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The necroptosis pathway and its role in age-related neurodegenerative diseases: will it open up new therapeutic avenues in the next decade?

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ABSTRACT

Introduction: Necroptosis is a programmed form of necrotic cell death. Growing evidence demonstrates that necroptosis contributes to cell demise in different pathological conditions including age-dependent neurodegenerative diseases (NDs). These findings open new avenues for understanding the mechanisms of neuronal loss in NDs, which might eventually translate into novel therapeutic interventions.

Areas covered: We reviewed key aspects of necroptosis, in health and disease, focusing on evidence demonstrating its involvement in the pathogenesis of age-related NDs. We then highlight the activation of this pathway in the mechanism of axonal degeneration. We searched on PubMed the literature regarding necroptosis published between 2008 and 2020 and reviewed all publications were necroptosis was studied in the context of age-related NDs.

Expert opinion: Axonal loss and neuronal death are the ultimate consequences of NDs that translate into disease phenotypes. Targeting degenerative mechanisms of the neuron appears as a strategy that might cover a wide range of diseases. Thus, the participation of necroptosis as a common mediator of neuronal demise emerges as a promising target for therapeutic intervention. Considering evidence demonstrating that necroptosis mediates axonal degeneration, we propose and discuss the potential of targeting necroptosis-mediated axonal destruction as a strategy to tackle NDs before neuronal loss occurs.

1. Introduction

Aging represents the most important risk factor for the development of neurodegenerative diseases (NDs). As life expectancy is increasing and birth rate decreasing, the world’s population is getting older at an unprecedented rate. It is estimated that by 2050, approximately 17% of the global population (1.6 billion) will be aged 65 and over [1]. Unfortunately, the fact that people are living longer has translated into an increase in NDs in the population, with enormous social and economic consequences. Most neurodegenerative conditions for which age constitutes the most important risk factor, including Alzheimer’s disease (AD), Parkinson’s disease (PD), and amyotrophic lateral sclerosis (ALS), have no cure yet and treatments only modestly decrease symptoms, without significantly delaying the progression of neurodegeneration and the associated functional impairment. Despite considerable advances toward the understanding of the etiology of NDs, the exact mechanisms underlying neuronal loss have not been completely elucidated.

Regulated necrosis, currently known as necroptosis, is a caspase-independent mechanism of cell death mediated by the kinase activity of receptor-interacting protein kinase-1 (RIPK1) and RIPK3, which ultimately leads to the activation of the pseudo-kinase mixed lineage kinase like (MLKL), the canonical necroptosis executor [2–4]. Necroptosis has crucial functions in development and tissue homeostasis, yet emerging evidence has implicated this pathway in the development of several pathological conditions including viral infections, inflammatory disorders, ischemic injury conditions, cancer, and various NDs including ALS, AD, PD, Huntington’s disease (HD), multiple sclerosis (MS), and glaucoma [5]. The discovery that the necroptosis pathway is activated in the context of neurodegeneration has brought excitement to the field as necroptosis appears to be an important driver for neuron cell death in NDs. Therefore, a deep understanding of the precise mechanisms underpinning the necroptosis pathway could provide important therapeutic insights.

In this review, we document the evidence that has arisen in the last decade demonstrating that the necroptotic signaling pathway is active in age-related neurodegenerative disorders. We then summarize key proof for the involvement of necroptosis as a mediator of axonal degeneration, an early event in most NDs, that constitutes a fundamental phase in the pathomechanism of this group of diseases. For a discussion on the role of necroptosis in other diseases, the following reviews have been published [5–10].

2. Necroptosis: a potential therapeutic target for age-related NDs

2.1. Necroptosis signaling pathway overview

Necroptosis is a regulated form of necrotic cell death. The necroptotic signaling pathway can be initiated by multiple stimuli.
Necroptosis is a recently identified mechanism of regulated cell death that can be activated by several stimuli including infection, inflammation, and activation of toll-like and cell death receptors. Necroptosis shares morphological and inflammatory features with non-regulated cell death by necrosis.

The necroptosis pathway requires as a first step the kinase activity of the proteins RIPK1 and RIPK3 to form the necrosome complex. Upon MLKL recruitment to this complex, its activation might result in the formation of oligomers that translocate and permeabilize the plasma membrane triggering cell death.

Although the necroptotic signaling pathway has crucial roles in development and tissue homeostasis, several reports have implicated necroptosis in the pathogenesis of numerous diseases, including various neurodegenerative conditions.

Aging is the most important risk factor for neurodegenerative conditions; therefore, the increased aged population worldwide represents an important social and economic burden as currently there is no cure for age-related neurodegenerative diseases.

Although the exact mechanisms of neurodegeneration in most neurodegenerative conditions are not clearly understood, recent evidence has demonstrated that necroptosis represents an important driver for neuronal loss in several of these diseases.

Recently published data have implicated the necroptotic machinery in the mechanisms of axonal degeneration, and the term neuronal necroptosis has been proposed to describe this process.

Degeneration of the axon represents a common, early feature of neurodegenerative conditions that occurs before the destruction of the neuronal soma; thus, necroptosis emerges as a novel target to treat neurodegenerative diseases before irreversible neuronal death occurs.

This box summarizes the key points contained in the article.

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Article Highlights

- Necroptosis is a recently identified mechanism of regulated cell death that can be activated by several stimuli including infection, inflammation, and activation of toll-like and cell death receptors. Necroptosis shares morphological and inflammatory features with non-regulated cell death by necrosis.
- The necroptosis pathway requires as a first step the kinase activity of the proteins RIPK1 and RIPK3 to form the necrosome complex. Upon MLKL recruitment to this complex, its activation might result in the formation of oligomers that translocate and permeabilize the plasma membrane triggering cell death.
- Although the necroptotic signaling pathway has crucial roles in development and tissue homeostasis, several reports have implicated necroptosis in the pathogenesis of numerous diseases, including various neurodegenerative conditions.
- Aging is the most important risk factor for neurodegenerative conditions; therefore, the increased aged population worldwide represents an important social and economic burden as currently there is no cure for age-related neurodegenerative diseases.
- Although the exact mechanisms of neurodegeneration in most neurodegenerative conditions are not clearly understood, recent evidence has demonstrated that necroptosis represents an important driver for neuronal loss in several of these diseases.
- Recently published data have implicated the necroptotic machinery in the mechanisms of axonal degeneration, and the term neuronal necroptosis has been proposed to describe this process.
- Degeneration of the axon represents a common, early feature of neurodegenerative conditions that occurs before the destruction of the neuronal soma; thus, necroptosis emerges as a novel target to treat neurodegenerative diseases before irreversible neuronal death occurs.

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including inflammation, activation of cell death and toll-like receptors, DNA damage, anti-cancer drugs, and by intracellular signals [11]. The most common – and therefore most investigated and better characterized – signaling pathway responsible for executing necroptosis is initiated by stimulation of the tumor necrosis factor type 1 receptor (TNFR1) by TNF-a ligation. Upon TNFR1 activation, recruitment of RIPK1 can result in the formation of the pro-survival signaling complex I to trigger cell survival via activation of NF-kB and MAPK pathways, which promote the expression of several pro-inflammatory and pro-survival genes [12]. Depending on the RIPK1 polyubiquitination level, detachment of the protein from complex I can lead to the formation of the cell death-inducing signaling complex II [2,12]. If caspase-8 is present, then RIPK1 promotes apoptosis activation [13]. In the absence of caspase-8 activity, RIPK1 in complex II recruits RIPK3, resulting in the assembly of the necrosome complex [3,14], where RIPK3 is phosphorylated at Ser227 [15]. Then, the necroptosis executioner MLKL is incorporated into the necrosome complex [4,16].

