

Review

Rapamycin and mTOR-independent autophagy inducers ameliorate toxicity of polyglutamine-expanded huntingtin and related proteinopathies

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The formation of intra-neuronal mutant protein aggregates is a characteristic of several human neurodegenerative disorders, like Alzheimer's disease, Parkinson's disease (PD) and polyglutamine disorders, including Huntington's disease (HD). Autophagy is a major clearance pathway for the removal of mutant huntingtin associated with HD, and many other disease-causing, cytoplasmic, aggregate-prone proteins. Autophagy is negatively regulated by the mammalian target of rapamycin (mTOR) and can be induced in all mammalian cell types by the mTOR inhibitor rapamycin. It can also be induced by a recently described cyclical mTOR-independent pathway, which has multiple drug targets, involving links between Ca^{2+} –calpain– G_{sx} and cAMP–Epac–PLC– ϵ – IP_3 signalling. Both pathways enhance the clearance of mutant huntingtin fragments and attenuate polyglutamine toxicity in cell and animal models. The protective effects of rapamycin *in vivo* are autophagy-dependent. In *Drosophila* models of various diseases, the benefits of rapamycin are lost when the expression of different autophagy genes is reduced, implicating that its effects are not mediated by autophagy-independent processes (like mild translation suppression). Also, the mTOR-independent autophagy enhancers have no effects on mutant protein clearance in autophagy-deficient cells. In this review, we describe various drugs and pathways inducing autophagy, which may be potential therapeutic approaches for HD and related conditions.

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Autophagy

Macroautophagy (henceforth referred to as autophagy) has become a field of rapidly growing interest in recent years, and has possible implications in several human diseases such as cancer, certain neurodegenerative disorders, myopathies and infectious diseases.¹ Autophagy is a non-specific, bulk degradation process involved in the clearance of long-lived proteins and organelles (Figure 1). The process begins with the formation of isolation membranes called phagophores at the phagophore assembly site. The phagophores then elongate and engulf a portion of the cytosol to form mature autophagosomes, which ultimately fuse with lysosomes to

form the degradative autophagolysosomes.² Hybrid intermediates called amphisomes are formed when autophagosomes fuse with the endosomes.³ The acidic lysosomal hydrolases aid in the degradation of the engulfed contents and the nutrients are recycled back to the cell.²

Autophagy is a highly conserved process from yeast to mammals. Genetic analysis in yeast has uncovered several *Atg* (autophagy) genes, many of which have mammalian orthologs.⁴ The formation of yeast autophagosomes requires the Atg9p and its cycling system, a phosphatidylinositol 3-kinase (PI3K) complex and two ubiquitin-like conjugation systems.^{2,5} The formation of autophagosome precursors requires class III PI3K and is prevented by PI3K inhibitors

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Abbreviations: AC, adenylyl cyclase; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; α_2 -AR, α_2 -adrenergic receptor; cAMP, cyclic AMP; CBZ, carbamazepine; CMA, chaperone-mediated autophagy; DAG, diacylglycerol; 2'5'-ddA, 2'5'-dideoxyadenosine; ER, endoplasmic reticulum; FIP200, focal adhesion kinase family interacting protein of 200 kDa; FKBP12, FK506-binding protein 12; $\text{G}\beta\text{L}$, G-protein β -subunit like protein; GSK-3 β , glycogen synthase kinase-3 β ; HAP1A, huntingtin-associated protein-1A; HD, Huntington's disease; hsc70, heat shock cognate protein of 70 kDa; IMPase, inositol monophosphatase; Ins, inositol; IP_3 , inositol 1,4,5-trisphosphate; IP_4 , inositol 1,3,4,5-tetrakisphosphate; IP_3R , IP_3 receptor; I1R, inidazoline-1 receptor; LAMP2A, lysosome-associated membrane protein type 2A; 3-MA, 3-methyladenine; MAP1-LC3, microtubule-associated protein 1 light chain 3; MEF, mouse embryonic fibroblast; mGluR1/5, class 1 metabotropic glutamate receptor; mhht, mutant huntingtin; mTOR, mammalian target of rapamycin; NMDAR, N-methyl-D-aspartate receptors; PACAP, pituitary adenylyl cyclase-activating polypeptide; PAS, phagophore assembly site; PD, Parkinson's disease; PI3K, phosphatidylinositol 3-kinase; PIP_2 , phosphatidylinositol 4,5-bisphosphate; PKA, protein kinase A; PLC, phospholipase C; polyA, polyalanine; polyQ, polyglutamine; raptor, regulatory associated protein of mTOR; SBMA, spinobulbar muscular atrophy; SCA, spinocerebellar ataxias; SMER, small-molecule enhancer of rapamycin; SMIR, small-molecule inhibitor of rapamycin; SOD1, superoxide dismutase 1; TSC, tuberous sclerosis complex; ULK, UNC (uncoordinated movement)-51-like kinase; VPA, valproic acid

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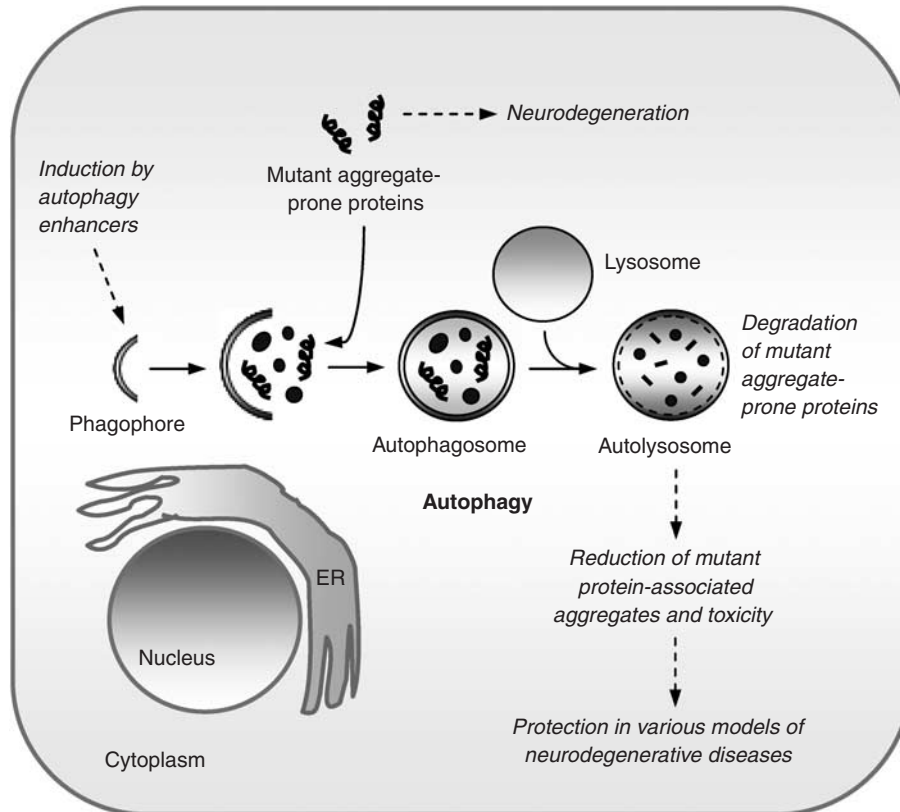


