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Genome-wide analysis reveals mechanisms modulating autophagy in normal brain aging and in Alzheimer's disease

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Dysregulation of autophagy, a cellular catabolic mechanism essential for degradation of misfolded proteins, has been implicated in multiple neurodegenerative diseases. However, the mechanisms that lead to the autophagy dysfunction are still not clear. Based on the results of a genome-wide screen, we show that reactive oxygen species (ROS) serve as common mediators upstream of the activation of the type III PI3 kinase, which is critical for the initiation of autophagy. Furthermore, ROS play an essential function in the induction of the type III PI3 kinase and autophagy in response to amyloid β peptide, the main pathogenic mediator of Alzheimer's disease (AD). However, lysosomal blockage also caused by $A\beta$ is independent of ROS. In addition, we demonstrate that autophagy is transcriptionally down-regulated during normal aging in the human brain. Strikingly, in contrast to normal aging, we observe transcriptional up-regulation of autophagy in the brains of AD patients, suggesting that there might be a compensatory regulation of autophagy. Interestingly, we show that an AD drug and an AD drug candidate have inhibitory effects on autophagy, raising the possibility that decreasing input into the lysosomal system may help to reduce cellular stress in AD. Finally, we provide a list of candidate drug targets that can be used to safely modulate levels of autophagy without causing cell death.

reactive oxygen species | type III PI3 kinase | neurodegeneration | signaling | transcriptional regulation

Autophagy, a lysosome-dependent catabolic process mediating turnover of cellular components, plays an important role in regulating cellular homeostasis in the nervous system (1). Even in the absence of any other risk factors, autophagy deficiency in the CNS has been shown to lead to the accumulation of protein aggregates and progressive neurodegeneration (2). Thus, autophagy has been established as an important mechanism mediating degradation of misfolded proteins in the CNS. Because accumulation of misfolded proteins is a common feature in multiple human neurodegenerative diseases, activation of autophagy has been proposed as a strategy for combating neurodegeneration (3). However, little is currently known about how defects in autophagy might be involved in specific neurodegenerative diseases. Furthermore, as induction of autophagy is frequently associated with cell death, it remains a challenge to identify molecular targets whose inhibition can specifically activate autophagy without compromising cell viability.

Pathological evidence supports the involvement of autophagy dysfunction in neurodegenerative diseases in humans. In Alzheimer's disease (AD), one of the earliest pathological changes include accumulation of autophagic vesicles (AVs) specifically within damaged neuritic processes and synaptic terminals (4). This phenotype is also observed in AD animal models and in cell-based models upon exposure to amyloid β peptide ($A\beta$). However,

the mechanisms leading to the accumulation of AVs and the causal relationship to neurodegeneration are not yet established.

We have recently conducted a genome-wide screen using siRNA library to identify genes regulating autophagy in human cells under normal nutritional conditions (5). In this image-based screen we took advantage of the autophagy specific GFP-LC3 reporter whose translocation from the cytosol to autophagosomes can serve as a quantitative measure of autophagy. In this study, we specifically explore the mechanisms that regulate autophagy in neural cells using the hits identified in our screen. We demonstrate that reactive oxygen species (ROS) play a general function in mediation of autophagy upstream of the type III PI3 kinase and that this pathway is essential for the up-regulation of autophagy by $A\beta$. Interestingly, our data show that genes regulating autophagy are differentially expressed in normal aging and in AD patient brains. Finally, we identify candidate molecular targets that may be safely manipulated to modulate autophagy to treat neurodegenerative diseases.

Results

ROS Functions Upstream of the Type III PI3 Kinase to Induce Autophagy. Genes whose knock-down induced autophagy in our screen included components of the ROS detoxification pathway GPx2 and SOD1, whose mutations are known to cause familial ALS. The screen hits also included several mitochondrial proteins, some of which are involved in oxidative respiration and electron transport (Table S1) as well as genes reported to be involved in ROS homeostasis based on a literature co-citation analysis (Table S2). This suggests a role for ROS in the induction of autophagy and a possible function for this pathway in neurodegeneration.

