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**REVIEW ARTICLE**

# P2X7 Receptor-Associated Programmed Cell Death in the Pathophysiology of Hemorrhagic Stroke

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**Abstract:** Hemorrhagic stroke is a life-threatening disease characterized by a sudden rupture of cerebral blood vessels, and cell death is widely believed to occur after exposure to blood metabolites or subsequently damaged cells. Recently, programmed cell death, such as apoptosis, autophagy, necroptosis, pyroptosis, and ferroptosis, has been demonstrated to play crucial roles in the pathophysiology of stroke. However, the detailed mechanisms of these novel kinds of cell death are still unclear. The P2X7 receptor, previously known for its cytotoxic activity, is an ATP-gated, non-selective cation channel that belongs to the family of ionotropic P2X receptors. Evolving evidence indicates that the P2X7 receptor plays a pivotal role in central nervous system pathology; genetic deletion and pharmacological blockade of the P2X7 receptor provide neuroprotection in various neurological disorders, including intracerebral hemorrhage and subarachnoid hemorrhage. The P2X7 receptor may regulate programmed cell death *via* (I) exocytosis of secretory lysosomes, (II) exocytosis of autophagosomes or autophagolysosomes during formation of the initial autophagic isolation membrane or omegasome, and (III) direct release of cytosolic IL-1β secondary to regulated cell death by pyroptosis or necroptosis. In this review, we present an overview of P2X7 receptor-associated programmed cell death for further understanding of hemorrhagic stroke pathophysiology, as well as potential therapeutic targets for its treatment.

**Keywords:** P2X7 receptor, apoptosis, autophagy, necroptosis, pyroptosis, intracerebral hemorrhage, subarachnoid hemorrhage.

## 1. INTRODUCTION

Hemorrhagic stroke is characterized by a sudden rupture of cerebral vessels, which leads to blood rapidly accumulating in the brain tissue, ventricular system or subarachnoid space, classified as intracerebral hemorrhage (ICH), intraventricular hemorrhage (IVH) and subarachnoid hemorrhage (SAH), respectively [1]. With a trend towards a growing incidence [2], hemorrhagic stroke accounts for 30-40% of all cerebrovascular diseases in the world, while that figure is as high as 40-50% in China [3]. Nearly half of the patients with hemorrhagic stroke will lose their lives in 5 years, and the quality of life for survivors is often very limited, requiring long-term hospitalization and rehabilitation [4]. Despite significant progress in its clinical treatment, as of yet, there have been no effective medical or surgical therapies to improve outcomes for hemorrhagic stroke patients. Therefore, basic studies to define the pathogenesis and targets for the prevention and treatment of hemorrhagic stroke are still needed. Damage following hemorrhagic stroke is triggered by the lysis of red blood cells, releasing hemoglobin, heme, and iron, and the activation of the coagulation cascade, which leads to irreversible destruction of the components of the neurovascular unit, and is followed by blood-brain barrier disruption and deadly brain edema with massive brain cell death [5, 6]. Although a number of factors contribute to the poor outcomes in patients, and different hemorrhagic stroke types have different styles of onset, cell death occurs throughout the whole pathophysiological processes after exposure to the stress events [7]. In contrast to necrosis, which is thought to be an unregulated process lacking a defined molecular pathway, programmed cell death (PCD), referring to apoptosis, autophagy, necroptosis, pyroptosis, and ferroptosis, occurs in an active way and is regulated by certain genes [8, 9]. Increasing researchers have studied PCD and attempted to find ways to provide neuroprotection after hemorrhagic stroke. There are many pathways that are believed to be important in relation to PCD including the death receptor pathway [10]; one of the "death receptors", the P2X7 receptor (P2X7R), is believed to play important roles in regulating PCD under central nervous system pathological conditions including hemorrhagic stroke. In this review, we will summarize the current understandings of PCD after hemorrhagic stroke, especially those associated with P2X7R. In addition, we will also discuss and summarize the thera-

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peutic P2X7R interventions for PCD after hemorrhagic stroke, which may contribute to the development of new therapeutic approaches.

## 2. PCD IN THE PATHOPHYSIOLOGICAL PROCESSES AFTER HEMORRHAGIC STROKE

After hemorrhagic stroke, many molecular events contribute to the occurrence of cell death, such as inflammatory responses [11], production of reactive oxygen species (ROS) [12], endoplasmic reticulum stress [13], axonal degeneration [14], excitotoxicity and loss of calcium homeostasis [15]. Necrosis occurs in the acute phase and is characterized by organelle and cytoplasm swelling, disruption of plasma membrane integrity and disturbed Ca2+ homeostasis, which leads to calpain activation [16, 17]. PCD may be a relatively late event after hemorrhagic stroke and manifests with apoptotic or non-apoptotic features depending on various insults and distinct sequences of molecular events [18-24]. Different types of PCD have different mechanisms, but they also interact with each other [25, 26]. Here, we briefly discuss the characteristics of PCD and its role in the pathophysiological processes after hemorrhagic stroke (Fig. **1**).

