NEUROSCIENCE BULLETIN

Neuroscience Bulletin

The Official Journal of The Chinese Neuroscience Society
神经科学通报

Volume 31
Number 4
August 2015

Special Issue on Autophagy in Neural Function and Neurodegeneration

SIBS Springer
www.neurosci.cn

NEUROSCIENCE BULLETIN

Vol.31 No.4 August 2015

NEUROSCIENCE BULLETIN (Bimonthly)

August 1, 2015 Vol. 31 No. 4

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Special Issue on Autophagy in Neural Function and Neurodegeneration

About the Cover

Illustrating the degradation of unnecessary and dysfunctional cellular components through the actions of lysosomes, the cover image showcases autophagy, an important process in the nervous system. In this special issue, we present a collection of articles that discuss the roles of autophagy in neuronal function and degeneration. (Cover art by Dr. Yefei Li)

Neurosci Bull August 1, 2015, 31(4): 379–381. http://www.neurosci.cn
DOI: 10.1007/s12264-015-1551-7

·Editorial·

Selective role of autophagy in neuronal function and neurodegenerative diseases

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Proteostasis is critical for neuronal maintenance and survival, and its imbalance leads to neurodegeneration with the hallmark of protein misfolding and aggregation[1]. Macroautophagy becomes a major route for the clearance of protein aggregates that are normally poor substrates for the proteasome, the other protein quality-control machinery[2]. As a flux process, macroautophagy (hereafter referred to as autophagy) involves the formation of the autophagosome, a double-membrane vesicle for engulfing unwanted cellular components such as protein aggregates, and the fusion of autophagosomes with lysosomes that contain many potent proteases for final degradation[3].

In the past decades, the mechanism of autophagy has been intensively studied under starvation conditions. Autophagy is a hierarchical process involving the activation of ULK1, the initiating kinase, and many downstream events such as Beclin 1 complex activation and LC3 lipidation[3]. It has been well established that, during amino-acid starvation, mTOR, the master nutrient sensor, dissociates from and in turn activates ULK1 kinase[4]. Interestingly, upon energy starvation such as glucose depletion, the main energy sensor AMPK activates ULK1 through antagonizing mTOR-mediated ULK1 inhibitory phosphorylation[5]. It is generally believed that starvation induces non-selective autophagy that targets non-essential cellular components for degradation to provide building blocks for cellular survival in unfavorable conditions.

In comparison with the well-characterized starvation-induced nonselective autophagy, the molecular mechanism of selective autophagy is largely unknown[6]. Selective autophagy is a comprehensive term that can be classified into many different types such as aggrephagy, mitophagy, and lipophagy[7]. In particular, aggrephagy plays an important role in neuronal survival, as neurons are non-dividing cells and cannot overcome cellular toxicity induced by protein aggregates through dilution effects. Therefore, aggrephagy becomes essential for neurons to preserve proteostasis. However, the underlying mechanism is yet to be uncovered. Recently, Huntingtin, the Huntington's disease gene product, was identified as a scaffold protein for selective autophagy including aggrephagy, mitophagy, and lipophagy in mammalian cells, paving the way for further investigation in this emerging young field[8]. In addition, cargo receptor proteins such as p62 and NBR1 bring cargos to autophagosomes by interacting with the autophagosomal protein LC3[9]. As both p62 and NBR1 contain ubiquitin-binding domains, the cargos modified by lineage-specific ubiquitin play an important role in cargo recognition in selective autophagy. For instance, ubiquitin K63-modified substrates preferentially bind to p62 for autophagic degradation[10].

Selective autophagy has been linked to a variety of human diseases including but not limited to neuro-degeneration and cancer[11]. For example, tau protein, implicated in Alzheimer's disease (AD), was shown to be an autophagy substrate, and increasing autophagy-

mediated tau degradation ameliorates disease progression in AD models[12, 13]. Autophagy dysregulation has also been reported in amyotrophic lateral sclerosis (ALS), another neurodegenerative disease that can be caused by SOD1 mutations, and enhancing autophagy by pharmaceutical or genetic methods can improve the clinical symptoms in animal models of ALS, supporting the notion that autophagy is a promising translational target [14-16].

In this special issue, "Autophagy in Neural Function and Neurodegeneration", we present 13 high-quality, peer-reviewed articles. Among them, 8 are reviews and 5 are original research papers, which cover a broad basic research on autophagy with regard to neuronal function, synaptic development, neuronal cell death and neuroprotection, and clinical relevant research on neurological and psychological disorders (AD, ALS and other motor neuron diseases, depression, multiple sclerosis, Parkinson's disease and stroke) [15, 17-28].

Selective autophagy is generally believed to be beneficial to neuronal development and functions[17-19]. However, in some contexts, dysregulation of autophagy may cause neurodegeneration and cell death[20, 21]. Rubinsztein and colleagues[20] have provided an excellent review of this important field by analyzing several key findings on autophagic cell death and they proposed a new model on how the extent of autophagy dictates the fate of neurons under stress, which quite nicely explains why autophagy can play opposing roles in neurons depending on the context. In addition to selective macroautophagy, chaperone-mediate autophagy (CMA), another type of selective autophagy, also plays an important role in neuronal survival, as many aggregate-prone proteins such as α-synuclein and tau serve as its targets. The detailed relationship between neuroprotection and CMA is reviewed by Dr. Yang[19]. Equally importantly, the members of Dr. Mao's lab fully discuss how to prevent neurodegeneration by targeting the CMA pathway[21]. In his review, Dr. Mao outlines the key molecular players in CMA and the stressors associated with it, and analyzed the role of CMA in neurodegeneration, concluding that it is a potential therapeutic target.

It is becoming clearer that autophagy dysregulation plays an important role in several major neurological and psychological disorders[15, 22-28]. In this special issue, Dr. Chen's group give an in-depth review on the role of mitophagy in ischemic brain injury[22]. They discussed several stressors that cause mitophagy activation including endoplasmic reticulum (ER) stress, oxidative stress, and excitotoxicity, as well as mitophagy mediated by the three pathways PINK1/Parkin, Bnip3/Nix, or FUNDC1[22]. Liang *et al.* [23] used meta-analysis to identify a critical autophagy signaling network that contributes to stroke in the ischemic rodent brain. Interestingly, dysfunction of mitophagy and CMA has recently been found to be involved in multiple sclerosis (MS), a permanent neurological impairment typical of chronic inflammatory demyelinating disorders [24]. As MS is an auto-immune disease of the central nervous system, the connection between neuro-immunology and autophagy is gaining increasing attention[24]. For example, the activation of autophagy may participate in Aβ vaccine-induced Aβ clearance in AD[25].

Non-selective or selective autophagy share the same "trash-can" – the lysosome – which means that if autophagic flux is aberrant, simply boosting the biogenesis of autophagosomes is not enough to solve the problem. For example, an autophagic flux defect in ALS is reported in the original article by Dr. Le's lab[15] and this is ameliorated by overexpression of histone deacetylase 6, an adaptor protein promoting autophagosome-lysosome fusion. CHIP, an E3 ligase, is also considered to be an important player in regulating autophagic flux associated with ALS and other motor neuron diseases[26]. In addition to these neurological diseases, autophagy dysregulation is believed to contribute to the pathophysiology of major depressive disorder and many anti-depressants are autophagy modulators[27].

We hope that the special issue presented here will attract attention and initiate extensive discussion of these new research directions including selective autophagy and cell death in neurons, eventually developing new translational targets and offering hope to the people who suffer greatly from these devastating neurodegenerative diseases. As the guest editor I thank all authors for their hard work in contributing to this special issue.

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Neurosci Bull August 1, 2015, 31(4): 382–394. http://www.neurosci.cn
DOI: 10.1007/s12264-015-1528-y

·Review·

Autophagic activity in neuronal cell death

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As post-mitotic cells with great energy demands, neurons depend upon the homeostatic and waste-recycling functions provided by autophagy. In addition, autophagy also promotes survival during periods of harsh stress and targets aggregate-prone proteins associated with neurodegeneration for degradation. Despite this, autophagy has also been controversially described as a mechanism of programmed cell death. Instances of autophagic cell death are typically associated with elevated numbers of cytoplasmic autophagosomes, which have been assumed to lead to excessive degradation of cellular components. Due to the high activity and reliance on autophagy in neurons, these cells may be particularly susceptible to autophagic death. In this review, we summarize and assess current evidence in support of autophagic cell death in neurons, as well as how the dysregulation of autophagy commonly seen in neurodegeneration can contribute to neuron loss. From here, we discuss potential treatment strategies relevant to such cell-death pathways.

Keywords: autophagy; autophagic cell death; programmed cell death; apoptosis; necrosis; autosis; neurodegeneration

Introduction

Autophagy is an intracellular process of 'self-eating', which provides homeostatic maintenance through the capture and degradation of aggregate-prone proteins and dysfunctional organelles. More specifically, autophagy encompasses three separate mechanisms – microautophagy, chaperone-mediated autophagy, and macroautophagy – but only the latter is discussed in this review, and is referred to simply as 'autophagy'[1].

Briefly, autophagy proceeds through the capture of portions of cytoplasm containing target material inside expanding membranes, which finally enclose to form double-membrane vesicles called autophagosomes. Fully-formed autophagosomes are shuttled along microtubules to lysosomes, whereupon fusion and degradation occur[2, 3]. This removal and recycling serves as an emergency energy supply during starvation, but autophagy has also been linked to a diverse range of other protective roles[4-7]. These include the capture of invading pathogens[8], context-dependent tumor suppressive and tumorigenic qualities[9-12], and the removal of toxic aggregate-prone proteins often linked to neurodegeneration[13-15]. From these findings, interest in autophagy research has surged over the past decade or so[16].

Despite these pro-survival roles, autophagy has also been implicated as a mechanism of programmed cell death (PCD)[17-19]. Numerous studies have reported instances of dying cells displaying accumulated autophagosomes, which engulf large portions of the cell's cytoplasm and which have been presumed to lead to excessive destruction of vital components[20, 21]. However, this notion of 'autophagic cell death' (ACD) has met with some skepticism, with critics arguing that these accumulations of autophagosomes may represent a failed rescue response to a lethal stress, as

opposed to a direct lethal mechanism in its own right[22-24].

Neurons have high energy demands, and as post-mitotic cells, quality control and homeostasis maintenance are vital[25]. From these traits, one would assume neurons to rely heavily on autophagy, yet these cells typically contain very few autophagosomes, suggesting little autophagic activity. However, impairing lysosomal function leads to the accumulation of autophagosomes, revealing that autophagy is highly efficient in neurons, with a quick progression from vesicle formation to degradation[26, 27]. Due to this high autophagic flux, it is possible that neurons are particularly sensitive to ACD. As pathologies like neurodegeneration are associated with a progressive loss of neurons, an appreciation of the diverse mechanisms of death in these cells may aid the design of future protective and preventative treatments for disease. In this review, after briefly covering the machinery of autophagy, we compare the seemingly paradoxical roles autophagy plays in both promoting survival and death in neurons. Then, we address the implications these findings have for our understanding of ACD, as well as potential applications for neuron therapy.

Regulation of the Autophagy Machinery

The autophagy machinery is highly conserved. Autophagy-related (Atg) genes were first reported in yeast[28, 29], and many of the 30+ members of this group have mammalian homologues[30]. The different Atg genes regulate each stage of autophagosome formation - from initiation of the process, to nucleation of the target membrane, and finally its subsequent elongation and fusion, forming the complete vesicle[31]. Various origins of the membrane have been suggested, with sites including the endoplasmic reticulum[32, 33], plasma membrane[34-36], mitochondrial membrane[37] and Golgi apparatus[38], all receiving support.

Under normal conditions, autophagy proceeds at a relatively low basal rate. Multiple regulators of autophagy have been identified, but in mammals the best characterised is the mammalian target of rapamycin (mTOR), which inhibits the initiation of autophagosome formation. However, upon certain stimuli such as starvation, mTOR is inactivated, allowing autophagy to proceed. The initial stages of the process are mediated by the un-coordinated 51-like kinase 1 (ULK1) complex, which activates the downstream phosphatidylinositide 3-kinase (PI3K) Class III complex[39]. Vps34, the only mammalian Class III PI3K, catalyzes PI(3)P generation, allowing for recruitment of additional facilitators of autophagosome nucleation. Recently, PI(5)P has been shown to be able to substitute for PI(3)P in this regard, and this lipid is particularly important in responses to glucose starvation[40]. Membrane elongation is completed through the action of two ubiquitin-like conjugation complexes: ATG12-5 and LC3-phosphatidylethanolamine (PE)[41-43]. Although the ATG12-5-16L1 complex dissociates from completed vesicles, the LC3-PE conjugate (LC3-II) remains, making it a commonly-used marker of autophagosomes[44]. At the end of the process, autophagosomes are shuttled to lysosomes and autophagosome-lysosome fusion occurs (Fig. 1). Lysosomal enzymes like cathepsins degrade the vesicles and their cargo, and permeases release amino-acids for recycling[45]. The successful progression from autophagosome formation to degradation is referred to as 'autophagy flux'.

Autophagy in Neuronal Survival

Perhaps the clearest demonstration of the importance of autophagy in survival is that complete knockout of several of the Atg genes (such as Atg3, 5, 7, 9, and 16L1) results in neonatal lethality in mice[30]. Neuron-specific Atg gene knockouts specifically reveal that basal rates of autophagy are required for normal neuronal survival[46, 47]. Autophagy is a protective mechanism in response to numerous stresses. As well as during harsh environmental states such as starvation[48, 49] or hypoxia[50], autophagic activity can also promote survival through the clearance of faulty intracellular material. For instance, the specialized subdivision of autophagy that targets mitochondria, mitophagy, serves as a form of quality-control for these organelles. Defective mitochondria are targeted by machinery including PTEN-induced kinase 1 and the ubiquitin ligase parkin, which ubiquitinates proteins on their outer membrane, allowing for their selective engulfment in autophagosomes. The removal of damaged mitochondria limits the risk of further damage from the generation of reactive oxygen species (ROS)[51-53]. In the event of ROS production, autophagy is triggered by upstream activators like AMPK, or by increased activity of ATG proteins, again affording protection to cells[53]. In

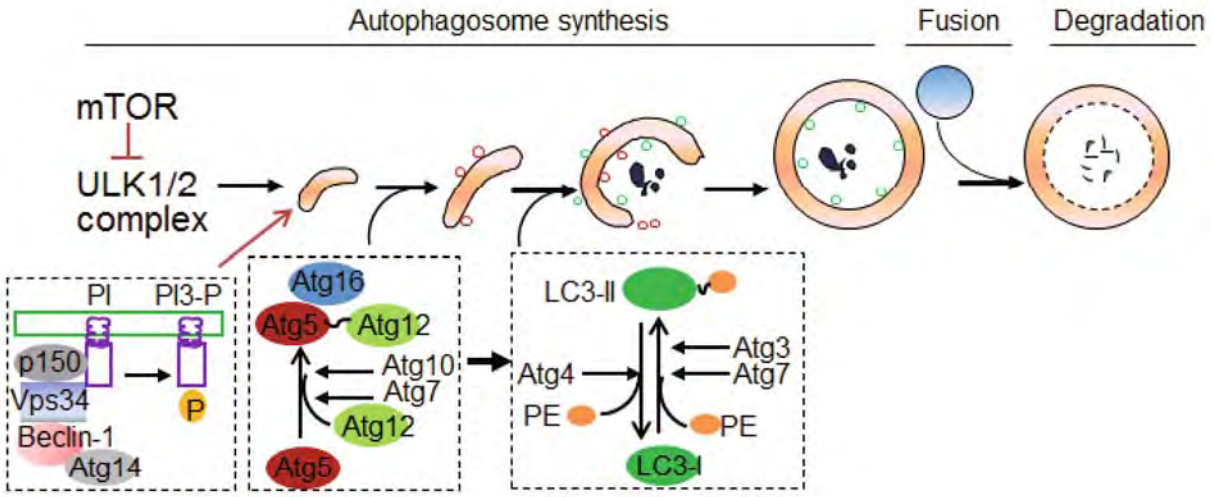

Fig. 1. Schematic diagram of the autophagy process. The autophagy process includes autophagosome biogenesis, autophagosome-lysosome fusion, and cargo degradation. The ULK1/2-Atg13-FIP200 complex, negatively regulated by mTOR, senses the signals for autophagosome initiation. The class III PI3-kinase complex, containing Vps34, beclin-1/Atg6, p150/Vps15, and Atg14, generates PI3P required for autophagosome nucleation. Autophagosome elongation involves two ubiquitin-like (UBL) conjugations: conjugation of the UBL protein Atg12 to Atg5, and conjugation of the UBL protein LC3 to PE. Atg5-Atg12 conjugation is catalyzed sequentially by the E1-like enzyme Atg7 and the E2-like enzyme Atg10. The Atg5-Atg12 complex then forms a larger complex with Atg16 and the site of Atg16 recruitment is enabled by its binding to WIPI2, which, in turn, is recruited to membranes enriched in PI3P or PI5P. The Atg5-12 conjugate is an E3 ligase that catalyzes LC3-PE (LC3-II) conjugation that also requires E1-like Atg7 and E2-like Atg3. LC3-II, the lipidated form of LC3, is required for the expansion and completion of pre-autophagosomal membranes.

some circumstances, autophagy suppresses apoptosis and necrosis[54-57]. Given these roles, it is of little surprise that autophagy has emerged as one of the central targets in anti-ageing studies. Regimens that enhance the process have led to reductions in pathologies that manifest with age across several models[58].

One of the branches of age-related disease that autophagy has been shown to influence is neurodegeneration. A common feature shared across these pathologies is the progressive accumulation of toxic aggregate-prone proteins. The identity of the aggregates varies between diseases: Alzheimer's disease (AD) features amyloid-β (Aβ) plaques and intracellular neurofibrillary tangles containing tau aggregates[59, 60]; Parkinson's disease (PD) is characterised by Lewy body inclusions that have α-synuclein as a major constituent[61, 62]; and Huntington's disease (HD) is the result of polyglutamine expansions of the huntingtin (Htt) protein[13, 63]. As neurons are post-mitotic, 'in-house' modes of waste clearance are imperative to prevent the formation of these aggregates[25]. Autophagy seems vital in this regard, as the narrow entry to the proteasome precludes it handling oligomeric assemblies[15]. Therefore, dysfunction in autophagy is likely to be a major contributor to the onset of neurodegeneration. Indeed, autophagic activity has been suggested to decrease with age in human tissues, including the brain[15, 64, 65]. In some cases, degenerating neurons show accumulations of non-degraded autophagosomes in addition to the aggregates, implicating a failure of the lysosomal clearance stage in these diseases[66-68]. An interesting exception is in the case of HD, where mutant Htt appears to reduce the recognition and capture of certain cargoes, as revealed by the recently-discovered roles that the protein plays in autophagosome-substrate interactions[69, 70].

Consistent with these observations, knockout of the autophagy regulators Atg5 or Atg7 in the mouse CNS results in pathologies comparable to the effects of neurodegeneration, including the presence of protein aggregates coupled with neuronal damage and loss[46, 47]. Beclin-1 (mammalian Atg6 homologue) activity decreases in ageing and neurodegenerative brains, and similarly, its loss enhances aggregate formation in models of AD, PD, and

HD[64, 71]. Strategies of beclin-1 overexpression complement these findings, with enhanced clearance of toxins and reduced neuronal damage[66, 72]. Mutations commonly associated with neurodegenerative pathologies have also been shown to affect autophagy. As examples, mutated presenilin-1 in AD alters the acidification of lysosomes, causing a blockade of autophagosome degradation[73]. The AD PICALM locus is a well-validated 'hit' from genome-wide association studies and loss of this protein impairs autophagy as well as tau clearance[74]. In PD, defects in parkin and PINK-1 result in insufficient labelling of damaged mitochondria for mitophagy, increasing the risk of ROS generation and further neuron damage[59]. Furthermore, the α-synuclein accumulation that characterizes this disease impairs autophagosome formation[75, 76], and the VPS35 D620N PD mutation has a similar impact on the pathway, impairing autophagy substrate clearance[77].

Autophagy in Cell Death

Are increases in autophagic activity always beneficial for neuronal health? The concept of ACD has persisted from the early days of autophagy research, representing Class II death in the recently-abandoned morphological classifications of PCD, alongside apoptosis (Class I), and necrosis (Class III)[23, 78]. Cells undergoing ACD are characterised by enhanced numbers of autophagosomes, resulting in extensive cytoplasmic vacuolization[17, 20]. This has largely been attributed to increases in autophagosome synthesis and flux, causing excessive degradation of important cell components[78]. However, this concept has courted controversy across the literature, with some groups proposing these increases in autophagic vacuoles are representative of roles more in keeping with autophagy as a pro-survival system[22, 24]. Suggestions include autophagy up-regulation as a failing salvage effort against lethal stresses, or a clearance system of dying cells, rather than a direct route of cell death in and of itself. To try and provide more clarity on this issue, various guidelines have been suggested that themselves have attracted criticism for being overly stringent[19, 23]. It has been proposed that for an instance of cell death to be truly mediated by autophagy, then autophagy ablation by pharmacological or genetic inhibitors should provide some protection from lethality. In addition, suppression of apoptotic or necrotic processes should provide no such alleviation. Several cases of ACD have been strongly supported, such as large-scale clearance during development[79-81], and the actions of some chemotherapeutic agents, at least *in vitro*[21, 82-85]. While a number of studies have supported the concept of autophagic cell death, for instance by showing that such death is attenuated by the loss of autophagic genes[21, 79, 85, 86], the interpretations of such studies are not always straightforward. It is possible that some autophagy is required to enable the execution of cell death after certain insults, and such experimental paradigms using autophagy-null states certainly support the concept that autophagy may be permissive in these scenarios. However, in order to test if the increased autophagy associated with certain forms of cell death is causal, one needs to ideally manipulate autophagy back to normality and not to the null state. As this type of manipulation is very challenging, most studies have not excluded the possibility that the increased autophagy they found does not cause the cell death but rather that some autophagy is required to execute cell death in a manner analogous to ATP being required for apoptosis. The extent of autophagic modulation of cell death may depend on cellular contexts and on the duration and strength of autophagy induction. Berry and Baehrecke[79] initially reported that both autophagy and caspase activity are required for the cell death in salivary glands during *Drosophila* development, and autophagy selectively degrades the caspase inhibitor dBruce to induce *Drosophila* ovary cell death[87]. These studies suggest that multiple possible mechanisms may be involved in ACD. In mammalian cells, the role of cellular contexts in ACD remains more elusive, while autophagy-relevant proteins such as beclin-1, Atg7, or DRAM have been reported to play a role in cell death in a variety of tumor cell lines[88].

ACD in Excitotoxic and Ischemic Neuronal Stress

Some of the strongest support for ACD as a pathological process has been found under conditions of excitotoxicity and hypoxia-ischemia, stresses that may result from trauma or stroke[89, 90]. Both conditions are potent inducers of autophagy, a response presumably associated with damage-limitation and the promotion of survival[91]. Some have reported that the pharmacological induction of autophagy with rapamycin reduces apoptotic and necrotic

death during hypoxia, while inhibition with 3-methyladenine (3-MA) and wortmannin enhances this loss[50, 91, 92]. Contrary to this, many groups have reported that this increase in autophagy can contribute to lethality. Using the glutamate receptor activator kainate as a model of excitotoxicity results in death in rat cortical neurons that is largely independent of apoptotic caspase activation. However, this cell death is reduced by inhibition of autophagosome formation using the PI3K inhibitor 3-MA, or *via* genetic knockdown of Atg7 and beclin-1[93]. These findings have been supported in other excitotoxic models[94, 95]. Similar approaches during hypoxia-ischemia have also aided in alleviating neuron loss both *in vitro* and *in vivo*. The administration of 3-MA has proven neuroprotective in several rodent hypoxia-ischemia models[96, 97], although it is worth bearing in mind that this agent inhibits many PI3 kinases and thus has many autophagy-independent effects. Interestingly, Atg7 loss in pyramidal neurons appears to suppress both caspase-dependent and -independent death, suggesting that apoptosis and ACD may both occur in neurons under hypoxia, with autophagy serving as a positive mediator of both processes[94]. The distinctive morphological changes and increases in autophagic vesicles following hypoxia in rat hippocampal neurons have even aided in the coining of a new subtype of ACD, autosis. Autosis has been characterised by an increase in both autophagosomes and autolysosomes, and displays other unique morphological characteristics, such as mild chromatin condensation and focal swelling of the perinuclear space[86, 98]. Notably, autotic death shows an independence from apoptosis and necroptosis, instead requiring the activity of the Na^+, K^+-ATPase. This pump can be blocked by cardiac glycosides. Neriifolin belongs to this class of compounds, and reduces cerebral infarct size in rodent ischemic models[99]. Importantly, these improvements in neuron survival are coupled with a decline in the number of autophagic vesicles, as well as the absence of other autotic features[86]. Therefore, autosis seems to be a distinct form of canonical ACD that occurs in neurons. The dependence of this phenomenon on the Na^+, K^+-ATPase may be relevant for the treatment of hypoxia-ischemia, as many cardiac glycosides have well-characterised safety profiles and are widely used in clinical medicine[99].

So, how can we explain these dramatically opposing results of autophagy activation on the susceptibility to cell death? One possibility is that the extent of autophagy induction dictates the outcome. Physiological levels likely still serve a protective role, providing an energy source and relief from oxidative stress. However, over-activation may lead to the destruction of cellular components, as well as exerting additional strain on the neuron through continued autophagosome formation[89, 100] (Fig. 2). This form of ACD by excessive autophagy stimulation has also been implicated in the neurotoxicity caused by drugs such as MDMA[101]. The involvement of other forms of PCD appears to vary under ischemic stresses, with both caspase-dependent and -independent cases documented[94, 97]. While neuron type seems an unlikely determinant of which scenario occurs (as both have been reported in the same population of pyramidal neurons[94]), the neuronal region or nature of the stress may shape the outcome[89]. It is important to consider that to date there is no evidence that the specific activation of autophagy induces cell death, and thus it is possible that other signaling pathways induced by different neuronal stressors may determine the impact of autophagic activity on cell survival. It has even been postulated that under certain circumstances, autophagy may promote apoptosis as a form of damage-limitation against inflammatory necrosis[91].

Autophagy-Lysosome Dysfunction in Neurodegenerative Disease

Rather than over-activation of autophagosome synthesis, the combination of autophagosome and aggregate accumulations seen in neurons in neurodegenerative diseases are frequently the result of impaired lysosomal degradation[63, 102]. Therefore, in such instances it seems unlikely that ACD occurs *via* excessive degradation of cellular components, as the autophagic flux is impaired. However, these accumulations may still have detrimental effects on cell survival. Without a means of waste removal, the hallmark toxic aggregates associated with conditions like AD, PD, and HD can accumulate[59]. Other important homeostatic processes, such as faulty organelle removal, are not fulfilled either in these conditions, which may exacerbate the damage. Neurotoxins like rotenone and MPTP mimic PD pathology by inhibiting complex I of the mitochondrial electron transport chain and causing ROS generation[103-105]. Defective mitochondria may disrupt the

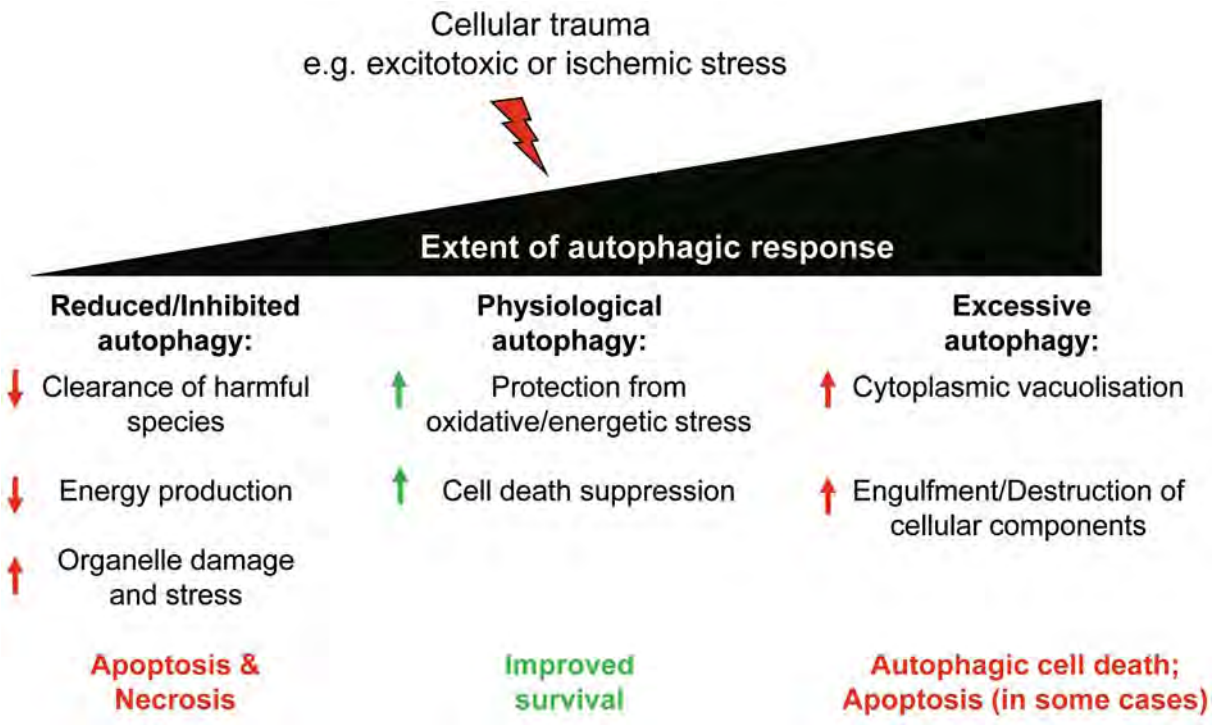

Fig. 2. One hypothetical model where the extent of autophagy dictates the fate of neurons under stress. When faced with a harsh stress, neurons rely on autophagy induction as a means of protection and damage limitation, suppressing cell death and promoting survival. This means an inefficient or inhibited level of autophagy can be detrimental to neuronal health. At the other end of the spectrum, an excessive autophagic response may result in the degradation of vital cellular components, culminating in ACD. Therefore, it seems that in order for autophagy to exert its protective effects, a balance needs to be maintained to avoid neuronal death.

microtubule-dependent trafficking of autophagosomes to lysosomes further, decreasing the clearance of aggregates and ROS even more[106]. These conditions also favor the mitochondrial release of cytochrome *c* into the cytosol, promoting cell death by apoptosis[107]. Elevated ROS have a negative impact on lysosomes: decreasing both their number and membrane integrity. Lysosomal membrane permeabilization can lead to leakage of proteases like cathepsins with damaging consequences[104, 105]. While these events can hardly be classified as a bona fide case of ACD, they still highlight how impairment of the system can lead to neuronal death (Fig. 3). Death associated with autophagy-lysosome dysfunction is likely to be of particular relevance to conditions like AD and PD, which commonly display defective autophagic flux[15, 59, 104].

In such cases, efforts should focus on salvaging lysosomal function and boosting autophagic flux. Pharmacological inducers of autophagy have been trialed across a variety of animal models, and can aid the removal of aggregates associated with AD, PD, and HD[59, 108, 109]. In some cases, these treatments provide a degree of cognitive restoration[71, 110]. Notably, a number of these therapies have been achieved with approved drugs, such as rapamycins[71, 111-114], rilmenidine[110]. Furthermore, benefits have been seen in a number of animal models with the polyamine spermidine, which induces autophagy[115].

A variety of strategies aimed at a number of targets have adopted this concept, with some encouraging findings. Agents like the small molecule GTM-1 and natural product arctigenin both enhance autophagic clearance, and are associated with reductions in toxic aggregates and improved cognition in AD mouse models[116, 117]. Boosting cathepsin activity also provides similar benefits, although the incidence of lysosomal membrane permeabilization in some disease states may make this mechanism unfeasible[118]. Glucosylceramide (GlcCer) has

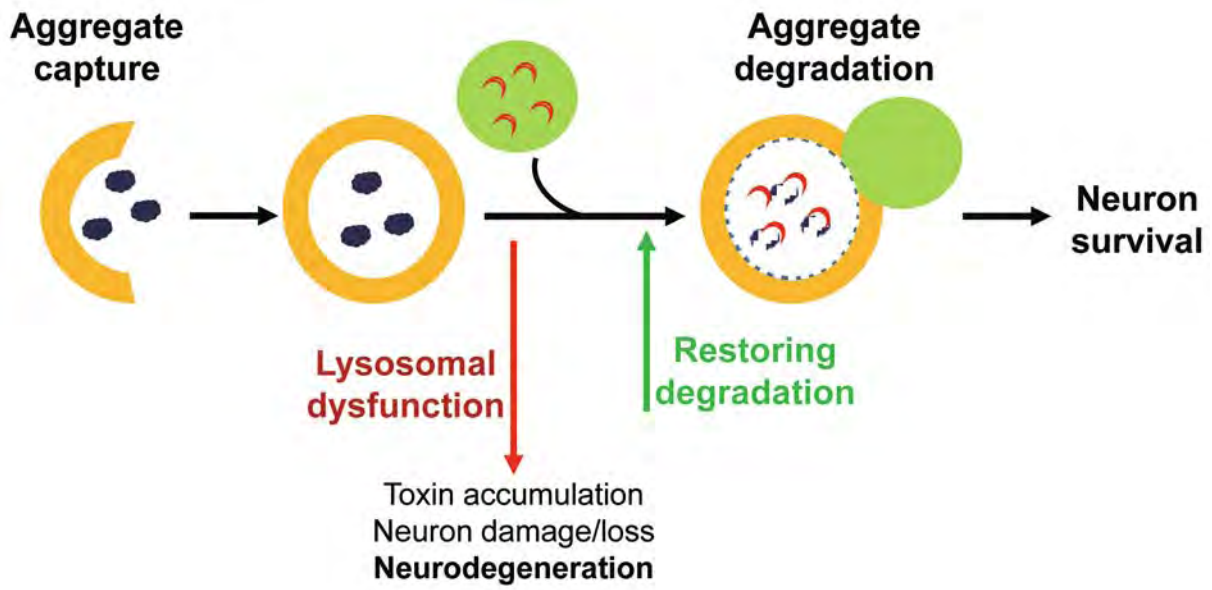

Fig. 3. Dysfunctional lysosomal clearance of autophagy promotes the accumulation of toxic aggregates associated with neurodegeneration. Efficient autophagic clearance is required to remove potentially toxic aggregate-prone proteins from neurons. In instances of lysosomal dysfunction, these accumulations can form, and, coupled with the loss of protective autophagy, cause further damage to neurons. Ultimately, these stresses can result in neuronal loss and neurodegeneration. Strategies for restoring lysosomal integrity and function allow autophagic degradation to resume, and may provide a means of prevention against these pathologies.

been associated with reductions in lysosomal activity, and inhibitors of GlcCer appear to have the desired restorative effects on organelles and improve PD pathology[119, 120]. Niemann-Pick Type C (NPC) is another neurodegenerative disease that has a pathology similar to AD[121]. In NPC mice, enhancing degradation aids neuron survival[122]. An exciting candidate that has emerged in recent years is the transcription factor EB (TFEB). TFEB is a positive regulator of a number of lysosomal and autophagy-related genes; its expression is associated with enhanced lysosome biogenesis and substrate clearance[123]. As the brains of mouse models of neurodegeneration have been reported to show reductions in TFEB, the effects of elevating its expression have been investigated[25, 67]. Importantly, increased TFEB aids in the degradation of misfolded tau[124], α-synuclein[67], and mutant Htt[25] both *in vitro* and *in vivo*. There has been less clear support for Aβ plaque removal[124]. Pharmacological autophagy inducers like rapamycin and trehalose also activate TFEB, and aid in the clearance of protein aggregates in mouse models of neurodegeneration, as well as reducing the damage associated with neurotoxins like rotenone and MPTP[67, 104, 105]. Selected pharmacological agents/strategies used for autophagy flux restoration in neurodegenerative models are listed in Table 1.

Concluding Remarks

The role of autophagy in neuronal survival appears complex. While its homeostatic functions seem vital for survival through protection against stress and the removal of toxins, imbalances in the pathway can promote lethality. Interestingly, it appears that both over-activation and inactivation of autophagy can lead to neuronal death. Support for ACD by over-activation has come from observations made during periods of harsh stress like excitotoxicity and hypoxia. In these situations, it is possible that autophagy acts as an out-of-control protective response, and induces death by excessive capture and destruction of intracellular components. Genetic ablation of the autophagy machinery can alleviate this phenomenon. However, it is important to consider that the experimental paradigms that lead to the conclusion of ACD generally use autophagy-null states or chemical inhibitors like 3-MA or wortmannin which have multiple autophagy-independent

Table 1. Selected strategies of autophagy flux restoration in models of neurodegeneration (mammalian where available)

Strategy	Neurodegenerative Disease	Changes to Pathology	Reference
Pharmacological			
Rapamycin	Alzheimer's disease	Autophagy induction; reductions in Aβ and cognitive recovery in AD mice	[71],[112]
	Huntington's disease	Reductions in Htt aggregate formation, improvements in behavioral tests in mice	[111]
	Parkinson's disease	Reductions in α-synuclein accumulation, alleviation of neurodegenerative behavior in mice	[113],[114]
Rilmenidine	Huntington's disease	Autophagy induction; enhanced clearance of mutant Htt, improved motor performance in mice	[110]
Spermidine	Parkinson's disease	Autophagy induction; Improved motor performance in fruit fly, reduced dopaminergic neuron loss in nematodes	[115]
Arctigenin	Alzheimer's disease	Autophagy induction; Reduction in Aβ plaques through inhibition of formation and enhanced clearance, improved memory in mice	[117]
GTM-1	Alzheimer's disease	Autophagy induction and increased flux; removal of Aβ oligomers, cognitive improvements in mice	[116]
Glucosylceramide inhibitors	Niemann-Pick Type-C 1	Correction of autophagic flux; improved clearance of cholesterol and autophagic vesicles in mouse and cat models, prolonged neuron survival	[119],[120]
Genetic			
TFEB	Alzheimer's disease	Upregulation of lysosomal and autophagy genes;	[124]
	Huntington's disease	enhanced clearance of tau, α-synuclein, and mutant	[25]
	Parkinson's disease	Htt aggregates	[67]

effects. Thus, autophagy may be permissive for certain forms of cell death, but may not be sufficient – we are not aware of any data showing that "specific" autophagic hyper-activation by overexpressing a complete Atg gene induces cell death. Lysosomal dysfunction can also lead to aberrant autophagosome accumulation through blockade of their degradation, and is associated with neurodegenerative pathologies. In such cases there is reduced autophagic flux and the failure to successfully clear intracellular protein aggregates and ROS means the toxins can propagate unchecked and cause further damage, ultimately leading to cellular demise. These differing mechanisms influence treatment strategies. While autophagy inhibitors may improve survival in ACD by over-activation of autophagosome biogenesis, their application is likely to be of little use in lysosomal dysfunction. In the latter instance, therapies should instead focus on the restoration of lysosomal function and autophagic flux. Therefore, it is clear that targeting autophagy to prevent cell death is not a case of 'one size fits all', but rather, careful consideration is needed before selecting a treatment strategy.

ACKNOWLEDGEMENTS

DCR is funded by a Wellcome Trust Principal Research Fellowship and the NIHR Biomedical Research Unit in Dementia at Addenbrooke's Hospital. This review is supported by Plymouth University and Northcott Medical Foundation.

Received date: 2015-04-14; Accepted date: 2015-05-11

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Neurosci Bull August 1, 2015, 31(4): 395–406. http://www.neurosci.cn
DOI: 10.1007/s12264-015-1544-6

·Review·

Regulation of mitophagy in ischemic brain injury

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The selective degradation of damaged or excessive mitochondria by autophagy is termed mitophagy. Mitophagy is crucial for mitochondrial quality control and has been implicated in several neurodegenerative disorders as well as in ischemic brain injury. Emerging evidence suggested that the role of mitophagy in cerebral ischemia may depend on different pathological processes. In particular, a neuroprotective role of mitophagy has been proposed, and the regulation of mitophagy seems to be important in cell survival. For these reasons, extensive investigations aimed to profile the mitophagy process and its underlying molecular mechanisms have been executed in recent years. In this review, we summarize the current knowledge regarding the mitophagy process and its role in cerebral ischemia, and focus on the pathological events and molecules that regulate mitophagy in ischemic brain injury.

Keywords: mitophagy; ischemic brain injury; mitochondria

Introduction

One of the first autophagy investigations was performed by Keith R. Porter, who in 1962 discovered an increased number of vacuoles in rat liver cells treated with glucagon[1]. Autophagy is a pivotal intracellular process for the bulk degradation and recycling of unnecessary or dysfunctional proteins and organelles by lysosomes[2]. There are three forms: microautophagy, chaperone-mediated autophagy, and macroautophagy[3]. Macroautophagy is the most prevalent form and here is referred to as autophagy. It is critical for maintaining cell functions and deciding cell fate, and its intracellular processes have been elucidated in recent decades[3, 4]. Generally, double-membrane vesicles termed autophagosomes sequester target intracellular cargoes and then fuse with endosomes or directly with lysosomes for degradation. Autophagy is canonically activated under starvation[5], hypoxia[6] and intracellular stress[7, 8]. The mammalian target of rapamycin (mTOR) kinase is a critical regulator of autophagy induction: activation of mTOR by AMPK-p53 signaling promotes it[9], while inhibition of mTOR *via* PI3K-Akt signaling is suppressive[10]. The current knowledge of cell signaling on the regulation of autophagy has been summarized in several comprehensive review articles[11-13].

Autophagy is widely implicated in central nervous system (CNS) disorders[2]. A well-supported theory is that dysfunctional autophagy leads to aberrant accumulations of toxic protein aggregates in specific sites within the CNS and thus causes neurodegenerative diseases[14]. Given that autophagy can promptly respond to insufficient nutrients and energy supply, it is not surprising that it is extensively observed in ischemic brain tissues[6, 15, 16], where it was primarily postulated to promote cell death[17], whereas lines of evidence indicated that it is required for neuronal survival during development and neurodegeneration, as well as in several models of cerebral ischemia[15, 18, 19]. Unlike apoptosis and necrosis, which certainly contribute to ischemic brain injury, autophagy differs in that it may serve as a potential therapeutic target against ischemia[20-22]. Therefore, although they remain controversial, these emerging data suggest that autophagy may play a vital role in neuroprotection against ischemic brain injury, but the underlying mechanisms await clarification.

Selective elimination of mitochondria by autophagy, termed mitophagy, may be a responsible mechanism. Mitophagy is highly evolutionarily conserved; many of its essential proteins in yeast have homologs in mammals[23]. As the powerhouse of cells, mitochondria have a much shorter average lifespan of 10–25 days, compared with neurons, which are postmitotic cells with high energy demands. As a result, mitochondrial turnover by mitophagy is extremely important for neuronal survival. Dysfunctional mitophagy has been implicated in several human CNS diseases, including Alzheimer's disease[24] and Parkinson's disease[25]. Intriguingly, it has also been reported in ischemic brain injury by several independent groups[15, 26-28]. By clearing dysfunctional mitochondria and promoting mitochondrial turnover, cell death resulting from ischemic insult is attenuated[15]. Thus, mitophagy seems to be involved in ischemic brain injury, but questions remain. In this review, we summarize the current progresses on the regulation of mitophagy and its specific role in ischemic brain injury.

Mitophagy in Ischemic Brain Injury

Stroke is the third leading cause of death and disability worldwide. Cerebral ischemia makes up 65%–80% of total stroke events. Ischemic brain injury is generally caused by the sudden block of blood supply, leading to decreased oxygen and glucose supply to the brain tissue. The pathological mechanisms include, but are not limited to, neuro-excitotoxicity, brain inflammation, microglial activation, and endothelial injury[29-32]. In general, apoptosis is primarily found in striatal and cortical neurons after ischemic reperfusion, followed by necrotic neuronal death in the ischemic core area[33]. Autophagy, however, is widely involved in ischemic brain injury[15-18], and the autophagosome marker LC3-II is strongly elevated at the onset of ischemia[15], indicating the robust activation of autophagy, which might represent a cell defense mechanism against ischemic insult. Further, within 6 h after reperfusion, electron microscopy has shown damaged mitochondria surrounded by autophagosomes in the ischemic penumbra[15], and the numbers of mitochondria assessed by the constitutively-expressed mitochondrial proteins TOMM20 and COX4I1 are significantly reduced[15], suggesting that mitochondria are degraded by autophagy. In primary cultured neurons with oxygen and glucose deprivation (OGD) treatment, mitophagy is observed within 1 h, revealed by the co-localization of Mito-DsRed-labeled mitochondria and GFP-LC3-labeled autophagosomes (Fig. 1). The data indicated that mitophagy may reach a maximum within 3 h of OGD. Mitophagy is mainly evident

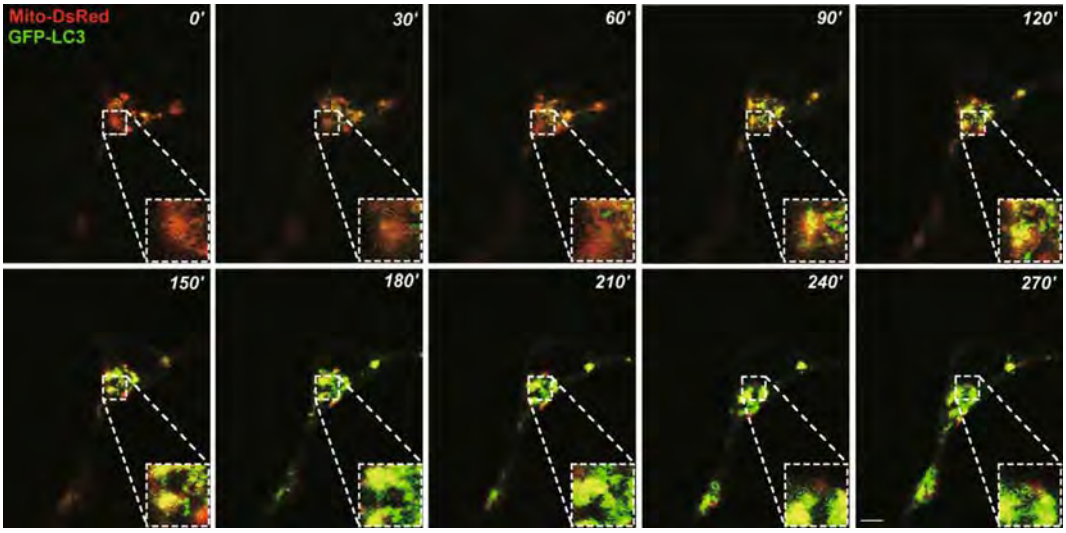

Fig. 1. Time-lapse imaging of mitophagy. Primary cultured mouse cortical neurons expressing Mito-DsRed and GFP-LC3 were exposed to oxygen and glucose deprivation for 2 h, and imaged at 1 frame/30 min. Mitophagy was determined by the co-localization of Mito-DsRed-labeled mitochondria and GFP-LC3-labeled autophagosomes. The boxed regions were amplified 4 times in the bottom right boxes. Scale bar, 10 μm.

in neurons undergoing brain ischemia[15, 19, 34], although astrocytes have also been documented[35, 36].

The contributions of autophagy and/or mitophagy to ischemic brain injury have been controversial for years[6, 16]. In long-term ischemia, for example, permanent middle cerebral artery occlusion (MCAO) models, the excessive activation of autophagy has been proposed to be associated with increased injury by inducing autophagic cell death, and inhibition of autophagy by 3-MA is neuroprotective[18]. Similarly, excessive induction of mitophagy leads to apoptosis in a neonatal ischemia/hypoxia model[37], in which the restoration of circulation is deficient due to intravascular coagulation. However, in the reperfusion phase after ischemia, mitophagy plays beneficial roles[15]. Inhibition of autophagy either pharmacologically or genetically rescues the ischemic brain[15] and conversely, injection of rapamycin, an autophagy inducer, has the opposite effects in rats with transient MCAO[28]. More interestingly, inhibition of autophagy immediately after reperfusion aggravates ischemic brain injury by reducing mitochondria-based apoptosis[15], while delayed inhibition of autophagy by 3-MA after reperfusion (3 h later) does not significantly increase brain damage (unpublished data), indicating that the protective action of autophagy occurs only at the initial step. In another study, rapamycin treatment was shown to reduce necrotic cell death in neonatal hypoxia-ischemia-induced brain injury[38]. Increasing evidence implies that autophagy may switch its contributions to ischemic stroke along with the pathological stages, but the reason is not clear. Energy restoration upon reperfusion[39] is perhaps the most plausible since autophagy/mitophagy is an energy-consuming process. But how the energy state may exquisitely regulate autophagy is far from clear. Alternation of mitochondrial dynamics during ischemia and reperfusion is an intriguing speculation[26, 40]. Upon reperfusion, mitochondria are fragmented by Drp1, a mitofission protein[26]. It is hypothesized that mitofission allows mitochondria to be engulfed by autophagosomes much more easily. The distinct mechanisms of permanent and transient ischemia may be another factor. In the reperfusion phase after ischemia, apoptosis is prone to be induced otherwise the prolonged ischemia normally results in necrosis[15, 41-44]. This may provide a chance for mitophagy to confer neuroprotection by clearing the damaged mitochondria. Subsequently, cytochrome *c* released from mitochondria is reduced and thus mitochondria-dependent apoptosis can be inhibited[15]. Although remaining enigmatic, the simplistic tagging of autophagy as 'bad' or 'good' in ischemic brains deserves to be reconsidered.

