysis was used to assess the connectivity patterns between plasma proteins in control and AD patients. **(a)** Unweighted connectivity matrix of control and AD patients with all 702 proteins on the x- and y-axis. Each black dot represents a connection between the two proteins (|rho|>0.4). **(b)** Connectivity score ki for the 702 proteins in the control (blue) and AD (red) samples. A higher score represents higher

connectivity. **(c)** Schematic representation of the differential connectivity. A given protein (dark grey circle in the center) is connected to six other proteins (outside circles). It is connected to three proteins under control conditions (blue lines) and to four proteins under AD conditions (red lines). One protein connection is maintained under both conditions (yellow line). This protein has a connectivity score of Ctrl:2/Both:1/AD:3. **(d)** Differential connectivity matrix for all 702 proteins. Connections that exist only in control samples (blue), only in AD samples (red), or in both samples (yellow) are indicated. **(e)** The mean unweighted connectivity score was significantly higher in AD samples. **(f)** The observed increase in higher connectivity in AD samples was due to a broad shift to more highly connected proteins. **(g)** Example list of the top ten proteins for each condition: Highest connectivity in control samples (blue), highest connectivity in both samples (yellow), and highest connectivity in AD samples (red). Grey boxes indicate published involvement in AD pathology. **(h)** TNFRSF21 (DR6) is shown as an example with all its connected partners under control (blue box), both (yellow box), or AD conditions (red box). Inset scatter graphs show the loss of connectivity between TNFRSF21 and CXCR3 in control versus AD samples.

Meta-analysis of the different statistical modules to rank proteins-of-interest and correlation with an independent measure of cognitive decline

With a variety of statistical measures available to rate the relevance of individual secreted signaling proteins, we then performed a meta-analysis by combining the different results into one meta analysis (see methods for details; Tab. 11). In addition to the methods described above we also included if the protein/gene had been found as a hit in one of the recent AD plasma/serum/CSF biomarker studies [433, 435, 438]. Changes in secreted signaling protein levels and connectivity are likely to contain important information on pathological pathway alterations during AD. Another important factor to consider is the correlation of secreted signaling protein levels with an independent and gradual clinical measure of memory performance. To achieve that, we used the mini-mental state exam (MMSE) scores of the AD patients that were recorded around the time of blood sample collection. The MMSE consists of a 30-point questionnaire test used to screen for cognitive impairment and to document dementia progression [439]. We found no significant correlation between the patient age and MMSE scores in our AD cohort (data not shown). When we performed Spearman's rank correlation between the MMSE-scores and the 702 proteins, we discovered 57 proteins with significant (either positive or negative) correlations to the MMSE scores (57/702=8.12%; Fig. 32a). Many of these MMSE correlated proteins were also among the top hits in the previous analyses (see Tab. 11, 9/25=36%). This represents a highly significant enrichment of cognition-correlated proteins in our hit list ($p<0.001$ by binomial test). This enrichment supports the possible role in AD pathology of the proteins we identified in our un-biased screen.

Finally, to allow the extraction of the most important factors, we computed a complete ranking of all proteins taking all analytical modules and confirmational results into account (Fig. 32b). There, factors are ranked based on their appearance as hits in the individual modules, their MMSE correlation and their external AlzGene or literature references. Only factors with at least three hits are considered part of this final list. To allow for a ranking across different analytical modules, we computed percentile significance values for each protein/module with lower values indicating higher significance.

		Experimental Percentile Rank							Confirmation	
Name	Gene	TT	SAM	eNet	Ctrl (ki)	Both (ki)	AD (ki)	Rank	MMSE	Lit.
VEGF R2 (KDR)	KDR	0.04	0.03	0.12				1	Yes	
GDF5	GDF5	0.07	0.03	0.23				2		
SMDF / NRG1Isoform	NRG1	0.14	0.03				0.31	3		
VEGF R3	FLT4	0.01	0.03		0.53	0.57		4		
GDF1	GDF1	0.18	0.03	0.42				5		
GCSF	CSF3	0.17	0.03	0.44				6		Yes
IL-31	IL31	0.06	0.03	0.58				7		
M-CSF	CSF1	0.14	0.03	0.56				8	Yes	Yes
TNF RI / TNFRSF1A	TNFRSF1A	0.10	0.49		0.16	0.41		9	Yes	
TRAIL R4 / TNFRSF10D	TNFRSF10D	0.08	0.49	0.40		0.37		10		Yes
TL1A / TNFSF15	TNFSF15	0.09	0.49	0.53				11	Yes	
Clusterin	CLU	0.61		0.26	0.78			12	Yes	
GDF8	MSTN	0.02	0.03					13		
Factor V pAb	F5	0.03	0.03					14	Yes	
SCGF/CLEC11a mAb	CLEC11A	0.03	0.03					15		
Furin mAb	FURIN	0.05	0.03					16	Yes	
GDF3	GDF3	0.08	0.03					17		
Netrin1 pAb	NTN1	0.11	0.03					18		
Inhibin A	INHBA	0.12	0.03					19		
FGF acidic mAb	FGF1	0.13	0.03					20	Yes	Yes
GLP-1	GLP1	0.15	0.03					21		
IL-29	IL29	0.45		0.12				22		
EGF R / ErbB1	EGFR	0.43		0.16				23		Yes
BIK	BIK	0.39		0.35				24	Yes	
BMP-13 pAb	GDF6	0.19	0.57					25		

Table 11: Meta-analysis of the plasma proteins (experimental data, top 25 hits)

Meta ranking of the different analysis methods. Hits in the individual analyses are ranked and then converted into a percentile value (smaller value = more significant). MMSE correlation is indicated as a measure of functional relevance enrichment within our top 25 factors. Previous occurrence in published literature is also indicated.

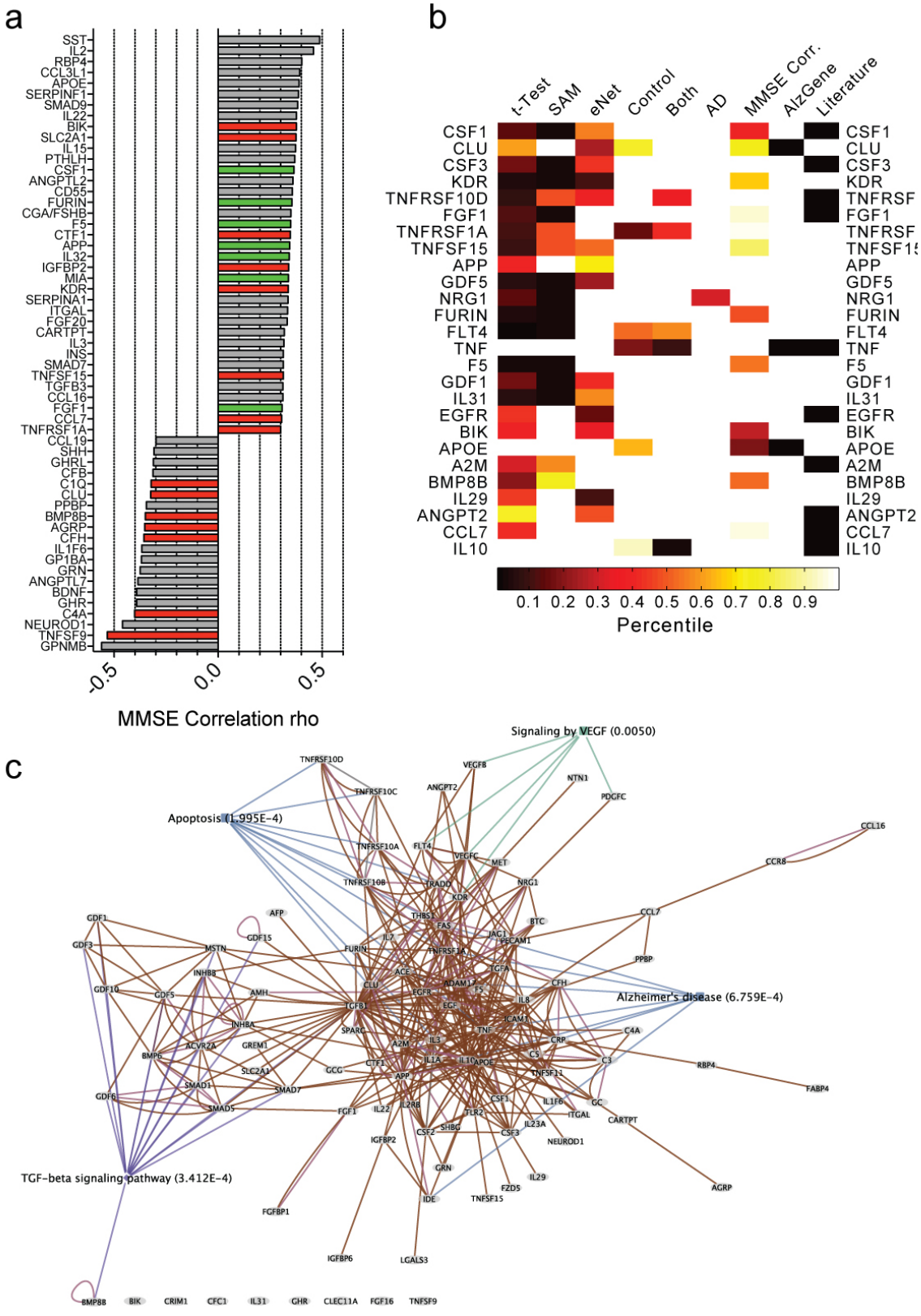

Figure 32: Meta analysis of the plasma protein hits

A number of different statistical and external sources were combined to compile a ranked list of plasma proteins of interest. **(a)** Factors that correlate with a measure of cognitive performance in the AD patients (MMSE). Bar color indicates the differential level between control and AD samples (red=increased in AD, green=decreased in AD, grey=no diff.). **(b)** After the combination of experimental results (Tab. 11), cognitive correlation, and literature comparison, a final ranked meta-analysis list was compiled with the most relevant factors descending from the top. Only entries with at least three hits across the analyses are shown. To directly compare the various different statistical rankings, the rankings were converted into percentile values with the lowest percentile value corresponding to the highest significance / most extreme value (see methods for details). AlzGene and literature hits were not ranked but are only yes/no represented (black/white). The list shown represents only the top factors with at least three hits. **(c)** EGAN network based on Pubmed co-occurance (brown) or protein-protein interactoins (pink). Selected biological pathways and functions are indicated (p-value based on all 702 factors as background). KEGG (blue), Panther (purple), Reactome (green). See text for details.

Protein-interaction, PubMed co-occurance, gene-ontology, miRNA target, and chromosome band analysis

To gain insight into the biological role of the factors that we had identified, we utilized Exploratory Gene Association Networks (EGAN, University of California San Francisco, CA). EGAN is a powerful database mining software that integrates large numbers of public data repositories and links them into a user-friendly, java-based software interface [440]. We performed protein-protein interaction, PubMed co-occurance (biological proximity or functional connectivity), gene-ontology (functional enrichment), miRNA target (co-regulation), and chromosome band analysis (genetic linkage) on our complete meta-hit list from Tab. 11 with the 702 factors as background for enrichment analysis. The resulting protein-protein (pink) and PubMed co-citation (brown) network is shown in Fig. 32c, with some of the ontology terms highlighted. Since each database has a distinctive emphasis, we used a number of different databases for the gene-ontology analysis. The top hits with the highest significance for each query were summarized in Tab. 12 (KEGG, NCI, Panther, Reactome). We found a strong indication of an involvement of the TGFβ-, TNFα-, and angiogenic signaling cascades in sporadic AD.

Protein expression can be regulated through miRNA mediated transcription control. To identify miRNA that might co-regulate a significant number of our hit proteins, we interrogated a miRNA sequence database against the sequence of our hit proteins and identified significantly enriched miRNAs that could potentially regulate a large number of the disturbed proteins we have identified (Tab. 12, miRNA targets). Interestingly, one miRNA that was significantly enriched in our meta-analysis had previously been implicated in AD (miR-298 [441]).

Finally, AD can be caused by a number of genetic mutations and risk for sporadic AD can be linked to certain genomic regions based on genome-wide-association studies [442]. We mapped the genomic locations of all our de-regulated hit proteins and identified significantly enriched regions (Tab. 12, Cytoband). Three of the regions we discovered in this un-biased secreted signaling protein analysis have previously been

linked to late-onset sporadic AD or other amyloidoses (8p21 [443-445], 4p32 [446], 10q23 [447]).

In summary, our ontology, signaling, and regulation meta-analysis suggests that, based on their disturbed plasma profiles, AD patients suffer from disease specific alterations in their TNFα-, TGFβ-, and angiogenic signaling.

Database	Description	Total in Dataset	Hits	p-value	Genes
KEGG	Apoptosis	18	10	0.0002	TNFRSF10D, TNFRSF10C, TNFRSF10A, TNFRSF10B, TRADD, FAS, TNFRSF1A, TNF, IL3, IL1A
	Alzheimer's disease	11	7	0.0006	FAS, TNFRSF1A, ADAM17, TNF, APOE, APP, IDE
	Natural killer cell mediated cytotoxicity	19	9	0.0019	TNFRSF10D, TNFRSF10C, TNFRSF10A, TNFRSF10B, FAS, TNF, ICAM1, CSF2, ITGAL
	TGF-beta signaling pathway	39	14	0.0029	BMP8B, GFD6, BMP6, GDF5, SMAD1, SMAD5, ACVR2A, INHBB, INHBA, AMH, SMAD7, TGFB1,THBS1, TNF
NCI Nature	Direct p53 effectors	18	10	0.0002	TNFRSF10D, TNFRSF10C, TNFRSF10A, TNFRSF10B, MET, FAS, TGFA, EGFR, AFP, GDF15
	Caspase cascade in apoptosis	4	4	0.0008	TRADD, TNFRSF1A, TNF, APP
	TRAIL signaling pathway	7	5	0.0021	TNFRSF10D, TNFRSF10C, TNFRSF10A, TNFRSF10B, TRADD
	TNF receptor signaling pathway	5	4	0.0036	TRADD, ADAM17, TNF, TNFRSF1A
Panther	TGF-beta signaling pathway	40	16	0.0003	GDF1, GDF3, GDF10, GDF5, BMP6, GDF6, MSTN, INHBB, ACVR2A, SMAD1, SMAD5, INHBA, SMAD7, GDF15, TGFB1, BMP8B
	p53 pathway	6	5	0.0007	TNFRSF10D, TNFRSF10A, TNFRF10B, THBS1, FAS
	Apoptosis signaling pathway	14	8	0.0007	TNFRSF10D, TNFRSF10A, TNFRSF10B, TRADD, FAS, TNFRSF1A, TNF, BIK
	Alzheimer disease-amyloid secretase pathway	3	3	0.0049	FURIN, ADAM17, APP
Reactome	Signaling by VEGF	8	5	0.0050	VEGFB, LT4, VEGFC, KDR, PDGFC
	Apoptosis	9	5	0.0097	TNFRSF10B, TRADD, FAS, TNFRSF1A, TNF
	Signaling by EGFR	4	3	0.017	EGFR, EGR, ADAM17
	Hemostasis	52	14	0.045	VEGFB, ANGBT2, VEGFC, CLU, TGFB1, SPARC, A2M, APP, EGF, F5, PECAM1, PPBP, ITGAL, THBS1
Cytoband	8p21	8	5	0.0050	TNFRSF10A, TNFRSF10B, TNFRSF10C, TNFRSF10D, CLU
	4q13	9	5	0.0097	AFP, BTC, PPBP, IL8, GC
	4q32	2	2	0.029	PDGFC, TLR2
	10q23.3	2	2	0.029	RBP4, IDE
	8p12	2	2	0.029	NRG1, CLU
miRNA targets	GUGUCAC [hsa-miR-597]	13	7	0.0025	VEGFB, INHBA, SPARC, CARTPT, IL10, ACVR2A, TNFRSF10B
	CCCACGU [hsa-miR-662]	5	4	0.0036	GDF10, FLT4, GCG, APP
	UAUAAUA [hsa-miR-374b, hsa-miR-374a]	60	17	0.016	MSTN, SMAD5, CLU, IL7, FAS, NTN1, MET, BTC, TGFA, IL22, IL10, IDE, FZD5, NEUROD1, CCL7, CRIM1, TNFSF9
	GCAGAAG [hsa-miR-298]	21	8	0.016	ACVR2A, SMAD5, APP, IL3, F5, JAG1, ANGPT2, IL31
	UCAUUUG [hsa-miR-579]	47	14	0.019	MSTN, ACVR2A, SMAD5, GREM1, FGF1, IL22, F5, JAG1, VEGFC, MET, NRG1, TLR2, FZD5, BMP8B

Table 12: Functional ontology analysis of the protein hits

Various public databases were interrogated for gene-ontology, signaling/interactions, genomic location, and miRNA regulation. The resulting significant terms are displayed with the corresponding total hits in our dataset of 702 proteins and the hits selected by our meta-analysis.

TNFα-, TGFβ-, and angiogenic signaling alterations in AD

Our present study identifies a number of systemic signaling pathways, a large set of proteins-of-interest, and various biological functions that appear highly altered in AD versus non-demented controls. While we cannot discuss all their implications in the context of this report, we would like to highlight some of the prominent changes, both established and novel. One of the pathways identified in our un-biased screen is the TNFα-signaling cascade. TNFα and related signaling molecules have been implicated in AD and CNS inflammation in the past [448, 449], however their precise role in the disease remains unclear. It appears that activated microglia play a role in the degradation of beta-amyloid and TNFα secretion, and their biochemical markers are elevated in human patients [450] and in transgenic mouse models [451]. Here we report the detection of a whole array of TNF-family molecules, most of which are elevated in AD plasma (Fig. 33a). Specifically, we detected elevated levels of the TNFSF10 (TRAIL) receptors TNFRSF10A (DR4), 10B (DR5), and the two decoy receptors 10C (DcR1) and 10D (DcR2). TNFSF10 and its receptors play a central role in CNS inflammation [452] and the neutralization of the TNFSF10 signaling can protect from beta-amyloid induced toxicity [453]. In addition, we found elevated levels of FAS and the TNFα-receptor TNFRSF1A in AD plasma, together with a number of other TNF-family ligands (Fig. 33a and data not shown) While TNFα-levels themselves appeared not different in AD compared to control, we did measure a significant alteration in the TNFα network connectivity. On the down-stream signaling side, we found elevated levels of TRADD, a protein that normally binds to the intracellular domain of TNFRSF1A. When levels of the pathway components were plotted against each other, they correlated significantly, indicating that the pathways are indeed affected as a whole (Fig. 33b). Overall, we found a predominant elevation of plasma levels from proteins in the TNF-family.

Another major signaling pathway that has been identified in our screen is the TGFβ-family signaling cascade (Fig. 33c). TGFβ-signaling in AD can be both, potentially beneficial [454, 455] and detrimental [456]. We detected elevated levels in some of the receptor-ligand pairs (VEGF) while others appeared reduced (INHB-ACVR2A). We also found a number of the more downstream signaling molecules, such

as SMAD1, SMAD5, or SMAD7, reduced in AD patients. This indicates a more complicated signaling picture than in the TNF-family context. Some components of the TGFβ family pathways might be elevated, while others might be reduced. Similarly to the TNF-family discussed above, we also found a strong correlation between the levels of the individual proteins in the TGFβ-pathway, which supports their intimate biological correlation (Fig. 33d).

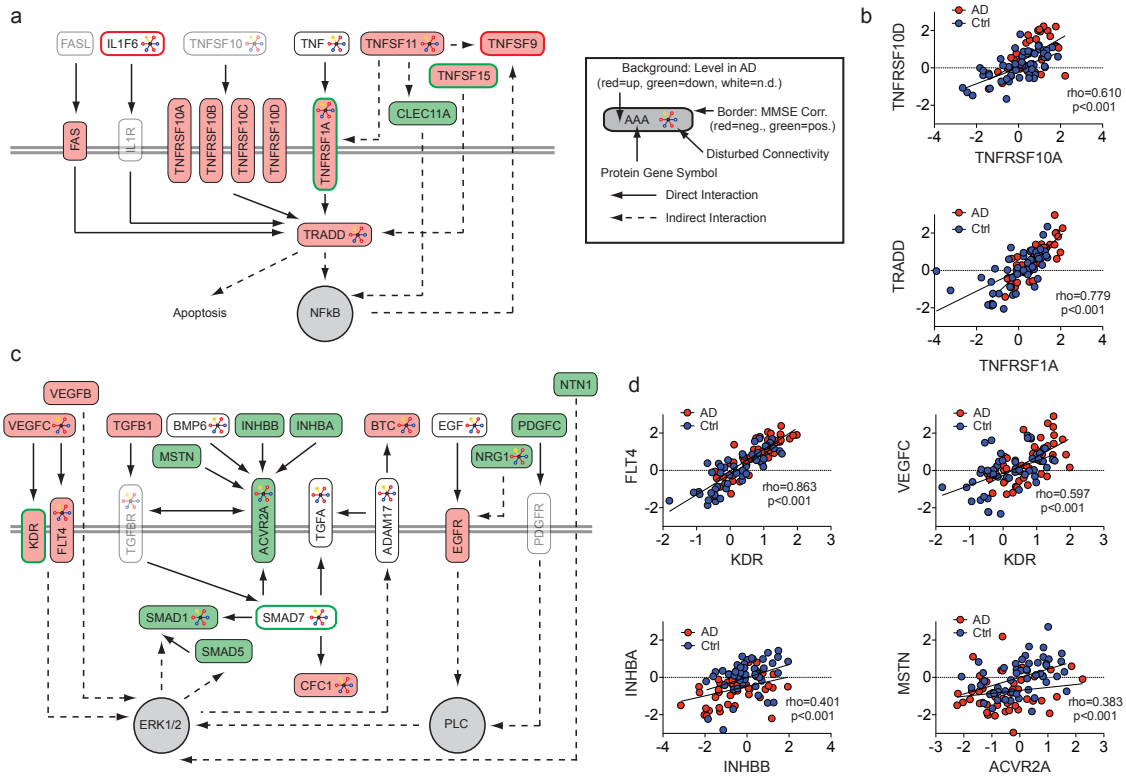

Figure 33: Ingenuity Pathway Analysis and biological correlation analysis

The hits from the meta-analysis were subject to Ingenuity Pathway Analysis (IPA). **(a)** Selected factors from the TNFα/NFkB signaling cascade, their interactions, and their deregulations in AD (pathways adapted from Ingenuity). **(b)** Correlation analysis of selected proteins that are part of the signaling cascade showed strong correlation, supporting that the identified pathways are indeed affected as a whole. **(c)** Selected factors from the TGFβ/GDF/VEGF/ERK signaling cascade, their interactions, and their deregulations in AD (pathways adapted from Ingenuity). **(d)** Correlation analysis of selected proteins that are part of the signaling cascade showed strong correlation, supporting that the identified pathways are indeed affected as a whole. Some proteins lose their correlation under AD conditions, such as MSTN-AVCR2A.

Discussion

Blood plasma represents the main transport medium for the signaling proteins in an organism. Previous mass-spectroscopy based studies, as part of the HUPO Plasma Proteome Project, have cataloged the proteins that can be identified in human plasma samples between 889 [457], 3'020 [458], and 9'504 (bioinformatics.med.umich.edu /hupo/ppp), depending on the stringency of the chosen cut-off values. Monitoring these, or a substantial fraction of them, will require medium to high throughput experiments, particularly when trying to avoid sample pooling. Our experimental and analytical approach allows measuring many hundreds or up to thousands of proteins simultaneously and across numerous individual samples and to analyze them in the context of biological significance.

Gene expression profiling has been conducted in a number of studies on tissue samples from AD patients [459-467]. These studies were performed on post-mortem brain tissue samples or peripheral cell samples (leukocytes, fibroblasts etc.). While these studies clearly add to our understanding of transcriptional changes that occur in defined regions of the AD brain or the patient's body, it is uncertain how well mRNA levels correlate with actual protein levels, both on the cellular and the tissue levels. While only few studies have been conducted to compare mRNA microarray measures to protein levels, the correlations observed vary between r=-0.025 to r=0.935 (mean around r=-0.4), indicating that protein expression cannot reliably be inferred from mRNA data [468]. Furthermore, since tissue is collected post-mortem, it is difficult to obtain accurate cognitive measures that are taken close to the time of death and no longitudinal studies can be performed. Peripheral plasma samples on the other hand, can be obtained at different disease stages and can easily be combined with other clinical measures such as cognitive tests or imaging studies.

