

Review

# Autophagy in Intracerebral Hemorrhage: From Mechanism to Regulation

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## Abstract

Intracerebral hemorrhage (ICH) is the most lethal type of stroke. Secondary injury from ICH determines the recovery, but there is still a lack of effective treatment. The identification of new therapeutic targets may address the current dilemma. The process of autophagy is mediated through the lysosomal pathway and is used to maintain cell homeostasis. Recent studies have advanced our knowledge of autophagy, and in particular its involvement in cell physiology and pathology. Autophagy involves multiple targets and signaling pathways and occurs in many brain cells. It also regulates oxidative stress and inflammation after ICH, both of which are important factors in secondary brain injury. An appropriate level of autophagy is protective in ICH, whereas excessive autophagy may be detrimental. In this review, we discuss the signaling pathways for autophagy in ICH and related factors that provide a theoretical basis for the discovery of new treatment targets.

**Keywords:** intracerebral hemorrhage; autophagy; signaling pathway; molecular mechanisms

## 1. Introduction

Intracerebral hemorrhage (ICH) accounts for 10–15% of all strokes and usually refers to non-traumatic brain parenchymal hemorrhage. ICH is the second most frequent type of stroke after ischemic stroke, but has a much higher mortality rate [1,2]. Hemorrhagic lacunar stroke accounts for 7.4% of ICH, with a symptom-free rate of 22.8% at discharge and a more favorable prognosis than ICH [3]. ICH survivors have a poor prognosis and a rate of high disability with reduced quality of life. Approximately 2.8 million people worldwide die from ICH every year [4]. The prevalence of ICH is higher in China than in Western countries and accounts for >25% of acute stroke patients [5]. Secondary brain injury (SBI) has been studied extensively with the aim of clarifying the damage mechanism and targets. This may lead to better clinical treatments and improved prognosis after ICH.

The lysosomal pathway mediates the process of autophagy, which breaks down macromolecules such as misfolded proteins in the cytoplasm and organelles. Moderate autophagy after ICH is protective against SBI, whereas excessive or insufficient autophagy causes harm. The key to discovering therapeutic targets is therefore to gain a better understanding of how to maximize the positive effects of autophagy. This paper focuses on the molecular mechanisms and regulation of autophagy.

## 2. Brain Injury after ICH

ICH occurs mostly in the brainstem, lobes, basal ganglia and thalamus, with the most common site being the basal ganglia. Hypertension, diabetes and oral anticoagulants are known risk factors for ICH [6]. Cerebral amyloid angiopathy (CAA) is a common cause, while hematological disorders are also a special cause of ICH, especially in young people and in patients with unexplained recurrence. Routine blood cell counts, prothrombin time and activated thrombin time are important factors in relation to ICH [7]. SBI after ICH is mainly caused by primary and secondary mechanisms. Direct injury can occur early after ICH and is mostly associated with the formation of hematoma, which occupies and compresses the brain parenchyma, thereby destroying peripheral neural structures. SBI after ICH is mainly caused by ischemia and hypoxia in the ischemic penumbra surrounding the hematoma. SBI is associated with cerebral edema, inflammatory response and brain herniation [8]. Activation of thrombin and dysfunction of the blood-brain barrier (BBB) cause cytotoxic edema and vasogenic edema, resulting in perihematomal edema (PHE). The PHE grows rapidly in the early stages after ICH and is an important cause of intracranial hypertension [9]. Hematoma formation results in the activation of cytotoxicity and excitotoxicity. Hemoglobin, iron and other blood components also induce injury, while oxidative stress and inflammatory responses induce SBI, leading to neurological dysfunction. SBI after ICH leaves the patient with severe sequelae and functional impairment.



### 3. Molecular Mechanisms of Autophagy

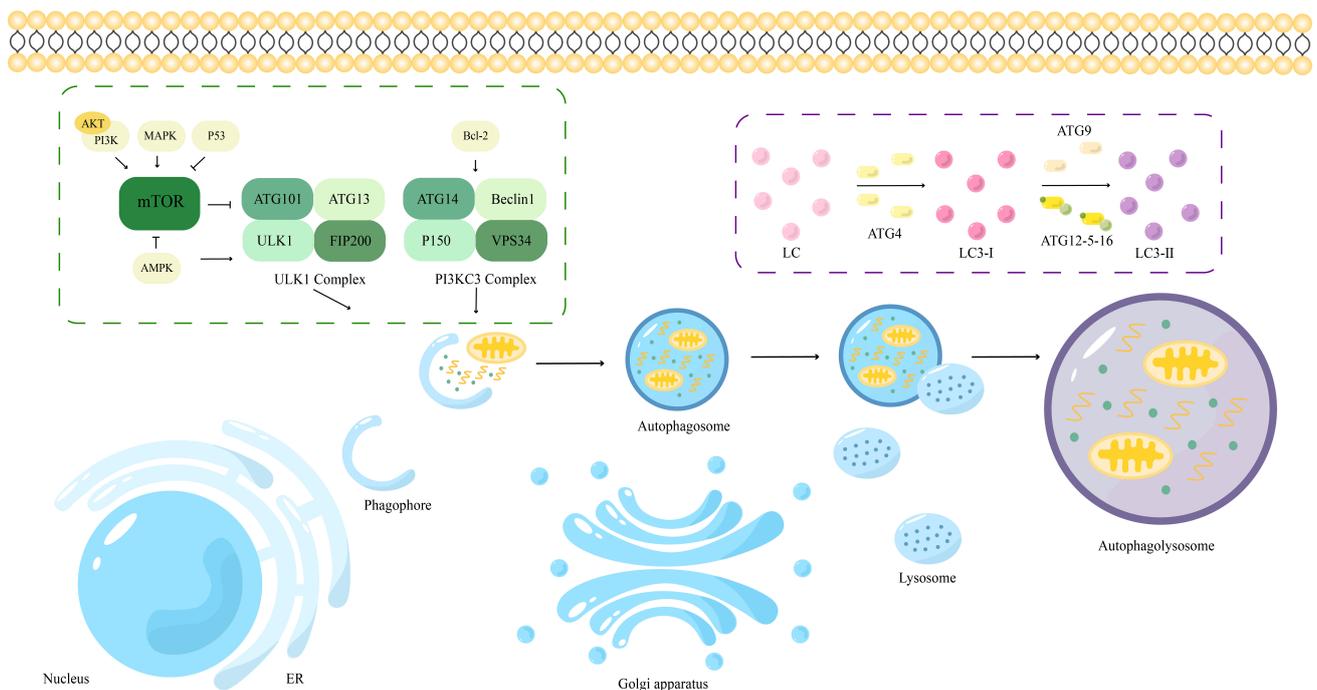
Autophagy is widespread in eukaryotic cells and was first detected in the 1950s by Christian de Duve using transmission electron microscopy. He observed that autophagy altered lysosome morphology and structure, and caused local degradation of the cytoplasm [10]. Autophagy is divided into three primary categories depending on the pathway by which substances enter lysosomes: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). These pathways coordinate, complement and work together to maintain cellular homeostasis. During macroautophagy, phagosomes with a bilayer membrane engulf part of the cytoplasm to form autophagosomes. These fuse with lysosomes to form autophagolysosomes, which are subsequently degraded by the action of lysosomal hydrolase. The degradation products are released and recycled for further use by the cells [11]. CMA is a highly selective process in which the molecular chaperone protein Hsc70 binds to a pentapeptide sequence target protein containing CMA recognition pentapeptide (KFERQ) and transports it to the lysosomal membrane. Here, it is recognized by the lysosomal-associated membrane protein 2A (LAMP-2A) and enters the lysosome to be degraded by lysosomal enzymes [12]. Microautophagy shares some similarities with CMA as a special type of autophagy, and some microautophagy is also associated with Hsc70 [13]. So far, microautophagy has mostly been studied in yeast, where lysosomal membrane invagination engulfs part of the cytoplasm directly into the lysosomes for degradation [14]. Most research on autophagy has been on macroautophagy, and hence this will be referred to here as autophagy.