### 2.1. Apoptosis in Hemorrhagic Stroke

Apoptosis is the first and best-characterized type of PCD, morphologically characterized by cell shrinkage and membrane blebbing with no changes in organelles and may be detected in various cells of the central nervous system, such as neurons, gliocytes and endothelium cells [27]. Apoptosis promotes cell renovation and elimination of injured cells, whereas dysregulation of cell apoptosis can induce cell death and tissue impairment, consequently leading to organ dysfunction [28]. Most often, apoptosis is driven by caspases, a collection of cysteine-aspartyl-specific proteases that specifically cleave a small subset of aspartic acid residues [29, 30]. There are two types of apoptotic caspases: initiators and effectors. The initiator caspases cleave inactive forms of effector caspases, thereby activating them; then, the effector caspases (for example, caspase-3, -6 and -7) activate endonucleases, leading to DNA fragmentation, ultimately resulting in the destruction of the structures of the whole cell [31, 32]. Under some circumstances, apoptosis can also be triggered in a caspase-independent way [33]. Even though apoptosis is not a new concept, the complex and intricately interwoven pathways of apoptosis are still being elucidated. For example, it has been shown that the caspase-dependent cascade may be particularly important in relation to ischemia damage, while the caspase-independent cascade relates more to neurotoxin-induced apoptosis [34, 35].

There are a number of pathways that are believed to be important in relation to hemorrhagic stroke; these include the following: 1. The death receptor-mediated apoptosis pathway-Hemorrhagic stroke can activate many death receptors, such as tumor necrosis factor receptor (TNF receptor), P2X7R, death receptor 4/5 and Fas, resulting in the activation of caspase-3, thereby activating apoptosis; 2. The mitochondrial apoptosis pathway-Hemorrhagic stroke promotes the release of cytochrome c from the mitochondrial matrix and the formation of the apoptosome with the binding of apoptotic protease activating factor-1 (APAF-1) to cytochrome c and procaspase-9. This Cyt-c-APAF-1-procaspase-