Pathological Events in Mitophagy Activation in Ischemic Brain

Although lines of evidence have implicated mitophagy in ischemic brains, the specific events initiating it and their integrated mechanisms remain largely unsolved. Recent work on endoplasmic reticulum (ER) stress, oxidative stress, and excitotoxicity may offer a solution to these questions.

Endoplasmic Reticulum Stress

ER stress has been reported to be widely involved in ischemic injury[45, 46]. However, consensus has not been reached on its role in this process. ER stress leads to apoptosis[47] and inhibition of the ER-associated pro-apoptotic factor CHOP confers remarkable neuroprotection in the mouse bilateral common carotid arteries occlusion model[48]. Consistently, several reagents that relieve ER stress protect against brain ischemia-reperfusion injury[49, 50], indicating that ER stress may be responsible for it. However, ER stress also triggers the unfolded protein response to restore ER functions by activating the ER transmembrane receptors PERK, IRE1, and ATF6[51]. It is well-accepted that increased ER stress leads to autophagy, and correspondingly autophagy helps to suppress ER stress[7, 52]. In the context of ischemic myocardial injury, treatment with ER stressors before operation induces autophagy[53]. Furthermore, mild induction of ER stress selectively reinforces ischemia-reperfusion-induced mitophagy *via* the PERK-EIF2S1-ATF4-Parkin signaling pathway[54]. Notably, the autophagic machinery does not accordingly increase with regard to the remarkable activation of mitophagy, as revealed by the numbers of autophagic vacuoles on mitochondria[54]. Since the stimulation of ER stress has been reported to result in mitochondrial dysfunction and thus impaired mitophagy in lung epithelial cells[55], it is of interest to explore why moderate ER stress reinforces mitophagy. The ER-mitochondria contact site (MAM) has recently been reported to be the location of autophagosome generation[56]. It is likely that mitochondria have priority to

be targeted by autophagosomes generated in the MAM, and appropriate stimulation of ER stress may accelerate the mitophagy originated by the ER. The mechanisms underlying ER stress-induced mitophagy, however, are not fully understood. Further, the extent to which ER stress favors mitophagy, and how to target mitophagy for therapy through ER stress activation, need to be further addressed.

Reactive Oxygen Species

Reactive oxygen species (ROS) are byproducts of oxygen metabolism, extensively and promptly generated in ischemic tissues after reperfusion[57-60]. ROS accumulation results in oxidative damage, opening of the mitochondrial permeability transition pore, and mitochondria-dependent cell death. Mitochondria produce most of the cellular ROS *via* the mitochondrial electron-transport chain[61], and play an important role in the pathogenesis of ischemic brain injury. ROS initiate autophagosome formation[62], and excessive ROS formation triggers bulk autophagy[63], while conversely, autophagy helps to reduce ROS levels by removing damaged organelles and abnormal proteins[64]. Interestingly, moderate ROS levels specifically induce mitophagy but not general autophagy to protect against cell death in a Drp1-dependent manner[63], indicating the ability of ROS to stimulate mitophagy. In support of this, in another study, increased ROS levels were shown to lower the mitochondrial membrane potential and activate Parkin-dependent mitophagy. Conversely, overexpression of superoxide dismutase-2, a mitochondrial antioxidant protein, blocks mitophagy by clearing photosensitizer-induced mitochondrial ROS in HeLa cells[62]. However, the association of ROS extension with the selective activation of mitophagy is still not clear.

In the context of ischemia, like ER stress, ROS may also be a double-edged sword with regard to cell survival (Fig. 2). On one hand, antioxidants that relieve ROS have been reported to help minimize ischemic injury[65-67]; on the other hand, ROS-induced mitophagy protects against ischemic myocardial injury. In p53-deficient mice, a p53-TIGAR-mediated decrease in the ROS signal reduces Bnip3-dependent mitophagy and enlarges the infarcted myocardium, while these are reversed by injection of the antioxidant N-acetylcysteine[68]. Therefore, it is likely that ROS are also responsible for brain ischemia-reperfusion-induced mitophagy, and serve as a potential target for clinical therapy.

Excitotoxicity

Excitotoxicity is evoked by excessive activation of neurotransmitters such as glutamate, which is the main neurotransmitter in the CNS. Excitatory neurotransmitters are widely involved in neurodegenerative diseases like Alzheimer's disease[24], Parkinson's disease[69], and stroke[59, 60, 70]. Excitotoxicity is induced by high levels of glutamate caused by over-activation of N-methyl-D-aspartate receptors (NMDARs) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors in ischemic brain injury. NMDA antagonists such as memantine are neuroprotective both *in vivo* and it *in vitro* in brain ischemia models[70, 71]. Excitotoxicity has been postulated to be associated with autophagy/mitophagy in mammals. In rats treated with the NMDAR agonist quinolinic acid, excitotoxicity is induced and it aggravates the neuronal injury by p53-activated autophagy[9]. A more recent study indicated that glutamate exposure results in the translocation of Parkin to mitochondria in a NMDAR-dependent manner, but is not sufficient to activate mitophagy. However, when co-treated with the antioxidant N-acetylcysteine, mitophagy is promoted[72], indicating that excitotoxicity can induce autophagy, but is insufficient to activate it alone. Therefore, excitotoxicity may not always be deleterious, since it facilitates the translocation of Parkin to mitochondria, which favors mitophagy, especially in the complex pathological process of ischemic brain injury.

With the progress made so far, multiple pathways could be involved in mitophagy in ischemic brain injury (Fig. 2). And more pathological events might be involved in mitophagy activation in the ischemic brain that need to be explored. How mitophagy occurs and how to promote it require further studies.

PINK1- and Parkin-Mediated Mitophagy

PINK1 (PTEN-induced kinase 1)- and Parkin-mediated mitophagy is perhaps the most extensively-studied mechanism underlying mitophagy. Both having loss-of-function mutations in familial Parkinson's disease, PINK1 and Parkin physically interact and work together in the same pathway in *Drosophila*[73]. In dysfunctional mitochondria, due to depolarization of the mitochondrial membrane, PINK1 is concentrated in the outer mitochondrial membrane, and then Parkin, an E3 ubiquitin

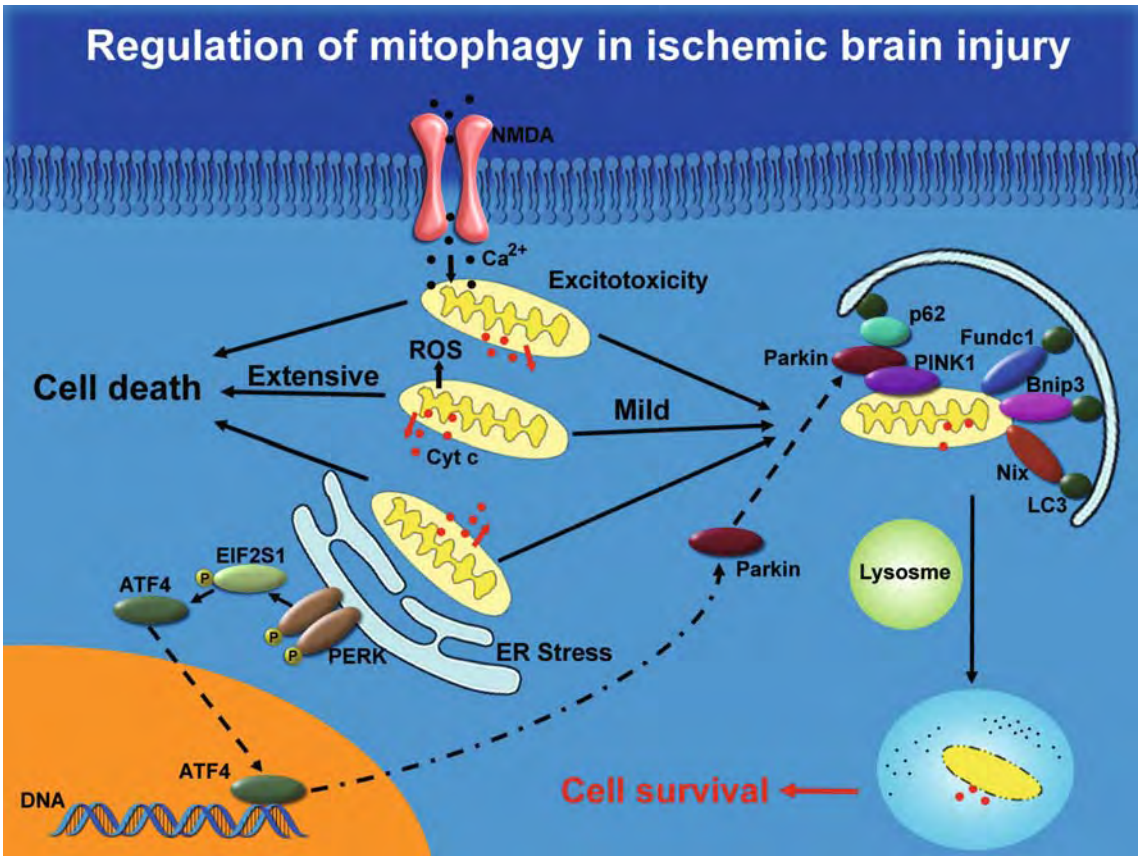

Fig. 2. Regulation of mitophagy in ischemic brain injury. Neuronal mitochondria are damaged by ER stress, the activation of oxidative stress, and/or excitotoxicity upon brain ischemia. Subsequently, the release of mitochondrial proteins such as cytochrome *c* ultimately leads to apoptotic cell death. However, on the other hand, mild ER stress, ROS generation, and excitotoxicity trigger the degradation of damaged mitochondria by mitophagy and therefore protect against ischemic brain injury. PINK1/Parkin, Bnip3, Nix, and Fundc1 are critical molecules responsible for mitophagy.

ligase, is phosphorylated by PINK1, and is further recruited to the damaged mitochondria[74] (for review, see [75]).

Lines of evidence have suggested that PINK1-Parkin are involved in ischemic brain injury. Parkin is markedly down-regulated after reperfusion for 3 h in a mouse model of transient MCAO[39]. This is temporally consistent with the peak of autophagic flux[15], implying that Parkin-labeled mitochondria can be eliminated by autophagy. Besides, ubiquitinated proteins are also present in the same model[76], regardless of whether Parkin is responsible for the ubiquitination or not, and this is now under discussion. Parkin is known to ubiquitylate several important substrates in cardiac injury[77], indicating that it may participate in ischemic brain injury by activating mitophagy. In ischemic preconditioning (IPC) of the rat heart, Parkin is translocated to mitochondria, and its ablation abolishes the cardioprotective effects of IPC[27], suggesting that Parkin-mediated mitophagy is required in this process. In support, we also found translocation of Parkin to mitochondria in primary cultured neurons exposed to OGD-reperfusion, and knockdown of Parkin impairs OGD-reperfusion-induced mitophagy[54]. Interestingly, the translocation of Parkin to mitochondria is accelerated by moderate activation of ER stress, ROS, and excitotoxicity as noted above, indicating that multiple stimulations may trigger Parkin-mediated mitophagy, which, from another perspective, ensures the integrity of execution of Parkin-mediated mitophagy in ischemic brain injury. Above all, Parkin-dependent mitophagy could be a target for ischemic brain injury therapy.

Bnip3- and Nix/Bnip3L-Mediated Mitophagy

Both Bnip3 and Nix (Bnip3L) are BH3-only proteins implicated in apoptosis and autophagy[78-80]. Bnip3 has been reported to participate in hypoxia-induced mitophagy in rat heart. Nix was first found to be essential for the induction of mitophagy during the maturation of red blood cells[79]. Both Bnip3 and Nix are primarily localized on the outer mitochondrial membrane, and share a similar mechanism to induce mitophagy (for reviews, see[80, 81]). The difference between the two proteins during the induction of mitophagy is of interest. Nix up-regulation cannot compensate for the loss of Bnip3-induced mitophagy[37], indicating clear differences between Bnip3- and Nix-dependent mitophagy. Bnip3 exclusively activates excessive mitophagy by interacting with LC3 in a mouse model of neonatal ischemia/hypoxia, and loss of Bnip3 significantly decreases mitophagy and reduces neuronal apoptosis. However, Nix has been proposed to function only under physiological conditions[37]. Nevertheless, a recent investigation found that Nix overexpression promotes carbonylcyanide-3-chlorophenylhydrazone (CCCP)-induced mitophagy in human embryonic kidney cells[82], suggesting that Nix also participates in stress-induced mitophagy. A possible hypothesis is that Nix may act as a substrate of Parkin, through which it senses damage to mitochondria[82]. Nevertheless, whether Nix-mediated mitophagy is involved in ischemic brain injury needs further investigation.

FUNDC1-Mediated Mitophagy

FUNDC1, which is located on the mitochondrial outer membrane, contains three transmembrane domains and has been newly identified as the mitophagy receptor in hypoxia[83]. FUNDC1 directly binds to LC3 *via* its conserved LIR motif. Both knockdown of FUNDC1 and mutation of the LIR motif inhibit mitophagy[83]. This binding is strengthened under hypoxic conditions, and is much stronger than that of Nix, suggesting that FUNDC1 has important implications for ischemia. Further, FUNDC1 can be phosphorylated by ULK1 at serine 17, which is critical for mitophagy induction under hypoxia. In contrast, FUNDC1 promotes the recruitment of ULK1 to damaged mitochondria[84]. Nonetheless, the role of FUNDC1 in brain ischemia has not been discussed.

Interestingly, increasing numbers of studies have indicated that these mitophagy pathways work cooperatively in pathological processes to ensure the effectiveness of mitophagy, especially in mammals. For example, Nix initiates autophagy and promotes the CCCP-induced translocation of Parkin[85]. In addition, Nix has very recently been reported to be the substrate of Parkin in PD[82], suggesting that Nix functions downstream of Parkin. Besides, mitophagy is nevertheless induced in HeLa cells that lack the Parkin protein[82]. Finally, Bnip3, Nix, and FUNDC1 have all been reported to be involved in hypoxia-induced mitophagy, suggesting that these proteins work together in specific mitochondrial diseases. However, how these pathways cooperate and which occupies a dominant role in ischemic brain injury need to be addressed.

Proteins That Potentially Regulate Mitophagy in Ischemic Brain Injury

Many mitophagy-related proteins have been implicated in ischemic brain injury (Table 1). However, whether they all participate in the process by regulating brain injury-induced mitophagy remains unclear. We summarize the features of several frequently-reported proteins below.

Beclin1

Beclin1 is an autophagy regulator and is implicated in mitophagy[86]. In myocardial ischemia, autophagy is activated by an AMPK-dependent mechanism, but in the reperfusion phase, Beclin1 is required[87]. Knockdown of Beclin1 by RNA interference protects against cerebral ischemic injury in rats by activating autophagy[88]. In addition, Beclin1 interacts with PINK1[86], and turns on autophagic flux. Nevertheless, mitophagy can also be triggered in a Beclin1-independent pathway with the parkinsonian neurotoxin MPP^+ treatment of neurons[89], suggesting that Beclin1 may not be as important in ischemic brain injury-induced mitophagy as in myocardial ischemia.

VDAC1

VDAC1 (voltage-dependent anion channel 1) is a poly-ubiquitination substrate of Parkin[90]. It is located on mitochondrial outer membrane, and has recently been reported to promote mitophagy and protect neurons in subarachnoid hemorrhage[34]. While VDAC1 is required for

Table 1. Mitophagy-related proteins involved in ischemic brain injury

Protein	Location	Function	Ref
SQSTM1	Cytosol	Autophagy receptor, ubiquitination substrate of Parkin	[98, 99]
ALOX15	Cytosol	Key enzyme in mitophagy induction in reticulocytes	[100]
CDC37	Cytosol	Co-chaperone of HSP90	[101]
P38	Cytosol	Phosphorylates LC3	[102, 103]
HSP90	Cytosol	Regulates ULK1- and ATG13-mediated mitophagy	[101]
TNFα	Cytosol	Induces mitophagy in mouse macrophages	[104]
RIPK2	Cytosol	Regulates mitophagy by phosphorylating ULK1	[105]
PINK1	Mitochondria or Cytosol	Involved in mitophagy by promoting translocation of Parkin	[75, 106]
Parkin	Mitochondria or Cytosol	E3 Ub ligase, ubiquitylates OMM proteins to promote mitophagy	[54, 75]
FUNDC1	Mitochondria	Receptor for hypoxia-induced mitophagy	[83]
VDAC1	Mitochondria	Ubiquitination substrate of Parkin	[34]
Drp1	Mitochondria	Required in mitochondrial division	[97]
Mitofusin1	Mitochondria	Mediates mitochondrial fusion	[107]
Mitofusin2	Mitochondria	Mediates mitochondrial fusion	[107]
PARL	Mitochondria	Prevents release of mitochondrial cytochrome C	[108, 109]
Beclin1	Mitochondria	Induces autophagy	[86, 88]
Bcl-2	Mitochondria	Regulates PINK1-Parkin-mediated mitophagy	[110]
Bcl-2l1	Mitochondria	Suppresses FUNDC1-mediated mitophagy	[111]
TRAF2	Mitochondria	E3 ubiquitin ligase	[112]
Nix	Mitochondria and ER	Mitophagy receptor, interacts with LC3 and GABARAP	[85, 113]
Bnip3	Mitochondria and ER	Mitophagy receptor, interacts with LC3	[78, 80]
HDAC6	Nucleus	Regulates Parkin-induced mitophagy	[114]
HMGB1	Nucleus	Regulates mitochondrial function and morphology	[115]
HSP27	Nucleus	Required for mitochondrial quality control	[116]

GABARAP, GABA receptor-associated protein.

Parkin-dependent mitophagy[90], whether it is dispensable for the induction of mitophagy is still under debate, since it cannot fully account for the mitochondrial K63-linked ubiquitin immunoreactivity after mitochondria depolarization[91].

Drp1

Mitochondrial dynamics and mitophagy are closely related (see reviews[92, 93]). Mitochondrial fission can produce an impaired unit that undergoes autophagic elimination[94]. Dynamin-related protein 1 (Drp1) is required for mitochondrial fission, and it controls the integrity of mitochondrial structure[95]. By interacting with Parkin, Drp1 is ubiquitylated and degraded by proteasomes, leading to mitofusion that blocks mitochondria from removal by autophagy[96]. Parkin and Drp1 may work synergistically, and interestingly when Drp1 is absent, Parkin becomes much more critical[97], suggesting that Parkin may help to initiate a compensatory pathway. Further, selective inhibition of Drp1 by mdivi-1 prevents mitochondrial division and mitophagy in brain ischemia-reperfusion, as well as exacerbating brain infarct volume[15].

Clinical Advances by Targeting Mitophagy

Molecular targets of autophagy have been identified for the discovery of inhibitors or enhancers (see review [117]), and promising patents are emerging[117]. For example, by targeting a functional region of Beclin1, Tat-Beclin1 peptide

interacts with GAPR-1, a negative regulator of autophagy, to induce protective autophagy[118]. Because of the crucial role of mitophagy in promoting cell survival, mitophagy has been considered as a clinical target for conquering diverse diseases[64, 119]. However, the specific selectivity of mitophagy, i.e., that specifically targets damaged mitochondria with autophagosomes, makes it much harder to take advantage of mitophagy for clinical applications. However, several mitophagy-related proteins, such as Parkin and Beclin1, have been proposed to be beneficial targets for ischemic brain injury treatment[54, 88]. Besides, the pathologic processes of ischemic brain injury have become increasingly clearer, and drugs that target mitophagy could be possible in the near future.

Conclusions and Future Directions

Looking deep into mitophagy helps to better understand the pathology of ischemic brain injury. The molecular regulation of mitophagy has achieved great progress, but many questions remain to be solved, for example, the role(s) of mitophagy in different models of brain ischemia, how mitophagy is initiated after ischemia-reperfusion, the detailed underlying mechanisms, and how to take advantage of mitophagy for clinical therapy. These questions still need further investigations in the future.

ACKNOWLEDGEMENTS

This review was supported by the National Natural Science Foundation of China (81373393 and 81273506), the Zhejiang Provincial Natural Science Foundation (LR15H310001), the Program for Zhejiang Provincial Leading Team of S&T Innovation Team (2011R50014), and the Fundamental Research Funds for the Central Universities, China (2015FZA7018). We are grateful to the Imaging Facilities, Zhejiang University School of Medicine, for help with confocal microscopy.

Received date: 2015-04-30; Accepted date: 2015-07-09

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Neurosci Bull August 1, 2015, 31(4): 407–415. http://www.neurosci.cn
DOI: 10.1007/s12264-015-1542-8

·Review·

Chaperone-mediated autophagy and neurodegeneration: connections, mechanisms, and therapeutic implications

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Lysosomes degrade dysfunctional intracellular components *via* three pathways: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Unlike the other two, CMA degrades cytosolic proteins with a recognized KFERQ-like motif in lysosomes and is important for cellular homeostasis. CMA activity declines with age and is altered in neurodegenerative diseases. Its impairment leads to the accumulation of aggregated proteins, some of which may be directly tied to the pathogenic processes of neurodegenerative diseases. Its induction may accelerate the clearance of pathogenic proteins and promote cell survival, representing a potential therapeutic approach for the treatment of neurodegenerative diseases. In this review, we summarize the current findings on how CMA is involved in neurodegenerative diseases, especially in Parkinson's disease.

Keywords: autophagy; chaperone-mediated autophagy; neurodegenerative disorder; Parkinson's disease; risk factors; mutant proteins; therapeutic target

Introduction

There are two major proteolytic systems in cells: the proteasome-based system and the lysosome-based system. The autophagy-lysosome pathway (ALP) is a dynamic process by which lysosomes dispose of subcellular components and has the capacity to degrade complete organelles in addition to proteins[1, 2]. This process has recently sparked great interest. Instead of being viewed as a passive process of degradation, the ALP is now recognized to be actively involved in the regulations of various cellular processes and the maintenance of cellular homeostasis. In mammals, depending on how the pathway substrates are delivered into the lysosomes, autophagy can be categorized into three main forms, macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) (Fig. 1). In macroautophagy, a newly-formed isolation membrane sequesters cytosolic components and organelles that need to be degraded. This membrane then matures and seals to become a double-membrane vesicle named the autophagic vacuole[3]. This allows degradation of the contents and the inner membrane of the autophagic vacuole by the lysosome enzymes for recycling. On the other hand, in microautophagy, intracellular components are directly captured by the lysosomal membrane *via* invagination[4, 5]. The engulfed cargoes are delivered into the lumen by vesicle scission for subsequent degradation[6, 7]. Microautophagy is considered to be the form that participates in the constant removal of organelles and intracellular proteins[5]. In contrast, CMA does not require

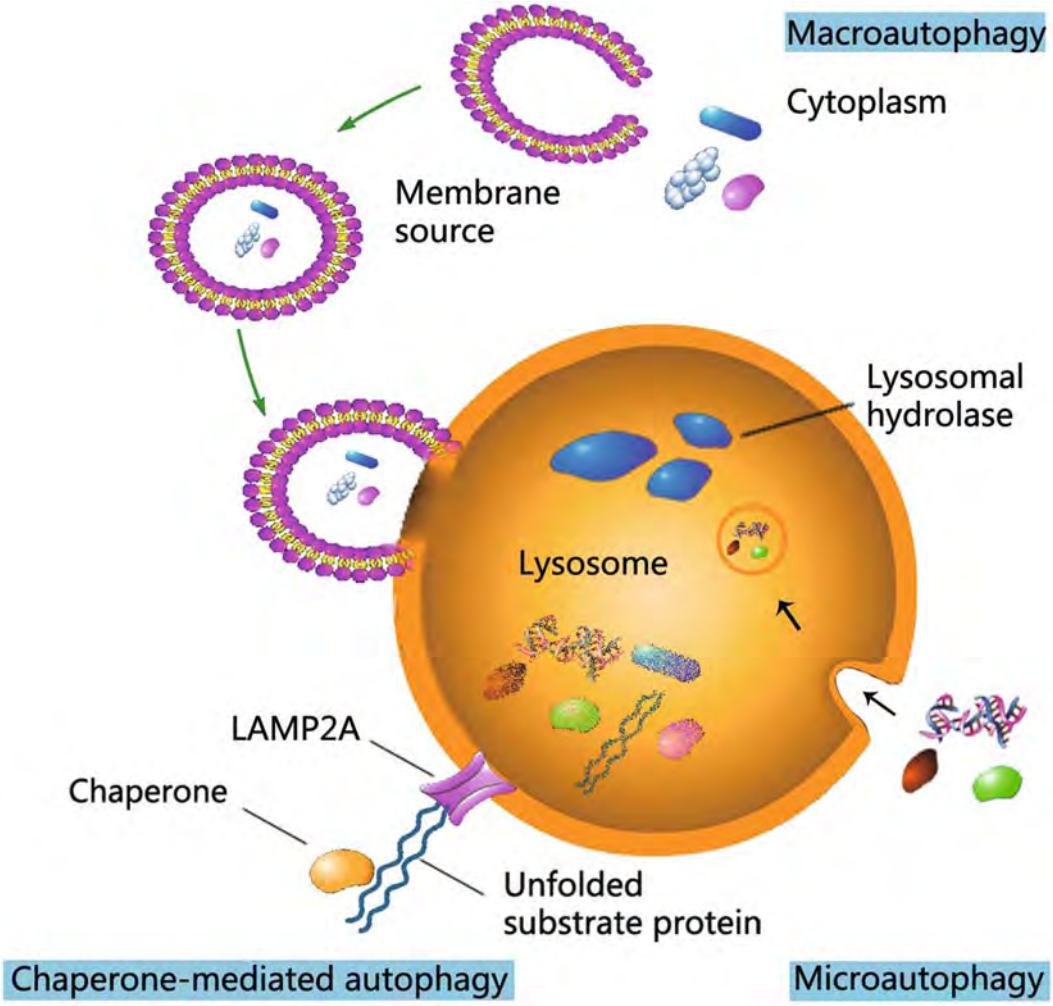

Fig. 1. A model of the three pathways for the degradation of intracellular proteins in lysosomes.

vesicle formation, but involves the capture and delivery of selected substrates to lysosomes for removal *via* chaperone proteins.

Dysfunction of the ALP plays critical roles in various pathological conditions including neurodegeneration. Many neurodegenerative diseases involve the aggregation of proteins, such as α-synuclein in Parkinson's (PD), amyloid-β and tau in Alzheimer's (AD), and huntingtin (Htt) in Huntington's disease (HD), which, in part, result from the failure of protein degradation systems. These proteins contain in their amino acid-sequences a KFERQ motif, the CMA substrate sequence, and have all been reported to be modulated by CMA[8, 9]. Changes of CMA activity, including both compensatory up-regulation and down-regulation, have been reported in models of neurodegeneration[10-12].

In this review, we summarize the current findings on CMA and neurodegeneration with a focus on PD, and highlight the potential of CMA modulation as a therapeutic approach for treating neurodegenerative diseases.

Chaperone-Mediated Autophagy

CMA described in mammalian cells is a highly selective form of autophagy. Its basic molecular components are known, but the detailed mechanisms by which it is regulated remain unclear[13]. One of the unique features of CMA is its selectivity and the fact that, in contrast to the other two autophagic pathways, it does not involve vesicle formation.

Instead, proteins with the recognition motif KFERQ in their amino-acid sequences are targeted individually by this process[14]. Through this unique mechanism, CMA functions as an efficient system to selectively remove damaged or abnormal proteins. Since ~30% of cytosolic proteins are reported to contain the KFERQ motif, it is reasonable to believe that CMA has the potential to regulate many cellular processes[15, 16].

Basic CMA Components

Chaperones Heat shock cognate 70 kDa protein (Hsc70) is a chaperone protein belonging to the heat shock protein 70 family[17, 18]. Cytosolic Hsc70 identifies a substrate protein *via* the KFERQ motif and transports it in complex to lysosomes for degradation[19]. Hsc70 in its ADP-bound form has a high affinity for CMA substrates[20] and also helps the docking of unfolded substrate proteins on the lysosomal membrane[21]. Besides Hsc70, other co-chaperones including Hsc40 and Hsc90 can interact with Hsc70 and facilitate this process[18, 22].

Receptor Lysosomal membrane glycoprotein (LAMP2A) functions as the receptor for CMA substrates[23]. Through its short cytosolic tail (GLKRHHTGYEQF), LAMP2A interacts directly with CMA substrate proteins and leads to their translocation into lysosomes[18]. The positively-charged residues located at the LAMP2A cytosolic tail are key to its interaction with substrates[24]. Overexpression of LAMP2A efficiently increases CMA activity, suggesting that its availability is rate-limiting for the entire process[18, 19, 25].

Substrates The KFERQ motif is the classic amino-acid sequence that mediates the interaction of a substrate protein with Hsc70[19, 26]. It includes a glutamine (Q) preceded or followed by a group of four amino-acids that contain a combination of acidic (D, E), basic (R, K), and bulky or hydrophobic (F, I, L, V) residues[27]. Such KFERQ motifs are found in ~30% of cytosolic proteins[28]. Some proteins such as the M2 isoform of pyruvate kinase have buried KFERQ motifs because of multimer formation. Other modifications such as ubiquitination can help dissociate the multimeric structure to expose the KFERQ motif, thus potentially regulating the spectrum of substrates[29].

Machinery

CMA uses a mechanism that is quite distinct from the other two autophagy pathways[19]. At the cellular level, the CMA process consists of four steps. The first includes substrate recognition and its targeting to the lysosomes. A typical CMA substrate protein with at least a KFERQ-like motif in its sequence is recognized and bound by Hsc70[20]. The second step involves substrate binding to LAMP2A and unfolding[24]. The CMA substrate protein in complex with Hsc70 binds to the C-terminal cytosolic tail of LAMP2A and this interaction quickly promotes the monomeric form of LAMP2A to assemble into a multimeric complex with a high molecular weight of ~700 KDa[24, 25]. The assembly of LAMP2A into this complex is dynamic and is in part initiated by the binding of the substrate to LAMP2A. The third step involves substrate translocation through the lysosomal membrane. It has been shown that both cytosolic and lysosome-associated Hsc70 participate in CMA translocation[13, 30-32]. Blockade of epitopes on lysosome-associated Hsc70 can interfere with the internalization. Thus, lysosome-associated Hsc70 plays an important role in substrate translocation after its delivery to the lysosomal surface by cytosolic Hsc70. The exact manner by which this translocation occurs has not yet been defined. The membrane complex may function actively to pull a substrate protein into the lumen or hold onto it to prevent its return to the cytosol[15]. The stability of lysosome-associated Hsc70 in lysosomes is highly influenced by the pH of this organelle, an increase in the lysosomal pH leading to its degradation. The fourth step is substrate degradation in the lysosomal lumen. After the substrate is pulled into the lumen of the lysosome, LAMP2A dissociates rapidly from the translocation complex into monomers, a state that allows it to bind the next substrate[25].

Regulation

CMA is influenced by multiple factors. For example, moderated levels of oxidative stress and neurotoxin treatment are known to increase the level of LAMP2A, the rate-limiting factor for CMA substrate flux, and enhance CMA activity[33]. Also, glial fibrillary acidic protein and elongation factor 1 alpha modulate CMA activity by regulating LAMP2A assembly/disassembly in a GTP-dependent manner[34]. Moreover, CMA can be regulated through the ubiquitin-proteasome system. When this system is compromised, part of the protective cellular response involves enhancing CMA to prevent the buildup of toxic species[19, 35]. On the contrary, blockade of CMA often leads to the accumulation of poly-ubiquitinated proteins[36].

In spite of these findings, the critical mechanisms by which CMA machinery responds to signals and is modulated remain to be determined.

Role of CMA in Neurodegeneration

Neurodegenerative diseases are associated with risk factors such as aging, exposure to neurotoxins, and oxidative stress[37, 38]. Some of these factors also interact with the CMA process. CMA dysfunction may be involved in the pathogenic process of several neurodegenerative diseases, and some of the more recent advances supporting this link are summarized below.

Effects of Neurodegeneration-Associated Risk Factors on CMA

Aging Aging is accompanied by a decline in the level of LAMP2A and CMA activity[39]. For example, in primary fibroblast cultures, CMA activity and lysosomal levels of LAMP2A are decreased in old-passage fibroblasts compared with early passages[40]. In the aged liver, although the transcription, synthesis, and lysosomal targeting of the LAMP2A protein appear to be the same in young and old rats, the stability of LAMP2A at the lysosomal membrane clearly decreases with aging[41]. The reason for this reduced stability is not clear, but it is possible that changes in the lipid microdomains of the lysosomal membrane contribute[41].

Neurotoxins Several neurotoxins modulate CMA activity. For example, 6-hyroxydopamine (6-OHDA) increases the levels of LAMP2A and Hsp90 in the nigral region in this rat model[42]. We showed that exposure to 6-OHDA increases the level of LAMP2A in a DA neuronal cell line and in the substantia nigra pars compacta (SNc) of mice and stimulates CMA activity[33]. Interestingly, toxins may alter the balance between CMA and substrate *via* mechanisms independent of a direct effect on lysosomes. Mitochondrial toxin also increases the levels of the CMA substrates α-synuclein and MEF2D[43]. But these changes seem to be due to changes in the regulation of their *de novo* synthesis but not their degradation by CMA. All of these findings provide examples of neurotoxin-induced changes in CMA activity and a shift of the balance between CMA and its substrates, suggesting a role for CMA as a cellular protective response to alleviate toxin-induced damage[44].

Oxidative stress Pro-oxidant compounds can cause changes in lysosomes isolated from cultured cells and rodent livers[45]. On the one hand, moderate oxidative stress seems to stimulate several key CMA components, including Hsp90, lysosomal Hsc70, and LAMP2A[33]. On the other hand, oxidative modifications of proteins may enhance their recognition by Hsc70. Together, these effects accelerate degradation *via* CMA, consistent with the evidence that antioxidants can partially prevent the CMA-mediated degradation of certain proteins following stress[46, 47]. In support of this, oxidative modifications of the neuronal survival factor MEF2D have been reported to promote its removal by CMA[33].

Role of CMA in PD

PD is one of the most common neurodegenerative diseases affecting the motor system. It results from the death of dopaminergic neurons in the SNc. Several gene mutations have been shown to cause familial PD[48]. Recent findings indicate that inhibition of CMA is a common mechanism by which the mutated proteins encoded by some of these genes exert their toxic effects.

α-synuclein and CMA PD is associated with an increase of α-synuclein protein[49]. α-synuclein contains a KFERQ-like motif and has been shown to be degraded by CMA[50]. Both monomers and dimers, but not oligomers, of α-synuclein are subjected to CMA regulation. Oxidation and nitration of α-synuclein may slightly hinder its degradation by CMA[51]. Furthermore, other post-translational modifications including phosphorylation and oligomerization may impair the turnover of α-synuclein, leading to its accumulation[52]. Interaction with oxidized dopamine (present in dopaminergic neurons) may induce a conformational change in the tertiary structure of α-synuclein, causing the protein to become misfolded and no longer internalizable by lysosomes. Pathogenic A30P and A53T α-synuclein mutants can bind to LAMP2A but are not internalized efficiently[8, 53]. Other mutant forms also hamper the CMA degradation pathway to varying degrees[54]. Therefore, all these conditions appear to impair CMA at the translocation and internalization step and may interfere with or inhibit the degradation of other CMA substrates.

Leucine-rich repeat kinase 2 (LRRK2) and CMA Mutation of the LRRK2 gene causes autosomal dominant PD. The LRRK2 protein is located in membrane microdomains, multivesicular bodies, and autophagic vesicles[55], and it

is also a substrate for CMA. However, the most common pathogenic mutant form of LRRK2, G2019S, is processed poorly through this pathway[56]. In the presence of other CMA substrates, the binding of both wild-type and several pathogenic mutant LRRK2 proteins to lysosomes is enhanced. This is thought to interfere with the organization of the CMA translocation complex, resulting in a CMA defect. In neuronal cultures, the brains of LRRK2 transgenic mice, induced pluripotent stem-cell-derived dopaminergic neurons, and the brains of PD patients carrying a LRRK2 mutation, cells respond to such LRRK2-mediated interference with CMA by increasing the level of LAMP2A. But this is not sufficient to overcome the blockade, resulting in the buildup of other CMA substrates including α-synuclein[56].

Mutant ubiquitin C-terminal hydrolase L1 (UCH-L1) and CMA UCH-L1 is a de-ubiquitinating enzyme expressed abundantly in neurons. Its oxidative/carbonyl damage has been reported in sporadic PD and its mutation is associated with familial PD[57]. UCH-L1 interacts with LAMP2A, Hsc70, and Hsp90. At the cellular level, the UCH-L1 I93M mutant shows an enhanced interaction with the CMA components independent of the mono-ubiquitin-binding activity but remains stuck in the membrane, blocking the CMA machinery[58]. Expression of the UCH-L1 I93M mutant in cells results in an increase of α-synuclein and possibly other substrates secondary to CMA inhibition. Therefore, the aberrant interactions between the UCH-L1 mutant and CMA machinery may underlie the pathogenesis of PD caused by this mutated protein[58].

Together, these findings indicate that several proteins whose mutation is associated with familial PD focus their toxic activity on CMA, implying that inhibition of CMA is a key and a common mechanism in the pathogenic process of familial forms of PD caused by the mutation of multiple genes.

Neuronal survival factor myocyte enhancer factor 2D (MEF2D) MEF2D, a transcription factor, plays an essential role in neuronal survival[59-61]. Our studies indicated that MEF2D is a CMA substrate[62]. Under normal conditions, non-functional MEF2D is transported to the cytoplasm and degraded by CMA and a decrease in CMA activity causes MEF2D accumulation in the cytoplasm. Both wild-type and A53T α-synuclein disrupt the CMA-mediated degradation of MEF2D and sensitize cells to stress[62, 63]. Consistent with this, cytoplasmic accumulation of MEF2D correlates positively with the level of α-synuclein in the brains of both α-synuclein transgenic mice and PD patients. Furthermore, our recent findings revealed that the neurotoxin 6-OHDA leads to oxidative modification of MEF2D and inhibits its activity[33]. Oxidatively-damaged MEF2D has a higher affinity for Hsc70 and is readily removed by CMA. Importantly, 6-OHDA induces MEF2D oxidation and increases LAMP2A in both cultured cells and the SNc region of the mouse brain. Similarly, the levels of oxidized MEF2D are much higher in postmortem PD brains than in controls. Functionally, reducing the levels of either MEF2D or LAMP2A exacerbates 6-OHDA-induced death in a dopaminergic neuronal cell line. An MEF2D mutant that is resistant to oxidative modification protects cells from 6-OHDA-induced death[33]. These findings indicate that removal of damaged MEF2D by CMA maintains cellular homeostasis and is protective against oxidative stress.

Alternations of CMA components The LAMP2 protein has three isoforms, LAMP2A, B, and C, which are affected differently in the early stages of PD[18]. The CMA receptor LAMP2A is selectively reduced related to the levels of α-synuclein. It has been reported that in PD brains, the levels of LAMP2A and Hsc70 are significantly reduced in the SNc and amygdala compared with those in age-matched AD and control brain samples[11, 64].

CMA in Other Neurodegenerative Diseases

Varying degrees of evidence indicate that dysfunctional CMA is also implicated in neurodegenerative diseases other than PD, including HD, AD, frontotemporal dementia, and amyotrophic lateral sclerosis.

In AD and tauopathies, CMA has been shown to influence the levels of neurofibrillary tangles resulting from the accumulation of mutant tau protein. The degradation of normal tau by CMA is mediated by its binding to Hsc70 *via* one of the two targeting motifs in its C-terminus[65]. Interestingly, it appears that it is the fragments of mutant tau, but not the full-length protein itself, that translocate to the lysosomal membrane through Hsc70 recognition to undergo CMA-dependent cleavage by cathepsin L[65].

Hsc70 and LAMP2A play roles in the degradation and clearance of Htt, whose gene mutation causes HD[52]. It has been reported that macroautophagic dysfunction triggers

an increase in CMA in the early stage of HD, which may be a protective measure[12]. Moreover, phosphorylation of Htt by inhibitor of kappa B kinase appears to promote its degradation in the lysosomes of unaffected neurons[66]. Therefore, a decline in CMA activity may function as one of the aggravating factors in the progression of HD[10, 19].

In frontotemporal dementia and amyotrophic lateral sclerosis, the pathogenic protein Tar-DNA binding protein 43 (TDP-43) forms cytosolic aggregates. TDP-43 is degraded by both ubiquitin proteasome and macroautophagy pathways, but there is also a report supporting the involvement of CMA in its degradation. This requires an interaction between Hsc70 and ubiquitinated TDP-43[67].

CMA as a Therapeutic Target in Treating Neurodegenerative Diseases

One of the most common characteristics among the different neurodegenerative diseases is the aggregation of deleterious proteins or inclusions in neurons[45, 68], including Htt protein in HD, amyloid-β in AD, and α-synuclein in PD. Many studies have established a strong link between a decline of CMA and the abnormal aggregation of such proteins in various models of neurodegenerative diseases[62, 69], suggesting that CMA is a new and promising target for treating multiple neurodegenerative disorders.

Increased or restored CMA activity could be protective in several pathological conditions. As summarized above, the involvement of CMA in neurodegenerative diseases is complex. It is responsible for the clearance of aggregated or harmful proteins, and it is often the target of the toxic effects of these deleterious proteins. Rigorously designed studies are needed to thoroughly evaluate and determine whether enhancing CMA activity can provide substantial therapeutic benefits.

For the pharmacological manipulation of CMA activity, several molecules and their analogues have been reported to enhance autophagy with verified efficacy in several animal models of disease[68, 69]. Genetically, LAMP2A overexpression in mouse liver improves lysosomal as well as liver functions[45]. This suggests that reversing the aging-related impairment of CMA could have functional consequences. Similarly, studies on models of neurodegeneration also confirm the efficacy of targeting LAMP2A. Overexpression of LAMP2A in both cell lines and primary neurons efficiently increases the CMA activity and decreases the amount of α-synuclein[70]. More remarkably, in the rat SNc, LAMP2A overexpression effectively reduces the levels of total α-synuclein and related aberrant inclusions, increases the survival of dopaminergic neurons, and alleviates the α-synuclein-induced neurodegeneration[71, 72]. In addition, experimentally increasing CMA activity seems to provide therapeutic benefits in HD models[10]. Overall, these findings strongly suggest that manipulation of CMA activity may be an effective therapeutic strategy for treating neurodegenerative diseases.

Summary and Direction

To date, over 30 CMA substrates have been identified, a significant portion of which is involved in the regulation of critical neuronal functions. This highlights the importance of CMA in the nervous system under both physiological and pathological conditions. As discussed above, there is a particularly strong link between CMA impairment and PD. Accumulating evidence indicates that loss of CMA activity is associated with multiple toxic and genetic conditions associated with PD, suggesting that a decrease in CMA activity may underlie in part the pathogenic process of PD. However, several issues remain to be resolved. First, a better understanding of the specific stage(s) when the CMA defect occurs in models and human tissues of PD is needed. Second, a better understanding of the critical mechanism(s) underlying how the CMA defect contributes to or even triggers the neurodegenerative process is needed. Third, although still preliminary, manipulating CMA has been shown to alleviate PD-related neurodegenerative changes in several experimental models. Future studies are clearly needed to validate these findings and optimize strategies aimed at selectively and potently modulating CMA function. The selective modulation of CMA will not only allow a more precise assessment of the possible side-effects of such therapies, but also aid investigations into the mechanisms of how the pathological factors cause degenerative alterations through inhibition of the CMA pathway.

ACKNOWLEDGMENTS

This review was supported in part by grants of NIH, USA (AG023695, NS079858, and ES015317).

Received date: 2015-05-05; Accepted date: 2015-06-27

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Neurosci Bull August 1, 2015, 31(4): 416–426. http://www.neurosci.cn
DOI: 10.1007/s12264-015-1536-6

·Review·

Autophagy in synaptic development, function, and pathology

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In the nervous system, neurons contact each other to form neuronal circuits and drive behavior, relying heavily on synaptic connections. The proper development and growth of synapses allows functional transmission of electrical information between neurons or between neurons and muscle fibers. Defects in synapse-formation or development lead to many diseases. Autophagy, a major determinant of protein turnover, is an essential process that takes place in developing synapses. During the induction of autophagy, proteins and cytoplasmic components are encapsulated in autophagosomes, which fuse with lysosomes to form autolysosomes. The cargoes are subsequently degraded and recycled. However, aberrant autophagic activity may lead to synaptic dysfunction, which is a common pathological characteristic in several disorders. Here, we review the current understanding of autophagy in regulating synaptic development and function. In addition, autophagy-related synaptic dysfunction in human diseases is also summarized.

Keywords: autophagy; synaptogenesis; synaptic elimination; synaptic function; synaptic pathology

Introduction

As the predominant form of autophagy, macroautophagy (hereafter "autophagy" for short) is an essential self-defense mechanism for the maintenance of cellular homeostasis. It operates by the sequestration of cytoplasmic materials and proteins into a double-membrane autophagosome, which fuses with a lysosome or late endosome whereby encapsulated materials are degraded. Under pathological conditions, autophagy functions as a critical quality-control system; damaged intracellular organelles, misfolded proteins, or protein aggregates are removed by autophagic clearance.

Maday and Holzbaur were the first to uncover the biogenesis of autophagosomes in neurons[1]. Under physiological conditions, autophagosomes are generated in a compartmentalized pattern, as most are synthesized in the axonal terminals[1]. Although both anterograde-directed motor kinesin and retrograde-directed motor dynein are tightly associated with axonal autophagosomes[2, 3], binding of the scaffolding protein JIP1 to the autophagosome adaptor LC3 ensures the robust retrograde transport of newly-formed autophagosomes along microtubules in axons[4]. Emerging lines of evidence suggest that autophagy regulates the development and function of axons, dendrites, and synapses. Besides, insufficient or excessive neuronal autophagy contributes to pathological changes in these polarized structures. The regulatory role of autophagy in axonal and dendritic degeneration was discussed in our previous review[5].

Synapses are dynamically organized elements[6]; the wiring and rewiring of neuronal circuits largely depend on orchestrated changes in the strengths of synaptic contacts in response to developmental and environmental cues. The synapse is the point of contact between the neurons, and plays a crucial role in the transmission of neuronal information. The integrity of synaptic structure and function is pivotal to ensuring that neurons acquire, transfer, process, and store information smoothly and systematically. Because of the high energy demand and protein turnover

ratio in the region of the synapse, the timely clearance of synaptic contents appears to be crucial for maintaining synaptic function[7]. Several lines of evidence point to the involvement of autophagy in synaptogenesis, synaptic elimination, and synaptic transmission. Besides, autophagy-related synaptic dysfunction has been implicated in neurodevelopmental disorders and neurodegenerative disorders. In this article, we review the recent experimental findings on how autophagy modulates the development, function, and pathology of the synapse.

Autophagy in Synaptic Development

Synapses are highly dynamic components of neurons, and persistent turnover of synapses occurs during development and in the adult brain. During development, the synaptogenesis and synaptic elimination are under delicate balance to maintain the normal functions of neuronal circuits[8-10]. Synaptic gain and elimination, proceeding by synaptic turnover, are key rearrangement events in learning, memory, and cognition[11]. Since the autophagic pathway plays a fundamental role in regulating protein turnover, we summarize the recent progress in understanding the regulatory effects of autophagy on synaptogenesis and synaptic elimination.