To determine whether ROS are sufficient to induce autophagy, we confirmed that transfection of SOD1 siRNA led to both an induction of autophagy in H4 human neuroblastoma cells, as well as to elevated levels of ROS (Fig. S1A–C). Consistent with a causal role of ROS, treatment with an antioxidant *N*-acetyl-L-cysteine (NAC) significantly attenuated the induction of autophagy (Fig. S1D). Therefore, interference with normal cellular ROS homeostasis by inactivation of a gene involved in neurodegeneration is sufficient to induce autophagy in the absence of any other noxious stimuli.

To determine whether ROS may have a general signaling function in autophagy, we compared the levels of autophagy induced by knock-down of our screen hit genes in the presence and

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The authors declare no conflict of interest.

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absence of NAC. We uncovered a large group of genes (117, or 54% of all genes tested, Fig. S24 and Table S3) that when knocked-down led to translocation of GFP-LC3 to autophagosomes in the absence but not the presence of NAC, suggesting that ROS were required for the induction of autophagy. Interestingly, the presence of NAC also reduced the vesicular accumulation of FYVE-dsRed, a reporter protein for PtdIns3P, induced by the knock-down of these genes (Fig. 1A). As PtdIns3P is the product of type III PI3 kinase, this result suggests that ROS may serve an important general signaling function in the early steps of autophagic pathway mediated by the type III PI3 kinase.

Knock-down any of the remaining 98 (46%, Table S4) genes was able to induce accumulation of GFP-LC3 in the presence of NAC, suggesting that, in these cases, autophagy can be induced independently of ROS. Knock-down of these genes was also able to induce comparable average levels of vesicular FYVE-dsRed in the presence and absence of NAC (Fig. 1A). Thus, knock-down of this group of genes led to induction of the type III PI3 kinase through a mechanism independent of ROS.

Because our data indicated that the type III PI3 kinase plays an especially crucial role in the mediation and regulation of autophagy downstream of ROS, we performed an additional screen to determine how our hit genes are influenced by the type III PI3 kinase activity. In addition to its prominent function in regulation of apoptotic cell death, Bcl-2 may negatively regulate autophagy through its interaction with beclin 1 and consequent inhibition of the type III PI3 kinase activity (6). Consistently, we

observed a significant increase in cell viability and a decrease in levels of PtdIns3P following knock-down of the hit genes in H4 cells expressing Bcl-2 as compared with WT controls (Fig. 1B and C and Fig. S2B). Knock-down of 124 (58%) of the 215 tested hit genes was unable to induce accumulation of vesicular GFP-LC3 in cells overexpressing Bcl-2, confirming that, in the majority of cases, up-regulation of the type III PI3 kinase activity is necessary for the induction of autophagy (Fig. S2C and Table S5). On the other hand, knock-down of the remaining 91 (42%) genes was able to induce translocation of GFP-LC3 to autophagosomes in the presence of Bcl-2 (Table S6). For 17 (19%) of these genes, induction of autophagy was accompanied by an increase in type III PI3 kinase activity, suggesting additional mechanisms that regulate production of PtdIns3P downstream of Bcl-2 (Fig. 1D). However, knock-down of the remaining 74 genes was able to induce autophagy without additional activation of the type III PI3 kinase. Knock-down of 31 (34%) of these genes led to the expansion of the lysosomal compartment as assessed by the accumulation of Lamp-1-RFP marker, indicating that, in these cases, a block in lysosomal degradation may contribute to the apparent increase in autophagy (Fig. 1D). No changes in the lysosomal function were observed for the remaining 43 (47%) genes. These genes may function either downstream or independently of the type III PI3 kinase, suggesting that the apparent inhibitory effect of Bcl-2 on the type III PI3 kinase is not always incompatible with the induction of autophagy, which, in these cases, can occur without an increase in PtdIns3P levels.

Interestingly, we observed a substantial overlap between genes whose knock-down was unable to induce autophagy in the presence of NAC or Bcl-2 (86, 40% of all genes tested; Fig. 1E). The overlapping genes include the majority (11 of 18) of the mitochondrial genes, confirming that mitochondria-generated ROS induce autophagy by positively regulating the type III PI3 kinase. Because overexpression of Bcl-2 has been shown to reduce stress induced generation of ROS (7), our data imply that, in addition to directly binding and suppressing the activity of Beclin1 (6), Bcl-2 may be able to contribute to the regulation of the type III PI3 kinase and autophagy through its ability to attenuate generation of ROS.