Of note, there is evidence of RIPK1-independent necroptosis activation. By instance, it was demonstrated that infection by murine cytomegalovirus triggers RIPK3-dependent necroptosis activation which proceeded independent of RIPK1 [17]. In another study, it was shown that deletion of caspase-8 during stimulation of toll-like receptor 3, induced RIPK3 and MLKL-dependent necroptosis that, depending on the cell type studied, did not involve RIPK1 activity [18]. Thus, under these conditions, fibroblasts and endothelial cells underwent necroptosis independent of RIPK1, however, macrophages did rely on RIPK1 for necroptosis activation [18]. Moreover, under certain conditions, RIPK1 can block necroptosis triggered by RIPK3. The observation that ablation of either Casp8 or Ripk3 could not prevent the lethality triggered by Ripk1 ablation, but ablation of both genes did provide protection, indicated that RIPK1 can suppress RIPK3-mediated necroptosis [19]. In this line, Dannappel et al. demonstrated that keratinocyte necroptosis induced by Ripk1 deficiency was prevented by Ripk3 ablation in vivo [20].

A role for mitochondria in the mechanism of necroptotic cell death was proposed following the observation of mitochondrial dysfunction and mitochondrial reactive oxygen species (ROS) accumulation upon necroptosis activation [21–24]. However, further studies showed that the involvement of mitochondrial ROS signaling is not indispensable for necroptosis execution as ROS scavengers or antioxidants did not protect certain cell types against TNF-mediated necroptosis [25,26] which suggests that this might depend on the cell type being studied. Evidence supporting a role for mitochondria in the necroptotic pathway demonstrated the involvement of the mitochondrial permeability transition pore (mPTP) in this process [27,28]; however, other studies showed that ablation of the mPTP regulator cyclophilin-D did not prevent necroptosis execution [29,30]. To test the contribution of mitochondria in necroptosis, Tait et al. depleted mitochondria via mitophagy and showed that TNF-induced necrotic cell death was not blocked, arguing against a crucial role for mitochondria as effectors of necroptosis [29]. However, RIPK1, RIPK3, and MLKL have been shown to be translocated to the mitochondria, altering energetic metabolism by activating enzymes of aerobic respiration pathways, leading to increased mitochondrial ROS production [24,31]. Although there is a clear crosstalk between mitochondria and necroptotic protein players, the exact mechanism remains to be clarified, which appears to depend on the context and cell type.

During the canonical necrosome assembly, MLKL undergoes conformational changes upon phosphorylation by RIPK3 [32], resulting in the formation of oligomers that translocate to the plasma membrane, leading to cell permeabilization and eventually, cell death [33–39]. Nevertheless, how exactly MLKL oligomers lead to cellular destruction is still under investigation, and different mechanisms have been suggested to operate, including direct binding to phospholipids at the cell membrane leading to the formation of membrane pores [33,35,39,40]. Furthermore, Cai and colleagues demonstrated the involvement of transient receptor potential melastatin related 7 (TRPM7) as an MLKL downstream effector of necroptosis, as ablation of this channel blocked necroptosis in HT29, Jurkat, and J774A.1 cells. Further experiments using a genetically encoded Ca2+ indicator showed that TRPM7 mediates extracellular Ca2+ influx during necroptosis [41]. However, it is important to mention that these results might represent a case that depends on a specific type of cell and cellular context, and do not imply that TRPM7 constitutes an obligate contributor to necroptosis in other cell contexts.
Indeed, another study demonstrated that cells cultured in ion-free media were still able to undergo necroptosis [33].

Rupture of the plasma membrane and the subsequent release of intracellular damage-associated molecular patterns (DAMPs), such as histones, mitochondrial DNA, ATP, HMGB1, and cytokines, cause a robust inflammatory response [42], which has been historically a signature for necrotic cell death. A schematic representation of the TNFR1-mediated necroptotic signaling pathway is shown in Figure 1.

2.1.1. Physiological role of necroptosis
Termination of cellular functions by genetically programmed cell death plays important roles in multiple physiological processes, including development, differentiation, host defense against microbial infection, and homeostasis of the immune system. The fact that mutant mice lacking indispensable components of the necroptotic machinery – such as Ripk3 and Mlkl knockout mice – develop normally [32,43], and the observation that Ripk1, Ripk3, and Mlkl genes are not present in primitive organisms [44], prompted Shan et al. to suggest that the necroptosis signaling is not involved in animal development or normal adult function and rather, they suggested that necroptosis operate as a mechanism to abort defective mutant embryos and preserve offspring health [45]. The researchers supported their hypothesis in studies demonstrating that genetic ablation of Ripk3 and Mlkl prevent the embryonic lethality triggered by mutations during different stages of embryonic development. By instance, mice lacking caspase-8 exhibit alterations in their cardiovascular system that leads to embryonic lethality, yet the lethality is rescued when these animals are bred in a Ripk3−/− or Mlkl−/− background [46,47]. Nonetheless, although these Casp8−/−Ripk3−/− and Casp8−/−Mlkl−/− double mutant mice are viable and develop into fertile adults, they exhibit severe lymphadenopathy. Additionally, Casp8−/−Mlkl−/− develops systemic autoimmune disease and thrombocytopenia suggesting that RIPK3 and MLKL might be involved in immune functions beyond their critical roles as canonical regulators of necroptotic cell death [47].

The necroptotic machinery has been increasingly recognized as participating in innate immunity, critical for the host defense response against many pathogens. Indeed, in contrast to Shan et al. premise, it was proposed that the necroptotic pathway evolved as an alternative mechanism to fight against infection when pathogens interfere with the apoptotic machinery [48]. Upon infection, cellular activation of necroptosis can contribute to pathogen clearance by triggering cell death, thereby blocking pathogen replication and spread, and also by releasing the