The blood-brain-barrier (BBB), a tight layer of endothelia cells lining the brain vasculature, regulates the passage of proteins, metabolites, and cells from the blood into the brain. A meta-analysis of available BBB permeability data concluded that permeability is increased in AD and other dementias [469] making it more likely that CNS derived proteins can leak out into the systemic bloodstream. Furthermore,

peripheral blood cells have been shown to play an important role in clearing brain amyloid plaques and these cells can cross the BBB [470]. Concordantly, local and systemic components of the immune system play an important role in AD pathology and it is likely that a continuous flow of inflammatory factors and immune signaling molecules travel from the CNS into the periphery and back [471, 472]. Thus, a whole range of secreted signaling proteins should be available to assess the tissue status in the CNS of AD patients.

Here, we show that AD patients have a network of secreted signaling proteins that is on many levels different from non-demented control patients. While small sets of secreted signaling proteins had been investigated before [433-438], this is the first report on a comprehensive analysis of systemic plasma factors in any neurodegenerative disease. We report changes in the levels of plasma factors, but also changes in their connectivity between each other. Furthermore, to show biological and clinical relevance, we demonstrate that many of the factors identified by changes in levels and/or connectivity correlate significantly with patient's memory performance. To explore some of our most highly enriched pathways in greater detail, we focused on the most significantly deregulated pathways (TGFβ-, TNFα-signaling, and angiogenesis). The two highest-ranking proteins (CSF1 and CLU) are also particularly noteworthy. CSF1 (M-CSF) is reduced in our AD plasma samples and in an earlier study by our laboratory [438]. Concordantly, administration of peripheral CSF1 has been shown to improve cognitive performance in an AD mouse model [473]. In strong support of these studies, we now find an additional positive correlation between increased plasma levels of AD patients and increase cognitive performance (MMSE). The second ranking protein CLU (ApoJ) is a novel risk factor for AD [443-445]. Here we demonstrate that CLU plasma levels appear elevated in AD patients and that CLU levels correlate negatively with cognitive performance. This might be related to the effects of CLU on beta-amyloid solubility [474]. While some of the individual factors that we identified in this study had been implicated in AD before, we expand this list of potentially disease relevant factors to a large and well-connected set of biological pathways. Importantly, the discovered pathway network encompasses a number of previously known major risk factors for AD,

such as APP, APOE, IDE, and CLU, and embeds them into a new picture of pathologically deregulated networks (Fig. 32c).

While we used our meta-analysis strategy to identify deregulated plasma proteins and networks, we have to emphasize that it is not possible to immediately deduce the levels of functional protein in diseased tissue by looking at its plasma levels. A mix of passive cell surface release, active secretion, and changed degradation influences plasma protein levels. Furthermore, secreted receptors can be release into the plasma because their expression is extremely high (due to very low ligand concentrations), or because they are actively secreted to bind and block their ligand (due to very high ligand concentrations). We believe that our multi-modular approach is highly capable to identify enriched de-regulated pathways. However, to determine the exact direction of deregulation on the individual protein level will likely require follow-up measurements *in situ*.

We hope that the increased understanding of systemic pathway changes presented in this study can be utilized to better comprehend pathological changes in AD patients, to follow that pathology over the course of the disease, and to discover novel treatment strategies to stop this devastating disorder.

Material and methods

Human Plasma Sample Acquisition: We collected a total of 99 archived human plasma samples with ethylene diamine tetra acetate (EDTA) as anticoagulant from the University of California San Francisco Medical School and the Mayo Clinics (Rochester, MN and Jacksonville, FL). Plasma was produced by standard blood processing, then frozen and stored in aliquots at –80°C. Informed consent was obtained from human subjects according to the ethics committee guidelines at the respective academic centers.

Plasma Protein Microarrays: Plasma protein levels were measured using antibody-based protein microarrays. An adapted commercially available microarray (L-Series, RayBiotech Inc., Norcross, GA) containing 507 antibodies against standard chemokines/cytokines was supplemented with another 61 antibodies against secreted signaling factors printed in triplicates by the company. The custom-made in-house array contained a separate set of 148 secreted signaling factor antibodies printed in quadruplicates. A total of 726 secreted signaling factors were measured. A comprehensive method to produce and quality control custom-made protein microarrays has been submitted elsewhere. In brief, antibodies of interest were selected based on their biological role as secreted signaling factors and the availability of ELISA-grade quality batches to ensure likely detection of the epitope in solution. The arrays were printed onto SuperEpoxy glass slides (ArrayIt, Sunnyvale, CA) using a custom-built robotic microarrayer fitted with sixteen SMP4B pins (ArrayIt). The human plasma samples were platelet- and lipid-reduced by centrifugation. The plasma was then diluted and dialyzed (96 well Dispodialyzer/5kDa, Harvard Apparatus, Holliston, MA) at 4°C in multiple over-night steps to yield a maximally pure plasma protein fraction. The plasma proteins were N-terminally biotinylated (NHS SulfoBiotin, Thermo Scientific, Rockford, IL) and incubated with blocked antibody arrays over-night at 4 °C. After multiple washing steps antibody-bound protein was detected using Alexa555 conjugated streptavidin on a GenePix Pro 4000B scanner (Molecular Devices, Sunnyvale, CA).

Microarray Data Preparation: Individual array spots were background subtracted locally and the mean intensity raw data were calculated from the replicates for each antibody. Negative spot intensities were set to 1 (overall range: 1-63170, median: 1768).

The mean array background was calculated and spots, which had an intensity of less than 1.68 standard deviations above mean background, were flagged as "not-detectable". Antibodies with more than 55% "not-detectable" spots were removed from the data set (11/148 for the in-house array and 2/568 for the RayBiotech array). Log2 transformation and iterative row- and column-wise median centering and normalization were performed 5 times for each array-type separately using CLUSTER 3.0 for OSX (M. de Hoon, Riken, Japan). The CLUSTER-normalized data was Z-scored (Z-scored Data) and the control cohort means were set to 0 for each center and sex combination to account for baseline differences (zeroed Data).

Microarray Data Analysis: The human plasma microarray data was prepared as described above and was then subject to a variety of statistical analysis tools: Network parameters to describe global changes in network structure and connectivity in controls and disease conditions were extracted based on tools developed for gene coexpression network analysis. Student's unpaired t-test was used to compile a list of all significantly changed proteins. Statistical Analysis of Microarrays (SAM) was used to identify significant group differences between control and disease cohorts and to estimate false positive discovery rates based on multi-comparison analysis (Stanford University, Stanford, CA). Unsupervised clustering was performed using CLUSTER 3.0 (M. de Hoon, Riken, Japan) with Spearman rank correlation for both, proteins and arrays, and average linkage. Connectivity analysis was performed based on Spearman rank correlation values between all proteins.

Thesis Discussion

Autophagy in Alzheimer Disease

Over the last ten years, autophagy has increasingly been recognized as a major protein degradation pathway, important not only for the maintenance of protein homeostasis, but also essential to avoid accumulation of potentially toxic intracellular protein aggregates. A number of studies have now shown that this is particularly true for neurodegenerative disease of the proteinopathy type. Previous work had established an involvement of autophagy in diseases such as Parkinson Disease and Huntington Disease (Ravikumar, Vacher et al. 2004; Hara, Nakamura et al. 2006; Komatsu, Waguri et al. 2007), however its role and contribution to Alzheimer Disease (AD) had not been well studied. Pathological studies had previously observed autophagosome-like structures in AD neurons (Yu, Cuervo et al. 2005) and thus it appeared imperative to us, to better understand autophagy and autophagosome turnover in this devastating disease.

Our laboratory's initial discovery that Beclin1 protein levels were reduced in Alzheimer Disease patients' brains (Pickford, Masliah et al. 2008) led us to explore its role in the disease in greater detail. We were especially interested in investigating, if Beclin 1 is involved in AD via autophagy or through some uncharacterized, autophagy-independent function. While our early mouse data had clearly shown that a reduction of Beclin 1 led to a more severe pathology in AD mouse models (Pickford, Masliah et al. 2008), we chose to use the more controllable *in vitro* environment of cell culture to test the autophagy hypothesis.

Utilizing different cell culture models for APP metabolism and Aβ accumulation, I was able to show that APP can be a substrate for autophagy and that autophagy activation can increase APP and Aβ turnover. Furthermore, inhibition of Beclin 1 expression using several different RNAi approaches, demonstrated that loss of Beclin 1 impairs proper autophagosomal-lysosomal flux and leads to increased accumulation of APP and its catabolites, both inside and outside of the cell. Accordingly, a mild over-activation of autophagy through moderate Beclin 1 overexpression can also facilitate APP degradation.

One of our important findings was that the presences of elevated levels of APP and Aβ by themselves are not responsible for the observed deregulation of autophagy. Both, *in vivo* AD-mouse-models and *in vitro* cell culture experiments with a wide range of hAPP overexpression show no changes in autophagy whatsoever. This seems to indicate that the observed autophagy disturbance is not a consequence of Aβ accumulation, but instead potentially a cause for its increased accumulation.

Changes in systemic plasma factors and their effects on autophagy

What could be the upstream mechanism that causes the initial disruption of autophagy? Autophagy is a major housekeeping pathway and tightly regulated by a large number of biological pathways that interact in a highly complex manner (Ravikumar, Sarkar et al. 2010). To obtain candidate pathways for further exploration, we decided to utilize a technology that had recently been established in our laboratory: antibody-based protein microarrays. We had previously used a similar technology to investigate AD-related plasma protein to discover disease biomarkers (Ray, Britschgi et al. 2007). This time however, we decided to adapt the technology not for biomarker discovery, but instead as an unbiased discovery tool for biological pathway alterations. To be able to monitor system-wide changes in biological pathways, we had to establish a method that was both, capable to eavesdrop on hundreds of different pathways, and practical, since it is impossible to measure the tens of thousands of different proteins that make up the human proteome.

The novel approach that we developed in response to this challenge, was to narrow down the number of measured proteins to between 600 and 1000, which addressed the practical part of the equation. To still being able to monitor hundreds of pathways simultaneously, we decided to focus on a special set of proteins: secreted communication factors. By measuring proteins that are involved in the intracellular and intra-organismal communication, such as chemokines, cytokines, growth factors etc., we were able to monitor pathological changes, even in inaccessible tissues such as the brain, by collecting peripheral plasma samples (Fig. 34).

The novelty of this approach demanded numerous innovations, both on the technological side, where we decided to rely on custom-printed arrays specifically tailored to our requirements, and on the analytical side, where most statistical and theoretical tools had been developed for gene microarrays and had to be adapted from scratch for the use with our medium-throughput proteomics data.

Ultimately, we succeeded in the identification of a variety of biological pathways that appear particularly de-regulated in our AD patients' samples. Some of these pathways had been suggested to play a role in AD in the past (such as TGF-β and TNF-α), however we are now able to not only expand these pathways from a single molecule to a complete signaling cascade, but we also were able to validate many of them through correlation to an independent measure of cognitive decline.

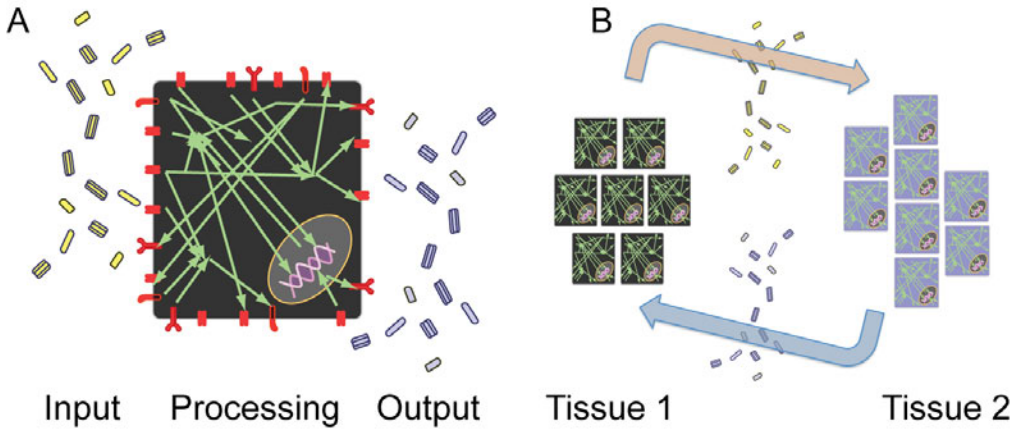

Figure 34: Concept of the signaling factor array

(A) A cell receives informational input through extracellular factors that bind to cell surface receptors [INPUT]. After binding to the receptors, these factors elicit complex intracellular responses [PROCESSING]. Ultimately, this leads to the release of a defined set of other communication and signaling factors [OUTPUT]. **(B)** This type of information flow is also occurring between tissues and across the whole organism. Monitoring of the various secreted factors and cleaved or shed receptors thus allows us to draw certain conclusions about the intracellular activities.

Outlook

Now that we have a number of candidate pathways at hand, the next question will be: Which of the pathways we identified are responsible for the autophagy disturbance that is apparent in AD brains? While we do not have the answer to this question yet, our research is now focusing on trying to understand the connection between some of our lead pathways, Beclin 1 levels, and autophagy activity.

One area that is of particular interest is the TNF-α/TRAIL pathway. Beclin 1 can be cleaved by caspase 3 (Rohn, Wirawan et al. 2010; Wirawan, Vande Walle et al. 2010) and caspase in turn can be activated via and TNF-α and TRAIL signaling (Mills, Reginato et al. 2004; Collison, Foster et al. 2009; Hoffmann, Zipp et al. 2009). Secreted TRAIL receptors were indeed one of the most prominent groups of factors that we found in our plasma analysis. Therefore it will be of great interest, to further explore the connection between increased inflammation, elevated and TNF-α/TRAIL signaling, decreased Beclin 1 levels, impaired autophagy, and APP metabolism.

Recent publications support our idea of autophagy driven APP degradation (Tamboli, Hampel et al. 2011) and we hope to draw further interest to this intriguing connection between two parallel, but currently biologically unconnected pathologies in AD: protein aggregation and inflammation.

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Appendix:

The following pages contain additional material for my thesis:

- Curriculum vitae
- the original publications.

Curriculum Vitae
Philipp Jaeger, Dipl.-Biochem.
PhD candidate

For reasons of data protection, the curriculum vitae is not included in the online version.

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

Theses:

PA. Jaeger: *The Role of Autophagy in Alzheimer Disease - from Cellular Mechanisms to Systems Proteomics* (Dissertation for Dr. rer. nat. / PhD), Free University, Berlin, expected graduation date May **2011**.

PA. Jaeger: *Identifikation und Charakterisierung potenzieller Aggregations-Inhibitoren zur Behandlung von MORBUS ALZHEIMER und CHOREA HUNTINGTON.* 'Diplom' (Master's) thesis in Biochemistry, Free University, Berlin **2004**.

Research Publications:

PA Jaeger, M Britschgi K Rufibach, B Burkholder, H Johns, CH Sun, S Pradhan, R Petersen, DS Knopman, BF Boeve, AL Boxer, A Karydas, BL Miller, R Rademakers, DW Dickson, S Yonnkin, N Graff-Radford, T Wyss-Coray. *Plasma Protein Changes in Sporadic Alzheimer Disease Patients are Linked to Cognitive Decline and Identify Disease-related Pathways*. (in preparation)

PA. Jaeger, M. Britschgi, CH Sun, H. Johns, S. Pradhan, T. Wyss-Coray: *Production, Quality Control, and Analysis of Plasma Protein Arrays to Study Alzheimer Disease and other Dementias.* (in preparation)

Y. He, H. Zhang, **PA. Jaeger**, O. Olayiwola, N. Fainberg, T. Wyss-Coray: *Neuronal TGF-β Signaling Regulates Survival and Maturation of Newborn Neurons in the Adult Dentate Gyrus.* (submitted to Cell Stem Cell)

PA. Jaeger, F. Pickford, CH. Sun, KM. Lucin, E. Masliah,T. Wyss-Coray: *Regulation of Amyloid Precursor Protein Processing by the Beclin 1 Complex*. PLoS One 5(6): e11102 Jun **2010**.

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F. Pickford, E. Masliah, M. Britschgi, K. Lucin, R. Narasimhan, **PA. Jaeger**, S. Small, B. Spencer, E. Rockenstein, B. Levine, T. Wyss-Coray: *The Autophagy-related Protein Beclin 1 is Reduced in Early Alzheimer Disease and Regulates Aβ Accumulation.* Journal of Clinical Investigation, 118(6):2190-9 Jun **2008**.

Review and Invited Publications:

PA. Jaeger, T. Wyss-Coray: *The Beclin 1 Complex in Autophagy and Alzheimer's Disease*. Archives of Neurology 67(10):1181-1184, Oct **2010**.

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Talks:

PA. Jaeger, M. Britschgi, CH Sun, H. Johns, S. Pradhan, RC. Petersen, DS. Knopman, BF. Boeve, AL. Boxer, A. Karydas, BL. Miller, R. Rademakers, DW. Dickson, N. Graff-Radford, T. Wyss-Coray: *The Communicome of a Disease: Application and Potential of Human Plasma Proteomics to Study Frontotemporal Dementia.* 40th Annual Meeting of the Society for Neuroscience 2010, San Diego/CA, USA, Nov 13-17, **2010**.

PA. Jaeger, M. Britschgi, H. Johns, CH. Sun, T-Wyss-Coray: *Exploring the Communicome of Frontotemporal Dementia Patients through Plasma Proteomics.* Consortium for Frontotemporal Dementia Research (CFR): Research in Progress Meeting, The J. David Gladstone Institute, San Francisco/CA, USA, Jun 4, **2010**.

PA. Jaeger, F. Pickford, CH. Sun, KM. Lucin, T. Wyss-Coray: *Beclin 1 Regulates APP Turnover in the Endosomal-lysosomal Pathway.* Cold Spring Harbor Meeting "Neurodegenerative Diseases", Cold Spring Harbor Laboratory, Cold Spring Harbor/NY, USA, Dec 4-7, **2008**.

F. Pickford, **PA. Jaeger**, E. Masliah, T. Wyss-Coray: *Amyloid Precursor Protein Processing, Autophagy, and Alzheimer's Disease.* Fall meeting of the German Society for Biochemistry and Molecular Biology (GBM), Hamburg, Germany, Sep 16-19, **2007**.

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Poster Presentations:

PA. Jaeger, M. Britschgi, CH. Sun, S. Pradhan, H. Johns, T. Wyss-Coray: *The Plasma Proteome of Secreted Cellular Communication Factors as a Tool to Study Dementia.* Keystone Symposium "Alzheimer's Disease Beyond Abeta", Copper Mt, CO, Jan 10-15, **2010**.

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Original Publications

The following pages contain the original publications in their final journal layout.

Molecular Neurodegeneration

BioMed Central

Review

Open Access

All-you-can-eat: autophagy in neurodegeneration and neuroprotection

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Abstract

Autophagy is the major pathway involved in the degradation of proteins and organelles, cellular remodeling, and survival during nutrient starvation. Autophagosomal dysfunction has been implicated in an increasing number of diseases from cancer to bacterial and viral infections and more recently in neurodegeneration. While a decrease in autophagic activity appears to interfere with protein degradation and possibly organelle turnover, increased autophagy has been shown to facilitate the clearance of aggregation-prone proteins and promote neuronal survival in a number of disease models. On the other hand, too much autophagic activity can be detrimental as well and lead to cell death, suggesting the regulation of autophagy has an important role in cell fate decisions. An increasing number of model systems are now available to study the role of autophagy in the central nervous system and how it might be exploited to treat disease. We will review here the current knowledge of autophagy in the central nervous system and provide an overview of the various models that have been used to study acute and chronic neurodegeneration.

Background

Cells have a constant need for the building blocks of life: amino acids, lipids, carbohydrates, and nucleic acids. To sustain this catabolic and anabolic need, they rely on uptake and recycling. While nutrient uptake is important, different degradation systems are in place to efficiently turnover recyclable intracellular material and provide quality control. The main pathways for protein degradation and recycling are the ubiquitin/proteasome pathway (for degrading short-lived cytosolic and nuclear proteins) [1], the lysosomal pathway (for cytosolic proteolysis), and autophagy (for bulk cytosolic degradation and organelle recycling) [2]. Deficits in any of these recycling pathways can result in uncontrolled accumulation of cellular debris or severe deficiencies in metabolic productivity, ultimately causing cell death.

The term autophagy, coined from the Greek words of αυτς ('autos', self) and φαγειν ('phagein'), meaning 'eating', was first used in 1963 by Christian de Duve to establish a nomenclature for different cellular pathways and compartments in the endosomal-lysosomal pathway [3]. Early work in autophagy research was done in rat liver cells and autophagy was characterized as a physiological response to starvation in order to degrade and recycle non-essential intracellular macromolecules [4-6]. Later, autophagy [7] and many of the autophagy genes [8] were identified in yeast, which gave the scientific community access to pow-

erful cloning and pathway analysis tools. Subsequent identification of mammalian homologues led to the investigation of the role of autophagy in cancer, programmed cell death, tissue remodeling, heart, liver and muscle diseases, and bacterial and viral infections [9].

In recent years, increasing attention has been focused on the role of autophagy in metabolism of misfolded proteins and neuronal cell death in neurodegeneration (for comprehensive reviews see [10-13]). Abnormal autophagy has been implicated in the pathology of numerous diseases of the central nervous system (CNS), both chronic disorders (such as proteopathies) and many acute injuries. While it is still early in our understanding of this pathway, autophagy seems to have both beneficial and detrimental effects in disease, and it will be key to define the context that determines the outcome.

Types of neuronal autophagy

Autophagy is involved in the intracellular turnover of proteins and cell organelles and has an important role in regulating cell fate in response to stress [14,15]. It is a highly conserved process that occurs in all species and cell types studied thus far. Two main types of mammalian autophagy have been identified and implicated in CNS injury and disease: macroautophagy and chaperone-mediated autophagy. Other more specialized forms of autophagy exist, such as mitophagy (direct targeting of mitochondria to lysosomes) [16], pexophagy (selective degradation of peroxisomes) [17,18], xenophagy (degradation of intracellular bacteria and viruses) [14,19], crinophagy (lysosomal fusion with re-directed exosomes) [20], microautophagy (direct engulfment of cytosol by lysosomes) [21,22], and piecemeal microautophagy of the nucleus (partial sequestration and degradation of the nucleus) [23], but most of them have only been observed in yeast or under special conditions and are not reviewed here.

Macroautophagy is a bulk degradation pathway and the only intracellular mechanism potentially capable of degrading large protein aggregates or damaged organelles. It is a well-understood process in yeast, but details about the exact sequence of events and the proteins involved are still uncertain in mammals. A cup-shaped isolation membrane forms around cytosolic components, eventually fusing to form a double membrane bound vesicle [24,25]. The origin of the membrane material for the formation of the isolation membrane is still under investigation, but recent evidence suggests that it might be derived from the endoplasmatic reticulum (ER) [26]. The protein MAP1LC3 is anchored via conjugated phosphatidylethanolamine (MAP1LC3-II) to the isolation membrane and is a specific marker for the so-called autophagosomes [27]. The autophagosome undergoes several microtubule-[28,29] and dynein-dependent maturation events [30,31], including fusions with multivesicular bodies (MVB), early and/or late endosomes [32,33], before it fuses with lysosomes [34,35] (Fig. 1, for a more comprehensive overview of autophagosome turnover see [28,36]).

At least 12 Atg (autophagy-related) and 4 other proteins are known to be involved in mammalian macroautophagy initiation and execution [37,38] (see Fig. 2). Whether direct autophagosomal-lysosomal fusion is possible, or endosomes first have to deliver essential enzymes to the maturating autophagosomes, is unclear. While the content of the autophagosome initially has the same pH as the surrounding cytosol, it becomes more acidic during its maturation [39,40]. For successful degradation of the autophagosomal content, autophagosomes need to migrate from their site of formation to lysosome rich perinuclear regions [29,41]. After fusion with the lysosome the outer autophagosome membrane can be reused, while lysosomal enzymes degrade the inner membrane and its cytosolic contents, enabling the recycling of macromolecules [42] (Fig. 1). It is unknown which markers, if any, label organelles or cytoplasm for sequestration and inclusion into autophagosomes. One possible marker for protein aggregates is the ubiquitin binding protein sequestosome 1 (SQSTM1, also known as p62) [43]. Almost all protein aggregates are poly-ubiquitinated and SQSTM1 binds both, MAP1LC3 and ubiquitin [44-46]. Macroautophagy components are expressed in neurons and neuronal cell lines (Tab. 1). While the function of autophagy-related proteins has been described for some, it is still unknown for others (Tab. 2). Macroautophagy has been implicated in chronic neurodegenerative diseases and acute neuronal injuries (Tab. 3 and 4).