Differential Expression of Autophagy Regulators in AD Brain Samples.

Accumulation of both ROS and AVs are early features in AD (4). To determine whether we could detect changes in the expression of genes involved in regulation of autophagy in this disease, we analyzed expression of the autophagy screen hit genes in six brain regions of 34 patients with AD and 14 age-matched normal controls (8). We observed an overall significant underexpression of the hit genes in AD patient samples compared with controls specifically in the hippocampus and entorhinal cortex, the brain regions most affected by the disease (Fig. 2A). Consistent trends were observed in other brain regions affected by AD (superior frontal gyrus, posterior cingulate, and medial temporal gyrus), but did not reach statistical significance. Notably, in the visual cortex, a brain region relatively resistant to AD pathology, these changes were absent. Further subdivision of the hit genes revealed that in the entorhinal cortex negative regulators of autophagy flux were specifically negatively enriched (Fig. 2B). A similar trend was also observed in other brain areas affected by AD. Conversely, positive regulators of autophagy were positively enriched in the entorhinal cortex (Fig. 2C). Such differential expression patterns of autophagy regulators suggest transcriptional up-regulation of autophagy in AD brains.

KD of Mitochondrial Complex IV Gene Cox5a Leads to ROS-Induced Autophagy.

The screen hits included Cox5a, a nuclear-encoded component of the mitochondrial electron transport complex IV (Table S1). Inactivation of this complex has been suggested to occur in AD and to contribute to accumulation of ROS (9). We determined that inhibition of complex IV by knock-down of Cox5a led to the accumulation of ROS (Fig. 3A and Fig. S3A and B) and induction of autophagy (Fig. 3B and Fig. S3C). Consistent with the requirement for ROS, induction of autophagy

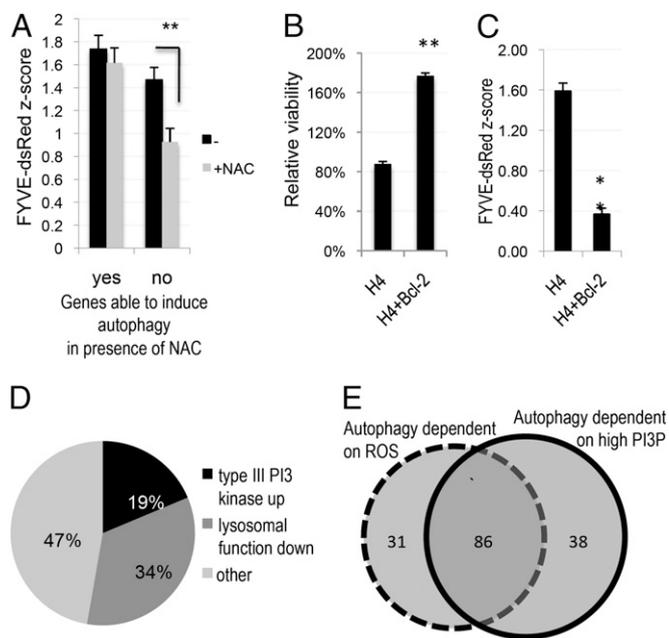


Fig. 1. Suppression of ROS and expression of Bcl-2 lead to decrease in the levels of autophagy and the type III PI3 kinase activity. (A) Quantification of average type III PI3 kinase activity following knock-down of genes able (yes) and unable (no) to induce autophagy in the presence of NAC. H4 FYVE-dsRed cells were transfected with hit siRNA for 72 h, followed by fixation and imaging on a high-throughput fluorescent microscope at 10 \times magnification. Average z-scores as compared with nontargeting siRNA are shown. (B) Comparison of the relative average viability of WT or pBabe-Bcl-2-expressing H4 cells transfected with hit gene siRNAs for 72 h. (C) Comparison of average type III PI3 kinase activity in WT or pBabe-Bcl-2-expressing H4 FYVE-dsRed cells following hit siRNA transfection for 72 h. (D) Subdivision of hits whose knock-down was able to induce autophagy under conditions of low PtdIns3P into functional categories based on their ability to up-regulate type III PI3 kinase activity or to alter lysosomal processing. (E) Subdivision of genes whose knock-down led to the induction of autophagy into functional categories based on their dependence on ROS and elevated levels of PtdIns3P (PI3P). * $P < 0.05$, ** $P < 0.01$ based on two-tailed t test with equal variance. All error bars indicate SEM.