**Figure 1.** TNFR1-mediated necroptosis activation. Stimulation of TNFR1 by TNF-α may promote multiple signaling pathways. TNFR1 activation results in the interaction between TNFR-associated death domain (TRADD) and RIPK1 in the pro-survival complex I. Poly-ubiquitinylation of RIPK1 by cellular inhibitor of apoptosis proteins (cIAPs) and linear ubiquitin chain assembly complex (LUBAC) promotes nuclear translocation and the activation of NF-κB mediated survival. Alternately, the de-ubiquitination of RIPK1 by ubiquitin carboxyl-terminal hydrolase (CYLD) can lead to the activation of complex IIa, where RIPK1 interacts with Fas-associated death domain (FADD) protein and pro-caspase-8 resulting caspase-8 activation and apoptosis. In the absence of caspase-8 activity, RIPK1 interacts with RIPK3 to form the complex IIb, which triggers necroptosis. Within the necrosome, MLKL is recruited and phosphorylated by RIPK3 which triggers its oligomerization and translocation to the plasma membrane, leading to cell permeabilization and the release of DAMPs. Depending on the context, the necrosome can enter mitochondria triggering ROS production and leading to enhanced necroptosis activation.
aforementioned DAMPs as a consequence of MLKL-induced cell permeabilization, which results in the recruitment of pro-inflammatory cells to sites of infection to promote anti-viral and anti-bacterial inflammation. In the context of infection, it has been shown that different DAMPs can stimulate pattern-recognition receptors that act as initial sensors for infection, orchestrating an inflammatory response that mediates pathogen elimination [49]. During infection, viruses use multiple strategies to evade the host apoptotic machinery, for example, inhibiting caspase-8 [50]. Hence, the cellular capability to induce necroptosis under this apoptosis compromised condition is a key mechanism used to limit viral replication. Supporting evidence of this was demonstrated by Cho et al., who showed that the RIPK1-RIPK3 complex is induced following vaccinia virus infection in mice. Notably, infected Ripk3 knockout mice failed to initiate virus-induced tissue necrosis and inflammation, resulting in an elevated viral replication and animal mortality [14]. Similarly, Ripk3−/− mice and mice expressing a loss-of-function mutant form of RIPK1, both failed to control vaccinia virus replication in vivo [51]. It was recently reported that mice lacking Ripk3 exhibited suppressed neuronal chemokine expression, decreased recruitment of T lymphocytes, and inflammatory myeloid cells and were susceptible to enhanced mortality compared to wild-type mice following West Nile virus infection. In contrast, mice lacking Mlkl behaved similarly to wild-type mice indicating that RIPK3 can limit viral pathogenesis independently of necrotic cell death by promoting neuroinflammation [52]. Moreover, in vivo studies performed by Kitur et al. using a model of Staphylococcus aureus infection in mice demonstrated that activation of the necroptosis pathway limited excessive inflammatory signaling and contributed to bacterial clearance, increasing the survival of infected mice [53]. Nonetheless, although under certain conditions of infection, necroptosis activation can promote pathogen removal and eliminate infected cells to prevent a detrimental disproportionate proinflammatory signaling, there is also evidence showing that by activating necroptosis, the pathogen can actually impair the host immunity [54]. In this line, the same research group recently demonstrated that infection with Staphylococcus aureus small colony variants (SCVs), pathogens commonly associated with chronic infection, triggered the activation of host glycolysis which led to increased mitochondrial ROS production and necroptosis activation, which interfered with SCVs elimination and promoted its persistence [54].

The role of necroptosis as a regulator of metabolic signaling was uncovered during initial studies on the underlying mechanisms of necroptosis activation, which revealed that by activating important metabolic enzymes including glycogen phosphorylase, glutamate ammonia ligase, and glutamate dehydrogenase 1, RIPK3 regulates TNF-induced ROS production [24]. Further studies demonstrated that upon TNF-induced necroptosis, the necrosome complex can translocate to the mitochondria and activate pyruvate dehydrogenase, upregulating aerobic respiration leading to increased ROS generation [31].

2.1.2. Current pharmacological necroptosis inhibitors

Following the discovery of crucial mediators of the necroptosis signaling pathway, a number of compounds that can target these proteins have been identified and further tested both in vitro and in vivo, showing its capability to effectively modulate necroptosis. A list of small molecules that have been characterized is presented in Table 1.

Necrostatin-1 (nec-1), was the first compound to be identified as an inhibitor of necroptosis [67]. The molecule allosterically inhibits RIPK1 and its effect has been validated in different in vitro and in vivo settings of necroptotic activation triggered by multiple stimuli [68–76]. However, due to its short half-life, in vivo studies have been limited. Also, nec-1 has some off-target effects, as it also inhibits indoleamine-2,3-dioxygenase, an enzyme that participates in immunomodulatory function [77]. 7-Cl-O-Nec-1 or nec-1s is a nec-1 that has a longer half-life in vivo and have not reported off-target effects. Whereas nec-1s can potently block RIPK1-dependent necroptosis, this molecule also inhibits RIPK1-dependent apoptosis [77]. GSK2982772 and GSK3145095 are selective RIPK1 inhibitors that are currently being tested in clinical trials for the treatment of rheumatoid arthritis, psoriasis, ulcerative colitis, and cancer [78–80]. Also, the RIPK1 inhibitor DNL747 is under clinical study for ALS and AD [80]. Other small molecules have been shown to inhibit RIPK1 including RIPA-56 [81], furo[2,3-d]pyrimidine, and GSK’963 [82,83].

In contrast to RIPK1, RIPK3 is an indispensable component of the necroptotic pathway. Hence, small molecules that target RIPK3 become critical to specifically block necroptosis. Several RIPK3 inhibitors have been described, including GSK’840, GSK’843, and GSK’872, which have shown to selectively inhibit RIPK3-dependent necroptosis; however, it was demonstrated that these molecules can induce apoptosis in a concentration-dependent manner, limiting their potential use in vivo [84]. Other compounds that have proven to target the kinase function of RIPK3 include dabrafenib [85], which is on clinical trial for melanoma treatment, GW440139B, which was shown to block RIPK3 activity and thus inhibiting downstream MLKL activation and oligomerization [86], and HS-137, which directly binds to RIPK3 in an ATP-competitive fashion suppressing its kinase activity [87].

As stated above, MLKL is known as the most downstream, final effector of necroptotic cell death. Therefore, tackling this molecule represents the most specific strategy to target necroptosis providing a powerful tool to study this pathway. A number of MLKL inhibitors have been characterized, yet the most widely used is necrosulfonamide (NSA). This compound covalently binds to human but not rodent Cys86 of MLKL inhibiting further oligomerization of the protein [4]. Another potent MLKL inhibitor that targets Cys86 of MLKL is TC13172 [82]. MLKL polymerization constitutes a critical step required by MLKL to execute necroptosis. In this line, one study showed that thioredoxin-1 can suppress disulfide bond-dependent MLKL polymer formation by reducing the protein and as consequence blocking necroptosis [88].