Autophagy can be initiated under specific circumstances, such as nutrient starvation and oxidative stress. The initiation mechanism is conserved, and the formation of autophagosomes and autolysosomes is crucial to the process of autophagy. Autophagy initiation depends on the regulation of multiple factors, such as autophagy-related genes (ATGs). Autophagosomes have a bilayer vesicular structure whose formation is inseparable from the role of the endoplasmic reticulum (ER). ATG2 was shown to transfer lipid molecules from within the ER to autophagosomes. This process often occurs near the ER and may also involve other organelles that contain membrane structures [15,16]. ATG9 located on the Golgi apparatus is the sole transmembrane protein amongst the autophagy-related proteins [17]. The Golgi apparatus plays a role in the formation of autophagosomes by regulating ATG9 transport, and the autophagic signaling pathway involves Golgi apparatus proteins [18]. Through a process of extension and expansion, phagosomes engulf and degrade small parts of the cytoplasm containing specific proteins and organelles to form autophagosomes. The uncoordinated 51-like kinase 1 (ULK1) complex and the Beclin-1 class III phosphatidylinositol-3-kinase (PI3KC3) complex are the two main protein complexes involved in the initiation of

autophagy [19]. Light chain 3 (LC3) is a soluble protein that regulates autophagic flux and can be used as a marker of autophagy. The two variants of LC3 are LC3-I, which is the cytosolic type, and LC3-II which is the membrane-bound type. LC3 transforms into LC3-I under the action of ATG4 [20]. Upon the initiation of autophagy, LC3-I is transformed into LC3-II, and the formation of autophagosomes is related to the ratio of LC3-II to LC3-I. One of the most crucial organelles in autophagy is the lysosome, since it provides an acidic environment and various hydrolysis enzymes to degrade specific substrates. The fusion of autophagosome membranes to lysosomes results in the formation of autophagolysosomes. This process is negatively regulated by mammalian target of rapamycin complex 1 (mTORC1) [21]. As shown in Fig. 1, the contents of autophagolysosomes can be reused after degradation.

### 4. Related Factors that Affect Autophagy in ICH

A number of pathogenic responses are induced by ICH. These include oxidative stress and inflammation [22, 23], as well as iron overload and ER stress [24,25]. These pathological reactions can affect autophagy when they occur. Neutrophils can also release proinflammatory proteases that aggravate the pathological damage from ICH. A high neutrophil-to-lymphocyte ratio is associated with poor prognosis in ICH patients [26,27]. Activated neutrophils, microglia, and macrophages produce excess reactive oxygen species (ROS) following ICH [28]. ROS accumulation can in turn cause oxidative stress and break the normal balance between oxidant and antioxidant, thereby aggravating SBI after ICH. ROS can also induce the onset of autophagy. Degradation of the products of oxidative stress by phagocytosis can regulate excess free radicals such as ROS, thus mitigating SBI after ICH. This process involves multiple pathways, including p62, CMA and the mitochondrial pathway [29,30]. Oxidative stress can also cause iron overload [31], and autophagy induced by ICH is significantly influenced by iron. Erythrocytes lyse following ICH, resulting in massive accumulation of free iron [32] that can lead to increased ROS, thus exacerbating SBI. Premenopausal women have higher survival rates after ICH than men. Estradiol protects brain tissue, participates in the inhibition of autophagy, and reduces SBI, which is also associated with iron overload [33]. Heme oxygenase-1 (HO-1) can also induce excessive iron production which then aggravates brain damage [34]. Autophagy is activated in various brain cells after ICH. Thrombin is a serine protease that can cause autophagy in astrocytes and brain cells [35] by increasing the conversion of LC3-I to LC3-II. The LC3-II to LC3-I ratio is increased on the third day after ICH, indicating that autophagy has begun. Hematoma is the main cause of inflammatory response after ICH, with microglia and other inflammatory cells such as macrophages also being significant contributors



**Fig. 1. Molecular mechanisms of autophagy initiation.** Autophagy is activated when the body undergoes adverse reactions such as oxidative stress and inflammatory responses. The uncoordinated 51-like kinase 1 (ULK1) complex and the class III phosphatidylinositol-3-kinase (PI3KC3) complex adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK), mTOR, and the B-cell lymphoma 2 (Bcl-2)/Beclin-1 signaling pathway. Phagosome extension engulfs misfolded proteins and organelles and fuses them with lysosomes to form autophagosomes. Light chain 3 (LC3) is converted to LC3-I by autophagy-related gene 4 (*ATG4*). LC3-I can be cleaved enzymatically to convert into LC3-II. LC3-II acts together with ATG9 and ATG12-5-16 complexes to expand and fuse autophagosome and lysosome membranes in a process that depends on the endoplasmic reticulum. mTOR, mammalian target of rapamycin; AKT, protein kinase B; PI3K, phosphatidylinositol-3-kinase; MAPK, mitogen-activated protein kinase.

[36]. Microglia activation and autophagy are mediated by toll-like receptor 4 (TLR4). Hematomas causes TLR4 to activate signaling pathways, resulting in inflammatory damage [37,38]. Interleukin-17A (IL-17A) is involved in mediating autophagy and inflammation during treatment for ICH [39]. NOD-like receptor thermal protein domain associated protein 6 (NLRP6) belongs to the family of NOD-like receptors (NLRs) that are involved in the regulation of inflammation. Han Xiao *et al.* [40] observed increased NLRP6 expression after the onset of ICH in an experimental model. Downregulation of NLRP6 expression can reduce autophagy and the inflammatory response, which is inextricably linked to oxidative stress [41]. Oxidative stress promotes the occurrence of an inflammatory response. Pro-inflammatory cytokines resulting from ROS can activate the c-Jun N-terminal kinase (JNK) and tumor necrosis factor (TNF) induced nuclear factor kappa-B (NF- $\kappa$ B) signaling pathway [42]. Conversely, inflammatory responses can affect oxidative stress. The TNF-induced NF- $\kappa$ B signaling pathway is important in reducing intracellular ROS levels, and the coordination between these is important after ICH. Experiments have confirmed that autophagy increases 6 h

after ICH. When ER stress is present, autophagy increases further and participates in caspase12-mediated apoptosis. One week after ICH, autophagy inhibits ER stress, reduces neuronal apoptosis and necrosis, and protects the cerebral nerves [43]. The incidence of ICH increases with age, but shows a downward trend after 85 years of age [44]. Yotam Raz *et al.* [45] reported that autophagy can still occur in centenarians. Both insufficient and excess autophagy may lead to neuronal cell death. Excessive autophagy in the early stages of ICH can harm the brain, while its inhibition can preserve the brain. Autophagy is involved in maintaining cellular homeostasis in the latter phases of ICH, thereby protecting against brain injury [46].