Chaperone-mediated autophagy (CMA) is distinctly different from macroautophagy in that no vesicular trafficking is involved (Fig. 1). Instead, a pentapeptide motif in substrate proteins allows their specific translocation to the lysosome membrane (reviewed in [47-49]). Thus, CMA degrades only proteins with the motif KFERQ or a biochemically related sequence, which is present in about 30% of all cytosolic proteins [50]. It has recently been suggested that 80% of aminoacyl-tRNA synthases are also substrates for CMA [48], indicating a possible role of CMA in protein synthesis control under starvation conditions.

To be targeted for CMA, substrate proteins first bind to a cytosolic complex containing the chaperone HSC70 (Fig. 1). This complex then interacts with a lysosomal membrane complex containing LAMP2A and HSP90 [51]. The substrate protein is finally degraded after unfolding and translocation into the lysosomal lumen (with the help of lys-HSC70, a luminal form of HSC70) [51]. The chaper-

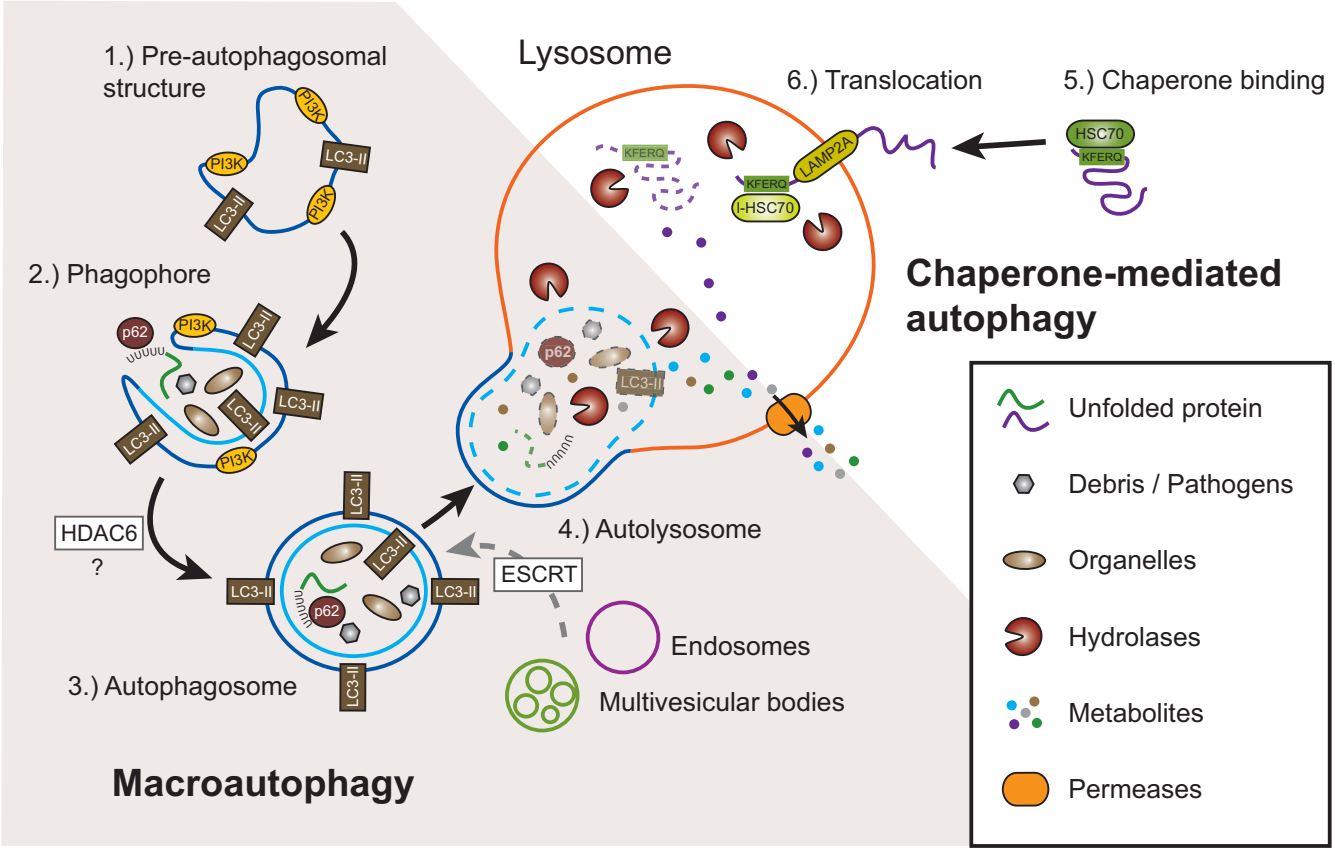

Figure 1

Steps in macroautophagy and chaperone-mediated autophagy (CMA). Macroautophagy: 1.) Nucleation. An unidentified membrane source delivers lipid bi-layers for the formation of the phagophore. In yeast this early structure is termed pre-autophagosomal structure (PAS), its identity in mammalian cells is uncertain. A class III PI3K complex consisting of at least BECN1, PIK3C3, PIK3R4, UVRAG, and AMBRA1 is required for PAS formation and MAP1LC3 is anchored to the membrane via a phosphoethanolamine (PE) anchor (LC3-II). 2.) Expansion. The PAS or a comparable structure in mammals sequesters cytosolic cargo (either specifically via SQSTM1 [p62] or nonspecifically) by invagination, forming a double-membranous vesicle. This stage is also called "isolation membrane". More membrane and LC3-II is being recruited to the developing vacuole. 3.) Maturation. The completed autophagosome undergoes multiple maturation steps and fusion events with multi-vesicular bodies (MVB) or endosomes. The exact nature and sequence of this maturation, and whether these steps are always required is currently unknown. The autophagosomal lumen becomes more acidified during this maturation. 4.) Docking and fusion. During docking and fusion the inner membrane compartment together with its content gets released into the lysosome/autolysosome and is being degraded by lysosomal hydrolases. The components of the outer membrane are available for re-usage. Chaperone-mediated autophagy: 5.) Recognition and binding. The HSC70 chaperone complex (consisting of HSC70, HSP90 and maybe other proteins) recognizes unfolded proteins with the KFERQ sequence and moves them to the lysosome. 6.) Translocation. LAMP2A and a lysosomal form of HSC70 (l-HSC70) translocate the substrate protein across the lysosomal membrane into the lumen for degradation. The autophagy delivered substrates get degraded inside the lysosomes and their macromolecular components are made available to the cell's metabolism via permeases that allow their transport back into the cytosol.

one complex consists of many more proteins but their exact localization and role in CMA is presently unclear [52].

Macroautophagy and CMA are interconnected, although the details of this crosstalk are not well understood. A possible connection is BCL2 associated athanogene (BAG1) which functions as a nucleotide exchange factor for HSC70 [53] and has been reported to bind MAP1LC3 [54]. Impairing macroautophagy, either genetically or pharmacologically, results in a compensatory up-regulation of CMA [55]. CMA components are expressed in neurons and neuronal cell lines (Tab. 1) and CMA has also been implicated in chronic neurodegenerative diseases (Tab. 3).

Autophagy in the healthy nervous system

The brain is well protected against short-term periods of systemic starvation. Selective transport of glucose, amino acids, and hormones across the blood-brain-barrier

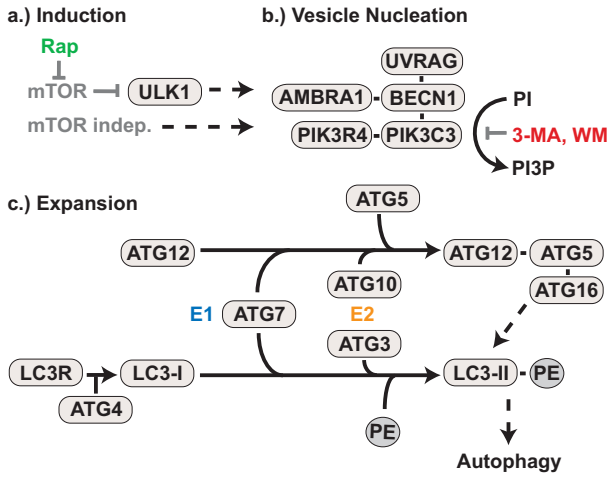

Figure 2
Autophagy pathway in mammals. The formation of autophagosomes appears to follow a pathway conserved across species and most findings made in yeast or other organisms also apply to mammalian autophagy. a.) Autophagy can be induced via mTOR dependent or independent pathways (for more information, see text and Fig. 3) which stimulate the nucleation and expansion of the phagophore/isolation membrane. b.) A multi-protein complex surrounding BECN1 with PI3K activity (mediated by PIK3C3) is important for the formation of the autophagosomal membrane. c.) Two ubiquitin-like modification systems are essential for mammalian autophagy; ATG12 is activated by ATG7 (E1 step), transferred to ATG10 (E2 step), conjugated to ATG5 and subsequently forms a complex with ATG16. This step is necessary early in autophagy for the formation of the phagophore or isolation membrane. MAP1LC3 (LC3) is cleaved by ATG4, activated by ATG7 (E1 step), transferred to ATG3 (E2 step), and conjugated to the phospholipid phosphoethanolamine (PE). This form known as MAP1LC3-II (LC3-II), localizes to the autophagosome membrane and is subsequently degraded in the lysosome. ATG4 cleaves off a C-terminal arginine (R) to expose a glycine residue that is then being linked to PE. Rapamycin (Rap) inhibits mTOR and activates macroautophagy, while 3-methyladenin (3-MA) and wortmannin (WM) inhibit the PI3K activity and de-activate macroautophagy.

ensures ample supply of metabolites and local populations of glia cells release trophic factors under normal or energy restricted conditions. High levels of constitutive autophagy in neurons may therefore not be necessary to maintain the cellular energy needs; indeed, forty-eight hours of food deprivation caused no apparent autophagy induction in the mouse brain [56].

Instead, autophagy probably supports local housekeeping functions within the neuron: macroautophagy is the only cellular mechanism capable of degrading expired organelles in neurons that can live for decades. In addition autophagy is a potential clearing mechanism for protein aggregates that occur frequently in aging neurons, but not in young and healthy cells. Consistent with such a role in the normal brain autophagosome numbers [57] and the levels of MAP1LC3-II protein [56,58,59] are low when compared with other tissues. Nevertheless, recent findings show that autophagy in neurons is indeed constitutively active [60,61] and autophagosomes accumulate rapidly when their clearance is blocked [62], indicating fast basal turnover.

A number of autophagy related genes are expressed (measured either by mRNA or protein analysis) in neuronal tissues of humans [58,59,63-78], rodents [56,59-61,65,75,76,79-107], and insects [97,108-116] (Tab. 1). Electron microscopy of human and mouse brain tissue shows the presence of lysosomes and autophagosomes in neurons further supporting a basal level of autophagy during normal neuronal homeostasis [57,58,117,118]. Model organisms have been crucial for the identification of genes that regulate autophagy and clarification of their function as detailed in Tab. 2[27,40,59-61, 79, 83, 85, 90, 91,97,99,100,107,111-116,119-146].

Age is a major risk factor for many neurodegenerative diseases and a number of studies suggest a role for autophagy in aging. Interestingly, protein degradation and specifically autophagy (both macroautophagy and CMA) decline with age, although to what extent that reduction occurs within the CNS is not clear [147-150]. An age related decline of Atg genes has been shown in *D. melanogaster*, and Atg8 overexpression increases the fly's lifespan [151,152] while RNAi of autophagy genes in *C. elegans* leads to decreased lifespan [136,153]. If and how decreasing autophagy activity in the aging human CNS contributes to the higher prevalence of neurodegenerative diseases and accumulation of various protein aggregates will have to be clarified in future studies.

Autophagy as a clearing mechanism for protein degradation

The strongest evidence for an active role of autophagy in maintaining neuronal homeostasis comes from engineered mutant mice lacking autophagy genes. While *Atg5* and *Atg7* knockout mice had been created before [128,130], their early developmental mortality made the study of the adult CNS impossible. To overcome this limitation, two landmark studies generated conditional knockout mice lacking *Atg5* and *Atg7* only in neurons [60,61].

The $Atg5^{flox/flox}$;nestin-Cre mice showed growth retardation, progressive motor and behavioral deficits, prominent neurodegeneration and axonal swelling in a number of brain regions. Histological examination also revealed abundant ubiquitin-positive inclusions in neurons, indicating a crucial role of autophagy in the turnover of diffuse cytosolic proteins labeled for degradation [60].

Table 1: Presence of autophagy related gene expression in neuronal tissue.

Gene	H. sapiens		M. musculus		R. norvegicus	Gene	D. melanogaster	Gene	C. elegans
	mRNA	Protein		AllenB					
ULK1	[67]		[99,100,107]	Yes	[92]	***Atg1***	[97]	***unc-51***	WoBa [109,110,112,113]
ATG3	[74]		[104]	Yes		***Atg4/Aut1***	[111]	***atg-3***	
ATG4	[68]		[90]	Yes	[105]	***Aut2/Atg4***		***atg-4.1-2***	WoBa
ATG5			[60,91,93,96]	Weak	[87]	***Atg5***		***atg-5/atgr-5***	
BECN1	[59,69]	[59,65,69]	[59,81,96]	Yes	[80,94,95,106]	***Atg6***	[97]	***bec-1***	WoBa [116]
PIK3C3	[66]	[77]		Yes		***Vps34/Pi3K59F***		***vps-34/let-512***	[114]
PIK3R4	[71]			Weak		***Vps15/ird1***		***ZK930.1***	
UVRAG	[72]			Yes					
AMBRA1			[83]	n.a.					
ATG7	[75]		[61,96]	Weak	[75]	***Atg7***		***atg-7/atgr-7***	
MAP1LC3		[58,65]	[56,60,65,79,84,86, 96]	Yes	[87-89,95,103,106]		[97]	***lgg-2***	
GABARAP	[78]			Yes	[89,92]	***Atg8a***		***lgg-1***	WoBa
GABARAPL2	[78]			Yes	[89]				
ATG12			[82,96]	Weak	[87]	***Atg12***		***lgg-3***	
CHMP4B			[85]	n.a.		***shrb/Vps32***	[108,115]	***vps-32.1***	WoBa
HSPA8	[70]	[63,73,76]		Yes	[76,98,101]	***Hsc70-4***		***hsp-1***	
LAMP2		[64]	[102]	Weak	[102,103]				

Examples of autophagy related gene expression in humans and common model organisms (mRNA and/or protein). For human, mouse, and rat genes the approved human gene symbol is used http://www.genenames.org, for D. melanogaster and C. elegans their respective gene symbols (if existent) are provided. *(AllenB):* Gene mRNA is detectable by hybridization as published in the Allen Brain Atlas http://www.brain-map.org; *(WoBa):* neuronal expression data available at WormBase http://www.wormbase.org; *(n.a.):* not available.

Table 2: Neuronal phenotype of autophagy related knockout/knockdown animal models.

Gene (Alias)	Protein function	Knockout/ knockdown	OE/TG	ES/M @ IMSR	Neuronal phenotype after k.o./k.d. (Animal model)	K.o. embryonic lethal
ULK1 (ATG1)	Ser/Thr protein kinase (regulation and vesicle formation)	[107,112,113] * [97,99,100,131,132,135,141,145]	[140] (OE)	ES M (GT)	Impaired endocytosis of nerve growth factor, excessive axon arborization, stunted axon elongation (MM) Paralysis, aberrant axon growth, abnormal vesicles, arrested differentiation (CE)	Yes (DM)
ATG3	Ubiquitin-conjugating-like enzyme (attaches MAP1LC3 to PE)	[111,143]		n.a.	Not reported	Yes (DM)
ATG4	Cystein protease (cleaves C-terminus of MAP1LC3 for conjugation)	[90,144]		ES M (GT/TG)	Not reported	Yes (CE)
ATG5	Unknown (conjugates to ATG12, binds ATG16)	[60] * [91,130,141]		ES M (MUT)	Progressive motor deficits, accumulation of inclusion bodies, neurodegeneration, aberrant vacuoles in Purkinje cells (MM)	No # (DM/MM)
BECN1 (ATG6)	Unknown (part of class III PI3K complex, anchor protein, autophagy initiation)	[59] * [97,116,124,137,146]	[119] (TG)	M (TG)	Neurodegeneration, lysosomal abnormalities (MM)	Yes (MM/CE/DM)
PIK3C3 (VPS34)	Class III PI3K complex (forms complex with BECN1/PIK3R4/AMBRA1/ UVRAG, autophagy initiation)	[123,142] * [114,126]		ES	Abnormal protein aggregation, abnormal locomotion (CE)	Yes (CE)
PIK3R4 (VPS15, P150)	Ser/Thr protein kinase (forms a complex with and activates PIK3C3)	[134]		ES	Not reported	Yes (DM)
AMBRA1	Unknown (component of the class III PI3K complex)	[83] *		ES	Neural tube defects, polyU aggregates, unbalanced cell proliferation, cell death (MM)	Yes (MM)
ATG7	Ubiquitin-activating-like enzyme (activates MAP1LC3 and ATG12 for conjugation)	[61,123,129] * [124,125,127,128,136,141]		ES	Behavioral deficits, neuronal loss, polyU inclusions, axonal dystrophy, axonal terminal degeneration (MM) PolyU aggregates, neuronal degeneration (DM) Abnormal protein aggregation (CE)	No # (DM/MM)

Table 2: Neuronal phenotype of autophagy related knockout/knockdown animal models. *(Continued)*

MAP1LC3 (LC3)	Unknown (similarity with ubiquitin, part of autophagosomal membrane)	[123,145] * [79,97]	[27] (TG)	ES M (TG)	Abnormal protein aggregation (CE)	Yes (CE) No (MM)
ATG12	Unknown (similarity with ubiquitin, conjugated to ATG5)	[123] *		n.a.	Abnormal protein aggregation (CE)	Unknown
CHMP4B (SNF7-2)	Unknown (part of the ESCRT-III complex, involved in surface receptor degradation, formation of MVBs and autophagosomes)	[85,115,138]		ES	Dendritic and axonal branching impaired, dendritic retraction, reduced cell viability, autophagosomes accumulate, increased htt toxicity (DM)	Yes (MM)
HSPA8 (HSC70)	Chaperone (recognizes CMA motif, lysosomal translocation)	[121,139] *	[120] (OE)	ES	Impaired transmitter release, o.e. rescues α-synuclein pathology, Bolwig's nerve projection abnormalities (DM)	Yes (DM)
LAMP2	Unknown (Lysosomal membrane glyco-protein, forms complex with HSPA8)	[40]		ES	Not reported	No (MM)

Examples of model organism with knockout, knockdown, or overexpression of autophagy genes and the corresponding neuronal phenotype. Approved human gene names are used http://www.genenames.org, in addition commonly used aliases are provided. # While non-neuronal *Atg5* and *Atg7* k.o. mice survive birth, they die within one day postnatal. *(MM):* M. musculus; *(DM):* D. melanogaster; *(CE):* C. elegans; *(OE):* overexpression; *(ES):* embryonic knockout stem cell; *(M):* mouse line; *(TG):* transgenic; *(GT):* gene-trap; *(MUT):* targeted mutation; *(IMSR):* knockout ES/mice available through the International Mouse Strain Resource http://www.informatics.jax.org/imsr/index.jsp; *(*):* neuronal tissue examined; *(n.a.):* not available.

In the *Atg7*$^{flox/flox}$;nestin-Cre mice, strikingly similar pathological changes occurred: reduced growth, motor and behavior changes, loss of Purkinje cells, activation of glia cells, and accumulation of ubiquitinated inclusions. Proteasomal function was not impaired by autophagy inhibition, which shows that autophagy has an important role in the basal turnover of poly-ubiquitinated (polyU) proteins together with the proteasome [61]. The ubiquitin-positive aggregates also contain abnormal amounts of SQSTM1 [127].

While polyU proteins themselves are sticky but not highly aggregating, the presence of large amounts of SQSTM1 might enhance their aggregation [43,154]. SQSTM1 can directly interact with MAP1LC3 [45] and tags ubiquitinated protein-aggregates for autophagic degradation [43,155]. It appears that impairment of autophagy leads to the accumulation of SQSTM1, which in turn increases the rate of aggregation for diffuse ubiquitinated proteins. Interestingly, the double knockout of *Atg7* and *Sqstm1* prevents the formation of ubiquitinated aggregates in neurons, but has no effect on the other observed neurodegenerative phenotypes [127], indicating that autophagy plays multiple roles in neuronal homeostasis, not just clearance. This crosstalk between autophagy and the ubiquitin-proteasome system (UPS) is supported by *in vitro* induction of autophagy in response to impaired UPS [156]. SQSTM1 is not the only protein facilitating the degradation of protein aggregates via autophagy, as HDAC6, a microtubule-associated histone deacetylase that interacts with polyU proteins, also provides a link to autophagy (see below [156,157]).

Additional evidence for a role of autophagy in protein turnover comes from mice lacking *Ambra1*, a recently discovered regulator of autophagy that interacts with Beclin 1 (BECN1) [83] (Fig. 2). *Ambra1* knockout mice show polyU inclusions and severe neural tube deficits, unbalanced cell proliferation, and excessive apoptotic cell death. Autophagy has a complex interplay with apoptosis, where it can serve both as an alternative cell-death and as an anti-apoptotic survival mechanism. More details of this relationship will be discussed at the end of this article and comprehensive reviews have been published on this topic elsewhere [133,158].

Autophagy in vesicle sorting and organelle turnover

Another set of important findings indicates that endosomal sorting and endosomal-autophagosomal fusion are impaired in certain neurodegenerative diseases. ESCRT-0

Table 3: Autophagy in common chronic neurodegenerative diseases.

Disease	Autophagosomal phenotype	Ref.
Alzheimer disease	Autophagy appears impaired, autophagosomes accumulate, endosomal-lysosomal abnormalities, increased mitophagy, reduction of macroautophagy enhances pathology, pharmacological activation of macroautophagy can promote the clearance of Aβ/APP and reduces tau pathology, autophagosomes contain APP/Aβ/secretases.	[206,208,59,62,204,207,203,209,205,57,58,118]
Parkinson disease	Autophagy/mitophagy appears impaired, autophagosome-like structures accumulate, pharmacological activation of macroautophagy enhances α-synuclein clearance and is neuroprotective, α-synuclein is a target of CMA and macroautophagy and the proteasome, dopamine-modified/mutated α-synuclein blocks CMA and dopamine induces autophagic cell death and α-synuclein accumulation, mutant UCH-L1 binds to LAMP2A and inhibits CMA.	[220,214,215,213,219,212,102,216,192,210,211,218,217,117]
Huntington diseases	Impaired sorting/degradation of autophagosomes, autophagosomes accumulate, BECN1 is recruited to htt inclusions and BECN1 reduction causes enhanced htt accumulation, pharmacological or signaling mediated activation of macroautophagy reduces htt toxicity, mTOR is sequestered into htt inclusions, which causes macroautophagy activation.	[225,227,228,216,229-231,203,221,226,224,195,223,222]
Frontotemporal dementia	Impaired endosome maturation, enlarged autophagosome accumulation, mutant CHMP2B disturbs the ESCRT-III complex for endosomal sorting which results in polyU/SQSTM1 aggregates.	[162,85]
Amyotrophic lateral sclerosis	Impaired early endosomes, impaired sorting/degradation of autophagosomes, CHMP2B disturbs the ESCRT-III complex for endosomal/MVB sorting which results in polyU/SQSTM1 aggregates, MVBs are required for TDP-43 clearance, Lithium activates protective autophagy.	[232,86,162,233]

to III (endosomal sorting complex required for transport) orchestrate the progression of endosomes along the endosomal-lysosomal pathway. Dysfunction of one of these complexes (ESCRT-III), either by RNAi depletion of its essential subunit *CHMP4B* (also known as *SNF7-2*) or by expression of a mutant CHMP2B protein (another subunit of ESCRT-III and associated with Frontotemporal dementia linked to chromosome 3), caused autophagosome and polyU protein aggregate accumulation, and dendritic retraction followed by neuronal death in cultured mature cortical neurons [85]. It has been suggested that the endosomal and autophagosomal pathways merge upstream of lysosomal fusion [159-161], in particular that intact multivesicular bodies (MVB) are essential for autophagosome maturation [138,162]. ESCRT-III seems to play an important role during this endosomal-autophagosomal fusion event and its dysfunction leads to impaired processing and accumulation of autophagosomes. In a recent paper, deletion of the *Hrs* (also known as *Hgs*) gene, a component of ESCRT-0, in the neurons of $Hrs^{flox/flox}$;SynI-cre mice caused apoptosis, loss of hippocampal CA3 pyramidal neurons, and accumulation of polyU proteins and SQSTM1 [163]. Accordingly, locomotor activity and learning ability were severely reduced in these mice.