Different agents have been shown to be able to block necroptosis by interacting with more than one target. Using a representative panel of Food and Drug Administration (FDA)-approved drugs, a screen for small-molecule inhibitors of necroptosis identified two anti-cancer agents, ponatinib and pazopanib. Both molecules were shown to inhibit MLKL phosphorylation targeting RIPK1 and RIPK3 in the case of...
Table 1. Pharmacological necroptosis inhibitors.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Target</th>
<th>ND for which has been tested</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>nec-1</td>
<td>RIPK1</td>
<td>PD (cells), ALS (cells), Glaucoma (mouse, rat, cells), Retinitis pigmentosa (cells, mouse),</td>
<td>[67-76,88-90,122]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ischemic injury (mouse, rat), Niemann-pick disease (cells, mouse), HD (cells, mouse), MS (mouse),</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spinal cord injury (rat, mouse), Traumatic brain injury (mouse), Intervertebral disc degeneration (cells)</td>
<td></td>
</tr>
<tr>
<td>nec-1s</td>
<td>RIPK1</td>
<td>AD (mouse), PD (cells, mouse), ALS (mouse), Glaucoma (rat), Retinitis pigmentosa (mouse)</td>
<td>[77,93,111,128,133,136]</td>
</tr>
<tr>
<td>GSK2982772</td>
<td>RIPK1</td>
<td>n/a</td>
<td>[78]</td>
</tr>
<tr>
<td>GSK3145095</td>
<td>RIPK1</td>
<td>n/a</td>
<td>[79]</td>
</tr>
<tr>
<td>DNL747</td>
<td>RIPK1</td>
<td>ALS (human), AD (human), MS (human)</td>
<td>[80]</td>
</tr>
<tr>
<td>RIPA-56</td>
<td>RIPK1</td>
<td>MS (mouse)</td>
<td>[57,81]</td>
</tr>
<tr>
<td>furo[2,3-d]pyrimidin</td>
<td>RIPK1</td>
<td>n/a</td>
<td>[82]</td>
</tr>
<tr>
<td>GSK963</td>
<td>RIPK1</td>
<td>n/a</td>
<td>[83]</td>
</tr>
<tr>
<td>GSK574</td>
<td>RIPK1</td>
<td>Niemann-pick disease (mouse)</td>
<td>[58]</td>
</tr>
<tr>
<td>pazopanib</td>
<td>RIPK1</td>
<td>n/a</td>
<td>[59]</td>
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<tr>
<td>GSK840</td>
<td>RIPK3</td>
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<td>[84]</td>
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<tr>
<td>GSK843</td>
<td>RIPK3</td>
<td>n/a</td>
<td>[84]</td>
</tr>
<tr>
<td>GSK872</td>
<td>RIPK3</td>
<td>Ischemic injury (mouse), Intervertebral disc degeneration (cells)</td>
<td>[56,60,84]</td>
</tr>
<tr>
<td>Dabrafenib</td>
<td>RIPK3</td>
<td>n/a</td>
<td>[85]</td>
</tr>
<tr>
<td>GW440139B</td>
<td>RIPK3</td>
<td>n/a</td>
<td>[86]</td>
</tr>
<tr>
<td>HS-1371</td>
<td>RIPK3</td>
<td>n/a</td>
<td>[87]</td>
</tr>
<tr>
<td>NSA</td>
<td>MLKL</td>
<td>ALS (cells), Spinal cord injury (rat), Intervertebral disc degeneration (cells)</td>
<td>[4,56,61,130]</td>
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<tr>
<td>TC13172</td>
<td>MLKL</td>
<td>n/a</td>
<td>[62]</td>
</tr>
<tr>
<td>thioredoxin-1</td>
<td>MLKL</td>
<td>n/a</td>
<td>[63]</td>
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<td>ponatinib</td>
<td>RIPK1,</td>
<td>n/a</td>
<td>[59]</td>
</tr>
<tr>
<td></td>
<td>RIPK3</td>
<td>n/a</td>
<td></td>
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<tr>
<td>GW806742X</td>
<td>RIPK1,</td>
<td>n/a</td>
<td>[34,64]</td>
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<tr>
<td></td>
<td>RIPK3,</td>
<td>MLKL</td>
<td></td>
</tr>
<tr>
<td>sorafenib</td>
<td>n/a</td>
<td>n/a</td>
<td>[65]</td>
</tr>
<tr>
<td>Necrosome</td>
<td>n/a</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>17AAG</td>
<td>hsp90</td>
<td>n/a</td>
<td>[66]</td>
</tr>
</tbody>
</table>

ponatinib and RIPK1 in the case of pazopanib. Both compounds specifically target necroptosis and not apoptosis [89]. Additionally, although GW806742X has been described to inhibit MLKL by targeting its pseudo-kinase domain, it has also shown to target RIPK1 and RIPK3. Therefore, the prevention of necroptosis mediated by this compound does not rely solely on MLKL inhibition [34,90]. The multi-targeting kinase inhibitor sorafenib, commonly used for the treatment of acute leukemia, was demonstrated to effectively inhibit necroptosis in cellular models of leukemia, whereas it was shown to inhibit MLKL phosphorylation via interfering with necosome assembly, its specific target remains unknown [91]. The heat shock protein 90 (hsp90) has been shown to interfere with the necrototic pathway at different levels. By instance, hsp90 can alter the stability of RIPK1 and promote apoptosis. Moreover, this chaperone can target the necrototic function of RIPK3 and inhibit MLKL phosphorylation. Finally, hsp90 was proven to be required for MLKL polymerization, and blocking its activity successfully prevented MLKL assembly into oligomers, therefore blocking its necrototic function as effector [89,92–96].

2.2. Preclinical evidence of necroptosis activation in age-related NDs

The activation of necroptosis as a mediator of inflammation and cell death has been implicated in numerous human diseases since its first description about two decades ago. Several studies in the last years have reported evidence that the necroptotic cascade is activated in postmortem tissue samples of patients affected by diverse neurological disorders, as well as in preclinical animal models of these conditions (Table 2). Remarkably, researchers have been able to show that blocking this signaling pathway might provide neuroprotection in the context of NDs, which has generated great interest in the field as there are still no effective therapeutic interventions for most NDs. In this section, an overview of published data evidencing a role of necroptosis in age-related neurological diseases is presented and summarized in Table 2.

2.2.1. AD

AD is a severe and progressive age-dependent neurodegenerative disorder and represents the most common type of dementia in the elderly population. Histopathologically, AD is characterized by the presence of senile plaques and neurofibrillary tangles, which are composed of aggregated amyloid-β (Aβ) and hyperphosphorylated tau, respectively [110]. Neuronal and synaptic loss, ultimately resulting in brain atrophy, are prominent features of this disease. Clinically, AD is characterized by progressive memory impairment which is followed by the deterioration of other cognitive functions and behavioral changes [110]. Currently available treatments are mainly focused on ameliorating symptoms but do not have a disease-modifying effect [110]. Whereas several risk factors have been associated with the pathophysiology of sporadic AD, its precise cause remains elusive.