## 5. Detrimental and Beneficial Roles of Autophagy in ICH

There are two sides to autophagy following ICH. On one hand it can induce programmed cell death, and on the other it can affect cell survival. This is closely related to the level of autophagy, where either insufficient or excessive autophagy can lead to neuronal cell death. Excessive autophagy leads to SBI early during the on-

set of ICH. It has been confirmed experimentally that autophagy increases 6 hours after ICH. This rises even further due to the involvement of ER stress, which participates in caspase12-mediated apoptosis [43]. Autophagy is induced by ROS, and it also regulates free radicals such as excess ROS to mitigate SBI after ICH. This process involves multiple pathways including p62, CMA, and the mitochondrial pathway. Inhibition of autophagy reduces microglia activation and the inflammatory response, and attenuates brain injury. Interleukin broadly regulates the immune response in ICH. Regulation of the expression of anti- and pro-inflammatory factors improves the prognosis of IL. IL-6 is a pro-inflammatory cytokine and elevated levels can increase the risk of hematoma expansion, resulting in impaired recovery and affecting patient outcome [47]. IL-10 is an anti-inflammatory mediator that regulates the levels of B-cell lymphoma 2 (Bcl-2) and Bax to reduce inflammation and apoptosis, thereby helping to repair nerve damage [48]. IL-33 often acts on microglia and macrophages and can increase the level of anti-inflammatory factors by decreasing the release of pro-inflammatory factors. IL-33 reduces neuronal apoptosis by increasing Bcl-2 and decreasing caspase-3. IL-33 can inhibit autophagy and reduce brain injury by increasing the level of p62, reducing the expression of autophagy-related proteins LC3-II and Beclin-1, and inhibiting expression of the pro-inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  [38]. Autophagy has a detrimental effect on ICH, and therefore inhibition of autophagy can have a protective effect in the brain.

The benefit of autophagy after ICH lies in its ability to maintain intracellular homeostasis, thus providing a neuroprotective effect in brain injury [46]. Although autophagy is activated in response to oxidative stress, it also degrades oxidative stress products through phagocytosis, regulates ROS levels, and attenuates SBI caused by oxidative stress. Thrombin is produced immediately after the onset of ICH and disrupts the blood-brain barrier, thus causing edema. Autophagy plays a protective role against thrombin-induced brain injury. One week after ICH, autophagy inhibits ER stress, reduces neuronal apoptosis and necrosis, and protects brain nerves.  $\alpha$ 7nAChR can trigger the cholinergic anti-inflammatory pathway (CAIP) to reduce the inflammatory response. Activation of  $\alpha$ 7nAChR increases Beclin expression, increases the LC3-II/LC3-I ratio, reduces p62/Sequestosome 1 (SQSTM1), and enhances autophagy. These confer cerebral protection through autophagy and improve cardiac dysfunction following ICH [49]. Another study [50] reported that hypoxic preconditioning could upregulate autophagy, promote stem cell proliferation, and enhance neural regeneration after ICH in a process that involved miR-326. MiR-326 mediated autophagy delayed the senescence of olfactory mucosa mesenchymal stem cells (OM-MSCs) during treatment for ICH.

## 6. Molecular Mechanisms Involved in Autophagy in ICH

Under normal physiological conditions, autophagy maintains the energy balance by regulating metabolism and maintaining body homeostasis. Autophagy can have both destructive and protective functions in ICH due to regulation by a variety of signaling pathways. These include the mTOR, adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK), PI3K, mitogen-activated protein kinase (MAPK), and Beclin-1/B-cell lymphoma 2 (Bcl-2) signaling pathways.

### 6.1 mTOR Signaling Pathway

mTOR is a serine/threonine protein kinase in the PI3K-associated protein kinase (PIKK) family and is crucial in the regulation of autophagy and the control of cellular metabolism [51]. Autophagy is typically downstream of mTOR [52,53]. mTOR is comprised of the mTORC1 and mTORC2 complexes. Both complexes contain mTOR, DEP domain-containing mTOR-interacting protein (DEPTOR), mammalian lethal with SEC13 protein 8 (mLST8), and the Tti1/Tel2 complex. mTORC1 also contain regulatory-associated protein of mTOR (RAPTOR) and 40 kDa Pro-rich Akt substrate (PRAS40). mTORC2 also contains rapamycin-insensitive companion of mTOR (RICTOR), mammalian stress-activated map kinase-interacting protein 1 (mSIN1), protein observed with RICTOR1 (PROTOR1) and PROTOR2 [54,55]. The different mTORC protein complexes recognize different substrates and thus transmit different signals in the cell. mTORC1 is rapamycin-sensitive and regulates cell growth, proliferation, apoptosis and autophagy. mTORC2 is insensitive to rapamycin and its primary functions are the cytoskeleton and cell survival [56]. mTORC1 is more closely involved in autophagy than mTORC2. It drives cell growth under nutrient-sufficient conditions, and inactivation stimulates autophagosome formation and thus autophagy [57]. Autophagy can also occur upstream of mTORC1. After the inhibition of mTOR, intracellular anabolism is affected and the autophagic flux increases. Lysosome-associated proteins and the autophagic cycle participate in the activation of mTORC1 [58].

### 6.2 AMPK Signaling

AMPK is a serine/threonine kinase with a heterotrimeric structure consisting of a catalytic  $\alpha$ -subunit, a non-catalytic  $\beta$ -subunit, and a  $\gamma$ -subunit [59]. AMPK protects the brain and reduces nerve injury after ICH. It is one of the main regulatory molecules in autophagy and can either directly activate autophagy or block the mTOR signaling pathway. ULK1 is critical in the AMPK signaling pathway that mediates autophagy. AMPK is sensitive to the energy state of the cell and is activated when the adenosine triphosphate/adenosine monophosphate (ATP/AMP) ratio decreases. Direct phosphorylation of ULK1 leads to the

activation of autophagy. mTOR can disrupt the interaction between AMPK and ULK1, thereby preventing autophagy [60]. Cytosolic  $\text{Ca}^{2+}$  can trigger autophagy. When  $\text{Ca}^{2+}$  levels within the cell are elevated, this activates the subordinate  $\text{Ca}^{2+}$ /Calmodulin-dependent protein kinase kinase $\beta$  (CaMKK $\beta$ ) effector. In reaction to CaMKK $\beta$ , AMPK is phosphorylated and activates ULK1 to initiate autophagy. ULK1 is an important upstream kinase of in autophagy and participates in autophagosome formation [61]. In addition to its involvement in the activation of autophagy,  $\text{Ca}^{2+}$  may sometimes be anti-autophagy under certain conditions [62–64]. The AMPK signaling pathway is involved in the secondary response after ICH.  $\alpha 7\text{nAChR}$  reduces the inflammatory response after ICH and improves cardiac-brain function through autophagy mediated by the AMPK/mTOR signaling pathway [49]. Deng Xiong *et al.* [65] confirmed that inhibition of AMPK/mTOR signaling by lovastatin reduces autophagy levels and mitigates nerve damage in a rat model of ICH.