While no evidence for the autophagosomal degradation of specific neuronal organelles (such as synaptic vesicles) in healthy neurons exists thus far, mitochondria were selectively degraded by macroautophagy in neurons exposed to experimental neurotoxins 1-methyl-4-phenylpyridinium (MPP+) or 6-Hydroxydopamine, which induce mitochondrial damage [164,165]. Autophagosomes were also observed in dopaminergic neurons treated with methamphetamine [166], supporting the idea that autophagy serves to clear damaged organelles in neurons. Together, these studies underline the critical role of autophagosomal-endosomal-lysosomal trafficking and sorting in neuronal homeostasis

Autophagosomes as transport vacuoles

Autophagosomes are not only found in the soma but also in the distal parts of the axon and dendrites and can be retrogradely transported to the cell soma for degradation [167]. Autophagy may thus support neurite and growth cone remodeling and clear axons and dendrites of defec-

Table 4: Autophagy in acute neuronal injury.

Injury	Autophagy related changes	Ref.
Hypoxia/Ischemia	Mixed results after hypoxic treatments: Knockout of Atg genes in C. elegans decreases survival after hypoxia and autophagy activation by rapamycin treatment leads to injury reduction in rat and rat tissue. On the contrary, *Atg7*$^{-/-}$ mice lacking functional autophagy in the CNS are largely protected from neurodegeneration.	[247,80,104,94,244,246,245]
Trauma	Macroautophagy appears to be beneficial: Autophagy can be activated for more than a month following brain trauma (elevated BECN1, MAP1LC3-II, ATG5-12 levels, increased AV numbers) in rodents, autophagy appears activated in human tissue samples. Rapamycin treatment is neuroprotective in mice.	[106,87,249,248,65,95,84,81,250]
Pharmacological injury	Autophagy appears to be deleterious: Transient activation of autophagy after injury (elevated MAP1LC3-II, p-mTOR, LAMP2, increased AV numbers) and activation of apoptosis in rodents and primary neuronal culture. 3-MA treatment or RNAi against *ATG5* or *BECN1* blocks cell death.	[96,252,166,103,253,251,254]
Trophic deprivation	Autophagy appears to be deleterious: Growth factor withdrawal leads to autophagic cell death in rodents or chicken, 3-MA blocks cytochrome C release and delays apoptosis.	[257,255,256,259,258]

tive larger structures. Efficient bi-directional transport along the axon is necessary for neuronal survival [168,169] and supports the clearing of protein aggregates by autophagosomes [31].

In addition, autophagosomes are retrogradely transported, making them potential transport vacuoles for the delivery of trophic factors from the synapse to the cell body. Autophagosomes can travel along microtubules, possibly facilitated through an interaction between MAP1LC3 and MAP1A/B [29,41]. Some evidence exists that signaling endosomes containing nerve growth factor (NGF) might be derived from or be related to autophagosomes, based on the microscopic association of fluorescently labeled LC3 with retrogradely transported NGF and the NGF receptors TrkA and p75 [170]. This finding could indicate that disturbed autophagy (for example, as a result of changes in APP expression or metabolism) might contribute to the reported impairment of NGF transport in neurodegenerative diseases such as Down's syndrome. In this condition, an extra copy of chromosome 21, which contains the *APP* gene, results in increased APP expression and the development of Alzheimer-like dementia. Intriguingly, in a trisomic mouse model of Down's syndrome deletion of one copy of *APP* led to a marked improvement in transport of signaling endosomes containing NGF, reduced neurodegeneration, and improved cognitive function [171].

Several studies point towards an important role of ULK1 in this trafficking role of autophagy. For example, knockdown of *ULK1* by RNAi in cultured mouse spinal sensory neurons leads to impaired endocytosis of NGF [107]. Axonal growth appears stunted in *C. elegans* in *unc-51* mutants [110,112] and after *ULK1* knockdown in mouse neuronal cells [107], while dominant negative *ULK1* mutants expressed in immature murine cerebellar granule cells lead to inhibition of neurite outgrowth and developmental arrest [99]. ULK1 is important for autophagy initiation and has been reported to interact with GABARAP and GABARAPL2 (also known as GATE16), two homologues of MAP1LC3, in mouse pyramidal, mitral, and Purkinje cells. This interaction indicates an involvement of autophagosome transport in some of the *ULK1* knockdown phenotypes [92], although it clearly has functions independent of autophagy [100,172].

Another interaction between autophagy and neuronal receptors was found in Lurcher mice, which have a mutation in the glutamate receptor GluRδ2 and are a model for ataxia. The mutated receptor GluRδ2^{Lc}, but not the wildtype receptor, bind to BECN1 and may thus trigger autophagy in dying Purkinje cells in Lurcher mice [173,174]. In this way, autophagy might serve as an early stress response to axonal dystrophy. Autophagosomes appear rapidly in axons in Lurcher mice and this is attributed to the induction and local synthesis of autophagosomes in axon terminals in response to stress [174]. How autophagosomes form so fast in distal cell parts is unclear, but early ultrastructural studies suggest that smooth ER in axons might be a source for quick membrane supply [175,176].

Regulation of autophagy

Because of its key function in cell homeostasis, multiple signaling cascades have been implicated in the regulation of autophagy (Fig. 3). A large amount of this knowledge has been acquired in yeast and it is unknown how much can be translated to mammalian cells (for reviews see [177-179]). One of the key regulators of autophagy is the level of amino acids, both extracellular and intracellular. Cells measure intracellular amino acid levels via the protein kinase EIF2AK4 (also known as GCN2), which is activated by unloaded transfer RNAs. Low levels of intracellular amino acids leading to free transfer RNAs thus activate autophagy through phosphorylation of the eukaryotic initiation factor eIFα2 [180]. Extracellular amino acids are sensed via a putative receptor in the cell membrane [181], which seems to signal through mammalian target of rapamycin (mTOR, also known as FRAP1). mTOR is a protein kinase that plays a central role in nutrient sensing, cell proliferation, and metabolism [182-184], integrating many signaling pathways. Activated mTOR promotes protein synthesis and inhibits autophagy via phosphorylation of the ULK1 binding partner ATG13, while deactivated mTOR activates autophagy [185]. Insulin and growth factors signal through AKT, activate mTOR [182,186] and deactivate autophagy, while energy depletion [187] or elevated intracellular calcium [188] inhibit mTOR through AMP-activated protein kinase (AMPK) and activate autophagy. Other signaling cascades implicated in the regulation of autophagy include Ras/Raf and ERK signaling (mTOR dependent [189] or independent [190]) and the mTOR independent inositol signaling pathway [191,192]. Lastly, autophagy may be induced "directly" through the presence of intracellular inclusions [193-195]. It is unclear which of these pathways are involved in neurodegenerative conditions.

Even less is known about the transcriptional control of autophagy, especially in neurons. Nevertheless, a number of important transcription factors have been associated with the regulation of autophagy genes in non-neural cell types. Since these processes are likely conserved, they may contribute to the control of autophagy in neurons as well.

In one study, a high-affinity E2F4 transcription factor-binding region in the *BECN1* promoter was identified [196]. A number of autophagy proteins are also controlled by the FOXO3 transcription factor in muscle cells [197,198] and potentially hepatoma and pheochromocytoma cells [198]. In these cell types, FOXO3 binds directly to the promoters of *MAP1LC3*, *ATG12*, and *GABARAP* genes to increase their expression and induce autophagy [197]. Indeed, FOXO3 increases the expression of ATG4, PIK3C3 and BECN1, but the exact mechanisms are unknown [198].

Members of the p53 family also play important roles in autophagy control: Cytosolic p53 inhibits autophagy [199], whereas nuclear p53 activates it [200]. The localization of p53 appears to be a sensor for genotoxic stress. In addition, p53 acts upstream of mTOR, inhibiting its activity through AMPK, thus stimulating autophagy. Recently, a p53 homologue, p73, has been identified by integrating whole-genome chromatin immunoprecipitation and expression profiling in cell culture that binds to regulatory regions of several autophagy genes (*ATG5*, *ATG7*, *UVRAG*, *GABARAP*, *AMBRA1*, *ATG16*, *PIK3C3*) presumably through its nuclear activity [201,202]. Further studies that investigate the upstream control of autophagy in neurons will greatly help to improve our understanding of the potential misregulation of autophagy during neurodegeneration.

The above findings suggest three main roles for autophagy in neuronal homeostasis: First, impaired autophagy results in abnormal protein aggregation across species, indicating an involvement of autophagy in the clearance of intracellular protein aggregates, especially when these aggregates are poly-ubiquitinated. Second, changes in vesicular appearance and trafficking point towards a crucial role of autophagy in maintaining the normal turnover and flux of vacuolar compartments and possibly trophic factors through the neuron. And third, disrupted autophagy leads to changes in neuronal morphology and connectivity, such as excessive axon arborization, stunted axon growth, axonal dystrophy, axonal terminal degeneration or impaired axonal projections, implicating autophagy genes and their gene products in neuronal shaping, connectivity, and development. Whether these observations are always directly linked to the gene's role in autophagy or are sometimes a result of non-autophagic functions remains to be determined.

Autophagy in CNS disease and injury

Several excellent reviews have recently covered the emerging relationship between autophagy and various neurodegenerative diseases [10-13] and we provide only a brief overview of the most prevalent diseases associated with histopathological changes in autophagy. Instead, we summarize here which aspects of autophagosomal pathology that have been observed in human disease are now being successfully replicated in model systems (Tab. 3 and 4).

In general, the effect of autophagy in neurons during disease can be broadly divided into two classes: autophagosomal degradation is either impaired or excessively activated, leading to an apparent disruption of the intracellular organelle organization and accumulation of autophagosomes in neurons over long periods of time (chronic conditions, Tab. 3), or autophagy genes are acti-

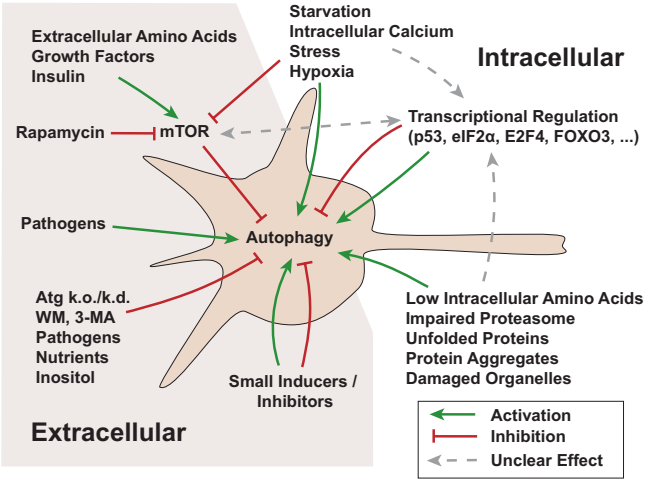

Figure 3
Control of autophagy. Autophagy is a major housekeeping pathway and under the control of many different signaling cascades. Mammalian Target of rapamycin (mTOR) plays a central role in the regulation of autophagic activity as it integrates signaling from different sensors of cellular homeostasis. When mTOR is active in yeast it keeps an important ULK1 binding partner (ATG13) phosphorylated, thus inhibiting the induction of autophagy. While signals indicating abundant nutritional and trophic support activate mTOR (and deactivate autophagy), signals of starvation or other stressors inhibit mTOR (and activate autophagy). Autophagy can be directly stimulated by intracellular debris (such as unfolded proteins and damaged organelles) or by indicators of an overwhelmed ubiquitin-proteasome system (UPS). Also certain pathogens activate autophagy. Autophagy can be directly inhibited by genetic ablation of important Atg genes, inhibitors of the class III PI3K-complex (WM, 3-MA), high nutrient levels, and inositol signaling. More recently screenings of small compound libraries have yielded inducers and inhibitors of autophagy, both mTOR dependent and independent. And last, transcriptional regulators, such as p53, eIF2α, E2F4, or FOXO3 regulate autophagy by controlling the expression levels of many Atg genes. For further details, please refer to the text.

vated in response to temporary injury/stress (acute response, Tab. 4).

Autophagy in chronic CNS diseases

Typical examples of the first class of diseases are Alzheimer (AD) [57-59,62,118,203-209], Parkinson (PD) [102,117,192,210-220], and Huntington disease (HD) [195,203,216,221-231] (Tab. 3). In these diseases, the pathological accumulation of autophagosomes/autophagosome-like structures and abnormalities in the endosomal-lysosomal pathway were documented by electron microscopy (EM) in human postmortem brain tissue [57,58,117,118,207]. Diseases with a seemingly more endosomal pathology, but an autophagic component, are Amyotrophic lateral sclerosis (ALS) and Frontotemporal dementia (FTD) [85,86,162,232,233].

In Alzheimer research, expression analysis revealed that *BECN1* mRNA is reduced in AD brain tissue [59,234], and BECN1 protein levels are significantly lower in the cortex of AD patients compared with age-matched controls [59]. This is despite the fact that an increase in autophagosome numbers in neurons from AD patients is obvious by EM, and AD brains also show increased levels of MAP1LC3-I and MAP1LC3-II [58]. A possible explanation for this apparent contradiction is that reduced BECN1 levels lead to changes in autophagosomal flux. This in turn could impair endosomal-lysosomal degradation, leading to a built-up of intracellular vesicular compartments over time. Changes in the endosomal-lysosomal pathway are amongst the earliest changes in AD [235] and a possible indicator for disturbed vacuolar trafficking.

While the aforementioned studies were descriptive, one of the first mechanistic insights into the possible role of autophagy in neurodegenerative diseases was provided by a study of primary neurons from a mouse model for HD. The authors observed increased autophagy, increased oxidative stress, and polyU aggregates in cultured striatal neurons from transgenic mice expressing mutant human huntingtin in response to a single exposure of a neurotoxic concentration of dopamine [223]. The results suggest that dopamine triggered free radical-mediated oxidation of macromolecules and stimulated autophagy. Subsequent studies demonstrated that SQSTM1 extensively decorates polyU protein aggregates, co-localizes with MAP1LC3 and becomes sequestered in autophagosomes. This highlights the importance of autophagy as a degradative pathway for polyU aggregates [43]. Another link between autophagy and protein aggregates was provided by a study showing that mTOR accumulates in huntingtin aggregates in cells, mice, and human brains [226]. The authors speculate that mTOR can be sequestered and inactivated in this way, leading to a protective induction of autophagic degradation of protein aggregates. Arguing against this interpretation is the observation that BECN1, a protein necessary for the induction of autophagy, is recruited into pathological huntingtin aggregates in human brain tissue as well [230].

The effect of autophagy on the degradation of protein aggregates was investigated further in cell culture and animal models using pharmacological inducers and inhibitors of autophagy (see Tab. 4). It was discovered that rapamycin, an inducer of autophagy, leads to the clearance of polyQ/polyA aggregates in cell culture, fly, and mouse models of HD [195,226]. This finding was confirmed for α-synuclein in cell culture [218] and wildtype tau in flies [203]. Together, these results triggered a con-

certed research effort to find mTOR dependent and independent pharmacological inducers of autophagy and led to the discovery of many small compounds that facilitate the clearing of aggregated proteins [216,219,229,236]. While pharmacological autophagy stimulation reduces the toxicity of many aggregate-prone proteins, experiments in cell culture demonstrate that α-synuclein can be degraded by both the proteasome and autophagy. Pharmacological inhibition of either pathway leads to increased intracellular α-synuclein levels [218]. Interestingly, pharmacological inhibition of microtubule formation by nocodazole treatment inhibits polyQ aggregate formation and at the same time increases its toxicity in cell culture [237,238]. This is at least partially due to the inhibition of autophagosome-lysosome fusion [239], demonstrating that intracellular transport is essential for proper aggresome/inclusion body formation and autophagosomal function. Furthermore, activation of autophagy through starvation in primary cortical mouse neurons expressing polyQ proteins protects against cell death [186]. In summary, autophagy might be especially effective in clearing aggregated proteins.

While these pharmacological studies increase our understanding of some aspects of autophagy in neurodegeneration, they mostly employ drugs that are rather nonspecific and they target proteins such as mTOR and AKT, which have broad functions outside autophagy. Genetic or RNAi-based methods overcome some of these limitations.

It has been shown, for example, that cytosolic protein aggregates can be specifically targeted by autophagy and that their aggregation increases after inhibition of autophagy by siRNA knockdown of *MAP1LC3* in cell culture [221]. In *C. elegans*, RNAi mediated deletion of *bec-1*, *atgr-7*, and *Ce-atg18* led to increased accumulation of polyQ aggregates in models for HD, confirming the earlier studies in mammalian cell culture systems [124].

The cytoplasmic histone deacetylase HDAC6, although not directly an autophagy related protein, plays an essential role in the microtubule- and dynein-dependent intracellular movement of polyU protein aggregates [240]. *HDAC6* RNAi impairs retrograde transport of autophagosomes and lysosomes [156]. HDAC6 overexpression, on the other hand, is sufficient to rescue neurodegeneration caused by proteasome mutations or polyQ toxicity in transgenic flies via autophagy, providing a direct link between UPS and autophagy [157]. HDAC6 activates autophagy by an unknown mechanism, leading to accelerated protein turnover. Potential mechanisms include modulation of HSP90 (and maybe CMA), a substrate of HDAC6 [241], accelerated transport of polyU-proteins into aggregates and to autophagosomes [240], and enhanced transport of lysosomes to autophagosomes [156]. The importance of autophagosomal transport for effective clearance of aggregated proteins has been demonstrated in HD fly and mouse models, where dynein mutations caused increased aggregate formation and decreased autophagosome-lysosome fusion [31].

Recently, autophagy was genetically manipulated in a mouse model of AD by crossing *Becn1* heterozygous knockout mice (*Becn1*$^{+/-}$) with human amyloid precursor protein (APP) transgenic mice. *Becn1* deficiency resulted in neurodegeneration and increased β-amyloid (Aβ) deposition in APP mice [59]. Based on these findings and new cell culture data from our lab (Jaeger *et al.*, manuscript in preparation) we propose that autophagosomes can degrade APP and thus lower Aβ accumulation [59]. On the other hand, autophagosomes contain the enzymes necessary for processing of APP into Aβ and are potential producers of this toxic peptide [58]. A decisive factor that determines whether autophagy reduces or promotes Aβ accumulation might be the speed of autophagosomal turnover and the clearance of autophagic vesicles. Both are impaired under disease conditions [62]. Disturbances in autophagy initiation due to insufficient BECN1 levels could cause expansion of the endosomal-lysosomal system, producing a high load of potentially Aβ generating vacuoles. Interestingly, two APP mouse models for AD have been analyzed for changes in Becn1 levels, but no differences were detected [59]. These findings hint at an autophagy dysfunction upstream of APP pathology in AD.

CMA is also clearly involved in chronic neurodegenerative diseases, most prominently in PD: HSP90 levels are increased in human PD brains and are correlated with the levels of insoluble α-Synuclein [242]. In the same study, immunohistochemistry and EM show that HSP90 co-localizes with α-synuclein in Lewy bodies, Lewy neurites, and glia cell inclusions, both in PD patients and α-synuclein transgenic mice. Furthermore, HSP90 and HSC70 co-immunoprecipitate with α-synuclein in cell culture [242]. While this could indicate increased (protective) CMA in PD, a recent gene expression profiling of substantia nigra tissue from sporadic PD patients revealed reduced expression of UPS proteins and reduced HSC70 [243]. At some point during disease progression, HSP90 may be sequestered into α-synuclein aggregates and deactivated, thus reducing CMA activity.

A landmark study identified α-synuclein as a target for CMA and demonstrated that the PD associated mutations A53T and A30P cause α-synuclein to bind to the CMA receptor and inhibit both the degradation of the receptor itself and that of other CMA substrates [210]. While these α-synuclein mutations are relatively rare, recent findings demonstrate that post-translational modifications of wildtype α-synuclein through dopamine can cause a similar toxic gain-of-function behavior [213]. Furthermore, inhibition of CMA by lentiviral RNAi against *LAMP2*

increases the level of endogenous α-synuclein in rat cortical neurons [102]. Additionally, a link has been suggested between the PD associated mutant ubiquitin carboxyl-terminal esterase L1 (UCH-L1) and the lysosomal receptor for chaperone-mediated autophagy. This mutant UCH-L1 interacts aberrantly with LAMP2, HSC70, and HSP90, inhibits CMA and causes an increase in α-synuclein in cell culture [212].

While the role of autophagy in neurodegenerative diseases is far from being understood, the available data indicate it plays an integral role in the cellular response to intracellular protein aggregation common to these diseases. Autophagy appears impaired in the final stages of neurodegenerative diseases, whereas alterations in vacuolar trafficking are apparent in early stages, often before other histopathological changes manifest themselves. It is therefore likely that autophagy, UPS, the endosomal-lysosomal pathway, and the escalating accumulation of toxic proteins are tightly connected. Whether mutant or misfolded proteins are causing the changes in vacuolar trafficking and later autophagy or whether abnormalities in these protein degradation pathways precede protein aggregation remains to be shown.

Autophagy in acute CNS diseases and injuries

The second class of brain insults that present with an autophagy phenotype are acute injuries or stressors which activate competing cellular death and pro-survival pathways (Tab. 4). Examples include hypoxia/ischemia [80,94,104,244-247], brain trauma [65, 81,84, 87,95, 106,248-250], experimental pharmacological injury models (kainate, methamphetamine, oxidative stress and others) [96,103,166,251-254], and trophic factor deprivation [255-259]. Similar to chronic neurodegenerative conditions, many observational studies find increased levels of autophagy proteins and/or numbers of autophagosomes after acute CNS injury such as hypoxia/ischemia or trauma [81,87,94,95,104,106,244,246,248,250].

As described in the previous chapter above, autophagy has beneficial functions in neurons that seem to be relevant for acute injury as well. For example, the autophagy inducing drug rapamycin reduced brain injury and protected neurons in a rat model of neonatal hypoxia/ischemia [80,249] or traumatic brain injury in mice [80,249]. Consistent with these findings, RNAi mediated knockdown of *bec-1*, *lgg-1*, and *lgg-2*, or mutation of *unc-51* reduced survival after hypoxia in *C. elegans* [247].

However, in contrast to most studies in chronic degenerative models, acute pharmacologically induced injury or withdrawal of trophic support triggered cell death that involved autophagy and signs of apoptosis (Tab. 4). In support for a role in promoting cell death, inhibition of autophagy by 3-methyladenine (3-MA) treatment, decreased the toxic effects or delayed neuronal loss after noxious treatments [103,253,254,260]. Likewise, knockdown of *ATG5* or *BECN1* by RNAi reduced cell death in photoreceptor cells that were exposed to oxidative stress [253]. Maybe most convincingly, $Atg7^{flox/flox}$;nestin-Cre mice lacking *Atg7* in the neuronal lineage are almost completely protected against stroke-induced neurodegeneration [245].

Why seemingly similar studies come to these opposing conclusions is not clear at this point but differences in the models, the tools used to analyze autophagy, or the time of analysis after injury could be responsible. In support of the last point, autophagy was still increased in surviving cells at the injury site one month after traumatic brain injury [106] while cells undergoing necrotic or apoptotic death (and possibly involving autophagy in its detrimental role) would likely have disappeared. It will therefore be interesting to explore whether inhibiting autophagy early or late after a traumatic brain injury may have different outcomes. In addition, a better understanding of how exactly autophagy contributes to cell death and how it interacts with necrotic and apoptotic death programs is necessary.