Figure 1 Autophagy as a protective pathway for the clearance of aggregate-prone proteins. Autophagy is one of the primary degradation pathways for various aggregate-prone proteins associated with neurodegenerative diseases. Induction of autophagy by an autophagy enhancer triggers formation of double-membrane, cytoplasmic structures called phagophores, which engulf mutant aggregate-prone proteins along with portions of the cytoplasm within autophagosomes. Autophagosomes then ultimately fuse with the lysosomes to form autolysosomes, where their contents are degraded by acidic lysosomal hydrolases. This enhanced autophagic clearance of the mutant aggregate-prone proteins causing neurodegeneration results in a reduction in mutant protein-associated aggregation and toxicity, which is protective in several models of neurodegenerative diseases

like 3-methyladenine (3-MA) or wortmanin.^{6,7} The only known mammalian protein that specifically associates with the autophagosome membrane is LC3 (microtubule-associated protein 1 light chain 3), which is the mammalian ortholog of yeast Atg8p. Two forms of LC3 have been described: the cytosolic LC3-I form that conjugates with phosphatidylethanolamine upon autophagy induction, resulting in the autophagosome-associated LC3-II form.⁸

Similar to yeast, the classical pathway that regulates autophagy involves the serine/threonine kinase, mammalian target of rapamycin (mTOR).⁷ However, several pathways seem to regulate autophagy in mammalian cells. Physiologically, autophagy is induced by amino acid deprivation.⁹ Although yeast cells induce autophagy during nutrient deprivation conditions, autophagy occurs at a basal level in mammalian cells. Although autophagy is thought to be predominantly a cell survival mechanism, a number of studies also implicate a role for autophagy in cell death.¹⁰

Aggregate-Prone Proteins Causing Neurodegeneration are Degraded by Autophagy

The formation of mutant protein aggregates is a characteristic feature of several human neurodegenerative disorders, like Alzheimer's disease, Parkinson's disease (PD), amyotrophic

lateral sclerosis (ALS), prion diseases and polyglutamine (polyQ) disorders, including Huntington's disease (HD) and various spinocerebellar ataxias (SCA).¹¹ It is debated whether the aggregates are toxic or protective; however, recent studies have implicated the pre-aggregate oligomers to be the most toxic species.^{11–14} Experiments in cell culture have suggested that cells with large mutant huntingtin inclusions visible by light microscopy may be less prone to cell death than cells with diffuse mutant protein expression (which may however be present in microaggregates).¹⁵ However, this study did not test if cells with mutant huntingtin inclusions were compromised compared with cells overexpressing wild-type huntingtin. Although the monomeric aggregate-prone proteins can be cleared by the ubiquitin–proteasome system, the narrow pore of the proteasome precludes entry of oligomers and larger structures. As autophagy involves degradation of protein complexes and organelles, and the aggregates occurring in PD and HD visible by light microscopy are not membrane-bound and are larger than typical autophagosomes, it is likely that the monomeric and oligomeric species of the aggregate-prone proteins are cleared by autophagy.¹⁶

Autophagy is a major degradation pathway for various intracytosolic, aggregate-prone, disease-causing proteins associated with neurodegenerative disorders, such as mutant huntingtin (both N-terminal fragments and full-length forms)

associated with HD, the A53T or A30P point mutants of α -synuclein causing familial PD, ataxin 3 involved in SCA3, mutant forms of superoxide dismutase 1 causing familial ALS and mutant forms of tau causing fronto-temporal dementias.^{17–22}

Another lysosomal pathway that may clear certain aggregate-prone proteins related to disease is chaperone-mediated autophagy (CMA). This pathway involves recognition of a pentapeptide motif in client proteins by cytosolic heat-shock cognate protein of 70 kDa (hsc70), which mediates their ability to be taken up into lysosomes for degradation by the lysosome-associated membrane protein type 2A (LAMP2A) that acts as a receptor. This pathway appears to be important for the clearance of wild-type α -synuclein.²³ However, Cuervo *et al.*²³ have shown that dopamine-modified or the point-mutated forms of α -synuclein cannot be cleared by this

pathway – in fact these α -synuclein species impaired the clearance of other CMA substrates.²⁴

Recent studies by Mizushima, Tanaka and coworkers^{25,26} have highlighted a strong link between autophagy and neurodegeneration, as loss of basal autophagy by conditional knockout of key autophagy genes (*Atg5* or *Atg7*) in mouse brains resulted in a neurodegenerative phenotype and the formation of protein aggregates. Thus, constitutive autophagy may play a pivotal role in the clearance of normally occurring misfolded proteins in the cells. We have previously shown that autophagy upregulation may be a possible therapeutic strategy for neurodegenerative diseases by enhancing the clearance of aggregate-prone proteins⁷ (Figure 1). In this review, we discuss various strategies for inducing autophagy, both by mTOR-dependent and -independent pathways (Table 1). These were identified using cell-based assays,

Table 1 List of autophagy enhancers and their protective effects in neurodegenerative diseases

Autophagy enhancer	Mode of action	Protective effects in neurodegenerative diseases	Reference
(1) Rapamycin (2) CCI-779 (3) Glucose (4) Glucose-6-phosphate	Inhibit mammalian target of rapamycin (mTOR)	Enhance clearance of mutant htt (1–4), α -syn (1, 3, 4), tau (1), ataxin 3 (1) and expanded polyA (1); reduce mutant htt (1–4) and expanded polyQ (1) aggregates; inhibit intrinsic apoptotic pathway (1); protect cell (1–4), fly (1) and mouse models (2) of HD; protect fly models of tauopathy (1), SBMA (1) and flies with expanded polyQ or polyA (1)	17–20,27–29
(5) Lithium (6) L-690,330	Inhibit IMPase and lower inositol and IP ₃ levels; mTOR-independent	Enhance clearance of mutant htt and α -syn (5, 6); reduce mutant htt (5, 6) and SOD1 (5) aggregates; protect cell (5, 6) and fly models of HD (5); protect and increase survival in ALS mouse models and patients (5)	21,30,31
(7) Carbamazepine (8) Sodium valproate	Lower inositol and IP ₃ levels; mTOR-independent	Enhance clearance of mutant htt and α -syn (7, 8); reduce mutant htt aggregates (7, 8); protect cell (7, 8) and fly (8) models of HD	30,32
(9) Verapamil (10) Loperamide (11) Amiodarone (12) Nimodipine (13) Nitrendipine (14) Niguldipine (15) Pimozide (16) Calpastatin (17) Calpeptin	Ca ²⁺ channel blocker; mTOR-independent	Enhance clearance of mutant htt and α -syn (9–13); reduce mutant htt (9–13) and expanded polyQ (10, 11, 14, 15) aggregates; protect cell (9–13), fly (9) and zebrafish (9) models of HD	32,33
(18) Clonidine (19) Rilmenidine	Imidazoline-1 receptor agonist; reduce cAMP; mTOR-independent	Enhance clearance of mutant htt and α -syn (18, 19); reduce mutant htt aggregates (18, 19); protect cell (18, 19), fly (18) and zebrafish (18) models of HD	32
(20) 2′5′-dideoxyadenosine	Adenylyl cyclase inhibitor; reduces cAMP; mTOR-independent	Enhances clearance of mutant htt and α -syn; reduces mutant htt aggregates; protects cell and zebrafish models of HD (20)	32
(21) NF449 (22) Minoxidil	G _s α inhibitor; mTOR-independent	Reduces mutant htt aggregates in cells (21)	32
(23) Penitrem A	K _{ATP} channel opener; mTOR-independent	Enhances clearance of htt and α -syn; reduces mutant htt aggregates; protects cell models of HD (22)	32
(24) Fluspiriline (25) Trifluoperazine (26) Trehalose	Inhibits high conductance Ca ²⁺ -activated K ⁺ channel; mTOR-independent	Reduces expanded polyQ aggregates in cells (23)	33
(27) SMER10 (28) SMER18 (29) SMER28 (30) 18 SMER analogues	Dopamine antagonist; mTOR-independent Unknown; mTOR-independent Unknown; mTOR-independent	Reduce expanded polyQ aggregates in cells (24, 25)	33
		Enhances clearance of mutant htt and α -syn; reduces mutant htt aggregates; inhibits intrinsic apoptotic pathway; protects cell and mouse models of HD (26)	34,35
		Enhance clearance of mutant htt and α -syn (27–30); reduce mutant htt aggregates (27–30); protect cell and fly models of HD (27–29)	36