### 6.3 p53 Signaling

The tumor suppressor protein p53 has an interactive role in autophagy. P53 can activate autophagy, while autophagy can inhibit p53 through negative feedback regulation [66]. Following cellular stress, p53 is phosphorylated at serine and acts on upstream regulators of mTOR such as tuberous sclerosis complex 2 (TSC2) and Sestrin1 and 2 to inhibit mTOR activation of autophagy in a process associated with AMPK. Damage-regulated autophagy modulator (DRAM), a target of p53, mediates autophagy associated with apoptosis. DRAM and Sestrin2 can increase autophagy levels in response to nuclear p53 [67,68]. P53 is included within the release of pro-inflammatory factors after ICH [69], while autophagy inhibits p53 to attenuate secondary neuronal cell death. ATG7 activates the p53 suppressor to inhibit p53-mediated neurogenic damage [70,71]. The regulation of autophagy by p53 has two sides. In the pathological state, p53 often has a pro-autophagy role. However, under normal circumstances p53 inhibits AMP-dependent kinase and activates mTOR to exert an anti-autophagy effect [67,72].

### 6.4 PI3K/Akt Signaling

PI3K/Akt signaling is a classical autophagic pathway. It can be divided into three types depending on structure and function. Class I PI3K is the most dominant isoform involved in autophagy, class II PI3K is not closely related to autophagy, while Class III PI3K is involved in early autophagy. Akt is a serine/threonine kinase that moves from the cytoplasm to the cell membrane in response to PI3K. Once Akt is activated by PI3K, its phosphorylation inhibits autophagy by influencing downstream mTOR [73–75]. The PI3K/Akt/mTOR signaling pathway has brain-protective effects after ICH by reducing blood-brain barrier damage, oxidative stress and the inflammatory response,

and by promoting axonal regeneration [76–79]. Phosphatase and tensin homolog (PTEN) are an autophagy regulator localized in the hippocampus that antagonizes the PI3K/Akt signaling pathway. Downregulation of PTEN activates PI3K/AKT signaling, thereby reducing the level of autophagy-related proteins after ICH. This suppresses the autophagy program, thus alleviating secondary hippocampal impairment and cognitive function deficits after ICH [50]. MiR-326 reduces PI3K and promotes autophagy, thus protecting the nerves after ICH. MiR-326 reduces PI3K and promotes autophagy, thus protecting the nerves after ICH [80].

### 6.5 MAPK Signaling

The MAPK signaling pathway is crucial for cell growth, apoptosis and autophagy, in addition to its involvement in oxidative stress and immune regulation. MAPK involves three major pathways, namely p38MAPK, extracellular-signal-regulated kinase1/2 (ERK1/2), and JNK [81]. P38MAPK has four isoforms:  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ . Upon cell stimulation, P38MAPK is activated by the upstream activators MAPK kinase 3 (MKK3) and MKK6, leading to its participation in the autophagic response. MKK4 activates P38MAPK ion lysosomes and participates in ER stress-related autophagy by phosphorylating lysosome-associated membrane protein 2A (LAMP2A) [82,83]. Lipopolysaccharide (LPS) can also activate the MAPK signaling pathway. P38MAPK phosphorylates ULK1 in response to LPS, thereby inhibiting its activity and preventing it from interacting with ATG13 to reduce autophagic flux levels [81,84]. P38MAPK and ERK1/2 play distinct roles in regulating autophagy. An *in vitro* study of realgar-induced oxidative stress in neurons revealed that P38MAPK was implicated in development of the autophagosome membrane and in autophagic degradation [85]. Autophagy initiation, vesicle production and degradation are all regulated by ERK1/2, while ERK inhibition increases the autophagic flux [86]. The inflammatory response to ICH is closely associated with P38 and ERK1/2. The p38 MAPK and JNK signaling pathways reduce nerve inflammation and attenuate brain edema after ICH [87]. Oleuropein regulates oxidative stress levels in a rat model of ICH by inhibiting the brain hemorrhage-mediated activation of ERK, p38 and JNK [88]. Activation of the C-C chemokine receptor type 1 (CCR1) promotes inflammatory responses after ICH through the ERK1/2 signaling pathway [89]. Albumin alleviates oxidative stress after ICH through the ERK/nuclear factor-E2-related factor 2 (Nrf2)/HO-1 pathway [90]. Albumin also increases the plasma colloid osmotic pressure, reduces intracranial and peripheral edema, and improves nerve injury. ICH induces hypoalbuminemia and systemic inflammatory response syndrome, leading to worse prognosis [91]. The MAPK signaling pathway is involved in cell apoptosis and cell death after ICH, which then reduces SBI [92].

The three types of JNK are JNK1, JNK2 and JNK3. The first two act in multiple systems and are widely expressed *in vivo*. JNK3 has a different site of action compared to JNK1 and JNK2 [93] and is mostly confined to central neurons, with low expression in the heart and testis [94,95]. Apoptosis and autophagy are both heavily influenced by the JNK signaling pathway, which decreases apoptosis by positively regulating autophagy [96,97]. The activation of JNK signaling is crucial for alleviating SBI after ICH [98]. Differentiation of microglia M1 is also enhanced by JNK signaling, while the differentiation of microglia M2 is enhanced by suppression of JNK signaling. The JNK signaling pathway attenuates the inflammatory response after ICH by regulating thrombin activation on microglia [99].

### 6.6 Beclin-1/Bcl-2 Signaling

Beclin-1 is a crucial autophagy-associated protein that contributes to formation of the PI3K complex and is essential during the early stages of autophagy. Bcl-2 is an upstream protein that is pro-survival and inhibits autophagy. Many proteins regulate autophagy by competitively binding to Bcl-2 or Beclin1 to disrupt or enhance the interactions between them [100,101]. The Beclin-1/Bcl-2 ratio is an indicator of autophagy regulation. Autophagy is initiated when Beclin-1 binding to Bcl-2 is inhibited. The Bcl-2 family is therefore heavily involved in the induction of autophagy by JNK. Stimulation of JNK leads to Bcl-2 phosphorylation, thereby disrupting its binding to Beclin and activating autophagy [100,102,103]. The Bcl-2-associated thymus gene 2 (*BAG2*) from the BAG family is also involved in the pathogenesis of neurodegenerative diseases [104]. BAG2 disrupts the interaction of Beclin-1 with BCL-2 to activate autophagy [105]. The pro-apoptotic proteins Bax and Bim can also influence the induction of autophagy by interacting with Beclin-1 [106]. The expression of LC3-II and of Beclin-1 is normally increased after ICH. Beclin-1 is the primary step in autophagy activation following ICH, particularly in microglia and astrocytes [107,108]. Shukun Hu *et al.* [109] confirmed that thrombin induces Beclin-1 and LC3 expression in glial cells and enhances autophagy. Decreased Beclin-1 expression following treatment with necrostatin-1 [110], H<sub>2</sub>S [111] and IL-33 [38] inhibits autophagy and reduces SBI.