Autophagy in Synaptogenesis

Each individual *Drosophila* neuromuscular junction (NMJ) contains hundreds of synapses and therefore is a well-established model system for studying synaptogenesis. Synaptogenesis is a multistep process, and a variety of molecules and signaling pathways have been identified to mediate early synaptogenesis[12]. Autophagy and the ubiquitin-proteasome system are major pathways for protein degradation in cells. Accumulating evidence has indicated the importance of protein degradation *via* the ubiquitin-proteasome system, which is mainly responsible for the turnover of short-lived cytosolic proteins, in regulating synaptic growth[13-16]. Highwire (Hiw), an E3 ubiquitin ligase that mediates key steps in the protein ubiquitination process, negatively governs synaptic growth at the *Drosophila* NMJ[17, 18]. It has been suggested that Hiw mediates presynaptic bone morphogenetic protein signaling through ubiquitination mechanisms and thereby controls the growth of neuromuscular synapses[19]. Recent studies have emphasized the involvement of autophagy, which is responsible for the degradation of long-lived proteins and damaged organelles, in synaptic development. Increased levels of the synaptic protein synaptotagmin 1 have been found along with upregulated autophagy proteins (Atg9a and LC3-II) during the differentiation of mouse neural stem cells[20]. Under transmission electron microscopy, autophagosomes are distributed in the synaptic terminals of cultured hippocampal neurons[21], indicating that autophagy is required during synaptogenesis.

In 2009, Shen and Ganetzky reported that autophagy plays a positive role in promoting the growth of the larval *D. melanogaster* NMJ[22]. Impaired autophagy significantly reduces the size of NMJ synapses and the number of boutons in larvae, whereas overexpression of the autophagy-associated gene *atg1* induces NMJ overgrowth by elevating autophagic activity[22]. In accordance with these results, Batlevi *et al.* also reported a decreased number of synaptic boutons in dynein light chain 1 (*ddlc1*) mutant *Drosophila* that exhibited attenuated autophagic activity and reduced protein clearance[23].

Although the molecular mechanism underlying autophagy-regulated synaptic growth is not entirely clear, it has been suggested that autophagy regulates NMJ growth by inducing the degradation of Hiw[22]. *D. melanogaster* Rae1, an Hiw cofactor, binds to Hiw and prevents its autophagy-regulated downregulation[24]. In addition, the mitogen-activated protein kinase signaling pathway also participates in autophagy-mediated synaptogenesis. The downstream signaling cascades of this pathway, including extracellular signal-regulated kinase (ERK), c-Jun-N-terminal kinase (JNK), and p38 mitogen activated kinase, are well-characterized mediators of synaptic development[25]. Wairkar *et al.* revealed that Unc-51, the *Caenorhabditis elegans* Atg1 ortholog, promotes synaptic formation and development by downregulating ERK signaling[26]. JNK and its transcriptional effector AP-1 can be activated in response to oxidative stress. The activation of JNK/AP-1 regulates synaptic development under oxidative stress by activating autophagy[25, 27, 28].

Autophagy in Synaptic Elimination

It is worthy of note that the phenomenon of increased synapse number can result from enhanced synaptic formation or decreased synaptic elimination. Synaptic elimination, also known as synaptic pruning, is the process of removing redundant or inappropriate synaptic connections. Synaptic elimination helps to fine-tune precise

neuronal connectivity and is as important as synaptogenesis during brain development[29].

The spine is a specialized postsynaptic protrusion on dendrites. The time course of spine development in primary cultured hippocampal neurons is similar to that of dendritic spines in mouse brain[30, 31]. In cultured neurons, the number of spines increases during 6–10 days *in vitro* (DIV), peaks at 14–21 DIV, and decreases after 21–28 DIV[32]. Tang *et al.* found that silencing the key autophagy gene *atg7* increases the spine density at 19–20 DIV[32]. Interestingly, unlike control cells in which the rates of synapse formation and elimination are approximately equivalent, hippocampal neurons deficient in *atg7* exhibit normal spine formation but greatly inhibited elimination, indicating that autophagy enables synaptic elimination in cultured hippocampal neurons during the "mature" developmental stage[32, 33]. On the other hand, deficits in autophagy leading to insufficient synaptic elimination are closely associated with several neurodevelopmental diseases that are discussed in detail below. As autophagy is required for development of the *Drosophila* NMJ, the normal spine formation in atg7-deficient cultured neurons might be due to species differences or the different conditions between *in vivo* and *in vitro* studies.

Autophagy in Synaptic Function

In neurons, the majority of autophagosomes are locally synthesized in the distal terminals of axons[3] (Fig. 1A). After generation, autophagosomes are transported towards the soma and the engulfed cytoplasmic materials are delivered to lysosomes for degradation[4, 5]. Although the molecular mechanism involved in the biosynthesis of neuronal

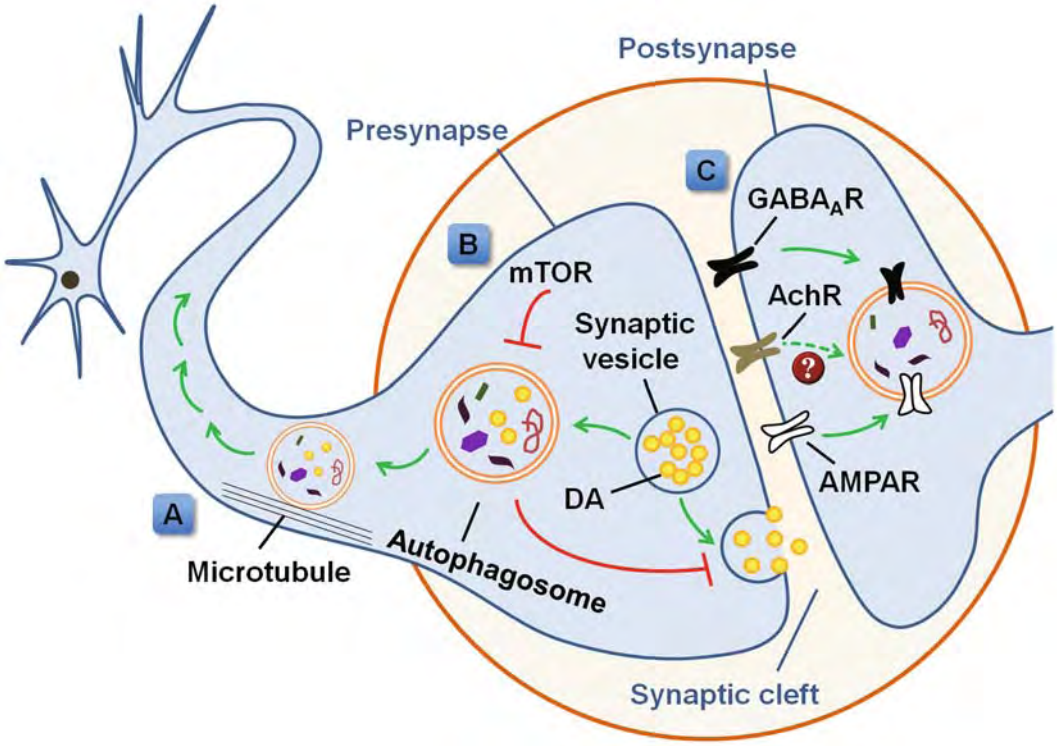

Fig. 1. Regulatory role of autophagy in synaptic terminals. (A) Cytoplasmic contents, including misfolded proteins and organelles, are engulfed into double-membrane autophagosomes. Most of the autophagosomes are locally synthesized in axons and are then transported along microtubules towards the cell body. (B) In the presynaptic terminals of dopaminergic neurons, autophagy mediates synaptic vesicle degradation and suppresses DA release. mTOR negatively regulates autophagic activation. (C) In postsynaptic terminals, autophagy contributes to the degradation of postsynaptic receptors, such as GABA$_A$Rs and AMPARs. Whether or not autophagy governs AChR degradation is unclear. AChR, acetylcholine receptor; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptor; DA, dopamine; GABA$_A$R, gamma-aminobutyric acid-A receptor; mTOR, mammalian target of rapamycin.

autophagosomes is not entirely understood, accumulating evidence indicates that the Rab family and related small GTPases are required for the formation and maturation of autophagosomes[34]. A recent study further showed that the GTPase Rab26 directs synaptic vesicles towards pre-autophagosomal structures[35], implying that autophagy participates in synaptic transmission. Synaptic transmission relies on neurotransmitters and their receptors. In this process, neurotransmitters are initially released from the presynaptic terminals, and subsequently bind to and activate their receptors located on the postsynaptic terminals, triggering a series of biochemical reactions.

A growing body of evidence has revealed that autophagy is capable of regulating synaptic function in presynaptic and postsynaptic terminals (Fig. 1B, C). Both basal and induced autophagy participate in the modulation of synaptic transmission and plastic remodeling. Autophagy-regulated synaptic function in GABAergic, dopaminergic, glutamatergic, and cholinergic neurotransmitter systems has been described in detail (Table 1).

Table 1. Autophagy-regulated synaptic function in GABAergic, dopaminergic, glutamatergic, and cholinergic neurotransmitter systems

Neurotransmitter system	Species	Tissues/Cells	Description	Reference
GABAergic	*C. elegans*	Non-innervated muscle cells	$GABA_A$ receptors target to autophagosomes for degradation	[49]
Dopaminergic	*Mus musculus*	DA neurons from DAT Cre mice	Autophagy activation depresses evoked DA secretion in dopaminergic neurons	[40]
	M. musculus	METH-treated ventral midbrain DA neurons	Perturbed DA release may in turn trigger autophagy	[45]
Glutamatergic	*Rattus norvegicus*	Primary cultured hippocampal neurons exposed to KCl	NMDAR-dependent autophagy contributes to AMPAR degradation	[52]
Cholinergic	*M. musculus*	Tibialis anterior muscle	Autophagy regulates the basal and atrophy-induced turnover of CHRN	[53]
	C. elegans	Non-innervated muscle cells	AChRs do not traffic to autophagosomes	[49]

AChRs, acetylcholine receptors; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptor; DA, dopamine; CHRN, muscle-type cholinergic receptor, nicotinic/nicotinic AChR; DAT, dopamine transporter; KCl, potassium chloride; METH, methamphetamine; NMDAR, glutamatergic N-methyl-*D*-aspartate receptor.

Autophagy in Presynaptic Terminals

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that acts as a key cell growth mediator *via* integrating the inputs from multiple upstream signals[36, 37]. mTOR blocks the activation of autophagy at an initial step during autophagosome formation[38]. Notably, mTOR regulates local RNA translation at the synapse and thus appears to be important for the synthesis of synaptic proteins[39]. Emerging lines of evidence highlight the crucial role of the mTOR signal in regulating synaptic transmission[40, 41] and synaptic plasticity[42]. Inhibition of the mTOR signaling pathway by rapamycin, which upregulates autophagic activity in mammalian cells, reduces the numbers of synaptic vesicle and depresses the evoked dopamine (DA) secretion from dopaminergic neurons[40] (Fig. 1B). Mice deficient in DA neuron-specific autophagy (*atg7* DAT Cre) exhibit enhanced DA release in response to stimulation and an increased rate of synaptic recovery[40]. Based on these findings, it has been speculated that autophagy acts as a brake on presynaptic activity by regulating the kinetics of DA release[43]. The perturbed neurotransmitter release may in turn trigger autophagy induction. For instance, dopaminergic terminals

are particularly vulnerable to methamphetamine (METH), a widely-abused psychostimulant[44]. In ventral midbrain DA neurons, METH promotes DA synthesis and subsequently elevates the cytosolic DA level[45]. The excessive DA metabolites may lead to the generation of damaged lipids and proteins, thereby inducing autophagic degradation[45].

Autophagy in Postsynaptic Terminals

In postsynaptic terminals, autophagy contributes to the degradation of special types of receptors. γ-aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the central nervous system (CNS). $GABA_A$ receptors ($GABA_A$Rs), the major postsynaptic components of GABAergic synapses, mediate fast synaptic inhibition in the brain[46]. These receptors, composed of different subunits, are distributed at both synaptic and extra-synaptic sites, where they play crucial roles in governing phasic and tonic inhibition, respectively[47, 48]. *C. elegans* is an ideal animal model for investigating neurotransmitter receptors because it can be genetically manipulated. In 2006, Rowland *et al.* for the first time reported that the cell-surface $GABA_A$Rs, but not acetylcholine receptors, targeted to autophagosomes for degradation[49] (Fig. 1C). In contrast to the simple and uniform distribution of $GABA_A$Rs in *C. elegans*[50], the structure of $GABA_A$Rs is rather complex in mammalian cells[51], and there is still no evidence that autophagy is required for the turnover of $GABA_A$Rs in mammalian cells.

In addition to GABARs, glutamatergic N-methyl-*D*-aspartate receptor (NMDAR)-dependent autophagy contributes to the degradation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptors (AMPARs) in cultured rat hippocampal neurons upon stimulation (Fig. 1C), suggesting that autophagy participates in NMDAR-dependent synaptic remodeling[52].

The regulatory effect of autophagy on cholinergic neurons has not yet been fully clarified (Fig. 1C). Rowland *et al.* showed that acetylcholine receptors do not traffic to autophagosomes in the non-innervated muscle cells of *C. elegans*[49]. In contrast, a recent report demonstrated that in mouse tibialis anterior muscles, autophagy contributes to the basal and atrophy-induced turnover of muscle-type cholinergic receptors, nicotinic/nicotinic acetylcholine receptors in a tripartite motif containing 63 (TRIM63)-dependent manner[53]. Such a discrepancy might be due to the different species used in experiments. Nevertheless, autophagy seems to act as a universal regulator for modulating receptor turnover in postsynaptic terminals, though the substrate-specificity of autophagosomes still needs to be well defined.

Autophagy and Synaptic Regulators

Despite the uncertainty of an association between synaptic regulators and autophagy, defects in certain synaptic proteins result in the failure of either autophagic induction or autophagic clearance. For example, loss of neuron-specific synaptic v-SNARE (soluble NSF attachment protein receptor) *n-syb* (*neuronal Synaptobrevin*) leads to increased autophagic activity in adult *D. melanogaster* photoreceptor neurons[54]. Such enhancement of autophagy is proposed to be a consequence of primary vesicle trafficking defects[54]. Snapin, initially identified as a neuronal SNARE-binding protein, is a crucial modulator of vesicle release and presynaptic homeostatic plasticity[55, 56]. Deleting *snapin* promotes the accumulation of autolysosomes in cortical neurons by impairing efficient autophagic turnover[57]. Therefore, synaptic regulators in turn may affect the autophagy-lysosomal degradative system.

Involvement of Autophagy in Synaptic Pathology

Functional autophagy participates in a variety of events in synapses, including dendritic spine elimination (Fig. 2A), local protein clearance and turnover (Fig. 2B), and synaptic growth. Morphological and functional impairment of synapse is a common theme in the pathogenesis of many neurological diseases. However, the potential impact of autophagy on synaptic pathology has not yet been explored in all neurological diseases. Here, we discuss the recent evidence supporting a role of autophagy in mediating synaptic pathology in human diseases, including neurodevelopmental disorders (e.g. autism spectrum disorders [ASDs]) and neurodegenerative disorders (e.g. Alzheimer's disease [AD][58, 59] and Parkinson's disease [PD][60, 61]). Moreover, the involvement of autophagy has also been noted in synaptic dysfunction upon aging and the burden of oxidative stress (OS), a condition involved in several neurological diseases.

Neurodevelopmental Disorders

Appropriate elimination of synapses is a crucial step for neuronal network refinement during brain development, while insufficient or abnormal synaptic elimination is linked to many neurodevelopmental disorders. ASD

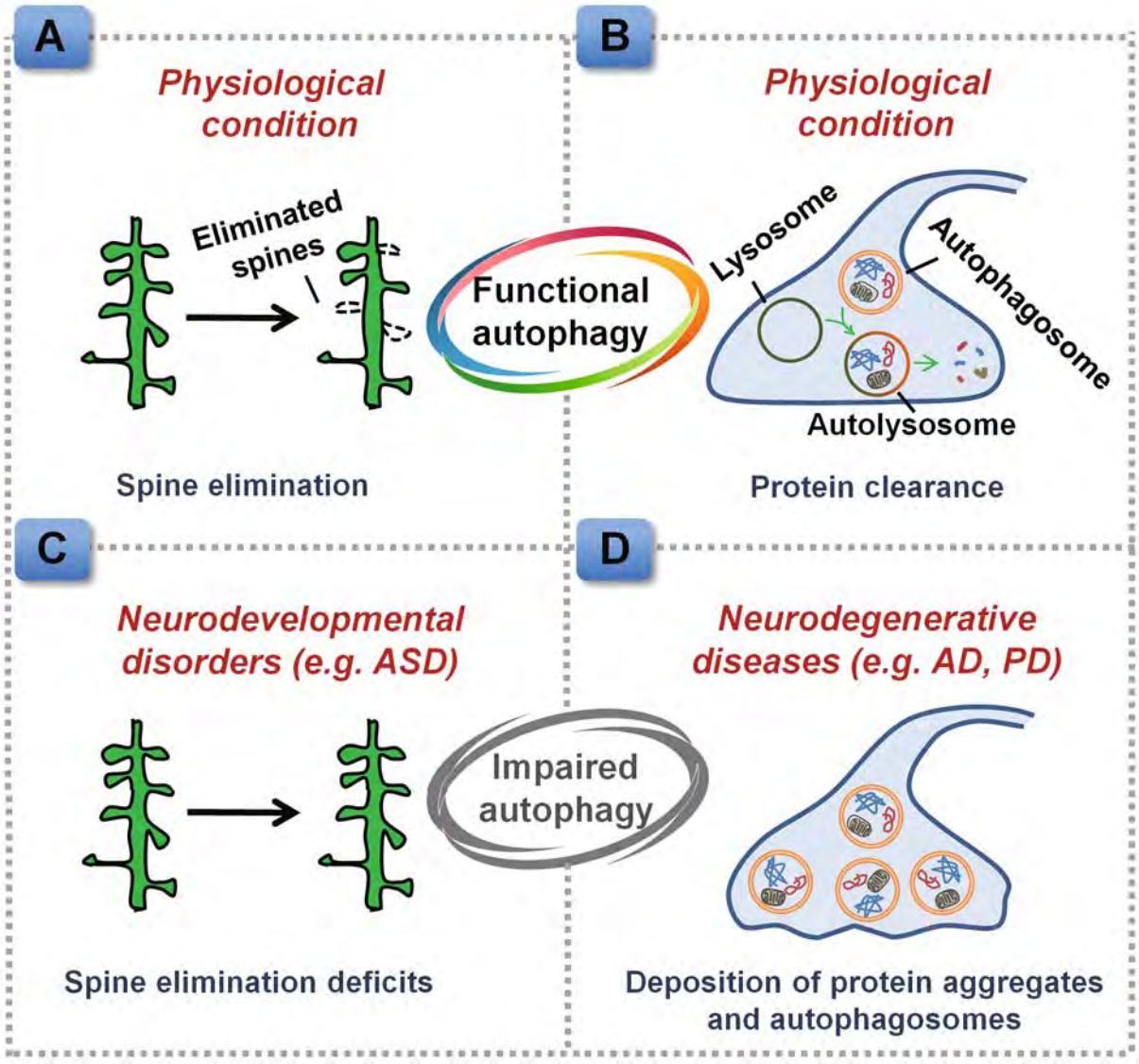

Fig. 2. Autophagy-related synaptic pathology in neurological diseases. Functional autophagy participates in dendritic spine elimination (A) and local protein clearance in the synapse (B). Impaired autophagy leads to spine elimination deficits in neurodevelopmental disorders, such as ASD (C). In addition, insufficient protein clearance caused by abnormal autophagy leads to the deposition of aberrant or misfolded protein aggregates and autophagosomes in synapses, which is a pathological feature of several neurodegenerative diseases such as AD and PD (D). AD, Alzheimer's disease; ASD, autism spectrum disorder; PD, Parkinson's disease.

is characterized by deficits in social interaction and communication, restricted interests, and repetitive behaviors, and the aberrant development and function of synapses is known to be involved in its pathogenesis[62]. Compared to age-matched control cases, increased dendritic spine densities occur in the frontal, temporal, and parietal lobe regions of ASD brains[63], and the greater spine densities are correlated with reduced cognitive function in individuals with ASD[63]. In addition, the increased dendritic spine density is predominantly caused by reduced developmental spine elimination. Most importantly, such spine pruning deficits result from hyperactivation of the mTOR signaling pathway and impaired autophagy[32]. Although the molecular mechanism by which autophagy

contributes to synaptic regulation has not yet been fully clarified, a recent report identified a key gene involved in this process. Ambra1 is a prominent upstream regulator of Beclin 1 (a principal mediator of autophagosome formation). Deficiency in Ambra1 results in autism-like phenotypes in female mice[64], implying that deregulation of the autophagic pathway causes the pathology of autism. Based on this evidence, it is possible that dysfunctional autophagy tends to contribute to synaptic pathology and leads to ASD phenotypes (Fig. 2C), while activation of autophagy may normalize the dendritic spine elimination and correct the synaptic pathology in ASD.

Neurodegenerative Diseases

In neurodegenerative diseases, synaptic loss and dysfunction commonly occurs before that in the soma. Besides, the formation and accumulation of aberrant or misfolded protein aggregates, owing to insufficient protein clearance by autophagy or other intracellular degradative pathways, is another pathological feature of neurodegenerative disorders[65]. There is no doubt that dysfunctional protein turnover in synapses is associated with the pathological protein accumulation. Indeed, excessive protein aggregates as well as autophagic vacuoles have been noted to accumulate locally in synapses.

Synaptic dysfunction is highly correlated with the cognition and memory decline in age-related neurobiological changes such as AD[66, 67]. In young AD mice (4–6-month-old PS1/APP mice), increased accumulation of autophagic vacuoles is correlated with aberrant presynaptic terminals[68]. In accord with this finding, senescence-accelerated prone 8 (SAMP8) mice, another AD model, exhibit elevated numbers of LC-3 positive cells in the hippocampus as well as prominent synaptic loss[69]. Generally, increased formation of autophagic vacuoles results either from induced autophagic activity or from autophagic flux defects. In primary cultured neurons with AD-like injury and in AD animal models, autophagy has been demonstrated to act as a protective mechanism, as the stimulation of autophagy or the recovery of lysosomal proteolysis is able to prevent AD-like neuritic degeneration, possibly by promoting the maturation of autophagosomes[5, 70]. In view of this point, we speculate that the accumulation of autophagic vacuoles, most likely caused by defective degradation of synaptic proteins (Fig. 2D), matches the synaptic dysfunction in AD and contributes to the cognitive and memory deficits in patients. This hypothesis is supported by a recent finding that oleuropein aglycone protects against pyroglutamylated-3 amyloid β peptide toxicity and synaptic dysfunction by activating neuronal autophagic machinery as determined by elevated Beclin 1 and LC3 immunoreactivity along with enhanced degradation of autophagy substrates[71].

PD is characterized by the accumulation of the aggregation-prone protein α-synuclein, which, under physiological conditions, functions in modulation of the presynaptic neurotransmitter vesicle pools[72, 73]. Wild-type α-synuclein is normally degraded by chaperone-mediated autophagy, another essential type of autophagy in which a pool of cytosolic proteins are targeted to lysosomes by chaperones for degradation[74, 75]. Macroautophagy is thought to be a compensatory mechanism for the failure of chaperone-mediated autophagy, and defective autophagy enhances the deposition of aberrant α-synuclein aggregation in Lewy bodies under the pathological conditions of PD[76]. The synapse is assumed to be the major target of α-synuclein, as aberrant α-synuclein deposition is found predominantly in presynaptic terminals and leads to synaptic pathology[77] (Fig. 2D). Impaired autophagic clearance results in the deposition of α-synuclein in presynaptic terminals of Atg7-deleted mice[78].

In addition to α-synuclein, a wealth of evidence highlights the importance of the mutations of another two PD gene products, leucine-rich repeat kinase-2 (LRRK2) and parkin, in synaptic pathology of PD[79, 80]. These PD gene products are involved in the maintenance of synaptic morphology and mediate synaptic protein trafficking. A detailed discussion of synaptic autophagy, LRRK2, and parkin in PD models can be found in another review[81]. Although the role of autophagy in the synaptic pathology of PD remains largely unexplored, it is known that parkin recruits damaged mitochondria for degradation through autophagic proteolysis. It is possible that impaired parkin may cause aberrant mitochondrial turnover regulated by selective autophagy (termed mitophagy), which contributes to abnormal synaptic homeostasis in PD[82].

Aging

The aging-associated reduction of synaptic number and function has been noted in the pathological changes in several neurodegenerative disorders; these changes

precede the memory impairment and cognitive decline in patients[83]. In addition, autophagic activity declines during aging, while autophagy augmented by genetic manipulation or by pharmacological interference (e.g. administration of rapamycin or spermidine) extends the lifespan of model organisms[84]. Studies of NMJ aging in *Drosophila* have revealed an abundant accumulation of early endosomes, multivesicular bodies, and autophagosomes in the synaptic boutons of old flies[85]. The enhanced autophagy might be closely associated with the misregulated recycling of synaptic vesicles in the motor terminals of old flies. Deficient autophagy in muscle leads to deterioration of neuromuscular synaptic function and precocious aging in mice[86]. Based on this evidence, it appears that age-related synaptic impairments are exacerbated by deficits in autophagy.

Oxidative Stress Burden

The cause of neuronal death in neurodegenerative diseases is known to be multifactorial, the OS burden caused by excessive generation of ROS being one of the most convincing theories of pathogenesis[87]. ROS not only leads to apoptotic cell death, but also regulates synaptic growth and function[28, 88]. Autophagy is the main cellular response to OS burden. In a *Drosophila* model of lysosomal storage disease, *spinster (spin)*, OS induces synaptic overgrowth[28]. Autophagy-related genes, such as *atg1* and *atg18*, are required for OS burden-triggered synaptic overgrowth in this model, and disturbance of autophagy is able to reverse synaptic overgrowth[28]. Therefore, it is hypothesized that upon OS burden, the overproduction of ROS may activate autophagy which plays a key role in mediating synaptic growth, function, and senescence[25]. Nevertheless, there is a lack of confirmatory data on the involvement of autophagy in regulating OS-induced synaptic pathology in mammalian cells. Owing to the importance of OS burden in a wide range of neurodegenerative disorders, a better understanding of the precise role of OS-activated autophagy in synaptic regulation may provide fundamental insights into pathogenesis and may offer novel targets for therapeutic interference.

In addition to the human disorders mentioned above, the impact of autophagy on synaptic pathology has also been addressed in other laboratory models of neurological diseases including ischemia[89], electroconvulsive seizures[90], and neurotoxicity[91].

Conclusions

Although in the past few years a wealth of evidence has been reported on this topic, the most crucial questions about how autophagy regulates synaptic development, function, and pathology have not yet been fully answered. Increased autophagy induction is found in synaptic terminals during pathogenesis. However, whether the excessive autophagy machinery is beneficial, harmful, or simply reflects an epiphenomenon, is yet to be finally determined. Hopefully, a clearer understanding of autophagy function in the physiological and pathological responses of synapses may open up new avenues for the development of therapeutic approaches targeting synaptic pathology in human disorders.

ACKNOWLEDGMENTS

This review was supported by the National Natural Science Foundation of China (81401043 and 81273491), Zhejiang Provincial Natural Science Foundation of China (LQ13H310004 and LY12H31010), the Health Bureau of Zhejiang Province (2013KYA147), a Key Laboratory of Hangzhou City Project (20090233T12), the Science Foundation of Hangzhou Normal University (2012QDL048), and the Program of “Xinmiao” Talents in Zhejiang Province, China (2015R423054).

Received date: 2015-04-24; Accepted date: 2015-05-30

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Neurosci Bull August 1, 2015, 31(4): 427–434. http://www.neurosci.cn
DOI: 10.1007/s12264-015-1548-2

·Review·

Molecular network of neuronal autophagy in the pathophysiology and treatment of depression

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Major depressive disorder (MDD) is a complicated multifactorial induced disease, characterized by depressed mood, anhedonia, fatigue, and altered cognitive function. Recently, many studies have shown that antidepressants regulate autophagy. In fact, autophagy, a conserved lysosomal degradation pathway, is essential for the central nervous system. Dysregulation of autophagic pathways, such as the mammalian target of rapamycin (mTOR) signaling pathway and the beclin pathway, has been studied in neurodegenerative diseases. However, autophagy in MDD has not been fully studied. Here, we discuss whether the dysregulation of autophagy contributes to the pathophysiology and treatment of MDD and summarize the current evidence that shows the involvement of autophagy in MDD.

Keywords: major depressive disorder; autophagy; mTOR; antidepressant

Introduction

Major depressive disorder (MDD) is a prevalent, heterogeneous illness characterized by depressed mood, anhedonia, low energy or fatigue, and altered cognitive function. Other symptoms, such as sleep and psychomotor disturbances, feelings of guilt, low self-esteem, suicidal tendencies, as well as autonomic and gastrointestinal disturbances, are also often present[1, 2]. If left untreated, it can be fatal. The lifetime prevalence of MDD is ~17% of the population and results in tremendous secondary costs to society[3, 4]. The 'gold standard' for depression treatment involves a combination of psychotherapy and medication. Unfortunately, current anti-depressant medications do not help everyone, and both normally take a number of weeks of regular treatment before they begin to have an effect[5]. Diagnosis of MDD is based on relatively subjective assessments of diverse symptoms representing multiple endo-phenotypes[6]. And most current treatments are based on monoamine neurochemical alterations in MDD[7]. Therefore, knowledge of the mechanism of MDD will help the development of effective treatment. As currently known, MDD is a complicated multifactorial induced disease associated with both genetic and environmental factors, and the detailed molecular mechanisms underlying the pathogenesis remain difficult to elucidate. The pathophysiology of MDD involves complex signaling networks[8], including alterations of cytokines, monoamine-deficiency in the central nervous system, and dysfunction of the glutamate system. Moreover, MDD is most often related to disturbed neurogenesis, structural and functional alterations of several limbic and cortical regions[9]. It is also proposed that dysfunction of synaptic plasticity is a basis of the etiology of MDD[10, 11]. Furthermore, postmortem brain tissues from MDD patients also display increased apoptotic stress and apoptosis-related factors[12, 13]. Recent studies

indicate that neuronal autophagic signaling pathways are also involved in MDD.

Autophagy is important for most cells in various tissues including the central nervous system; it is sensitive to the accumulation of toxic proteins/damaged organelles[14]. Therefore, alteration of autophagy during neurodevelopment and synaptic plasticity might cause abnormal development and synaptic malfunction. In addition, impairment of autophagy pathways may lead to the accumulation of pathogenic proteins and damaged organelles, which may finally result in neurological disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD)[15, 16]. Recently, many antidepressants were found to be involved in the neuronal autophagy signaling pathway. The co-chaperone FKBP5/FKBP51 (FK506 binding protein 5) acting as an antidepressant plays a role in autophagy[17]. These findings suggest that neuronal autophagy signaling pathways play an important role in MDD, so this review focuses on neuronal autophagy that is involved in MDD and is affected by antidepressants.

Neuronal Autophagy Dysregulation in Neurodegenerative Diseases

Basal Autophagy May Be Beneficial and Required for Normal Function of Neurons

Autophagy is extremely important in maintaining cellular homeostasis, which requires the continuous turnover of nonfunctional proteins and organelles[18]. Neurons are highly postmitotic, with specialized structures for intercellular communication. Therefore, neuronal integrity is more sensitive to alterations in basal autophagy than that of non-neurons[14]. Recent findings show that autophagy in neurons is indeed constitutively active, and that autophagosomes accumulate rapidly when their clearance is blocked[19, 20].

Many studies have shown that autophagy protects neurons under stress conditions. Jeong *et al.* showed that sirtuin 1 (SIRT1) overexpression prevents prion peptide neurotoxicity by inducing autophagy, while preventing autophagy by knock-down of autophagy-related 5 (Atg5) abolishes SIRT1-induced neuroprotection[21]. Shen *et al.* discovered that neuroautophagy positively regulates synaptic development, and overexpression of Atg1, a key regulator of autophagy, is sufficient to induce high levels of autophagy and subsequent enhancement of synaptic growth. In contrast, reducing autophagy results in the reduction of synapse size[22]. Moreover, the autophagosomal marker LC3-II and Akt and mammalian target of rapamycin (mTOR) dephosphorylation have a time-course coincident with degradation of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor GluR1 in neurons, which indicates that autophagy is a positive regulator of N-methyl-D-aspartate receptor (NMDAR)-dependent synaptic plasticity[23]. Neuronal autophagy may play important roles in the structural refinement of neurite growth, neuronal differentiation, synaptic growth, or synaptic plasticity, which ensures the formation of appropriate neuronal connections and their functions[24, 25].

Aberrant Autophagy Leads to Neurodegenerative Diseases, Including Depression

Dysregulation of autophagy might cause a cellular traffic-jam during neuronal development and synaptic plasticity, leading to neurodevelopmental disorders; it also might lead to the accumulation of misfolded protein aggregates and damaged organelles, leading to neuronal dysfunction and even death. Disruption of autophagy after spinal cord injury may contribute to endoplasmic reticulum-stress-induced neuronal apoptosis[26]. Neuron-specific knockout of the key autophagic gene Atg5 or Atg7 leads to accumulation of intracellular protein aggregates and neuronal death[19, 20].

Autophagy-lysosome defects occur early in the pathogenesis of AD and have been proposed to be a significant contributor to the disease process[27]. Nixon *et al.* provided evidence from electron microscopy that autophagy is extensively involved in the neurodegenerative process in AD[28]. And the transport of autophagic vacuoles and their maturation to lysosomes might be impaired in AD[29].

Autophagy of mitochondria can be regulated by parkin, PINK1 and DJ-1, and importantly, mutations in these proteins are thought to cause familial PD[30]. Xilouri *et al.* showed that α-synuclein, a major constituent of Lewy bodies, is degraded by autophagy in PD[31]. And recently, researchers have established an essential link between mitochondrial autophagy impairment and dopamine neuron degeneration in an *in vivo* model based on genetic deletion of either parkin or PINK1 (known PD genes)[32].

An autophagy defect has also been suggested by genetic studies of amyotrophic lateral sclerosis (ALS) and frontotemporal lobe dementia (FTD). For instance, mutations in UBQLN2 and SQSTM1/p62 have been reported in ALS and FTD[33-35]. In fact, UBQLNs are present in autophagosomes and bind LC3 in a complex while SQSTM1/p62 binds ubiquitinated proteins and LC3[36, 37]. Besides, evidence of direct alteration of the autophagic pathway, bypassing mTOR modulation has been shown in epileptogenesis[38]. As for depression, increased autophagosomal marker LC3-II has been reported in a cellular model of chemically-induced long-term depression (LTD)[18].

Interestingly, in the parkinsonian mimetic 6-hydroxydopamine (6-OHDA) model, the 6-OHDA-induced apoptosis is prevented by treatment with the early-phase inhibitor of autophagy, 3-methyladenine, but the late-phase inhibitor of autophagy, bafilomycin A1, aggravates this apoptosis[39]. In fact, most evidence points to autophagy as a protective process in neurons, but other studies also provide genetic and cellular evidence that otherwise argues for a role of autophagy in promoting neuronal death[40]. Autophagy might show a Janus face, too much or not enough would lead to disorders like neurodegenerative diseases[41].

Autophagy-Related Pathways in Depression

Dysregulation of the autophagic pathway in neurons may result in depression[42]. Autophagy is regulated by intracellular and extracellular signals *via* at least three pathways: (1) The mTOR-dependent pathway: the Atg1/unc-51-like kinase complex acts downstream of mTOR complex 1 (mTORC1); (2) the PI3K/beclin1 pathway; and (3) the Ca^{2+} pathway[43]. mTORC1 integrates nutrients, energy, growth factors, and amino-acid signaling; once activated, mTORC1 inhibits autophagy by acting on the Atg1 kinase complex, while the beclin complex positively regulates autophagy[44, 45]. It is now well established that Ca^{2+} is a regulator of autophagy, while it is still unclear whether Ca^{2+} is a positive or a negative regulator[46].

The PI3K-Akt-mTOR pathway is related to depression[47]. Decreased AKT1/mTOR mRNA expression has been reported in short-term bipolar disorder[48]. A recent study showed that neuronal stimulation induces NMDAR-dependent autophagy through the PI3K-Akt-mTOR pathway in a cellular model of LTD[25]. Simultaneously, metabotropic glutamate receptor (mGluR) activation results in increased PI3K-mTOR signaling and activation of protein synthesis near synapses in a mouse model of LTD[25]. And inhibition of protein synthesis or mTOR signaling blocks mGluR-dependent LTD[49]. In fact, mTOR signaling lies at the crossroads of multiple signals involved in protein synthesis and impairment of autophagy during neurodegeneration[50, 51]. Activation of mTOR has been functionally linked with local protein synthesis in synapses, resulting in the production of proteins required for the formation, maturation, and function of new spine synapses[50]. In the meantime, mTORC1 inhibits autophagy, an essential protein-degradation and recycling system[52]; for example, PI3K-Akt-mTOR is associated with autophagy impairment and is impaired in mild cognitive impairment and AD[53]. mTOR regulates both neuroprotective (*via* autophagy) and neuroregenerative (*via* protein synthesis) functions in various diseases of the central nervous system[42].

Brain-derived neurotrophic factor (BDNF) plays an essential role in neuronal plasticity, and downregulation of its expression/function is reproduced in a variety of animal models of MDD[54]. Indeed, the neuroprotective effect of BDNF not only prevents apoptosis by inhibiting caspase activation but also promotes neuron survival through modulation of autophagy[55]. And BDNF can be mediated by autophagy through the PI3K-Akt-mTOR pathway[56]. These results also suggest that autophagy plays an important role in MDD.

In addition, Cummings *et al.* showed that the minimal requirements for inducing LTD involve simply a transient influx of Ca^{2+} into the postsynaptic cell *via* either NMDARs or voltage-dependent Ca^{2+} channels[57].

We assume that cellular stress in the form of reactive oxygen species or other factors causes proteins to misfold and aggregate. Under normal conditions, this would in turn diminish or overwhelm degradation *via* the autophagy or ubiquitin–proteasome system. However, with autophagy impairment, cells would be unable to clear aggregates and damaged organelles. And additional mitochondrial dysfunction, excitotoxicity, and pore formation lead to increased intracellular Ca^{2+} levels, ultimately resulting in necrosis and apoptosis[58]. But excessive autophagy also induces apoptosis[59]. In fact, injury and apoptosis of

hippocampal tissue is the reason for MDD[60]. From this point of view, autophagy would be an accomplice of MDD (Fig. 1).

Antidepressants and Autophagy

Beyond their impact on monoaminergic neurotransmission, recent reports have evidenced that many antidepressants affect autophagy pathways in the process of anti-depression[17, 61]. Several studies have demonstrated that cellular autophagy markers are upregulated upon treatment with antidepressants[62]. Many antidepressants like sertraline activate mTOR. However, antidepressant activity of rapamycin (an mTOR inhibitor) has also been reported in an animal model[63]. Moreover, autophagic markers, such as beclin1, are increased following antidepressant treatment in mouse brain[64]. Autophagy might be a double-edged sword in MDD, which may be the reason why some MDD patients remain resistant to certain antidepressant medications.

Antidepressants Affect the Autophagic Pathway

The antidepressant drug amitriptyline (AMI) and the selective serotonin re-uptake inhibitor citalopram (CIT) have been reported to increase the expression of the autophagic markers LC3-II and beclin1, but venlafaxine fails to exert these effects[65]. AMI- and CIT-induced autophagy is functional in terms of autophagic flux, and is partially mediated by class III PI3K- and ROS-dependent pathways[62]. FKBP51 can synergize with antidepressants by binding beclin1, changing its phosphorylation and enhancing markers of autophagy and autophagic flux as well as triggering autophagic pathways[17]. Chronic paroxetine treatment of a depression-relevant stress model revealed that the physiological effects of antidepressants on behavior and autophagic markers depend on FKBP51[64]. Trehalose may have antidepressant-like properties through its enhancement of autophagy[66]. Lithium, which has been used for several decades to treat manic-depressive illness (bipolar affective disorder), induces autophagy, thereby promoting the clearance of mutant huntingtin and alpha-synucleins from experimental systems[67]. Thus some but not all antidepressants affect autophagy.

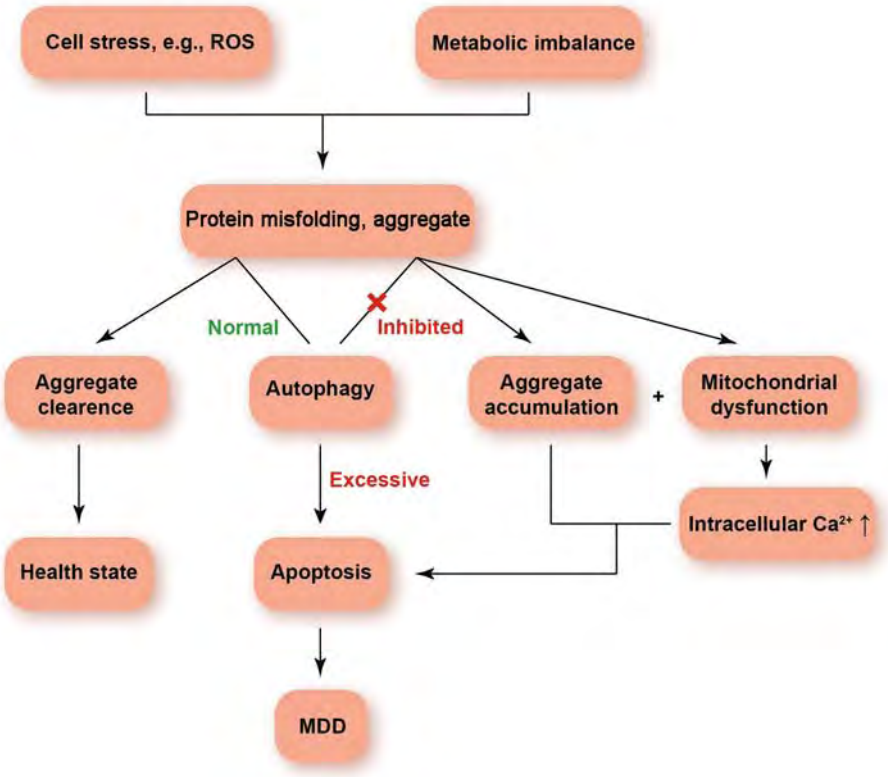

Fig. 1. Possible involvement of autophagy in the pathogenesis of MDD.

Autophagy Is a Potential Mechanism of Antidepressant Action

Antidepressants are commonly used in the treatment of cancer patients with depression, and the underlying mechanisms are also related to inducing autophagy. Elevated levels of the autophagic protein beclin-1 and the cellular redistribution of the marker LC3 have been found in C6 glioma cells treated with the antidepressant desipramine (DMI), which induces autophagic cell death by the formation of autophagosomes[61]. Moreover, activation of the PI3K-AKT-mTOR pathway, which is considered to be a negative regulator of autophagy, is also inhibited by DMI. Furthermore, DMI activates PERK-eIF2α and ATF6 of the endoplasmic reticulum stress pathway to induce autophagy in C6 glioma cells[61]. As another example, the tricyclic antidepressant imipramine stimulates the progression of autophagy, and exerts antitumor effects on PTEN-null U-87MG human glioma cells by inhibiting PI3K-Akt-mTOR signaling and by inducing autophagic cell death[68]. The antidepressants maprotiline and fluoxetine, which are novel pro-autophagic agents, induce autophagic programmed cell death (PCD) in the chemoresistant Burkitt's lymphoma (BL) cell line DG-75; this does not involve caspases, DNA fragmentation, or PARP cleavage, but is associated with the development of cytoplasmic vacuoles, all consistent with an autophagic mode of PCD[69]. Therefore, autophagy-initiating mechanisms should be considered as a pharmacological target to improve the treatment of depression.

Antidepressants and the mTOR-Dependent Signaling Pathway

The classic antidepressant drugs inhibit the PI3K-Akt-mTOR signaling pathway[68]. Fluoxetine, an antidepressant that inhibits the reuptake of serotonin in the central nervous system, promotes neurogenesis and improves the survival rate of neurons. A further study suggested that the improvement of neuron survival is achieved by upregulated expression of the phosphorylated AKT protein, which is a key factor in the PI3K-Akt-mTOR signaling pathway[70]. Another study showed novel *in vitro* evidence that some antidepressant drugs promote dendritic outgrowth and increase synaptic protein levels through mTOR signaling[71]. Warren *et al.* demonstrated that administration of fluoxetine in combination with methylphenidate induces mTOR activity in rats[72]. A rapid antidepressant and nonselective NMDAR antagonist, ketamine, activates the mTOR signaling pathway, leading to increased synaptic proteins in the rat prefrontal cortex[73].

In fact, most previous reports focused on the mTOR synaptogenesis by antidepressants[74]. Probably, neuronal autophagy-related mTOR signaling pathways could also explain the mechanism of antidepressant function, for mTOR signaling is at the crossroads between protein synthesis and impairment of autophagy in neurodegeneration[50, 51]. However, more studies are definitely needed.

Antidepressants and the mTOR-Independent Pathway

Besides, some antidepressant drugs seem to act *via* an mTOR-independent pathway to affect autophagy. For example, autophagy triggered by AMI and CIT is partially mediated by beclin pathways since 3-methyladenine slightly diminishes the effects of AMI. The antidepressant maprotiline has been shown to inhibit dendritic γ-amino-butyric acid- and NMDA-induced increases in Ca^{2+} in primary cultured rat cortical neurons[75] and in human prostate cancer cells[76]. Further, calcium channel blockade affects the processes related to antidepressant-induced changes in the crosstalk between α1- and β-adrenergic receptors[77]. In fact, it is now well established that intracellular Ca^{2+} is one of the regulators of autophagy[46]. All these results showed that many antidepressants are involved in autophagy *via* diverse pathways to synergize with antidepressant action. However, more detailed studies are needed to characterize the autophagic pathways in depression and their participation in antidepressant mechanisms[42].

Conclusion

In summary, MDD is one of the most prevalent debilitating public health problems worldwide. The current review summarized and discussed the possible involvement of neuronal autophagy in MDD. Although the molecular mechanisms underlying MDD are still largely unclear, we proposed that neuronal autophagy signaling network is also implicated in the pathogenesis of MDD and the mechanisms of some antidepressant actions. Further understanding of neuronal autophagy regulation in MDD is expected to contribute to the development of therapeutic interventions in MDD.

Received date: 2015-04-30; Accepted date: 2015-07-17

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Neurosci Bull August 1, 2015, 31(4): 435–444. http://www.neurosci.cn
DOI: 10.1007/s12264-015-1545-5

·Review·

Role of autophagy in the pathogenesis of multiple sclerosis

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Autophagy plays an important role in maintaining the cellular homeostasis. One of its functions is to degrade unnecessary organelles and proteins for energy recycling or amino-acids for cell survival. Ablation of autophagy leads to neurodegeneration. Multiple sclerosis (MS), a permanent neurological impairment typical of chronic inflammatory demyelinating disorder, is an auto-immune disease of the central nervous system (CNS). Autophagy is tightly linked to the innate and adaptive immune systems during the autoimmune process, and several studies have shown that autophagy directly participates in the progress of MS or experimental autoimmune encephalomyelitis (EAE, a mouse model of MS). Dysfunction of mitochondria that intensively influences the autophagy pathway is one of the important factors in the pathogenesis of MS. Autophagy-related gene (ATG) 5 and immune-related GTPase M (IRGM) 1 are increased, while ATG16L2 is decreased, in T-cells in EAE and active relapsing-remitting MS brains. Administration of rapamycin, an inhibitor of mammalian target of rapamycin (mTOR), ameliorates relapsing-remitting EAE. Inflammation and oxidative stress are increased in MS lesions and EAE, but Lamp2 and the LC3-II/LC3-I ratio are decreased. Furthermore, autophagy in various glial cells plays important roles in regulating neuro-inflammation in the CNS, implying potential roles in MS. In this review, we discuss the role of autophagy in the peripheral immune system and the CNS in neuro-inflammation associated with the pathogenesis of MS.