ALS, amyotrophic lateral sclerosis; α -syn, α -synuclein; camp, cyclic AMP; HD, Huntington's disease; htt, Huntingtin; IMPase, inositol monophosphatase; IP₃, inositol 1,4,5-trisphosphate; mTOR, mammalian target of rapamycin; polyA, polyalanine; polyQ, polyglutamine; SBMA, spinobulbar muscular atrophy; SOD1, superoxide dismutase 1; SMER, small molecule enhancer of rapamycin

either by measuring the clearance of autophagy substrates or by analysing autophagic flux.

Rapamycin Attenuates Toxicity of Aggregate-prone Proteins by Enhancing Autophagy, with Minimal Influence via Autophagy-independent Effects Like Translation

Autophagy is negatively regulated by mTOR, whose activity can be inhibited by rapamycin, a lipophilic macrolide antibiotic that is a well-established inducer of autophagy³⁷ (Figure 2). The mTOR pathway involves two functional complexes: mTORC1 consisting of mTOR, raptor (regulatory associated protein of mTOR) and GβL (G-protein β-subunit like protein); and mTORC2 comprising mTOR, rictor and GβL.³⁸ Rapamycin forms a complex with the immunophilin FK506-binding protein 12 (FKBP12), which then stabilizes the raptor-mTOR association and inhibits the kinase activity of mTOR.³⁹ The binding of GβL to mTOR stimulates its kinase activity, and GβL is necessary for the formation of a rapamycin-sensitive interaction between raptor and mTOR.⁴⁰ Rapamycin is a potent inducer of autophagy in a diverse range of cell lines from yeast to mammalian cells tested to date, including neuron-like cells.^{17,18,37,41} However, its downstream targets that regulate autophagy are still unknown in mammalian cells.

We have previously shown that rapamycin treatment in cells expressing several aggregate-prone mutant proteins, such as huntingtin, α-synucleins, ataxin 3 and tau, enhanced the degradation of the target proteins and reduced the number of aggregates, and also protected cells and flies from mutant protein-associated toxicity.^{17–20} As mTOR regulates a

number of cellular functions distinct from autophagy-like translation,³⁸ it is important to test if the protective effects of this drug against aggregate-prone intracytosolic proteins are autophagy-dependent. This is not feasible in mice, but can be tested in *Drosophila*, where one can overexpress an aggregate-prone protein in the context of a wild-type genetic background or a genetic background where an autophagy gene is modified. The protective effect of rapamycin in these situations appear to be autophagy-dependent *in vivo*, because its ability to protect against the toxicity of a polyalanine (polyA)-expanded aggregate-prone protein (which was previously shown to be an autophagy substrate) was abolished in flies expressing the mutant protein on a genetic background with 50% loss of the key autophagy gene, *Atg1*.²⁰ This conclusion was reinforced by an independent subsequent study that showed that the ability of rapamycin to reduce the toxicity of a polyQ-expanded protein was abrogated in flies where a different autophagy gene (*Atg12*) was knocked down by RNAi.²⁷ These studies strongly suggest that the protective effects of rapamycin *in vivo* are through autophagy, and that the effects of other consequences of rapamycin treatment, like inhibition of translation,⁴² are minimal. Consistent with the fly data, the rapamycin analogue CCI-779 ameliorates and delays the onset of a range of behavioural abnormalities and reduces aggregate load in a mouse model of HD.¹⁸

Rapamycin and autophagy upregulation also protects cells against pro-apoptotic insults by clearing mitochondria, which are endogenous autophagy substrates.²⁸ As a consequence of reduced mitochondrial load, the intrinsic apoptotic pathway was inhibited because of reduced cytochrome *c* release,