The first report demonstrating necroptosis activation in AD, which was recently published, generated great expectation in the field, as raised the possibility that this novel mechanism might underlie neurodegeneration in AD, thereby providing a novel target for neuroprotection. Using AD-derived postmortem brain tissue, Caccamo et al. were able to detect an
Table 2. Evidence of necroptosis in NDs.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Evidence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>Elevated necrotic markers and necrosome formation in AD brains that correlate with Braak stage. RIPK1 regulates expression of AD-related genes. Nec-1s prevents neuron death in 5XFAD mice. GVD-associated necroptosis markers inversely correlate with neuron density in AD brains. Increased levels of RIPK1 and RIPK3 in OXYS rats.</td>
<td>[111,112,117]</td>
</tr>
<tr>
<td>PD</td>
<td>Nec-1 increased 6-OHDA-treated PC12 cell viability. Elevated necroptosis markers in OPA1−/− JPC-derived neural cells and increased survival by nec-1s. Elevated necrotic markers in PD brains. Nec-1s and Ripk3 and Mlkl ablation provide neuroprotection to MPTP-treated mice. GW80 protects 6-OHDA-mediated axonal degeneration in vitro. Nec-1s and Ripk3 and Mlkl ablation protect axons and improve motor performance in 6-OHDA-treated mice.</td>
<td>[68,122,123,128]</td>
</tr>
<tr>
<td>ALS</td>
<td>Nec-1, RIPK1-shRNA and NSA prevent MN loss in in vitro models of ALS. Elevated necroptotic markers in the spinal cord of Optineurin−−/− mice. Targeting necroptosis protects axons and improves motor performance to Optineurin−−/− mice. Elevated necrotic markers in the spinal cord of mutant SOD1 mice. Targeting necroptosis protects axons and delays onset of motor alterations in mutant SOD1 mice. Elevated necrotic markers in ALS-derived tissue.</td>
<td>[130,133,134]</td>
</tr>
<tr>
<td>Glaucoma</td>
<td>Nec-1 protects RGCs from retinal ischemia in mouse. Increased MLKL levels in RGCs and nec-1s-induced protection in rat blunt ocular injury model. Inhibition of necroosome assembly protects mitochondria in RGCs after ischemia in vitro and attenuates RGC loss in rat glaucoma model.</td>
<td>[88–90,136]</td>
</tr>
<tr>
<td>Retinitis pigmentosa</td>
<td>Elevated necrotic markers in the retina of rd10 mice. Ripk3 ablation rescues cone cell death and photoreceptor cell loss following NaIO3 administration in mice. Elevated necroptosis markers in the retina of Irbp−−/− mice. Nec-1 and nec-1s prevent rod and cone cell death.</td>
<td>[91–93,97]</td>
</tr>
<tr>
<td>Ischemic injury</td>
<td>Nec-1 reduces infarct volume after MCAO. Nec-1 reduces inflammation and prevents cognitive decline induced by stroke in mice. Focal ischemia induces necroptosis-mediated neuron death [60,94,98] and alters motor performance in mice, which is inhibited by Ripk3 and Mlkl ko. Ripk1 ko or nec-1 reduces MCAO-induced neuron and astrocyte death, reduce infarct volume and improve cognition in mice and rats. GSK872 alleviates cell death and neurological deficits after MCAO in mice.</td>
<td>[67,70,71]</td>
</tr>
<tr>
<td>Niemann-pick disease</td>
<td>Elevated necroptotic markers in brains from patients. Elevated necroptotic markers and necroptosome formation in fibroblasts and neuronal stem cells derived from patients and in genetic mouse model of the disease. Nec-1 and Ripk1 and Ripk3 ablation prevented patient-derived fibroblasts death. Nec-1 delays onset and prolongs lifespan of transgenic mice.</td>
<td>[58,95]</td>
</tr>
<tr>
<td>HD</td>
<td>Nec-1 prevents cell death induced by caspase inhibitors in an in vitro model of the disease. Nec-1 delays disease onset and improves motor performance in the R6/2 HD mouse model.</td>
<td>[72]</td>
</tr>
<tr>
<td>MS</td>
<td>Elevated necroptotic markers in MS brains. RIPK1 inhibition protects against oligodendrocyte loss and reduces disease severity in a mouse model of MS. RIPK1 inhibition by RIPA-56 halts progression of demyelination and disease development in a cuprizone-induced MS model.</td>
<td>[57,73,99]</td>
</tr>
<tr>
<td>Spinal cord injury</td>
<td>Nec-1 protects neurons, reduces cytokines and ROS and restores physiological function after SCI. Nec-1 improves functional recovery after SCI in mice. miRNA-223-3p protects spinal Neurons from H2O2-induced death by blocking RIPK3. Oligodendrocyte necroptosis activation after SCI in rats. NSA protects neurons and improves motor performance in rats after SCI. Astrocyte necroptosis activation in humans and mice after SCI.</td>
<td>[74,75]</td>
</tr>
<tr>
<td>Traumatic brain injury (TBI)</td>
<td>Increased necroptotic markers in brains of humans, mice and rats after TBI. Ripk3 ko improves cognition and decreases inflammation and oxidative stress in mice after TBI. 2-BFI prevents TBI-induced neuropathology and downregulates necroptotic markers. Nec-1 decreases brain damage improves cognitive and motor performance and decreases inflammation after TBI in mice.</td>
<td>[76,104–107]</td>
</tr>
<tr>
<td>Gaucher’s disease (GD)</td>
<td>Elevated necroptotic markers in brains from transgenic GD mice. Ripk3 deficiency prevents neuron loss and inflammation and increases survival and motor coordination of GD mice.</td>
<td>[108]</td>
</tr>
<tr>
<td>Intervertebral disc degeneration stress</td>
<td>Nec-1, GSK872 and NSA increases viability of rat nucleus pulposus cells after mechanical stress.</td>
<td>[56]</td>
</tr>
<tr>
<td>Chronic brain hypoperfusion</td>
<td>Elevated necroptotic markers in brain tissue from rats under chronic cerebral hypoperfusion.</td>
<td>[109]</td>
</tr>
</tbody>
</table>

increase in the necroptosis markers RIPK1, MLKL, and pMLKL in AD-brains compared to age-matched control individuals. Confocal analysis revealed higher colocalization between RIPK1 and RIPK3, RIPK3, and MLKL, and RIPK1 and MLKL, and co-immunoprecipitation experiments showed higher interaction between these molecules, providing evidence of necroosome complex formation in human AD brains [111]. Double immunostaining revealed that most pMLKL was present in neuronal cells and in a lesser extent in glial cells. Interestingly, by performing a genome-wide mRNA screening the authors found that Ripk1 and Mlkl mRNA levels were significantly higher in AD than in control brains and positively correlated with the Braak stage. Additionally, a link between necroptosis activation and tau pathology was evidenced as necrotic and tau markers exhibited significant colocalization, implicating tau as a potential trigger of the necroptotic machinery. To model RIPK1 regulatory activity in AD brains, the authors generated a causal gene regulatory network using transcriptomic data from postmortem AD- and control-derived brain tissue, identifying a large set of genes regulated by RIPK1, many of which were also differentially expressed in AD versus controls, indicating that RIPK1 activity may account for the gene expression changes reported in AD brains. Further experiments using the AD mouse model 5XFAD revealed increased amounts of necroptotic markers in the brains of these mice compared to controls. Treatment with nec-1s reduced the pMLKL/MLKL ratio and prevented neuronal loss, evidencing the contribution of necroptosis in the mechanism of neuron death in this model [111].

In a newly published work, a link between necroptosis activation in postmortem AD-derived brain samples and AD-related neuropathological changes was for the first time demonstrated [112]. The researchers found positive immunoreactivity for pRIPK1, pRIPK3, and pMLKL in AD-derived tissue. Interestingly, the expression of these necroptotic markers was restricted to the granulovacuolar degeneration (GVD),
a common histopathological lesion observed in AD brains that correspond to cytoplasmic vacuoles believed to be derived from the macroautophagic pathway [113]. Co-expression of these proteins suggested the assembly of the necroosome complex, which was always found to be localized in neuronal GVD. The authors did not find pMLKL in microglia, which differed from Caccamo et al. observations [111]. Although Koper et al. did observe active MLKL in astrocytes, it was not associated with neuropathological markers of AD progression. However, in the case of GVD-bearing pMLKL-positive neurons, an inverse correlation of these lesions with neuronal density in hippocampal and cortical areas was demonstrated, as well as partial colocalization with tau pathology [112]. As the neurodegeneration process may take years in AD [114], the authors proposed a new concept of delayed necroptosis, which might underlie neuronal loss in AD [112].