Keywords: autophagy; multiple sclerosis; neuro-inflammation

Introduction

Autophagy, a lysosome-dependent degradation pathway, contributes to maintaining cellular homeostasis. Clearance of long-lived proteins, unnecessary organelles, and aggregate-prone proteins is mainly executed by autophagy for recycling cellular materials[1]. There are three subtypes of autophagy, macroautophagy, chaperone-mediated autophagy (CMA), and mitophagy. Macroautophagy is the major type for selective degradation of protein aggregates or misfolded proteins. The ubiquitin-labeled proteins are recognized by autophagy receptors, such as p62, neighbor of BRCA1 gene 1 (NBR1), and NIP3-like protein X (NIX), to form autophagosomes that then fuse with lysosomes for degradation. Many autophagy-related genes function during this process, such as Unc51-like kinase (ULK1) and autophagy-related gene (ATG) 5. Mammalian target of rapamycin (mTOR) represses autophagy by regulating ULK1 phosphorylation, while AMPK activates this process. CMA mediates the degradation of large molecular-weight proteins containing the KFERQ motif recognized by heat shock cognate protein of 70 kDa (HSC70). The substrate is then escorted to lysosome-associated membrane protein 2 (LAMP2A) for lysosomal degradation. Mitophagy is responsible for the degradation of damaged mitochondria. Parkin and PINK1 play critical roles in this process[2]. More

attention has been paid to autophagy in the pathogenesis of neurodegenerative diseases, such as Alzheimer's disease (AD)[3], Parkinson's disease (PD)[4] and amyotrophic lateral sclerosis (ALS)[5].

Multiple sclerosis (MS) is a non-inherited neurodegenerative disease characterized by the involvement of innate and adaptive immunity. MS occurs mainly in young adults and its incidence in females is two to three times higher than that in males. About 85% of MS patients show relapse-remission before irreversible progression. It is widely accepted that MS is an auto-immune disease caused by autoreactive T-cells that target the myelin sheath in the central nervous system (CNS)[6, 7]. These myelin sheath-targeting T-cells infiltrate the CNS *via* breakdown of the blood brain barrier, attack the myelin sheath, and initiate chronic inflammatory responses, leading to the loss of axons and neurons. The fact that adoptive transfer of activated myelin-specific $CD4^+$ T-cells can induce experimental autoimmune encephalomyelitis (EAE) demonstrates that auto-immunity is indispensable in the pathogenesis of MS[8]. Genome-wide association studies have shown that immunologically-relevant genes, especially T-helper-cell differentiation genes, are significantly overrepresented in the pathogenesis of MS[9]. However, it is still unclear what exactly causes the disease. One of the initiators of MS is antigen from virus or bacteria that is similar to the myelin basic protein peptide[10, 11]. The auto-immunity hypothesis is now being challenged since the finding that, like many other neurodegenerative disorders, gray matter lesions and brain atrophy are detectable before MS onset[12, 13]. So the initiators of MS still need further investigation. An important factor in MS development is neuro-inflammation, which is also one of the main characteristics of MS (chronic inflammation, infiltration of peripheral immune cells, demyelination, transected axons, and gliosis). The frail neurons die when they are exposed to the inflammatory environment. Pro-inflammatory factors such as IL17 impair the differentiation of oligodendrocyte progenitor cells (OPCs) in MS[14].

Autophagy is tightly linked with auto-immune diseases, including MS[15-17]. The extracellular matrix molecule fibronectin aggregates in chronically demyelinating MS and the relapsig phase of EAE; it may be resistant to degradation and affect the remyelination process[18]. Soluble oligomers have been detected in the brain and cerebral spinal fluid of MS patients[19]. Meanwhile, oxidized protein aggregates increase in EAE, probably due to the reduced autophagy level[20]. Moreover, autophagy intensively regulates inflammation in various diseases. To unravel the potential role of autophagy in the pathogenesis of MS, we highlight its possible importance in both the generation of auto-reactive lymphocytes and the regulation of neuro-inflammation. Differential cell-type specificity of autophagy in the peripheral immune system and the CNS might modulate the onset and progression of MS (Fig. 1).

Autophagy and Mitochondria in MS

Mitochondria play important roles in general and selective autophagic flux, including mitophagy. Dysfunction of mitochondria during stress is one of the important factors involved in the pathogenesis of MS and EAE. Autophagy maintains the architecture of mitochondria, restores their function, and prevents their dysfunction, which are critical for cell survival and the pathogenesis of MS[21-23]. In MS, the decreased expression of cytochrome *c* oxidase subunit 5b may impair the function of mitochondria[24]. Dysfunction of mitochondria produces reactive oxygen species (ROS), which contribute to demyelination and axonal loss[25]. Autophagy clears depolarized mitochondria to reduce the excessive production of ROS by increasing BECN1 or regulating ATG4 activity[26, 27], which is protective in MS.

Autophagy and MS in the Peripheral Immune System

Mutual Regulation of Autophagy and Inflammation

The connections between autophagy and inflammation are complex. Each regulates the other by different mechanisms. Pattern-recognition receptors for pathogen recognition, such as toll-like receptors (TLRs) and NOD-like receptors (NLRs) can elicit autophagy for pathogen clearance[28]. However, autophagy negatively regulates inflammation to prevent the harmful amplification of inflammatory factors. For instance, ATG9 suppresses the stimulator of IFN genes protein (STING) in type-I IFN signaling to reduce the inflammatory response[29]. Blockade of autophagy leads to the activation of inflammasomes which control the proteolytic processing and secretion of IL-1β and IL-18 under inflammatory stress[30, 31]. Also, ablation of autophagy-related 16-like 1 (ATG16L1) increases the production of

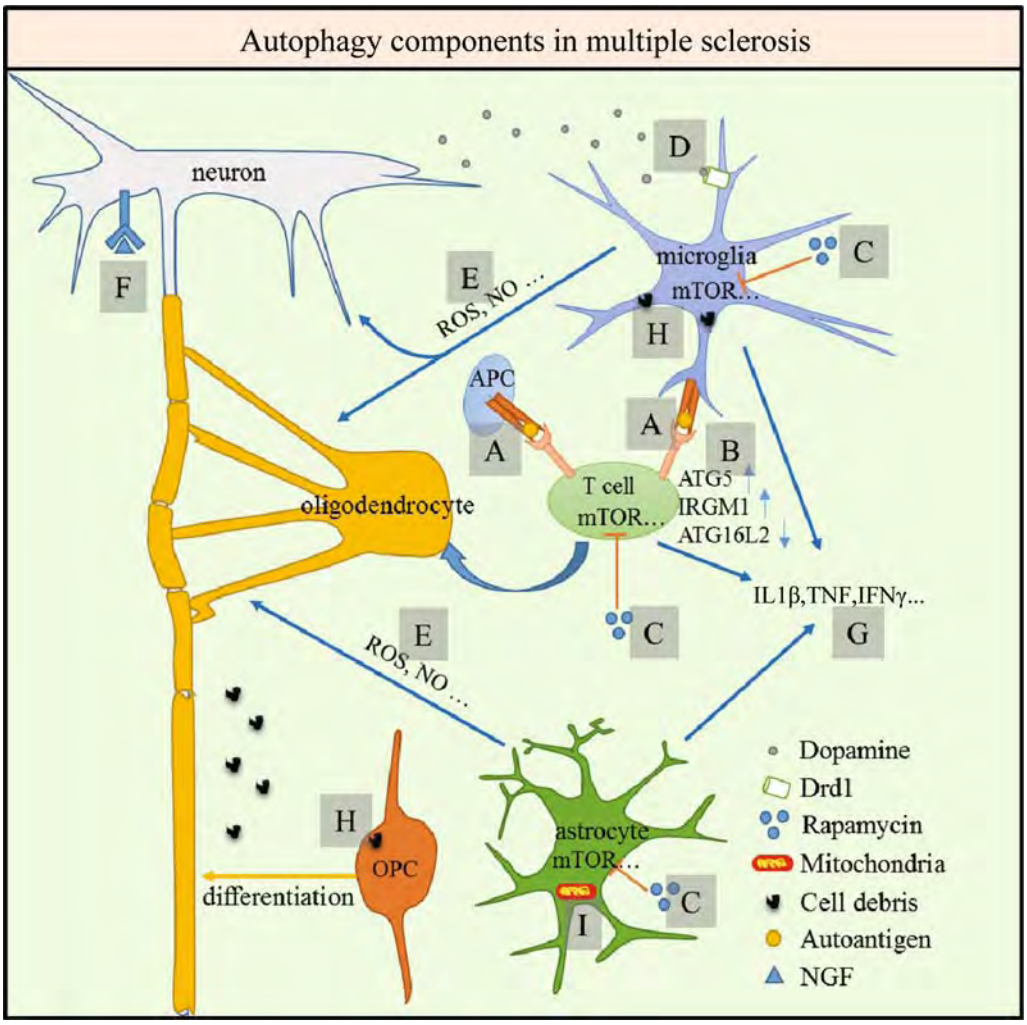

Fig. 1. Autophagy in the pathogenesis of multiple sclerosis. A: Autophagy is indispensable for antigen presentation in antigen presenting cells (APC, e.g. thymic epithelial cells, microglia). B: In MS patients and EAE mice, ATG5 and IRGM1 increase in T-cells to enhance their survival. Downregulation of ATG16L2 may perturb the homeostasis of T-cells. C: Rapamycin, the inhibitor of mTOR that represses autophagy, regulates inflammation and benefits EAE. D: Drd1 in microglia or macrophages activated by dopamine triggers autophagy to inhibit inflammasome activation during neuro-inflammation. E: ROS and NO produced by microglia and astrocytes in neuro-inflammation regulate autophagy in various cell types, including astrocytes. F: In MS patients, NGF increases in cerebrospinal fluid and represses autophagy in neurons to prevent cell death. G: Pro-inflammatory factors, such as IL-1β, TNF, and IFNγ, can induce autophagy in macrophages or microglia. H: Cell debris or protein aggresomes in MS or EAE are removed by microglia or macrophages through phagocytosis or autophagy. OPCs may also clear cellular debris and protein aggresomes by endocytosis and autophagy. I: Autophagy is necessary to maintain the architecture and restore the function of mitochondria in astrocytes during neuro-inflammation. Depolarized mitochondria are also removed by autophagy.

IL-1β and IL-18 in the mouse model of Crohn's disease[32]. Moreover, infection of ATG5-deficient myeloid cells with *M. tuberculosis* increases the expression of IL-17, a potent mediator in autoimmunity[33]. In primary effective T-cells, p62 serves as a receptor for autophagic degradation of ubiquitinated BCL10 during TCR activation to protect cells from excessively activated NF-κB pathway[34].

Inflammatory factors in turn regulate the progress of autophagy. The T-helper 1 (Th1) cell cytokine IFNγ induces autophagy in macrophages to suppress the survival of mycobacteria[35]. Another pro-inflammatory factor, Tnf, also induces autophagy in monocytes/macrophages[36].

However, the Th2 cell cytokines IL-4 and IL-13 ablate autophagy in macrophages[36].

Autoreactive T-Cell Regulation by Autophagy

Besides innate immunity, autophagy also participates intensively in shaping the adaptive immune system. Th1 cells are crucial at the early stage of MS pathogenesis, and Th17 cells are indispensable in the later stage. Autophagy is necessary for the generation of autoreactive T-cells. It participates in multiple functions of the adaptive immune system, such as antigen presentation and maintenance of the homeostasis of T-cells.

Autophagy mediates antigen-presentation during the generation of autoreactive T-cells. Autophagy enhances antigen-presentation to $CD4^+$ T-cells *via* major histocompatibility complex class II molecules[37, 38]. In thymic epithelial cells, autophagy contributes to the positive and negative control of the T-cell repertoire, playing a critical role in the elimination of self-reactive T-cells and exotic pathogens. Mice thymi with ATG5 ablation show infiltration of autoreactive $CD4^+$ T-cells into multiple organs and induce autoimmune colitis similar to Crohn's disease-related phenotypes[38]. Atg7 ablation in dendritic cells (DCs), the most potent antigen-presenting cells, ameliorates EAE. Blockade of autophagy in DCs by chloroquine also delays the onset or reduces the severity of EAE[39]. DCs from patients with Crohn's disease with ATG16L1 and NOD2 variants are defective in antigen presentation[40]. The presentation of citrullinated peptides by DCs, macrophages, and thymic DCs is blocked by inhibition of autophagy with 3-methyladenine[41].

Autophagy regulates the homeostasis, proliferation, differentiation, maturation, and apoptosis of T-cells. It also maintains the calcium flux and the pro-survival process and counteracts pro-apoptotic action to maintain T-cell homeostasis[42-45]. Cyt1 induces autophagosome formation when it binds to CD46, which may function in maintaining the homeostasis of T-cells during adaptive immunity[46]. Moreover, the expression of ATG16L2 in T-cells is reduced, and this is likely to perturb the homeostasis of T-cells in MS[47]. ATG5 plays important roles in T-cell survival and controls the proliferation, differentiation and maturation of $CD4/8^+$ T-cells and B-cells[48-50]. mTOR-deficient T-cells fail to differentiate into T-helper cells, Th1, Th2, and Th17 effector cells[51, 52]. ATG5 is significantly elevated in T-cells in MS lesions and EAE mice compared to controls. The increase of autophagy in T-cells may promote their survival, contributing to the pathogenesis of MS[53]. Immune-related GTPase M1 is highly expressed in MS lesions in the CNS and EAE models, and contributes to the pathogenesis of MS by increasing the survival of autoreactive $CD4^+$ T-cells[17]. Rapamycin, an inhibitor of mTOR, ameliorates the relapsing-remitting EAE (RR-EAE) in SJL/j mice by increasing the Treg cell population[54]. However, the contributions of autophagy in different T-cell populations to cell death depend on the context. Upon activation of autophagy, Th2 cells undergo growth factor-withdrawal-mediated cell death[55]. The induction of autophagy through binding of HIV-1 envelope glycoproteins to CXC chemokine receptor 4 of $CD4^+$ T-cells is necessary for apoptosis[56]. FADD or casp8 in T-cells limits autophagy and protects against cell necrosis and inflammation[57, 58]. Alteration of the proportions of different T-cell groups affects the immune response[59].

Pathological Role of Autophagy in MS

Autophagy and Neuro-inflammation

CNS autophagy is linked with serious neurodegenerative diseases. Deficiency of basal autophagy in neurons leads to protein aggregation and finally neurodegenerative diseases[60, 61]. Neuro-inflammation is one of the important factors that cause neurodegenerative diseases, especially MS. Autophagy and neuro-inflammation regulate each other to affect the progression of various diseases in the CNS.

Neuro-inflammation is negatively regulated by autophagy to avoid its harm to the CNS. Microglia, resident immune cells in the CNS, play important roles in the initiation and sustenance of neuro-inflammation to clear pathogens. They produce pro-inflammatory factors and neurotoxic factors including ROS and nitric oxide (NO). Subsequently-activated astrocytes further amplify the inflammatory response to produce more pro-inflammatory factors, chemokines, and NO, which have toxic effects on primary neurons[7, 62]. However, autophagy plays an essential role in maintaining neuro-inflammation at a harmless level. Activation of Drd1 by dopamine inhibits NOD-LRR-containing pyrin domain 3 (NLRP3) inflammasome activation through autophagic degradation

of NLRP3 *via* the E3 ubiquitin ligase MARCH7[63]. The dopamine system also regulates lymphocyte activity in lupus and MS, suggesting an important role of dopamine in the regulation of peripheral or CNS infiltrated lymphocytes during the pathogenesis of MS[64, 65]. In addition, inhibition of neuro-inflammation by autophagy has great benefits on ischemia[66]. Autophagy may be a stress response to the negative feedback regulation of neuro-inflammation. In the chronic LPS model, pro-inflammatory factor IL-1β in cortex is positively correlated with the autophagy markers Beclin-1 and LC3-II and inversely correlated with p62[67].

Autophagy can be deleterious depending on the disease. Activation of the autophagic pathway in ischemia leads to the death of astrocytes and neurons, which can be rescued by autophagy inhibitors[68, 69]. Meanwhile, blockade of microglial autophagy in permanent middle cerebral artery occlusion by autophagy inhibitors reduces the severity of the disease[70]. Thus, autophagy in different diseases and cell types in the CNS seems to function differently. Autophagic cell death is still under debate and needs further investigation[71].

Autophagy in Glial Cells

Until recently, most studies on autophagy in the CNS focused on neurons, with less on glial cells. The activation of glial cells plays important roles in neuro-inflammation, and these cells are indispensable for the development of MS[7]. MS progression occurs between glial activation and gliosis. Autophagy in various glial cells may play a role in responding to stress in MS. Therefore, it is urgent to study the effect of glial autophagy on neurological diseases, including MS.

Microglia are the first line of defense in the CNS, monitoring exotic and intrinsic pathogens. During demyelination, cell debris is mainly phagocytized by microglia or microphages, a process that requires autophagy-related genes[72]. Impairment of autophagy in microglia may hinder debris clearance, leading to damaged remyelination and augmentation of persistent neuro-inflammation. The rapid activation of microglia produces pro-inflammatory cytokines and free radicals, and acts as the major antigen-presenting cell[73, 74]. Pro-inflammatory factors such as IL-1β can trigger microglial autophagy, suggesting links between autophagy and inflammation activation[75]. mTOR plays a pivotal role in autophagy suppression and promotes inflammation in microglia, and rapamycin may have a beneficial effect in the treatment of MS[76]. mTOR inhibitors reduce neuro-inflammation by inhibiting microglial activation or viability, decreasing pro-inflammatory cytokines[77]. Simian immunodeficiency virus-infected microglia attenuate the survival of neurons by inhibiting neuronal autophagy[78], which implicates a cell-cell type regulation of autophagy in the CNS. However, microglial autophagy in middle cerebral artery occlusion makes the disease worse[70]. It is possible that the role of microglial autophagy depends on the disease.

Astrocytes are the primary supportive elements for neuronal structure and neurotrophy, comprising ~50% of cells in the adult mammalian brain. Autophagy is necessary for the differentiation and maturation of astrocytes[79], and can be initiated in inflammatory stress and may be important in the development of MS. During pro-inflammatory stimuli, autophagy is induced to maintain mitochondrial networks[22]. Impairment of the autophagy pathway by ablation of Atg7 in astrocytes in the inflammatory environment amplifies the response to produce more neurotoxic factors such as ROS, which induces astrocytic cell death[22, 80]. mTOR in the astrocyte may play a protective role in ischemia *via* its downstream kinase S6K1[81]. mTOR can also regulate the stability of iNOS mRNA in astrocytes, reducing the neurotoxic effect of NO which impairs autophagy by disrupting the BECN1 complex and activates mTORC1[82-84].

OPCs are indispensable during oligodendrocyte differentiation and myelin remyelination; they are activated for remyelination in MS lesions[85, 86]. They also play an important role in MS pathogenesis by modulating immune activity in the CNS. Act1 deletion in OPCs blocks the IL-17 pathway, attenuating neuro-inflammation and increasing OPC maturation[14]. OPCs can also clear β-amyloid peptides by triggering endocytosis and autophagy[87], implying an autophagic role of OPCs in MS. However, the exact functions of OPCs in MS need further investigation, including those of autophagy and its differentiation, nutritional support, debris clearance, and inflammation regulation.

Roles of Autophagy in MS-Associated Demyelination and Remyelination

Autophagy is closely linked to demyelination and remyelination. It plays potential roles in improving

Schwann cell remyelination in demyelinating peripheral neuropathies[88]. Rapamycin, a potent autophagy inducer, improves myelination, leading to enhanced neuronal survival in tuberous sclerosis[89]. Meanwhile, in Long–Evans shaker rats, an increased autophagy level increases the number of myelinated axons and myelin sheath thickness during dysmyelination and demyelination, which implies that the autophagy pathway is a direct target for therapy for demyelination[90]. Furthermore, high mobility group box chromosomal protein 1 (HMGB1), which promotes autophagy, is elevated in MS and EAE[91]. However, the progression of MS and EAE involves many cell types. Different cell types in the lesion site might show different patterns of autophagy. In MS, especially in the acute phase, nerve growth factor (NGF) is dramatically increased in the cerebrospinal fluid[92]. NGF inhibits autophagy and cell death of neurons through the p75 neurotrophin receptor, suggesting a protective role in MS[93]. In MS lesions, Lamp2 expression is reduced[94], implying an impairment of autophagy. The mTOR signaling pathway that inhibits autophagy restores the regrowth of axons in the CNS, which is important for remyelination in MS[95, 96]. Moreover, in acute and chronic EAE, protein aggresomes in the spinal cord and a reduced LC3-II/LC3-I ratio suggest that the protein turnover mechanism through autophagy is impaired[20]. In fact, the mechanism of autophagy in the pathogenesis of MS in the CNS is still superficially understood. Autophagy in different stages of the disease and different cell types needs further investigation to determine the causal relationship between autophagy and MS.

Conclusive Remarks

So far, little is known about the exact mechanism of autophagy in MS. It is still controversial whether autophagy leads to cell death in MS or is a rescue mechanism activated as part of an endogenous neuroprotective response, since autophagy plays opposite functions in inflammation and cell survival, depending on the context. Drugs modifying the immune system and remyelination have been used to help with relapse management, reduce the degree of disability, and improve the quality of life[97, 98]. The effects of rapamycin and chloroquine in the therapy of EAE shed light on the autophagy pathway as a potential target for drug development. The inhibition of autophagy in mesenchymal stem cells provides a novel strategy to improve therapeutic effects by enhancing the suppression of $CD4^+$ T-cells[99]. In order to develop effective and more specific therapeutic strategies, we need to fully understand the complex interplay between autophagy and MS in different cell types.

ACKNOWLEDGEMENTS

This review was supported by a grant from the National Natural Science Foundation of China (81430021 and 81370470), China Postdoctoral Science Foundation (2014M551465) and the Collaborative Innovation Center for Brain Science.

Received date: 2015-05-20; Accepted date: 2015-07-05

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Neurosci Bull August 1, 2015, 31(4): 445–451. http://www.neurosci.cn
DOI: 10.1007/s12264-015-1541-9

·Review·

Dysfunction of autophagy as the pathological mechanism of motor neuron disease based on a patient-specific disease model

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Autophagy is the main catabolic pathway in cells for the degradation of impaired proteins and organelles. Accumulating evidence supports the hypothesis that dysfunction of autophagy, leading to an imbalance of proteostasis and the accumulation of toxic proteins in neurons, is a central player in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS). The clinical pathology of ALS is complex and many genes associated with autophagy and RNA processing are mutated in patients with the familial form. But a causal relationship between autophagic dysfunction and ALS has not been fully established. More importantly, studies on the pathological mechanism of ALS are mainly based on animal models that may not precisely recapitulate the disease itself in human beings. The development of human iPSC techniques allows us to address these issues directly in human cell models that may profoundly influence drug discovery for ALS.

Keywords: motor neuron disease; iPSC; autophagy; amyotrophic lateral sclerosis; spinal muscular atrophy

General Introduction to Motor Neuron Diseases

Motor neuron (MN) diseases are a heterogeneous group of sporadic or familial disorders of the nervous system that mostly lead to a progressive loss of MNs and the subsequent impairment of neuromuscular function, such as spinal muscular atrophy (SMA) and amyotrophic lateral sclerosis (ALS). To date, there is no effective treatment for these diseases[1]. SMA is an autosomal recessive disease caused by loss or mutation of the SMN1 gene and retention of the SMN2 gene, which results in reduced levels of survival motor neuron (SMN) protein. With a casualty rate of 0.01%, it is the number one inherited killer of children below the age of two[2]. Currently, it is still largely unclear why low levels of SMN protein result specifically in an MN disease, although it has been proposed that loss of SMN may result in alteration of the splicing of a specific gene or the disruption of mRNA transport, which eventually leads to SMA[2, 3]. ALS is another devastating neurodegenerative disorder affecting upper and lower MNs that leads to death within 2–3 years from diagnosis. Between ~90% and 95% of cases are sporadic in origin, whereas the remaining 5%–10% are familial and usually of autosomal dominant inheritance. Of these, the first ALS gene, superoxide dismutase 1 enzyme (SOD1), accounts for ~20%. TAR DNA-binding protein (TDP-43/TADBP) has been identified as a major component of ubiquitinated inclusions in ALS and other neurodegenerative diseases. Hexanucleotide GGGGCC intronic expansions in the newly-identified C9ORF72 gene (chromosome 9 open reading frame 72) are the most common cause of both familial and sporadic ALS, and are responsible for up to 50% of the familial and ~10% of the sporadic form[4]. Other genes, such as FUS/TLS, angiogenin, SQSTM1, valosin-containing protein

(VCP), UBQLN2, optineurin (OPTN)[5], and very recently Matrin3, have also been found to be linked to familial ALS[6, 7]. The pathophysiology of ALS is complex and various mechanisms have been proposed for MN injury including protein misfolding and aggregation, mitochondrial dysfunction, oxidative stress, defective axonal transport, excitotoxicity, defects in RNA processing and protein degradation by autophagy, dysregulated transcription and RNA processing, endoplasmic reticulum stress, and apoptosis, as well as toxicity caused by non-neuronal cells[8]. Of these mechanisms, autophagic dysfunction is considered to play a key role[6, 8, 9].

Defective Autophagy May Be a Central Player in MN Diseases and Other Neurodegenerative Diseases

Autophagy, an intracellular degradation pathway for the clearance of damaged organelles and aggregation-prone proteins by lysosomes, is essential for the maintenance of protein homeostasis[9, 10]. Generally, there are three distinctive types: macroautophagy, microautophagy, and cheparone-mediated autophagy. Of these, macroautophagy (here referred to as autophagy) is the main catabolic pathway for maintaining protein homeostasis and sustaining neuronal functions[11, 12]. Recent studies have revealed that autophagy is involved in many essential biological functions including cell survival, cell death, cellular metabolism, development, aging, antigen-presentation, and anti-infection[13]. Autophagic degradation can be divided into several steps: induction, autophagosome formation, cargo recognition/sequestration, and autophagosome clearance. Failure at each of these steps can lead to neurodegenerative diseases[12, 14]. The common pathological hallmark of several major neurodegenerative diseases is the accumulation of misfolded proteins in both sporadic and genetic cases. In Alzheimer's disease (AD), the misfolded protein inclusions are mainly plaques of amyloid β peptide and intracellular neurofibrillary tangles comprising hyperphosphorylated tau. Parkinson's disease (PD) is characterized by the presence of Lewy bodies composed of aggregated α-synuclein and polyubiquinated proteins. In Huntington's disease (HD), mutant huntingtin forms protein aggregates in the cytoplasm[15]. Similarly, in ALS, TDP43 aggregates have been identified in almost all sporadic and genetic cases[9, 10, 15-20]. Although it is still not conclusive that these cytoplasmic inclusions themselves are toxic or merely serve to precipitate toxic cellular components and minimize their detrimental consequences, accumulating evidence reveals that the perturbation of autophagy primarily contributes to their existence and the pathogenesis of these diseases[21].

The very first link between the deregulation of autophagy and neurodegeneration came from studies showing that genetic inactivation of autophagy-related protein 5 (Atg5) or Atg7, which are key regulators of autophagosome formation, leads to massive neuronal death and eventually animal lethality accompanied by evident protein aggregation[22, 23]. Recent studies have established a tight connection between the pathogenesis of AD and the deregulation of autophagy. For example, Beclin1-deficiency can cause the aggregation of Aβ and amyloid precursor protein and subsequent neuronal loss attributable to autophagic dysfunction[19]. Under normal conditions, Aβ stimulates autophagy and facilitates its own degradation, which is important for maintaining a relatively low level of Aβ. Under pathological conditions, the cellular Aβ aggregates can block autophagosome clearance and lysosomal degradation, which results in exacerbation of the pathogenetic condition of AD[24]. Presenilin 1 (PS-1) is known to be an essential regulator for maintaining the acidic environment in lysosomes. PS-1 mutation interferes with the acidification of lysosomes and leads to impaired cargo clearance by autophagolysosomes. Similarly, many PD-associated genes, such as PINK1, Parkin, and LRRK2, have also been found to interact with Beclin1, and their mutant forms can alter the induction of PD and may contribute to its progress[25-27]. As a matter of fact, treatment with rapamycin, which enhances autophagic activity, can improve the symptoms of several neurodegenerative diseases including AD, PD, and HD[20, 28].

With regard to the link between proteinopathy and the development of MN diseases, autophagic dysfunction is considered to be one of the major factors in MN degeneration. Typical pathological features of ALS include protein inclusions enriched in ubiquitin, TDP-43, FUS, and SOD1. Especially, TDP-43-positive inclusions have been shown to be common to 97% of ALS cases, both sporadic and familial[7]. Growing evidence suggests that many ALS-

linked genetic mutations actually affect autophagy receptor proteins (ubiquilin-2, OPTN, SQSTM1/p62) and regulators (VCP) and eventually result in alteration of the autophagic process[29]. Such mutations may impair cargo recognition and the clearance of autophagy substrates, with severe consequences. TDP-43 proteostasis is normally maintained by the coordinated action of the ubiquitin–proteasome system and autophagy, which is particularly important for clearing TDP-43 oligomers and aggregates. The linkage of SQSTM1, VCP, UBQLN2, and OPTN with ALS suggests the impaired turnover of TDP-43 by autophagy may have profound impact on the pathogenesis of ALS[6, 7]. Similarly, deposition of TDP-43 is also the major feature of tau-negative frontotemporal dementia. In addition, the most common ALS-linked mutation is an intronic GGGGCC repeat expansion in C9ORF72, the pathology of which is also characterized by classical TDP-43 inclusions in the motor cortex and spinal cord, which are decorated by ubiquitin, p62, and/ or ubiquilin 2[16, 30]. Currently, it is not clear whether mutant TDP43 and C9ORF72 affect MN degeneration through similar pathways by modulating autophagy. Another common form of familial ALS is correlated with mutant SOD1. It has been demonstrated that mSOD1 is capable of unmasking the inhibition of Beclin1 by BCL-XL through association with this complex, further suggesting that perturbation of autophagy induction profoundly affects the fate of MNs in ALS[31]. However, in contrast to other neurodegenerative diseases, the pathological mechanism of MN diseases seems to be more complex given the facts that rapamycin treatment causes diverse responses in various ALS models with detrimental consequences in mSOD1 VCP transgenic mice but beneficial results for mutant TDP-43 mice[16, 32, 33]. Nassif and colleagues also reported that autophagic activity compromised by reducing the level of Beclin1 in the mSOD1 ALS model is potentially protective for the progress of ALS[31]. Coincidentally, in a recent study, upregulation of autophagy and the accumulation of autophagosomes have been thought to be pathogenic in a cellular model of SMA[18]. It is conceivable that in some forms of MN disease autophagy has become malfunctional, and a further boost of autophagy under this circumstance can only lead to overproduction of defective autophagosomes and exhaust the capacity to maintain protein homeostasis, which may have detrimental consequences and result in eventual cell loss[34]. Therefore, understanding the exact nature of autophagic dysfunction in different forms of human ALS as well as SMA is critical for finding proper therapeutic approaches.

Potential Link between Autophagy and the Pathogenesis of MN Diseases and Other Neuro-degenerative Diseases Based on Patient-Specific iPSC Models

A major challenge in studying the molecular pathological mechanisms underlying MN diseases is the limited access to disease-affected human tissue from the central nervous system. Over the years, several cell and transgenic animal models of ALS/SMA have been developed in yeast, zebrafish, *Drosophila*, mice, and rats. Although these models have made contributions of inestimable importance for molecular studies and drug testing, or for gene therapy, in terms of genetic background they often have limitiations in replicating human ALS/SMA-like phenotypes. It has been reported that results obtained in transgenic animals cannot always be directly transferred to humans[35].

Human induced pluripotent stem cells (iPSCs) are a source of great hope, because they provide access to virtually unlimited numbers of patient-specific cells for modeling MN diseases *in vitro*. Several groups have generated iPSCs from patients with various MN disease-specific mutations. Importantly, the pathology of ALS/SMA has been partially recapitulated in MNs derived from patient-specific iPSCs. Dimos *et al.* obtained fibroblast lines from two elderly siblings with early and late manifestations of ALS, including weakness of the arms and legs, caused by a disease-associated mutation in the SOD1 gene[36]. These cells were then used to generate iPSCs after infection with retroviruses containing human OCT3/4, SOX2, C-MYC, and KLF4. The resulting iPSC lines appeared to be pluripotent *in vitro*, as they spontaneously differentiated into representative phenotypes of the three embryonic germ-cell layers. Furthermore, they were driven to a spinal MN-like phenotype and expressed key MN markers, including HB9 and ISLET1/2. These first showed the possibility of modeling disease using iPSC techniques, although they were not functionally characterized for basic

neuronal properties and disease phenotypes[36].

Egawa *et al.* obtained MNs carrying disease-causing mutations in the gene encoding TDP-43[37]. The ALS MNs in culture recapitulated cellular and molecular abnormalities associated with ALS. Of particular interest, cytosolic aggregates were found in ALS patient-specific iPSC-derived MNs similar to the postmortem tissue from ALS patients, suggesting that deregulation of autophagy plays a role in its pathogenesis. Mutant TDP-43 in the ALS MNs was bound to the spliceosomal factor SNRPB2 and formed aggregates in the nucleus, resulting in perturbed RNA metabolism. It is likely that disturbances in autophagy and RNA processes are intrinsically connected and jointly lead to the disease phenotypes. It will be very interesting to explore whether the dynamics of stress granule formation is altered in this cellular disease model. Consequently, the ALS iPSC-derived MNs were more vulnerable to cellular stressors such as arsenite. The researchers then used the ALS MNs in a drug-screening assay and identified a compound called anacardic acid, a histone acetyltransferase inhibitor that reversed some of the ALS phenotypes in MNs. The new work provides an encouraging step toward using MNs generated from iPSCs derived from ALS patients to learn more about what triggers the death of MNs in this disease and to identify new candidate drugs that may be able to slow or reverse the devastating loss of MNs[37].

In addition, very recent studies with ALS C9ORF72 iPSC lines provide compelling evidence that the human cellular model can have utility that exceeds prior disease models[38]. In their new study, Sareen *et al.* reported a cellular model of C9ORF72-ALS with MNs differentiated from iPSCs derived from ALS patients carrying the C9ORF72 repeat expansion. Transcription of the repeat was increased, leading to the accumulation of GGGGCC repeat-containing RNA foci selectively in C9ORF-ALS iPSC-derived MNs[38]. Repeat-containing RNA foci co-localized with hnRNPA1 and Pur-a, suggesting that they may be able to alter RNA metabolism. Consistently, other groups have also reported that C9ORF72 interacts with both hnRNPA1 and hnRNA2B1 and may regulate the formation of stress granules, which in turn profoundly influence RNA processing[17, 39]. C9ORF72-ALS MNs show altered expression of genes involved in membrane excitability including dipeptidyl-peptidase 6, and demonstrate a diminished capacity to fire continuous spike trains upon depolarization compared to control MNs. Antisense oligonucleotides targeting the C9ORF72 transcript suppress the formation of RNA foci and reverse the gene expression alterations in C9-ALS MNs. These data show that patient-derived MNs can be used to delineate pathogenic events in ALS. Donnelly *et al.* demonstrated that fibroblasts and iPSNs from patients contain intranuclear GGGGCC RNA foci similar to those found *in vivo*, and which are toxic due to the sequestration of RNA-binding proteins such as ADARB2[34]. Simultaneously, cytoplasmic GGGGCC foci have been found in C9ORF72-ALS MNs and in C9ORF72 ALS postmortem motor cortex, suggesting that the deregulation of autophagy is likely to be the next important issue to be addressed regarding the pathogenesis of the disease.

The study by Ebert *et al.* took a similar approach to address the major spinal MN degenerative disorder SMA, typically caused by a genetic mutation in the SMN1 gene[40, 41]. The iPSCs obtained could in turn differentiate into a variety of cell types, including cells with a spinal MN phenotype that harbor the SMN1 mutation. Ebert *et al.* reported a specific reduction in the accumulation and size of these cells in patient cultures *versus* normal controls. In addition, the patient iPSCs displayed a predicted deficiency in nuclear SMN protein aggregates. Treatment of the cells with inducers of SMN protein expression—valproic acid or tobramycin—suppressed this phenotype. It will be of interest to determine whether patient iPSC-derived MN reduction can be similarly suppressed by these compounds or by novel SMN inducers.

In parallel, iPSC techniques have been implemented to generate human AD models for mechanistic studies. Yagi *et al*. recently discovered that the iPSCs derived from fibroblasts from AD patients carrying mutations in PS1 and PS2 can differentiate into neurons that show increased Aβ42 secretion compared to healthy controls[42]. Similarly, neurons derived from iPSCs carrying a duplication of the amyloid precursor protein express higher amounts of both Aβ40 and Aβ42 proteins, and form insoluble intracellular and extracellular amyloid aggregates with augmented phosphorylated tau protein, recapitulating the progress of AD[43]. However, it remains to be determined in these patient-specific AD models whether autophagy is defective

and correlated to the pathophysiology. More importantly, it would be of great value to test the efficacy of the compounds such as rapamycin that have been shown to be beneficial for treating the disease through the experiments mainly carried out in animal models.

With regard to PD, iPSC-derived dopaminergic neurons from patients carrying an LRRK2 mutation have increased expression of oxidative stress response genes and α-synuclein. The PD iPSC-derived neurons are also more susceptible to cell death when exposed to hydrogen peroxide, MG-132, and 6-hydroxydopamine than healthy controls[44], mimicking the pathology of PD, so they can be potentially used as an alternative model to explore the molecular mechanisms of neurodegeneration. Moreover, neurons derived from PD iPSCs carrying LRRK2, G2010S, and PINK1 mutations have been shown to exhibit higher mitochondrial superoxide formation and mitochondrial DNA lesions that may explain their susceptibility to cell death in responses to oxidative stress[45]. Increased expression of α-synuclein protein and augmented sensitivity to oxidative stress have also been reported in the dopaminergic neurons derived from iPSCs bearing a triplication of SNCA genes[46]. Most importantly, dopaminergic neurons derived from both sporadic-PD and LRRK2-PD iPSCs manifest impaired maturation of autophagosomes and defective autophagosome clearance compared to healthy controls, directly linking the pathophysiology of PD to autophagic dysfunction[47].

Conclusions

Taken together, recent advances in iPSC techniques provide a good opportunity for researchers to directly explore the pathological mechanisms underlying neurodegenerative diseases, especially MN diseases, in human cell-based models with almost unlimited sources. However, several major obstacles remain to be overcome for better understanding the kind of role autophagic dysfunction plays in the clinical pathology of these diseases. First, efforts need to be made to optimize the methods to obtain purer populations of MNs either by increasing the differentiation efficiency or through a better sorting strategy so that detailed molecular studies can be implemented using newly-developed methods such as genome-wide sequencing techniques. Second, a large consortium of patient-specific iPSC lines carrying all kinds of mutated genes should be generated, allowing comprehensive examination of the pathogenesis of each individual gene. As noted above, despite direct links between autophagic malfunction and pathophysiology in the PD-iPSC model[47], a clear correlation between a disturbance of autophagy and the pathogenesis of MN diseases has not been established, not to mention exploration of the molecular mechanisms in great detail. This is a major challenge in the field of MN diseases, given the complexity of mutant genes and the diversity of the potential mechanisms as well as the potential involvement of multiple steps of autophagy. In spite of these difficulties, the preliminary studies based on iPSC disease models in the past few years have already shed light on the potential involvement of proteostasis and RNA metabolism as central players in the pathogenesis of MN diseases. Elucidation of the disease mechanism based on these human disease models will strengthen our understanding of the roots of the diseases, how they progress, and eventually provide an ideal system to evaluate drugs that may provide new therapeutic solutions.

ACKNOWLEDGEMENTS

This review was supported by the Ministry of Science and Technology of China (2011CB966200), the National Natural Science Foundation of China (81401053, 31471029, 81461138037, and 81472141), the National Program for Support of Top-notch Young Professionals, the Program for Young Excellent Talents in Tongji University (2014KJ049), and the Public Health Bureau of Tianjin Municipality, China (13KG127).

Received date: 2015-05-18; Accepted date: 2015-06-22

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Neurosci Bull August 1, 2015, 31(4): 452–458. http://www.neurosci.cn
DOI: 10.1007/s12264-015-1540-x

·Review·

Chaperone-mediated autophagy: roles in neuroprotection

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Chaperone-mediated autophagy (CMA), one of the main pathways of lysosomal proteolysis, is characterized by the selective targeting and direct translocation into the lysosomal lumen of substrate proteins containing a targeting motif biochemically related to the pentapeptide KFERQ. Along with the other two lysosomal pathways, macro- and micro-autophagy, CMA is essential for maintaining cellular homeostasis and survival by selectively degrading misfolded, oxidized, or damaged cytosolic proteins. CMA plays an important role in pathologies such as cancer, kidney disorders, and neurodegenerative diseases. Neurons are post-mitotic and highly susceptible to dysfunction of cellular quality-control systems. Maintaining a balance between protein synthesis and degradation is critical for neuronal functions and homeostasis. Recent studies have revealed several new mechanisms by which CMA protects neurons through regulating factors critical for their viability and homeostasis. In the current review, we summarize recent advances in the understanding of the regulation and physiology of CMA with a specific focus on its possible roles in neuroprotection.

Keywords: chaperone-mediated autophagy; cellular homeostasis; neuroprotection; neuronal death; neurodegenerative disease

Introduction

Maintaining the balance between protein synthesis and degradation contributes to cellular homeostasis[1-3]. The ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathway (ALP) are major systems present in almost all cell types to mediate the degradation of intracellular proteins into their constitutive amino-acids[4, 5]. The UPS is a multi-subunit protease complex that degrades proteins tagged with one or more covalently-bound ubiquitin molecules, and most proteasome substrates are proteins with a short half-life[6, 7].

In contrast to the UPS, the ALP is mainly responsible for the degradation of long-lived proteins and organelles. On the basis of the mechanism used for delivery of intracellular cargoes to lysosomes, autophagy can be divided into three types: macroautophagy (MA), microautophagy, and chaperone-mediated autophagy (CMA)[8, 9]. Both CMA and MA have been identified in mammals as processes important for damage and diseases of the central nervous system[10]. MA is a bulk degradation system that involves the formation of a double-membrane structure (autophagosome) that sequesters damaged organelles and proteins. The autophagosome acquires the hydrolytic enzymes necessary to degrade its cargo by fusing with lysosomes[11]. Microautophagy traps nonspecific cytoplasm inside vesicles *via* direct invagination of the lysosomal membrane. These vesicles "pinch off" into the lumen and are degraded by lysosomal hydrolases[12].

CMA is the third type of autophagy, and has so far been found only in mammalian cells[13, 14]. One of its intrinsic features is the selective targeting and direct translocation into the lysosomal lumen of substrate proteins containing a targeting motif related to the pentapeptide KFERQ[15, 16]. This selectivity allows for the removal of misfolded, oxidized, or damaged cytosolic proteins under physiological or

pathological conditions without perturbing normally-functioning forms of the same proteins[17-19]. CMA potentially regulates multiple cellular processes by the selective removal of inhibitors of transcription, enzymes, and cell-maintenance proteins[17, 20].

In this review, we first briefly summarize the main mechanisms of CMA under physiological conditions and then discuss its roles in neuroprotection and the therapeutic potential of targeting this pathway for the treatment of brain diseases.

Molecular Mechanisms of CMA

CMA is a complex process that can be divided into four distinct steps: (1) binding of substrates to the chaperone protein Hsc70 and targeting to lysosomes; (2) binding of substrates to the lysosomal receptor LAMP2A (lysosome-associated membrane protein type 2A) and unfolding; (3) substrate translocation into lysosomes; and (4) substrate degradation by hydrolytic enzymes in the lysosomal lumen[21].

During the first step, CMA substrates containing the pentapeptide motif KFERQ bind to the constitutive chaperone Hsc 70 in the cytosol[22-24]. Many other chaperones are also involved in this process, such as Hsp40, Hsp90, and Hip[25-27]. Although amino-acid sequence analysis shows that almost 30% of cytosolic proteins contain a KFERQ-like sequence, only a few have been experimentally confirmed to be degraded through this process[28]. Some characteristics associated with this motif are notable: (1) the CMA motif is based on the charge of the amino-acids; (2) post-translational modifications such as phosphorylation and acetylation can change the amino-acid charge and enable them to acquire a more effective motif recognizable by the CMA process[24, 29]; and (3) alternatively, a string of imperfect and overlapping motifs can also serve to mediate the CMA process[30]. Thus, containing a functioning KFERQ-like motif is the first requirement for a protein to be considered as a possible CMA substrate. The 'gold standard' to validate a protein as a CMA substrate is the lysosomal binding and uptake assay.

During the second step, the Hsc70-co-chaperone/substrate complex translates to the lysosomal membrane and binds to the cytosolic tail of LAMP2A[31]. The levels and conformational status of LAMP2A are critical for the CMA process, and this is a rate-limiting step for the process. LAMP2A exists as a monomer at the lysosomal membrane and forms a multimeric complex in association with other proteins[26]. CMA substrates first bind to monomeric LAMP2A present at the lysosomal membrane and this interaction drives LAMP2A multimerization to produce a 700-kDa complex required for the translocation of substrate into the lysosome. Once substrate proteins translate into the lysosomal lumen, lysosomal Hsc70 (lys-Hsc70) promotes disassembly of the LAMP2A multimerization complex to enable monomeric LAMP2A to bind to other substrates[26]. A portion of the LAMP2A may be transported into the lysosomal lumen and degraded by cathepsin A[32]. Many factors participate in regulating the assembly/disassembly of the LAMP2A multimerization complex, such as changes in the fluidity of the lysosomal membrane or protein and lipid composition[33]. GFAP and EF1-alpha have been shown to regulate the assembly/disassembly of the LAMP2A multimerization complex[34].

Translocation of substrate proteins into the lysosomal lumen requires lys-Hsc70[25]. The mechanisms by which lys-Hsc70 contributes to translocation remain to be elucidated. It may function by pulling substrate proteins or may immobilize them to prevent their return to the cytosol. The stability of lysosomal Hsc70 is regulated by lysosomal pH and small increases in pH are sufficient to exacerbate its degradation[35]. However, the mechanisms by which Hsc70 translates to lysosome remain largely unknown.

Although the molecular mechanisms by which CMA is modulated are poorly understood, CMA activity is closely associated with the levels of LAMP2A at the lysosomal membrane, and modulation of the LAMP2A content by cells can rapidly change the activity of this pathway[31]. The synthesis/degradation of LAMP2A and its redistribution from the lysosomal lumen to the lysosomal membrane all contribute to its levels at the membrane. In addition, lipid microdomains and cathepsin A play an important role in regulating the levels of this protein[32]. Previous studies have shown that lys-Hsc70 is a limiting factor in the modulation of CMA[35, 36]. Without lys-Hsc70, substrate protein cannot be translocated into the lysosomal lumen, and the level of lys-Hsc70 increases gradually with upregulation of CMA activity.

Physiological Role of CMA

As one of the cellular quality-control systems, CMA was first proposed to participate in amino-acid recycling[37]. When cells or animals are exposed to serum deprivation or prolonged starvation, CMA is maximally activated due in part to increased LAMP2A transcription, decreased LAMP2A clearance, or increased levels of lys-Hsc70. Although removal of serum from cell cultures or prolonged starvation in animals can activate both MA and CMA, the kinetics of these reactions appears to be different. MA is maximally activated shortly after these treatments (~4–6 h) and persists for a short period of time. However, CMA is activated later (~8–10 h) and persists much longer[37]. The selectivity of CMA may promote cellular survival under serum deprivation or prolonged starvation by maintaining essential proteins and removing non-essential proteins, such as glycolytic enzymes and inhibitors of transcription factors containing a KFERQ-like motif in their amino-acid sequences[38].

In addition, CMA contributes to the selective removal of aberrant or damaged proteins in order to maintain cellular homeostasis. Under oxidative stress, up-regulation of CMA promotes the degradation of oxidatively-damaged proteins[39]. In support of this idea, CMA increases under oxidative stress or exposure to toxic compounds[40]. Inhibition of CMA significantly exacerbates the accumulation of oxidatively-damaged proteins and decreases cell viability[40]. Furthermore, CMA can specifically remove the damaged subunits of cytosolic protein complexes[41]. Recent studies have shown that hypoxia induces CMA, and up-regulation of CMA effectively protects cells from hypoxia-induced cell death[42]. Although the mechanisms remain poorly understood, CMA may directly target hypoxia-inducible factor 1[43]. Recent studies have shown that CMA regulates tubular cell growth by modulating the degradation of the transcription factor Pax2[44]. Furthermore, CMA is involved in the immune response. Previous studies showed that only MA and UPS participate in processing antigens. However, recent studies have revealed that CMA is involved in antigen processing and presentation. The levels of autoantigen presentation are closely related to CMA activity[45-47].

As the two major proteolytic systems mediating the degradation of intracellular proteins[48, 49], the ALP and the UPS are not isolated and independent but tightly coordinated. CMA may affect the UPS and the other two autophagic pathways, and the UPS and the other two autophagic pathways can also modulate CMA activity. When MA and the UPS are reduced, CMA is usually activated[50]. Conversely, reducing CMA contributes to remarkable up-regulation of MA[19].