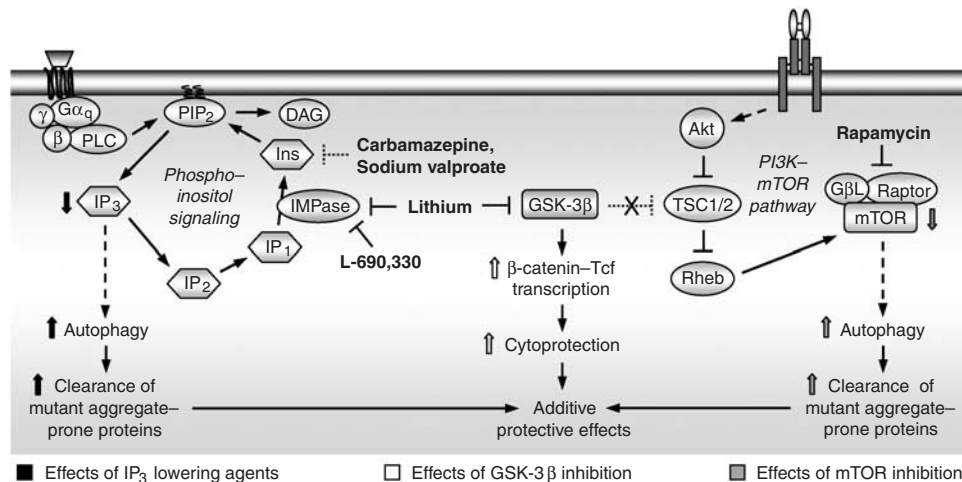


Figure 2 Combinatorial strategy for upregulating autophagy by mTOR and phospho-inositol-signalling pathways. The mammalian target of rapamycin (mTOR), which is a downstream target in the phosphatidylinositol 3-kinase (PI3K) pathway, negatively regulates autophagy. A diverse range of signals, such as growth factors and amino acids, regulate the raptor-mTOR pathway by inhibiting tuberous sclerosis complex (TSC1/2), thereby alleviating the inhibitory effect of TSC1/2 on rheb, which subsequently activates the raptor-mTOR complex. Several kinases, such as Akt, signal to raptor-mTOR by phosphorylating TSC2 and inhibiting the activity of the TSC1/2 heterodimer. Rapamycin forms a complex with the immunophilin FK506-binding protein 12 (FKBP12), which then stabilizes the raptor-mTOR association and inhibits the kinase activity of mTOR. Inhibition of mTOR by rapamycin induces autophagy and enhances the clearance of mutant aggregate-prone proteins associated with neurodegenerative diseases. Autophagy can also be induced with compounds that decrease inositol (Ins) or inositol 1,4,5-trisphosphate (IP₃) levels in the phosphoinositol-signalling pathway in an mTOR-independent manner. These agents include lithium and L-690,330 by inhibiting inositol monophosphatase (IMPase), and carbamazepine and sodium valproate by inhibiting inositol synthesis. However, lithium also inhibits glycogen synthase kinase-3β (GSK-3β) that activates mTOR by inhibiting TSC1/2, thereby impairing autophagy. Combination treatment with lithium and rapamycin alleviates the block in autophagy by GSK-3β inhibition (shown by a cross, X), and hence additively enhances autophagy and facilitates greater clearance of the aggregate-prone proteins. Furthermore, GSK-3β inhibition by lithium increases β-catenin-Tcf-mediated transcription, which is cytoprotective and may contribute to additional protective effects of this combination treatment for neurodegenerative diseases. Colour-coded block arrows denote increase or decrease

leading to decreased activation of caspases 9 and 3. Again, the protective effects of rapamycin in this context were autophagy-dependent and its benefits against paraquat toxicity in *Drosophila* were abrogated in flies with a 50% loss of *Atg1*.²⁸ This anti-apoptotic effect of autophagy upregulation may be beneficial in neurodegenerative disorders where there are secondary apoptotic insults.

An mTOR-Independent Pathway Regulating Autophagy Through Inositol or IP₃ Levels

We previously demonstrated that autophagy can be induced by lowering intracellular inositol or inositol 1,4,5-trisphosphate (IP₃) levels³⁰ (Figure 2). This was the first demonstration of the existence of an autophagy pathway in mammalian system independent of mTOR. Mood-stabilizing drugs for affective disorders, such as lithium, carbamazepine and sodium valproate, all of which reduce inositol levels,⁴³ induced autophagy and enhanced the clearance of autophagy substrates, such as mutant huntingtin and the A53T and A30P mutants of α -synuclein.³⁰ On the contrary, increasing cellular inositol levels by myo-inositol or elevating IP₃ levels by an inhibitor of prolyl oligopeptidase activity,⁴³ retarded the clearance of autophagy substrates and abrogated the effects of lithium, but not of rapamycin.³⁰ Moreover, drugs that decrease IP₃ levels did not reduce mTOR activity, and rapamycin did not affect inositol levels, further suggesting that the control of autophagy by IP₃ and rapamycin is mediated by two independent pathways. The autophagy-inducing ability of lithium was due to inhibition of inositol monophosphatase (IMPase), which prevents inositol recycling, leading to depletion of cellular inositol and inhibition of the phospho-inositol cycle.^{30,44,45} Likewise, L-690 330, a specific IMPase inhibitor, induced autophagy and mimicked the effects of lithium on the clearance of autophagy substrates.³⁰ Consistent with a role of IP₃ on autophagy, a recent study by Kroemer and coworkers⁴⁶ has shown that genetic knockdown or pharmacological inhibition of the IP₃ receptor (IP₃R) induces autophagy.

Although lithium has been used for decades for treating bipolar disorder patients, its mechanism of action is still not fully understood. Inositol depletion is one of the most widely accepted hypotheses for its therapeutic action.⁴⁷ It is tempting to speculate whether the beneficial effects of inositol-lowering agents used in the treatment of bipolar disorder are mediated by the clearance of long-lived autophagy substrates (this does not implicate protein aggregation is a feature of this disease), as lithium has acute effects on free inositol levels before showing clinical effects.⁴⁸ This idea⁴⁸ has recently been followed up in a study that demonstrated antidepressant effects of rapamycin in rat and mouse models, leading to the suggestion that autophagy inducers may be helpful in the treatment of these disorders.⁴⁹

The autophagy-inducing effects of lithium facilitated the clearance of mutant huntingtin in HD cell models, and reduced mutant protein-associated aggregation and toxicity.³⁰ Lithium also reduced neurodegeneration in HD *Drosophila* models.³¹ However, lithium is likely to have various antiapoptotic effects by inhibiting another intracellular target, glycogen synthase kinase-3 β (GSK-3 β), resulting in β -catenin upregulation.^{50–52}

A recent study by Fornai and coworkers²¹ has shown a striking effect of lithium in increasing survival and attenuating the disease progression in ALS patients and mouse models. All the ALS patients on lithium treatment for 15 months survived, but approximately 30% of age-, disease duration- and sex-matched controls receiving riluzole died.²¹ Apart from lithium's neuroprotective effects,⁵⁰ this fascinating result was suggested to be at least partially due to induction of autophagy.²¹ Importantly, the above study also demonstrated that lithium can trigger autophagy in neurons.²¹ Consistent with the protective role of IP₃-lowering agents through autophagy induction, sodium valproate, which inhibits inositol synthesis and thereby decreases intracellular IP₃ levels,^{43,53} also protected against neurodegeneration in a *Drosophila* model of HD.³²

Combination Treatment for Enhancing Autophagy by mTOR-Dependent and -Independent Pathways

The availability of mTOR-dependent and -independent pathways allows for a combination treatment strategy that enables greater upregulation of autophagy by simultaneously inducing autophagy using both rapamycin and lithium³¹ (Figure 2). Apart from the ability of lithium to induce mTOR-independent autophagy by inhibiting IMPase,³⁰ GSK-3 β , another intracellular target of lithium, has opposing effects on autophagy in an mTOR-dependent fashion.³¹ Guan and coworkers⁵⁴ reported inhibition of GSK-3-activated mTOR signalling by preventing GSK-3-dependent phosphorylation of tuberous sclerosis complex (TSC) protein TSC2. As activation of mTOR negatively regulates autophagy, we confirmed that autophagy was impaired by a specific GSK-3 β inhibitor, SB216763, or in GSK-3 β -deficient mouse embryonic fibroblasts (MEFs) compared with the wild-type MEFs.³¹ Inhibition of GSK-3 β retarded the clearance of aggregate-prone proteins, whereas a constitutively active GSK-3 β enhanced clearance and autophagic flux. To counteract the autophagy-inhibitory effects of mTOR activation resulting from lithium treatment because of GSK-3 β inhibition, we used rapamycin in combination with lithium. Rapamycin abrogates the mTOR activation mediated by lithium and the combination allows autophagy stimulation by mTOR inhibition and by lowering of IP₃ levels. Combination treatment with lithium and rapamycin had additive protective effects on the autophagic clearance of mutant huntingtin and against cell death, compared with the effects of either drug alone.^{30,31} We have further demonstrated proof-of-principle for this rational combination treatment approach *in vivo* by showing greater protection against neurodegeneration in an HD *Drosophila* model with TOR inhibition and lithium, or in HD flies treated with rapamycin and lithium, compared with inhibition of either pathway alone.³¹ Moreover, this strategy may also benefit from the cytoprotective effects of GSK-3 β inhibition, because of activation of the β -catenin/Tcf pathway^{31,51,52} (Figure 2).