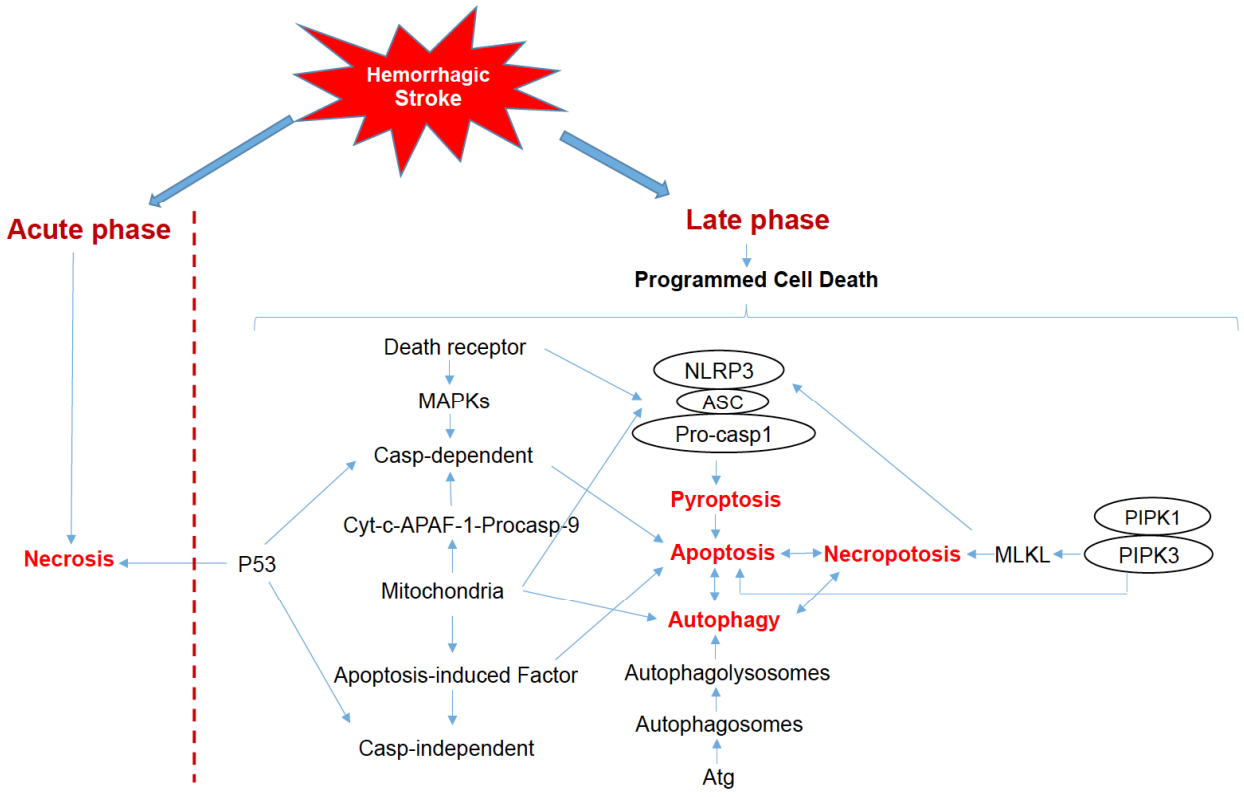


**Fig. (1).** Schematics for the programmed cell death after hemorrhagic stroke.

9 complex further activates downstream effector molecules to trigger apoptosis; 3. The p53 pathway-p53 is a transcription factor that is stabilized in the cytosol and functions as the central regulator of the apoptotic cascade in response to hemorrhagic stroke; 4. The caspase-independent pathway-In the absence of APAF-1, p53 can regulate the release of apoptosis-inducing factor (AIF), which is a mitochondrial intermembrane protein, thereby activating caspase-independent pathways after hemorrhagic stroke [36-40]. A number of studies have shown that modulating the apoptotic-related pathways could improve the outcome of central nervous system diseases including hemorrhagic stroke [36, 41, 42].

### 2.2. Autophagy in Hemorrhagic Stroke

Autophagy is a lysosomal degradation pathway that is essential for survival, development, and homeostasis and plays a key role in diverse pathologies [43]. Through secretion of damaged organelles by cells such as macrophages/microglia, autophagy can either initiate cell death or be pro-survival [44-48]. Increasing evidence confirms that autophagy can coexist or occur sequentially with apoptosis, whereas its effects on apoptotic cell death remain indefinite, indicating the complicated relationship between the two [49-51]. Several studies have shown an early upregulation of autophagy markers after both ICH and SAH [52-54]. However, the positive or negative contribution of autophagy to brain damage after hemorrhagic stroke remains controversial. For example, the suppression of autophagy has been demonstrated to contribute to a reduction in the severity of iron-induced brain injury [55, 56], whereas autophagy has been shown to have a protective effect in thrombin-induced brain injury [57]. In ICH, preventing autophagy exerted neuroprotective effects, which may have been related to the inhibition of subsequent apoptotic insults [58] and suppression of microglia activation [59]. However, activation of autophagic pathways could reduce early brain injury by anti-apoptotic mechanisms in SAH [60]. The different roles of autophagy in hemorrhagic stroke may depend on the specific conditions in different models of injury, and some studies have also suggested that autophagy may play different roles in the pathogenesis at different stages of hemorrhagic stroke [61].

### 2.3. Necroptosis in Hemorrhagic Stroke

Recently, another mechanism of PCD has been described as necroptosis, which manifests with similar morphological features as passive necrosis, but death is executed in a cell-autonomous fashion *via* distinct biochemical processes [62]. Necroptosis is activated when apoptosis is inhibited during host defense against infection and during inflammation [63], and receptor-interacting protein kinase 3 (RIPK3) is the major controller that switches between necroptosis and apoptosis [64]. Activated RIPK3 dimerized with interacting protein kinase 1 (RIPK1) has been proposed to phosphorylate and recruit mixed lineage kinase domain-like (MLKL), a pseudokinase, to the plasma membrane, thereby executing cell death in a caspase-independent way [65, 66]. However, RIPK3 can also induce caspase-dependent death when MLKL is absent, suggesting that the final mechanism leading to cell death depends on the availability of downstream effectors of apoptosis or necroptosis [67]. A novel role of necrostatin-1, a potent inhibitor of necroptosis, in limiting neurovascular injury both in tissue culture and animal models of hemorrhagic injury has been reported [21, 68, 69]. What is more, necrostatin-1 could exert its neuroprotective effects by suppressing apoptosis and autophagy after ICH [22], which further demonstrates the existence of a cross-talk among necroptosis, apoptosis, and autophagy.

### 2.4. Pyroptosis in Hemorrhagic Stroke

One last mechanism of programmed cell death is pyroptosis. Pyroptosis is executed by gasdermin D (GSDMD), which is processed and activated by caspase-1 and caspase-11 in mice and caspase-1, caspase-4, and caspase-5 in humans [70]. The downstream effects of caspase-1 activation are typically accompanied by the maturation and release of the cytokines interleukin-1β (IL-1β) and IL-18 as well as other activators of the immune system. Caspase-1 activation depends on the assembly of multiprotein complexes called inflammasomes that, with the exception of procaspase-1, are constituted by pattern recognition receptors and the apoptosis speck-like adaptor protein (ASC) that recognizes a wide range of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) [71]. Some pattern recognition receptors have been implicated in brain injury including nucleotide-binding oligomerization domain-like receptor containing pyrin domains 1 and 3 (NLRP1 and NLRP3), CARD domain containing 4 (NLRC4) and absent in melanoma 2 (AIM2) [72-75]. Moreover, inflammasome sensors, such as NLRP3 and AIM2, utilize the adaptor protein ASC, which, other than recruiting and activating caspase-1, can also bind and activate caspase-8 to induce apoptotic cell death [76]. Caspase-11-executed cell death appears mainly to be a physiological function and does not efficiently process IL-1β or IL-18 [77]. However, caspase-11 activation can cause $K^+$ efflux to indirectly activate NLRP3 and IL-1β processing and secretion [78]. Recently, this type of cell death has gained attention as a therapeutic target for hemorrhagic stroke since inhibiting NLRP3 inflammasome activation has been proven efficient in reducing neurological dysfunction and neuronal death in experimental hemorrhagic stroke [79, 80].