CMA and Neuroprotection

Because neurons are postmitotic, they are especially sensitive to homeostatic changes. CMA plays an important role in maintaining cellular homeostasis. Recent studies have shown that the key components of CMA, LAMP2A and Hsc70, are robustly expressed in the CNS[51, 52]; CMA is involved in the regulation of neuronal survival[30]; and CMA dysfunction has been linked to the pathogenic processes of several human disorders[21].

Parkinson's Disease (PD)

PD is the second most common degenerative disease, characterized by the specific loss of dopaminergic neurons in the substantia nigra pars compacta[53]. Although the etiologies of PD remain elusive, protein dyshomeostasis is the critical mechanism responsible for the neuronal death and may be involved in the pathogenesis. Analysis of postmortem brain tissue from PD patients shows that the level of LAMP2A in the substantia nigra is lower than in controls[52]. Cuervo *et al.* reported a link between CMA and the protein α-synuclein, which is the key component of Lewy bodies and whose mutation and level changes are involved in the pathogenesis of PD[54]. They showed that the α-synuclein amino-acid sequence contains a KFERQ-like CMA-targeting motif and confirmed it as a CMA substrate. They found that mutant forms of α-synuclein (A53T and A30P), which cause familial PD, are defective in their uptake by lysosomes due to their tight binding to LAMP2A and cannot be efficiently degraded. Although the study showed how mutation of α-synuclein may contribute to the dysfunction of CMA, it did not identify a direct mechanism by which CMA dysfunction may lead to neuronal death.

We recently showed that CMA directly targets for removal of non-functional myocyte enhancer factor 2D (MEF2D), a factor critical for the survival of dopaminergic neurons. This is critical for maintaining the homeostasis of MEF2D under basal conditions[30, 55]. Overexpression of

wild-type or disease-causing mutant α-synuclein in cells leads to an inhibition of its degradation by CMA. The levels of both MEF2D and α-synuclein are higher in the neuronal cytoplasm in the brains of PD patients than in controls[30, 55]. Thus, dysregulation of MEF2D homeostasis by CMA is a feature of PD. We tested the function of MEF2D that had accumulated in the cytoplasm due to defective CMA and found that the accumulated MEF2D had a much lower DNA-binding activity than controls[30]. Since increased MEF2D in the nucleus attenuates α-synuclein-induced cellular toxicity, our findings established a direct relationship between CMA and the nuclear survival machinery, indicating that its disruption may underlie the toxic effects of both wild-type and mutated α-synuclein.

Since increased oxidative stress has been proposed to play a critical role in the pathogenesis of PD, we further investigated its role in the CMA-mediated maintenance of MEF2D homeostasis. Our results showed that oxidative stress leads to direct oxidative modification of MEF2D and a significant decrease in its level[40]. This decrease is in part due to the accelerated removal of oxidized MEF2D by CMA. Consistently, the levels of oxidized MEF2D are much higher in the postmortem PD brain than in controls, consistent with the notion that reduced CMA in PD leads to the accumulation of damaged MEF2D, disrupting its homeostasis and function.

A recent investigation of leucine-rich repeat kinase 2 (LRRK2), mutation of which is linked to PD, showed that it is a CMA substrate[56]. LRRK2 G2019S, the most common mutant form, is poorly degraded by this pathway. Lysosomal binding of both wild-type and several pathogenic mutant LRRK2 proteins is enhanced in the presence of other CMA substrates, which may interfere with the organization of the CMA translocation complex, resulting in defective CMA. Similarly, ubiquitin C-terminal hydrolase L1 (UCH-L1), mutation of which is linked to familial PD, is degraded by CMA[57, 58]. It has been shown that the PD-related mutant UCH-L1 I93M binds much more tightly to Hsc70/hsp90 and LAMP2A present at the lysosomal membrane than the wild-type protein. Therefore, it may block CMA *via* mechanisms similar to those proposed for α-synuclein or LRRK2.

Alzheimer's Disease (AD)

AD is the most common neurodegenerative disease and is closely associated with aging. CMA gradually decreases with physiological aging. Previous studies have shown that dyshomeostases of intracellular proteins are critical factors in the pathogenesis of AD, one of which is abnormal tau metabolism[59]. Tau pathology in AD is characterized by its aggregation and cleavage[60]. How tau protein is degraded remains controversial. Wang *et al.* showed that CMA contributes to tau fragmentation into pro-aggregating forms and to the clearance of tau aggregates[61]. Tau_{RD}DK280 and its F1 fragment interact with the cytosolic chaperone Hsc70 and with the CMA receptor LAMP2A. Unlike typical CMA substrate proteins, these forms of tau are not translocated into lysosomes by CMA. Instead, they aggregate on the outer membrane of lysosomes, leading to the disruption of lysosomal membrane integrity and blockade of other CMA substrates.

Huntington's Disease (HD)

HD is caused by polyQ repeat expansion of huntingtin (Htt)[62]. The accumulation of aggregated mutant Htt protein in the affected neurons is its hallmark[63]. Clarifying the mechanisms that influence the cellular degradation of Htt may help to understand the pathology of the disease and identify a cure. However, the mechanisms by which Htt is degraded remain largely unknown. Several studies have demonstrated that MA regulates the degradation of Htt and an increase in MA activity may protect neurons[64]. Recent studies have shown that CMA may also be involved in Htt degradation[65, 66]. When post-translationally modified by phosphorylation/ubiquitination/SUMOylation and acetylation, Htt may bind to Hsc70 and LAMP2A with higher affinity[24]. Consistent with the involvement of CMA in HD, the activity of this pathway appears to increase in mouse models of HD.

Therapeutic Perspectives

Dyshomeostasis of proteins may be the cause of many diseases. A decrease in CMA plays an important role in the dyshomeostasis of various proteins. Therefore, restoration of CMA activity may be a strategy for treating many diseases, especially neurodegenerative diseases. Combinations of different types of approaches, including genetic methods and CMA-modulating drugs, have succeeded in slowing down the neurodegeneration in mouse models of HD and PD. It has been reported that

retinoic acid receptor alpha (RARα) negatively regulates CMA activity, and inhibition of RARα with synthetic derivatives of all-trans retinoic acid can specifically activate CMA. Activation of CMA by RARα can protect cells from oxidative stress and proteotoxicity[67].

Conclusions

CMA is an important proteolytic pathway characterized by selectivity and direct translocation of substrate proteins across the lysosomal membrane. It is critical for maintaining cellular homeostasis and for many different aspects of cell physiology, especially in the central nervous system. The roles of CMA in the physiological functions of neurons and in the pathogenesis of neurodegenerative diseases are largely unknown. Further investigation of its functions should help understand the pathogenic mechanisms underlying these diseases. Since a decrease in CMA is associated with many neurodegenerative diseases of aging, increasing CMA by various means should be explored as a treatment strategy.

ACKNOWLEDGEMENTS

We thank Professor Zixu Mao for critical reading of the manuscript. This review was partially supported by the FMMU Research Foundation, National Basic Research Development Program of China (2011CB510000), and the National Natural Science Foundation of China (31371400).

Received date: 2015-05-04; Accepted date: 2015-06-23

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Neurosci Bull August 1, 2015, 31(4): 459–468. http://www.neurosci.cn
DOI: 10.1007/s12264-015-1539-3

·Original Article·

Histone deacetylase 6 delays motor neuron degeneration by ameliorating the autophagic flux defect in a transgenic mouse model of amyotrophic lateral sclerosis

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by the selective loss of motor neurons. Abnormal protein aggregation and impaired protein degradation are believed to contribute to the pathogenesis of this disease. Our previous studies showed that an autophagic flux defect is involved in motor neuron degeneration in the SOD1^{G93A} mouse model of ALS. Histone deacetylase 6 (HDAC6) is a class II deacetylase that promotes autophagy by inducing the fusion of autophagosomes to lysosomes. In the present study, we showed that HDAC6 expression was decreased at the onset of disease and became extremely low at the late stage in ALS mice. Using lentivirus-HDAC6 gene injection, we found that HDAC6 overexpression prolonged the lifespan and delayed the motor neuron degeneration in ALS mice. Moreover, HDAC6 induced the formation of autolysosomes and accelerated the degradation of SOD1 protein aggregates in the motor neurons of ALS mice. Collectively, our results indicate that HDAC6 has neuroprotective effects in an animal model of ALS by improving the autophagic flux in motor neurons, and autophagosome-lysosome fusion might be a therapeutic target for ALS.

Keywords: motor neuron disease; motor neuron; neurodegenerative disease; amyotrophic lateral sclerosis; autophagy; histone deacetylase 6

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal, adult-onset neurodegenerative disease[1-3]. Pathologically, it is characterized by progressive, selective motor neuron loss in the brain and spinal cord[2,3]. Mutations in copper/zinc superoxide dismutase (*SOD1*) account for ~20% of familial cases of ALS[3, 4]. In this study, mice overexpressing the G93A mutation of *SOD1* were used to model ALS. The underlying pathogenic mechanisms, although still largely unknown, probably involve the aggregation of misfolded proteins[5, 6].

There are two main systems for cytoplasmic protein degradation: the ubiquitin-proteasome system and the autophagy-lysosome system[7]. Autophagy is a lysosome-based bulk-degradation pathway to clear misfolded proteins and toxic aggregates. Dysfunction of the autophagy-lysosome system may contribute to neuronal degeneration in several neurodegenerative diseases[6,8,9]. Our previous study and those of others have shown the accumulation of autophagic vacuoles in the spinal cord of SOD1^{G93A} mice and ALS patients, suggesting a role of autophagy in the pathogenesis of ALS[5, 10]. We further reported that targeting autophagy with rapamycin, a classic autophagy activator, significantly exacerbates motor neuron loss and fails to

remove the abnormal mutant SOD1 aggregates, raising the possibility of autophagic flux defects in SOD1^{G93A} mice[11]. Our latest study demonstrated that trehalose, an mTOR (mammalian target of rapamycin)-independent autophagy inducer, is able to decrease SOD1 and ubiquitinated protein accumulation as well as improve autophagic flux in the motor neurons of ALS mice, suggesting that the strategy of improving autophagic flux may have potential in ALS treatment[12].

Histone deacetylase 6 (HDAC6) is a unique class II deacetylase containing a ubiquitin-binding domain; it copes with excessive levels of misfolded proteins by recruiting them to dynein motors for transport to aggresomes[13, 14]. Furthermore, HDAC6 is a central component of the aggresome that promotes autophagy by inducing the fusion of autophagosomes to lysosomes and regulates the protective response to the formation of cytotoxic protein aggregates[15, 16]. Accumulating evidence has shown that HDAC6-induced autophagy rescues neurons from degeneration in animal models of Huntington's disease, Alzheimer's disease, and Parkinson's disease[17, 18]. However, little is known about the effects of HDAC6 on ALS.

In this study, we investigated the effects of HDAC6 on motor neuron degeneration in a mouse model of ALS through intracerebroventricular injection of lentivirus-HDAC6 into SOD1^{G93A} transgenic mice, and explored the underlying mechanisms.

MATERIALS AND METHODS

Mice and Treatments

Transgenic SOD1^{G93A} mice expressing mutant human SOD1 with a Gly93Ala substitution (B6SJL-Tg-SOD1G93A-1Gur) were originally obtained from Jackson Laboratories (002726, Sacramento, CA). All mice were housed under constant temperature and controlled light. Animal use was approved by the Ethics Committee of Shanghai Jiaotong University School of Medicine and all procedures in our experiments were conducted in accordance with the guidelines of National Institute of Health for animal care.

The genotypes of the transgenic mice were identified by PCR as in our previous reports[11, 12]. Male SOD1^{G93A} mice were randomly divided into 2 groups (Tg-Control and Tg-HDAC6, 18 mice in each). Those in the Tg-HDAC6 group were injected with HDAC6 lentivirus and those in the Tg-Control group were injected with the same volume of control lentivirus. Meanwhile, 36 age-matched wild-type (WT) littermates were divided into WT-Control and WT-HDAC6 groups. All the mice were weighed every four days from 94 to 146 days after birth. The mean body weight for each group at each time point was recorded.

Plasmids and Clones

Full-length human *HDAC6* (NM_006044) was cloned by PCR. The primers for *HDAC6* target sequences were as follows: forward 5'-CTCAAGCTTCGAATTCGCCACCATG-ACCTCAACCGGCCAG-3'; reverse 5'-CCTTGTAGTCGGA-TCCGTGTGGGTGGGGCATATC-3'. Full-length cDNA of HDAC6 was cloned into the pLVX-3Flag-puro lentiviral vector. All constructs were verified by sequencing. The pLVX-3Flag-puro lentiviral vector itself was used as the control. The lentiviral and help vectors (SBO Medical Biotech, Shanghai, China) were transfected into 293FT cells for viral packaging.

Intracerebroventricular Injection of Lentiviruses

Large-scale lentivirus production and purification were as described previously[19]. In brief, 293FT cells were infected with each lentiviral vector and help vector for viral packaging. After harvesting and shaking, the PEG-800-NaCl system (Sigma-Aldrich, St. Louis, MO) was used to purify and concentrate the lentivirus. The titers of the virus preparation were 6–8 × 10^8 vector genomes per milliliter. The mice were anesthetized with chloral hydrate and 8 μL HDAC6 lentivirus or control lentivirus was injected into the right cerebral ventricle (AP: –0.22 mm, ML: –1.0 mm, DV: –2.35 mm) at 1 μL/min.

Behavioral Tests

Assessment of Disease Onset (Rotarod Test)

SOD1^{G93A} transgenic mice showing disease onset were used for lentivirus injection. Rotarod performance was measured every day in these mice starting at 72 days of age. The date of disease onset was defined as when a mouse could not stay on the rotating rod for 5 min[20]. Based on rotarod performance, lentivirus injection was started at 95 days of age.

Assessment of Lifespan

For lifespan analysis, the date of "death" was defined as the day when a mouse could not right itself within 30 s after being placed on its back[21]. All the mice were tested every day and the date of "death" was recorded.

Immunofluorescence Staining

Each mouse was anesthetized, perfused with PBS, and fixed with 4% paraformaldehyde. The spinal cord was removed and postfixed in 4% paraformaldehyde at 4°C overnight, then cryoprotected in 15% sucrose for 24 h and 30% sucrose for another 24 h. The spinal cord was cut into 10-μm-thick sections on a sliding microtome (Leica 3050s, Nussloch, Germany). Slides were incubated at 4°C overnight with primary antibodies against LC3B (1:200; Cell Signaling, Danvers, MA), SOD1 (1:200; Abcam, HK), or p62 (1:100; BD Transduction Laboratories, Waltham, MA). After washing with PBS, the slides were incubated with secondary antibody Cy2 or Cy3 (Jackson Immunoresearch, West Grove, PA), and then visualized at 600× magnification under a fluorescence microscope (Nikon Eclipse 80i, Tokyo, Japan). Motor neurons were identified in the anterior horn, with cell bodies >20 μm. Slides incubated with secondary antibody Cy2 or Cy3 alone were used as a negative control. In each animal, the images of 20 randomly-selected motor neurons were captured and the number of LC3- or p62-positive puncta in each motor neuron was counted by a researcher who was blinded to the experimental design.

Analysis of Motor Neuron Survival

The fixed L4–5 segments were cut into 10-μm sections, which were Nissl stained with 1% cresyl violet (Sigma-Aldrich). Every fifth section was selected from the total 250 serial sections from each mouse. The 50 sections from each mouse were photographed under a microscope (Olympus IX81, Tokyo, Japan) and the anterior horns on both sides were examined by a technician who was blinded to the experimental design. The number of motor neurons was counted as described previously[21, 22].

Immunoblotting

For immunoblotting, 40 mg of lysed protein was separated on SDS-PAGE gel and transferred to PVDF membrane. Membranes were incubated with the primary antibodies against HDAC6 (1:1 000, Cell Signaling), LC3B (1:500, Cell Signaling), p62 (1:500, MBL, Nagoya, Japan), or Flag (1:1 000, Cell Signaling). After incubation overnight, secondary antibodies were applied and protein bands were visualized using chemiluminescent horseradish peroxidase substrate (ECL, Pierce, Waltham, MA) and quantified with an image analyzer (Image Lab 4.2, Bio-Rad, Hercules, CA). β-actin (1:6 000; Sigma-Aldrich) was used as an internal control.

Quantitative Real-time PCR

Total RNA was extracted from homogenized spinal cord samples (from 60-, 90-, and 120-day-old Tg and WT mice) with TRIzol reagent (Invitrogen, Carlsbad, CA). Two milligrams of total RNA were used for cDNA synthesis using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). Real-time PCR was performed using the SYBR premix Ex Taq TM II kit (Takara, Shiga, Japan) and analyzed using a real-time PCR System (ABI7500). The sequences of primers were as follows: HDAC6, forward 5'-ATAGCAGCACTACCTGGGAAAG-3' and reverse 5'-TTAGGGAAGTTTTCAGGAGCAG-3'; GAPDH, forward 5'-CCAATGTGTCCGTCGTGGATCT-3' and reverse 5'-GTTGAAGTCGCAGGAGACAACC-3'.

Electron Microscopy

Electron microscopy (EM) was performed as reported previously[11, 12]. In brief, L4–5 segments were fixed in 2.5% glutaraldehyde and cut into 50-μm sections. Then, the sections were postfixed in 1% OsO4, dehydrated, embedded, and further cut into 70-nm sections. The ultra-thin sections were stained with uranyl acetate and evaluated with a CM-120 EM (Philips, Eindhoven, The Netherlands). EM images were captured at a final magnification of 10 000×. Autolysosome was defined as single-membrane structures containing cytoplasmic material and/or organelles[23].

Statistical Analysis

Kaplan-Meier analysis (SPSS 17.0) was performed for lifespan data and the data were analyzed using the log-rank test, generating a χ^2 value to test for significance. Data of Flag-HDAC6 expression were analyzed using one-way ANOVA followed by Tukey's *post hoc* test. Other data were analyzed with two-way ANOVA (Prism 5, GraphPad

Software, La Jolla, CA). All values are presented as mean ± SEM. *P* <0.05 was defined as statistically significant.

RESULTS

HDAC6 Expression in the Spinal Cord of SOD1^{G93A} and WT Mice

Immunoblotting revealed that the level of HDAC6 markedly decreased in an age-dependent manner in the spinal cord of SOD1^{G93A} mice. This started at 90 days of age and became more evident at 120 days, while there were no significant differences between the levels in 60-, 90-, and 120-day-old WT mice (Fig. 1A). Analysis showed that the level of HDAC6 protein in the spinal cord of SOD1^{G93A} mice fell to 70.44% at 90 days and to 40.88% at 120 days of that in age-matched WT mice (Fig. 1B). In addition, there was a significant reduction in HDAC6 mRNA levels in the spinal cord of 90- and 120-day-old SOD1^{G93A} mice compared with age-matched WT mice (Fig. 1C). These results showed that HDAC6 expression decreases in SOD1^{G93A} mice at both the transcriptional and translational levels in an age-dependent manner.

To further investigate the expression of lentivirus-HDAC6 in the mouse spinal cord, immunoblotting was used to assess the levels of Flag 2, 4, 6, and 8 weeks after HDAC6 lentivirus injection. The Flag-HDAC6 expression in the spinal cord increased from 2 weeks to 4 weeks after HDAC6 lentivirus injection, then decreased with time (Fig. 1D, E).

HDAC6 Overexpression Prolonged the Lifespan of SOD1^{G93A} Mice

We injected the HDAC6 lentivirus into SOD1^{G93A} mice at disease onset based on rotarod performance, which was usually at the age of 95 days[11, 12]. We found that Tg-HDAC6 mice had a 17-day extension in lifespan compared with Tg-Control mice (140.00 ± 4.33 *vs* 123.00 ± 2.60,

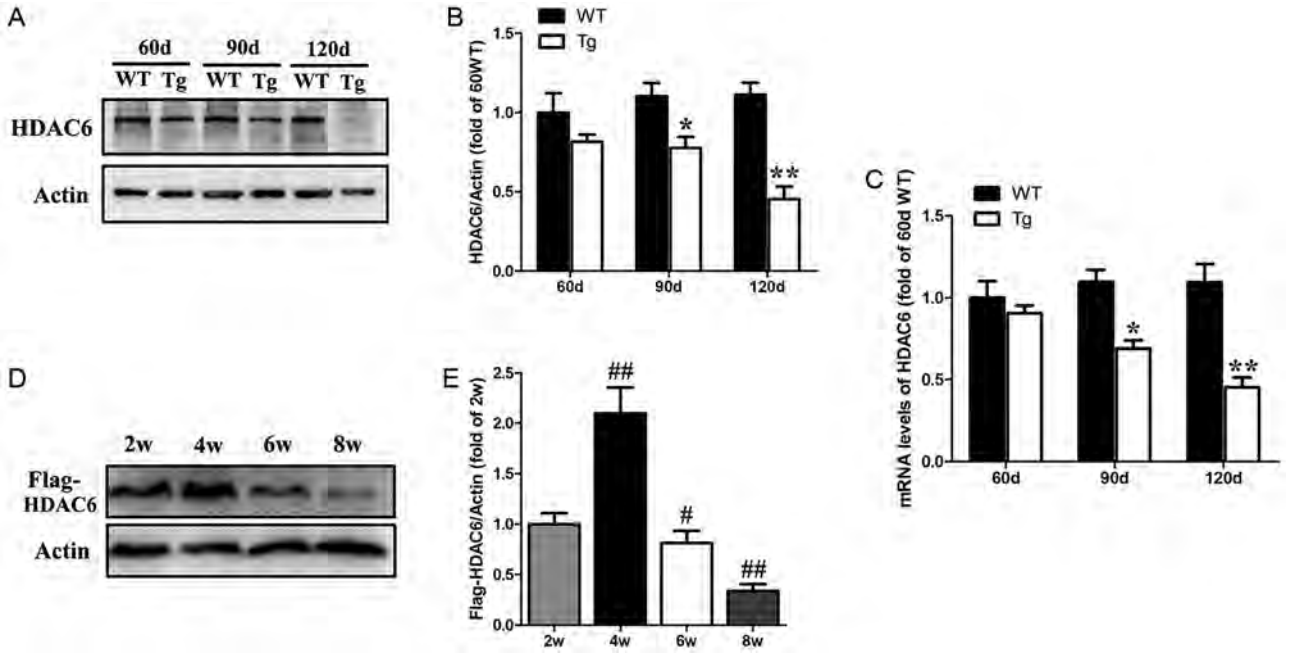

Fig. 1. HDAC6 expression in wild-type (WT) and transgenic (Tg) mice. (A) Immunoblots of HDAC6 expression in the spinal cord of 60-, 90-, 120-day-old G93A Tg and age-matched WT mice. (B) Analysis of HDAC6/actin in WT and Tg mice. (C) HDAC6 mRNA expression in the spinal cord as assessed by real-time PCR in WT and Tg mice. (D) Immunoblots of Flag-HDAC6 expression in the spinal cord of Tg mice at 2, 4, 6, and 8 weeks after lentivirus injection. (E) Analysis of Flag-HDAC6/actin at 2, 4, 6, and 8 weeks after lentivirus injection. *n* = 3 mice/group. Data in B and C were analyzed using two-way ANOVA with repeated measures. Data in E were analyzed using one-way ANOVA followed by Tukey's *post hoc* test. All values are presented as mean ± SEM. **P* <0.05 compared with 90-day-old WT mice; ***P* <0.01 compared with 120-day-old WT mice; $^{\#}$*P* <0.05, $^{\#\#}$*P* <0.01 compared with 2-week group.

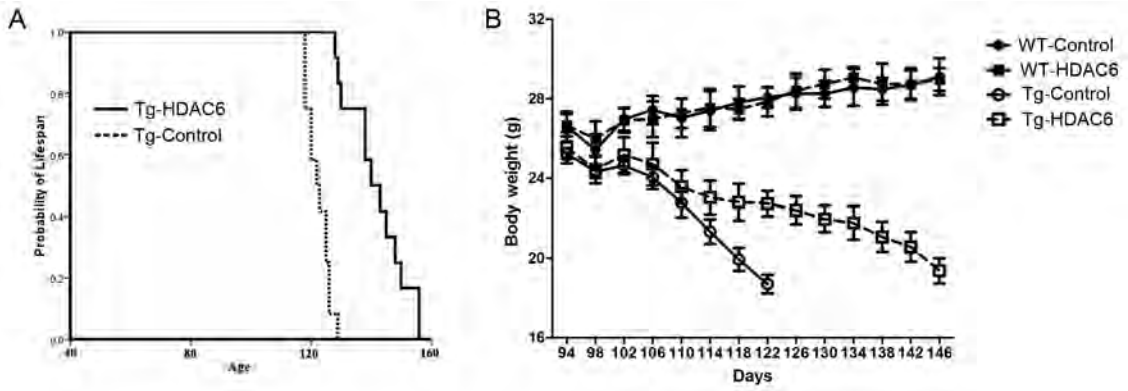

Fig. 2. Effects of HDAC6 overexpression on lifespan and body weight in WT and Tg mice. (A) Kaplan-Meier survival analysis showing the probability of survival in Tg-Control (dotted line) and Tg-HDAC6 mice (solid line). (B) Body weight curves in the 4 groups. *n* = 10/group.

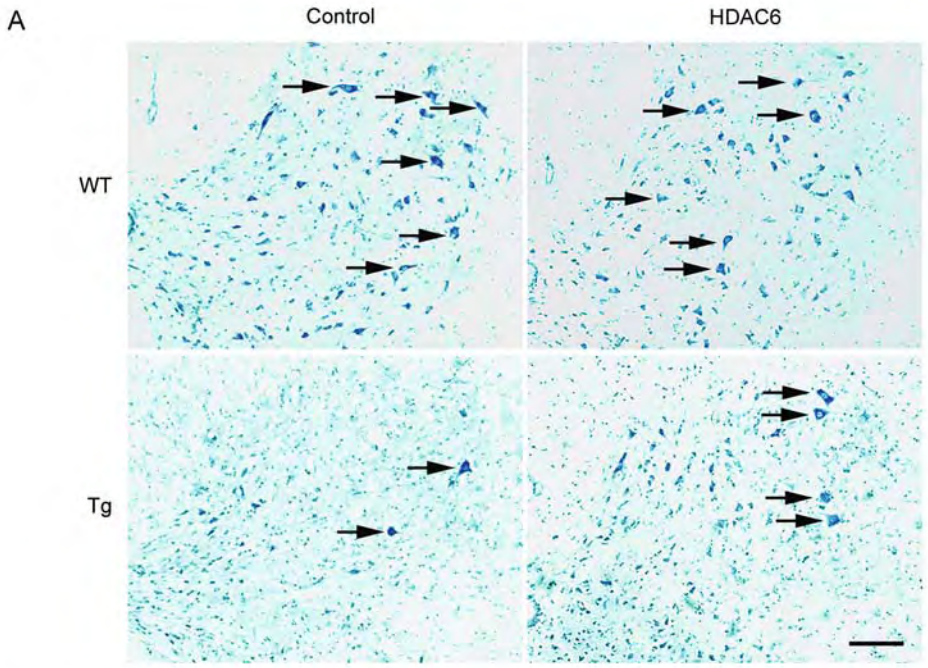

Fig. 3. Effects of HDAC6 overexpression after disease onset on motor neuron survival in Tg mice and in WT siblings at 120 days of age. (A) Representative photomicrographs of Nissl-stained motor neurons in the 4 groups of mice (arrows, motor neurons; *n* = 3 mice/group; scale bar, 100 μm). (B) Numbers of Nissl-stained motor neurons in L4–5 segments in the 4 groups of mice (mean ± SEM; **P* <0.05, ***P* <0.01 compared with 120-day-old Tg-Control mice; two-way ANOVA).

χ^2 = 23.76, P <0.01) (Fig. 2A). Moreover, Tg-HDAC6 mice exhibited a significant reduction in body weight loss compared with age-matched Tg-Control mice (Fig. 2B). However, there was no difference in body weight between WT-Control and WT-HDAC6 mice.

HDAC6 Overexpression Protected Motor Neurons in SOD1^{G93A} Mice

Nissl staining showed a significant loss of motor neurons in the L4–5 segments of 120-day-old SOD1^{G93A} mice compared with WT mice (Fig. 3A, B). In addition, the number of motor neurons in the Tg-HDAC6 mice was markedly higher than that in Tg-Control mice (423.0 ± 31.25 *vs* 257.5 ± 21.32, P <0.01; Fig. 3B). Motor neuron survival did not differ between WT-Control and WT-HDAC6 mice (817.30 ± 36.03 *vs* 824.80 ± 42.03, P >0.05) (Fig. 3A, B).

HDAC6 Overexpression Reduced the Aggregation of LC3-Positive Puncta in Motor Neurons of SOD1^{G93A} Mice

Previous studies have reported that HDAC6 plays an important role in the process of autophagy[15]. To determine the effect of HDAC6 in this process, we used immunoblotting to determine the LC3-II turnover in the spinal cord of 120-day-old SOD1^{G93A} mice, and found that HDAC6 overexpression decreased the elevated LC3-II level by ~30.2% (Fig. 4A, B). Immunofluorescence staining further revealed a decrease of LC3-positive puncta in the motor neurons of Tg-HDAC6 mice compared with the Tg-Control mice (Fig. 4C, D). These results showed that HDAC6 overexpression at disease onset reduces the premature autophagy in the motor neurons of SOD1^{G93A} mice.

HDAC6 Overexpression Improved Autophagic Flux in SOD1^{G93A} Mice

To further investigate the effects of HDAC6 overexpression on autophagic flux, we determined the protein level of p62, a biomarker of autophagic flux[24], in the spinal cord of SOD1^{G93A} mice. Immunoblotting showed an abnormally high level of p62 protein in the spinal cord of SOD1^{G93A} mice compared with WT mice; HDAC6 overexpression reduced the elevated p62 level in the SOD1^{G93A} mice (Fig. 5A) by 34.08% (Fig. 5B). Further, p62 immunostaining showed a significant decrease of p62-positive aggregation in the motor neurons of Tg-HDAC6 mice compared with

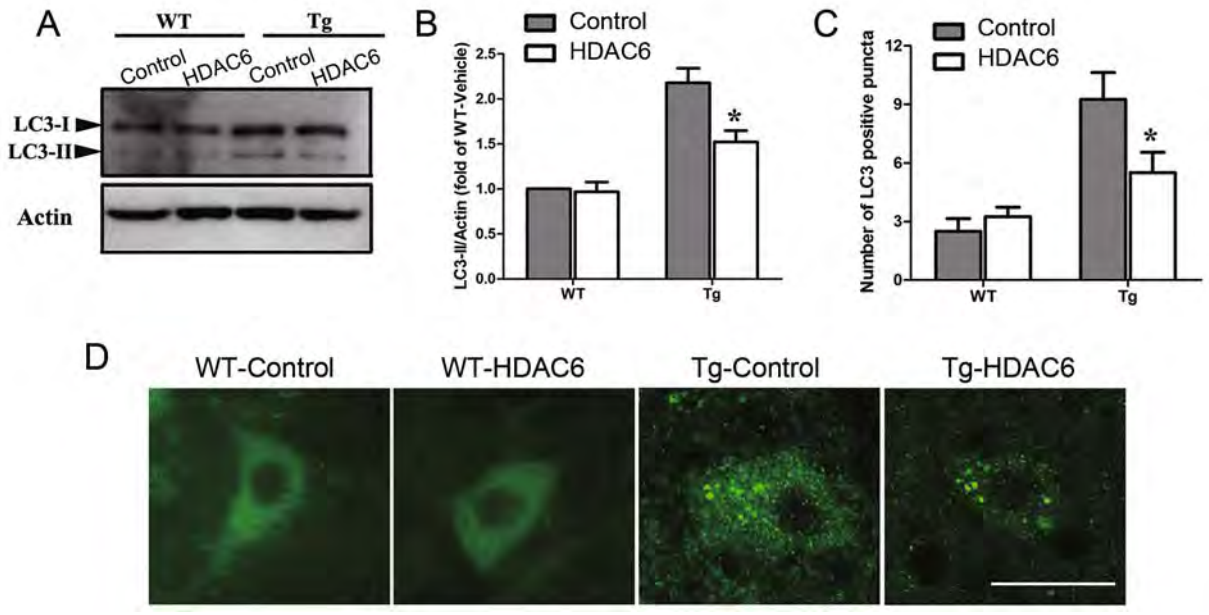

Fig. 4. Effects of HDAC6 overexpression on the number of autophagosomes in G93A Tg mice. (A) Immunoblots of LC3-II expression in the spinal cord of 120-day-old WT and Tg mice. (B, C) Analysis of LC3-II/actin in 120-day-old WT and Tg mice (B) and numbers of LC3-II-positive puncta (C) in the 4 groups of mice (mean ± SEM.; *P <0.05 compared with 120-day-old Tg-Control mice; two-way ANOVA). (D) Immunostaining of LC3 in motor neurons of the 4 groups of mice (scale bar, 20 μm).

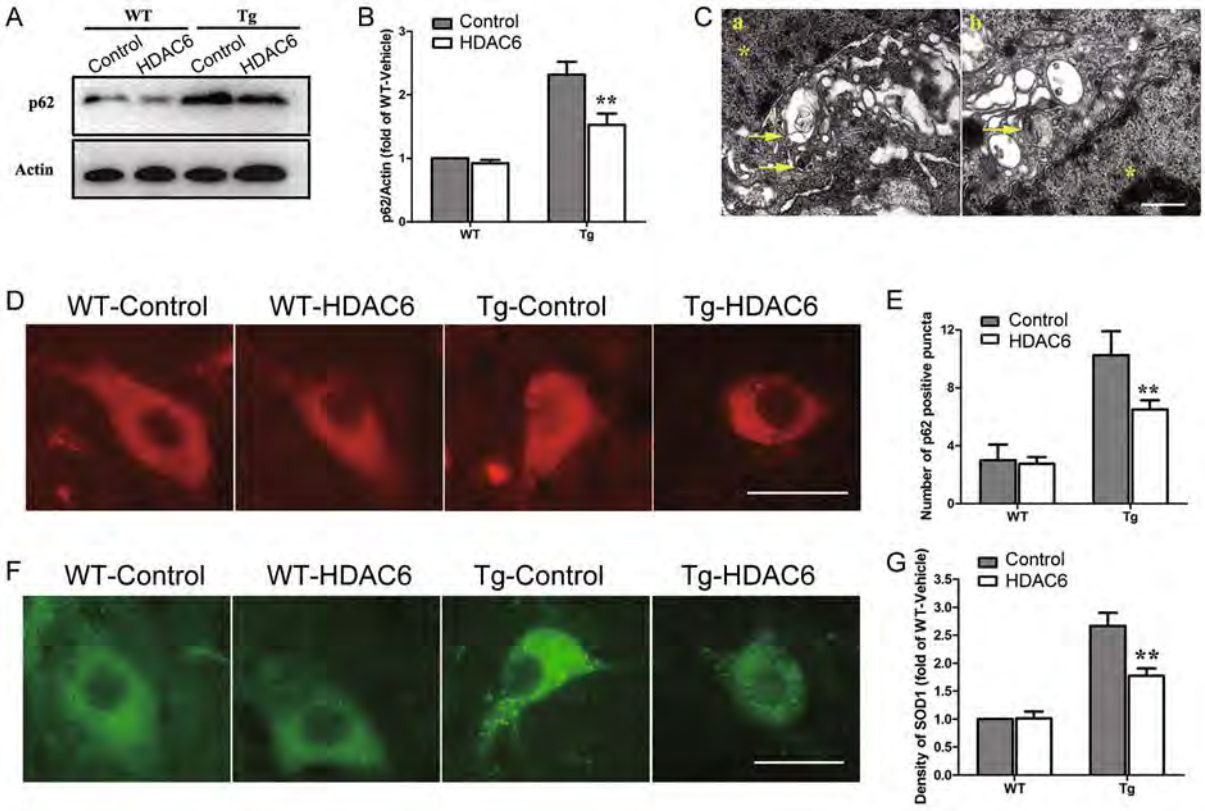

Fig. 5. Effects of HDAC6 overexpression on autophagic flux and SOD1 protein degradation in G93A Tg mice. (A) Immunoblots of p62 expression in the spinal cord of 120-day-old WT and Tg mice. (B) Analysis of p62/actin in 120-day-old WT and Tg mice. (C) Representative electron microscopic images of autolysosomes in the spinal motor neurons of 120-day-old Tg-HDAC6 mice (arrows, autolysosomes; *nucleus; scale bar, 0.5 μm). (D-G) Immunostaining of p62 (D) and SOD1 (F) in motor neurons from the 4 groups of mice (scale bars, 20 μm), and analysis of p62-positive puncta (E) and SOD1 density (G) in the motor neurons of 120-day-old WT and Tg mice (mean ± SEM; *****P* **<0.01 compared with 120-day-old Tg-Control mice; two-way ANOVA).**

Tg-Control mice (6.50 ± 1.71 *vs* 10.25 ± 2.95) (Fig. 5D, E). Moreover, EM examination further showed single-membrane structures of autolysosomes in the motor neurons of Tg-HDAC6 mice (Fig. 5C), providing more evidence for HDAC6-mediated autophagic protein degradation in SOD1^{G93A} mice.

We further found a significant decrease in SOD1 aggregates in the Tg-HDAC6 mice compared with the Tg-Control mice (Fig. 5F). Analysis showed that the density of SOD1 immunostaining in the motor neurons of Tg-HDAC6 mice was reduced to 66.6% of that in Tg-Control mice (Fig. 5G).

DISCUSSION

In this study, we first reported that the level of HDAC6 expression in the spinal cord of the SOD1^{G93A} mouse model of ALS decreased, starting at disease onset, and became extremely low in later stages. Furthermore, HDAC6 overexpression at disease onset prolonged the lifespan by delaying motor neuron degeneration in the ALS model. Moreover, the neuroprotective action of HDAC6 might be related to its effect on autophagic flux and then the clearance of abnormal protein aggregates in the motor neurons of SOD1^{G93A} mice.

According to previous reports, HDAC6 is highly conserved and the structure and function of mouse HDAC6 are similar to its human ortholog[25, 26]. The double catalytic domain, a ubiquitin-binding domain, and a nuclear export-signal domain are functionally conserved in mouse and human HDAC6, mediating the same deacetylase,

ubiquitin protein-binding, and nuclear protein export activities[27]. It has been documented that the ubiquitin-binding domain plays an important role in HDAC6-mediated autophagosome maturation and autophagosome-lysosome fusion[14, 15]. In this study, human HDAC6 was selected for overexpression, as it could provide evidence relevant to the clinical treatment of ALS.

Accumulating evidence has suggested that autophagic flux impairment plays a critical role in ALS[28, 29]. Our previous studies showed that the mTOR-dependent autophagic inducer rapamycin accelerates motor neuron loss and aggravates autophagic flux dysfunction in the SOD1^{G93A} mouse model of ALS[11]. Our latest report provided strong evidence for an autophagic flux defect, especially impairment in the fusion of autophagosomes and lysosomes in the ALS model[12]. In the present study, we found that HDAC6 overexpression after disease onset reduced p62 and SOD1 aggregation, which was accompanied by decreased LC3-positive aggregates in the motor neurons of SOD1^{G93A} mice. Furthermore, EM analysis showed the formation of autolysosomes in the motor neurons of Tg-HDAC6 mice. Otherwise, a previous study demonstrated that an autophagic protein marker is specifically increased in the spinal motor neurons of SOD1^{G93A} mice, and not in microglia or astrocytes[11]. In the present study, we further found that the alterations of LC3-II and p62 puncta were mainly in the motor neurons after HDAC6 overexpression in SOD1^{G93A} mice. These results suggested that HDAC6 improves autophagic flux by inducing the formation of autolysosomes in the motor neurons of ALS mice even at late stages of the disease.

It has been reported that microtubule-based vesicle trafficking, especially the fusion of autophagosomes and lysosomes, is critical for the autophagy process[30]. And impairment of dynein-mediated trafficking might be associated with the autophagosome-lysosome fusion defect[31]. Mutant SOD1 alters the cellular localization of dynein and inhibits the dynein-mediated trafficking in neurons, which might affect the microtubule-based autophagic fusion step in ALS[32]. HDAC6 acts as a multivalent adapter to bind both ubiquitinated proteins and dynein motors, recruiting misfolded protein cargos to the autophagosomes along the microtubules[14]. It is worth pointing out that HDAC6 stimulates the fusion of autophagosomes to lysosomes and substrate degradation in neurons[15]. Several lines of evidence have documented that HDAC6 deficiency leads to autophagosome maturation failure, protein aggregation, and neurodegeneration[15, 33]. On the other hand, expression of HDAC6 at the normal level is sufficient to rescue neurodegeneration in an autophagy-dependent manner[17]. In our study, we found that overexpressing HDAC6 in Tg-HDAC6 mice significantly improved the autophagic flux and enhanced the clearance of protein aggregation in motor neurons, further supporting a role of HDAC6 in autophagy-dependent protein degradation in ALS.

Although recent studies have indicated that the induction of HDAC6 can be neuroprotective by removing protein aggregates in neurodegenerative diseases, the therapeutic strategy of targeting HDAC6 is still controversial[33, 34]. Specific inhibitors of HDAC6 or genetic knock-down of HDAC6 has shown neuroprotective effects by increasing the acetylation levels of α-tubulin to improve axonal transport in neurons[35, 36]. It is not known whether inhibiting or deleting HDAC6 affects the expression of mutant SOD1^{G93A}. The variable results may be due to the different functions of HDAC6 at different stages of the disease [37, 38]. It is likely that HDAC6 inhibition at the early stage is beneficial for ALS by restoring axonal transport, while HDAC6 is required to maintain autophagosome-lysosome fusion at the later stages of ALS when protein aggregation is dominant in the motor neurons of SOD1^{G93A} mice. However, more studies are required to explore the detailed mechanisms of HDAC6 activity at different stages of ALS and to investigate the mechanisms underlying the age-dependent decrease of the HDAC6 level in ALS.

In summary, we first report that the level of HDAC6 is significantly decreased at disease onset in the SOD1^{G93A} mouse model of ALS. HDAC6 overexpression at disease onset prolonged the lifespan and delayed the motor neuron degeneration in ALS mice. Moreover, we found that HDAC6 induced the formation of autolysosomes and accelerated the degradation of SOD1 protein aggregates in the motor neurons of ALS mice. Collectively, these results indicate that HDAC6 is a potential target for ALS treatment.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Natural Science Foundation of China (81200977), the Shanghai

Natural Science Foundation (14ZR1446400), Shanghai Rising-Star Program (15QA1403000), the PhD Innovation Fund of Shanghai Jiaotong University School of Medicine (BXJ 201218), and the PhD Tutor Fund of the Ministry of Education of China (20120073110077).

Received date: 2015-05-12; Accepted date: 2015-06-10

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Neurosci Bull August 1, 2015, 31(4): 469–479. http://www.neurosci.cn
DOI: 10.1007/s12264-015-1543-7

·Original Article·

Regulation of autophagic flux by CHIP

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ABSTRACT

Autophagy is a major degradation system which processes substrates through the steps of autophagosome formation, autophagosome-lysosome fusion, and substrate degradation. Aberrant autophagic flux is present in many pathological conditions including neurodegeneration and tumors. CHIP/STUB1, an E3 ligase, plays an important role in neurodegeneration. In this study, we identified the regulation of autophagic flux by CHIP (carboxy-terminus of Hsc70-interacting protein). Knockdown of CHIP induced autophagosome formation through increasing the PTEN protein level and decreasing the AKT/mTOR activity as well as decreasing phosphorylation of ULK1 on Ser757. However, degradation of the autophagic substrate p62 was disturbed by knockdown of CHIP, suggesting an abnormality of autophagic flux. Furthermore, knockdown of CHIP increased the susceptibility of cells to autophagic cell death induced by bafilomycin A1. Thus, our data suggest that CHIP plays roles in the regulation of autophagic flux.

Keywords: CHIP/STUB1; autophagic flux; neurodegeneration; mTOR; AKT

INTRODUCTION

The autophagy-lysosome pathway (ALP) and the ubiquitin proteasome system (UPS) are major intracellular protein-degradation systems. Autophagy mediates the lysosomal degradation of cytoplasmic components including proteins with longer half-lives, aggregates, and damaged organelles[1]. The process of autophagy includes autophagosome formation, a step in which the cytoplasmic components are sequestered in a double membrane; autolysosome formation by fusion of mature autophagosomes with lysosomes; and substrate degradation in which the cargo-containing substrates are degraded by proteases in the lysosome[2]. Aberrant autophagy contributes to the pathogenesis in many neurodegenerative diseases[3-5].

Many neurodegenerative disease-related proteins act either as key regulators of autophagy or as autophagic substrates[3,4]. For example, amyloid β (Aβ) can be generated in autophagic vacuoles and degraded by the ALP[6,7]. Wild-type and mutant α-synuclein, expanded polyglutamine Huntingtin, and mutants of SOD1 are degraded through the ALP[8]. Moreover, neurodegenerative disease-related proteins such as presenilin 1, α-synuclein, DJ-1, parkin, PINK1, LRRK2, and SOD1 are involved in the regulation of autophagy either at the initiation, maturation, or substrate-recognition steps[4, 9,10].

The carboxy-terminus of Hsc70-interacting protein (CHIP), the product of the *STUB1* gene, has been identified as a co-chaperone and U-box type E3 ubiquitin ligase that has multiple functions in many cellular processes such as protein degradation, trafficking, transcription, signaling, and apoptosis[11]. CHIP is involved in many pathological processes, especially those of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) and several types of ataxia[12,13]. For example, CHIP exists in Lewy bodies, promotes the degradation of phosphorylated α-synuclein[14,15], binds to Parkin and enhances its E3 ligase activity[16], enhances

the degradation of phosphorylated tau, and reduces Aβ toxicity[17,18]. CHIP also represses the toxicity induced by LRRK2[19,20], expanded polyglutamine[21,22], and mutant SOD1[23]. Thus, CHIP has protective effects against the toxicity of neurodegenerative disease proteins. Recently, loss-of-function mutations in CHIP were identified as a causative genetic factor for a group of autosomal recessive cerebellar ataxias[24-28]. However, the precise mechanism of the involvement of CHIP in neurodegeneration remains unclear. In this study, we set out to address the role of CHIP in the regulation of autophagy, which may be associated with CHIP deficiency-related neurodegeneration.

MATERIALS AND METHODS

Plasmid Constructs and siRNAs

The EGFP-LC3 and FLAG-p62 expression plasmids were described previously[29,30]. mCherry-EGFP-LC3B was kindly provided by Jayanta Debnath (University of California at San Francisco, USA) (Addgene plasmid #22418)[31]. The fidelity of all constructs was confirmed by sequencing. siRNAs against human or mouse CHIP were synthesized with the following sequences: human CHIP, sense: 5'-UGCCGCCACUAUCUGUGUAAUTT-3', anti-sense: 5'-AUUACACAGAUAGUGGCGGCATT-3'; mouse CHIP, sense: 5'-AUACAUGGCAGAUAUGGAUTT-3', anti-sense: 5'-AUCCAUAUCUGCCAUGUAUTT-3'; negative control siRNA (si-NC), sense: 5'-UUCUCCGAACGUGUCACGUTT-3', anti-sense: 5'ACGUGACACGUUCGGAGAATT-3'.

Cell Culture, Transfection, and Drug Treatment

Human embryonic kidney 293 (HEK293) cells and mouse neuroblastoma Neuro 2a (N2a) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Gibco). Cells were transfected with siRNAs using lipofectamine RNAiMAX transfection reagent (Invitrogen, La Jolla, CA), or transfected with expression plasmids using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were treated with DMSO or 100 nmol/L bafilomycin A1 (Sigma, St. Louis, MO).

Immunoblot Analysis and Antibodies

Cells were lysed in lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl pH 7.5, 0.5% deoxycholate, 1% NP40, and protease inhibitor cocktail (Roche, Indianapolis, IN)]. Proteins were separated by 10% or 15% SDS-PAGE and transferred onto a PVDF (polyvinylidene difluoride) membrane (Millipore, Bedford, MA). The PVDF membrane was incubated with the following primary antibodies overnight at 4°C: anti-FLAG, anti-ATG1/ULK1 (Sigma), anti-GAPDH (Chemicon, Temecula, CA), anti-CHIP/STUB1, anti-p70S6K, anti-PTEN (Epitomics, Burlingame, CA), anti-phospho-p70S6K (S371), anti-phospho-AKT (S473), anti-phospho-mTOR (S2448), anti-phospho-ULK1 (S757), anti-mTOR, anti-cleaved caspase-3 (Cell Signaling Technology, Beverly, MA), anti-LC3 (Novus Biologicals, Littleton, CO), anti-AKT, and anti-p62/SQSTM1 (Santa Cruz, Santa Cruz, CA). Then the following secondary antibodies were used: horseradish peroxidase-conjugated sheep anti-mouse, anti-rabbit, or anti-goat IgG (Amersham Pharmacia Biotech, Peapack, NJ). Proteins were visualized with an ECL detection kit (Amersham Biosciences, Piscataway, NJ) using a chemiluminescence imaging system (Bioshine ChemiQ 4800) (Shanghai, China).