The rationale for enhancing autophagy using the modulators of mTOR-dependent and mTOR-independent pathways is also seen by the use of saturating doses of rapamycin with other mTOR-independent autophagy inducers that have been subsequently identified.^{30,32,34,36} Such combination treatments greatly facilitate the clearance of aggregate-prone

proteins and reduce toxicity in cell models, compared with either compounds alone. Combination therapy with lower doses of each drug may be safer for long-term treatment and may lessen the drug-specific side-effects if used for neurodegenerative diseases, compared with using doses of either compound alone that result in more severe perturbations of a single pathway. However, demonstration of this combination treatment strategy in mouse models of neurodegenerative disorders is challenging and remains to be investigated.

An mTOR-Independent Cyclical Autophagy Pathway with Multiple Drug Targets

Apart from the classical mTOR pathway regulating mammalian autophagy, we have recently described many of the players in an mTOR-independent pathway³² (Figure 3). We screened for autophagy modulators with 253 FDA-approved drugs and pharmacological probes by assessing the clearance of autophagy substrates, such as the A30P and A53T mutants of α -synuclein and mutant huntingtin. This screen yielded various autophagy enhancers, such as L-type Ca^{2+} channel antagonists, a K_{ATP} channel opener and a G_i -signalling activator, whose mechanisms of action were found to be linked in a cyclical fashion. The L-type Ca^{2+} channel modulators regulate autophagy by influencing intracytosolic Ca^{2+} levels, thereby regulating calpain activity, which in turn regulates the cleavage and activity of G_{sx} that subsequently generates cyclic AMP (cAMP). The K_{ATP} channel opener reduces cytosolic Ca^{2+} levels (like the L-type Ca^{2+} channel blockers) and is likely to work by the same pathway. On the

other hand, the G_i -signalling activator reduces cAMP, which regulates phospholipase C- ϵ (PLC- ϵ) activity through the Epac pathway, thereby influencing the generation of IP_3 , which subsequently regulates calpain activity.³² The data from this screen was consistent with previous findings where lowering intracellular IP_3 levels triggered autophagy.³⁰ This cyclical autophagy pathway has multiple drug targets for chemically inducing autophagy, and drugs acting on this pathway were protective in cell, *Drosophila* and zebrafish models of HD³² (Figure 3). The mTOR-independent autophagy enhancers identified in our screen may be of importance for neurodegenerative diseases like HD or other conditions where autophagy acts as a protective pathway. We will discuss the two arms of the pathway (Ca^{2+} –calpain– G_{sx} pathway and cAMP–Epac–PLC- ϵ – IP_3 pathway) separately.

Regulation of autophagy by Ca^{2+} –calpain– G_{sx} pathway.

The regulation of autophagy by intracytosolic Ca^{2+} levels was first suggested by Seglen and coworkers⁵⁵ more than a decade ago. The data from our recent screen was consistent with their earlier findings, in which an increase in intracytosolic Ca^{2+} was found to inhibit autophagy in rat hepatocytes. They found that autophagy was inhibited with agents like thapsigargin (an endoplasmic reticulum (ER) $\text{Ca}^{2+}/\text{Mg}^{2+}$ ATPase inhibitor that releases Ca^{2+} from ER stores) or ionomycin (Ca^{2+} ionophore that releases Ca^{2+} from intracellular stores), which increase intracytosolic Ca^{2+} levels.⁵⁵

The first category of compounds that our screen revealed as autophagy enhancers are L-type Ca^{2+} channel

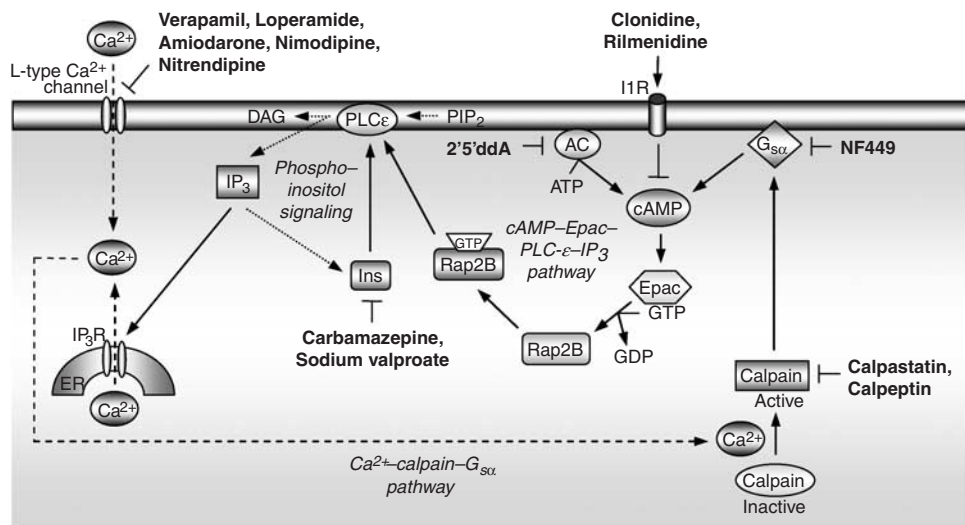


Figure 3 A cyclical mTOR-independent autophagy pathway. A cyclical mTOR-independent pathway regulating mammalian autophagy, comprising cAMP–Epac–PLC- ϵ – IP_3 and Ca^{2+} –calpains– G_{sx} pathways, which has multiple drug targets for neurodegenerative diseases. Intracellular cAMP levels are increased by adenylyl cyclase (AC) activity, thereby activating Epac. Epac then activates a small G-protein Rap2B, which activates PLC- ϵ , resulting in the production of IP_3 consequently releasing Ca^{2+} from the endoplasmic reticulum (ER) stores. Intracytosolic Ca^{2+} levels are also increased by L-type Ca^{2+} channel agonists. An increase in intracytosolic Ca^{2+} activates a family of Ca^{2+} -dependent cysteine proteases called calpains, which cleave and activate G_{sx} . Activation of G_{sx} , in turn, increases adenylyl cyclase activity to elevate cAMP levels, thereby forming a loop. Activation of this pathway inhibits autophagy. Multiple drug targets acting at distinct stages in this pathway induce autophagy, such as imidazoline-1 receptor (I1R) agonists (clonidine and rilmenidine) and adenylyl cyclase inhibitor [2'5'-dideoxyadenosine (2'5'ddA)], which decrease the levels of intracellular cAMP, agents reducing inositol and IP_3 levels [carbamazepine (CBZ) and valproic acid (VPA)], L-type Ca^{2+} channel antagonists (verapamil, loperamide, amiodarone, nimodipine and nitrendipine), calpain inhibitors (calpastatin and calpeptin) and G_{sx} inhibitor (NF449). Upregulating autophagy through this pathway has been shown to be protective in cell, fly and zebrafish models of Huntington's disease (HD)