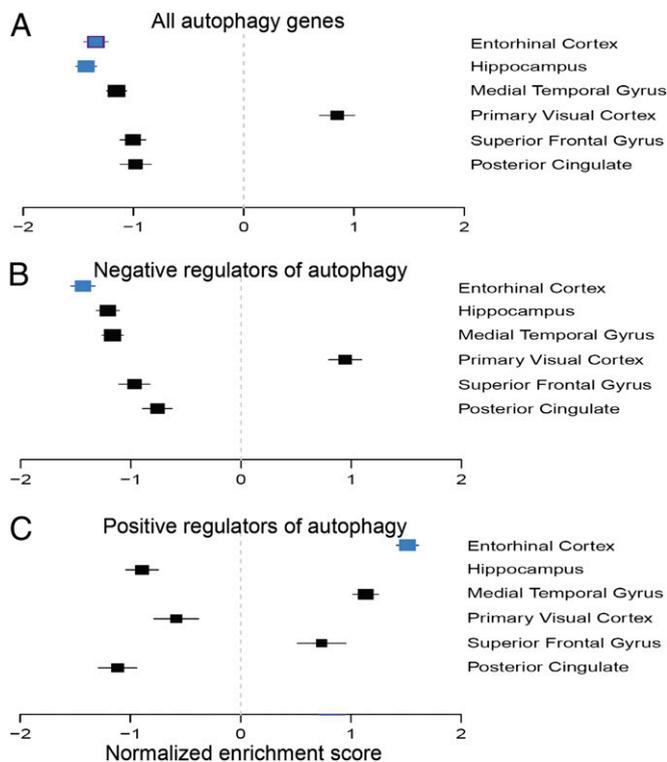


Fig. 2. Differential gene expression leads to transcriptional up-regulation of autophagy in Alzheimer's disease. Forrest plots of NES estimates with SD for the screen hit gene sets are shown. (A) GSEA analysis of overall screen hit gene expression in different regions of AD brain as compared with unaffected age-matched controls. (B and C) GSEA analysis of hit genes determined to function as negative (B) or positive (C) regulators of autophagy flux. Blue squares indicate enrichment signals with $P \leq 0.05$ in an individual comparison. The size of a square is inversely proportional to the respective SD.

agy following knock-down of Cox5a was attenuated in the presence of NAC (Fig. 3C).

Our screen data indicated that ROS is regulating autophagy by activating the type III PI3 kinase. In agreement, knock-down of Cox5a led to the increase in the levels of PtdIns3P (Fig. 3D). Levels of PtdIns3P were suppressed in the presence of the type III PI3 kinase inhibitor, 3MA, confirming the involvement of this kinase. In addition, 3MA, as well as the knock-down of the catalytic subunit of the kinase, Vps34, attenuated the induction of autophagy in response to the loss of Cox5a (Fig. 3E and F and Fig. S3D). Together, these data indicate that knock-down of Cox5a leads to the induction of autophagy by increasing ROS dependent type III PI3 kinase activity.

ROS Mediate Autophagy in Response to Amyloid β . A β , the main pathogenic factor in AD, has been proposed to cause mitochondrial damage leading to the generation of ROS (9). We hypothesized that induction of autophagy by A β may also be mediated by ROS. We observed increased levels of autophagy following treatment of H4 cells with A β (Fig. 4A and Fig. S4A). To determine whether this was due to an increase in the initiation of autophagy or to a block in lysosomal degradation, we compared the accumulation of LC3-II following A β treatment in the absence and presence of lysosomal protease inhibitor, E64d (Fig. 4A). Up to 8 h after treatment the accumulation of LC3-II could be observed only in the presence of E64d. At 48 h after the addition of A β , the increased levels of LC3-II were observed even without E64d but were further increased in the presence of E64d. In addition, we observed increased conjugation of Atg12-Atg5 starting 4 h after A β treatment. Together these data suggest increased initiation of autophagy in response to A β . However, as previously reported (4), lysosomal proteolysis also appeared to