Fluorescence and Immunofluorescence

Transfected HEK293 cells were washed with PBS (Gibco, pH 7.4) and fixed in 4% paraformaldehyde for 5 min, then the fixed cells were visualized under an inverted microscope (IX71, Olympus, Tokyo, Japan) or a confocal microscope (LSM710, Zeiss, Oberkochen, Germany). For immunofluorescence assays, after permeabilization with 0.25% Triton X-100 in PBS, the cells were washed 3 times in PBS and pre-blocked with 4% fetal bovine serum for 1 h in PBST (0.1% Tween 20 in PBS). Then the cells were incubated with primary antibodies overnight, followed by incubation with fluorescent secondary antibodies (Alexa Fluor 594-conjugated Affinipure donkey anti-rabbit or anti-mouse IgG, Alexa Fluor 488-conjugated Affinipure donkey anti-mouse IgG, Invitrogen) for 2 h. The labeled cells were visualized under the confocal microscope.

MTT Cell Viability Assay

Cells were washed with PBS and incubated with 0.5 mg/mL MTT (Sigma) dissolved in DMEM without phenol red. After 3 h, the medium was removed and the formazan crystals were dissolved in DMSO by incubation at 37°C for 30 min. The absorbance was measured by a photometer at 570

nm by subtracting background at 630 nm. The data were normalized to the control and the ratios are presented as mean ± SEM from three independent experiments.

Statistical Analysis

Immunoblot densitometric analysis of three independent experiments was performed using Photoshop 7.0 software (Adobe, San Jose, CA). The data were analyzed by one-way analysis of variance (ANOVA) using Origin 6.0 software (OriginLab, Northampton, MA).

RESULTS

Knockdown of CHIP Induces Autophagosome Formation

To investigate whether CHIP is associated with autophagy, CHIP was knocked down in two cell lines, N2a and HEK293, and LC3 protein levels were measured. LC3 is cleaved at its carboxyl termini to form LC3-I, which is conjugated with phosphatidylethanolamine to generate LC3-II during the initiation of autophagy[29, 32-33]. LC3-II binds tightly to the autophagosomal membrane and its level or the ratio of LC3-II to LC3-I is a useful marker of autophagosome numbers[33]. Knockdown of CHIP increased the LC-II level in both N2a and HEK293 cells in three independent experiments (Fig. 1A and B), suggesting that CHIP affects autophagosome formation across diverse types of cells. Consistently, the fluorescence of EGFP-LC3 showed significantly more puncta in CHIP knockdown cells than in control cells (Fig. 1C). The ubiquitin-binding autophagic adaptor p62/SQSTM1 (p62) binds to LC3 and mediates the engulfment of autophagic cargoes into autophagosomes, so the co-localization of p62 and LC3 puncta also serves as a marker of autophagosome formation[34-36]. Here, we found dramatically increased co-localization of FLAG-p62 with endogenous LC3 or EGFP-LC3 puncta in CHIP-knockdown cells (Fig. 1D and E). These data indicate that CHIP deficiency induces autophagosome formation.

AKT/mTOR/p70S6K Is Involved in CHIP-Mediated Autophagosome Formation

PI3K/AKT/mTOR is the major pathway for the regulation of autophagosome formation[37,38]. Considering that CHIP is an E3 ligase for PTEN degradation[39,40] and that PTEN is a key negative regulator of the PI3K/AKT/mTOR pathway[37], we investigated whether CHIP has effects on the PI3K/AKT/mTOR pathway. With CHIP-knockdown, the phosphorylation of AKT, mTOR, and p70S6K decreased in both N2a and HEK293 cells (Fig. 2A and B). We also assessed the protein level of PTEN, as well as the phosphorylation of ULK1 on Ser757, which is phosphorylated by activation of mTOR[41,42]. Increased PTEN protein levels and decreased phosphorylation of ULK1 on Ser757 were detected in both N2a and HEK293 cells (Fig. 2C and D). These results indicated that loss of CHIP affects the PTEN/AKT/mTOR pathway, leading to the induction of autophagosome formation.

CHIP Influences Autophagic Flux and p62 Degradation

Increased LC3-II levels and puncta with a co-localization of LC3 and p62 may reflect increased autophagic flux towards lysosomes or accumulation of autophagosomes by impaired autophagosome-lysosome fusion[33]. To further confirm the effect of CHIP on autophagic flux, we performed CHIP knockdown experiments in cells expressing mCherry-GFP-LC3B. Tandem mCherry-GFP-LC3B is a useful tool for monitoring autophagic flux. Both mCherry (red) and GFP (green) emit fluorescence resulting in yellow fluorescence in autophagosomes; however, when autolysosomes are formed, pH-sensitive GFP fluorescence is lost while red fluorescence is preserved[43,44]. Interestingly, an increased autophagosome (yellow) ratio and a decreased red fluorescence ratio were present in CHIP knockdown cells compared with controls (Fig. 3A and B), giving an appearance similar to treatment with bafilomycin (Baf) A1, a vacuolar-type H^+-ATPase inhibitor that decreases lysosome acidification and affects the fusion of autophagosomes with lysosomse (Fig. 3A and B). In addition, the yellow fluorescence further increased in CHIP-depleted cells compared with controls under Baf A1 treatment (Fig. 3A and B). Next, we examined the effect of CHIP on autophagic substrate degradation, and found that the autophagic substrate p62 protein was increased in CHIP-depleted cells (Fig. 3C). Together, these results suggested that knockdown of CHIP leads to abnormal autophagosome accumulation and substrate degradation with increased autophagosome formation but inhibition of autophagosome-lysosome fusion and substrate degradation.

Knockdown of CHIP Increases Cell Death in Response to Inhibition of Autophagic Flux

Inhibition of autophagic flux leads to cell death in response

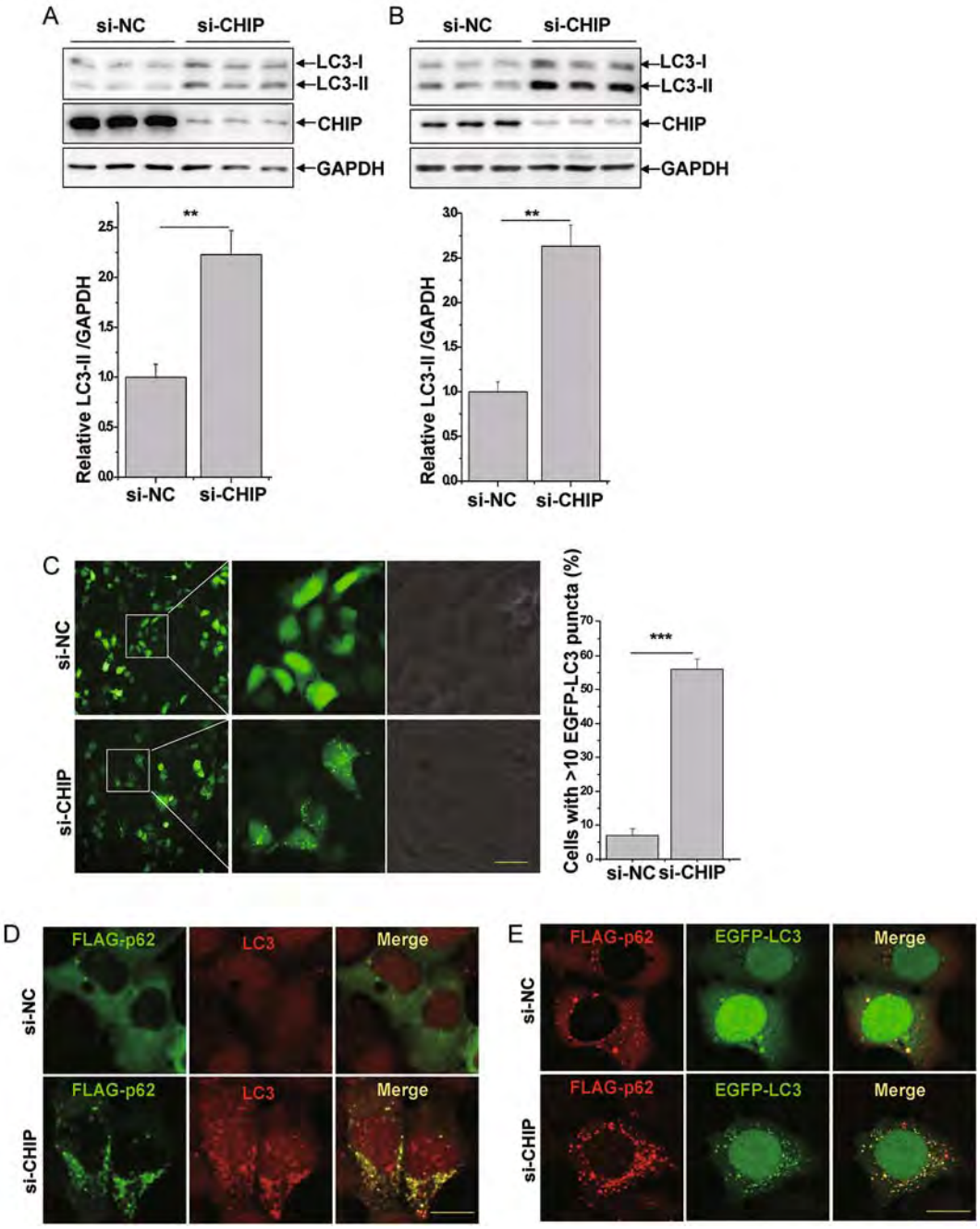

Fig. 1. Autophagosome formation induced by knockdown of CHIP. (A-B) Upper panels, HEK293 cells (A) and N2a cells (B) were transfected with indicated siRNAs. Seventy-two hours later, cells were lysed and the lysates subjected to immunoblot analysis using anti-LC3, anti-CHIP, and anti-GAPDH antibodies. Lower panels, relative ratios of LC3-II to GAPDH (mean ± SEM; *****P* **<0.01, one-way ANOVA). (C) HEK293 cells expressing EGFP-LC3 were transfected with indicated siRNAs. Seventy-two hours later, the green fluorescence was visualized under an inverted fluorescent microscope. Scale bar, 10 μm. The quantitative data presented the percentages of cells with >10 EGFP-LC3 puncta (mean ± SEM; ******P*** **<0.001, one-way ANOVA). (D) HEK293 cells expressing FLAG-p62 were transfected with indicated siRNAs. Seventy-two hours later, cells were immuno-stained with anti-FLAG (green) and anti-LC3 (red) and were visualized under a confocal microscope. Scale bar, 10 μm. (E) HEK293 cells expressing FLAG-p62 and EGFP-LC3 were transfected with indicated siRNAs. Seventy-two hours later, cells were immuno-stained with anti-FLAG (red), then visualized with a confocal microscope. Scale bar, 10 μm.**

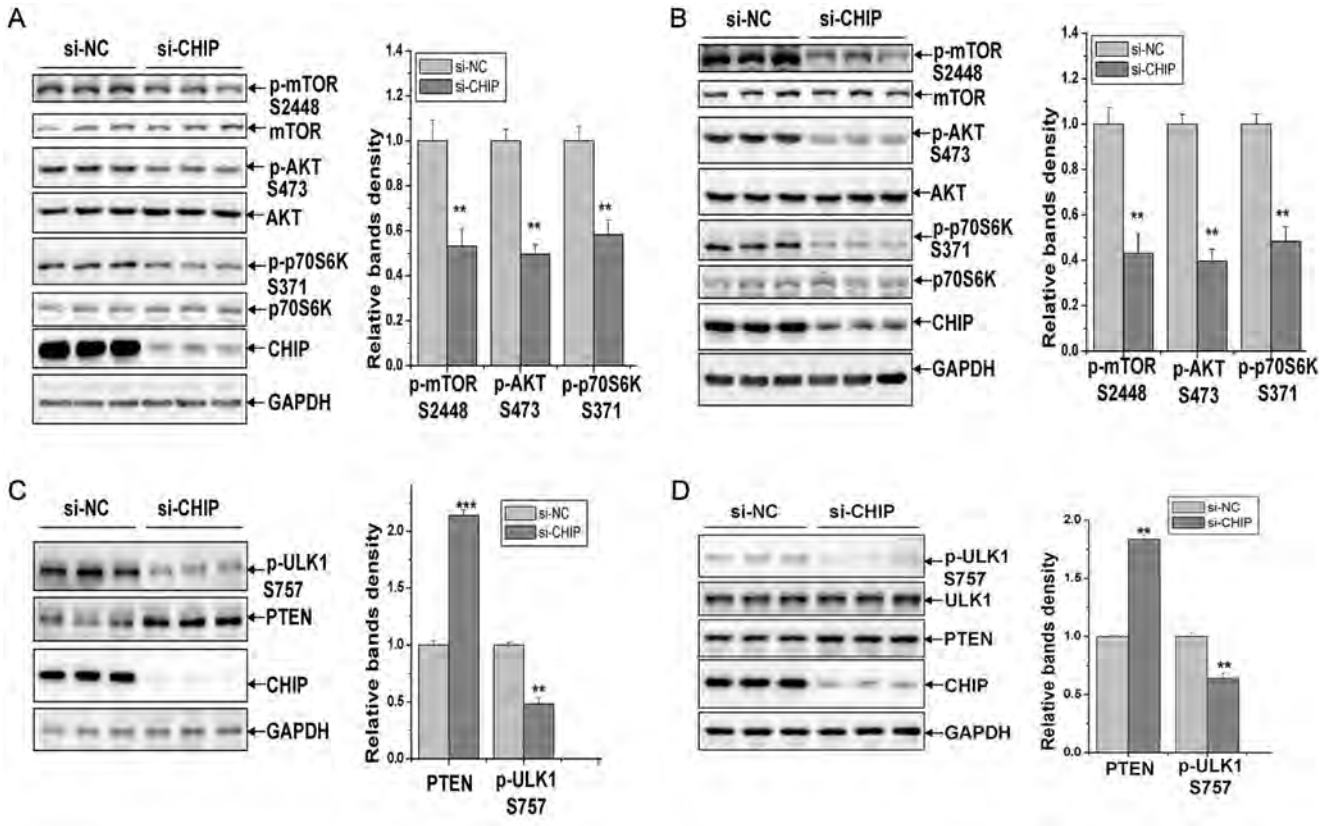

Fig. 2. Down-regulation of AKT/mTOR phosphorylation by knockdown of CHIP. (A–D) Left panels, HEK293 cells (A, C) and N2a cells (B, D) were transfected with the indicated siRNAs. Seventy-two hours later, cells were lysed and the lysates subjected to immunoblot analysis using the indicated antibodies. Right panels, relative ratios of phosphorylation of AKT/mTOR/p70S6K to total protein (A, B) and phosphorylation of ULK1 S757 as well as the protein level of PTEN to GAPDH (C, D) (mean ± SEM; *****P* **<0.01, ******P*** **<0.001 *vs* si-NC, one-way ANOVA).**

to autophagy inhibitors such as Baf A1[45,46]. Therefore, we examined the effect of CHIP on cell survival with or without Baf A1 treatment, and found that knockdown of CHIP alone did not significantly influence cell survival despite accumulation of LC3-II (Fig. 4A and B). However, under Baf A1 treatment, more robust caspase-3 cleavage and LC3-II accumulation were found in CHIP-depleted cells than in control cells (Fig. 4A and B). Besides, although knockdown of CHIP alone had little effect on cell viability, it significantly decreased viability in response to Baf A1 treatment (Fig. 4C and D). These results suggested increased susceptibility to cell death by knockdown of CHIP in response to inhibition of autophagic flux.

DISCUSSION

CHIP is ubiquitously expressed in tissues and has multiple functions[11]. CHIP is a U-box-type E3 ligase containing tetratricopeptide repeat (TPR) domains, with a co-chaperone property that functions in combination with several chaperones such as HSP70 and HSP90[47-52]. CHIP plays a crucial role in cytoplasmic homeostasis and cell viability by decreasing or stabilizing its substrates[11,12]. It participates in a number of biological processes, especially in neurodegeneration including AD, PD, HD, ALS, and several types of ataxia[12,13, 24-28]. Here, we showed that the neurodegenerative disease-related protein CHIP plays a role in the regulation of autophagic flux. CHIP deficiency resulted in autophagic flux inhibition and autophagosome accumulation by increasing autophagosome formation through the AKT/mTOR/p70S6K pathway and by inhibiting autolysosome maturation.

Impairment of autophagic flux with increased

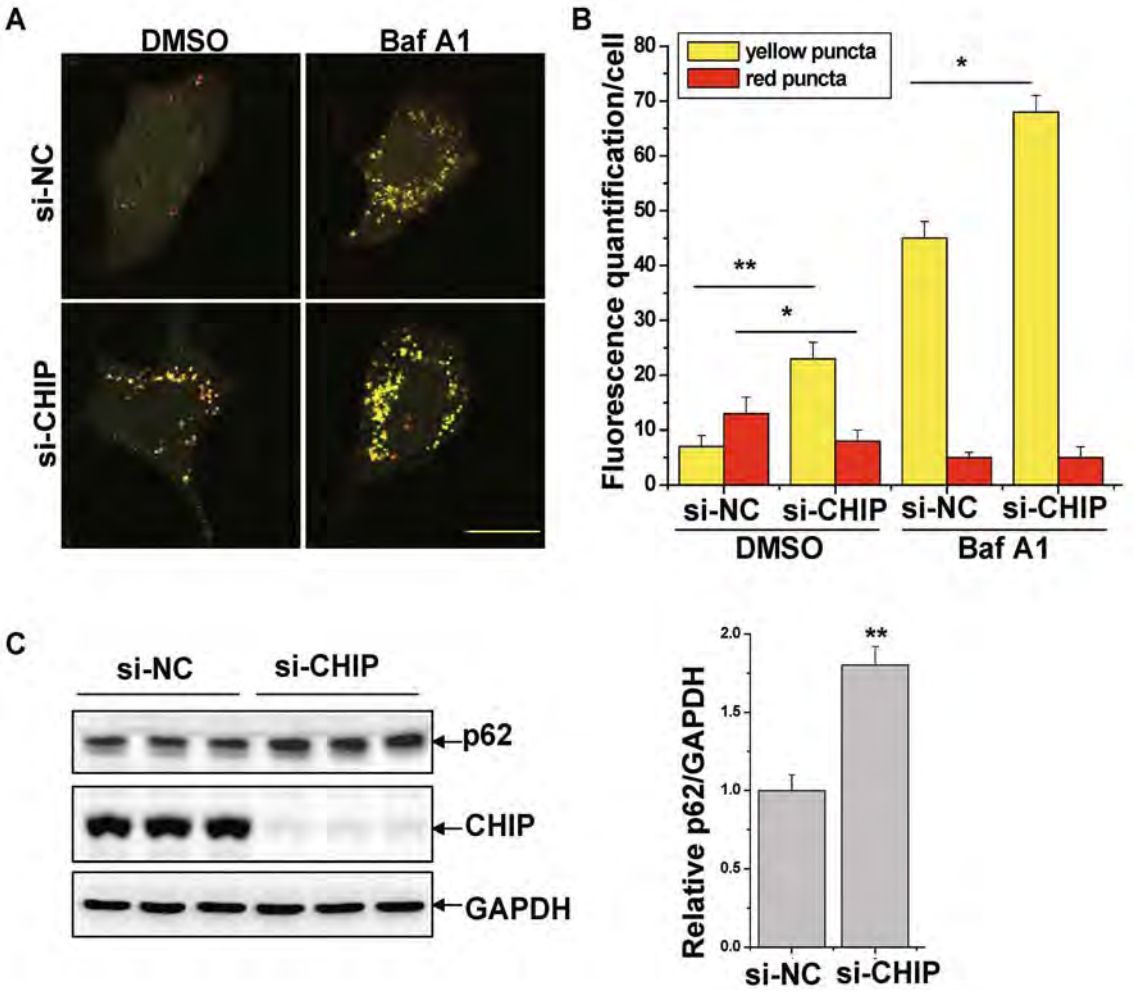

Fig. 3. Inhibition of autophagosome-lysosome fusion and p62 degradation by knockdown of CHIP. (A) Confocal images of HEK293 cells expressing mCherry-EGFP-LC3B, transfected with indicated siRNAs. Seventy-two hours later, cells were treated with DMSO or 100 nmol/L Baf A1 for 4 h as indicated. Scale bar, 10 μm. (B) Quantitative data presented yellow and red puncta in control or CHIP knockdown with or without Baf A1 treatment (mean ± SEM; *P <0.05, **P<0.01, one-way ANOVA). (C) Left panel, HEK293 cells were transfected with indicated siRNAs. Seventy-two hours later, cells were collected and the total cell lysates were subjected to immunoblot analysis using the indicated antibodies. Right panel, relative ratios of p62 to GAPDH (mean ± SEM; *P <0.05, **P <0.01, one-way ANOVA).

autophagosome formation and impaired autolysosome clearance occurs frequently in neurodegenerative diseases[3,4]. For example, massive autophagosome accumulation occurs in AD brains because of increased initiation of autophagy but decreased maturation of autophagosomes[53-55]. In PD, the dysregulation of autophagy occurs at multiple steps[3,4,9]. In HD, a deficit in cargo recognition leads to inefficient autophagy although the autophagosome formation and clearance are normal[56]. Increased autophagosomes with an impaired autophagic flux also occur in ALS patients and animal models[10, 57-59].

Besides CHIP, many E3 ligases including parkin, SCF (β-TrCP), MDM2, RNF5, TRAF6, and TRIM13 are involved in autophagy by modifying different targets[60]. For example, the PD-associated protein parkin is an E3 ligase that inhibits autophagy by stabilizing Bcl-2 through mono-ubiquitination[61]. SCF inhibits autophagy through activating mTOR signaling by ubiquitinating DEPTOR, an mTOR inhibitor[62]. TRIM13 induces autophagy by interacting with p62/SQSTM1 during ER stress[63]. Here, we showed that

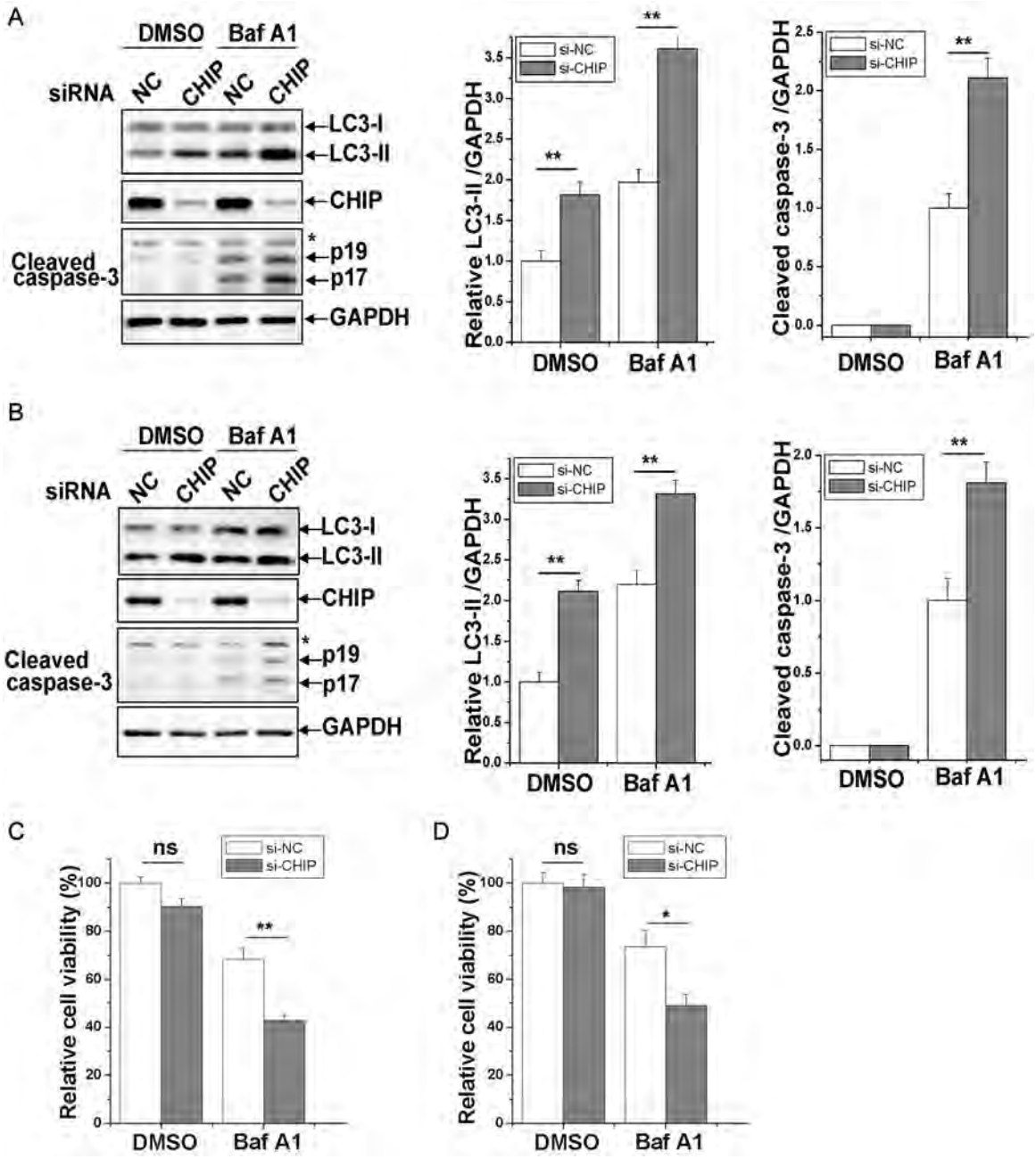

Fig. 4. Increased cell death in response to autophagic flux inhibition by CHIP knockdown. (A–B) Left panels, HEK293 cells (A) and N2a cells (B) were transfected with indicated siRNAs. Forty-eight hours later, cells were treated with DMSO or 100 nmol/L Baf A1 for 4 h as indicated. Then total cell lysates were subjected to immunoblot analysis using the indicated antibodies (*nonspecific band). Right panels, relative ratios of LC-II (left) and cleaved caspase-3 (right) to GAPDH (mean ± SEM; *****P* **<0.01, one-way ANOVA). (C–D) HEK293 cells (C) and N2a cells (D) were transfected with indicated siRNAs. Forty-eight hours later, cells were treated with DMSO or 100 nmol/L Baf A1 for 4 h as indicated. Then cells were subjected to MTT assay (ns, not significant; ****P*** **<0.05; *****P*** **<0.01).**

knockdown of CHIP increases autophagosome formation by decreasing AKT/mTOR signaling. mTOR signaling is considered as the master regulator of autophagy initiation. mTOR acts as a sensor of cellular nutrients and growth

factors, promoting cell growth by activating biosynthetic pathways and inhibiting autophagy under nutritional sufficiency[37,38,64]. Inactivation of mTOR by starvation or mTOR inhibitors such as rapamycin promotes autophagy initiation and autophagosome nucleation by affecting the phosphorylation of multiple autophagy-related proteins including ULK1/2, ATG13, and FIP200[65-67]. The ULK1/2-ATG13-FIP200 complex acts as the major autophagy initiator[68]. Under starvation, the mTOR-dependent phosphorylation site Ser757 in mouse ULK1 (equivalent to Ser758 of human ULK1) is dephosphorylated and this subsequently leads to phosphorylation of ULK1 at Ser 317 and Ser777, as well as phosphorylation of ATG13 and FIP200. Then the activated complex initiates autophagosome formation[41,42,68]. p70S6K, a downstream target of mTOR, also acts as a negative regulator of autophagy[69]. In our studies, the phosphorylation of p70S6K was decreased in CHIP-depleted cells. AKT is a crucial positive mediator of mTOR activity and a downstream effector of PI3K[70]. Decreased phosphorylation of AKT in CHIP-depleted cells indicates that CHIP may influence the upstream of AKT. Interestingly, several findings have suggested that CHIP binds to and degrades PTEN, which is a major negative regulator of the PI3K/AKT/mTOR pathway[39,40,71]. CHIP-regulated autophagy may depend on its E3 ligase activity by targeting PTEN[39,40]. Our results also verified that knockdown of CHIP increases PTEN protein levels (Fig. 2C and D). Taken together, we propose that knockdown of CHIP triggers autophagosome formation by increasing PTEN protein levels and negatively regulating AKT/mTOR activity.

Abnormal activity of the PI3K/AKT/mTOR pathway commonly occurs in neurodegenerative diseases[38]. In AD, a significant loss of PTEN and hyperactivation of the PI3K/AKT/mTOR pathway are associated with impaired clearance of Aβ and tau[72]. Inhibition of mTOR signaling with rapamycin improves cognitive impairment and promotes Aβ and tau clearance by inducing autophagy[73]. However, in PD brains, as well as in SOD1 G93A transgenic mice, the PI3K/AKT/mTOR activity is downregulated[57,74,75].

CHIP is an HSP90/HSP70-interacting E3 ligase that ubiquitinates many unfolded proteins to enhance their degradation and mitigate severe endoplasmic reticulum (ER) stress[76,77]. Given that the unfolded protein response (UPR)-induced ER stress can lead to the induction of autophagy[78], the increased autophagy initiation by CHIP deficiency may also be caused by its effects on the UPR and ER stress.

Although autophagosome formation is increased after CHIP knockdown, the autophagic flux is apparently blocked, as indicated by the tandem mCherry-GFP-LC3B fluorescence and p62 level, similar to the results of Baf A1 treatment, suggesting the impairment of fusion of autophagosomes with lysosomes, or interference with lysosomal degradation. Inhibition of autophagic flux can trigger cell death[45,46]. However, knockdown of CHIP alone does not decrease cell viability. We reason that inhibition of autophagosome-lysosome fusion by CHIP deficiency is limited compared with Baf A1 treatment. However, significant cell death can be induced in CHIP-depleted cells compared with control cells under Baf A1 treatment, suggesting that loss of CHIP increases susceptibility to autophagic cell death.

In summary, we demonstrated that CHIP deficiency leads to abnormal autophagy followed by susceptibility of cells to autophagic cell death.

ACKNOWLEDGMENTS

This work was supported in part by the National Natural Science Foundation of China (31330030 and 31471012), the National Basic Research Development Program of China (2012CB947602), and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Received date: 2015-05-04; Accepted date: 2015-07-01

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Neurosci Bull August 1, 2015, 31(4): 480–490. http://www.neurosci.cn
DOI: 10.1007/s12264-015-1547-3

·Original Article·

Identification of autophagy signaling network that contributes to stroke in the ischemic rodent brain *via* gene expression

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ABSTRACT

Autophagy plays a vital role in cerebral ischemia and may be a potential target for developing novel therapy for stroke. In this study, we constructed an autophagy-related pathway network by analyzing the genes related to autophagy and ischemic stroke, and the risk genes were screened. Two autophagy-related modules were significantly up-regulated and clustered to influence cerebral ischemia. Besides, three key modular genes (NFKB1, RELA, and STAT3) were revealed. With 5-fold cross validation, the ROC curves of NFKB1, RELA, and STAT3 were 0.8256, 0.8462, and 0.8923. They formed a complex module and competitively mediated the activation of autophagy in cerebral ischemia. In conclusion, a module containing NFKB1, RELA, and STAT3 mediates autophagy, serving as a potential biomarker for the diagnosis and therapy of ischemic stroke.

Keywords: cerebral ischemia; autophagy; functional module; stroke; gene expression profile

INTRODUCTION

Ischemic stroke is one of the major causes of death and disability[1-5]. It is generally considered to be a heterogeneous and multifactorial disorder and caused by both conventional environmental risk factors and genetic factors[6, 7]. It can cause acute neuronal death *via* oxygen and nutrient depletion, and result in disruption of the blood-brain barrier[8]. Acute ischemic stroke resulting from intracranial vessel occlusion is associated with high morbidity and mortality[9]. Therefore, it is pivotal to explore the pathogenesis of ischemic stroke. Moreover, since most ischemic strokes (~80%) occur in the territory of middle cerebral artery (MCA), many animal stroke models of middle cerebral artery occlusion (MCAO) have been developed for studies of ischemic stroke[10].

Recent research has shown that ischemic insult activates autophagy, and an autophagic mechanism may contribute to ischemic neuronal injury[11]. Autophagy can be induced by pattern-recognition receptors and stresses such as nutrient depletion, closed head injury, or focal cerebral ischemia. Cerebral ischemia-induced microglial autophagy contributes to ischemic neuronal inflammation and injury. Autophagy is a process for the intracellular bulk degradation of cellular constituents that has multiple effects on immunity[12-14]. Blocking autophagy in epithelial cells enhances host cell death and finally leads to tissue destruction and inflammation[15]. Moreover, autophagy eliminates the abnormal protein aggregates and the damaged organelles in neurons after transient cerebral ischemia[16]. Besides, ischemic stroke increases autophagosomes and activates the autophagy-related pathways (ARPs)[17]. After neonatal hypoxia-ischemia, autophagy increases in neurons, indicating that over-activation of autophagic pathways is a potential protective mechanism in the early stage of brain injury[18]. It has been demonstrated that protective autophagy is induced and further promotes the neuroprotective effect on ischemic stroke by regulating mitogen-activated protein kinase

(MAPK) signals[19]. It has also been reported that global ischemia increases the autophagosomes *via* decreasing autophagosome degradation[16]. The activation of autophagic and lysosomal pathways has been implicated in neuronal injury in a rat model of permanent focal cerebral ischemia[11]. Furthermore, by regulating the TSC2-mTOR-S6K1 signaling pathway, autophagy is induced and further promotes neuronal survival during cerebral ischemia[20].

Based on the above findings, autophagy plays a vital role in cerebral ischemia, and may be a potential target for developing novel therapies for stroke. Thus, it is essential to explore the mechanism of autophagy in cerebral ischemia. In the current study, by using gene-expression and network information, we predicted autophagy-related genes that are highly correlated with ischemic stroke.

MATERIALS AND METHODS

Screening for Differentially-expressed Genes in Ischemic Brain

The National Center for Biotechnology Information (NCBI) GEO database[21] (http://www.ncbi.nlm.nih.gov/geo/) was screened to retrieve the gene expression profiles of the ischemic brain. Through searching for "ischemic brain" in the NCBI GEO database, 3007 results in 158 series were obtained from the DataSets database. Subsequently, we selected "series" for Entry type and "Expression profiling by array" for Study type. Consequently, 54 candidate series of *Mus musculus* and 40 candidate series of *Rattus norvegicus* were acquired. Then, several criteria were applied to screen the candidate series: (1) the tissue must be brain; (2) the study was not related to the reaction of ischemic brain; (3) eliminating series exposed to drug research; (4) eliminating series dealing with hypoxia-ischemia/reperfusion; (5) eliminating samples subjected to MCAO for >24 h. Finally, three series (GSE38037, GSE32529, and GSE58720) were screened for further study, and the samples with MCAO were the test groups. In GSE38037, there were eight samples (4 normal and 4 test samples)[22]. In GSE32529, there were 224 samples including 6 experimental conditions such as LPS treatment + ischemic challenge, and CpG treatment + ischemic challenge[23, 24]. In this study, the samples of "brain-unhandled" (i.e. non-treated) were selected as the normal control, and the brain-ischemia challenged (3 h and 24 h) samples were used as test groups. Finally, 6 normal and 8 test groups were obtained. In GSE58720, the sham and MCAO groups were measured at 24 h after reperfusion. Here, we considered the sham samples as the control group and the MCAO samples as the test groups. Finally, 3 test samples and 3 control groups were selected in GSE58720. The gene expression of each sample was estimated and normalized. Each probeset ID was mapped to gene symbol according to the corresponding platform. If multiple probsets were mapped to the same gene, the expression value for the gene was summarized as the arithmetic mean of the values of multiple probesets (on the log2 scale).

Gene Expression Significance Analysis

With the "MetaDE" package of R project, we merged the three expression profiles and screened the differentially-expressed genes (DEGs) by false discovery rate (FDR) <0.01. Then, Database for annotation, Visualization and Integrated Discovery 6.7 (DAVID, http://david.abcc.ncifcrf.gov/home.jsp) software was used to explore the functions of the DEGs, including biological processes and pathways. P <0.05 was set as the threshold used for enrichment analysis of the GO_BP and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

Construction of Autophagy-Related Pathway Network

There were 7 ARPs in KEGG (http://www.kegg.jp/kegg/pathway.html): Regulation of autophagy, Lysosome, FoxO signaling pathway, AMPK signaling pathway, Prion diseases, Shigellosis, and mTOR signaling pathways. All the autophagy-related pathways and pathway genes were extracted. To systematically analyze the role of autophagy genes in cerebral ischemia, the STRING v10 database (http://string-db.org/) was used to obtain a protein-protein interaction network of *Mus*. By integrating the protein interaction information of DEGs and autophagy-related pathway-gene information, we constructed an ARP[13] complex network. Four topological properties (degree, average shortest path length, closeness centrality, and clustering coefficient of the ARP network) were analyzed by the "network analyzer" plugin in Cytoscape software (www.cytoscape.org/).

Functional Module Analysis

The "MINE" plugin of Cytoscape was used to identify functional modules in the ARP networks, using a cutoff value for the connectivity degree of nodes (proteins in the network >2). Then, we set the module as a unit and achieved the module expression in different samples by calculating the average expression value of genes that were in the module.

$$M_{ik} = \frac{\sum_{j=1}^{n} S_{jk}}{n} \quad (1)$$

where M_{ik} is the expression value of module i in sample k. n is the number of genes in module i. S_{jk} is the expression value of module gene j in sample k.

For the "module expression profile", we further utilized the "limma" package of R project to screen the differentially-expressed modules between the disease and normal groups. Modules with P <0.05 were considered to be significantly expressed.

Identification of Potential Risk Genes

The important autophagy genes in ischemic stroke were screened based on the network topological properties in the ARP network, using Neighborhood scoring (NS) and Interconnectivity (ICN) optimization algorithms. The NS algorithm was based on the distribution of genes in the network and the expression level to screen the candidate genes[25]. Based on the NS, we weighted every node in the network by combining the fold change of the candidate node and its neighborhoods. If the node was differentially expressed and was linked directly to multiple nodes that were also DEGs, then we considered that the node and its neighbors were correlated. Thus, we inferred that the node was a potential target or diagnosis marker. We followed formula (2) to score the node.

$$Score(i) = \frac{1}{2} * FC_i + \frac{1}{2} * \frac{\sum_{j=1}^{N_i} FC_j}{|N_i|} \quad (2)$$

in which i is the candidate gene i in the network. FC_i represents the fold change of gene i between disease samples and normal samples. N_i is the number of neighbors for node i. j is the neighbor node of candidate gene i. Considering the properties of node self, if node i or its neighbors were not differentially expressed, then the $Score(i)$ was 0.

The ICN[26] mainly calculates the correlation between the node and all the DEGs, so we followed formula (3) to weight the genes.

$$Score(i) = \frac{1}{n} * \sum_{j=1}^{n} \frac{2 + N_{i,j}}{\sqrt{N_i * N_j}} \quad (3)$$

where n is the number of all DEGs. N_i is the number of interacting genes for gene i, and N_j is the number of interacting genes for gene j. $N_{i,j}$ is the number of genes shared by gene i and DEG j. In this algorithm, single nodes were rejected. All the genes were ranked in descending order, and a node with a higher score was considered to be more important.

The NS focuses on the expression variation of the node itself and the affected nodes. If more genes are affected and the fold change is more evident, then the gene is more important than other genes in disease development. The ICN focuses on the correlation between the candidate gene and DEGs. If the degree of correlation of a gene with all DEGs is higher, then that gene would be considered more important than others.

RESULTS

Differentially-Expressed Genes

By combining the three expression profiles (GSE38037, GSE32529 and GSE58720), we acquired 15 ischemic samples and 13 normal samples. With the "limma" package of R project, we finally acquired 337 common significant DEGs (P <0.05 and FDR <0.01) among GSE38037, GSE32529, and GSE58720 (Fig. 1A; Table S1). After functional enrichment, the common DEGs were involved in the Ribosome, Toll-like receptor signaling pathway, MAPK signaling pathway, and the Chemokine signaling pathway, among others (Fig. 1B). Besides, these DEGs played important roles in such processes as vasculature development, blood vessel development, and the regulation of angiogenesis (Fig. 1C).

ARP Network Analysis

Four hundred and ninety-two genes related to the autophagy-associated pathway were acquired from the KEGG database. By integrating the DEGs and autophagy-related genes, an ARP network was constructed that included 1356 nodes and 1983 edges (Fig. 2). We calculated the closeness centrality, average clustering

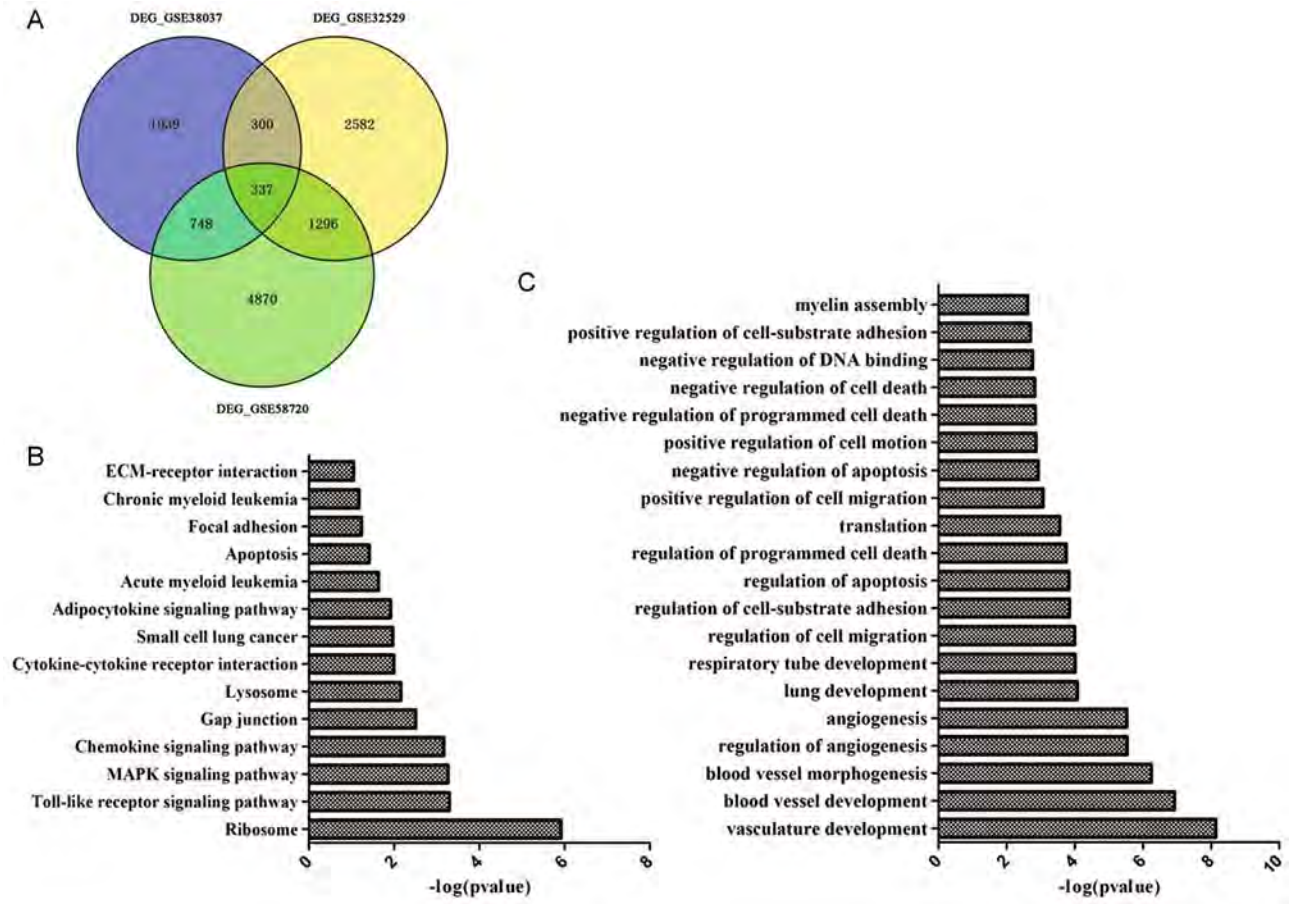

Fig. 1. Differentially-expressed genes (DEGs) acquired from different studies and function analysis of common DEGs. A, relationships of DEGs among GSE38037, GSE32529, and GSE58720; B, significantly enriched pathways of common DEGs; C, significant biological processes regulated by common DEGs. The larger –log (*P* value), the more important the function.

coefficient, degree distribution, and average shortest path length (Fig. 3). The degree of nodes in the ARP network followed a power-law distribution, and had small-world network characteristics such as a large average clustering coefficient and a short average shortest path length.

Module Analysis in ARP Network

The functional module expression was built for 15 functional modules (Fig. 4A), and two differentially-expressed modules were identified including modules 4 and 10 (Fig. 4B). Using the five-fold cross-validation test, we acquired the ROC curves of all modules (Table 1). Modules 4 and 10 had the highest classification accuracy, with ROC curves >0.85.

Key Autophagy Genes

We ranked the genes acquired by NS and ICN in descending order and screened the top 50 genes. Subsequently, we analyzed these 50 genes and obtained five common genes: NFKB1, RELA, STAT3, JAK2, and SHC1. Genes in the same module had similar or the same biological functions to affect the development of cerebral ischemia. Thus, it was likely that the three genes STAT3, NFKB1, and RELA in modules 4 and 10 affect autophagy in the development of ischemic stroke. Here, we focused on the analysis of STAT3, NFKB1 and RELA. At the gene-expression level, the three genes were up-regulated in the disease groups compared with the normal groups (Fig. 4). Five-fold cross validation showed that the ROC curves of all the risk genes were >0.80 (Fig. 4C). Therefore, these genes may be potential risk genes in the development of ischemic stroke, which was highly correlated with the

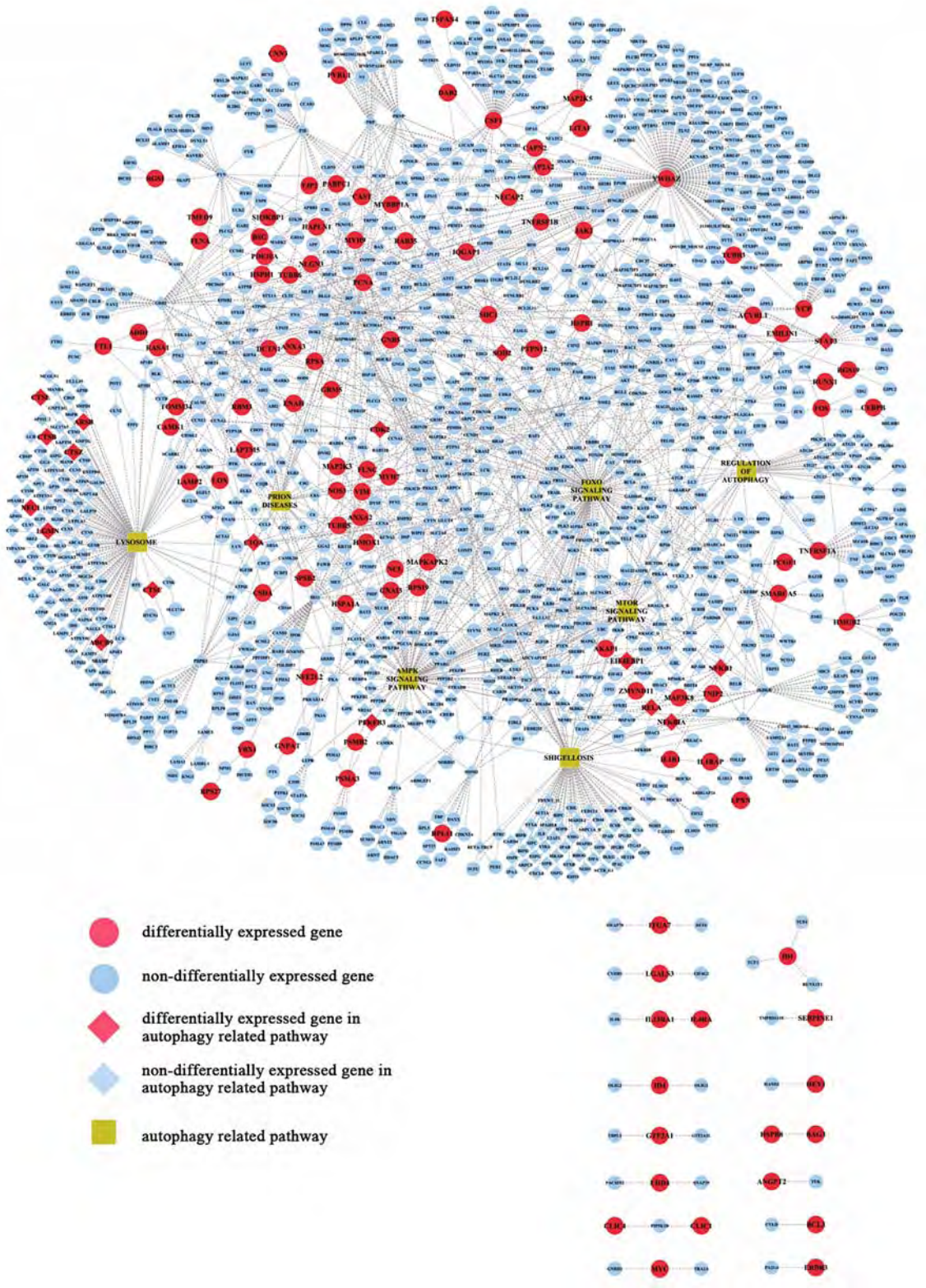

Fig. 2. The autophagy-related pathway network. Red circles, autophagy-related DEGs; blue circles, autophagy-related genes that were not differentially expressed between disease and normal groups; yellow squares, autophagy-related pathways.