antagonists, such as verapamil, loperamide, amiodarone, nimodipine and nitrendipine³² (Figure 3). These compounds prevent the influx of extracellular Ca^{2+} by inhibiting the L-type Ca^{2+} channels on the plasma membrane, thereby decreasing intracytosolic Ca^{2+} levels. Conversely, the L-type Ca^{2+} channel agonist (\pm)-Bay K8644, which increases intracytosolic Ca^{2+} , inhibited autophagy. The L-type Ca^{2+} channel antagonists enhanced the clearance of mutant aggregate-prone proteins and reduced mutant huntingtin aggregates in an autophagy-dependent manner, whereas the L-type Ca^{2+} channel agonist had opposite effects.

L-type Ca^{2+} channel antagonists, such as verapamil, have been used for treating hypertension for many years.⁵⁶ This drug was previously shown by electron microscopy to increase the number of autophagosomes in rat myocardial tissues.⁵⁷ Further to showing the effect of verapamil on autophagy in various cellular models, we also demonstrated that it protected against neurodegeneration in *Drosophila* and zebrafish models of HD.³² Whereas verapamil reduced mutant huntingtin aggregates in a zebrafish model of HD, the L-type Ca^{2+} channel agonist (\pm)-Bay K8644 increased aggregation.³² As verapamil has minimal side effects, it may be a potential therapeutic candidate for diseases where autophagy acts as a protective pathway.

Some of the L-type Ca^{2+} channel antagonists found in our screen as autophagy inducers were also reported by Yuan and coworkers³³ in an independent screen where the number of GFP-LC3 vesicles was measured as readout. The Ca^{2+} channel blockers from their screen, such as loperamide, amiodarone, niguldipine and pimozone, also reduced polyQ aggregates in cell models by inducing autophagy independently of mTOR.³³

Another class of compounds obtained from our recent screen that modulate cytosolic Ca^{2+} levels are K_{ATP}^+ channel openers, such as minoxidil.³² Minoxidil decreases whole-cell L-type Ca^{2+} channels,⁵⁸ induces autophagy and promotes the clearance of autophagy substrates.³² Our screen also revealed some K_{ATP}^+ channel blockers, such as quinine sulphate and tolazamide, which retarded the clearance of autophagy substrates, in contrast to minoxidil.³²

An increase in intracytosolic Ca^{2+} activates a family of Ca^{2+} -dependent cysteine proteases called calpains, including the ubiquitously-expressed calpain 1 (μ -calpain) and calpain 2 (m-calpain).⁵⁹ Calpain activation by L-type Ca^{2+} channel agonists or overexpression of constitutively active m-calpain inhibits autophagy and retards the clearance of aggregate-prone proteins.³² Likewise, inhibition of calpain by inhibitors such as calpastatin and calpeptin, or genetic knockdown, induces autophagy and enhances the clearance of autophagy substrates. Furthermore, calpastatin increased autophagic flux in cells and reduced mutant huntingtin aggregates in a zebrafish model of HD.³² The effects of L-type Ca^{2+} channel antagonists or agonists were abolished in the presence of calpain activation or inhibition, respectively, suggesting that calpain is acting downstream of the autophagy-regulatory effects of free cytosolic Ca^{2+} levels³² (Figure 3).

Calpain activation inhibits autophagy by cleaving the α -subunit of heterotrimeric G-proteins (G_{sx}),³² whose activity

increases leading to greater adenylyl cyclase activation and thus cAMP production.⁶⁰ Chemical inhibition of G_{sx} with NF449 or with siRNA knockdown increased autophagic flux and enhanced the clearance of autophagy substrates.³² The autophagy-inhibitory effects of calpain activation were abolished by knockdown of G_{sx} with siRNA. On the other hand, activation of G_{sx} with its natural ligand, pituitary adenylyl cyclase-activating polypeptide (PACAP), retarded the clearance of autophagy substrates. The autophagy-inhibitory effect of PACAP was abolished by an adenylyl cyclase inhibitor 2'5'-dideoxyadenosine that reduces cAMP levels, but not by the calpain inhibitor calpastatin.³² This suggests that G_{sx} is a downstream mediator of calpain regulating autophagy, which creates a link between Ca^{2+} -calpain and the cAMP pathway. Moreover, the autophagy-inhibitory effects of constitutively active m-calpain were rescued by 2'5'-dideoxyadenosine, further suggesting that cAMP is a downstream target of calpain and G_{sx} .³² (Figure 3).

An apparent conflict with our data which suggests that cytosolic Ca^{2+} inhibits autophagy by activating calpains, may be found in a study that reported that elevations in the levels of intracytosolic Ca^{2+} , after treatments with Ca^{2+} -mobilizing agents like thapsigargin or ionomycin, induced autophagy.⁶¹ However, the authors measured only GFP-LC3 vesicle (autophagosome) numbers and the study lacked data on autophagic flux. Consistent with the autophagy-inhibitory effects of thapsigargin as reported previously by Seglen and coworkers,⁵⁵ we found that thapsigargin actually inhibits autophagic flux by two different mechanisms.³² We found that thapsigargin increased LC3-II levels or LC3-positive vesicles (surrogates for autophagosome number), but that this was because of a block in autophagosome-lysosome fusion, rather than an increase in autophagosome synthesis. (These possibilities were distinguished by assessing autophagosome numbers under conditions where autophagosome degradation is inhibited.) Thapsigargin also inhibited autophagosome formation and retarded the clearance of autophagy substrates in an autophagy-dependent manner, which were largely rescued by calpain inhibition, consistent with our model and data suggesting that calpains are the main mediators of the autophagy-inhibitory effects of elevated intracytosolic Ca^{2+} levels.³² However, these data also suggest that thapsigargin may have complex effects on autophagy – some mediated by calpains (decreasing autophagosome synthesis) and some mediated by other effects (possibly by ER-related effects) resulting in impaired autophagosome-lysosome fusion.