## 3. THE P2X7 RECEPTOR AND ITS ROLE IN HEMORRHAGIC STROKE

The P2X7 receptor (P2X7R) is an adenosine triphosphate (ATP)-gated, non-selective cation channel that belongs to the P2X superfamily (P2X1-7) of purinoreceptors [81]. P2X7R is ubiquitously expressed in cells of hematopoietic lineage, such as peripherally localized macrophages and monocytes and centrally localized microglia and astrocytes [82]. However, its presence on peripheral or central neurons has been controversial owing to the poor selectivity of antibodies against P2X7R [83, 84]. P2X7R functions in a homotrimeric form and is considered to be the largest protein of the P2X family due to its subunits comprising 595 amino acids [85]. The activation of P2X7R requires a submillimolar to millimolar concentration of ATP, which is far greater than the nanomolar concentration required for activation of other P2X receptors (EC50 of ATP for P2X7R = 2–4 mM and for

other P2XRs = 1-10 μM) [86, 87]. Moreover, P2X7R has a higher affinity for 2'(3')-O-(4-benzoylbenzoyl)-ATP (BzATP) than ATP [88, 89]. Brief exposure of P2X7R to its agonist ATP allows for the passage of small cations ($Na^+$, $Ca^{2+}$, and $K^+$), whereas repeated or prolonged stimulation of P2X7R induces the formation of a non-selective pore allowing the entry of solutes up to 900 Da in size [90, 91].

Currently, two hypotheses have been proposed for the conversion of a non-selective cation channel to a cytolytic pore. One hypothesis suggests that the formation of the large pore occurs due to the dilation of the cation channel itself (intrinsic property of P2X7R), and the second one suggests that additional components are required for the opening of the non-selective membrane pore, such as connexins and pannexins [92-95]. ATP is released in large quantities following any kind of cell injury, and subsequent activation of P2X7R couples to multiple signaling cascades, such as activation of phospholipase D, p38 MAPK, cytoskeletal rearrangements, and L-selectin shedding, which eventually leads to membrane blebbing, release of cytokines and cell death [96-99]. P2X7R antagonists are recognized as potential therapeutics in nervous system diseases, such as traumatic brain injury (TBI) [100, 101], ischemia stroke (IS) [102], epilepsy [103], neuropathic pain [104], and neurodegenerative diseases [105], because, in these cases, secondary cell damage accompanies the primary pathological condition. In addition to antagonists, some other substances also have the ability to affect P2X7R function; for example, multiple nu-

**Table 1. The roles of P2X7R in hemorrhage stroke and other acute brain injuries.**

| Type of Injury | Experimental Species | Inhibition Methods | Mechanisms | Functions in Acute Brain Injury | Refs. |
|---|---|---|---|---|---|
| Intracerebral hemorrhage | Rats | A438079; siRNA | Inhibit RhoA activation | Preserve the blood-brain barrier and neurological function | [110] |
| Intracerebral hemorrhage | Rats | BBG; siRNA | Inhibit NLRP3 inflammasome activation | Reduce inflammation damage and neurologic deficits | [111] |
| Subarachnoid hemorrhage | Rats | BBG; siRNA | Inhibit NLRP3 inflammasome activation | Reduce inflammation damage and neurologic deficits | [79] |
| Subarachnoid hemorrhage | Rats | BBG; siRNA | Inhibit p38 MAPK activation | Ameliorate neuronal apoptosis and neurologic deficits | [109] |
| Traumatic brain injury | Mice | BBG; gene knockout | Decrease the levels of AQP4 and IL-1β | Attenuate cerebral edema and neurologic function | [100] |
| Traumatic brain injury | Rats | BBG | Reduce the levels of PKCγ and IL-1β | Attenuate cerebral edema and neurologic function | [101] |
| Traumatic brain injury | Rats | OxATP | Inhibit glutamate transport and reduce neural autophagy | Promote cognitive deficit repair | [143] |
| Ischemic stroke | Rats | None | Activate caspase-3 | Involved in cell apoptosis | [102] |
| Ischemic stroke | Rats | RB2 | Block the function of activated microglia in the infarct area but promote reactive microglia expression | Restrict the volume of infarction but may affect reparative processes | [176] |
| Ischemic stroke | Rats | OxATP | Decrease penumbral region and expand neuronal loss | Exacerbate ischemic brain damage | [181] |
| Ischemic stroke | Rats | BBG | Reduce $Ca^{2+}$ overload | Reduce infarct lesion and degenerated neurons | [177] |
| Ischemic stroke | Mice | BBG; gene knockout | Reduce $Ca^{2+}$ overload | Reduce microglial cell death | [182] |
| Ischemic stroke | Mice | BBG | Increase ciliary neurotrophic factor expression but not neurogenesis in the subventricular zone | Cannot reduce the lesion area or apoptosis in the penumbra | [180] |
| Ischemia reperfusion injury | Mice | BBG | Abolish the neuroprotective effect of ischemic postconditioning | Aggravate ischemia-reperfusion injury | [178] |
| Ischemic stroke | Mice | gene knockout | Affect the neuroprotective function of microglia | Aggravate brain edema development | [179] |

A438079, BBG, OxATP are P2X7R selective antagonists; RB2 is a P2 unselective antagonist.