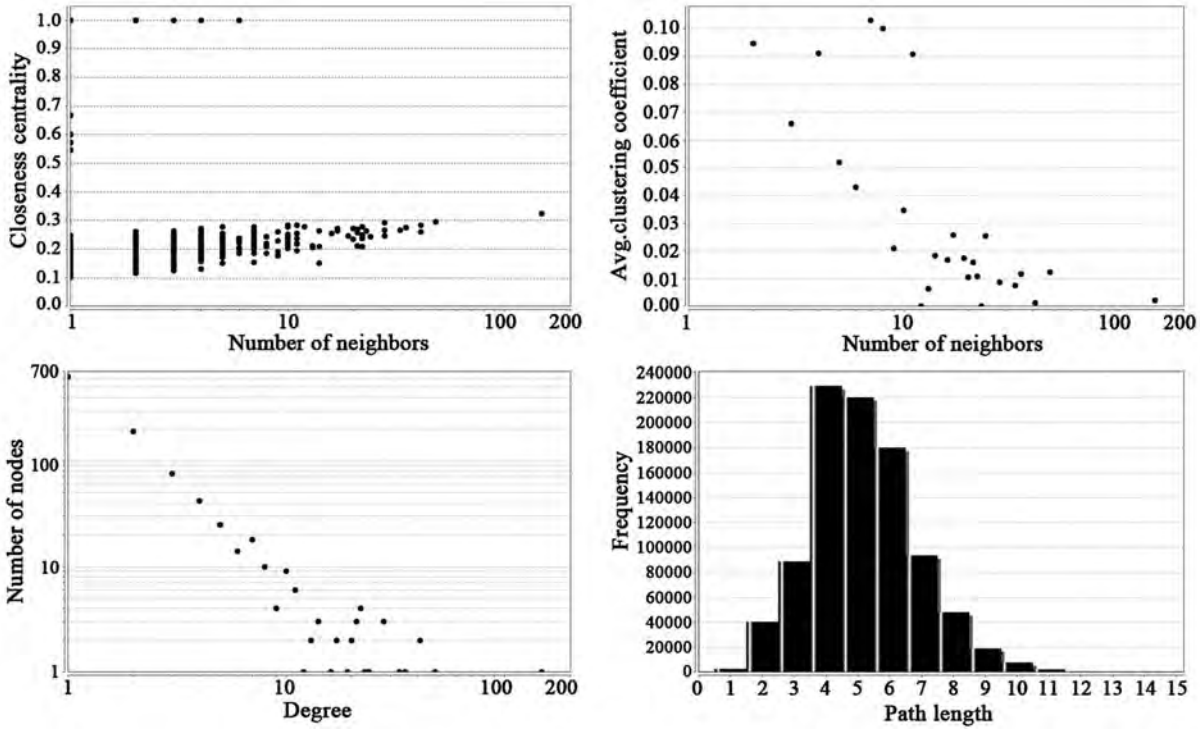

Fig. 3. Topological properties of the ARP network. Closeness centrality, average clustering coefficient, degree distribution, and average shortest path length.

Table 1. Properties of all modules in the ARP network

Modules	Nodes	Edges	P Value	ROC curve	Modular genes
module_1	5	8	0.0313	0.7282	TGFBR2, ACVRL1, CDH5, ENG, TGFBR1
module_2	6	9	0.0019	0.7641	INPP5D, CD22, SOS1, CBL, SHC1, GRB2
module_3	5	7	0.2016	0.5538	PRNP, HSPA5, APP, KCNMA1, PRP
module_4	**5**	**7**	**0.0006**	**0.8769**	**IL6ST, GHR, SHC1, JAK2, STAT3**
module_5	4	5	0.0851	0.6718	CCND1, CDKN1B, CDK4, P27
module_6	4	5	0.1274	0.7641	TNFSF6, FASL, PSTPIP1, PTPN12
module_7	4	5	0.1055	0.6872	PSMA1, PPP2R1A, PSMA3, PSMB2
module_8	4	5	0.1799	0.6256	AP2B1, DNAJC6, AP2A2, YWHAZ
module_9	4	5	0.1373	0.6718	LMNA, WDFY2, AKT, FOXO1
module_10	**3**	**3**	**0.0053**	**0.8513**	**HDAC1, RELA, NFKB1**
module_11	3	3	0.0044	0.7128	BLNK, GRB2, SH3KBP1
module_12	3	3	0.2375	0.6154	RICTOR, MAPKAP1, FRAP
module_13	3	3	0.1318	0.6769	AP1B1, CALM3, YWHAZ
module_14	3	3	0.2311	0.6564	CCNA2, ABL1, CDK2
module_15	3	3	0.0599	0.7231	CLTA, KCNMA1, YWHAZ

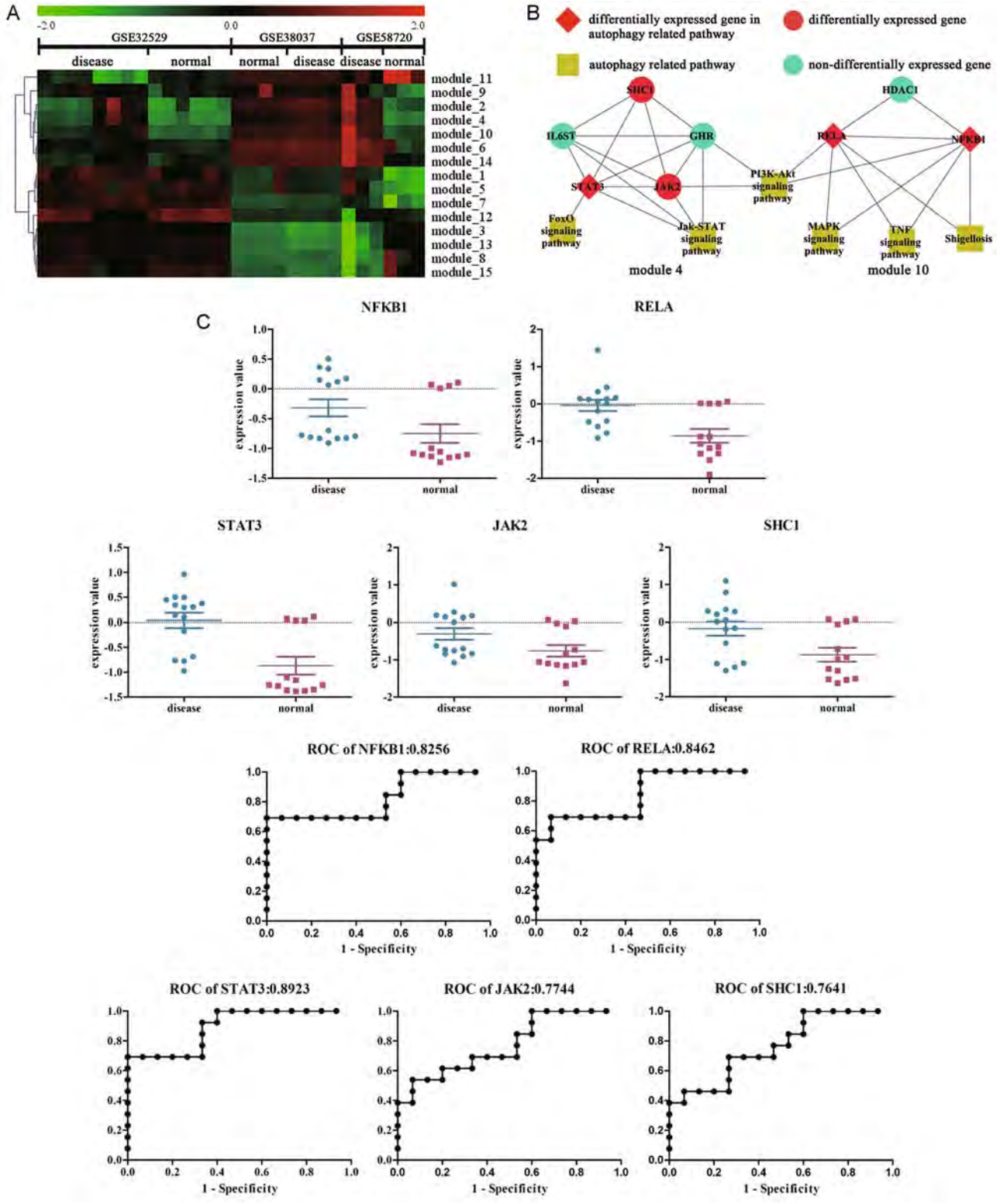

Fig. 4. Modules in the ARP network. A, heat map analysis of module expression. Green to red represents low to high expression. B, differentially-expressed modules in the ARP network. C, expression levels and ROC curves of NFKB1, RELA, STAT3, JAK2, and SHC1.

autophagy related pathways in the ARP network.

DISCUSSION

Autophagy is involved in neuronal death following cerebral ischemia, and plays a pivotal role in the course of ischemic stroke[27-29]. Moreover, autophagic pathways such as the mTOR pathway are closely related to the ischemic brain[30]. However, the regulatory network has not been fully explained. In this study, we used the gene expression profile of ischemic stroke from the GEO to explore possible pathogenesis associated with autophagy. By constructing a gene regulation network, we mined highly-correlated modules, and screened autophagy risk genes. Fifteen modules were obtained from the new network, among which modules 4 and 10 were significantly dysregulated, with ROC values >0.8. These two modules contained 5 DEGs, including RELA, NFKB1, and STAT3, and these three genes are closely associated with the autophagic pathway.

The autophagy-related modules were significantly up-regulated and clustered to influence cerebral ischemia. These modules are involved in several pathways including the FOXO signaling pathway and MAPK signaling pathway. The FOXO pathway was associated with most of the modules, and positive regulation of modules would promote the activation of FOXO pathways. Indeed, activation of FOXO pathways is neuroprotective against transient global cerebral ischemia[31, 32]. The MAPK signaling pathway plays a significant part in cerebral ischemia[33-35]. MAPK is an upstream regulator of mTORC1 and autophagy can be induced *via* the MAPK-mTOR signaling pathway[19]. In fact, the mTOR signaling pathway that induces autophagy has neuroprotective effects on ischemic stroke[19]. This protective autophagy is favorable in the treatment of stroke and avoids unfavorable side-effects[19]. In addition, our results indicated that genes in two modules interacted with each other and commonly stimulated the PI3K-Akt signaling pathway. Thus, our results verified previous studies showing that autophagy-related modules may play vital roles in cerebral ischemia.

Among all the modular genes in the autophagy-related pathway, three risk genes (NFKB1, RELA, and STAT3) that were differentially expressed in cerebral ischemia were extracted. NFKB1 (also known as NFKB or p50) and RELA form a complex in the NF-κB signaling pathway. Commonly, they form a dimer. IκBα, an inhibitor of NF-κB, combines with the dimer, sequestering this complex outside the nucleolus. Once IκBα is degraded, the dimer would be translocated into the nucleolus, initiating transcription[36] and regulating the expression of numerous genes involved in immunity, inflammation, proliferation, and apoptosis[37, 38]. Several studies have indicated that dysregulation of these three factors is significantly involved in brain diseases, while suppression of the NF-κB signaling pathway contributes to neuroprotection against cerebral ischemia[39-41]. NF-κB is activated after cerebral ischemia and can potentiate ischemic injury, activating many genes involved in the pathogenesis of cerebral ischemia, such as TNF-α, IL-1β, IL-6, ICAM-1, iNOS, and COX-2[42]. Results from immunohistological assay and western blot for NF-κB (p65) in the ischemic penumbra of rats showed that flurbiprofen inhibits NF-κB to protect against cerebral ischemia/reperfusion injury[40]. Brain tissue damage has also been correlated with the dysregulated NFKB1[43], blockade of which protects the brain against ischemic damage by regulating inflammatory responses[44, 45]. NFKB1 combines with RELA to form the NFKB1/ RELA complex that is a key molecule in the progression of ischemic stroke[46]. Notably, NFKB1/RELA induces pro-apoptotic transcription in acute brain ischemia and its activation is an important event in ischemic neuronal injury[47]. Blocking NF-κB activation or knocking out the p50 subunit of NF-κB can protect against infarct injury or can result in the development of a smaller infarct volume[48]. Moreover, IκBα is an inhibitor of NF-κB, and its dysregulation prevents brain damage after stroke. The activation of signal transducer and activator of transcription-3 (STAT3), a pivotal part of the JAK-STAT signaling pathway, is reportedly induced by cerebral ischemia[49-51]. Increased expression of the transcripts of IL-6 and JAK2, which are the essential components of STAT3 activation, is also related to the pathogenesis of cerebral ischemic damage[51]. All three risk genes were differentially expressed in cerebral ischemia. It is likely that their dysregulation modulates the development of cerebral ischemia and targeting the three genes would reduce post-ischemic brain injury.

Recent findings have demonstrated that NF-κB complex-related factors (NFKB1 and RELA) also affect the autophagy pathway in cerebral ischemia[52]. Previous studies have shown that RELA and NFKB1 modulate

canonical autophagy and their activation mediates the repression of autophagy[53-55]. RELA has been reported to modulate an increase of autophagy by BECN1[56]. However, in mice with focal cerebral ischemia and NFKB1 knockout, there are significantly more Beclin-1/TUNEL-positive cells than in wild type mice[52], indicating that cerebral ischemia-induced autophagy-like injury is regulated by the NF-κB pathway. It has also been found that a high-fat diet is cardioprotective against ischemia-reperfusion injury involving NF-κB dependent enhancement in autophagy and decreased apoptosis[57]. Moreover, variations within NFKB1 and IκBα can potentially influence the function of NF-κB[58] and in turn the autophagy process. As autophagy participates in the neuronal death and functional loss induced ischemia/reperfusion injury[59-61], genes involved in autophagy would be associated with cerebral ischemia. Both RELA and NFKB1 were up-regulated in cerebral ischemia according to our research and further activated the NF-κB pathway. It has been validated that the NF-κB pathway regulates the autophagy-like injury induced by cerebral ischemia[52]. Besides, inhibition of NF-κB *in vivo* prevents cerebral ischemic injury[62]. IκBα interacts with RELA and NFKB1 to form a complex and competitively regulates autophagy in the development of cerebral ischemia. Autophagy induced by the complex in cerebral ischemia would protect against neuronal injury.

In conclusion, Autophagy is a basic catabolic progress for cell survival under stress. Using a computational bioinformatics approach, we obtained two autophagy-related modules. In accordance with previous reports, our result also verified that the FOXO and MAPK signaling pathways are important in cerebral ischemia. Besides, NF-κB complex-related factors (NFKB1 and RELA) and STAT3 play a key role in inducing autophagy in the development of cerebral ischemia, and are potential targets of therapy.

ELECTRONIC SUPPLEMENTARY MATERIAL

Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s12264-015-1547-3.

ACKNOWLEDGEMENTS

This work was supported by a Shanghai Leading Talents Project (54[2012]) and a Pudong New Area Leading Talents Project of Health System (PWR12011-02).

Received date: 2015-04-30; Accepted date: 2015-07-09

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Neurosci Bull August 1, 2015, 31(4): 491–504. http://www.neurosci.cn
DOI: 10.1007/s12264-015-1546-4

·Original Article·

Autophagy is involved in oral rAAV/Aβ vaccine-induced Aβ clearance in APP/PS1 transgenic mice

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ABSTRACT

The imbalance between β-amyloid (Aβ) generation and clearance plays a fundamental role in the pathogenesis of Alzheimer's disease (AD). The sporadic form of AD is characterized by an overall impairment in Aβ clearance. Immunotherapy targeting Aβ clearance is believed to be a promising approach and is under active clinical investigation. Autophagy is a conserved pathway for degrading abnormal protein aggregates and is crucial for Aβ clearance. We previously reported that oral vaccination with a recombinant AAV/Aβ vaccine increased the clearance of Aβ from the brain and improved cognitive ability in AD animal models, while the underlying mechanisms were not well understood. In this study, we first demonstrated that oral vaccination with rAAV/Aβ decreased the p62 level and up-regulated the LC3B-II/LC3B-I ratio in APP/PS1 mouse brain, suggesting enhanced autophagy. Further, inhibition of the Akt/mTOR pathway may account for autophagy enhancement. We also found increased anti-Aβ antibodies in the sera of APP/PS1 mice with oral vaccination, accompanied by elevation of complement factors C1q and C3 levels in the brain. Our results indicate that autophagy is closely involved in oral vaccination-induced Aβ clearance, and modulating the autophagy pathway may be an important strategy for AD prevention and intervention.

Keywords: oral vaccination; autophagy; Akt/mTOR pathway; Aβ clearance; Alzheimer's disease

INTRODUCTION

Alzheimer's disease (AD) is characterized by increased amounts of β-amyloid (Aβ) species and has threatened millions of aged individuals. There is an urgent need for the development of novel therapeutic strategies for the prevention of AD, considering the increasingly serious aging trend[1]. The amyloid hypothesis proposes that dyshomeostasis in Aβ generation and clearance is essential for disease progression[2]. The central role of the Aβ cascade in the etiology of AD provides an important target for drug research and development. The sporadic form of AD, which accounts for >95% of all AD cases[3], has

been reported to show impaired clearance of Aβ from the brain[4], indicating that Aβ clearance is a potent target for the treatment of AD.

Aβ immunotherapy was initially developed in 1999 by Schenk *et al.*[5] and has attracted much interest for its therapeutic effects on brain Aβ pathology[6]. However, the failure of AN1792 due to the emergence of severe adverse reactions in clinical trials calls for the development of safer vaccines[7]. Phase III clinical trials of bapineuzumab and solanezumab also found no improvement in clinical outcomes in AD patients, while suggesting that anti-Aβ intervention should focus on the early stage of disease progression[8,9]. We previously developed an oral vaccine with a recombinant adeno-associated viral vector carrying Aβ1-43 cDNA (referred to as rAAV/Aβ), and animal studies demonstrated that oral vaccination significantly reduces the brain Aβ burdens without causing neuroinflammation and lymphocytic infiltration[10,11], suggesting it is a safe and effective vaccine for the treatment of AD. However, the underlying mechanism responsible for oral vaccination-induced Aβ clearance is still incompletely understood.

Autophagy plays a vital role in maintaining intracellular homeostasis and degrading abnormal protein aggregates[12]. Autophagy deficiency in the brain may contribute to Aβ accumulation and worsen cognition in AD mouse models[13]. We previously found that a presenilin-1 mutation results in pronounced intracellular Aβ42 deposition in neurons without causing amyloid plaque formation, which is correlated with accelerated neurodegeneration in aged mice[14]. The enhancement of autophagy by chemical or genetic approaches can protect against Aβ-induced neuronal damage and improve cognition[15,16]. Recent findings have uncovered a novel function of autophagy in the immune response, including the processing or elimination of antigens[17]. Besides, there is increasing evidence that autophagy can be activated in the context of viral infection or vaccination, which is in turn beneficial to the immune response[18,19].

We have previously found a dramatic reduction in brain Aβ content in Tg2576 mice with oral vaccination. However, whether autophagy is involved in the oral vaccination-induced Aβ clearance from the brain remains to be elucidated. The present study was designed to address the hypothesis that activation of autophagy participates in oral vaccination-related Aβ clearance.

MATERIALS AND METHODS

Construction of the Vaccine

The rAAV/Aβ vaccine and rAAV/GFP were designed and kindly provided by Takeshi Tabira (Juntendo University) and Hideo Hara (Saga University). The construction procedures are described in detail in previous studies[10,11].

Cell Culture and Treatment

Human embryonic kidney (HEK) 293 cells were cultured in DMEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) at 37°C in an atmosphere of 5% CO_2. Cells were passaged at a ratio of 1:6 when grown to 90% confluence. To verify that the vaccine was successfully constructed, cells at 70% confluence were divided into 3 groups, two were incubated with 10 μL rAAV/Aβ vaccine (containing 5×10^{11} genomes) or the same titer of rAAV/GFP for 48 h. Cells in the control group were treated with the same volume of PBS (pH 7.4). Sodium butyrate (Sigma-Aldrich, St. Louis, MO) at a final concentration of 2 mmol/L was added to all three groups of cells to promote gene expression[20]. Media and cells were harvested at the indicated time points. Complete proteinase inhibitor cocktail (Roche, Indianapolis, IN) was added into the media to prevent Aβ degradation and the cell debris was removed by centrifugation at 3000 × g for 10 min at 4°C[21].

Animals and Oral Vaccination

Male APPswe/PS1dE9 (referred to as APP/PS1) transgenic mice (5–6 months old) were raised in groups of 3 to 4 per cage under standardized housing conditions on a 12 h/12 h light/dark cycle, with food and water *ad libitum*. APP/PS1 mice develop pathology typical of Aβ deposition and cognitive impairment at the age of 5 to 6 months[22,23]. The experimental procedures were approved by Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch. Mice were divided into 3 groups (n = 10/group): PBS (control); rAAV/GFP (vehicle), and rAAV/Aβ (vaccine).

Oral immunization was carried out based on our previous research[10,11]. In brief, rAAV/GFP and rAAV/Aβ were diluted in PBS to give 5×10^{11} genomes/100 μL. APP/PS1 mice (5 to 6 months old) in the vaccine group were given 5×10^{11} genomes of rAAV/Aβ once through an orogastric tube. Mice in the vehicle group were treated with

5×10^{11} genomes of rAAV/GFP in the same manner. Age-matched control mice received 100 μL PBS once *via* an orogastric tube.

Sample Preparation

For ELISA and immunoblotting analysis, mice were sacrificed 4 weeks after oral vaccination. Brain tissues were dissected immediately and stored at −80°C until use. Tissues for ELISA assessment were further prepared as described[21,24]. In brief, brain tissues were homogenized in 5 volumes (*w*/*v*) of 1% Triton X-100 in Tris-buffered saline (25 mmol/L Tris and 137 mmol/L NaCl, pH 7.6) containing protease inhibitor cocktail (Roche). Homogenates were centrifuged at 100 000 g for 60 min at 4°C and the supernatant was collected as the Triton X-100-soluble fraction. The pellets were sonicated in 5 mol/L guanidine HCl in 50 mmol/L Tris (pH 8.0) in the presence of protease inhibitor cocktail (Roche). After incubation for 2 h at 25°C, the mixture was centrifuged at 13 000 g for 20 min at 4°C. The supernatant was 10-fold diluted and used as the guanidine HCl-soluble fraction.

ELISA

Aβ40 and Aβ42 levels in media and cell lysates were measured by sandwich ELISA according to the manufacturer's instructions (Wako, Japan). The Aβ concentration was normalized based on the number of cells in each culture (as determined by protein content in cell lysates)[21,25].

Aβ40 and Aβ42 levels in the detergent-soluble and guanidine HCl-soluble fractions were measured using commercially-available ELISA kits as noted in previous reports[21,25], with minor modifications, and calibrated with synthetic Aβ peptides. The described method is able to measure the Aβ40 or Aβ42 contents in brain samples with high specificity[21,25,26]. The final values of Aβ content were expressed as pmol per wet weight of brain tissue.

Complement C1q and C3 levels in brain tissues were assessed according to the manufacturer's instructions (Cloud-Clone Crop, China). Data were normalized to control. All ELISA assays were performed in duplicate.

Detection of Serum Anti-Aβ Antibody and Characterization of Antibody Isotypes

The concentration of anti-Aβ antibody in serum was determined as described[11], with minor modifications. Plates were coated with 4 μg/mL synthetic human Aβ42 dissolved in 55 mmol/L $NaHCO_3$ (pH 8.3) overnight at 4°C. Plates were washed three times with washing buffer and blocked with 1% BSA and 2% normal goat serum in PBS for 1 h at 37°C. After three washes, plates were incubated with mouse serum samples diluted in blocking buffer for 1 h with shaking. Then plates were washed and incubated with HRP-conjugated goat anti-mouse IgG for 1 h at 37°C. The chromogen substrate 3,3',5,5'-tetra-methylbenzidine was added to the plates after three washes, and incubated at 37°C for 30 min in the dark. H_2SO_4 (2 mol/L, 50 μL) was added to stop the reaction. The absorbance of each well at 450 nm was read with a microplate reader. The isotypes of IgG antibodies were further determined using mouse immunoglobulin isotyping ELISA kits (BD Bioscience PharMingen, San Jose, CA) according to the manufacturer's instructions. All measurements were performed in duplicate and data were normalized to control.

Immunofluorescence Imaging

Samples for immunofluorescence imaging were prepared as reported[27]. At 4 weeks after oral vaccination, mice were anesthetized with 5% chloral hydrate and perfused intracardially with 0.9% saline followed by ice-cold phosphate-buffered 4% paraformaldehyde (PFA, Sigma-Aldrich) at pH 7.4. Tissue samples were post-fixed overnight in 4% PFA and equilibrated in phosphate-buffered 30% sucrose for 48 h at 4°C. The brains were then sectioned on a frozen microtome (Leica, Bannockburn, IL) at 30 μm in the coronal plane. Sections were collected in 12-well plate and stored at −20°C in an antifreeze solution of phosphate buffer containing 30% glycerol and 30% ethylene glycol.

Immunofluorescence staining was conducted as described previously, with minor modifications[25,27]. Free-floating sections were incubated in PBS at room temperature for 30 min, then rinsed with PBS-Triton X-100 before incubation in 0.3% H_2O_2 for 30 min. Sections were then incubated overnight at 4°C with 6E10 (1:120; Covance, Princeton, NJ), a monoclonal antibody against Aβ, followed by incubation with Alexa-Fluor 488 conjugated secondary antibodies (Molecular Probes, Eugene, OR) for 1 h at room temperature. Nuclei were stained with Hoechst 33258 (Invitrogen, Carlsbad, CA). Sections were

washed and then imaged using a confocal laser scanning microscope (Olympus, Japan). No signal was detected when the primary antibody was omitted.

Western Blotting Analysis

Western blotting analyses were performed according to previous reports[10,11,28]. The dissected brain tissues were lysed on ice in lysis buffer containing 50 mmol/L Tris-HCl, pH 6.8, 8 mol/L urea, 5% β-mercaptoethanol, 2% SDS, and protease inhibitors. Lysates were collected and centrifuged at 12 000 g for 10 min at 4°C. The protein content was determined using a BCA protein assay kit (Pierce, Rockford, IL). Proteins (40 to 60 μg) were separated by SDS-PAGE electrophoresis and then transferred to 0.45 μm polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked for 1 h in 5% (*m*/*v*) nonfat milk in Tris-buffered saline (pH 7.5) supplemented with 0.1% Tween-20, and incubated with primary antibodies against LC3B (1:4 000, Novus, Littleton, CO), p62 (1:5 000, MBL, Nagoya, Japan), mTOR (1:1 000, CST, Danvers, MA), pmTOR (Ser2448, 1:1 000, CST), Akt (1:1 000, CST) or pAkt (Ser473, 1:1 000, CST) overnight at 4°C. β-actin (1:5 000, Sigma-Aldrich) was used as an internal control. The membranes were then incubated with peroxidase-conjugated secondary antibodies, and protein bands were developed with the ECL system (Millipore).

Statistical Analysis

Data are expressed as mean ± SEM and were analyzed by GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). All data were tested for normal distribution and for homogeneity of variance before analysis of variance (ANOVA). Data that fit a normal distribution and with homogeneous variance were analyzed using one-way ANOVA followed by Tukey's *post hoc* test. Otherwise, data were analyzed by the Kruskal-Wallis non-parametric test with Dunn's Multiple Comparison Tests. P <0.05 was considered statistically significant.

RESULTS

rAAV/Aβ Vaccine Transfection Increased Aβ Secretion in HEK293 Cells

To verify the successful construction of the vaccine, we transfected it into HEK 293 cells and evaluated Aβ expression. We found that co-incubation of 5×10^{11} genomes of vaccine particles (rAAV/Aβ) for 48 h resulted in a significant increase in Aβ40 and Aβ42 content in both cell lysates and the media (Fig. 1, P <0.001). No significant difference was found in Aβ40 and Aβ42 levels between the rAAV/GFP and PBS groups (Fig. 1). Therefore, the constructed vaccine was able to express large amounts of Aβ peptides in mammalian cells, indicating that it may be an efficient antigen.

Oral Vaccination with rAAV/Aβ Significantly Attenuated Brain Aβ Burden in APP/PS1 Transgenic Mice

ELISA analysis (Fig. 2A, B) showed a remarkable reduction in soluble Aβ40 and Aβ42 contents in the brain of mice with oral rAAV/Aβ vaccination, compared with rAAV/GFP- and PBS-treated mice (Fig. 2A, P <0.01). Similar results were found for insoluble Aβ40 and Aβ42 (Fig. 2B, P <0.001). Mice treated with rAAV/GFP had a brain Aβ burden similar to PBS-treated mice (P >0.05). The decrease in the brain Aβ burden was further supported by immunofluorescent imaging. The brain Aβ load in the cerebral cortex and hippocampus of APP/PS1 mice following oral rAAV/Aβ vaccination was markedly lower than that in rAAV/GFP- and PBS-treated mice (Fig. 2C). These results together demonstrated that the developed vaccine was capable of improving Aβ pathology in APP/PS1 mice.

Oral Vaccination with rAAV/Aβ Increased Concentrations of Serum Anti-Aβ Antibody and Elevated Brain Complement Levels in APP/PS1 Mice

Antibodies against Aβ are known to be capable of preventing Aβ deposition and disrupting Aβ assembly[29]. Here, serum anti-Aβ antibodies were significantly elevated in APP/PS1 mice with rAAV/Aβ vaccination (Fig. 3A). The titers of the antibodies were much higher than those in the vehicle and control groups (Fig. 3A; P < 0.001), indicating the efficacy of the developed vaccine in triggering the immune response in APP/PS1 mice. We next determined the isotypes of the antibodies in sera from all three groups. We found that IgG1, IgG2a, and IgG2b, main immunoglobulins of the IgG family, were increased after oral vaccination (Fig. 3A). IgG2b was the predominant Ig isotype in rAAV/Aβ-vaccinated mice, followed by IgG1, while the concentration of IgG2a was slightly increased. The pattern of antibody isotypes

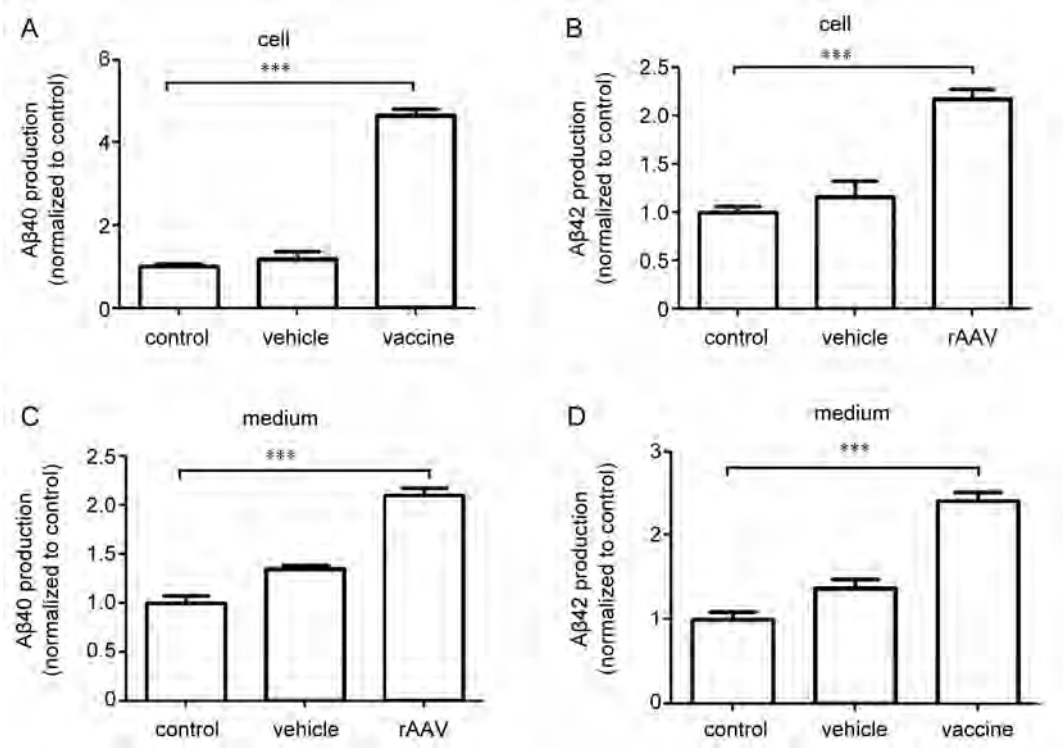

Fig. 1. ***In vitro*** **characterization of recombinant AAV/Aβ vaccine. HEK293 cells were co-incubated with rAAV virus for 48 h and cell lysates and media were harvested to quantify Aβ levels using specific ELISA kits. Aβ40 (A) and Aβ42 levels (B) in cell lysates; Aβ40 (C) and Aβ42 (D) in the media were assessed and compared in all three groups (mean ± SEM; ******P* **<0.001** ***vs*** **control,** ***n*** **= 3; one-way ANOVA).**

implied that oral vaccination induced Th2-based immune responses.

Activation of the complement system is a common physiological reaction in the immune response[30]. It has been reported that brain Aβ deposits are surrounded by small amounts of IgG and complement factors C1q and C3[31], indicating a possible role of these components in limiting Aβ pathology[32]. To determine whether oral vaccination with rAAV/Aβ activates the complement system, we assessed the content of C1q and C3 in the brains of mice from all three groups. The C1q and C3 content was notably higher in rAAV/Aβ-treated mice than in rAAV/GFP- and PBS-treated mice (Fig. 3B, P <0.05; Fig. 3C, P < 0.01). There was no difference in the C1q and C3 content between the rAAV/GFP and PBS groups. The changes of brain C1q and C3 levels after oral vaccination also indicate a possible participation of the complement system in vaccine-induced Aβ clearance.

Activation of Autophagy Function in Brains of APP/PS1 Mice with Oral rAAV/Aβ Vaccination

Autophagy has been found to closely participate in Aβ metabolism[33]. However, little is known about the role of autophagy in AD immunotherapy. In order to clarify whether oral vaccination with rAAV/Aβ vaccine enhanced autophagy in the brain, we examined several markers of autophagy (LC3B and p62) in all the three groups. We found that the p62 levels in the brains of rAAV/Aβ-vaccinated mice were strikingly lower than those of the rAAV/GFP and PBS mice (Fig. 4B, P <0.05). The autophagosome marker LC3B-II and the LC3B-II/LC3B-I ratio were markedly increased in brains of mice undergoing rAAV/Aβ vaccination (Fig. 4C, P <0.05). Note that the rAAV/GFP (vehicle) itself did not alter autophagy as the LC3B and p62 levels were unchanged after its administration (compared with PBS-treated mice). These results imply that the activation of autophagy may play a part in oral vaccination-induced Aβ clearance.

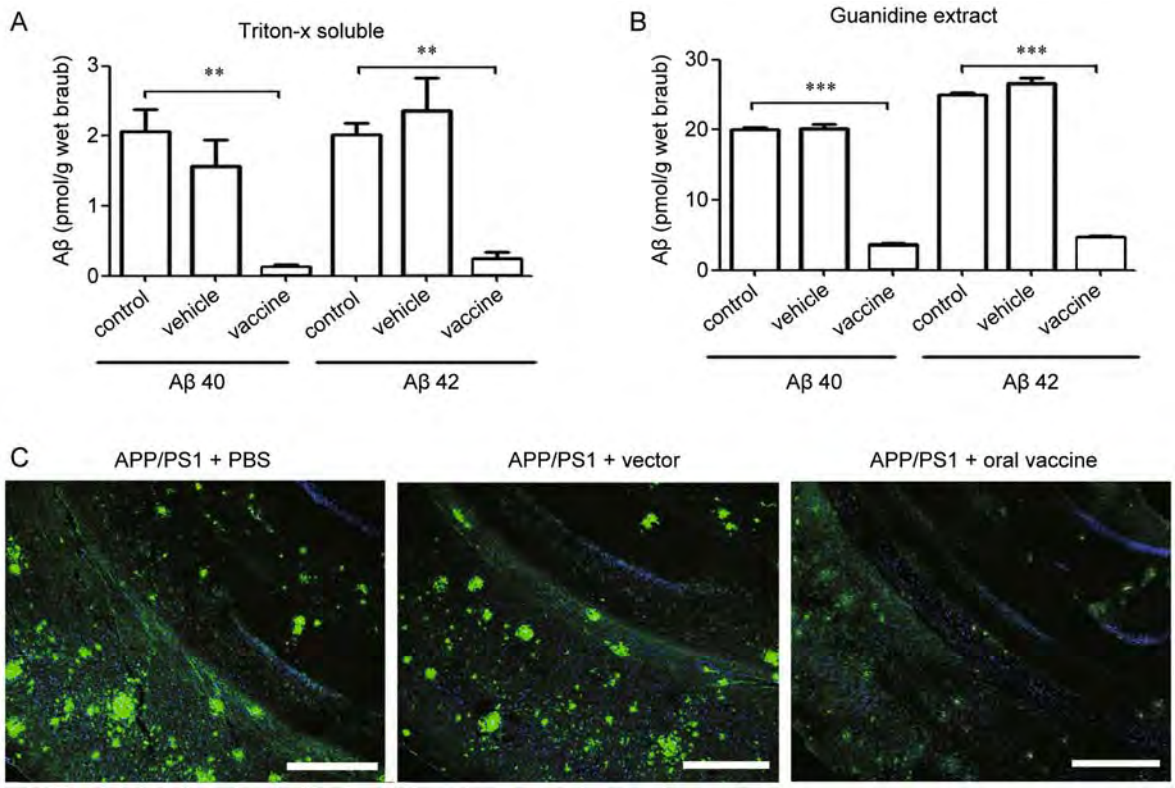

Fig. 2. Oral vaccination with rAAV/Aβ vaccines effectively reduced the brain Aβ burden in APP/PS1 mice. (A,B) The changes in brain Aβ content at 4 weeks after oral vaccination were examined by ELISA and immunofluorescence staining. ELISA quantification of Triton X-soluble Aβ (A) and guanidine HCl-soluble Aβ (B) content in brain tissues from control, vehicle and vaccine groups. (C) Representative immunofluorescence staining of Aβ load in brain regions (cerebral cortex and hippocampus) using 6E10 antibody in coronal sections from APP/PS1+PBS (control), APP/PS1+rAAV/GFP (vehicle), and APP/PS1+rAAV/Aβ (vaccine) groups (mean ± SEM, normalized to control; *****P* **<0.01, ******P*** **<0.001 *vs* control, *n* = 5; one-way ANOVA; scale bar, 200 μm).**

Akt/mTOR Pathway Suppressed in Brains of APP/PS1 Mice with Oral rAAV/Aβ Vaccination

The Akt/mTOR pathway is well-recognized in regulating autophagy[34]; recent studies have also proposed a role of this pathway in the virus-related immune response[35]. To further investigate the potential mechanisms associated with oral vaccination-induced autophagy, we assessed the Akt/mTOR pathway in the brains of all three groups of mice. We discovered that the phosphorylated Akt (Ser473) level was significantly down-regulated following oral vaccination (Fig. 5A, C, P <0.01). Consistent results were obtained for phosphorylated mTOR (Ser2448) levels (Fig. 5B, D, P <0.01). The total protein levels of Akt and mTOR were not altered in the three groups. The rAAV/GFP had no influence on the Akt/mTOR pathway (Fig. 5, P >0.05). These results were in line with the finding that oral vaccination induced autophagy activation in the brain, suggesting that such activation may depend on Akt/mTOR inhibition.

DISCUSSION

Oral Vaccination with rAAV/Aβ Initiated the Immune Response and Increased Serum Anti-Aβ Antibody in APP/PS1 Mice

Amyloidopathy is one of the major pathological changes in the progression of AD. It is generally accepted that Aβ imbalance occurs in the early stage of disease progression[36], and appears to act as a trigger for neuronal damage[37]. An overall dysfunction in Aβ clearance in the brain has been emphasized in the pathogenesis of late-onset AD, the

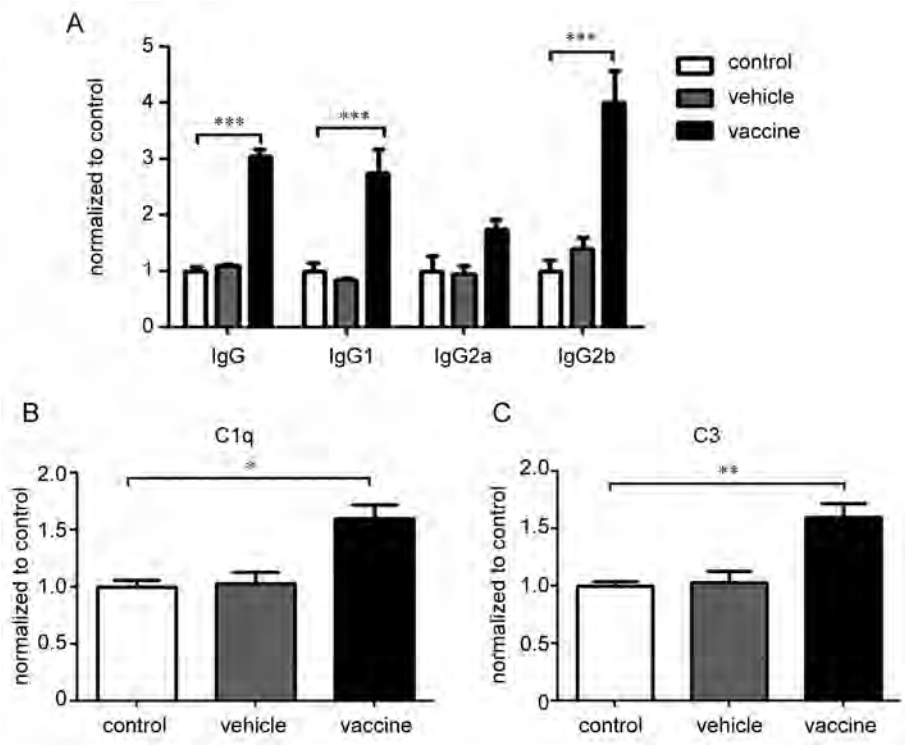

Fig. 3. Oral vaccination with rAAV/Aβ generated a strong immune response by increasing serum anti-Aβ antibodies and elevating brain complement levels. The titers of anti-Aβ antibody and the subclasses of these antibodies in serum samples from all three groups of mice were determined using specific ELISA. (A) Concentrations of antibody against Aβ and the contents of IgG1, IgG2a, and IgG2b in sera from control, vehicle, and vaccine groups were measured. The levels of complement factors C1q (B) and C3 (C) in brains from control, vehicle, and vaccine groups were measured by ELISA (mean ± SEM, normalized to control; *P <0.05, **P <0.01, ***P <0.001 *vs* control, n = 5; one-way ANOVA).

major form of dementia[4]. Targeting Aβ cascades has been the focus of AD drug development. Among the ongoing research projects directed at Aβ clearance, immunotherapy has emerged as a promising strategy[38]. Our previous studies have shown that oral Aβ vaccine using an rAAV vector is effective in decreasing the Aβ burden and improving cognitive ability in Tg2576 mice[10,11]. In parallel, the present study on APP/PS1 mice obtained similar results showing that oral vaccination reduced both the soluble and insoluble Aβ species in the brain. Oral vaccination with rAAV/Aβ may stimulate the immune response *via* gut-associated lymphoid tissues[39] since rAAV/Aβ-induced Aβ expression is detectable in the lamina propria of the stomach and duodenum of mice, and transduction of rAAV occurs in intestinal cells[10,11]. Antibodies against Aβ are regarded to be necessary for Aβ clearance[40], and we indeed found an increase in serum IgG antibodies against Aβ species in a previous study[10]. Similarly, Zhang *et al*. reported that single oral immunization with an Aβ vaccine induces the strong production of anti-Aβ antibodies in a mouse model of AD[39]. In accordance with these findings, we found a significant increase in serum anti-Aβ antibody titers in APP/PS1 mice treated with rAAV-Aβ, confirming that oral vaccination successfully initiated an immune response. Of note, we characterized the antibody isotypes after oral vaccination, and revealed that IgG1 and IgG2b were the predominant isotypes rather than IgG2a. The robust elevation of IgG1 and IgG2b, which are typically used as markers to determine the types of humoral immune response[41], indicates a Th2-based immune response induced by this vaccine. Besides, the gut immune system, a possible mediator for initiating the oral vaccination-induced

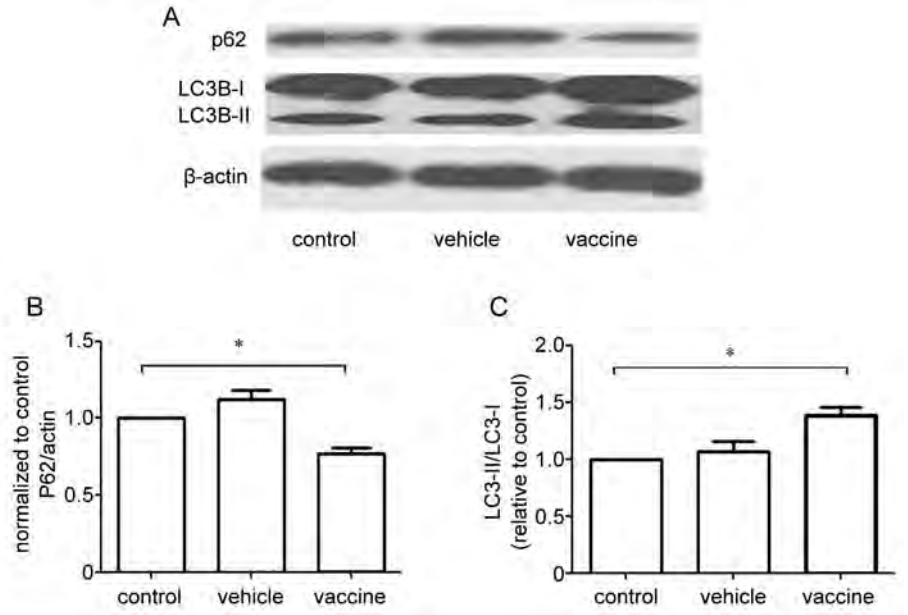

Fig. 4. Activation of autophagic functions in the brains of mice following oral vaccination. The autophagic markers LC3B and p62 in brain tissues were determined by western blotting analysis. The ratio of LC3B-II/LC3B-I and p62 protein levels were evaluated and compared in all three groups. Representative LC3B and p62 western blotting images are shown in (A); quantitative results of p62 protein levels in (B); and the ratio of LC3B-II/LC3B-I in (C) (mean ± SEM, normalized to control; *P <0.05 *vs* control, n = 5; one-way ANOVA).