Regulation of autophagy by cAMP–Epac–PLC- ϵ –IP₃ pathway. Another category of autophagy enhancers that our recent screen revealed was a G_i -signalling activator clonidine, which reduces cAMP levels and thereby links with the Ca^{2+} –calpain– G_{sx} pathway³² (Figure 3). Clonidine reduces cAMP levels by binding to α_2 -adrenergic (α_2 -AR) receptors, thereby activating the G_i -signalling pathway and inhibiting adenylyl cyclase.⁶² It also lowers cAMP by binding to imidazoline-1 (I1R) receptors,⁶³ but whether this is mediated through G-proteins is disputed. Our study revealed that clonidine was triggering autophagy by reducing cAMP levels through I1R. Likewise, rilmenidine, a clinically approved

centrally acting drug that binds to I1R at a much greater affinity than to α_2 -AR, also induced autophagy and enhanced the clearance of mutant aggregate-prone proteins.³²

Furthermore, agents that modulate cAMP levels, such as the adenylyl cyclase inhibitor 2′5′-dideoxyadenosine that reduces cAMP, induced autophagy and enhanced the clearance of autophagy substrates; whereas cAMP activators like forskolin or cAMP analogues like dibutyryl cAMP had opposite effects.³² Consistent with our data, Ohsumi and Noda³⁷ have previously shown that high levels of cAMP inhibited autophagy in yeast. The autophagy-inducing effects of clonidine and 2′5′-dideoxyadenosine protected against rod photoreceptor degeneration and reduced mutant huntingtin aggregates in a zebrafish model of HD. Clonidine also protected against neurodegeneration in an HD *Drosophila* model.³²

The two major targets of cAMP are Epac, which is a guanine nucleotide exchange factor and protein kinase A (PKA)⁶⁴ (Figure 3). The regulation of autophagy by cAMP was found to be mediated through Epac, and not through PKA, as an Epac-specific cAMP analogue 8-CPT-2-Me-cAMP retarded the clearance of autophagy substrates whereas the PKA-specific cAMP analogue 6-Bnz-cAMP had no effect.³² Activation of Epac by increased intracellular cAMP levels activates Rap2B, a small G-protein, which subsequently activates PLC- ϵ , and this established pathway is well conserved in various mammalian cell lines.^{32,65} Autophagy was induced by a dominant-negative Rap2B, which also abrogated the inhibitory effects of the Epac-specific cAMP analogue 8-CPT-2-Me-cAMP, suggesting that Rap2B is a downstream autophagy regulator of Epac.³² Furthermore, overexpression of PLC- ϵ inhibited autophagy and retarded the clearance of autophagy substrates.³² Activation of PLC- ϵ hydrolyzes phosphatidylinositol 4,5-bisphosphate to form IP₃ and diacylglycerol. Thus, the link from cAMP to Epac to Rap2B to PLC- ϵ culminating on the generation of IP₃ was consistent with our earlier findings where elevated intracellular IP₃ levels were shown to negatively regulate autophagy³⁰ (Figures 2 and 3). Indeed, overexpression of cytosolic IP₃ kinase A, which lowers IP₃ levels by phosphorylating it to inositol 1,3,4,5-tetrakisphosphate (IP₄),⁶⁶ enhanced the clearance of autophagy substrates.³²

Regulation of autophagy by reduced intracellular IP₃ levels is likely because of inactivation of the signal for ER Ca²⁺ release, as raised cytosolic IP₃ levels binds to its receptors (IP₃R) on the ER to trigger depletion of the ER Ca²⁺ store.⁴⁵ As a rise in cytosolic Ca²⁺ activates calpain, we found that the inhibitory effects of PLC- ϵ overexpression, which generates IP₃, could be abolished by a calpain inhibitor.³² Furthermore, calpain inhibition by calpastatin rescued the autophagy-inhibitory effects of raised cytosolic Ca²⁺ induced by an L-type Ca²⁺ channel activator or thapsigargin.³² This links IP₃ regulation of autophagy to calpain through intracytosolic Ca²⁺ levels. Also, overexpression of constitutively active m-calpain completely abolished the protective effects of verapamil (L-type Ca²⁺ channel blocker) and clonidine (reduces cAMP),³² suggesting that calpain is a downstream mediator of the autophagy signals converging from the L-type Ca²⁺ channels and the cAMP–Epac–PLC- ϵ –IP₃ pathway. This creates an interesting cyclic pathway where calpain

regulates autophagy through G_{sx} that signals through the cAMP–Epac–PLC- ϵ –IP₃ pathway, which modulates calpain activity by influencing Ca²⁺ levels³² (Figure 3).

Neurodegeneration, Excitotoxicity and Autophagy

Ca²⁺ excitotoxicity results from excessive neuronal excitation by the neurotransmitter glutamate, leading to cell stress or death primarily because of Ca²⁺ influx.⁶⁷ A number of studies have suggested that increased cytosolic Ca²⁺ contributing to excitotoxicity may be an important mediator of a variety of neurodegenerative diseases. For example, abnormally high cytosolic Ca²⁺ levels were detected in cultured CA1 pyramidal neurons in transgenic HD mice expressing full-length human mutant huntingtin.⁶⁸ Mutant huntingtin increased Ca²⁺ levels in cultured HD mouse neurons by potentiating the activity of NR2B subtype of N-methyl-D-aspartate receptors (NMDARs),⁶⁹ class 1 metabotropic glutamate receptors (mGluR1/5) and type 1 inositol 1,4,5-trisphosphate receptors (IP₃R1)⁷⁰ (Figure 4). Stimulation of glutamate receptors (NMDARs and mGluR1/5) and IP₃R1 causes excessive Ca²⁺ overload that leads to excitotoxicity in HD mouse neurons.⁶⁷ Furthermore, increased intracellular Ca²⁺ can be toxic by activating caspases that trigger apoptosis by mitochondrial Ca²⁺ overload.^{69,71} Increased caspase activity catalyzes mutant huntingtin proteolysis, leading to generation of toxic N-terminal fragments.⁷² Moreover, elevated Ca²⁺ levels activate calpains, which has been reported by Ellerby and Gafni in the caudate of human HD brains.⁷³

These data suggest that autophagy is inhibited by elevated intracytosolic Ca²⁺ levels caused by increased influx of Ca²⁺ through Ca²⁺ channels on the cell membrane or due to depletion of the intracellular Ca²⁺ stores. Increased cytosolic Ca²⁺ inhibits autophagy by calpain activation.³² If this occurred in cells with no mTOR inhibition, then one would expect that autophagy would be inhibited. This would impair the clearance of aggregate-prone proteins, which may, in turn, augment neurodegeneration by creating a positive feedback loop resulting in more Ca²⁺ excitotoxicity and apoptosis (Figure 4).

Other mTOR-Independent Small Molecule Regulators of Autophagy

We have recently identified a number of small molecule enhancers and inhibitors of mTOR-independent mammalian autophagy by a chemical screening approach.³⁶ A primary screen in yeast with 50 729 compounds revealed various enhancers (small-molecule enhancers of rapamycin (SMERs)) and suppressors (small-molecule inhibitors of rapamycin (SMIRs)) of the cytostatic effects of rapamycin in yeast. A secondary screen in mammalian cells with SMERs and SMIRs, tested independently without rapamycin, identified 3 SMERs that induced autophagy and enhanced the clearance of aggregate-prone proteins.³⁶ Interestingly, all the autophagy-inducing SMERs (SMERs 10, 18 and 28) protected against mutant huntingtin fragment toxicity in HD *Drosophila* model,³⁶ and two of them enhanced killing of mycobacteria by primary human macrophages in models of