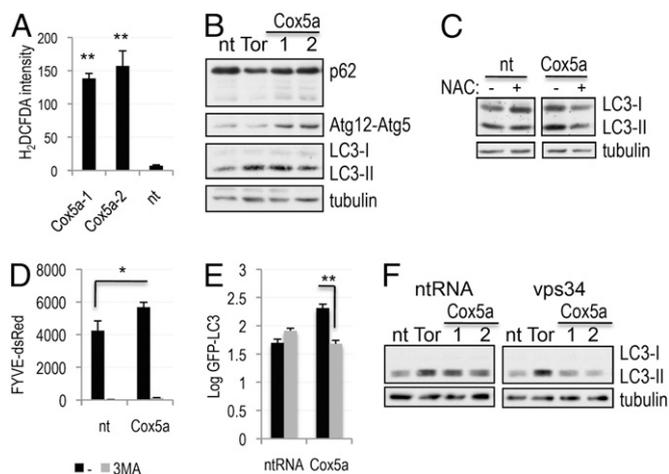


Fig. 3. Knock-down of the mitochondrial gene Cox5a leads to generation of ROS and induction of autophagy. (A) Induction of ROS in H4 cells transfected with two independent siRNAs against Cox5a or nontargeting siRNA for 72 h. Cells were stained in 25 mM carboxy-H2DCFDA and Hoechst and imaged at 40 \times . (B) Induction of autophagy in cells transfected with siRNAs against Cox5a or controls for 72 h; autophagy levels were assessed with antibodies against p62, Atg5 (Atg12-Atg5 complex is shown), and LC3. (C) Induction of autophagy by Cox5a knock-down is suppressed in the presence of the antioxidant NAC. Cells were prepared as in B, except that, where indicated, 10 mM NAC was added to the culture media 24 h after transfection. (D) Knock-down of Cox5a leads to the accumulation of PtdIns3P in a manner dependent on the function of the type III PI3 kinase. Quantification of FYVE-dsRed reporter is shown. Cells were transfected as in B; where indicated, the type III PI3 kinase inhibitor 3MA (10 mM) was added for 8 h before fixation and imaging. (E) Induction of autophagy following Cox5a knock-down depends on the function of the type III PI3 kinase. GFP-LC3 H4 cells were prepared and imaged as in D. (F) Levels of autophagy induced following knock-down of Cox5a were assessed in H4 cells transfected with control siRNA (nt, nontargeting; Tor, mTOR) or siRNA against Vps34 for 72 h by Western blot. * $P < 0.05$, ** $P < 0.01$ based on two-tailed *t* test with equal variance. All error bars indicate SEM.

be inhibited, as demonstrated by the expansion of the lysosomal compartment (Fig. 4B) and altered mobility of lysosomal proteins, cathepsin D (CtsD) and Lamp-2 on a Western blot, starting at 24 h after A β treatment (Fig. S4B, Left). Therefore, A β regulates the apparent levels of cellular autophagy by both enhancing initiation of autophagy and decreasing the rate of autophagosome clearance due to lysosomal inhibition.

To determine whether ROS are involved in the induction of autophagy by A β , we first checked that A β induced ROS (Figs. 4C and S4C). Confirming the essential function of ROS, NAC attenuated induction of autophagy in response to A β (Fig. 4D). Interestingly, NAC was unable to suppress the A β -induced changes in lysosomal proteins (Fig. S4B, Right), suggesting that lysosomal damage may be mediated by a different mechanism.

We then investigated the involvement of the type III PI3 kinase in the induction of autophagy by A β . We observed increased accumulation of PtdIns3P (Fig. 4E), which was suppressed in the presence of 3MA (Fig. 4E), confirming the involvement of the type III PI3 kinase. In agreement with a causal role of ROS, accumulation of PtdIns3P was suppressed in the presence of NAC (Fig. 4F). Finally, treatment with 3MA (Fig. 4G) or knock-down of Vps34 (Fig. S4D) was able to attenuate induction of autophagy in response to A β . On the other hand, an A β induced change in Lamp-2 mobility was not affected (Fig. S4D). Therefore, A β influences autophagy by two distinct mechanisms: by increasing initiation, and by blocking lysosomal degradation. The initiation of autophagy is an earlier event and is dependent on the accumulation of ROS and up-regulation of the type III PI3 kinase activity. The blockade of lysosomal degradation is mediated by a ROS and type III PI3 kinase independent mechanism.