Different cell death mechanisms underpinning neuronal cell death in AD have been reported [111,115,116]. With the purpose of clarifying the interplay between these different mechanisms, Telegina et al. utilized non-transgenic senescence-accelerated OXYS rats, which spontaneously develop a phenotype similar to human AD [117]. Neuropathological assessment of the cerebral cortex of this rat model revealed an early activation of apoptosis, at an age where this model starts to present behavioral alterations. Increased levels of apoptotic cells were observed as the animals aged, which was concomitant with an increase in the amount of both RIPK1 and RIPK3 protein levels. As alterations in autophagy markers were detected, the authors suggested that a decline in autophagy-mediated proteostasis may trigger the accumulation of misfolded proteins observed in this model, which in turn promotes both apoptosis and necroptosis [117].

2.2.2. PD

PD is considered the second most common age-related ND, with no available cure. It is estimated that PD affects 7 to 10 million people worldwide and considering the increasing aged population, it is predicted that cases will double over the next generation [118]. Clinically, this disease is characterized by motor symptoms that include resting tremor, bradykinesia, rigidity of the limbs, and irregular gait. Neuropathologically, PD exhibits intraneuronal accumulation of modified a-synuclein termed Lewy bodies as well-progressive degeneration of dopaminergic neurons from the substantia nigra pars compacta (SNpc), which triggers impairment of motor control due to reduction in dopamine release. Several genetic mutations have been identified in familial PD cases; yet, more than 90% of the cases are considered idiopathic [119–121].

Currently, the mechanisms that mediate neurodegeneration in PD are not clearly understood. To explore the possible role of necroptosis in the disease, and therefore the protective potential of nec-1, Wu et al. treated cultured PC12 cells with 6-hydroxydopamine (6-OHDA), a toxic stimulus that triggers degeneration of dopaminergic neurons. 6-OHDA-treated cells underwent increased autophagy, loss of mitochondrial membrane potential, and cell death. Interestingly, pre-treatment with nec-1 provided significant protection and increased cell viability [68], raising the possibility that the necrototic machinery might be involved in dopaminergic neuronal death. A couple of years after this publication, new evidence supporting these findings was demonstrated by another research group. In this case, the authors used induced pluripotent stem cells (iPSC)-derived neural cells from PD patients with mutations in dynamin-related GTPase optic atrophy type 1 (OPA1), a PD-causative gene that encodes a mitochondrial membrane protein. These neuronal cells exhibited marked mitochondrial dysfunction, respiration impairment, ATP deficits, and enhanced oxidative stress, which translated into cell death [122]. Additionally, these cells exhibited increased expression of RIPK1, RIPK3, and MLKL proteins, which were also enhanced in PD-derived postmortem tissue from the substantia nigra compared to control individuals. To assess the contribution of necroptosis to the alterations observed in this cell line, cultures were differentiated in the presence of nec-1s, which indeed promoted neuronal survival. To test in vivo the role of necroptosis in the pathogenesis of PD, Iannielli et al. used 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice, an established model of the disease that displays mitochondrial dysfunction, increased ROS production, energetic failure, and dopaminergic neuronal loss. Interestingly, pharmacological inhibition of necroptosis using nec-1s reduced oxidative stress and attenuated nigrostriatal degeneration induced by MPTP [122]. Using the same PD model, another group recently demonstrated that MPTP treatment triggers a dramatic decrease of the striatal level of dopamine as well as a reduction in the number of dopaminergic neurons in the SNpc, which is associated with increased expression levels of the necroptotic markers RIPK1, RIPK3, MLKL, pRIPK3, and pMLKL [123]. All the observed changes were reversed when treating the mice with nec-1. Further experiments performed using MPTP-treated Ripk3 and Mlkl knock out mice showed that the absence of these necroptosis mediators prevents the decrease of dopamine and ameliorates neuronal loss. In addition, the authors proved that although MPTP treatment induces apoptosis in treated mice, necroptosis inhibition did not alter the activation of apoptosis, indicating that both mechanisms of cell death act independently in this PD model [123].

Previous studies indicate that the progression of PD occurs from striatum to SNpc, suggesting that degeneration of terminals and axons are early pathological events, and therefore attractive therapeutic targets for the disease [124,125]. Importantly, we have previously demonstrated that axonal degeneration after diverse stimuli, including mechanical damage and excitotoxicity, proceeds by necroptosis [126,127]. Whether necroptosis is involved in the early stages of dopaminergic neuron degeneration in PD was not explored so far. To investigate the participation of necroptosis in the process of axonal degeneration, our group utilized 6-OHDA to treat cultured mesencephalic and cortical neurons, which showed increased pMLKL levels and underwent extensive axon degeneration evidenced by beading and retraction of neuronal projections. Significant protection to the 6-OHDA-dependent neurodegenerative effect was observed when treating cells with nec-1s or GW80, suggesting the involvement of necroptosis in 6-OHDA-mediated axonal degeneration [128]. In addition, the injection of 6-OHDA in the striatum of wild-type mice led to an early phase of axonal degeneration in both striatum and nigrostriatal pathway, characterized by the upregulation of RIPK3 and the necroptotic effector pMLKL [128]. Moreover, by performing co-immunoprecipitation
assays, Oñate et al. were able to detect the formation of the necosome induced by 6-OHDA. Remarkably, genetic ablation of Ripk3 or Mlkl attenuated axonal damage translating into delayed motor impairment induced by 6-OHDA injection. Similar results were obtained when nec-1s was administered to the animals previous to the toxic insult, which also delayed axon degeneration and motor impairment in this neurotoxic PD animal model [128]. Altogether, these findings suggest that axonal degeneration in PD is mediated by the necroptosis machinery.

2.2.3. ALS

ALS is a progressive neurodegenerative disorder of the motor system characterized by the degeneration of both upper and lower motor neurons (MN) which leads to muscle atrophy and eventual paralysis. With a risk of developing ALS that increases with age, the disease represents the most common paralytic disorder in adults and its onset appears between the ages of 40 and the mid-60s, whereas a subset of ALS cases has a genetic cause; in 90% of the patients, the cause underlying the development of the disease remains unknown [129].

Prompted by previous reports demonstrating the critical role of non-neuronal cells in the mechanisms of neurodegeneration in ALS, Re et al. established a humanized in vitro model of the disease by co-culturing human adult primary sporadic ALS astrocytes and human embryonic stem cell-derived MN. Notably, the researchers were able to recapitulate the disease as cultured astrocytes from ALS patients triggered MN death whereas control astrocytes did not [130]. Mutations in superoxide dismutase-1 (SOD1) cause a rare form of familial ALS, and earlier studies from this group showed that mutant SOD1 mouse astrocytes can trigger mouse MN loss [131]. Thus, using both mouse mutant SOD1 and the human ALS in vitro disease models, the authors demonstrated that the mechanism underlying neurodegeneration was caspase independent and rather was dependent on RIPK1 and MLKL. Indeed, targeting these necroptotic proteins by pharmacological and genetic interventions using nec-1, RIPK1-shRNA, and NSA largely prevented MN demise in these co-culture systems [130], revealing for the first time a role for necroptosis in the pathogenesis of ALS and defining a novel mechanism of non-cell-autonomous MN death.