Autophagy and Apoptosis

As described in the previous chapters, autophagy in the CNS can be protective under some circumstances, while it leads to cell death in others. Furthermore the resulting cell death can be either apoptotic (type I cell death) or autophagic (type II cell death), depending on the cellular setting and inducing stressor (see also reviews [133,158]). This dichotomous role of autophagy is the result of a complex relationship between the autophagy and apoptosis pathways (Fig. 4). While some mixed phenotypes have been reported [261-263], autophagy and apoptosis ultimately develop in a mutually exclusive way and appear to inhibit each other [264-267].

Strong evidence for a role of autophagy as an alternative cell death mechanism comes from mice deficient in apoptosis. One of the key features of apoptotic cell death is the mitochondrial outer membrane permeabilization (MOMP), which requires the two BCL2 family proteins BAX and BAK1. Cells from $Bax^{-/-}Bak^{-/-}$ knockout mice are resistant to various apoptotic stimuli, but can die through a delayed autophagic cell death in response to DNA damage [268]. Autophagic cell death can also be observed after caspase inhibition, a treatment that disrupts normal apoptosis [266]. Conversely, inhibition of autophagy via RNAi targeting various autophagy genes (*ATG5*, *ATG7*, *BECN1*) can reduce autophagic cell death in certain situations [268-270].

In contrast to its function as a cell death mechanism, autophagy is induced under starvation conditions to supply the cell's metabolic needs. Under these conditions, inhibition of autophagy results in cell death [8]. Even without starvation, loss of autophagy itself (as in the *Atg5*$^{-/-}$ or *Atg7*$^{-/-}$ knockout mice) is sufficient to cause neuronal apoptosis [60,61], and it has been suggested that autophagy is primarily a pro-survival pathway [271].

It has been shown that autophagy and apoptosis share common inducers such as reactive oxidative species (ROS), ceramide, and intracellular calcium [188,272-275]. The two pathways are further linked through ATG5 proteolysis [275], the transcription factor p53 [276], and the BCL2 protein family (via BECN1) [277] (Fig. 4). How the balance between autophagy and apoptosis is maintained in neurons requires further investigation.

Concluding remarks

Unknown to most neuroscientists just a few years ago, autophagy has gained increasing attention not only from translational researchers but also from basic neuroscientists interested in neuronal cell biology. Consequently, there are few answers as to the role and relevance of autophagy in neurons, let alone in glia cells, and very few genetic *in vivo* studies have been conducted to investigate its role in neurological disease. Nevertheless, it seems clear that neurons require autophagy for normal function and that neuronal stress will rapidly trigger this pathway (see Appendix 1: Key Observations). There is growing consent that intraneuronal protein aggregates trigger autophagy and that this response is beneficial – at least in its intent. This notion is supported by a limited number of pharmacological and genetic studies in animal models, which demonstrate that reduced autophagy promotes neurodegenerative disease while increased autophagy is beneficial. In contrast, work from stroke models and other acute forms of neural injury indicate that autophagy can be detrimental in such circumstances and promotes cell death. It will be necessary to employ state of the art genetic and molecular tools to dissect the role of autophagy in normal and pathological conditions in cell culture and in mammalian disease models (see Appendix 2: Critical Next Steps). Conditional knockout mice are being developed or are already available to target autophagy not only in neurons but also in astrocytes, oligodendrocytes and microglia. Such studies are likely to add additional complexity to our understanding of autophagy but they may also uncover new therapeutic opportunities. Self-eating, after all, does not equate with self-destruction but may in fact be a powerful survival pathway for the cell, and as such, of key importance to neurodegeneration or neuroprotection.

Abbreviations

3-MA: 3-Methyladenine; Atg: Autophagy related genes; AD/PD/HD: Alzheimer/Parkinson/Huntington disease; APP: Amyloid precursor protein; AV: Autophagic vesicles; CMA: Chaperone-mediated autophagy; CNS: Central nervous system; EM: Electron microscopy; ER: Endoplasmatic reticulum; htt: Huntingtin; MOMP: Mitochondrial outer membrane permeabilization: MVB: Multivesicular body; NGF: Nerve growth factor; PE: Phosphoethanolamine; PI3K: Phosphoinositide 3-kinase; polyQ/polyA/polyU: Proteins with long sequences of Glu/Ala or that are ubiquitin decorated; Rap: Rapamycin: ROS: Reactive oxidative species: UPS: Ubiquitin-proteasome system; WM: Wortmannin;

Competing interests

The authors declare that they have no competing interests.

Appendix 1

Key Observations

- Autophagy plays a crucial role in maintaining neuronal homeostasis through clearance of defective organelles and unfolded/aggregating proteins. Knockout of autophagy pathway genes leads to accumulation of poly-ubiquitinated protein aggregates and can result in neurodegeneration, and motor and behavioral deficits in mice.

- Autophagy interacts with other protein degradation and vesicular trafficking pathways. While autophagy can at least partially substitute for reduced proteasomal activity and vice versa, the disturbance of the endosomal-lysosomal system disrupts autophagy and reduced autophagy impairs endosomal-lysosomal trafficking.

- Autophagy clears neurotoxic proteins. Activation of autophagy reduces the toxicity of aggregation prone proteins, while inhibition of autophagy impairs their clearance and causes enhanced cellular stress and neurodegeneration.

- Autophagy can be a cellular death pathway, which is activated in neurons after acute injury and inhibition of autophagy under those conditions can reduce neurodegeneration.

- Autophagy is impaired in the final stages of most neurodegenerative diseases.

Appendix 2

Critical Next Steps

- What is the sequence of events? Impaired autophagy is a histopathological hallmark of many neurodegen-

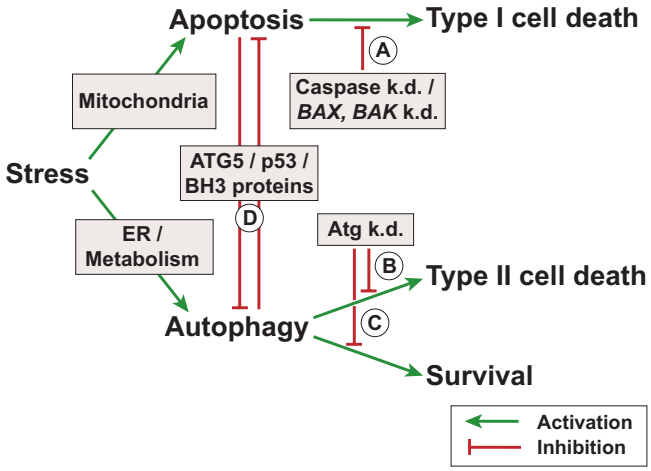

Figure 4

Interaction between autophagy and apoptosis. Cellular stressors can lead to mitochondria outer membrane permeabilization (MOMP) and subsequent cytochrome c release and apoptosis, while nutrient deficiency or ER stress can cause autophagy activation. Under physiological conditions autophagy and apoptosis keep each other inactive through mutual inhibition. A strong apoptotic stimulus (for example DNA damage, death-receptor stimulation, or cytokine deprivation) can drive a cell into apoptotic 'type I' cell death. If apoptosis is inhibited under such conditions (by caspase knockout or *Bax*/*Bak* knockout, [A]), autophagy can become activated and result in a delayed 'type II' cell death through degradation of most cytoplasmic cell components and organelles. Under these circumstances the knockdown of autophagy related genes [B] reduces cell death. Autophagy can become activated through ER stress (for example accumulation of misfolded proteins in the ER, intracellular calcium release from the ER) or nutrient deficiency. The cell then ensures survival by enhancing metabolic recycling through autophagy and adapting to the new nutrient conditions. Knockdown of autophagy genes in such a situation leads to an increase in apoptotic 'type I' cell death [C]. The crosstalk between autophagy and apoptosis [D] is mediated via proteolytic processing of ATG5, the transcription factor p53, and the binding and subcellular localization of BCL2 family proteins with BH3 domains. For further details, please refer to the references in the text.

erative diseases. But it is unknown if autophagy is first impaired, contributing to the disease early on, or if autophagy is highly active to fight the disease and is overwhelmed in the end. The use of inducible knockout animals crossed with traditional disease models or RNAi against autophagy genes in different disease stages could help to elucidate this problem.

• Which autophagy genes are involved? Autophagy is mediated through an evolutionary conserved pathway involving more than 20 proteins. Several of them link autophagy to other important cellular pathways such as apoptosis, the ubiquitin/proteasome system, the endosomal-lysosomal system, and vesicle and receptor trafficking. Which proteins are involved in neurodegeneration is not well understood. Careful analysis of autophagy activity, and mRNA and protein levels of central autophagy genes in tissue from human patients and animal models could help us identify the key players.

• What genetic mutations are associated with autophagy and altered susceptibility to neurodegeneration? While some data exist about mutations in disease-associated genes that interact with autophagy, no mutations in human autophagy genes that cause neurodegeneration are known so far. If autophagy plays a central role in protein clearance, the identification of change-of-function mutations in autophagy genes would be essential to define "autophagosomal diseases".

• How can autophagy be modulated to enhance clinical outcome? The discovery of drugs beyond rapamycin to enhance autophagy has made substantial progress. Because autophagy is linked with multiple intracellular pathways, the identification and functional characterization of key proteins that specifically control only limited aspects of this interplay could help design more precise modulators of autophagic activity, with lessened effects on connected pathways.

Authors' contributions

PAJ collected references, generated the artwork, and wrote the manuscript with input from TWC.

Note

Throughout this review approved human gene and protein names are used to describe experiments and general observations (independent of the actual source species of the cells or the findings discussed). Only for targeted disruption of endogenous genes (such as knockout mice) species-specific nomenclature is used.

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Regulation of Amyloid Precursor Protein Processing by the Beclin 1 Complex

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Abstract

Autophagy is an intracellular degradation pathway that functions in protein and organelle turnover in response to starvation and cellular stress. Autophagy is initiated by the formation of a complex containing Beclin 1 (BECN1) and its binding partner Phosphoinositide-3-kinase, class 3 (PIK3C3). Recently, BECN1 deficiency was shown to enhance the pathology of a mouse model of Alzheimer Disease (AD). However, the mechanism by which BECN1 or autophagy mediate these effects are unknown. Here, we report that the levels of Amyloid precursor protein (APP) and its metabolites can be reduced through autophagy activation, indicating that they are a substrate for autophagy. Furthermore, we find that knockdown of *Becn1* in cell culture increases the levels of APP and its metabolites. Accumulation of APP and APP C-terminal fragments (APP-CTF) are accompanied by impaired autophagosomal clearance. Pharmacological inhibition of autophagosomal-lysosomal degradation causes a comparable accumulation of APP and APP-metabolites in autophagosomes. *Becn1* reduction in cell culture leads to lower levels of its binding partner Pik3c3 and increased presence of Microtubule-associated protein 1, light chain 3 (LC3). Overexpression of *Becn1*, on the other hand, reduces cellular APP levels. In line with these observations, we detected less BECN1 and PIK3C3 but more LC3 protein in brains of AD patients. We conclude that BECN1 regulates APP processing and turnover. BECN1 is involved in autophagy initiation and autophagosome clearance. Accordingly, BECN1 deficiency disrupts cellular autophagy and autophagosomal-lysosomal degradation and alters APP metabolism. Together, our findings suggest that autophagy and the BECN1-PIK3C3 complex regulate APP processing and play an important role in AD pathology.

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Introduction

Alzheimer Disease (AD) affects a growing number of the elderly and results in dramatic loss of cognitive function. It is characterized pathologically by the presence of extracellular beta amyloid (Aβ) assemblies called plaques [1,2], and intracellular accumulation of Aβ [3] and tau [4]. These lesions are hallmarks of the disease and are associated with neurodegeneration and inflammation [5]. Currently it is unclear how these lesions form, and how protein aggregation and neuronal loss are connected [6]. While much research has centered on abnormal proteolytic processing of Amyloid precursor protein (APP) and tau, less focus has been placed on the possibility that slow, progressive dysfunction of intracellular protein sorting and degradation pathways, such as macroautophagy, may drive pathogenesis steadily over time, especially in cases of sporadic AD [7,8].

APP is a type I transmembrane protein that can be processed by one of two mutually exclusive cleavage pathways: α-secretase (non-amyloidogenic processing) or β-secretase (amyloidogenic processing) followed by γ-secretase cleavage. Non-amyloidogenic cleavage occurs mainly at the cell surface, whereas amyloidogenic processing takes place in intracellular compartments, including endosomes, lysosomes, and autophagosomes [9,10]. Amyloidogenic processing releases Aβ which can subsequently be secreted from cells. In addition, APP C-terminal fragments (APP-CTF) of both cleavage pathways can translocate to the nucleus and induce nuclear signaling [11,12,13,14]. Both, Aβ and APP-CTF potentially contribute to AD pathology and can exhibit neurotoxic properties through multiple pathways [15,16].

APP levels, Aβ levels, and neurodegeneration are tightly coupled. Less than 1% of all AD cases are autosomal dominant early-onset familial AD (FAD) and are caused by mutations in one of three major genes APP, Presenilin-1 (PSEN1), or Presenilin-2 (PSEN2) [17]. These mutations lead to the predominant amyloidogenic cleavage of APP. Additionally, FAD can be caused by APP locus duplication [18] and polymorphisms in the APP promoter region that increase APP levels have been linked to an increased risk for AD [19]. In Down Syndrome an additional copy of chromosome

21, which harbors the *APP* gene, leads to overexpression of APP protein, elevated Aβ levels, plaque deposition and AD-like disease in all older Down's patients [20,21,22]. While this illustrates the importance of *APP* gene regulation and APP protein levels in AD, little is known about the regulation of APP metabolism in sporadic AD cases. The levels of APP protein and APP mRNA in AD cases versus control has been reported in the past with conflicting results, but more recent research indicates increased levels of APP and APP-CTFs in sporadic AD brains [23,24,25,26].

Macroautophagy (in this paper referred to as 'autophagy') is a major pathway involved in the degradation of long-lived proteins, protein aggregates, and organelles, cellular remodeling, and survival during starvation [27,28]. Autophagy is characterized by the formation of a cup-shaped isolation membrane that develops around cytosolic components and eventually fuses to form a double membrane bound vesicle [29,30,31,32]. The protein Microtubule-associated protein 1, light chain 3 (LC3) is anchored via conjugated phosphatidylethanolamine to the vesicle's membrane. While the un-conjugated LC3 is called LC3-I, the phosphatidylethanolamine conjugated LC3 is referred to as LC3-II and is a specific marker for these so-called autophagosomes [33]. Autophagosomes then undergo several microtubule- [34] and dynein-dependent maturation events [35,36], including fusions with multivesicular bodies, early and/or late endosomes [37], before eventually fusing with lysosomes [38,39].

Autophagy has recently been implicated in a number of diseases including neurodegenerative conditions and it appears that autophagy can exert both a pathological or protective role, depending on the setting [40]. While it is still largely unknown how dysfunction of the autophagy pathway might contribute to neurodegeneration and AD, recent papers suggest a role for Beclin 1 (BECN1) in AD and mild cognitive impairment [41,42,43]. Haploinsufficiency of *Becn1* in mice decreases neuronal autophagy and promotes neuronal degeneration [41]. Moreover, in a mouse model for AD genetic reduction of *Becn1* expression results in increased accumulation of APP fragments and Aβ, increased neurodegeneration and increased inflammation [41]. In addition, Autophagy has been shown to protect neurons from Aβ induced cytotoxicity [44,45,46].

BECN1 plays an important role in autophagy [47,48,49,50,51] and is the human homolog of the yeast autophagy protein Atg6/Apg6 [52]. BECN1 forms a core complex with the class 3 phosphoinositide-3-kinase PIK3C3 (also known as Vps34) [51,53,54]. Other proteins such as UVRAG, Atg14L, PIK3R4/Vps15, Ambra1, Rubicon, or Bif-1, join this complex depending on its physiological function in autophagy or endosomal trafficking [55,56,57]. *Becn1* and *Pik3c3* mRNA and protein are expressed in human and mouse brains [40] (Fig. S1, from the Allen Mouse Brain Atlas. Seattle (WA): Allen Institute for Brain Science. Available from: http://mouse.brain-map.org [58]). Knockout mice lacking *Becn1* (*Becn1*$^{-/-}$) die during embryogenesis [48,50].

To date, the mechanism describing how deficiency in BECN1 can cause changes in APP processing and amyloid accumulation are unknown. Here we characterize the relationship between BECN1 levels, autophagy, and APP processing in cell culture and in human brain tissue. We show that intracellular APP, APP-CTFs, and Aβ can be reduced by autophagy activation and that the BECN1-PIK3C3 complex regulates APP processing and accumulation.

Results

Activation of autophagy promotes APP, APP-CTF, and Aβ degradation

Activation of autophagy can lead to degradation of α-synuclein, huntingtin, and poly-ubiquitinated proteins [59,60,61,62]. To test whether APP and APP-CTFs can also be reduced through this mechanism, we induced autophagy in B103 rat neuroblastoma cells which lack endogenous rat APP and are stably transfected with wildtype human APP695 (B103/hAPP) [63]. To induce autophagy we used either starvation [64] or rapamycin treatment which inhibits mTOR and activates autophagy [65] (Fig. 1A). APP and APP-CTF levels were significantly reduced in starved B103/hAPP cells and further reduced in rapamycin treated B103/hAPP cells (Figure 1B–C). Rapamycin treatment did not affect APP mRNA levels analyzed by qRT-PCR (data not shown). Furthermore, inhibition of autophagy through lenti-viral Atg5 siRNA significantly impaired starvation-induced autophagosomal clearance of APP (Fig. S2). Similar to the findings in neuronal cells, Chinese hamster ovary (CHO) cells stably transfected with human APP695 (CHO/hAPP) [12] and treated with the autophagy inducer thapsigargin [66] showed a more than 50% reduction in APP and APP-CTF levels (Fig. 1D–F) and significantly reduced levels of Aβ in the cell supernatant (Fig. 1G). Consistent with these biochemical findings, microscopy (Fig. 1H) revealed reductions both in intracellular APP (detected with CT20 antibody) and in cell surface APP (detected on non-permeabilized cells with 8E5 antibody). These findings indicate that autophagy activation can reduce levels of APP and APP metabolites.

Becn1 knockdown increases APP, APP-like proteins, APP-CTFs and Aβ

The reported reduction in BECN1 in AD brains [41,42] and the increased plaque formation and neurodegeneration in *Becn1*$^{+/-}$*APP* mice [41] led us to investigate whether Becn1 deficiency affected APP production, processing, or degradation *in vitro*. Reduction of *Becn1* by siRNA in B103/hAPP cells more than doubled the levels of cellular APP and APP-CTFs (Fig. 2A and S3). Moreover, the reduced levels of Becn1 also increased the amount of secreted Aβ in the cell culture supernatant when compared to cells treated with a scrambled control siRNA (Fig. 2B). Similar results were obtained with two different siRNA sequences (data not shown). CHO/hAPP cells treated with *Becn1* siRNA also showed twofold increases in APP and APP-CTFs (Fig. 2 C and D). This prominent increase in APP protein in *Becn1* siRNA treated cells could also be visualized and quantified with fluorescent microscopy showing increased immunoreactivity for both, C-terminal (CT20) and N-terminal (8E5) APP antibody stains (Fig. S4).

Reduced autophagic activity could be specific for APP degradation or it could also affect the processing of amyloid precursor-like proteins. Both, Amyloid precursor like protein-1 (APLP1) and Amyloid precursor like protein-2 (APLP2) are substrates of α-, γ-, and ε-secretase cleavage in a similar manner as APP, while APLP2 can also be cleaved by β-secretase [67]. APP, APLP1, and APLP2 can form homo- and heterodimers [68], making it possible that they are affected similarly by processing alterations. To test if autophagy plays a role in APLP1 and APLP2 degradation, we applied *Becn1* siRNA to cell lines stably expressing human APLP1 or APLP2 [12]. Reducing Becn1 in CHO/hAPLP1 and CHO/hAPLP2 cells resulted in significant increases in APLP1 (Fig. 2E–F) and APLP2 levels, respectively (Fig. 2G–H).

To exclude the possibility that the observed cellular changes in APP, APP-CTF, and Aβ levels in response to *Becn1* siRNA could be accounted for by transcriptional up-regulation of APP mRNA levels, we performed qRT-PCR on *Becn1* or control siRNA treated B103/hAPP cells. APP mRNA levels decreased slightly in *Becn1* siRNA treated B103/APP cells (Fig. 2J), therefore increases in APP, APP-CTFs, and Aβ cannot be attributed to increased transcription of the precursor.

To reduce potential transfection related effects, we transduced CHO/hAPP cells with a lentivirus (LV) containing either a control

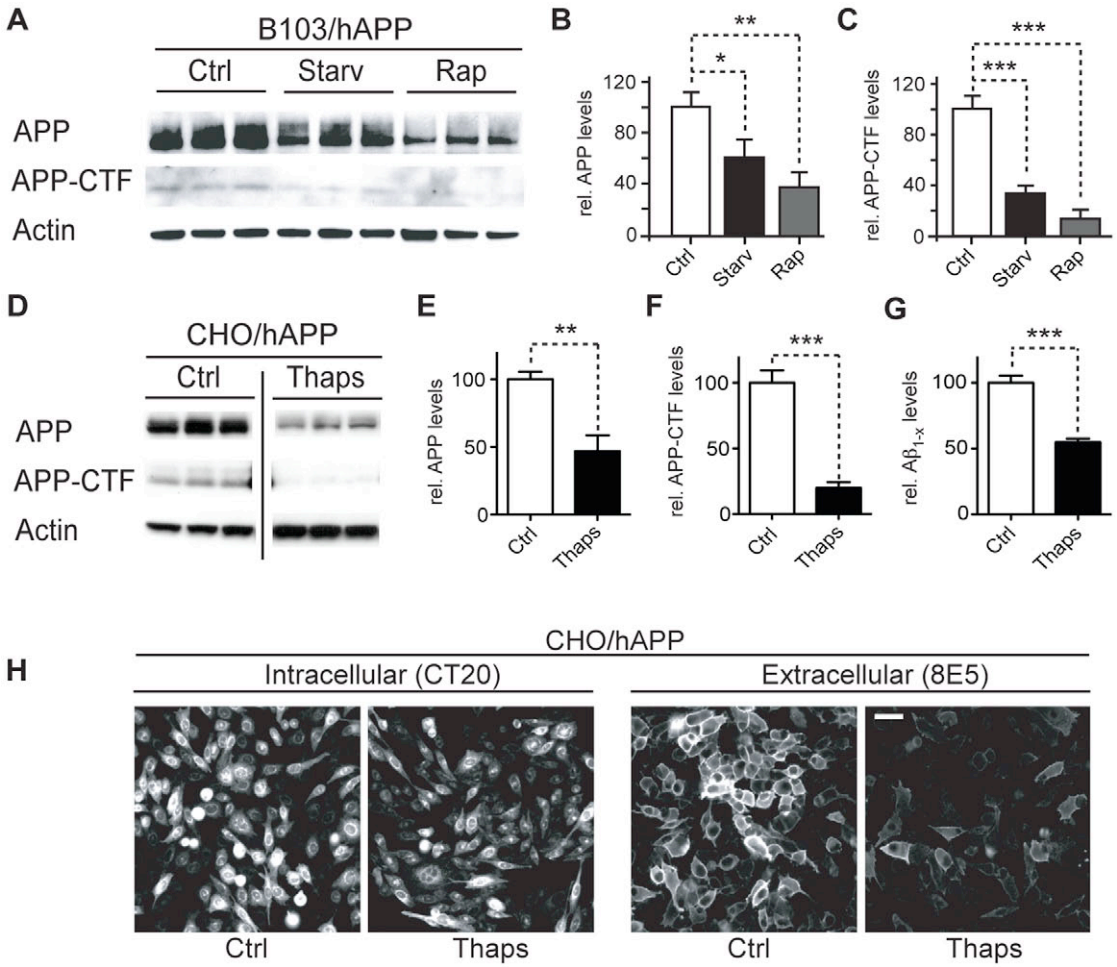

Figure 1. Activation of autophagy promotes APP, APP-CTF, and Aβ degradation. A–C. B103/hAPP cells were left untreated (Ctrl), starved for 90 min in HANKS solution (Starv), or treated with 100 nM rapamycin in DMEM (Rap) for 90 min. Western blots (A) and quantification (B, C) of RIPA cell lysates probed with the CT15 antibody recognizing full-length APP and APP-CTFs and with an actin antibody as a control for loading. **D–F.** CHO/hAPP cells were left untreated (Ctrl) or treated for 12 hrs with 3 μM thapsigargin (Thaps) in DMEM/10%FBS. Western blots (D) and quantification (E, F) of RIPA cell lysates probed with antibodies as in A. (Data from the same blot. The vertical line indicates removal of three lanes not part of this experiment.) **G.** Secretion of Aβ into the cell supernatant was measured by ELISA (12 hrs/1 μM Thaps) **H.** Epifluorescence microscopy images of CHO/hAPP cells treated as in D, permeabilized with Tween and stained with antibody CT20 to label all cellular APP, or not permeabilized and stained with antibody 8E5 which recognizes the ectodomain of APP at the cell surface (scale bar represents 25 μm). Bars are mean ± SEM from triplicate cultures of at least two independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by unpaired Student's t test.
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plasmid encoding only GFP (GFP LV) or a GFP plasmid encoding a *Becn1* shRNA sequence (*Becn1* shLV; different sequence from the siRNA's used above). The *Becn1* shRNA LV treated cells exhibited a significant increase in APP immunofluorescence when compared to GFP LV treated control cells (Fig. 2K–L).