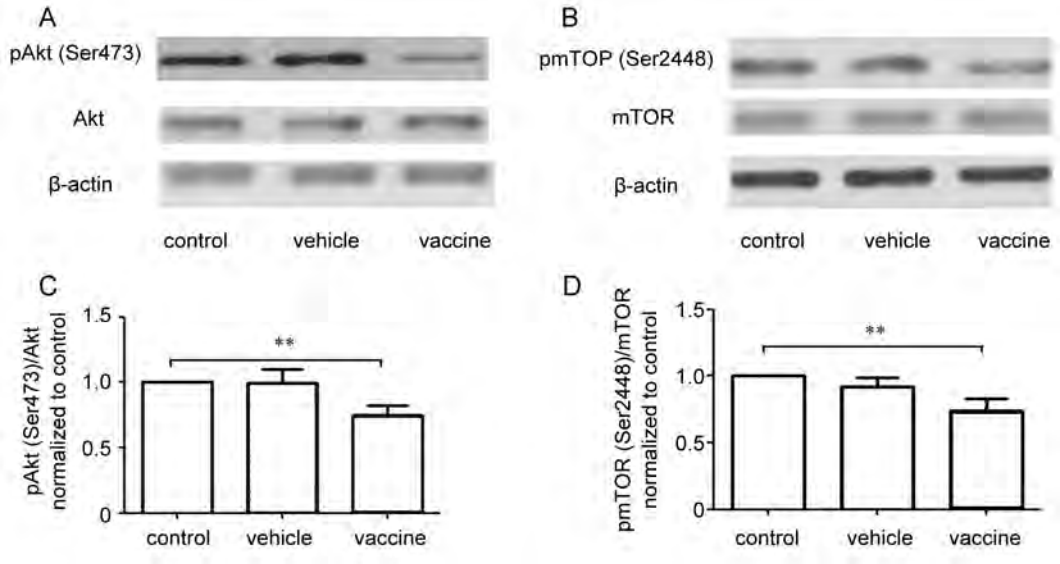

Fig. 5. Oral vaccination with rAAV/Aβ inhibited the brain Akt/mTOR pathway in APP/PS1 mice. The changes in pAkt (Ser473)/Akt and pmTOR (Ser2448)/mTOR were analyzed by western blotting. Representative immunoblotting images of pAkt (Ser473)/Akt (A) and pmTOR (Ser2448)/mTOR (B). Quantitative results of the alteration in pAkt (Ser473)/Akt (C) and pmTOR (Ser2448)/mTOR (D) (mean ± SEM, normalized to control; **P <0.01 *vs* control, n = 5; one-way ANOVA).

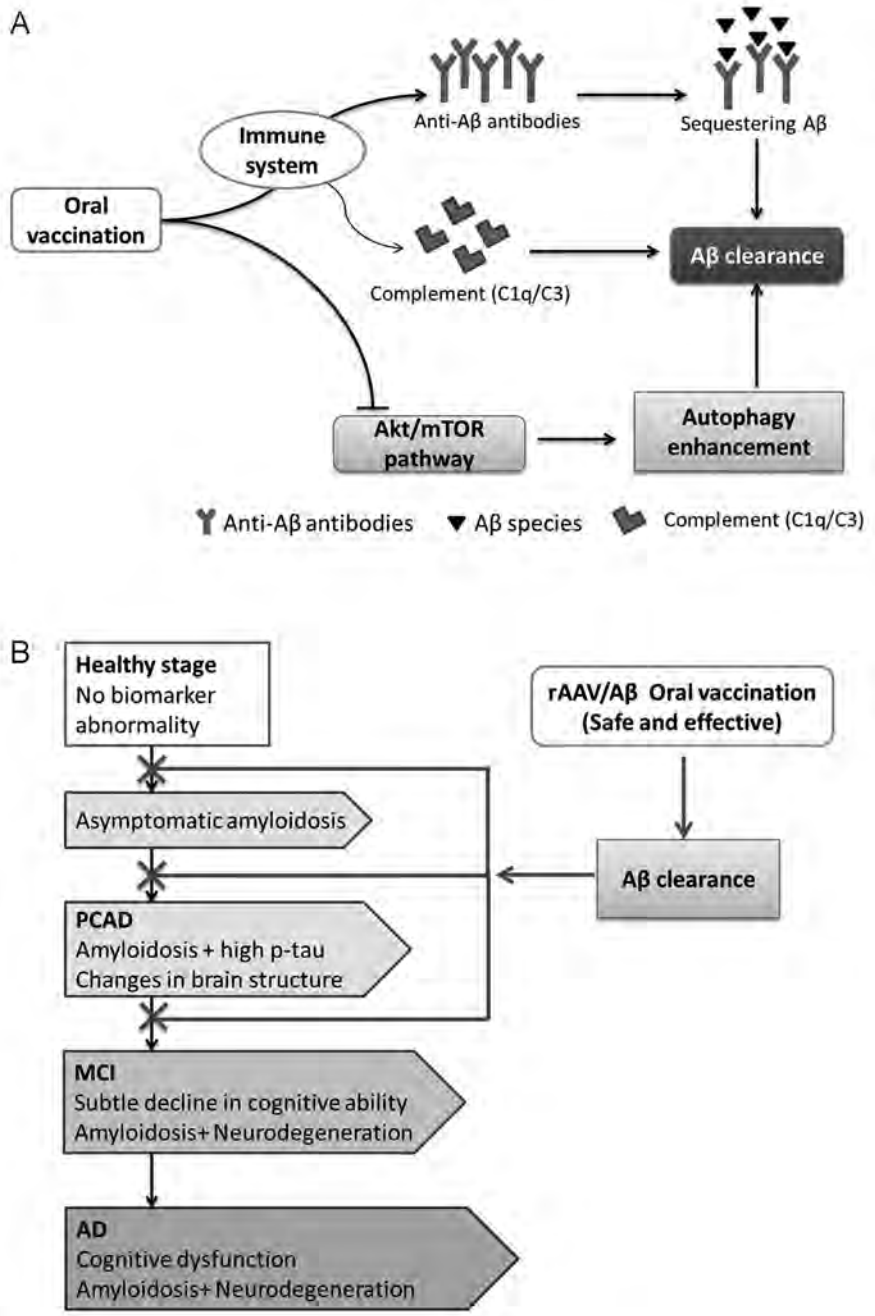

Fig. 6. (A) Schema of the established effects of oral rAAV/Aβ vaccination on Aβ clearance. Here, we demonstrated that activation of brain autophagy was closely involved in oral vaccination-induced Aβ clearance, and this effect might be mediated by inhibition of the Akt/mTOR pathway. We also found that the decrease of Aβ was accompanied by elevation of C1q and C3. In addition, elevation of antibodies against Aβ also played an important part in sequestering and eliminating Aβ species. All three pathways may play a role in oral vaccination-induced Aβ clearance. (B) Oral vaccination is likely to be a safe and effective strategy for early intervention in AD. The hypothetical staging of AD based on the progression of the pathological changes is shown. Amyloidopathy has been regarded to be a key early event in the pathophysiological process of AD[71,74], and recent evidence suggests that aberrant clearance of Aβ species may be critical to the etiology of AD[4]. Based on our study on rAAV/Aβ, we propose that oral immunization with this vaccine might be effective in improving Aβ pathology and retarding disease progression.

immune response, can also suppress the Th1 response and enhance the Th2 response[42]. The Th2 immune response is regarded to be non-inflammatory and is closely linked to the safety of the vaccine[40,43]. Our results that oral vaccination preferentially induced a Th2-based immune response suggested that rAAV/Aβ has the potential to avoid adverse reactions such as neuroinflammation and microhemorrhage.

The complement system is an essential element in the immune response for its function in regulating immune reactions and antigen elimination[44]. It has also been demonstrated that complement factors are widely expressed in the central nervous system[45]. The function of complement in AD is controversial regarding its influence on Aβ pathology, while accumulating studies support a protective effect of complement on Aβ clearance[46]. C1q and C3 are key factors in the complement cascade. Activation of C1q in the early stage protects AD mouse models from Aβ-related neurotoxicity[47]. In addition, C3 deficiency in AD mice models results in age-associated increase in brain Aβ deposition[48], and elevated brain C3 levels can reduce Aβ accumulation[49]. Complement factors are known to activate microglial cells and elevate phagocytic capacity of macrophages or microglia by binding with their receptors, which may contribute to Aβ clearance[30]. We showed here that oral vaccination increased brain C1q and C3 levels, indicating that complement activation might be an auxiliary pathway in oral vaccination-induced Aβ clearance (Fig. 6A).

Potential Mechanisms Responsible for Oral Vaccination-Induced Antibody- Mediated Aβ Clearance

Several hypotheses have been proposed to explain the mechanism by which active immunization removes Aβ from the central nervous system. One possible route is the "peripheral sink hypothesis": anti-Aβ antibodies generated following vaccination are able to sequester and eliminate peripheral Aβ species, and this process may further facilitate the efflux of Aβ peptides, favoring the decline of Aβ burden[50]. It is known that the activation of microglia is related to Aβ pathology in the progression of AD. The microglial attraction induced by Aβ species may exert a positive effect on the clearance of Aβ by phagocytosis[51–53]. The presence of anti-Aβ antibodies in the brain could bind with Aβ and form antibody-Aβ complexes, which would further interact with Fc receptors on phagocytic microglia, thereby promoting the uptake and internalization of Aβ species[54-56]. In addition, the complement factors C1q and C3 were able to facilitate the phagocytic response of microglia as noted above (Fig. 6A). Therefore, microglia-dependent phagocytosis may also be an additional mechanism for the oral vaccination-induced elimination of Aβ from the brain, regarding the elevation of both antibodies and complement factors induced by the rAAV/Aβ vaccine.

Enhancement of Autophagy in the Brain May Play a Novel Role in Oral Vaccination-Induced Aβ Clearance

Increasing evidence supports the pivotal role of autophagy in Aβ metabolism[12,33]. During normal aging or the progression of AD, autophagy gradually declines in the central nervous system[57,58], likely causing the accumulation of aberrant proteins. The impairment of autophagy in AD has been substantiated by the accumulation of autophagosomes in dystrophic neurites[59,60]. Autophagy enhancement is beneficial in ameliorating Aβ-related pathological changes by promoting Aβ clearance[61]. Here, we discovered that rAAV/Aβ vaccination enhanced autophagy as demonstrated by an increase in the LC3B-II/LC3B-I ratio, suggesting the up-regulation of autophagosome formation. Meanwhile, the p62 protein levels were significantly decreased in mice subjected to rAAV/Aβ vaccination; this could furthermore support the enhancement of autophagy. Lysosomal function in the brain plays an essential role in regulating autophagy[62], but whether oral immunization with rAAV/Aβ influences lysosomal function in mouse brains remains to be determined. Enhanced autophagy is closely associated with Aβ clearance[15]. Based on our results, we tentatively conclude that activation of autophagy is an important pathway for mediating oral vaccination-induced Aβ clearance (Fig. 6A).

The Akt/mTOR pathway is fundamental in modulating various cellular responses, including autophagy[63]. Suppression of the Akt/mTOR pathway can improve autophagy function[63,64]. A recent clinical study found that the Akt/mTOR pathway is hyperactive in patients with mild cognitive impairment or Alzheimer's disease[34], and such hyperactivation is closely linked to autophagy failure in these individuals[34]. Genetic or chemical approaches that inhibit the Akt/mTOR pathway successfully rescue

Aβ pathology and improve cognitive function in AD mouse models[61,65]. Consistent with these findings, the Akt/mTOR pathway was also inhibited in the brains of mice following oral rAAV/Aβ vaccination. Given the mechanistic link between autophagy and the Akt/mTOR pathway, it is likely that oral vaccination may induce autophagy *via* Akt/mTOR inhibition.

The detailed functions of autophagy in the vaccination-related immune response are largely unknown, although it is well-established that autophagy is actively involved in antigen-processing or elimination in immune cells, and in maintaining immunological memory[66–68]. It has been reported that viral infections may activate autophagy with unknown mechanisms[69]. Recent research on a recombinant BCGΔureC::hly (rBCG) vaccine pointed out that rBCG vaccination induces autophagy in macrophages *via* an AIM2-dependent pathway[18]. Since our rAAV was detected only in the gut after oral vaccination[10], and rAAV/GFP did not activate autophagy, it is unlikely that oral administration of rAAV directly activated autophagy in the brain. Nevertheless, the molecular mechanism behind the inhibition of the Akt/mTOR pathway by oral vaccination requires further study.

Oral Vaccination Appears to Be a Safe Strategy for the Early Prevention of AD

Amyloidosis, which occurs in the preclinical stage of AD[70,71], has been regarded to be an upstream trigger for many neuropathological changes[72]. Although the relationship between Aβ dysmetabolism and neurodegeneration has not been totally clarified, the prolonged existence of Aβ pathology that begins in the preclinical stage suggests that targeting Aβ clearance could be a potential approach for early intervention[73,74]. The safety of the developed vaccine is also of particular importance, considering the adverse reactions of AN1792[75]. In previous animal studies we did not observe lymphocytic infiltration and microhemorrhage in mouse brains[11]. In the present study, we monitored the changes in body weight following oral vaccination and found no significant reduction compared with mice treated with PBS (data not shown). The survival rate in the rAAV/Aβ group was 100% during the experiment. These results support the safety of the developed vaccine and the route of vaccination. Moreover, oral vaccination may have a better tolerability than other immunization routes in aged patients with mild cognitive impairment or AD, especially when considering the long-term course of the disease[76]. We propose that oral rAAV/Aβ vaccination may be a safe and potent approach for early intervention in AD (Fig. 6B). Further investigations on the safety of the vaccine are underway to better address this concern.

Taken together, the present study demonstrated for the first time that autophagy activation is closely involved in oral rAAV/Aβ vaccination-induced Aβ clearance, and this activation is likely due to the inhibition of the Akt/mTOR pathway. Our research may provide new insights into vaccine-induced autophagy-dependent Aβ clearance and the development of early interventional strategies for AD.

ACKNOWLEDGEMENTS

This work was supported by the National Basic Research Development Program of China (2012CB911000 and 2012CB911004), the NSFC-JSPS Cooperation Program (81211140047), and the National Natural Science Foundation of China (81171015).

Received date: 2015-04-29; Accepted date: 2015-07-14

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Neurosci Bull August 1, 2015, 31(4): 505–513. http://www.neurosci.cn
DOI: 10.1007/s12264-015-1549-1

·Original Article·

Duration-dependent regulation of autophagy by isoflurane exposure in aged rats

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ABSTRACT

Current evidence suggests a central role for autophagy in many inflammatory brain disorders, including Alzheimer's disease (AD). Furthermore, it is also well accepted that some inhalation anesthetics, such as isoflurane, may cause AD-like neuropathogenesis and resultant postoperative cognitive dysfunction, especially in the elderly population. However, the impact of inhalation anesthetics on autophagic components in the brain remains to be documented. Hence, our objective was to investigate the effects of different durations of isoflurane exposure on hippocampus-dependent learning and hippocampal autophagy in aged rats. Aged Sprague-Dawley rats (20 months old) were randomly exposed to 1.5% isoflurane or 100% oxygen for 1 or 4 h. Animals were then trained in the Morris water maze (4 trials/day for 5 consecutive days). Hippocampal phagophore formation markers, beclin 1 and protein microtubule-associated protein 1 light chain-3B (LC3B), as well as p62, an indicator of autophagic flux, were quantified by western blotting. There was no significant difference in the escape latencies and time spent in the target quadrant, as well as hippocampal expression of beclin 1, LC3B-II, and p62 at 24 h post-anesthesia between the 1-h isoflurane-exposed rats and their controls (P >0.05). Four-hour exposure to isoflurane resulted in spatial learning and memory deficits, as evidenced by prolonged escape latencies on days 4 and 5 post-anesthesia and less time spent in the target quadrant than sham-exposed animals (P <0.05). These events were accompanied by a decline in hippocampal expression of LC3B-I, LC3B-II, and beclin 1 24 h after isoflurane (P <0.01 and P <0.05). Nevertheless, no significant change in p62 expression was found. Further kinetics study of autophagic changes induced by 4 h of isoflurane showed a transient upregulation of LC3B-I, LC3B-II, and beclin 1 at the end of exposure and a subsequent striking decrease within 12–24 h post-anesthesia (P <0.05). Hippocampal p62 peaked at 6 h but subsequently resolved. These results from our pilot *in vivo* study support a duration-dependent relationship between 1.5% isoflurane exposure, and spatial cognitive function as well as hippocampal phagophore formation.

Keywords: autophagy; phagophore formation; cognitive dysfunction

INTRODUCTION

Dementia is a neurodegenerative syndrome in

which there is deterioration in cognition, behavior, and daily living activity[1]. As the population ages, the impact of dementia on the social and clinical burden will increase. Dementia is caused by a variety of diseases and injuries, such as Alzheimer's disease (AD), Parkinson's diseases, multiple sclerosis, and stroke[1, 2]. Recently, surgery and anesthesia have been proposed to increase the incidence of AD[3, 4]. Surgical procedures and administration of anesthesia are associated with a transient or permanent decline in cognitive function, termed postoperative cognitive dysfunction (POCD). POCD delays rehabilitation and increases morbidity and early mortality[5], and also has emerged as a major health concern, especially in the geriatric population[6].

In examining the role of surgery and anesthesia on POCD, it is difficult to discriminate the effects of anesthetics from surgical stress. Nevertheless, increasing numbers of animal studies have indicated that inhalation anesthetics, such as isoflurane, may cause or increase the risk of developing POCD[7, 8]. Specifically, inhalation anesthetics may alter cognitive function *via* NF-κB-dependent neuroinflammation[9, 10], amyloid β (Aβ) accumulation[8, 11-13], tau phosphorylation[13, 14], modified neurotransmission[15] and deregulated calcium homeostasis[16]. Among these, the first three appear to be the shared pathological markers of AD, thereby implying an emerging link between POCD and dementia.

Autophagy is a major catabolic pathway in eukaryotic cells and is a vital pathway for degrading normal and aggregated proteins and altered or unwanted organelles, particularly under stress conditions[17]. Autophagy at an appropriate level not only plays a crucial role in protein quality control, but also acts as a defense response to stress, thus maintaining cellular homeostasis[17]. Previous studies have demonstrated that Aβ is generated in Aβ precursor protein-rich organelles during autophagic turnover[18]. Generally speaking, both increased autophagy induction and defective clearance of Aβ-generating autophagic vacuoles can act together to facilitate Aβ accumulation in AD[19, 20]. In addition, the relationships between autophagy and inflammation, another hallmark of POCD, have also been recently explored in both *in vitro*[21] and *in vivo*[22] models of AD. The results indicated that there is crosstalk between the impairment of autophagy and substantial neuroinflammation in AD.

Nevertheless, the linkages between autophagy and POCD remain largely unknown, and whether the inhalation anesthetics represented by isoflurane modulate autophagy has never been investigated in the aged brain. Based on the above findings and the similarity between POCD and AD, we therefore preliminarily investigated the effects of exposure to isoflurane for different durations on hippocampus-dependent learning and memory and the autophagy response in the hippocampus of aged rats.

MATERIALS AND METHODS

Experimental Animals

Seventy-six 20-month-old male Sprague-Dawley rats weighing 550–650 g were purchased from the Dongchuang Laboratory Animal Center (Changsha, Hunan, China) and housed in standard barrier facilities. They were maintained under a 12-h light/dark cycle (lights on at 07:00) with food and water *ad libitum*. All animals had a recovery period of at least 7 days before experiments. All work was conducted with the approval of Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch (Approval No. 20150041).

Experimental Protocols

To study the effects of different durations of isoflurane exposure on spatial learning and memory, rats were randomly exposed to 1.5% isoflurane or vehicle gas for 1 or 4 h. On day 1 post-anesthesia, 9 rats under each experimental condition were randomly selected and performed the Morris water maze (MWM) task to evaluate hippocampal-dependent spatial learning and memory. In addition, we sought to determine the effects of different durations of isoflurane exposure on the autophagy response in the aged hippocampus. So, the hippocampal expression levels of beclin 1 and protein microtubule-associated protein 1 light chain-3B (LC3B), markers of phagophore formation, and p62, an indicator of autophagic flux, were examined at 24 h after isoflurane using western blotting (n = 4 in each condition). In preliminary studies, we found that the longer duration (4 h) of isoflurane exposure impaired spatial leaning, so we further examined the hippocampal expression levels of beclin 1, LC3B, and p62 at 0, 3, 6, 12, and 24 h after a 4-h isoflurane exposure using western blotting (n = 4 per time-point).

Isoflurane Exposure

Isoflurane exposure was performed as previously described[9, 15]. In brief, animals were placed in a transparent anesthetic chamber resting in a temperature-controlled water bath (38°C). In the chamber, rats were exposed to 1.5% isoflurane (Baxter Healthcare, Deerfield, IL) in 100% oxygen carrier gas for 1 or 4 h. The control animals were treated with the same duration of 100% oxygen exposure. Volatile anesthetics at 0.5–1.3 minimum alveolar concentration (MAC, the anesthetic concentration at which 50% of animals do not have a motor response to painful stimuli) is commonly used in clinical practice. The isoflurane was titrated to 1.5% as this represents one MAC in aged rats[23]. Gas composition (isoflurane, oxygen, and carbon dioxide) within the chamber was measured using a gas monitor (Datex-Ohmeda, Louisville, CO). After 1 or 4 h of isoflurane exposure and complete recovery, rats were then returned to their home cages.

Morris Water Maze Experiments

As previously described[24] with minor modifications, animals were tested for spatial learning and memory using MWM tests by two investigators blinded to the group allocation. In brief, the rats received four training trials daily for five consecutive days. On each trial, rats were gently placed in a fixed position into the water facing the wall of the maze. Each rat was allowed 120 s to locate the platform submerged ~1 cm below the water surface. The time to reach the platform and swimming speed were recorded. After each trial, the rats were allowed to remain on the platform for 20 s before being removed from the pool. On day six, the platform was removed. Probe trials were conducted to evaluate memory retention. The rats were allowed to swim for 90 s, and the percentage of time spent in the previous platform quadrant was determined.

Western Blotting

Hippocampal tissues were homogenized in RIPA buffer (Applygen Technologies Inc., Beijing, China). Protein concentrations were determined using BCA protein assay (Applygen). Sixty micrograms of protein per lane was loaded on 10% SDS-PAGE for separating beclin 1 and p62, and on 15% acrylamide for separating LC3B. After transfer onto nitrocellulose membranes (0.45 μm pore size for beclin 1 and p62 and 0.22 μm for LC3B), the following primary antibodies were used: anti-LC3B (#3868; 1:1 000; CST, Danvers, MA), anti-beclin 1 (#3495; 1:1 000; CST), and anti-p62 (#5114; 1:1 000; CST). Fluorescently-labeled secondary antibodies (1:1 0000; LI-COR Biosciences, Lincoln, NE) were used.

Immunohistochemical Analysis

Twenty-four hours after 4-h isoflurane exposure, immunofluorescence staining of hippocampal sections was performed as previously described[25] using an anti-LC3B primary antibody (#3868; 1:200; CST) that detects endogenous levels of total LC3B protein, followed by incubation with Alexa-Fluor 488 conjugated secondary antibody (#ab150077; 1:200; Abcam, Cambridge, UK). Cell nuclei were counterstained with Hoechst 33258 (Invitrogen, Carlsbad, CA). Sections were imaged using a confocal microscope (Olympus FV1000, Tokyo, Japan).

Statistical Analysis

Statistics were calculated using SPSS (SPSS Inc., Chicago, IL). All data are expressed as mean ± SEM. Data on escape latency in the MWM tests were assessed with two-way analysis of variance (ANOVA) for repeated measures (treatment condition × day). Data from the kinetics study of autophagic changes induced by 4 h of isoflurane were analyzed using one-way ANOVAs with least significant difference *post hoc* test. Other quantitative data from the two groups were tested by the independent samples *t*-test. Statistical significance was set at $P < 0.05$.

RESULTS

Effects of Different Durations of Isoflurane Exposure on Spatial Learning and Memory

All animals swam normally. There was no significant difference in swimming speeds among different experimental conditions (data not shown). After a 1-h isoflurane exposure, repeated factor (day) ($F = 142.80$, $P < 0.01$), but not group factor (treatment) ($F = 1.13$, $P > 0.05$) significantly affected the escape latency, and no interaction was found ($F = 0.30$, $P > 0.05$). After a 4-h isoflurane exposure, comparison of rats that received isoflurane with their controls revealed a significant effect of days ($F = 42.90$, $P < 0.01$), but a non-significant effect of

groups (F = 1.55, P >0.05) and interaction between groups and days (F = 1.01, P >0.05). The reduced latency over the five daily sessions suggested that all rats were able to learn the task successfully. As shown in Fig. 1B, statistical analyses showed that on days 4 (33.62 ± 5.94 *vs* 17.73 ± 3.29 s, n = 9, P <0.05) and 5 (29.94 ± 4.82 *vs* 17.73 ± 2.73 s, n = 9, P <0.05), the 4-h isoflurane-exposed rats took longer to reach the platform than the sham-exposed rats. In the probe test, the time spent in the target quadrant by rats exposed to 1.5% isoflurane for 4 h was much shorter than that of control rats (22.33 ± 2.79 *vs* 41.10 ± 2.89 s, n = 9, P <0.05; Fig. 1D), thus validating the memory impairments after a 4-h isoflurane exposure. However, there were no significant differences in the time spent in the target quadrant between the rats treated with 1.5% isoflurane and vehicle gas for 1 h (45.67 ± 2.51 *vs* 43.29 ± 2.57 s, n = 9, P > 0.05; Fig. 1C).

Effects of Different Durations of Isoflurane Exposure on Autophagy

Exposure to isoflurane for only 1 h had no effect on the hippocampal expression levels of LC3B-I, LC3B-II, beclin 1, and p62 at 24 h post-anesthesia compared with their controls (Fig. 2A, P >0.05). Nevertheless, 24 h after a 4-h isoflurane exposure, quantitative analysis of western blots revealed a marked decrease in the expression levels of both LC3B-I and LC3B-II (Fig. 2B), which was consistent with the results from LC3B immunofluorescence staining (Fig. 3). In the hippocampal CA1 region, rats exposed to isoflurane for 4 h, but not 1 h, exhibited decreased staining for LC3B compared with vehicle-treated animals at 24 h after gas exposure. Beclin 1 was also significantly downregulated

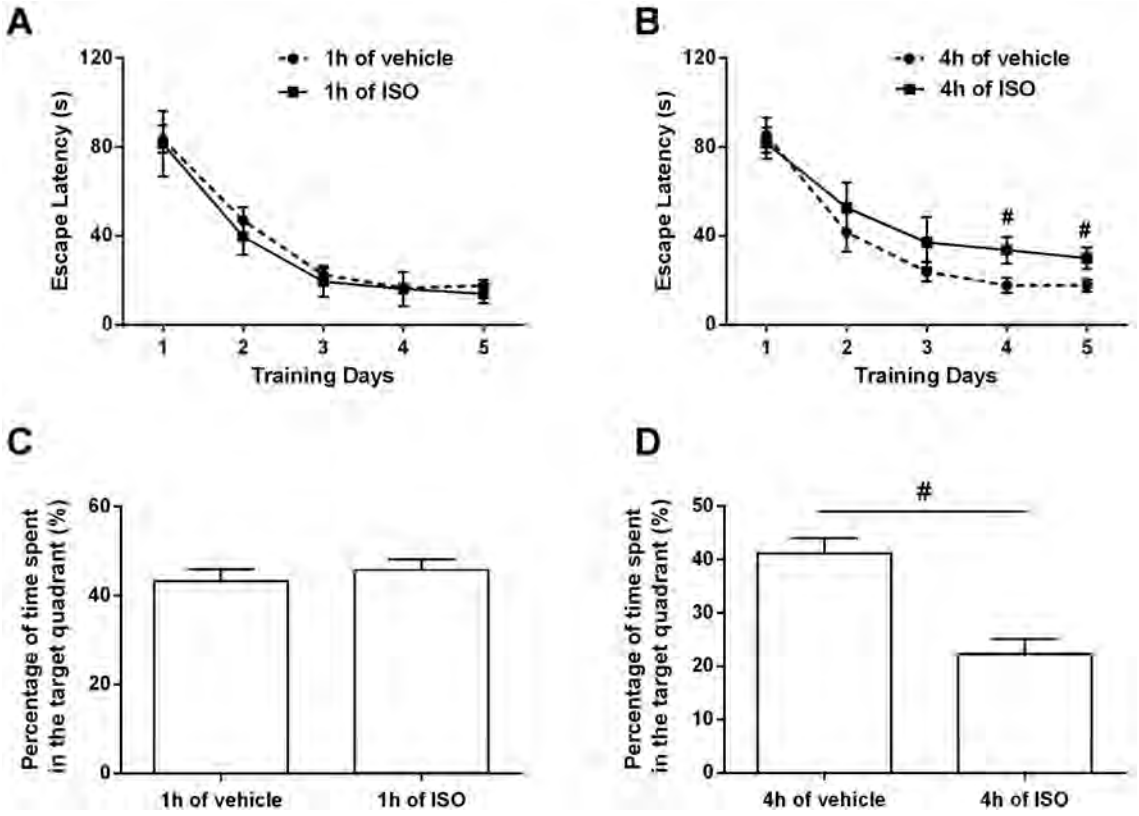

Fig. 1. Effect of different durations of isoflurane exposure on spatial learning and memory in the Morris water maze test. The diagrams show the escape latency and the percentage of time spent in the previous platform quadrant after exposure to 1.5% isoflurane or vehicle gas for 1 or 4 h. A–B: Acquisition trial demonstrating the latency for rats to reach the platform, measuring spatial information acquisition. C–D: During the probe trial, the time spent in the target quadrant was calculated. Data are mean ± SEM; $^{\#}P$ <0.05 *vs* 4 h of vehicle (n = 9). ISO, isoflurane.

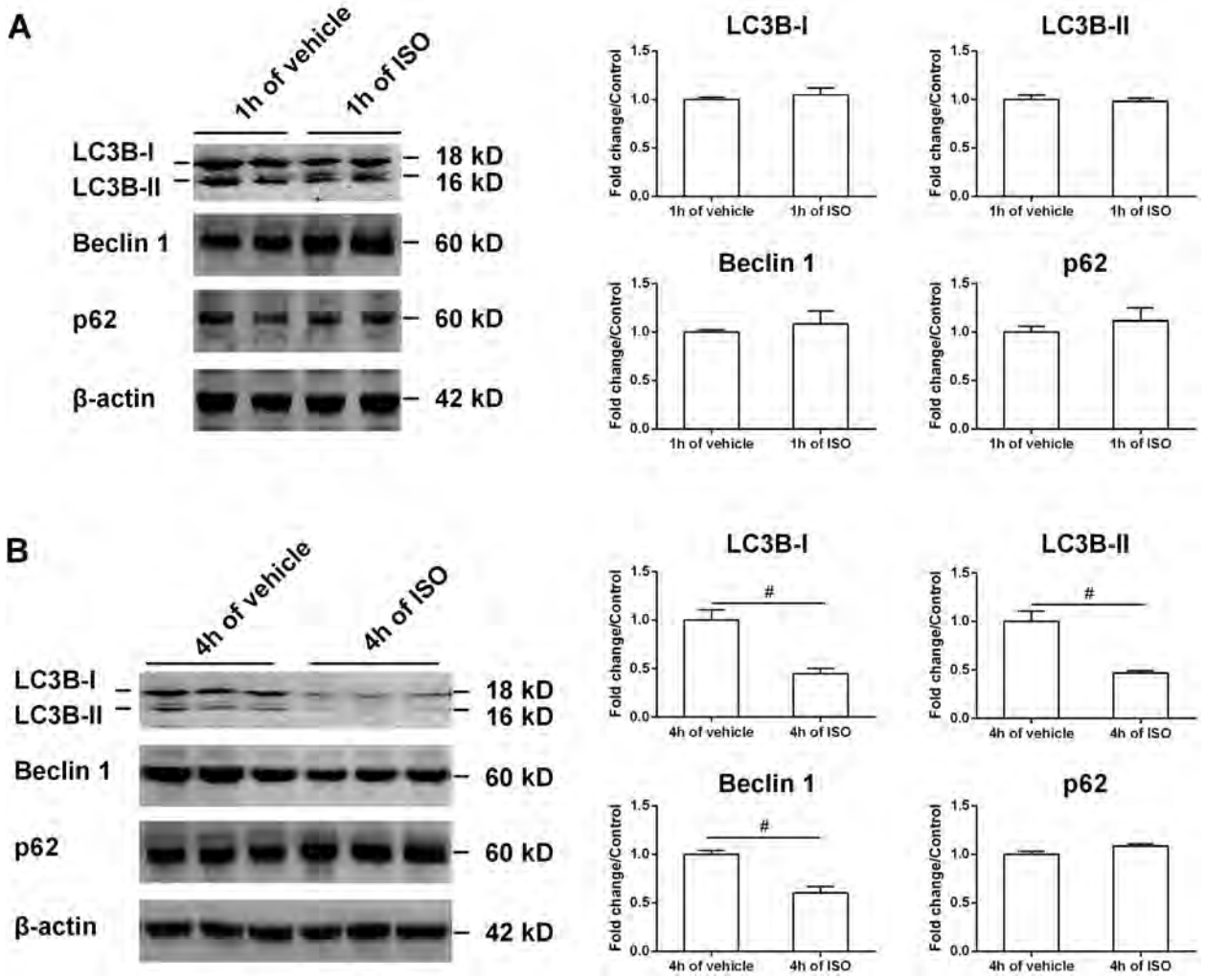

Fig. 2. Effect of different durations of isoflurane exposure on the protein levels of autophagy markers in the hippocampus of aged rats at 24 h post-anesthesia. Left panels in A and B: western blots showing expression of LC3B and beclin 1, phagophore formation markers, and p62, an indicator of autophagic flux, in the hippocampus of aged rats after exposure to 1.5% isoflurane or vehicle gas for 1 h (A) or 4 h (B). Right panels in A and B: semi-quantitative data showing protein expression levels of LC3B-I, LC3B-II, beclin 1, and p62. Values are mean ± SEM of protein densities normalized to β-actin; $^{\#}P$ <0.05 *vs* 4 h of vehicle (*n* = 4). ISO, isoflurane.

in rats after a 4-h isoflurane exposure compared with controls (Fig. 2B, P <0.01). No difference was found in the expression of p62 (Fig. 2B, P >0.05).

Kinetics of Autophagy Response Induced by 4 Hours of Isoflurane

Since exposure to isoflurane for 4 h, but not 1 h, impaired spatial learning and memory, we therefore further examined the dynamics of the hippocampal expression levels of beclin 1, LC3B, and p62 after a 4-h isoflurane exposure. The expression of both LC3B-I and LC3B-II significantly increased, first at the end of exposure, and then decreased within 12 to 24 h in rats exposed for 4 h compared with vehicle gas (Fig. 4C and D, P <0.05). Similarly, compared with controls, beclin 1 expression increased immediately after isoflurane exposure and remarkably decreased 12 and 24 h after isoflurane (Fig. 4E, P <0.05). p62 peaked at 6 h but subsequently resolved at 12 to 24 h after isoflurane (Fig. 4F, P <0.05), suggesting that the defect in autophagic flux is temporary.

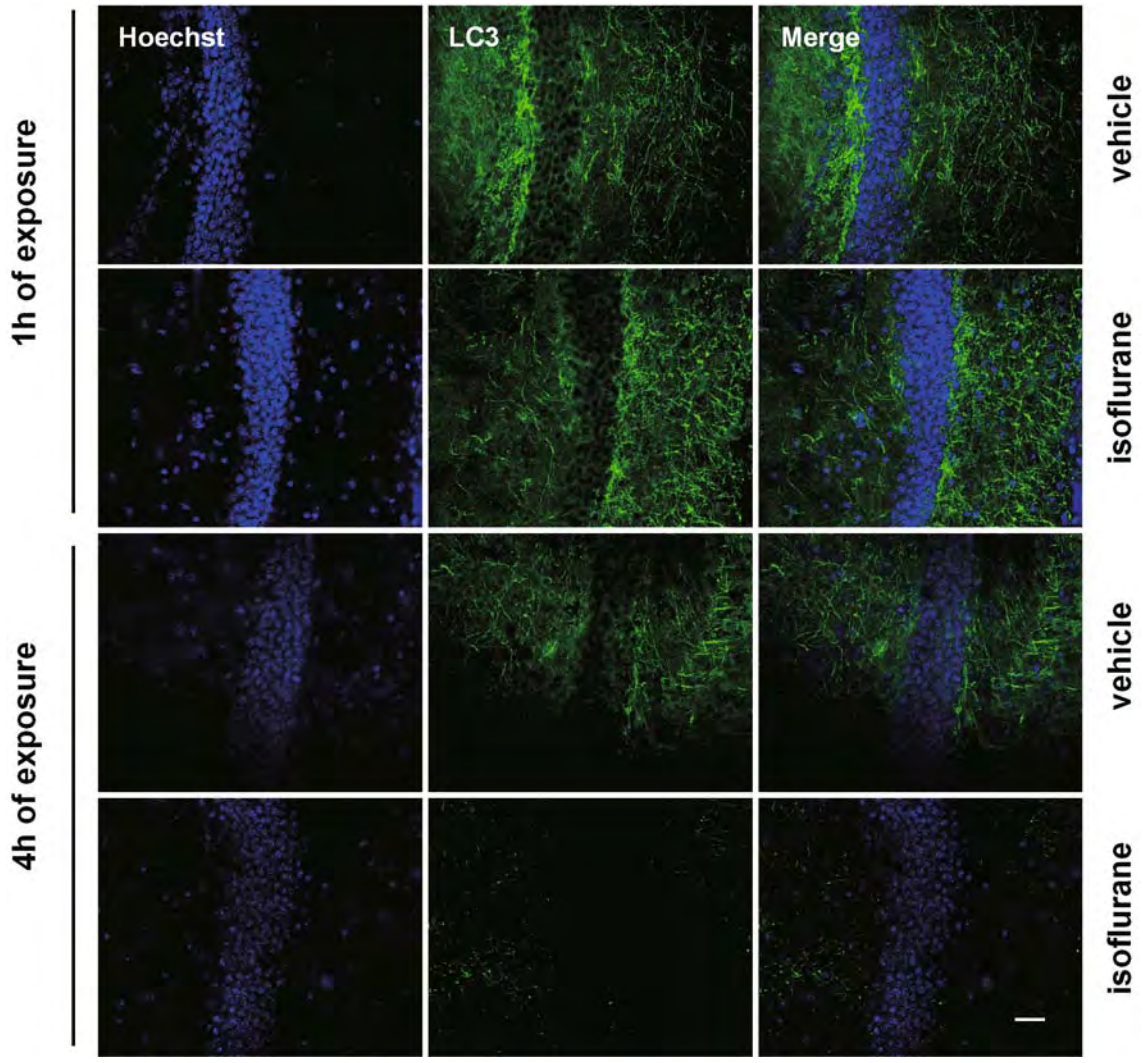

Fig. 3. Representative confocal images of LC3 staining in the hippocampus of vehicle and isoflurane-treated animals. (A) Exposure to isoflurane for 1 h did not alter the expression of LC3 in the CA1 region. (B) Rats exposed to 4 h of isoflurane exhibited significantly decreased staining of LC3 compared with vehicle-treated animals in the CA1 region at 24 h after gas exposure (*n* = 4/group; scale bar, 50 μm).

DISCUSSION

Longer but not Shorter Isoflurane Exposure Inhibits Phagophore Formation

Alteration of autophagy is a central feature common to various brain diseases such as AD, Pakinson's disease, and Huntington's disease[26], all of which are accompanied by cognitive dysfunction. However, the relationship between autophagy and isoflurane-induced acute cognitive dysfunction remains unknown. The current study therefore aimed at determining whether isoflurane could modulate the autophagic process in the aged brain. Here, we first found that isoflurane influenced autophagy in the hippocampus of aged rats and spatial cognitive function in a time-dependent manner. Specifically, exposure to 1.5% isoflurane for 4 h impaired spatial learning and memory, accompanied by impaired autophagy following a transient activation; however, exposure for only 1 h did not affect cognition and autophagy.

Strictly speaking, the direct effects of isoflurane on

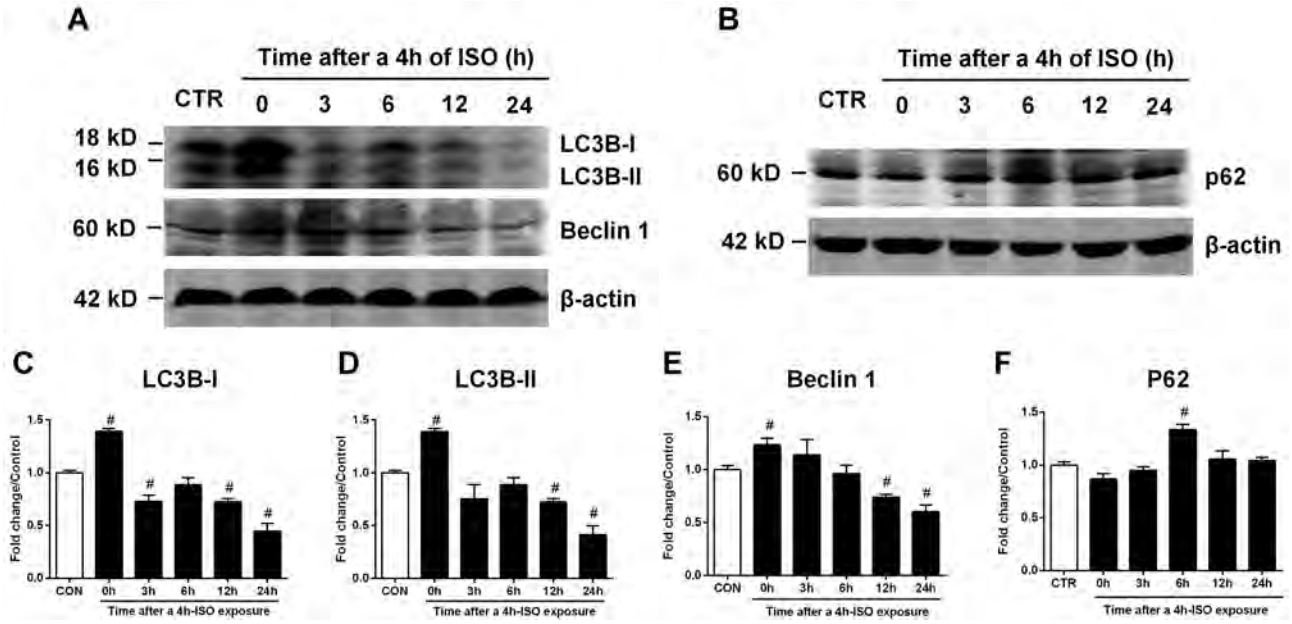

Fig. 4. Kinetics of autophagy induced by 4 h of isoflurane exposure. A–B: Western blots showing expression of autophagic components LC3B, beclin 1, and p62 in the hippocampus of aged rats after exposure to 1.5% isoflurane or vehicle gas for 4 h. C–F: Semi-quantitative data showing protein expression levels of LC3B-I (C), LC3B-II (D), beclin 1 (E), and p62 (F). Values are mean ± SEM of protein densities normalized to β-actin; $^{\#}P$ <0.05 *vs* 4 h of vehicle (n = 4). ISO, isoflurane.

autophagy have been described in only two studies. At the peripheral level, Kashiwagi *et al.* reported that several anesthetics including isoflurane (1.5% for 2 h) lead to autophagy upregulation in skeletal muscle[27]. In the brain, Sheng *et al.* found that isoflurane preconditioning (2% for 0.5 h) up-regulates LC3 in primary cortical neurons at 24 h post-anesthesia, and that 1% isoflurane exposure for 3 h induces autophagy activation 24 h later in the cortex and striatum, but not in the hippocampus of adult mice[28]. On the contrary, in the present study, we found that exposure to 1.5% isoflurane for 4 h induced a decrease in autophagy at 24 h post-anesthesia, as evidenced by decreased LC3B and beclin 1, phagophore markers in the early stage of autophagy. This discrepancy could be due to the age of the animals as there is an age-dependent deficit of autophagy[29]. Furthermore, the protein level of p62, an autophagic flux marker, was not significantly changed, suggesting that a 4-h isoflurane exposure mainly inhibits phagophore formation.

Here, we did not find any of the autophagy markers (LC3B, beclin 1, and p62) to be significantly changed 24 h after a 1-h isoflurane exposure. These results indicate that a short isoflurane exposure may not be sufficient to induce autophagy in the aged hippocampus. These events were accompanied by unchanged spatial learning and memory in the MWM tests.

Longer Isoflurane Exposure Induces Suppression of Autophagy Following Brief Activation

The kinetics study of autophagic changes induced by 4 h of isoflurane revealed that it suppressed autophagy following a transient activation, as evidenced by increased LC3B-II and beclin 1 immediately after isoflurane for 4 h, followed by decreased levels 12 h later. Taking the time-course pattern of the autophagy response into consideration, our results suggested that the whole process occurs in the aged hippocampus within 6 h after isoflurane. Support for this time-window comes from the increased p62 level at 6 h, which would be a consequence of the decreased autophagic flux that would result in inhibition of the degradation of p62 by lysosomes. Our results also suggested that the effect of modulating autophagy by isoflurane is context-dependent, and the level and duration of modulation should be fully considered. Since the

polyubiquitin-binding protein p62/SQSTM1 is degraded by autophagy[30], further investigation of ubiquitination levels may reinforce this hypothesis.

Impaired Hippocampus-dependent Spatial Learning and Memory after Longer but not Shorter Isoflurane Exposure

Volatile anesthetics have long been considered neuroprotective. Nevertheless, available evidence has also suggested that there are growing concerns about potential neurotoxicity. Logically, it is easy to understand how volatile anesthetics can provide both neuroprotection and neurotoxicity. The most popular theory in this field is that almost all general anesthetics have dual effects, depending on the concentration and duration[32, 33]. Short exposure to general anesthetics can provide neuroprotection at low concentrations but become lethal stress factors at high concentrations for prolonged durations[32]. In accordance with this, we found that exposure to isoflurane for a longer duration (4 h) impaired hippocampus-dependent spatial learning and memory in aged rats. Nevertheless, we did not find any cognition-improving effects in the aged rats after a short (1 h) exposure. This does not deny the possibility of neuroprotection since there are conflicting findings in the literature regarding the effect of isoflurane on cognition. Two research groups reported improved cognitive performance in adult mice after exposure to 1 MAC isoflurane for 2 h[34,35]. In contrast, impaired cognitive performance after the same dose and duration of isoflurane has been reported in adult rats (4 months)[36] and in aged rats (18–20 months)[37]. Different methodologies, including animal age, strain, anesthetic carrier gas, outcome measurement time, or behavioral method may have contributed to these different findings.

Summary

In conclusion, although time-dependent spatial cognitive changes concomitant with different autophagic responses were found in aged rats, our results did not establish an *in vivo* link between central autophagy and cognitive dysfunction in the aged hippocampus after isoflurane challenge. Further research is warranted to determine the functional relationship between impaired autophagy and cognitive dysfunction, as well as the crosstalk between autophagy, neuroinflammation, and Aβ accumulation in the aged brain induced by isoflurane.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Natural Science Foundation of China (81371205) and the National Basic Research Development Program (973 Program) of China (2012CB911000 and 2012CB911004)

Received date: 2015-05-23; Accepted date: 2015-07-17

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NEUROSCIENCE BULLETIN

NEUROSCIENCE BULLETIN

Impact Factor 2.509

2014 Journal Citation Reports (Thomson Reuters, 2015)

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NEUROSCIENCE BULLETIN 神经科学通报(Bimonthly)

Vol.31 No.4 August 1, 2015

Sponsored by: Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, the Chinese Neuroscience Society and the Second Military Medical University

Editor-in-Chief: Shumin Duan

Edited by: Editorial Board of *Neuroscience Bulletin*
319 Yueyang Road, Building 31B, Room 405, Shanghai 200031, China
Phone: +86-21-54922863; Fax: +86-21-54922833
E-mail: nsb@sibs.ac.cn; http://www.neurosci.cn

Editors: Bin Wei, Xu Jiang, Yefei Li

Published by: Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (319 Yueyang Road, Shanghai 200031)

Printed by: Shanghai Tu Yu Printing Co., Ltd. (No.28 Lane 23 Yingao Road, Shanghai 200439)

Overseas Distributed by: Springer Science+Business Media

Home Distributed by: Local Post Offices

ISSN 1673-7067
CN 31-1975/R

Post Office Code Number: 4-608

Permit of Ad. Number: 3100420080068

Price: ¥50.00

ISSN 1673-7067