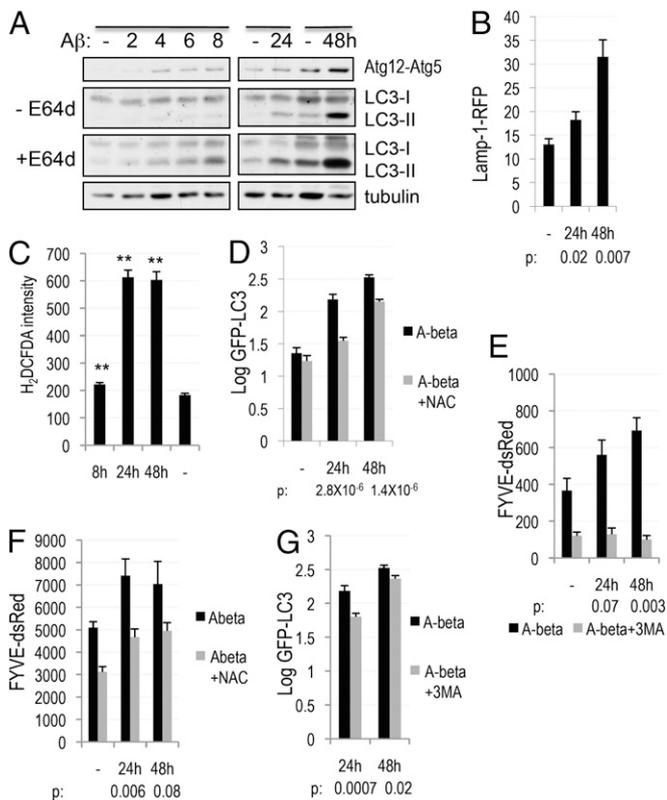


Fig. 4. Amyloid β up-regulates autophagy by inducing accumulation or ROS and the type III PI3 kinase activity. (A) Comparison of levels of LC3-II accumulation in the presence or absence of 10 μ M E64d following treatment of H4 cells with 5 μ M A β . (B) Expansion of the lysosomal compartment following A β treatment based on the Lamp-1-RFP lysosomal reporter. Cells were treated as in A, then fixed and imaged at 10 \times . (C) Treatment with A β leads to generation of ROS. Cells were stained in 25 mM carboxy-H2DCFDA and imaged at 40 \times magnification. (D) Induction of autophagy by A β is suppressed in the presence of the antioxidant NAC. GFP-LC3 cells were prepared as in B except that, where indicated, 10 mM NAC was added. (E) A β induces accumulation of PtdIns3P. FYVE-dsRed cells were prepared and imaged as in B; where indicated, the type III PI3 kinase inhibitor 3MA (10mM) was added for 8 h before fixation. (F) Induction of the type III PI3 kinase activity by A β is suppressed in the presence of antioxidant. Cells were prepared as in D. (G) Induction of autophagy by A β is dependent on the type III PI3 kinase activity. H4 GFP-LC3 cells were treated and imaged as in E. $**P < 0.01$ based on two-tailed *t* test with equal variance. All error bars represent SEM.

Modulation of Autophagy as Treatment Against Neurodegeneration.

To explore the feasibility of modulating autophagy as treatment for neurodegenerative diseases, we compared our screen hits with the known neurodegenerative disease drug targets. This revealed that several of the genes identified in the screen are targets of drugs currently in use or in clinical trials against neurological and neurodegenerative diseases, including AD (Table S7). Consistent with the predicted negative effect of the growth hormone secretagogue receptor activity on the levels of autophagy, treatment of H4 cells with its ligand, Ghrelin, a candidate drug for AD, led to a dose-dependent suppression of autophagy (Fig. 5A). Similarly, galanthamine hydrochloride, an agonist of the nicotinic acetylcholine receptor (CHRND) and an AD drug, led to the suppression of autophagy levels (Fig. 5B). Neither drug significantly affected cell viability (Fig. S5A and B). Therefore, these drugs may be able to partially suppress up-regulation of autophagy.