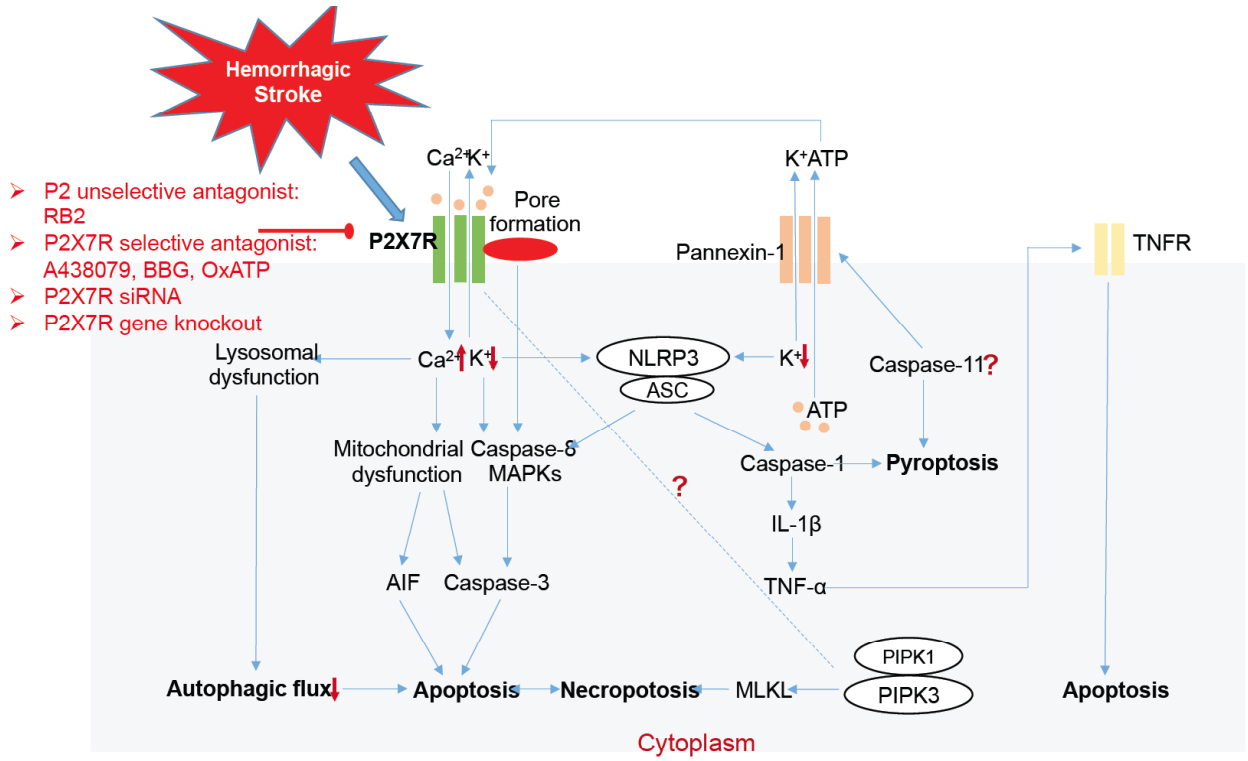


**Fig. (2).** Schematics for the P2X7 receptor-associated programmed cell death.

cleoside reverse transcriptase inhibitors (NRTIs), widely used to treat human immunodeficiency virus infection, have been reported to be therapeutic in a mouse model of dry age-related macular degeneration (AMD) by virtue of their intrinsic anti-NLRP3 inflammasome activity *via* affecting P2X7R pore function [106]. What is more, a recent study found that hydrogen sulfide may be protective in rats with local cerebral ischemia/reperfusion injury by downregulating the expression of P2X7R [107, 108], and our ongoing work also found that hydrogen sulfide administration could significantly reduce NLRP3 inflammasome activation and brain injury induced by ICH through suppressing P2X7R expression.

Studies have demonstrated that activation of P2X7R can also initiate downstream responses such as activation of mitogen-activated protein kinases (MAPKs) [109], RhoA [110], and the NLRP3 inflammasome [111] after hemorrhagic stroke, further aggravating brain functions. Therefore, preventing activation of P2X7R shows a particularly attractive option for alleviating hemorrhagic stroke, as well as other acute brain injuries with similar pathophysiological processes (Table **1**).