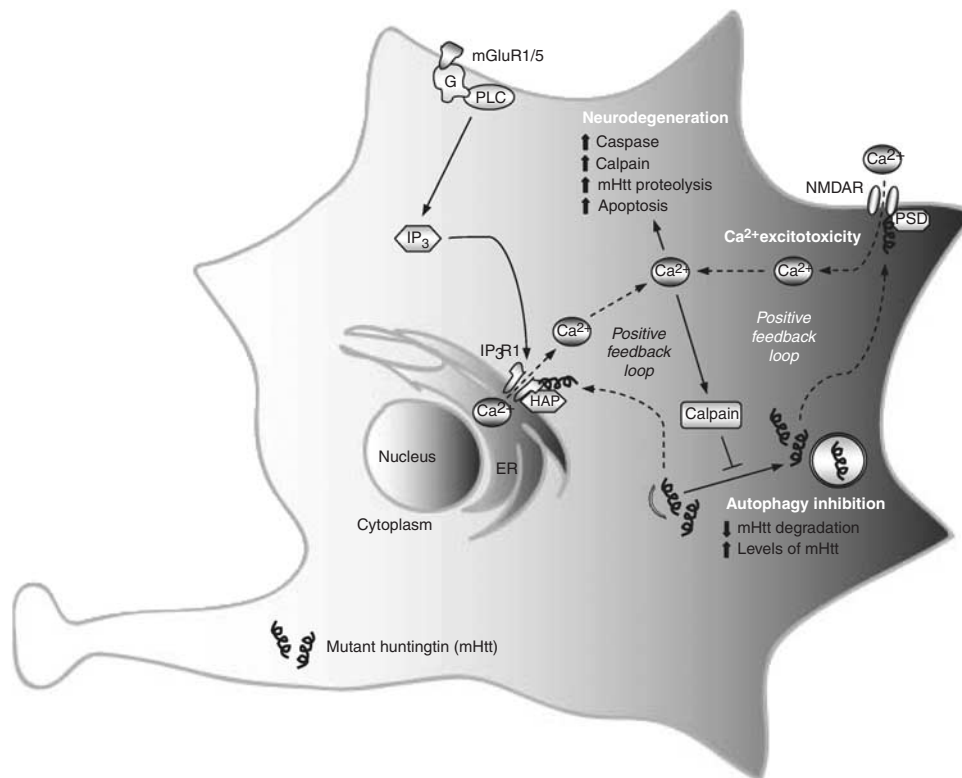


Figure 4 Ca^{2+} excitotoxicity impairs autophagy and augments neurodegeneration. Mutant huntingtin (mHtt) associated with Huntington's disease (HD) has been suggested to cause Ca^{2+} excitotoxicity in neurons. Mutant huntingtin increases NMDA receptor (NMDAR) function, possibly through altered interaction with PSD95–NR1A/NR2B complex, which results in abnormally high Ca^{2+} influx. Mutant huntingtin also binds to inositol 1,4,5-trisphosphate receptor (IP_3R) on the endoplasmic reticulum (ER) through huntingtin-associated protein-1A (HAP1A). This binding of the mutant protein sensitizes IP_3R and depletes ER Ca^{2+} stores in response to low levels of IP_3 generated by the heterotrimeric G-protein-linked phospholipase C (PLC) signalling from the metabotropic glutamate receptors (mGluR1/5). Increased intracellular Ca^{2+} levels activate caspases and calpains, which cleave mutant huntingtin into toxic N-terminal fragments, thereby triggering apoptosis and augmenting neurodegeneration. Calpain activation also inhibits autophagy and impairs the clearance of mutant aggregate-prone proteins. This block in autophagy results in high levels of mutant proteins in cells, which in turn lead to more Ca^{2+} excitotoxicity and neurodegeneration in a positive feedback loop. This model assumes no change in mTOR activity

tuberculosis.⁷⁴ Further screening of structural analogues of these SMERs identified 18 additional candidates that enhanced the clearance of aggregate-prone proteins.³⁶

An image-based screen for autophagy inducers carried out by Yuan and coworkers³³ with 480 bioactive compounds identified a number of compounds, which induced mTOR-independent autophagy and reduced expanded polyQ aggregates in a cell-based assay. Their hits include fluspirilene, trifluoperazine (dopamine antagonist), pimozide, niguldipine, amiodarone, loperamide (Ca^{2+} channel blockers) and penitrem A (inhibitor of high conductance Ca^{2+} -activated K^+ channels), which provide a number of candidates for neurodegenerative disorders as most of these compounds were FDA-approved drugs.³³

Another mTOR-independent autophagy enhancer is trehalose,³⁴ a disaccharide, which is also a 'chemical chaperone' because of its ability to influence protein folding through direct protein-trehalose interactions.⁷⁵ Although we have shown that trehalose enhanced the clearance of mutant huntingtin and protected against pro-apoptotic insults in cells,³⁴ it was earlier shown to be protective in HD mouse model.³⁵ The protective properties of trehalose as an 'autophagy inducer' and 'chemical chaperone', coupled with its lack of toxicity, may be of great benefit in the treatment of neurodegenerative disorders.

Downstream Targets of mTOR and mTOR-Independent Regulators

Many of the molecular mechanisms of the autophagic machinery have been dissected in yeast, where the kinase Atg1p was shown to be required for autophagosome formation acting downstream of TOR.² Atg1 homologues have been reported in several species, including mammals, where there are two Atg1 homologues, UNC (uncoordinated movement)-51-like kinase 1 (ULK1) and ULK2.^{76,77} Tooze and co-workers⁷⁸ have shown that siRNA-mediated knockdown of ULK1, but not ULK2, inhibited rapamycin-induced autophagy, further suggesting a role downstream of mTOR. This is consistent with our earlier finding in which rapamycin failed to rescue neurodegeneration in *Drosophila* with heterozygous loss of *Atg1*.²⁰ Interestingly, Neufeld and coworkers⁷⁹ have shown that overexpression of Atg1 in *Drosophila* induced autophagy. Mizushima and coworkers⁸⁰ have recently identified the focal adhesion kinase family-interacting protein of 200 kDa (FIP200) as a murine ULK-interacting protein. ULK1 and ULK2, as well as FIP200, localized to phagophores upon starvation and FIP200 is necessary for membrane targeting and functioning of ULKs. Using FIP200-deficient MEFs, the authors have shown that autophagy induced by starvation,

rapamycin, lithium or ceramide was impaired, as assessed by LC3 conversion.⁸⁰ These interesting data suggest that FIP200 is a downstream mediator of both mTOR-dependent and mTOR-independent autophagy pathways.

Although the autophagic machinery in yeast has been extensively studied, pathways regulating mammalian autophagy are not fully understood. An important question that needs to be elucidated is how our recently described mTOR-independent cyclic pathway connects to the autophagic machinery. However, we cannot exclude the possibility that this pathway may have several branching points that link to the autophagic machinery. This remains a daunting task as it is still unclear how mTOR regulates autophagy. Furthermore, the targets of various autophagy-inducing small molecules reported so far are still unknown, and the mechanisms of how they trigger autophagy require further studies. Nonetheless, the growing number of small molecule autophagy enhancers may have possible benefits for neurodegenerative disorders or other diseases where autophagy acts as a protective pathway, and may also shed light on important regulatory pathways governing autophagy in mammalian systems.

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