Candidate Autophagy Drug Targets Against Neurodegeneration. To modulate autophagy as a treatment against neurodegenerative diseases, we need to identify novel molecular drug targets that can up-regulate autophagy flux without affecting cell viability. As ROS can be the cause of cellular damage, these genes should up-

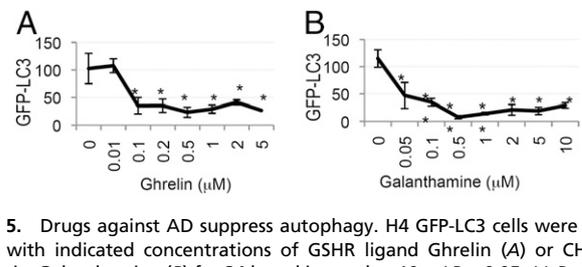


Fig. 5. Drugs against AD suppress autophagy. H4 GFP-LC3 cells were treated with indicated concentrations of GSHR ligand Ghrelin (A) or CHRND agonist Galanthamine (B) for 24 h and imaged at 10 \times . $*P < 0.05$, $**P < 0.01$ based on two-tailed *t* test with equal variance. All error bars represent SEM.

regulate autophagy in a ROS-independent manner. To this end, we analyzed our screen data and identified 26 such candidate genes (Table 1 and Table S8). We propose these genes as candidate inhibitory drug targets against neurodegenerative diseases where up-regulation of autophagy is beneficial.

In addition, our screen included nine genes whose inhibition down-regulated autophagy without affecting cell viability and

Table 1. Candidate inhibitory drug targets for modulation of autophagy in neurodegenerative disease

Gene symbol	Gene ID	Gene name
Drug targets for up-regulation of autophagy		
PPFIA4	8497	Protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 4
ADMR	11318	Adrenomedullin receptor
PTGER2	5732	Prostaglandin E receptor 2 (subtype EP2), 53kDa
EP300	2033	E1A binding protein p300
CXCL12	6387	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
NCR3	259197	Natural cytotoxicity triggering receptor 3
GABBR2	9568	Gamma-aminobutyric acid (GABA) B receptor, 2
GJA4	2701	Gap junction protein, alpha 4, 37kDa
KRT18	3875	Keratin 18
SDHB	6390	Succinate dehydrogenase complex, subunit B, iron sulfur (lp)
GTF2IRD2	84163	GTF2I repeat domain containing 2
GNRH2	2797	Gonadotropin-releasing hormone 2
HIVEP2	3097	HIV type 1 enhancer binding protein 2
USP19	10869	Ubiquitin specific peptidase 19
EPHA6	203806	EPH receptor A6
TACR2	6865	Tachykinin receptor 2
MYL3	4634	Myosin, light chain 3, alkali; ventricular, skeletal, slow
CAPN1	823	Calpain 1, (mu/1) large subunit
SORCS2	57537	Sortilin-related VPS10 domain containing receptor 2
BAIAP2	10458	BAI1-associated protein 2
THBS2	7058	Thrombospondin 2
PCGF1	84759	Polycomb group ring finger 1
MMP10	4319	Matrix metalloproteinase 10 (stromelysin 2)
CHRND	1144	Cholinergic receptor, nicotinic, delta
CDKN2D	1032	Cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)
FBXL20	84961	F-box and leucine-rich repeat protein 20
Drug targets for down-regulation of autophagy		
ATG7	10533	ATG7 autophagy related 7 homolog (<i>S. cerevisiae</i>)
MEGF10	84466	Multiple EGF-like-domains 10
KIF5C	3800	Kinesin family member 5C
SMYD3	64754	SET and MYND domain containing 3
LOC285647	285647	Suppressor of defective silencing 3 pseudogene
STIM1	6786	Stromal interaction molecule 1
GAB1	2549	GRB2-associated binding protein 1
GPR18	2841	G-protein-coupled receptor 18
CATSPER4	378807	Cation channel, sperm associated 4

regulatory networks mediating actin dynamics and endocytic processes involved in axonal guidance suggests that autophagy may directly contribute to the regulation of growth and maintenance of neuronal processes. Therefore, progressive transcriptional down-regulation of autophagy in normal human brain aging may also contribute to age related decline in synaptic plasticity, memory, and cognitive function.