## 4. POSSIBLE EFFECTS OF P2X7R ON PCD AFTER HEMORRHAGIC STROKE

After hemorrhagic stroke, cells suffer from a complex bleeding environment, including mechanical stress, blood and its metabolites, free radicals, inflammatory cytokines, electrical pulses, *etc*. [5, 112, 113]. All of these factors may cause cell death by necrosis, apoptosis, autophagy, necroptosis and pyroptosis. Over the recent years, P2X7R-mediated regulation of innate immunity has appeared to be a common avenue of many neurologic disorders of different etiology [114, 115]. A further interesting function of P2X7R is to regulate differentiation and cell fate under pathological conditions [116, 117]. Here, we try to summarize its possible roles in PCD processes regarding the mechanisms underlying brain injury, especially in hemorrhagic stroke (Fig. **2**).

### 4.1. P2X7R and Apoptosis

In most cases, apoptosis is driven by caspase activation as mentioned earlier [29]. P2X7R-mediated changes in intracellular $K^+$ concentrations could induce activation of caspase-8 followed by activation of caspase-3, which is a typical effector of apoptosis [118, 119]. Caspase-3 activation underlies cytolytic mechanisms, which have also been reported to be activated through P2X7R pore formation [97]. Moreover, P2X7R stimulation can activate caspase-1, which causes the rapid maturation and release of IL-1β, and increased IL-1β concentrations, in turn, trigger the induction of tumor necrosis factor-α, which also has pro-apoptotic effects leading to the expansion of cell apoptosis [120]. Therefore, prolonged activation of P2X7R involves the activation of caspase-1, -3 and -8, suggesting the recruitment of apoptosis pathways.

As mitochondrial pathways are known to be an important contributor to apoptosis, persistent mitochondrial permeability transition pore (mPTP) opening can enhance mitochondrial outer membrane permeabilization and cytochrome c release from mitochondria into the cytoplasm, finally inducing caspase-3 activation [121]. A previous study found that activation of P2X7R evokes $Ca^{2+}$ influx *via* ion channels and subsequent mitochondrial dysfunction and caspase-3 activa-

tion in ATP-treated neurons, which were all inhibited by P2X7R antagonism, indicating that caspase-3-dependent apoptosis *via* P2X7R activation can be triggered by mitochondrial dysfunction [122]. Mitochondrial outer membrane permeabilization also results in the mitochondrial release of AIF, which translocates to the nucleus and participates in caspase-independent apoptosis [123]. A recent study reported that ligation of P2X7R by extracellular ATP may accelerate AIF translocation to the nucleus and participate in caspase-independent apoptosis in age-related macular degeneration with subretinal hemorrhage [124], suggesting that this mechanism may also exist in hemorrhagic stroke due to the similar bleeding environment.

Stimulation of P2X7R may also increase protein tyrosine phosphorylation, ultimately leading to activation of mitogen-activated protein kinases (MAPKs) [125, 126], including extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPKs. ERKs are essential for cell survival, whereas JNKs and p38 MAPKs are stress activated and thus involved in apoptosis [127, 128]. Previous studies have demonstrated that JNKs can phosphorylate Bcl-2 and Bcl-xL and diminish their anti-apoptotic activity [129], whereas activation of p38 MAPKs leads to the production of caspase-3 and reduction of Bcl-2, which results in apoptotic cell death [130].

A hematoma rapidly forms in the brain parenchyma after ICH, resulting in necrosis within the local hematoma, while in the peri-hematoma tissue, apoptosis occurs more often, indicating that the cellular damage is reversible [131, 132]. In SAH, inhibition of apoptosis in the cortex and hippocampus near the bleeding site is one of the important neuroprotective ways as well [133]. In combination with the sequential upregulation of P2X7R in microglia after ICH, brain inflammation and neuronal apoptosis have been shown to be reduced by either P2X7R siRNA or the selective P2X7R antagonist BBG *via* alleviating NLRP3 inflammasome activation and subsequent IL-1β/IL-18 release [111]. Another study found that P2X7R can be expressed on the neurons after SAH, and treatment with BBG or gene silencing effectively reversed SAH-induced p38 MAPK activation, thus reducing neuronal apoptosis [109]. Although the close relationship between P2X7R and apoptosis after hemorrhagic stroke has been demonstrated, a more detailed mechanism needs to be verified in the future.

### 4.2. P2X7R and Autophagy

Autophagy serves as a fundamental defense mechanism to eliminate intracellular pathogens in the innate immune system and is involved in a number of central nervous system disease processes including hemorrhagic stroke [58, 134-136]. Given the critical role of P2X7R during various immunologic functions (*i.e*., caspase activation and IL-1β secretion) and in the pathophysiological processes of central nervous system diseases, it is reasonable to propose that P2X7R may play a significant role in regulating autophagy after hemorrhagic stroke. A previous study demonstrated that activation of P2X7R by ATP could result in an elevation of lysosomal PH in both MG6 mouse microglial cells and primary microglia; thereby, accumulated autophagosomes were released into the extracellular space rather than being subjected to degradation *via* fusion with lysosomes. Therefore, the P2X7R pathway may be linked to the reduction in autophagic flux in microglial cells *via* the impairment of lysosomal functions [115, 137]. Similarly, another study demonstrated that stimulation of P2X7R by ATP would alkalinize lysosomes and impair lysosomal function in cultured human retinal pigmented epithelial cells [138]. However, whether inhibition of P2X7R could provide a protective effect for lysosomal functions and normalize autophagic flux in microglia after hemorrhagic stroke needs to be explored in future studies.