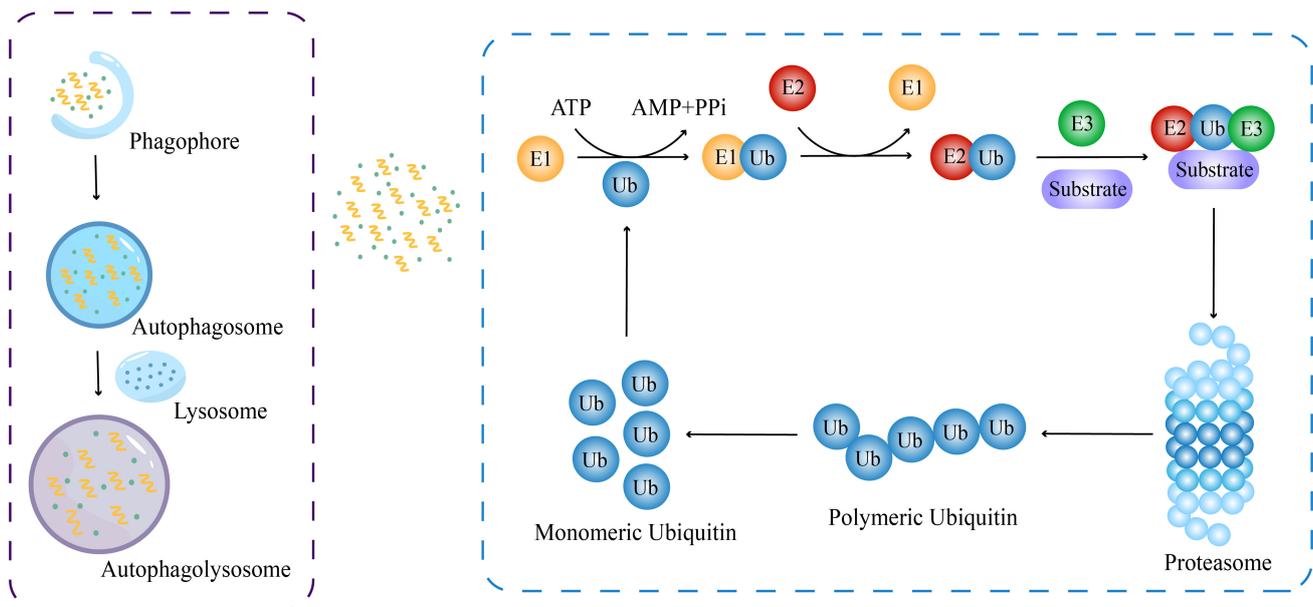
## 7. UPS and Autophagy

Autophagy and the ubiquitin-proteasome system (UPS) are two pathways involved in the metabolism of damaged proteins (Fig. 2). UPS acts on the proteasome, while autophagy acts on proteins within the lysosome. Both processes are highly specific and maintain protein homeostasis under both physiological and pathological conditions [112,113]. UPS is distinct from autophagy in that it is a non-lysosomal pathway consisting of ubiquitin, ubiquitin-activating enzyme (E1), ubiquitin-conjugating

enzyme (E2), ubiquitin ligase (E3), deubiquitinating enzyme (DUB) and the proteasome. Ubiquitin contains 76 amino acid residues covalently bonded to the protein substrate following a ubiquitination cascade reaction that labels the target protein [114,115]. ATP supplies the energy and ubiquitin is activated to produce a thioester link with E1. E1 transfers ubiquitin to E2 to form an E2-ubiquitin complex via a generated thioester bond. E3 selectively recognizes the substrate and acts on the E2-ubiquitin complex to bind it to ubiquitin. The resulting ubiquitinated protein substrate is eventually recognized by the proteasome for degradation in a reversible process. DUBs act specifically between the ubiquitin and the protein substrate to sever the ubiquitin chain structure [116,117]. Although they act independently, the UPS and autophagy pathways nevertheless intersect with each other [118]. The autophagy receptor p62 links UPS with autophagy. P62 is a ubiquitin-binding protein involved in proteasomal degradation while also simultaneously activating Nrf2 signaling [119]. The triangle motif (TRIM) family member TRIM44 is known to connect UPS to autophagy. De-ubiquitination of TRIM44 oligomerizes SQSTM1, which acts on autophagy-associated structures to activate autophagy [120]. Regulators involved in autophagy, such as the ubiquitin ligase TRAF6, ubiquitinate K63 ULK1 to promote the initiation of autophagy. TRAF6 ubiquitinates K63 of Beclin-1 to prevent it from binding to Bcl-2, thus promoting autophagy [121,122]. Luteolin has neuroprotective effects and reduces brain edema. It can be used to treat brain damage caused by the inflammatory response and oxidative stress after ICH. Luteolin inhibits ubiquitination of TRAF6 and Nrf2, enhances autophagy, and activates the p62/Keap1/Nrf2 pathway to exert neuroprotective effects [123,124].

## 8. Selective Autophagy

The selective recognition, isolation and degradation of specific protein aggregates or damaged organelles via the autophagic pathway is called selective autophagy. This process serves to remove unwanted material from the cell and maintain intracellular homeostasis. Selective autophagy includes mitophagy, peroxisomal autophagy, ER autophagy, ribosomal autophagy and lipid autophagy. These represent different types of autophagy in different cellular organelles and in response to different stresses [125,126]. Ubiquitin can also degrade protein aggregates via selective autophagy. The p62/SQSTM1 protein can act as a selective autophagy receptor and is important in signaling, differentiation, and the removal of protein aggregates. P62 can bind directly to LC3 at the site of autophagic vesicle production to mediate selective autophagy. It associates with ubiquitinated proteins, delivers ubiquitinated protein aggregates to autophagic vesicles, and degrades ubiquitinated substrates [127]. mTORC1 and the Keap1/Nrf2 signaling pathway are both crucially activated by p62 during the process of selective autophagy. Phosphorylated p62 competes for Nrf2