In the *Becn1* siRNA treated cells there was a significant correlation between Aβ and APP, and between Aβ and APP-CTFs ($R = 0.619$, $p = 0.03$ and $R = 0.698$, $p = 0.01$, respectively, data not shown), suggesting that the increase in secreted Aβ was due to increased levels of its precursor, APP. The Aβ/APP ratio was similar in control and *Becn1* siRNA treated B103/APP cells (data not shown), suggesting unchanged γ-secretase activity. To further test the role of γ-secretase in the observed effects, we treated control or *Becn1* shLV transduced B103/hAPP cells with DAPT, a γ-secretase inhibitor. This treatment had no significant effect on the accumulation of full-length APP in control cells (Fig. 2M and S5) and did not significantly enhance the levels of full-length APP in *Becn1* shLV treated cells any further. The APP-CTF levels on the other hand were significantly increased after DAPT treatment (indicating successful γ-secretase inhibition) and this effect was additive when DAPT was applied together with *Becn1* shLV. These results indicate that the accumulation of APP and APP-CTFs in the Becn1 deficient cells are unlikely the result of substantial changes in γ-secretase activity.

In summary, these findings show that reduced Becn1 levels can cause intracellular accumulation of APP and its metabolites and increased secretion of Aβ. This accumulation appears not to be restricted to APP but also affects other APP-family members, suggesting that the observed accumulations are due to changes in shared processing and trafficking pathways. Finally, the buildup of APP and APP-CTFs mediated by Becn1 deficiency appears to be independent of γ-secretase activity.

Overexpression of APP does not change Becn1 or Pik3c3 protein levels

Brains from AD patients contain less BECN1 protein and mRNA than non-demented controls [41,42,43]. This reduction

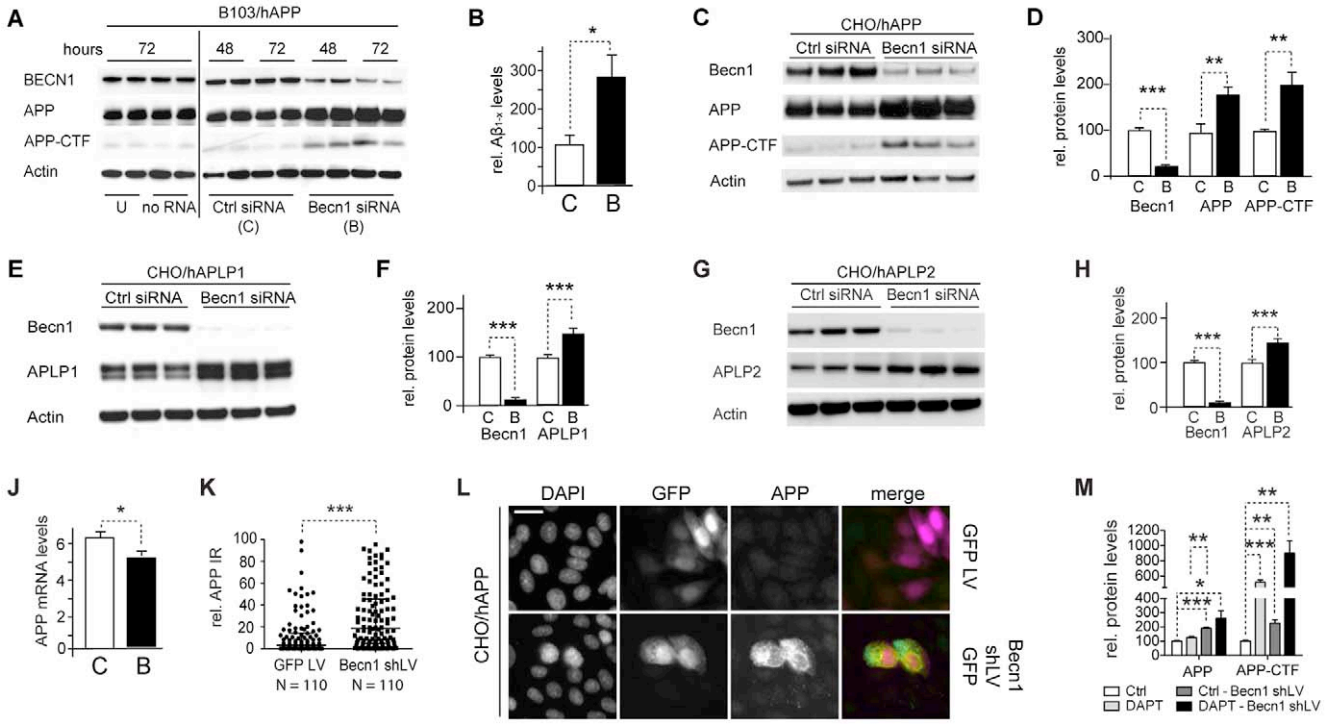

Figure 2. *Becn1* knockdown increases APP, APP-like proteins, APP-CTFs and Aβ. A–B. B103/hAPP cells were treated with *Becn1* siRNA for 48–72 hrs. Cells were left untreated (U), treated with transfection reagent alone (no RNA), treated with scrambled siRNA (Ctrl siRNA [C]), or treated with Becn1 siRNA (Becn1 siRNA [B]). Western blots (A) of RIPA cell lysates were probed with a Becn1 antibody, the CT15 APP antibody, and with an actin antibody as a control for loading. For quantification see Fig, S2. (Data from two blots with identical exposure times. Blot border indicated by vertical black line.) Total $A\beta_{1-x}$ concentrations measured by ELISA in cell culture supernatant from the same cells at 72 hrs (B). **C–D.** CHO/hAPP cells were treated with *Becn1* siRNA for 48 hrs. Western blots (C) and quantification (D) of RIPA cell lysates that were probed with a Becn1 antibody, the CT15 APP antibody, and with an actin antibody as a control for loading. **E–F.** CHO/APLP1 cells were treated with *Becn1* siRNA for 48 hrs. Western blots (E) and quantification (F) of RIPA cell lysates that were probed with a Becn1 antibody, an APLP1 antibody, and with an actin antibody as a control for loading. **G–H.** CHO/APLP2 cells were treated with *Becn1* siRNA for 48 hrs. Western blots (G) and quantification (H) of RIPA cell lysates that were probed with a Becn1 antibody, an APLP2 antibody, and with an actin antibody as a control for loading. **J.** Levels of APP mRNA were compared by qRT-PCR in scrambled [C] or *Becn1* [B] siRNA treated B103/hAPP cells. **K–L.** CHO/hAPP cells were treated with either GFP lentivirus or *Becn1* shRNA-GFP lentivirus. Quantification of the relative APP immunofluorescence (K) and epifluorescence microscopy (L) of GFP lentivirus or *Becn1* shRNA-GFP lentivirus treated permeabilized CHO/hAPP cells, probed with DAPI and CT20 APP antibody (scale bar represents 10 μm). **M.** Inhibition of γ-secretase activity through 100 nM DAPT treatment had no significant effect on APP levels and an additive effect on APP-CTF accumulation with *Becn1* shLV treatment. Bars are mean ± SEM from duplicate/triplicate cultures of at least two independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by unpaired Student's t test.

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could be caused by a disease-related (BECN1-independent) increase in APP levels. To measure the effects of APP expression on Becn1 and Pik3c3 levels, we compared B103 cells that were stably transfected with a mock vector and express no endogenous APP (B103/mock) with cells that were stably transfected with human APP (B103/hAPP; these cells express close to endogenous levels of APP [69]) (Fig. 3A). While APP and APP-CTF levels were strongly increased, Becn1 and Pik3c3 levels were unchanged in B103/hAPP cell compared to B103/mock cells (Fig. 3B).

Expression levels of APP that are chronically much higher than normal could have an effect on Becn1 and Pik3c3 levels. To measure the effects of higher than endogenous levels of APP expression on Becn1 and Pik3c3 levels, we compared CHO cells that were stably transfected with a mock vector and express only endogenous hamster APP (CHO/mock) with cells that were stably transfected with a hAPP vector and express high hAPP levels (CHO/hAPP) (Fig. 3C). Becn1 and Pik3c3 levels remained unchanged despite a strong elevation in APP and APP-CTF levels in these cells (Fig. 3D). These findings indicate that the levels of cellular APP or APP-CTF do not directly influence Becn1 and Pik3c3 levels.

Reduction of Becn1 impairs degradation of autophagosomes and reduces Pik3c3 levels

To investigate how the observed effects of Becn1 reduction on APP-family protein processing can be linked to autophagy, we measured the levels of the autophagosomal marker LC3-II in *Becn1* siRNA treated CHO/hAPP, CHO/hAPLP1, and CHO/hAPLP2 cells (Fig. 4A and data not shown). A 75% knockdown of Becn1 (Fig. 4B) caused a significant shift in the LC3-II/LC3-I ratio indicating an accumulation of autophagosomes in all three cell lines (Fig. 4C and data not shown).

Becn1 is a core component of the class 3 PI3 kinase complex [70]. Reduction of Becn1 levels could affect the stability of this complex and influence the levels of other proteins in the complex. To address this possibility we measured the levels of Pik3c3 in response to *Becn1* siRNA treatment, and the levels of Becn1 in response to *Pik3c3* siRNA (Fig. 4D). The cellular levels of both proteins, Becn1 and Pik3c3, appear to be linked, with the reduction of one leading to a comparable reduction of the other (Fig. 4E).

These findings led us to investigate if Pik3c3 reduction by itself can cause a change in APP processing, similar to *Becn1* siRNA

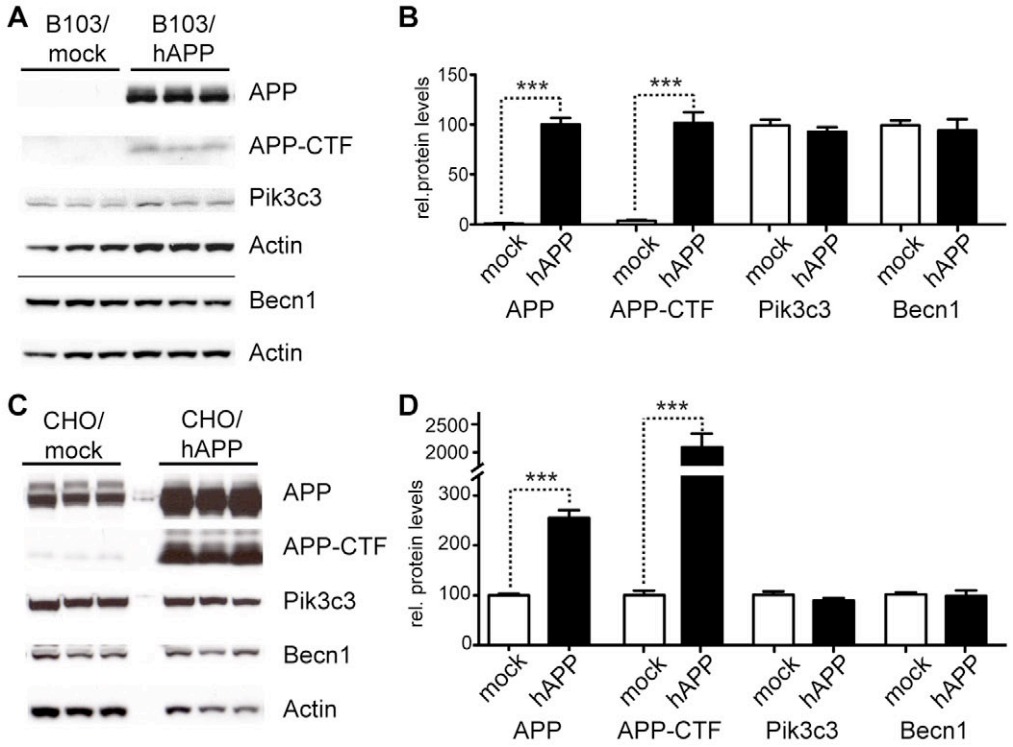

Figure 3. Overexpression of APP does not change Becn1 or Pik3c3 protein levels. A–B. B103 cells were stably transfected with an empty plasmid (mock) or a hAPP encoding plasmid. Western blots (A) and quantification (B) of RIPA cell lysates that were probed with the CT15 APP, a Becn1, and a Pik3c3 antibody. An actin antibody was used as a loading control. **C–D.** CHO cells were stably transfected with an empty plasmid (mock) or a hAPP encoding plasmid. Western blots (C) and quantification (D) of RIPA cell lysates that were probed with the CT15 APP, a Becn1, and a Pik3c3 antibody. Actin antibody was used as a loading control. Bars are mean ± SEM from triplicate cultures of at least two independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by unpaired Student's t test.
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(Fig. 4A). While we observed a trend towards increased APP-CTF in *Pik3c3* siRNA treated cells, we found no significant differences (Fig. 4F–G). These data support a central role for Becn1 in modulating APP levels.

Inhibition of autophagosome turnover leads to a reduction in Becn1 and Pik3c3 levels

BECN1 is reduced in AD brains [41,42,43], however the mechanism behind this reduction is unknown. One hypothesis is that impaired autophagosomal-lysosomal function may activate a negative feedback loop that subsequently reduces BECN1 levels. It is conceivable that this homeostatic loop could become activated after autophagy is impaired in order to prevent apoptosis or autophagic cell death [71]. An accumulation of autophagosomes in AD brain tissue (indicating impaired autophagosomal degradation) has been reported previously [7,10,72,73]. To test this hypothesis we inhibited autophagosomal-lysosomal fusion using bafilomycin A1 (BafA) [74,75]. BafA treatment has been shown to lead to accumulation of APP and APP-CTFs in late endosomes and multivesicular bodies (MVB) [76]. We tested if BafA treatment can also lead to APP and APP-CTF accumulation in autophagosomes and if the accumulation of these autophagosomes has any effects on Becn1 or Pik3c3 levels.

In B103/hAPP cells BafA treatment led to a strong increase in APP and APP-CTFs compared to vehicle treated cells (Fig. 5A–C). It also led to a significant accumulation of LC3-I and LC3-II (Fig. 5A), indicating a successful inhibition of autophagosomal degradation through BafA treatment. This impairment of autophagy caused a significant decrease in Becn1 (Fig. 5D, $p = 0.025$) and reduced, but not significantly changed, Pik3c3 levels (Fig. 5E, $p = 0.063$). Microscopy revealed that APP accumulates primarily in large vacuoles in the perinuclear space (Fig. 5F). Some APP containing vesicles stained positive for LC3 (Fig. 5F, arrowheads) but APP also accumulated in large non-LC3 positive vesicles (Fig. 5F, arrow). In vehicle treated cells only very little APP was found in LC3 positive compartments and these compartments were small in size (Fig. 5F).

Similar results were obtained for CHO/hAPP cells, where treatment with BafA also led to a reduction in Becn1 and Pik3c3 protein levels respectively (Fig. 5G, 5K–L). While CT20 full length APP immunoreactivity slightly decreased (Fig. 5H), a strong increase in APP-CTFs (Fig. 5J) and in sAPP (Fig. 5M–N) were observed. The reduction of full-length APP in CHO/hAPP cells (Fig. 5G–H) can be attributed to elevated intracellular and extracellular cleavage of APP. The antibody used in Fig. 5G (CT20) does not recognize the N-terminal cleavage product (Fig. 5P) and enhanced APP processing will lead to an apparent reduction in intracellular (full-length) APP (CT20) levels. Accordingly, the N-terminal sAPP cleavage product accumulates both in the cell supernatant (Fig. 5M–N) and in intracellular, LysoTracker-positive vesicles (Fig. 5O) when probed with the N-terminal antibody 8E5. Total APP and its metabolites accumulate in CHO/hAPP cells, consistent with a disruption in autophagosomal degradation.

To explore alternative inhibitors of autophagosomal-lysosomal degradation and rule out unspecific BafA effects, we compared control, BafA, chloroquine (CQ), and ammonium-chloride/leupeptin (NL) treated CHO/hAPP and B103/hAPP cells (Fig.

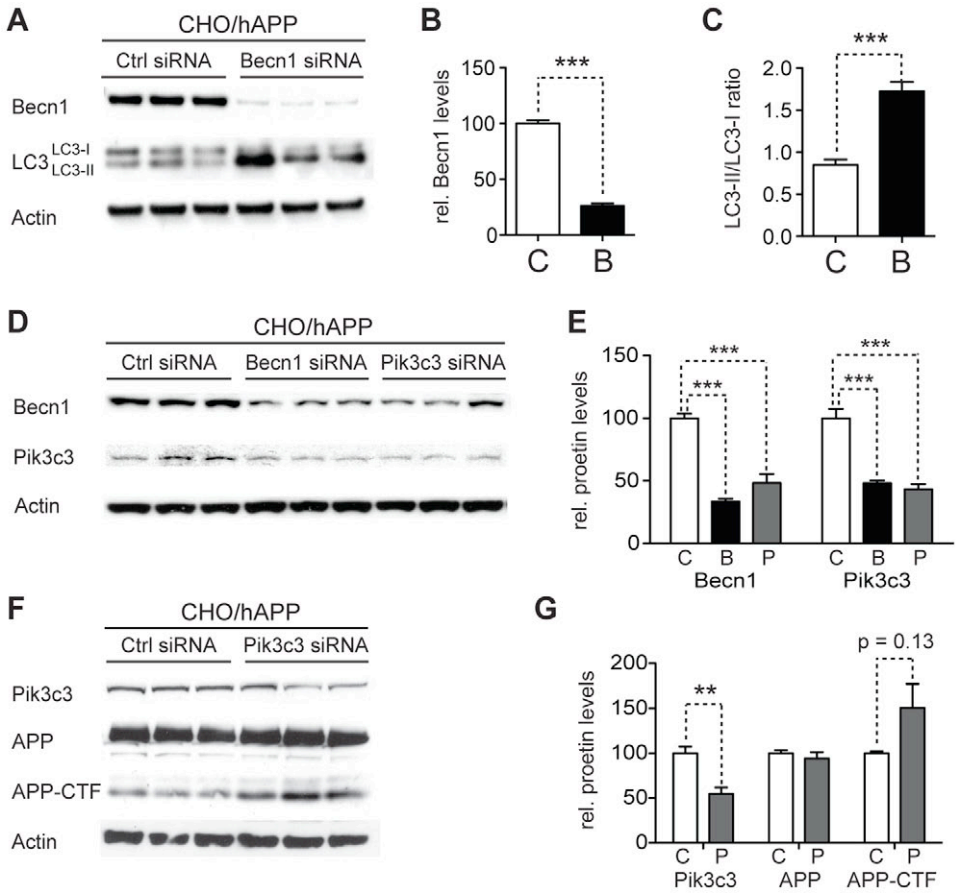

Figure 4. Reduction of Becn1 impairs degradation of autophagosomes and reduces Pik3c3 levels. A–C. CHO/hAPP cells were treated with *Becn1* siRNA for 48 h. Western blots (A) of RIPA cell lysates were probed with a Becn1 and LC3 antibody. An actin antibody was used as a loading control. Quantification (B) of the Becn1 band intensity and the ratio of LC3-II to LC3-I (C). **D–E.** CHO/hAPP cells were treated with *Becn1* and *Pik3c3* siRNA for 48 h. Western blots (D) and quantification (E) of RIPA cell lysates that were probed with a Becn1 and Pik3c3 antibody. An actin antibody was used as a loading control. **F–G.** CHO/hAPP cells were treated with *Pik3c3* siRNA for 48 h. Western blots (F) and quantification (G) of RIPA cell lysates that were probed with the CT15 APP antibody and with an actin antibody as a control for loading. Bars are mean ± SEM from triplicate cultures of at least two independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by unpaired Student's t test.
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S6). We found that both CQ and NL cause an accumulation of APP and APP-CTFs, very similar to BafA. This strongly supports the hypothesis that APP is indeed processed through the autophagosomal-lysosomal pathway. Interestingly, we observe the highest reduction in Becn1 and Pik3c3 levels after BafA treatment. CQ treatment causes a slight ($p = 0.06$) reduction in Becn1 and a significant reduction in Pik3c3, while NL has no significant effect on Becn1 or Pik3c3. BafA inhibits autophagosomal-lysosomal fusion, while the two other treatments primarily inhibit autolysosomal degradation. This supports the hypothesis that the accumulation of autophagosomes, rather then the inhibition of lysosomal degradation, affects Becn1 and Pik3c3 levels in a negative feedback-loop.

We conclude that inhibition of autophagosomal-lysosomal fusion through pharmacological treatments leads to accumulation of APP, APP-CTF, sAPP, and autophagosomes. This accumulation results in a reduction of Becn1 and Pik3c3 levels, possibly through a negative feedback mechanism.