The elevation of excitatory amino acids caused by many central nervous system diseases may contribute to excitotoxic neuronal death *via* abnormal stimulation of glutamate receptors, and autophagy might be involved in this process [139-141]. It has been demonstrated that P2X7R stimulation in primary cultures of rat spinal microglia could downregulate the activity of glutamate transporters [142]. Another study found that, in traumatic brain injury *in vivo*, P2X7R stimulation could promote neural autophagy by downregulation of glial glutamate transporter expression in the hippocampus, finally resulting in cognitive impairments including spatial cognitive deficits and long-term potentiation [143]. As the involvement of excitotoxicity following hemorrhagic stroke also contributes to excitotoxic neuronal death and brain damage [144, 145], it is possible that strategies targeting the prevention of P2X7R activation may inhibit glutamate transport activity, thereby reducing neural autophagy to alleviate the neurologic injury after hemorrhagic stroke. However, further study of the interaction between P2X7R and autophagy in different cell types and different stages after hemorrhagic stroke needs to be done.

### 4.3. P2X7R and Necroptosis

Increasing evidence demonstrates that there exists considerable cross-talk between apoptosis and necroptosis, and components of both may be activated simultaneously [146-148]. Therefore, the process of PCD may be a continuum, with apoptosis and necroptosis representing two extremes of biochemically overlapping death pathways. In spite of little specific evidence illuminating the relationship between P2X7R and necroptosis, one previous study reported that activation of P2X7R leads to initial pro-apoptotic cell shrinkage with subsequent large necroptotic cell swelling and plasma membrane disintegration [148]. In fact, as apoptosis induced by P2X7R stimulation shows a rise in cytosolic $Ca^{2+}$ and ROS along with endoplasmic reticulum stress, all of these signals are equally well known to induce necroptosis [149]. Necroptotic cell death may be underestimated at present, due to the lack of simple detection assays [150, 151]. Moreover, our unpublished work proved that necroptotic cell death occurs even more extensively than apoptosis after experimental SAH. Therefore, it is reasonable to speculate that P2X7R-mediated cell death is at least partially *via* the necroptotic pathway after hemorrhagic stroke.

### 4.4. P2X7R and Pyroptosis

As P2X7R directly interacts with the NLRP3 inflammasome [152], stimulation of P2X7R activates inflammasome-associated caspase-1, leading to pyroptosis, as well as cleav-

age of pro-IL-1β to mature IL-1β and its release from the cell [153, 154]. We and others have observed that P2X7R suppression significantly reduces NLRP3 inflammasome-associated caspase-1 activation either in ICH or SAH [79, 111]. Therefore, P2X7R mediated-pyroptosis is obviously involved in the pathological process of brain injury after hemorrhagic stroke.

Caspase-11-induced cell death resembles caspase-1-mediated pyroptosis [155, 156]. What is more, *in vitro* and *in vivo* evidence has suggested that activated caspase-11 may cleave the C-terminal portion of the membrane channel pannexin-1 to cause ATP release and $K^+$ efflux [157]. Extracellular ATP further activates P2X7R, which may lead to the formation of membrane pores and subsequent cytolysis and DAMP release [158]. Meanwhile, pannexin-1 mediated $K^+$ efflux can also activate NLRP3-caspase-1 activation independent of P2X7R activity [159]. Although the role of caspase-11 in hemorrhagic stroke is not clear, its role has been explored at the level of brain cells and some other brain disorders. For example, hypoxic exposure can induce expression and activation of caspase-11, which is accompanied by activation of caspase-1 and secretion of mature IL-1β and IL-18 in brain microglia [160], and caspase-11 also participates in endoplasmic reticulum stress-dependent astrocyte death in ischemic conditions [161]. What is more, neutralization of NLRP1 inflammasomes could reduce its associated caspase-1 and caspase-11 activation and subsequent pyroptosis after traumatic brain injury [162]. Nevertheless, whether and how caspase-11-mediated pannexin-1 activation interacts with P2X7R still needs to be clarified.