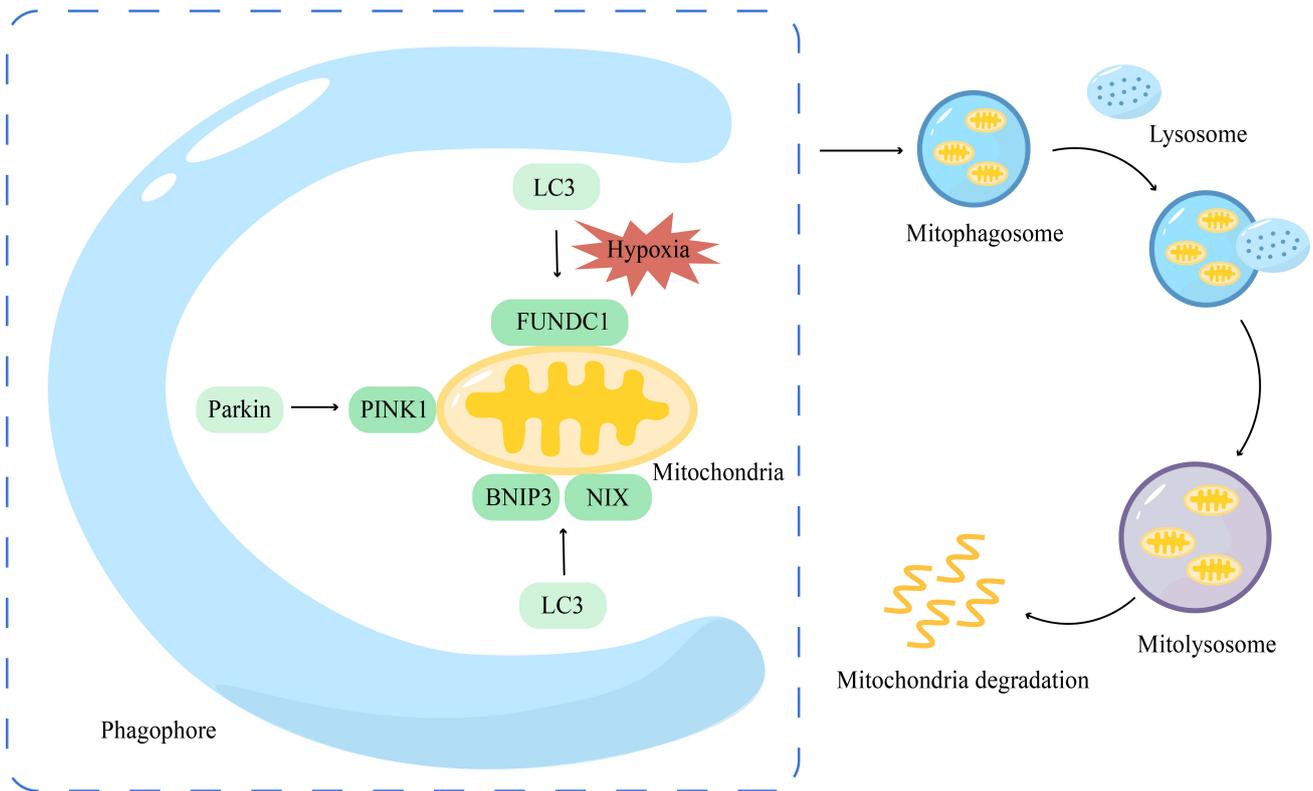
**Fig. 2. Autophagy and UPS.** Autophagy and UPS are important protein degradation pathways in human cells. In UPS, ubiquitin molecules covalently bind to the substrate protein under the cascade catalysis of ubiquitin-activating enzyme (E1), ubiquitin-conjugation enzyme (E2) and ubiquitin-protein ligase (E3). UPS, ubiquitin-proteasome system; ATP, adenosine triphosphate; AMP, adenosine monophosphate.

binding sites during Keap1/Nrf2 signaling under the mediation of mTORC1 to inhibit Nrf2 degradation and induce the expression of cytoprotective Nrf2 targets [128,129]. Toll-like receptor-4 (TLR4) stimulates the transcription of p62/SQSTM1 via the NF- $\kappa$ B signaling pathway and hence its participation in autophagy [130]. P62 can also interact with ATG8 attached to the phagosomal membrane. ATG8 is coupled to phosphatidylethanolamine (PE) and modifies autophagosomes [131,132]. Moreover, ATG8 is involved in the recruitment of specific autophagy receptors with an ATG8-interacting motif (AIM). Excessive or damaged mitochondria, peroxisomes, ER, ribosomes, and lipid receptors can act as ATG-binding proteins and induce selective autophagy through high affinity ubiquitin-interacting motif (UIM)-targeted binding to ATG8 [133,134]. Selective autophagy plays a broad role in physiology and pathology, including the removal of protein aggregates and damaged mitochondria. It is also critical for maintaining functional stability of the nervous system [135,136].

### 8.1 Mitophagy

Mitochondrial autophagy, or mitophagy, is a specific type of autophagy that maintains mitochondrial homeostasis by selectively identifying and removing dysfunctional and damaged mitochondria [137]. Mitophagy is the most common type of selective autophagy in neurological diseases and occurs in numerous physiological and pathological conditions. Lemaster first proposed mitophagy as a quality control mechanism in 2005 [138]. Mitochondria is involved in SBI after ICH, while oxidative stress,

inflammatory response, mitochondrial permeability transition pore (mPTP) opening, mtDNA damage, and  $\text{Ca}^{2+}$  overload are closely associated with mitochondrial dysfunction. Mitophagy is therefore an important therapeutic target for ICH [139]. It can be activated via a number of different pathways (Fig. 3), with the classical one being the PINK1/Parkin pathway. Mitochondrial-targeted PINK1 acts upstream of the E3 ubiquitin ligase Parkin. Phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1) is normally located in the mitochondria, but mitochondrial damage leads to its activation and accumulation in the outer mitochondrial membrane. Parkin is located in the cytoplasm, phosphorylated by PINK1, and is recruited to damaged mitochondria. The conditions for initiation of autophagy are provided by the subsequent ubiquitination of Parkin [140–142]. Jingchen Li *et al.* [143] showed that PINK1 promotes mitophagy in microglia, thus protecting against brain injury after ICH. NLRP3 inflammatory vesicles are closely related to mitophagy and can be triggered by excess ROS and calcium overload. Parkin activates mitophagy and inhibits ROS production, thereby suppressing NLRP3 inflammatory vesicle-mediated inflammation [144]. The AMPK, FUN14 domain containing 1 (FUNDC1), PI3KIII and Bcl2/E1B 19 kDa-interacting protein 3/NIP3-like protein X (BNIP3/NIX) pathways can also activate mitophagy. AMPK phosphorylates ULK1 to promote mitophagy and is also involved in regulation mitochondrial homeostasis. FUNDC1 is an outer mitochondrial membrane (OMM) protein that is activated under hypoxic conditions. FUNDC1 inhibits the activation of



**Fig. 3. Molecular mechanisms of microglia.** When mitochondria are damaged, the main receptors on microglia are PINK1, BNIP3, NIX and FUNDC1. PINK1 accumulates in the outer mitochondrial membrane and recruits Parkin to the mitochondria, where it is then activated. BNIP3 and NIX are also located on the outer mitochondrial membrane and their levels increase in response to upstream signaling factors. The LIR site of BNIP3 and NIX is on the cytoplasmic side and activates microglia by binding to LC3. FUNDC1 enhances the interaction with LC3 under hypoxic conditions and upregulates microglia. PINK1, Phosphatase and tensin homolog (PTEN)-induced kinase 1; LC3, Light chain 3; FUNDC1, FUN14 domain containing 1; NIP3-like protein X (NIX); BNIP3, Bcl2/E1B 19 kDa-interacting protein 3-like protein.

NOD-like receptor protein 3 (NLRP3) inflammatory vesicles by promoting mitophagy, thereby reducing brain damage after ICH [145]. BNIP3 and its analogue NIX are both OMM proteins that contain an LC3-interacting region (LIR). BNIP3/NIX binds to ATG8 via the LIR sequence and recruits ATG8 to targeted mitochondria, thus activating mitophagy [146–148]. Scalp acupuncture can prevent brain tissue damage after ICH and reduce apoptosis by activating mitophagy through the PI3KIII, PINK1/Parkin and NIX pathways [149].

The mechanism underlying mitophagy involves many complex pathways, while the specific mechanisms involved in SBI after ICH are still to be clarified. The role of mitophagy is two-fold. Moderate autophagy is beneficial to the organism, whereas excessive autophagy can have harmful effects. Mitophagy may therefore be a valuable therapeutic target in the future.

### 8.2 ER-Phagy

Degradation of the ER is usually tightly regulated by multiple mechanisms. ER-phagy is selective autophagy of the ER. In neurological disorders, ER-phagy maintains ER

homeostasis and balances the ER network. Under normal conditions, ER-phagy is normally maintained at a low level. However, nutrient starvation and the accumulation of large amounts of unfolded proteins can induce ER-phagy, with mitochondrial metabolism playing a role in this process. ER-phagy regulates the number, shape, size and function of the ER. ER stress occurs after ICH, thereby promoting ER-phagy to form autophagic vesicles [150].