Becn1 overexpression reduces APP immunoreactivity

To determine if *Becn1* overexpression alone can reduce APP baseline levels we transduced CHO/hAPP cells with either a *Becn1* LV or a control GFP LV (Fig. 6A). While baseline Becn1 levels give only very faint immunoreactivity in fluorescent microscopy, the *Becn1* LV treated cells exhibited a wide range of Becn1 expression levels (from baseline to strong overexpression, Fig. 6A). We randomly selected N = 214 *Becn1* LV treated cells covering the whole spectrum of Becn1 expression from both, the Becn1 (red outline) and APP channel (yellow outline), and measured their relative Becn1 and APP immunofluorescence (Fig. 6B). Next, we grouped these cells into low (<20th percentile), medium (20–80th percentile), and high (>80th percentile) Becn1 expressing cells and compared the median APP immunofluorescence in these groups (Fig. 6C). While no or low overexpression of Becn1 has no effect on APP immunoreactivity (Fig. 6C, 0–20), medium overexpression significantly reduces baseline APP levels (Fig. 6C, 20–80). Very strong, and likely non-physiological overexpression of Becn1 (Fig. 6C, 80–100) had no lowering effect on APP immunoreactivity, but led to either abnormally decreased or increased cell size, indicating that these very high levels of Becn1 expression might impair cellular homeostasis (Fig. S7A–B). This last finding is similar to very high overexpression of GFP protein and probably an artifact. For more details on the effects of GFP overexpression in the control cells, see supplemental Fig. S7 B. These results

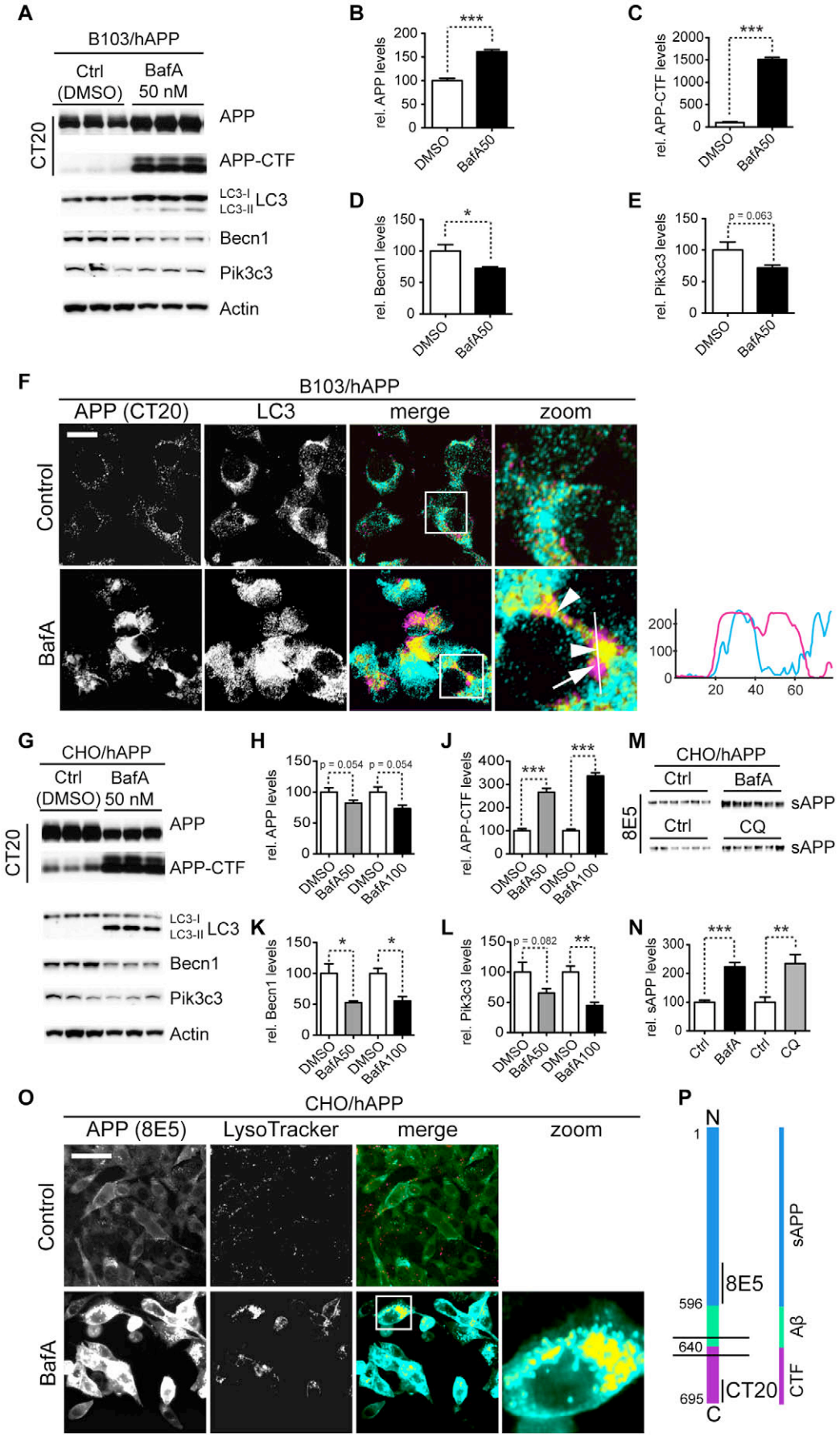
A
B103/hAPP
Ctrl (DMSO)
BafA 50 nM
CT20
APP
APP-CTF
LC3-I
LC3-II
LC3
Becn1
Pik3c3
Actin
B
rel. APP levels
***
DMSO
BafA50
C
rel. APP-CTF levels
***
D
rel. Becn1 levels
*
E
rel. Pik3c3 levels
p = 0.063
F
B103/hAPP
APP (CT20)
LC3
merge
zoom
Control
BafA
G
CHO/hAPP
H
rel. APP levels
p = 0.054
p = 0.054
DMSO
BafA50
DMSO
BafA100
J
rel. APP-CTF levels
***
***
M
CHO/hAPP
Ctrl
BafA
8E5
sAPP
Ctrl
CQ
sAPP
K
rel. Becn1 levels
*
*
L
rel. Pik3c3 levels
p = 0.082
**
N
rel. sAPP levels
***
**
Ctrl
BafA
Ctrl
CQ
O
CHO/hAPP
APP (8E5)
LysoTracker
merge
zoom
Control
BafA
P
N
1
8E5
596
640
CT20
695
C
sAPP
Aβ
CTF

Figure 5. Inhibition of autophagosomal turnover leads to a reduction in Becn1 and Pik3c3 levels. A–E. B103/hAPP cells were treated with vehicle (DMSO) or 50 nM BafA for 24 hrs to inhibit autophagosomal degradation. Western blots (A) and quantification (B–E) of RIPA cell lysates that were probed with CT15 APP, LC3, Becn1, and Pik3c3 antibody. An actin antibody was used as a loading control. **F.** Confocal microscopy of B103/hAPP cells treated with vehicle (DMSO) or 100 nM BafA for 24 hrs. Cells were stained with CT20 APP antibody (magenta) and LC3 antibody (cyan). Co-localization is indicated in yellow. Arrowheads indicate LC3 positive APP containing vesicles. The arrow indicates an APP containing LC3 negative vesicle (scale bar represents 10 μm). The line indicates cross-section. Cyan line in the cross-section represents APP intensity, magenta line represents LC3 intensity (AU). **G–L.** CHO/hAPP cells were treated with vehicle (DMSO), 50 nM, or 100 nM BafA (WB data not shown) for 24 hrs. Western blots (G) and quantification (H–L) of RIPA cell lysates that were probed with the CT15 APP, LC3, Becn1, and Pik3c3 antibody. An actin antibody was used as a loading control. **M–N.** BafA and CQ treatment cause increased APP processing which in turn leads to elevated levels of secreted APP (sAPP) in the cell supernatant (M). This is quantified in (N). **O.** Epifluorescence microscopy of CHO/hAPP cells treated with vehicle (DMSO) or 100 nM BafA for 12 hrs. Cells were stained with the 8E5 APP antibody (magenta) and LysoTracker (cyan). Co-localization is indicated in yellow (scale bar represents 25 μm). **P.** Schematic representation of the APP antibody epitopes. Bars are mean ± SEM from triplicate cultures of at least two independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by unpaired Student's t test.
doi:10.1371/journal.pone.0011102.g005

suggest that moderate increases in Becn1 levels alone can have an APP-lowering effect in CHO/hAPP cells, as long as Becn1 is not expressed at extremely high and probably non-physiological levels.

AD brains have less BECN1 and PIK3C3 and more LC3

BECN1 and PIK3C3 form a complex with PI3 kinase (PI3K) activity that is necessary for the classical autophagy-activating pathway through mTOR. We and others have previously shown that BECN1 is strongly and specifically reduced in affected regions of Alzheimer's disease (AD) brains [41,42,43]. Heterozygous deletion of *Becn1* in an AD mouse model caused increased neurodegeneration, decreased autophagy, and disruption of the lysosomal system [41]. Our cell culture findings presented above indicate that BECN1 plays an important role in APP processing and trafficking and that BECN1 reduction has effects on the PI3K complex stability and autophagosomal degradation. To understand if the observed reduction of BECN1 in AD patients is an isolated finding or if it could causes a more general disturbance of the autophagosomal system (similar to our *in vitro* findings) we measured multiple key proteins involved in autophagy (Fig. 7A) in human brain samples. Protein was extracted from cortical gray matter of confirmed Alzheimer disease patients (N = 7, age 81±12.6 years, MMSE 4.3±6.1) and non-demented control subjects (N = 10, age 77.7±8.1 years, MMSE 28.3±3.0), using a detergent containing extraction buffer (RIPA). We found PIK3C3 and, consistent with our previously published findings [41], BECN1 to be strongly reduced in AD brains when compared to non-demented age-matched controls (Fig. 7B–C). There was a highly significant correlation between the amount of BECN1 and PIK3C3 (Fig. 7D, R = 0.86, $p<0.0001$) in agreement with their

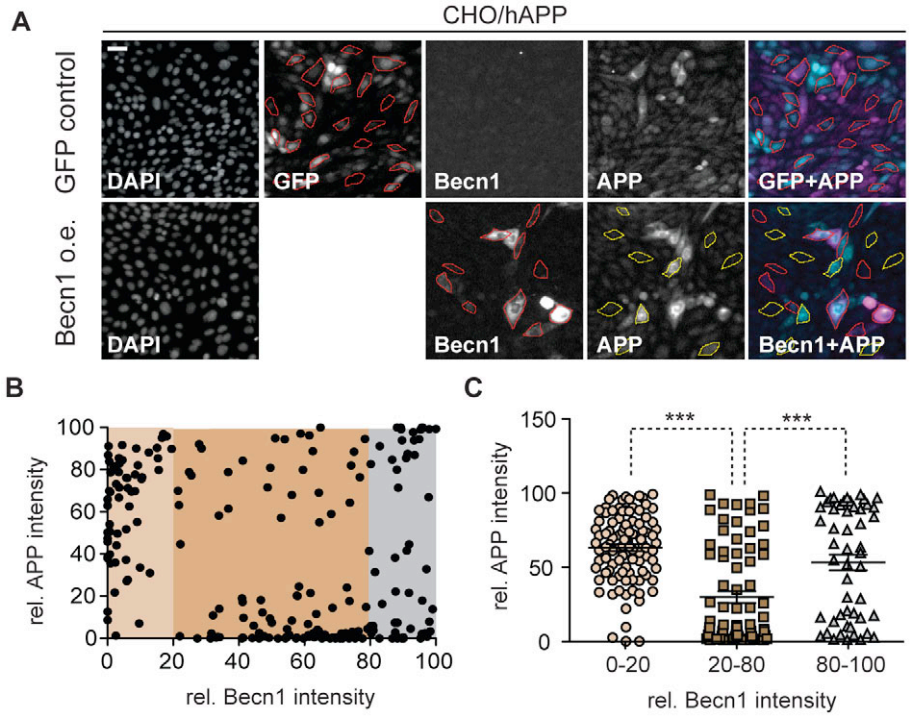

Figure 6. Becn1 overexpression reduces APP immunoreactivity. A. CHO/hAPP cells were transduced with either a *GFP* LV (GFP control) or a *mBecn1* LV (Becn1 o.e.). Epifluorescence microscopy was performed after staining with Becn1 and APP CT15 antibodies (Scale bar represents 25 μm). *GFP* LV transduced cells show very faint Becn1 immunoreactivity, while *Becn1* LV transduced cells exhibit a range of Becn1 signal intensity. No GFP signal is present in the *Becn1* LV cells. A random selection of cells (N = 214) was picked from the *GFP* LV cells and the *Becn1* LV cells. The *Becn1* LV cells were randomly selected in both, the APP (yellow outline) and the Becn1 (red outline) channel. **B.** Relative immunofluorescence of the selected cells (AU). They can be divided in low, medium, and high Becn1 expressing cells. **C.** Quantification of the relative APP immunofluorescence in the three cohorts. Medium Becn1 overexpression leads to a significant reduction in APP levels. Medians were compared by Man-Whitney U test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$
doi:10.1371/journal.pone.0011102.g006

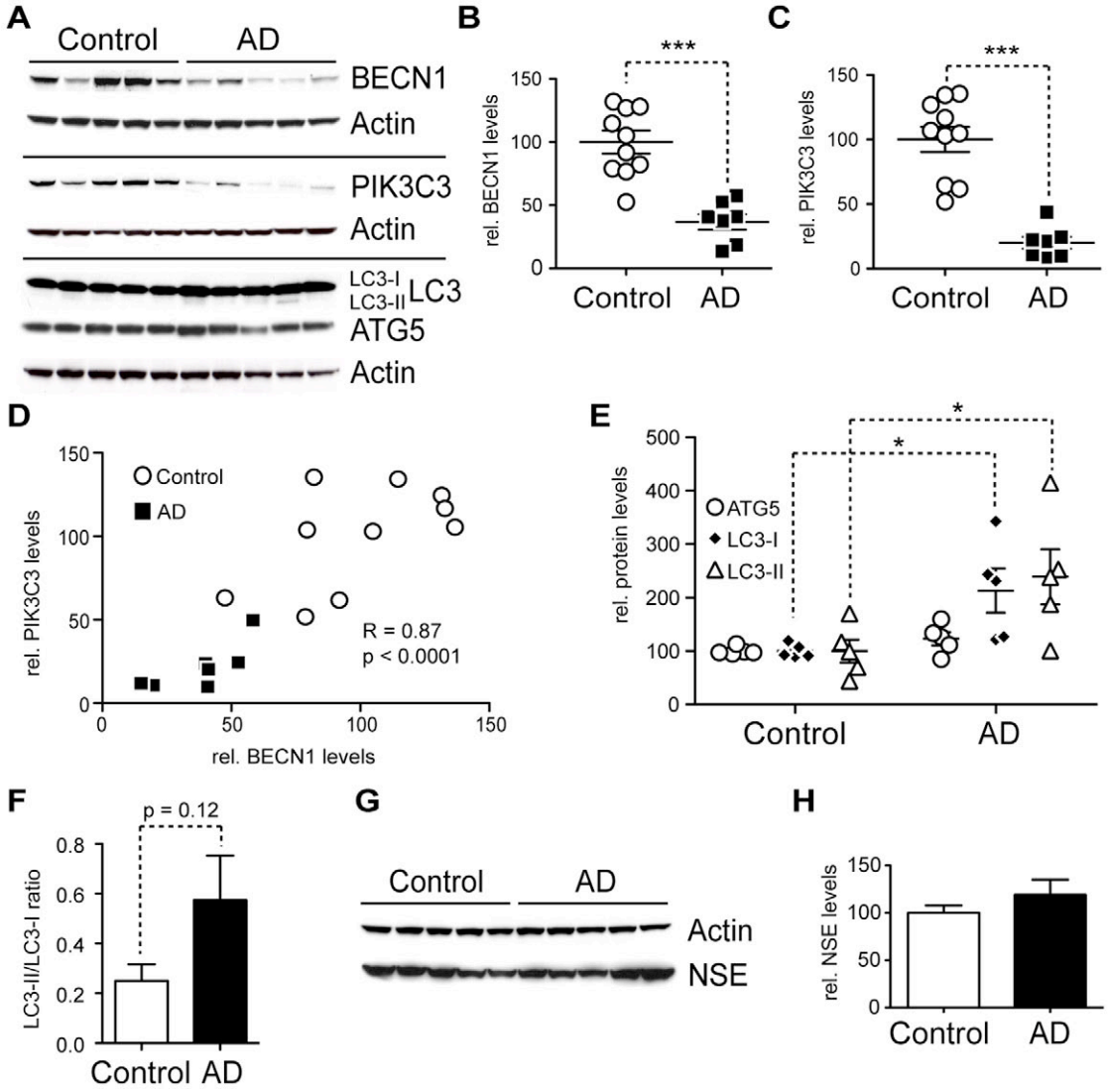

Figure 7. AD brains have less BECN1 and PIK3C3 and more LC3. A–H. Comparison of protein levels in frontal cortex (gray matter) from AD brains and age matched, non-demented, non-pathological controls. Western blots (A) and quantification (B–F) of RIPA lysates that were probed with the CT20 APP, LC3, Becn1, Pik3c3, and Atg5 antibody. An actin antibody was used as a loading control. 7 AD and 10 control cases were used. BECN1 and PIK3C3 levels were significantly reduced in AD cases (B–C). A significant linear correlation exists between BECN1 and PIK3C3 levels ($R = 0.86$, $p < 0.0001$), consistent with them functioning in a complex (D). While ATG5 levels appear unchanged, LC3-I and LC3-II levels are significantly elevated (E). A slight trend was detected in LC3-II/LC3-I ratio change (F). No significant difference could be detected in the levels of a neuronal marker NSE between the control and AD brains, indicating that the observed changes are not due to gross neuronal loss (G and H). All scattergrams show mean ± SEM. Means were compared by unpaired Student's t test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$
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combined role in forming the autophagy inducing PI3K complex. In support of previous findings by others [10], we measured elevated levels of LC3-I and LC3-II in AD patient brains (Fig. 7E) and we observed a trend towards higher LC3-II/LC3-I ratios (Fig. 7F). In contrast, expression levels of another autophagy protein, ATG5 were unchanged in AD brains, indicating that only portions of the autophagy pathway are de-regulated in AD (Fig. 7A&E). To ensure that the observed reduction in BECN1 and PIK3C3 levels cannot be attributed to a gross decrease in neuronal mass, we measured the levels of the marker neuron-specific enolase (NSE) in lysates from AD and non-demented control brains and found no significant difference (Fig. 7G–H).

Tissue protein measurements are very sensitive to the extraction method used. To rule out extraction artifacts, we extracted a different set of human gray matter tissue (AD $N = 10$, age 77.9 ± 7.7 years, MMSE 4.9 ± 5.4/Ctrl $N = 10$, age 77.0 ± 8.2 years, MMSE 29.3 ± 1.0) with sequential extraction buffers yielding a cytosolic fraction (RAB buffer) and a membrane bound fraction (RIPA buffer). BECN1, PIK3C3, and ATG5 were predominantly found in the membranous protein fraction with BECN1 and PIK3C3 again significantly reduced in AD brain tissue and ATG5 levels unchanged (Table S1, $p = 0.003$ and $p = 0.019$).

Discussion

Recent advances in our understanding of intracellular protein trafficking have shown that subtle alterations in the intracellular distribution of APP can have strong effects on whether it is processed by the non-amyloidogenic or amyloidogenic pathways

[77]. In the current study we present data showing that autophagy is a degradative pathway that has the capacity to reduce cellular levels of APP and its metabolites when activated either physiologically (starvation), through pharmacological treatment (rapamycin or thapsigargin), or by lentiviral overproduction of Becn1. Conversely, reduced expression of Becn1 or pharmacological inhibition of autophagosomal degradation (bafilomycin A1, chloroquine, ammonium-chloride/leupeptin) led to an increase in APP and its metabolites. We conclude that Becn1 is a key regulator of cellular APP turnover.

Autophagy is a physiological mechanism that can have both beneficial and detrimental effects on neurons, depending on the circumstances [40]. Whether or not autophagy is increased in AD and whether such an increase reflects a protective attempt by cells to possibly degrade APP and Aβ, or a neurotoxic process promoting autophagic cell death has been debated. However, recent publications indicate that pharmacological stimulation of autophagy can be beneficial and reduce Aβ mediated toxicity [44,45,46]. In human brains and AD mouse models autophagosomes can be readily detected by electron microscopy and they appear to accumulate in swollen dystrophic neurites [7,10,72,73]. This is most commonly interpreted as a sign of impaired autophagosomal degradation [7]. Furthermore APP-cleaving secretases and Aβ have been localized to autophagosomes and the accumulation of autophagosomes in AD brains and APP/PS1 mice has been interpreted as evidence that autophagy could promote AD pathology [10]. In agreement with these neuropathological findings, we observed that APP transgenic mice accumulate lysosomal and autophagosomal vesicles and that Becn1 deficiency in APP mice further promotes this pathology [41]. In addition, we confirm here that autophagy is activated in AD by detecting increased levels of LC3-II in AD brains (Fig. 7E).

However, at the same time, we and others found BECN1 [41,42,43] and in the current study PIK3C3, reduced in AD tissue (Fig. 7B–C and Table S1), suggesting an impairment in the initiation of autophagy. To reconcile these apparently contradictory findings we postulate a dual role for BECN1: one in autophagy initiation, in a complex with PIK3C3, and another in autophagosomal flux and degradation, potentially in a complex with other proteins (Fig. 8). BECN1 has been shown to execute various functions depending on its binding partners and siRNA mediated knockdown of *Becn1* has been demonstrated to impair autophagosomal degradation and cause LC3-II accumulation at the same time [57], similar to our findings (Fig. 4A&C). Different experimental models therefore appear to reflect different aspects of this dual role. On one hand *Becn1* heterozygous knockout mice have reduced autophagosomes and reduced LC3-II [41], reflecting impaired autophagosomal initiation. On the other hand, *Becn1* siRNA treated CHO cells have increased LC3-II levels (Fig. 4A&C). This reflects impaired autophagosomal degradation similar to the pathology observed in AD brains. In either role, reducing BECN1 leads to pathological accumulations of APP and its metabolites through impaired autophagy (Fig. 8).

Our observations regarding the effect of *Becn1* siRNA on autophagy confirm that the BECN1-PI3K complex has a crucial role during the initiation stages of autophagy, but they also show that reduction of Becn1 protein levels can have effects on the availability of PIK3C3 and vice versa (Fig. 4D–G). This is supported by recent findings of similar Becn1 reduction after *Pik3c3* knockdown [78], although a reduction of Pik3c3 after *Becn1*

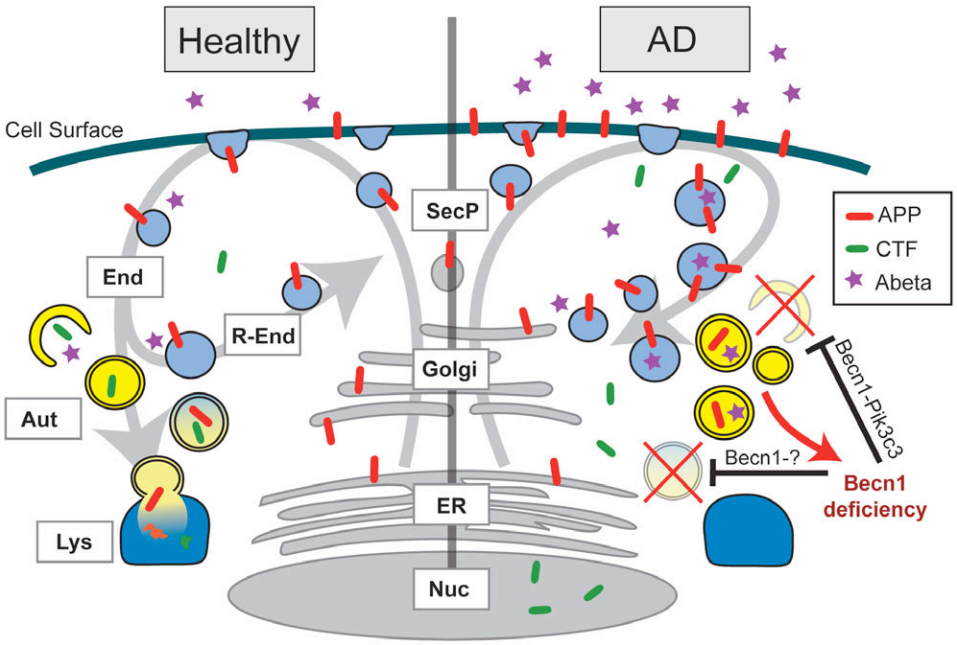

Figure 8. Effects of BECN1 deficiency in AD. In healthy individuals, APP is transcribed in the endoplasmatic reticulum (ER, grey), modified in the golgi network (Golgi, grey) and then shuttled to the cell surface through the secretory pathway (SecP, grey). The cell takes up APP through endocytosis (End, light blue). From here, APP can either be degraded via autophagy (Aut, yellow) and the lysosomes (Lys, dark blue) or APP can be recycled via the recycling endosomes (R-End, light blue) and enter the cycle again. In AD brains and Becn1 deficient cells BECN1 deficiency impairs both induction of autophagy (through the complex with PIK3C3) and autophagosomal degradation (potentially through a complex with an unknown binding partner). APP containing vesicles (endosomes, autophagosomes, and others) build up inside the cell. APP is increasingly cleaved by secretases and large amounts of APP-CTF and Aβ are being released, causing neurotoxic events. The disruption of autophagosomal degradation includes an increasing accumulation of autophagosomes. This accumulation can further inhibit autophagy and BECN1 expression (red arrow), worsening the reduction in APP turnover and degradation.
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knockdown had not been reported. It will be important do determine if other proteins that are part of the BECN1 complex (Atg14L, PIK3R4, UVRAG, Ambra1, Vps15, Bif-1, or Rubicon) are also reduced in AD or in response to BECN1 reduction, respectively, as this could help explain the (possibly indirect) effects of BECN1 reduction on autophagosomal degradation (Fig. 8). Atg14L and UVRAG are especially interesting candidates for this since both proteins have been shown to determine the stability of Becn1 [78] and Atg14L knockdown causes LC3-II accumulation similar to Becn1 siRNA [57]. Further studies will be needed to precisely determine the role of Becn1 and its binding partners in the modulation of autophagic flux and autophagosomal maturation. Nevertheless, with respect to APP metabolism, Becn1 seems to play central role, since Pik3c3 siRNA does not cause a comparable effect on APP accumulation in our *in vitro* system (Fig. 4F–G).