Degeneration of nerve terminals occurs early during ALS development and precedes MN cell body loss, constituting an important contributor for neuronal dysfunction in ALS [132]. In an attempt to define the mechanism of axonal demise in ALS, Ito et al. developed a transgenic mouse model deficient in Optineurin, a gene implicated in both familial and sporadic ALS [133]. Based on the prior discovery by the same group that Optineurin deficiency sensitizes cells to necroptosis, the authors went on to explore the potential activation of necroptosis in these experimental settings and indeed, they found increased levels of RIPK1, RIPK3, and pMLKL in the spinal cord of Optineurin−/− mice. Targeting these proteins ameliorated axonal degeneration and improved motor performance providing support to the role of necroptosis in Optineurin-mediated axonal loss [133]. Additionally, Optineurin−/− oligodendrocytes were sensitized to die by TNFα-induced necroptosis, which was prevented by nec-1s. To evaluate the involvement of necroptosis-mediated axonal pathology in the context of familial ALS, the researchers utilized the mutant SOD1 mouse model, which indeed exhibited RIPK1, RIPK3, and pMLKL upregulation in the spinal cord. Inhibition of the necroptotic signaling provided protection against axonal dysmyelination as well as delayed the onset of motor alterations. Finally, the authors demonstrated the upregulation of necroptotic markers in pathological samples from ALS patients [133]. Taken together, this work suggested an instrumental role for necroptosis in this animal model of ALS. However, shortly after this publication, two different studies challenged the view of necroptosis being necessary for MN degeneration. In the first one, Dermentzaki et al. analyzed the expression levels of necroptotic mediators in the spinal cord of the Tg SOD1 model and in contrast to the results from Ito et al., only RIPK1 protein levels were found to be elevated [134]. When analyzing RIPK1 levels in human ALS motor cortex samples, no differences were found compared to control-derived samples. As Ito et al. showed necroptosis activation in glial but not neuronal cells [133], Dermentzaki et al. sought to determine whether necroptosis was activated specifically in MNs in ALS. To this end, MNs derived from Ripk3−/− mice and astrocytes from mutant SOD1 mice were co-cultured and notably, RIPK3 deficient MNs showed a longer survival time compared to control neurons. However, when extending these results to an in vivo model, the Ripk3−/− transgenic SOD1 males exhibited a delayed onset (defined as the age at which mice lose 10% of its peak weight) and extended survival, but this was not associated with improvements in either motor performance or neuropathological hallmarks of the disease [134]. Thus, in light of their observations, the authors concluded that further investigation is needed to unquestionably implicate necroptosis in ALS pathogenesis. To address this, another research group focused their investigation on the necroptosis executioner MLKL [135]. In contrast to Ito et al. report [133], the disease onset, locomotor function, and lifespan of Mlkl knockout Tg SOD1 mice did not differ from Tg SOD1 mice [135]. Moreover, Mlkl−/− mice were neuropathologically normal as their MN count was similar to wt mice. Also, both astrocyte and microglia activation were not modified by Mlkl ablation. Whereas RIPK1 levels were significantly elevated in spinal cords of Tg SOD1 mice compared with wt mice (independent of Mlkl genotype), RIPK3 or MLKL was not detected in the frontal cortex or spinal cord of mice of any genotype [135], which differed from Ito et al. results [133]. Altogether, these data argue against a role for the necroptosis signaling in ALS pathology.

2.2.4. Glaucoma

Glaucoma is the second leading cause of blindness worldwide, affecting around 70 million people. The mean age of onset is 60 years and the risk rise with age. The predominant cause of glaucoma is intraocular pressure elevation that leads to the loss of retinal ganglion cells (RGCs) and progressive optic nerve atrophy [88,89]. Currently, lowering the intraocular pressure is the usual treatment for the disease, yet this only slows down the progression of RGC degeneration. Apoptosis has been shown to contribute to RGC death; however, interventions aimed at halting the apoptotic signaling pathway have failed to provide neuroprotection and to stop disease progression [88].

Ischemia-reperfusion injury has shown to occur in different retinal disorders including glaucoma. Thus, based on studies
showing neuroprotection by nec-1 following ischemic brain injury, Rosenbaum et al. sought to determine if necroptosis contributes to neuronal damage and functional impairment in a rat model of retinal ischemia. The authors demonstrated that nec-1 treatment prevented ischemia-induced neuronal death, preserved retinal tissue, and reduced functional impairment of the retina [90]. In line with this work, another group of researchers confirmed that nec-1 increases RGC survival in a mouse model of retinal ischemia. Also, nec-1 administration suppressed the induction of a pro-inflammatory response, as decreased levels of inflammatory markers were detected by qPCR [88]. Although these studies suggest that the necroptotic cascade is associated with the pathology of retinal neuronal degeneration, in both cases only RIPK1 was targeted, and considering that this protein can participate in a variety of cellular pathways including inflammation, it became crucial to study other necroptotic molecules that act downstream RIPK1. Subsequent works shed light into this implicating other key necroptotic molecules as contributors of RGC loss. One such study demonstrated increased levels of MLKL in RGCs in a blunt ocular injury model in rats [136]. Moreover, Thomas et al. were able to show focal RGC neuroprotection in rats treated with nec-1s. Nevertheless, nec-1s did not provide functional rescue of the retina as measured by electroretinogram, which might be explained by the fact that ischemic injury leads to both apoptotic and necrotic cell death in the retina and therefore both mechanisms act in parallel during RGC degeneration [136]. In another publication, a new RIPK1 inhibitor (RIC) was introduced and its protective properties against glaucoma neuropathology were tested [89]. The researchers used different ischemic stimuli and showed that RIC suppressed ischemia-induced necroptosis in RGCs through blocking RIPK1-RIPK3 interaction, indicating that necrosome formation was blocked by this molecule. Notably, RIC prevented mitochondrial dysfunction by suppressing mitochondria ROS generation. Thereafter, Do et al. went on to validate these results in vivo and demonstrated that intraperitoneal RIC administration blocked RIPK1 phosphorylation and attenuated ischemia-induced retinal neuron loss in a rat glaucoma model [89].

2.3. The role of necroptosis in axonal degeneration

The degeneration of axons constitutes a salient feature shared by several NDs that contributes to neuronal dysfunction and cell death [132]. Evidence for this has been demonstrated in the context of AD, PD, ALS, HD, and glaucoma [132,137–140]. Notably, axonal degeneration arises as an early event in the context of both the cellular progression of neurodegeneration and the disease development. In vivo optical imaging techniques constitute potent tools to study the dynamics of axonal degeneration with high temporal resolution [141]. The use of optical imaging techniques has been instrumental to understand the mechanisms involved in axonal destruction in different mouse models of injury and disease, including spinal cord injury [142], MS [143], optic nerve crush [144] and AD [145], and importantly, it has allowed to demonstrate that the degeneration of nerve fibers at the central nervous system constitutes a potentially reversible process [142,143,146].

To date, the precise molecular and cellular mechanisms of axonal degeneration in NDs are under intense investigation. The morphological evidence indicates that axon degeneration as a consequence of aging and NDs takes place in a retrograde fashion also known as the dying back degenerative process, which precedes cell death of the neuronal soma [124,137,147–149]. Importantly, accumulating evidence demonstrates that axonal degeneration occurs through a self-destructive program independent from the mechanism of cell body death [148–150]. Axon degeneration shares several characteristics with cell death by activation of the necrotic signaling pathway, including mitochondrial dysfunction, ROS production, and intracellular calcium increase [151,152], and recently we reported that pharmacological inhibition of RIPK1 using nec-1 strongly delayed axonal degeneration in neurons from the peripheral and central nervous systems in mice [126]. Also, nec-1 protects in vitro sensory axons from disintegration triggered by axotomy or vincristine, a toxic insult associated with chemotherapy-induced neuropathies [126]. To definitively demonstrate whether necroptosis mediate axon degeneration, and considering the aforementioned off-target effects of nec-1, inhibition of RIPK3 and MLKL was then assessed. These experiments showed that the protective effects of nec-1 were also observed following genetic knock-down of Ripk3 or Mlkl in vitro and inhibition of necroptosis also delayed the loss of the electrophysiological nerve function after nerve injury [126].