### 8.3 Other Types of Autophagy

Iron metabolism is an important part of internal environmental stability. Imbalance of iron metabolism after stroke leads to hypoxia, lipid peroxidation, damage to mitochondria and neurons, and aggravation of brain injury [151]. Ferritinophagy is a mechanism for transferring ferritin to lysosomes for degradation. In nervous system diseases, iron homeostasis in the body is maintained by degrading the ferritin stored in the cytoplasm so as to inhibit the ferroptosis of neuronal cells. Nuclear receptor coactivator 4 (NCOA4) is the main receptor for iron autophagy. NCOA4 binds to ferritin and degrades it, thus releasing free iron and regulating iron metabolism [152,153]. NCOA4

also mediates iron autophagy, activates oxidative stress, promotes autophagy and apoptosis, and aggravates brain injury. Peroxisomes are involved in the generation and removal of ROS, and are important organelles for regulating redox. When the body is under stress conditions or there is peroxisome dysfunction, specific organelle membranes are ubiquitinated and pexophagy is initiated. Autophagy decreases with age, resulting in excessive accumulation of peroxisomes. Disruption of peroxisome homeostasis is associated with neurological disorders [154]. The selective removal of aggregated proteins is called aggrephagy. Autophagy mediated by ubiquitin-binding receptors such as p62, neighbor of BRCA1 gene 1 (*NBR1*) and human T cell leukemia virus type I (Tax1) binding protein 1 (*TAX1BP1*) plays an important role in mediating protein transport in nervous system diseases [155].

## 9. Cellular Autophagy in ICH

Autophagy occurs in different cell types after ICH, but is mainly concentrated in microglia, astrocytes and neurons. Autophagy protects injured neurons but also affects the clearance of harmful cells, leading to SBI.

### 9.1 Microglia

Microglia are immune effector cells located in the neurological system. They are associated with inflammatory responses, phagocytosis, and the maintenance of brain homeostasis. Microglia respond rapidly to brain injury and are sensitive inflammatory cells [156]. Following ICH, microglia are rapidly activated by hematoma and both of the polarized phenotypes are involved in the secondary response. The M1 phenotype has a pro-inflammatory role and participates in inflammation, oxidative stress and cytotoxicity, thus leading to SBI. The M2 phenotype has the opposite effect to the M1 phenotype. It has anti-inflammatory effects, acts on red blood cells to clear hematomas and inhibits inflammatory responses, thereby reducing brain damage [157,158]. Autophagy in microglia regulates inflammation of the nervous system [159]. After ICH, the Beclin1-Atg5 signaling pathway promotes the activation of autophagy in microglia and aggravates the inflammation of neuronal cells [107]. Microglia can activate TLRs and the TREM2 signaling pathway in response to brain cell injury, and TREM2 upregulates autophagy through the inhibition of mTOR [160]. miRNA-144 acts directly on mTOR to promote microglial autophagy following ICH [161]. miRNA-144 is different to miRNA-23b, which uses Akt/mTOR signaling to downregulate autophagy, inhibit microglial activation, and protect neurons [162]. Multiple factors induce an inflammatory response by activating microglia, thus causing inflammatory damage. Hematomas form after ICH and the lysed red blood cells activate autophagy in microglia via TLR4 [37]. IL-33 often acts on microglia and macrophages. Experimental work has shown that IL-33 can increase anti-inflammatory factors by reducing pro-

inflammatory factors. IL-33-induced increases in Bcl-2 and decreases in caspase-3 reduce neuronal apoptosis. Increased levels of p62 reduce LC3-II and Beclin-1, which then inhibit autophagy and reduce SBI [38]. The proinflammatory cytokine IL-17A activates microglia and mediates autophagy activation via the autophagy genes ATG5 and ATG7 [39]. Microglia contain NLRP3 inflammatory vesicles, and the proinflammatory role of microglia after ICH results from the activation of NLRP3, leading to cerebral edema and an inflammatory response. The AMPK/Beclin-1 signaling pathway activates autophagy, which exacerbates brain damage in an NLRP3-mediated process. Inhibition of the pro-inflammatory mediator NLRP3 is critical to anti-inflammatory treatment after ICH [163]. Autophagy in microglia has two sides, and  $\alpha 7nAChR$  reduces inflammatory responses by activating autophagy in microglia [49].

### 9.2 Astrocytes

Astrocytes are the most abundant cell type in the central nervous system (CNS) and interact with microglia to secrete a variety of cytokines, including proinflammatory and anti-inflammatory cytokines [158]. Under normal conditions, astrocytes nourish neurons and regulate neuronal metabolism. These cells are activated in various pathological states, participate in neuronal protection and reconstruction, and secrete inflammatory factors to clear damage. Astrocytes are important brain cells involved in autophagy after ICH. Thrombin, a serine protease, is an important link in the coagulation cascade reaction. It is produced immediately following ICH and exerts strong hemostatic effects. Excessive accumulation of thrombin leads to edema formation, disruption of the blood-brain barrier, and cell death. Thrombin also promotes the recovery of neurological functions and activates autophagy in the brain, especially in astrocytes and neurons. It is involved in brain injury after ICH and induces autophagy in astrocytes [35]. Inhibition of autophagy exacerbates thrombin-induced cell death. Low doses of thrombin can protect astrocytes and neurons, while high doses promote brain edema and neuronal death [109].

### 9.3 Neurons

Neuronal death in ICH involves multiple mechanisms including oxidative stress, excitotoxicity, hematotoxicity, apoptosis, necrosis, autophagy, and inflammation. A large number of red blood cells lyse after ICH, and extracellular hemoglobin releases heme. The neurotoxicity of hemoglobin causes damage to neurons and oxidative stress-induced SBI. Zhao Yang *et al.* [164] demonstrated that heme and iron induce ER stress in neurons and promote autophagy in neuronal cells. Autophagy is upstream of apoptosis and induces cell death in a process that may be associated with the DDIT3/ATF4 pathway. Neurons often undergo apoptosis after ICH, and autophagy occurs in the neurons surrounding the hematoma. Autophagy protects neurons and maintains normal neurological functions, which in

**Table 1. Relevant experiments involving autophagy in the treatment of ICH.**

Therapy	Pathway	Conclusion	Reference
Mild hypothermia therapy		Mild hypothermia inhibits the up-regulation of autophagy after ICH and exerts neuroprotective effects.	[168]
Hypoxic preconditioning	miR-326/PTBP1/PI3K pathway	Hypoxic preconditioning up-regulates autophagy and enhances neuroprotective effects after ICH.	[50]
IL-17A	ATG5, ATG7	IL-17A promotes microglia autophagy and inflammation after ICH.	[39]
Hesperadin	MST4/AKT pathway	MST4 kinase inhibitor hesperadin attenuates autophagy.	[169]
Acupuncture	mTOR pathway	Acupuncture inhibits mTOR pathway and activates autophagy to improve neurological function after ICH.	[170]
Minocycline		Minocycline effectively treats ICH by inhibiting autophagy.	[108]
Luteolin	TLR4/TRAF6/NF- $\kappa$ B pathway	Luteolin reduces neuroinflammation after ICH.	[123]

ICH, intracerebral hemorrhage; PTBP1, polypyrimidine tract-binding protein 1; mTOR, mammalian target of rapamycin; TLR4, toll-like receptor 4; TRAF6, TNF receptor-associated factor 6; NF- $\kappa$ B, tumor necrosis factor (TNF) induced nuclear factor kappa-B.

turn can cause neuronal damage. Using transmission electron microscopy, Chenghan Wu *et al.* [165] observed a large number of autophagic vesicles and autophagic lysosomes in neurons surrounding the hematoma. Thrombin contributes to the formation of autophagic vacuoles in neurons, which were positively correlated with the degree of brain injury. Qian Li *et al.* [166] observed cellular necrosis and iron sagging in conjunction with autophagy in neuronal cells from the perihematoma region. Iron is one of the factors that influences autophagy in brain cells, and iron pro-lapse leads to neuronal death.