## 5. THERAPEUTIC SIGNIFICANCE

It is now well established that ATP acting at P2X7R serves as an efficient stimulus for inflammation and PCD in the pathogenesis of many diseases, ranging from inflammatory to autoimmune disorders and from altered neurological conditions to cancer, suggesting a high pharmacological potential for P2X7 blocking drugs in a broad range of settings [1, 163]. A number P2X7R antagonists have been developed, including various compounds and biologics, and some of them are in clinical trials, as previously highly reviewed by Manju Tewari [89] and Jin-Hee Park [164]. Although end-products of the pioneering developments of P2X7R antagonists, such as AstraZeneca's AZD9056 [165] and Pfizer's CE-224,535 [166], have not proven efficacious in Phase II trials in rheumatoid arthritis patients, clinical studies have revealed an acceptable safety and tolerability profile of such antagonists as a whole [165-167], opening up the possibility of developing P2X7R-targeting compounds in new areas, such as central nervous system disorders. The ability to penetrate the blood-brain barrier is the key to a great amount of pharmaceutical research and development in the field of central nervous system diseases. Currently, with the considerable progress of P2X7R antagonist development, some new and high-affinity P2X7R antagonists readily enter the central nervous system. GSK1482160 was reported to have good brain-penetrating properties from positron emission tomography (PET) studies [168]. In addition, Janssen has consistently released new brain-penetrating and triazolopyridine-based P2X7R antagonists, such as JNJ-47965567 (hP2X7 pKi = 7.9, rP2X7 pKi = 7.9), JNJ-42253432 (hP2X7 pKi = 7.9, rP2X7 pKi = 9.1), and JNJ-54232334 (rP2X7 pKi = 9.3) for use in central nervous system diseases [169-174]. Among them, JNJ-54232334, an orally bioavailable compound with good drug-like properties, showed markedly improved target engagement for the rat P2X7R and appropriate physical properties, including effective penetration of the central nervous system [171]. Furthermore, recently identified negative allosteric modulators of P2X7R (*e.g.*, certain phenothiazine type antipsychotic drugs), already registered for human use may also become important therapeutic tools in the field of central nervous system disorders [175].

Although the neuroprotective effects of P2X7R suppression remain controversial in ischemic stroke [176-182], inhibiting P2X7R has only been reported to be useful in the prevention of acute neuroinflammation and cell death after hemorrhagic stroke [79, 109-111], owing to the possibility that hemorrhagic stroke strikes the cells more directly and severely, thereby causing rapid release of ATP and its accumulation in a larger volume. Therefore, P2X7R could be an ideal target for developing novel preventive and therapeutic strategies by aiming to modulate the inflammatory responses and various types of PCD, thus consequently alleviating neurologic defects after hemorrhagic stroke. In the future, based on a deeper comprehension of the roles of P2X7R in human pathological processes translated from animal disease models, rigorous clinical trials are highly warranted for the evaluation of the efficacy of P2X7R suppression after hemorrhagic stroke.

## CONCLUSION

Recent studies have revealed that PCD appears to be an important pathophysiological event in many central nervous system disorders including hemorrhagic stroke. Ferroptosis, also a newly recognized form of PCD characterized by iron-dependent cell death and regulated by ferroptosis-related genes, such as lipocalin-2, a protein that participates in iron homeostasis and enhances brain iron clearance, has been reported to occur after ICH [183, 184]. However, existing evidence for ferroptosis is nonspecific and seems to lack a close relationship with P2X7R after hemorrhagic stroke, so we did not discuss it here in detail.

Based on the tight involvement and current understanding of P2X7R in PCD after hemorrhagic stroke, we believe that suppression of P2X7R would be a novel contribution to the regulation of PCD, thereby alleviating neurologic defects in a hemorrhagic stroke-induced complicated environment. However, the exact mechanism of P2C7R suppression is not fully understood for the following reasons: 1. The degree of ATP overflow and P2X7R activation after hemorrhagic stroke has not been measured, although extracellular ATP is believed to increase as a result of leakage from damaged or dying cells after hemorrhagic stroke; 2. The roles of different types of PCD have not been evaluated after hemorrhagic stroke due to the lack of specific markers; therefore, the exact processes of apoptosis, autophagy, necroptosis, and pyroptosis in various cell types after hemorrhagic stroke are not clear; 3. As many downstream pathways are affected by P2X7R activation, other potential mechanisms that contribute to PCD after hemorrhagic stroke still need to be deter-

mined; 4. Current P2X7R selective antagonists and even gene-deficient mouse models are not fully capable of probing P2X7R function; therefore, the regulation of PCD by P2X7R after hemorrhagic stroke may require the use of more advanced gene editing techniques, such as cell-type specific and/or inducible knockouts, optogenetic constructs, and humanized mouse models reproducing human gene polymorphisms in rodents [82]. Hence, further studies are still needed to figure out the close cross-talk between P2X7R and PCD.

As this article illustrates, with increasing evidence that P2X7R antagonists exert benefits to central nervous system pathology in early clinical investigations [185, 186], the discovery of receptor-selective antagonists for P2X7R has shown significant therapeutic potential after hemorrhagic stroke. However, from a pharmacological perspective, it is worth noting that there is presently a wider structural diversity of nucleotide P2X7R antagonist pharmacophores than have been described for other P2X receptors [187, 188]. Therefore, the discovery and application of selective P2X7R antagonists in the field of hemorrhagic stroke need to differentiate the respective roles of these receptors. Furthermore, further comprehension of the roles of P2X7R in human pathological processes translated from animal models after hemorrhagic stroke should be taken into account.

## CONSENT FOR PUBLICATION

Not applicable.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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