Conversely, our data indicate that autophagy is specifically up-regulated in AD, due both to ROS-dependent activation of the type III PI3 kinase as well as at the transcriptional level. We hypothesize that this may represent, respectively, an acute and a long-term attempt by the affected neuronal cells to rid themselves of the harmful effects of A β exposure, such as accumulation of defective mitochondria and protein aggregates.

Surprisingly, our study also demonstrated that drugs in clinical trials or used against AD lead to the inhibition of autophagy. Because increasing autophagy is predicted to be beneficial for the treatment of neurodegenerative diseases, one may wonder if this inhibition may be an unintended side effect, as autophagy was not a parameter tested when developing these drugs. Alternatively, inhibition of autophagy could contribute to the efficacy of those drugs. Our data indicate that treatment with A β leads to both an increase in the initiation of autophagy as well as a decrease in the lysosomal degradation. We hypothesize that as the downstream degradative pathway is partially blocked, increasing the autophagic input may further exacerbate the stress on the system. In this case, reducing the initiation of autophagy might be beneficial by decreasing input into the lysosomal system, thus allowing for gradual turnover of the accumulated material. Therefore, although up-regulation of autophagy might be beneficial as a preventive measure during normal aging, as well as to combat some neurodegenerative diseases including early stages of AD (21, 22), increasing autophagic input may backfire if applied to later stages of AD, in which the lysosomal blockage is preeminent.

Dissociation of Autophagy from Cell Death for Clinical Benefit. Another important consideration in developing autophagy-based therapies is the ability to change levels of autophagy without inducing neuronal cell death. As a first important step toward this goal, we provide a list of potential molecular drug targets for the modulation of autophagy in neurodegenerative diseases. Interestingly, this list includes calpain 1 which, as we have pre-

viously shown, can be inhibited by fluspirilene, an inhibitor of intracellular Ca²⁺ flux, to induce autophagy by reducing cleavage of full-length Atg5 and increasing conjugated Atg12-Atg5 without inducing cell death (23). We propose that by developing inhibitors against these gene targets, autophagy can be safely manipulated without harming neuronal cells, thus providing potential novel therapies against neurodegenerative diseases.

Materials and Methods

Cell Lines and Culture Conditions. H4 human neuroblastoma cells were cultured and treated as described elsewhere (5). GFP-LC3, FYVE-dsRed, GFP-LC3 pSRP-Beclin1 knock-down (11) and Lamp-1-RFP (5) H4 cells have been previously described.

For antioxidant assay, cells were treated with 2.5 mM NAC (Sigma). A β was prepared using a modified method from ref. 24.

Imaging and Image Quantification. Cells were imaged as previously described (5) on a CellWoRx microscope (Applied Precision) at $\times 10$ magnification. Images were quantified using VHSscan and VHSview software (Cellomics).

Quantification of Cellular ROS Levels. ROS were quantified using Image-iT LIVE Green ROS Detection Kit for microscopy (Molecular Probes) according to manufacturers instructions. Images were acquired on a Nikon Eclipse E800 microscope at $\times 40$ magnification and quantified using CellProfiler software (25).

Bioinformatics Analysis. Gene set enrichment analysis. Associations of autophagy gene sets with AD were evaluated using GSEA (26) and gene expression data from laser-capture microdissected non-tangle-bearing neurons of 34 late-onset AD-afflicted individuals with a mean age at death of 79.9 ± 6.9 y and 14 neurologically normal healthy elderly controls (27) (GEO accession number GSE5281).

Analysis of hit gene expression in aging. Analysis was based on Affymetrix HG-U133_Plus_2 microarray data of young (≤ 40 y old) and old (≥ 70 y old) human brain samples (10). Array normalization, expression value calculation and clustering analysis were performed using the dChip software (28) (www.dchip.org).

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