## 10. Trial that Target Autophagy Post-Stroke

Chenghan Wu *et al.* [165] used transmission electron microscopy and autophagy-related protein labeling to observe autophagy of neurons around the hematoma in patients with ICH. The level of autophagy was positively correlated with the degree of nerve cell dysfunction and the amount of bleeding. Thrombin is involved in the activation of autophagy. Targeted autophagy intervention in clinical practice may lead to better patient outcomes. Central post-stroke pain is due to vascular injury caused by bleeding or ischemia and can be relieved by oral antidepressants. Acupuncture regulates the activation of glial cells and apoptosis of neuronal cells, while affecting the transmission of neurotransmitters that have analgesic effects. Ling Zheng *et al.* [167] used electroacupuncture to inhibit autophagy in the hippocampus, which proved effective in relieving post-stroke pain. Yue Su *et al.* [49] showed that activation of  $\alpha$ 7nAChR could improve brain edema and neurological function after ICH.

## 11. Treatment after ICH

The treatment of cerebral hemorrhage and mitigation of SBI remain crucial to the survival and recovery of patients with ICH, but the pathophysiological processes involved are complex. Increasing experimental evidence has confirmed that autophagy is involved in oxidative stress and

inflammatory response, and that autophagy could be used as a potential therapeutic target to reduce SBI. Relevant trials on the treatment of ICH are shown in Table 1 (Ref. [39,50,108,123,168–170]). However, the role of autophagy is inconsistent and it may have different effects at different stages of ICH. Knowledge gaps remain in how to regulate autophagy, how to avoid the harmful effects of autophagy, and how to avoid damage to the brain and other organs so that it can be used optimally in the treatment of ICH.

## 12. Summary and Outlook

In conclusion, autophagy is a double-edged sword with varying outcomes depending on the stage of ICH. Autophagy is used to clear necrotic tissue cells. On the flip side, an excess of autophagy results in more severe brain injury. Autophagy does not have a single role and is instead an important part of a series of pathological reactions secondary to ICH. It is influenced by a number of factors including initiating events, areas of activity, and related effects. Research in this area has advanced significantly, but many of the mechanisms remain to be clarified. For the effective treatment of ICH, it is essential to control autophagy by specific initiation or inhibition at specific sites in order to prevent harmful effects and to avoid damage to the brain and other organs. Key to the outcome of ICH is the identification and selection of critical time points, having effective means to control harmful autophagy, and using autophagy in a beneficial manner. Elucidation of the mechanism of autophagy in nerve cells requires further research. Neuroinflammation and autophagy are important mechanisms in the synergistic treatment of ICH, and the aim is to reduce the inflammatory response through autophagy to obtain better patient outcomes. Autophagy and selective autophagy are regulated by a variety of cytokines, and their molecular mechanisms and signaling pathways show considerable diversity. With the exception of mitophagy, there have so far been few studies on selective autophagy following cerebral hemorrhage. The binding receptor could also be explored

as a potential therapeutic target. Autophagy could therefore be clinically useful as a therapeutic target for SBI after ICH. However, current research is still insufficient and further studies in this area are needed.

## Abbreviations

ICH, intracerebral hemorrhage; SBI, secondary brain injury; CAA, cerebral amyloid angiopathy; BBB, blood-brain barrier; PHE, perihematomal edema; CMA, chaperone-mediated autophagy; LAMP-2A, lysosomal-associated membrane protein 2A; ATGs, autophagy-related genes; ER, endoplasmic reticulum; ULK1, uncoordinated 51-like kinase 1; PI3KC3, Beclin1 class III phosphatidylinositol-3-kinase; LC3, light chain 3; mTORC1, mammalian target of rapamycin complex 1; AKT, protein kinase B; ROS, reactive oxygen species; HO-1, heme oxygenase-1; TLR4, toll-like receptor 4; NLRs, nod-like receptors; JNK, c-Jun N-terminal kinase; TNF, tumor necrosis factor; NF- $\kappa$ B, nuclear factor kappa-B; IL-17A, interleukin-17A; CAIP, cholinergic anti-inflammatory pathway; SQSTM1, Sequestosome 1; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; MAPK, mitogen-activated protein kinase; Bcl-2, B-cell lymphoma 2; PI3K, PI3K-associated protein kinase; DEPTOR, DEP domain-containing mTOR-interacting protein; mLST8, mammalian lethal with SEC13 protein 8; RAPTOR, regulatory-associated protein of mTOR; PRAS40, 40 kDa Pro-rich Akt substrate; RICTOR, rapamycin-insensitive companion of mTOR; mSIN1, mammalian stress-activated map kinase-interacting protein 1; PROTOR1, protein observed with RICTOR1; ATP/AMP, adenosine triphosphate/adenosine monophosphate; CaMKK $\beta$ , Ca<sup>2+</sup>/Calmodulin-dependent protein kinase kinase $\beta$ ; TSC2, tuberous sclerosis complex2; DRAM, damage-regulated autophagy modulator; PTEN, phosphatase and tensin homolog; ERK1/2, extracellular-signal-regulated kinase1/2; MKK3, MAPK kinase 3; LAMP2A, lysosome-associated membrane protein 2A; LPS, Lipopolysaccharide; CCR1, C-C chemokine receptor type 1; Nrf2, nuclear factor-E2-related factor 2; BAG2, BCL2-associated thymus gene2; UPS, ubiquitin-proteasome system; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; DUB, deubiquitinating enzyme; TRIM, triangle motif; PE, phosphatidylethanolamine; AIM, ATG8-interacting motif; UIM, ubiquitin-interacting motif; ALS, amyotrophic lateral sclerosis; mPTP, mitochondrial permeability transition pore; PINK1, phosphatase and tensin homolog (PTEN)-induced kinase 1; FUNDC1, FUN14 domain containing 1; OMM, outer mitochondrial membrane; BNIP3/NIX, Bcl2/E1B 19 kDa-interacting protein 3-like protein; NLRP3, NOD-like receptor protein 3; NCOA4, Nuclear receptor coactivator 4; NBR1, neighbor of BRCA1 gene 1; TAX1BP1, Tax1 (human T cell leukemia virus I) binding protein 1; LIR, LC3-interacting region;

CNS, central nervous system; PTBP1, polypyrimidine tract-binding protein 1; TRAF6, TNF receptor-associated factor 6.

## Author Contributions

JZ, WZ and XPY conceptualized and designed the study. JZ designed the figures and conducted a literature review. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

Not applicable.

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## Conflict of Interest

The authors declare no conflict of interest.

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