Aiming to validate our cell culture findings in AD brain tissue, we measured the levels of PIK3C3, LC3, and ATG5. We found a reduction not only of BECN1, but also of its binding partner PIK3C3, similar to our cell culture model using *Becn1* siRNA (Fig. 7C). Importantly, we observed a linear relationship between the levels of these two proteins (Fig. 7D) similar to the cell culture studies, supporting the idea that reduction in one of the proteins can cause instability of the PI3K complex and increased degradation or reduced production of the respective binding partner. The levels of ATG5 on the other hand were not significantly changed, arguing for a specific disruption of the PI3K complex in AD rather than a general deficiency in the autophagy pathway and signaling cascade. The reduction in PI3K complex components appear to have an inhibiting effect on the degradation rate of autophagosomes, which may lead to the build-up of LC3 protein in brain tissue and a subsequent accumulation of APP and its metabolites.

Which comes first, BECN1/PIK3C3 deficiency or APP accumulation? While the data from the transgenic mice suggested an important role of Becn1 levels on AD pathology [41], it was unclear if this effect is upstream of APP pathology or partially a consequence of disrupted intracellular trafficking due to APP overexpression. Our cell culture data from wildtype human APP overexpressing cell lines demonstrate now that APP overexpression alone does not lead to reduced Becn1 and Pik3c3 levels, leaving the possibility that autophagy disturbance could precede APP/Aβ pathology *in vivo*, and that the observed reduction of BECN1 in human AD brain tissue is unlikely due to elevated levels of APP or its metabolites alone. Instead, it suggest that an escalating disturbance in autophagosomal flux and degradation could have a negative impact on BECN1 and/or PIK3C3 levels, presumably via a negative feedback loop, downregulating autophagy induction in response to abundant autophagosome numbers (Fig. 8). Such a loop could be in place to prevent an uncontrolled run-off activation of autophagy with potentially disastrous consequences for the cell. In support of such a model, pharmacological inhibition of autophagosomal-lysosomal fusion using BafA causes a strong accumulation of autophagosomes, accompanied with APP and APP-CTF accumulation in those autophagosomes and other intracellular vesicles. This in turn leads to decreased levels of Becn1 and, at least under some treatment conditions, of Pik3c3 (Fig. 5L). These findings suggest that disturbances in autophagosome turnover can further inhibit proper induction and execution of autophagy, potentially worsening the cellular capacity to degrade APP and its metabolites.

The initial factor that impairs autophagy in AD and reduces BECN1/PIK3C3 still has to be determined. This study however identifies autophagy as an important degradative pathway for APP and suggests that once autophagosomal flux and turnover is impaired an escalating cycle of APP/APP-CTF/Aβ accumulation and further reduced initiation of autophagy occurs (Fig. 8). Future studies of conditional knockout mice for proteins that are part of the BECN1-PI3K complex will help to deepen our understanding of the sequence of events that lead to the disruption of autophagy and how this contributes to the development of AD pathology.

Materials and Methods

Cell culture

B103/hAPPwt rat neuroblastoma cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad/CA, USA) containing 10% (v/v) fetal bovine serum and 5% (v/v) horse serum at 37°C with 5% CO_2. Selection was maintained with 400 µg/ml geneticin/G418 (Invitrogen). CHO/hAPPwt, APLP1 and APLP2 hamster ovary cells were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum and selection maintained using 500 µg/ml hygromycin (Invitrogen).

Drug treatments/Starvation

Cells were washed once in warm PBS and covered with fresh medium containing drugs at the indicated concentrations/for the indicated periods: 100 nM rapamycin for 90 min (Calbiochem, San Diego/CA, USA); 3 µM/1 µM thapsigargin for 12 hrs (Calbiochem, San Diego/CA, USA); 50 nM/100 nM bafilomycin A1 for 24 hrs (LC Laboratories, Woburn/MA, USA); 20 mM ammoniumchloride and 10 µg/ml leupeptin (Sigma-Aldrich, St. Louis/MO, USA) for 24 hrs; 30 µg/ml chloroquine (Sigma-Aldrich, St. Louis/MO, USA) for 16 hrs; 100 nM DAPT (Sigma-Aldrich, St. Louis/MO, USA) for 24 hrs. Control cells were treated with the corresponding amount of vehicle. At the end of the incubation period the cells were harvested or imaged as described below. For starvation experiments, the cells were washed twice in warm PBS and then incubated for 90 min in HANKS or 4 hrs in DPBS (Invitrogen, Carlsbad/CA, USA) solution.

Antibodies

The following primary antibodies were used: BECN1 antibody #612112 1:500 (BD Biosciences, San Jose/CA, USA); LC3 antibody #PD014 1:500 WB/1:200 IHC (MBL International, Woburn/MA, USA); PIK3C3 antibody #38-2100 1:500 (Zymed, San Francisco/CA, USA); Actin antibody #A-5060 1:10000 (Sigma-Aldrich, St. Louis/MO, USA); Atg5 antibody 1:2000 (gift from Dr. Noburo Mizushima, Tokyo Metropolitan Institute of Medical Science, Japan); N-terminal APP 8E5 antibody 1:5000(WB)/1:200(IHC) (gift from Elan, South San Francisco/CA, USA); C-terminal APP CT15/CT20 antibody 1:1000(WB)/1:200(IHC) (gift from Dr. Todd Golde, Mayo Clinic, Jacksonville/FL, USA); APLP1 antibody #171615 1:5000 (Calbiochem, SanDiego/CA, USA); APLP2 antibody #171616 1:5000 (Calbiochem, SanDiego/CA, USA); NSE antibody #MS-171-P1 1:1000 (LabVision, Fremont/C, USA).

RNAi and LV particles

B103/hAPPwt, CHO/hAPP, CHO/hAPLP1 or CHO/hAPLP2 cells were transfected with 40 nM synthetic Stealth siRNA (Invitrogen) using Lipofectamine 2000 (Invitrogen, Carlsbad/CA, USA) following manufacturers instructions. The siRNA sequences used were as follows:

BECN1: CCCAGCCAGGAUGAUGUCUACAGAA and GCUAACUCAGGAGAGGAGCCAUUUA.

PIK3C3: CAUUGCCGUUAGAGCCACAGGUGAA and GGAGCCUACCAAGAAGGAUAGUCAA.

Control: GCUACUCGAGGAGGAACCGUAAUUA.

For LV experiments the cells were transduced with virus containing a shRNA plasmid against mBecn1 targeting the nucleotides 405–423 (or against mAtg5) and a GFP-marker. The control LV contained the empty plasmid with only the GFP-marker. For the Becn1 overexpression experiments, the LV particles contained a plasmid encoding mBecn1 alone. Cells were transduced in 96 well plates at 50 MOI in the presence of polybrenen (8 μg/ml). Successful transduction was monitored by GFP expression. Following the transduction and expansion the cells were stained or lysed after 36–96 hrs. All LV particles were provided by Dr. E. Masliah, University of California San Diego/CA, USA.

Protein extraction

Samples from human brain tissue were homogenized in extraction buffer (see blow) by pulsed ultrasonification at 4°C, followed by centrifugation at 10000×g at 4°C for 30 min. The resulting supernatant was used for protein analysis. For cell culture samples, cells were washed once with PBS (Invitrogen, Carlsbad/CA, USA) and scraped off the plate. After a brief centrifugation at 4500×g at 4°C for 5 min, the cell pellets were re-suspended in extraction buffer and homogenized by pipetting, three freeze-thaw cycles on dry ice, and 30 min incubation on ice. Insoluble particles were pelleted by centrifugation with 10000×g at 4°C and the resulting supernatant was used for analysis. Proteins were extracted using RIPA buffer (50 mM HCl, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM Na_2VO_4, 1% NP40, 0.5% Sodium deoxycholate, 1 mM PMSF, 0.1% SDS, pH 7.4) containing 2x Complete proteinase inhibitor (Roche Diagnostics, Mannheim, Germany). When sequential extraction was performed the samples were first extracted with detergent free RAB buffer (MES 100 mM, EGTA 1 mM, $MgSO_4$ 0.5 mM, NaCl 750 mM, NaF 20 mM, EDTA 100 mM, Na_2VO_4 1 mM, PMSF 1 mM, pH 6.5) containing 2x Complete proteinase inhibitor (Roche Diagnostics, Mannheim, Germany) followed by a RIPA extraction of the pellet.

Western blotting

A pre-cast bis-tris gel (Invitrogen, Carlsbad/CA, USA) and a MOPS buffer system were used and standard Western blotting protocols were followed. 10–20 μg of total protein were loaded. Gels were transferred onto 0.4 μm nitro-cellulose membranes (BioRad, Hercules/CA, USA) and pre-incubated with MISER antibody extender solution (Pierce, Rockford/IL, USA). Total protein was measured with the BCA Protein Assay Kit (Thermo Scientific, Rockford/IL, USA) against a BSA standard. Antigen specific primary antibodies were incubated 1 hr at room temperature or overnight at 4°C and detected with species-specific horseradish-peroxidase coupled secondary antibodies. The ECL Western Blotting Detection kit (GE Healthcare, Buckinghamshire, UK) was used to obtain a chemiluminescence signal, which was then detected using Amersham Hyperfilm ECL (GE Healthcare, Buckinghamshire, UK) at varying exposure times to obtain images with optimal density within the dynamic range of the film (30 s–30 min). The films were digitalized at 300dpi and arranged in Photoshop CS4 (Adobe, San Jose/CA, USA) as TIFF files. Band quantification was performed using ImageJ software (NIH, Bethesda/MD, USA). Bands of interest were normalized to a loading control using Microsoft Excel 2008 (Microsoft Corporation, Seattle/WA, USA) and statistical analysis and graph production was performed in Prism5 (GraphPad Software, La Jolla/CA, USA).

Aβ ELISA

ELISAs were performed as described [41] using antibody 266 ($A\beta_{13-28}$, Elan) as the capture antibody for total Aβ, or antibody 21F12 ($A\beta_{37-42}$, Elan, South San Francisco/CA, USA) as the capture antibody for $A\beta_{x-42}$ and biotinylated 3D6 ($A\beta_{1-5}$, Elan, South San Francisco/CA, USA) as the detection antibody. After incubation with the secondary antibody, samples were incubated with avidin-HRP and the signal developed using "1-step slow TMB ELISA solution" (Thermo Scientific, Rockford/IL, USA). For the thapsigargin-treatment experiments, we used a MesoScale detection system (Gaithersburg/MD, USA) and followed the standard protocol with the above antibodies.

Fluorescence Microscopy

For epifluorescence microscopy cells were grown in 12 well tissue culture plates (Becton Dickinson Labware, Franklin Lakes/NJ, USA). They were washed with ice-cold PBS and then fixed in cold 4% PFA in phosphate buffer for 5 min at 4°C followed by 10 min at RT. Cells were then washed three times with ice-cold PBS and PFA fluorescence was quenched with ice-cold 100 mM tris-HCl pH 8.0 for 3 min. The cells were then either washed three times in ice-cold PBS and stained (for cell surface APP) or permeabilized with ice-cold methanol for 6 min at −20°C, followed by three washes of ice-cold PBS and staining (for intracellular proteins). Staining was performed by blocking cells in blocking buffer (4% donkey serum, 2% bovine serum albumin, 2% fetal calf serum, 0.2% fish gelatin in PBS) for 1 hr at RT. Primary antibodies in blocking buffer were applied to the cells for 1 hr at RT, followed by three 5 min washes in PBS. Fluorescent secondary antibodies in blocking buffer were added and incubated for 1 hr at RT, followed by three washes in PBS for 5 min. Cells were visualized with a Olympus IX71 microscope with a CoolSnapHQ camera (Roper Scientific, Tucson/AZ, USA). Image analysis was done with MetaMorph 6.1r6 (Molecular Devices, Sunnyvale/CA, USA). For confocal microscopy cells were grown on glass cover slips (Fisher Scientific, Hampton/NH, USA) in 12 well plates, and fixed and stained similar to the epifluorescence protocol above. The glass coverslips were mounted in MoWiol and visualized on a Zeiss LSM 510 confocal microscope. Image analysis was done with the Zeiss LSM software package.

RT-PCR

RNA was extracted from B103/hAPP cells (n = 5 wells per treatment group) using Trizol and cleaned using RNAeasy mini kit (Qiagen, Valencia/CA, USA). cDNA was synthesized using TaqMan reverse transcriptase (Applied Biosystems, Branchburg/NJ, USA). cDNA was amplified in triplicate on a MyiQ single color real time PCR detection system using primers specific to human APP (F 5′CACCAATGTGGTAGAAGCCAACC3′, R 5′GGGCAACACACAAACTCTACCCC3′), and GAPDH (F 5′TGCGACTTCAACAGCAACTC3′, R 5′ATGTAGGCCATGAGGTCCAC3′). The PCR cycle was as follows: 10 min at 95°C, 45 x (30 s at 95°C, 2 min at 60°C, 30 s at 72°C). Cycle numbers for amplification to exceed a pre-set threshold were used to determine the APP mRNA copy number. cDNA prepared without reverse transcriptase was amplified to ensure no genomic DNA contamination of the samples.

Human brain tissue

Brain tissues from confirmed AD and age-matched, non-demented, non-pathological controls were obtained from ADRC at the University of California - San Diego, The Institute for Brain Aging and Dementia Tissue Repository at the University of California - Irvine, and Stanford Brain Bank at Stanford University in strict accordance with all ethical and institutional guidelines. Cortical mid-frontal gray matter tissue was cut out of frozen tissue blocks and subject to protein extraction as described above.

Statistics

Human brain tissue protein data consists of one-sample measurements for each case. The data was normalized against actin and differences calculated using Student's unpaired t-test. Cell culture western blots experiments were conducted in two to three independent experiments consisting of duplicates or triplicates. All measurements were normalized by actin intensities and then calculated as levels relative to control conditions. Differences between treatment conditions were established using student's unpaired t-test (with two conditions) or one-way ANOVA followed by Dunnett's test for multiple comparisons (for more than two conditions). For fluorescence microscopy, stains were done in independent duplicates and representative images chosen.

Supporting Information

Figure S1 Expression of Becn1 and Pik3c3 in the mouse brain, especially in the hippocampus, indicates an important function of autophagy in neuronal homeostasis (from the Allen Mouse Brain Atlas. Seattle (WA): Allen Institute for Brain Science. Available from http://mouse.brain-map.org).
Found at: doi:10.1371/journal.pone.0011102.s001 (0.38 MB TIF)

Figure S2 Control or Atg5 shLV treated B103/hAPP cells were starved in DPBS for 4 hours. Atg5 and APP levels were measured by Western-blotting and quantified. Atg5 reduction significantly impairs starvation induced autophagosomal APP degradation (Data is from the same blot. The vertical line indicates removal of three lanes not part of this experiment.) Bars are mean ± SEM from triplicate cultures. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by unpaired Student's t test.
Found at: doi:10.1371/journal.pone.0011102.s002 (0.16 MB TIF)

Figure S3 Quantification of B103/hAPP RIPA cell lysates, 72 hours after siRNA kockdown. All bars are mean ± SEM. Means from at least two independent experiments were compared by unpaired Student's t test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$
Found at: doi:10.1371/journal.pone.0011102.s003 (0.14 MB TIF)

Figure S4 Epifluorescence microscopy of CHO/hAPP cells treated with Becn1 siRNA for 48 hours. All bars are mean ± SEM. Means from at least two independent experiments were compared by unpaired Student's t test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$
Found at: doi:10.1371/journal.pone.0011102.s004 (0.85 MB TIF)

Figure S5 Western-blot of control or Becn1 shLV transduced B103/hAPP cells that were treated with vehicle or 100 nM DAPT for 24 hours. An anti-luciferase shLV was used as control.
Found at: doi:10.1371/journal.pone.0011102.s005 (0.30 MB TIF)

Figure S6 Western-blots and quantification of CHO/hAPP and B103/hAPP cells treated with chloroquine (CQ) or ammoniumchloride/leupeptin (NL). Means from three independent experiments were compared by unpaired Student's t test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$
Found at: doi:10.1371/journal.pone.0011102.s006 (0.45 MB TIF)

Figure S7 A–B. Control experiments for the LV overexpression of Becn1. Control for cell size as a measure of physiological cell health (A). High Becn1 overexpressors exhibit either swollen or shrunken cell bodies, indicating non-physiological stress. Quantification (B) of APP, Becn1 immunofluorescence, and cell size in GFP LV control cells (N = 100) shows no difference in APP or Becn1 levels for low and medium overexpression of GFP. High GFP expression induces non-physiological conditions leading to an unspecific accumulation of Becn1 and APP and cell shrinkage. This led us to not further explore the effects of the highest Becn1 or GFP expressing cells.
Found at: doi:10.1371/journal.pone.0011102.s007 (0.19 MB TIF)

Table S1 Human cortical gray matter tissue was subject to sequential RAB/RIPA buffer extraction and Western blotting. Control (N = 10) and AD (N = 10) cases were compared regarding their relative BECN1, PIK3C3, and ATG5 levels. While BECN1 and PIK3C3 levels were significantly reduced in AD brains when compared to controls, no difference was detectable in ATG5 levels.
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Acknowledgments

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Author Contributions

Conceived and designed the experiments: PAJ FP TWC. Performed the experiments: PAJ FP CHS KML. Analyzed the data: PAJ FP KML. Contributed reagents/materials/analysis tools: EM. Wrote the paper: PAJ TWC.

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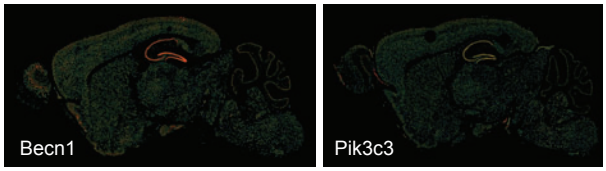

Supplementary Figure 1:
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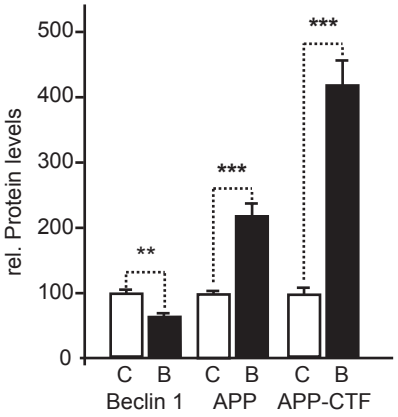
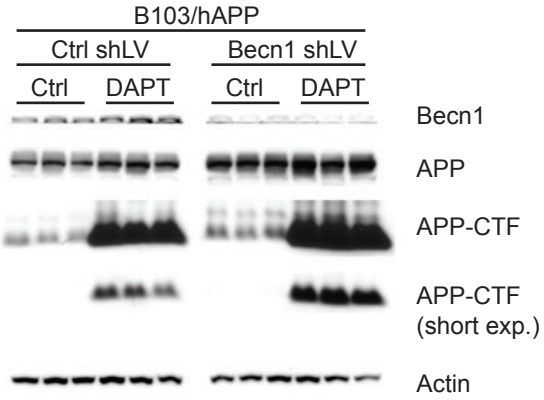


Supplementary Figure 3:
Quantification of B103/hAPP RIPA cell lysates, 72 hrs after siRNA kockdown. All bars are mean ± SEM. Means from at least two independent experiments were compared by unpaired Student's t test. * p<0.05, ** p<0.01, *** p<0.001



Supplementary Figure 5:
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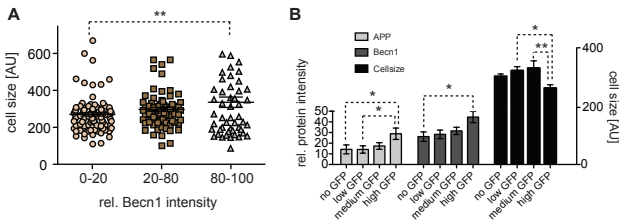
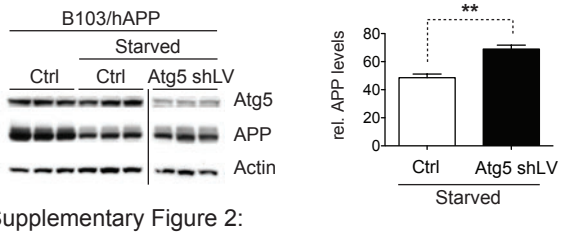
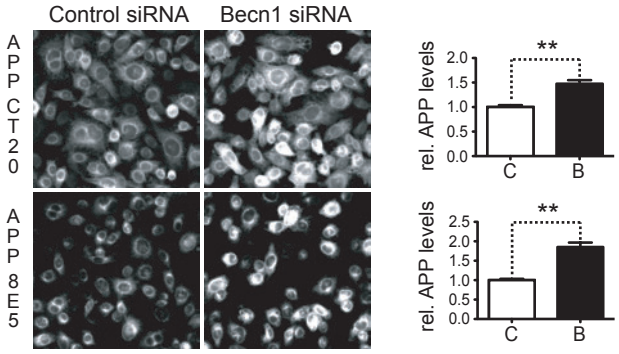


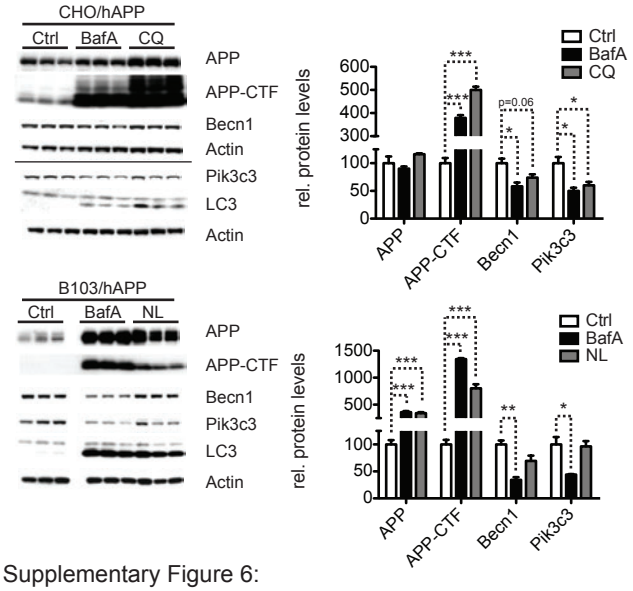
Supplementary Figure 7:
A-B. Control experiments for the LV overexpression of Becn1. Control for cell size as a measure of physiological cell health (A). High Becn1 overexpressors exhibit either swollen or shrunken cell bodies, indicating non-physiological stress. Quantification (B) of APP, Becn1 immunofluorescence, and cell size in GFP LV control cells (N=100) shows no difference in APP or Becn1 levels for low and medium overexpression of GFP. High GFP expression induces non-physiolgical conditions leading to an unspecific accumulation of Becn1 and APP and cell shrinkage. This led us to not further explore the effects of the highest Becn1 or GFP expressing cells.



Supplementary Figure 2:
Control or Atg5 shLV treated B103/hAPP cells were starved in DPBS for 4 hrs. Atg5 and APP levels were measured by Western-blotting and quantified. Atg5 reduction significantly impairs starvation induced autophagosomal APP degradation (Data from the same blot. The vertical line indicates removal of three lanes not part of this experiment.). Bars are mean ± SEM from triplicate cultures. * p<0.05, ** p<0.01, *** p<0.001 by unpaired Student's t test.



Supplementary Figure 4:
Epifluorescence microscopy of CHO/hAPP cells treated with Becn1 siRNA for 48 hrs. All bars are mean ± SEM. Means from at least two independent experiments were compared by unpaired Student's t test. * p<0.05, ** p<0.01, *** p<0.001



Supplementary Figure 6:
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		Cytosolic (RAB)			Membranous (RIPA)		
Protein	**Disease**	**Mean**	**Stdev**	**p value**	**Mean**	**Stdev**	**p value**
BECN1	Control AD	n.d.			0.51 0.30	0.05 0.03	0.003 **
PIK3C3	Control AD	n.d.			0.76 0.41	0.10 0.07	0.019 *
ATG5	Control AD	0.37 0.19	0.11 0.03	0.168	1.79 2.10	0.14 0.19	0.20

Supplementary Table 1:

Human cortical gray matter tissue was subject to sequential RAB / RIPA buffer extraction and Western blotting. Control (N=10) and AD (N=10) cases were compared regarding their relative BECN1, PIK3C3, and ATG5 levels. While BECN1 and PIK3C3 levels were significantly reduced in AD brains when compared to controls, no difference was detectable in ATG5 levels.

The article "***Beclin 1 Complex in Autophagy and Alzheimer Disease***" by Philipp Jaeger and Tony Wyss-Coray can be found at the publisher's website following the link below. The original article had to be removed from the online version of this thesis to comply with the publisher's copyright guidelines.

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