Glutamate excitotoxicity represents a deleterious contributing factor in various neurodegenerative conditions. Glutamate receptors are expressed along the soma and neuronal projections, therefore excessive glutamate release can trigger excitotoxicity in different neuronal compartments, leading to soma apoptosis as well as degeneration of axons and dendrites by a process associated to calcium increase and mitochondrial dysfunction [153]. Using a compartmentalized cell culture system, our group showed that excitotoxicity-induced axonal degeneration of hippocampal neurons proceeds by necroptosis [127]. Pharmacological inhibition of RIPK1 as well as knocking-down Ripk3 or Mlkl prevented key steps in the axon disintegration cascade including mitochondrial depolarization, opening of the permeability transition pore and calcium dysregulation in the axonal compartment, delaying axonal degeneration and consequently neuronal cell death. Moreover, Hernández et al. showed that excitotoxicity led to canonical apoptosis in the cell soma, demonstrating the co-activation of two independent death mechanisms in different compartments of the neuron [127].

As mentioned, early axonal damage is a prominent feature of ALS neuropathology [132] and the engagement of key necroptotic mediators in spinal MN axon degeneration was demonstrated in in vitro models of ALS, implying necroptosis as a driver for axon loss and MN death in ALS [130,133]. However, other studies using in vivo models challenged these findings [134,135]. This conflicting evidence highlights the intricate nature of the necroptotic pathway. While RIPK1 and RIPK3 might be involved in proinflammatory events leading to MN disruption, it is possible that MLKL and consequently necroptosis as a cell death mechanism is not necessarily engaged [135]. Hence, functional studies tackling
the final effector of the necroptotic cascade become fundamental.

At the time of motor symptoms appearance in PD, the loss of striatal dopaminergic markers exceeds that of dopaminergic neurons in the SN. From different estimates, motor symptoms appear after 50–70% of striatal dopamine has been depleted and 30–50% of the nigral dopaminergic cells have died \cite{154,155}. These studies suggest that striatal dopamine depletion, rather than loss of dopaminergic neurons, better correlates with the severity of PD symptoms. Therefore, the progression of PD is likely to occur retrogradely, from axons and terminals in the striatum to neuronal cell bodies in the SN. In addition, immunohistochemical analyses have shown that the greatest abundance of α-synuclein aggregates is not found in cell bodies, but in presynaptic terminals \cite{156}. Moreover, several animal models of PD support the idea that axonopathy is an early event in PD \cite{124}. Expression of mutant and wild-type α-synuclein in murine dopaminergic neurons leads to striatal axonal pathology and an inflammatory response in the striatum \cite{124}. In mice expressing the human disease-causing LRRK2, histochemical analysis showed fragmented axons, associated with spheroids and dystrophic neurites with no loss of mesencephalic dopamine neurons \cite{157,158}. Additional in vitro evidence demonstrated that in primary neurons exposed to α-synuclein preformed fibrils, the damage is found initially in axons and propagated to form inclusions in the soma, leading to neuronal cell death \cite{159}. Chu et al. used human PD tissue to demonstrate an early decline in the expression of motor proteins involved in axonal transport associated with α-synuclein aggregation, before the alteration of dopaminergic cell bodies \cite{125}. This result agrees with previous studies demonstrating alterations in axonal transport associated with α-synuclein mutations in vitro \cite{160} and in vivo \cite{161}. Together, these data suggest that both axonal dysfunction and degeneration correspond to an early event during the progression of PD. As discussed in Section 2.2.2, our group recently evidenced a novel function of the necroptosis machinery in controlling the mechanisms of axonal destruction in PD \cite{128}. In line with these results, we proposed that the necrototic cascade operates to induce axonal degeneration by a process referred to as necroapoptosis, a prospective target to prevent dopaminergic neuronal loss in PD \cite{128}.

3. Conclusion

One of the most critical consequences of the global increase in life expectancy that we have witnessed in the last 20 years has been the exponential rise in the incidence of age-related neurodegenerative conditions, which are among the leading causes of disability worldwide. The cause of most of these diseases remains elusive and none of these disorders have a cure yet. While the etiology of the majority of the neurodegenerative disorders has a genetic component, environmental factors play an important role and therefore the origin of these disorders is considered multifactorial which sums complexity in the search for an effective disease-modifying treatments. Important advances in neuroscience research in the last two decades have impacted enormously our current knowledge concerning the complex cellular pathways that contribute to neurodegeneration during the development of NDs. The discovery of necroptosis activation in different brain diseases sheds light into the mechanisms that contribute to neuronal loss, providing new avenues for potential therapies.

4. Expert opinion

In this article, we reviewed the evidence that demonstrates activation of the necroptosis signaling pathway in age-related NDs. Taken together, this evidence shows that inhibition of key players of the necroptotic cascade, namely, RIPK1, RIPK3, and MLKL can provide therapeutic benefits in the context of these group of diseases. Multiple studies have validated necroptosis as a target with therapeutic value for diseases such as AD, PD, ALS, and glaucoma, by using different models, including in vitro settings, animal models, and most recently clinical trials. Although there are still several gaps in our knowledge regarding the participation of necroptosis in NDs, especially whether the activation of the pathway takes place in neurons, glial cells, and/or immune cells, there is accumulating literature demonstrating necroptosis is a pathway activated in different conditions and likely contribute to brain pathologies.

Each ND exhibits unique neuropathological hallmarks including the specific neuronal type affected. Furthermore, a combination of other common pathological components that include misfolded protein aggregation, neuroinflammation, and oxidative stress contributes to the onset and progression of these diseases. Thus, a myriad of triggering stimuli has been described to promote neurodegeneration. Unfortunately, clinical trials aimed at targeting many of these stimuli have largely failed to reach clinical endpoints. As mentioned, one of these pathological elements are protein aggregates, which accumulate in the diseased brains, constituting a shared feature of NDs \cite{162}. Examples of these proteins are Aβ and α-synuclein, which build up in AD and PD brains, respectively. Whether these misfolded proteins take part in the mechanism for necroptosis activation represents an intriguing hypothesis to be investigated. Necroptosis signaling requires the formation of amyloid-like fibrils composed by the necosome complex, which constitutes a functional amyloid signaling complex \cite{3}, and also, active MLKL undergoes oligomerization. Thus, it can be speculated that these necrototic aggregates could serve as a seeding platform for misfolded proteins such as Aβ and α-synuclein to promote their aggregation and that way accelerate the process of neurodegeneration. However, the crosstalk between these proteins in the context of cross-seeding has not yet been studied. In this line, an interesting work that was recently published demonstrated that RIPK1-mediated inflammation promotes the development of disease-associated microglia, with impaired phagocytic capacity that induces the accumulation of Aβ \cite{163}.

Degeneration of neuronal compartments including axons, dendrites and neuronal soma are the ultimate consequence of all neurodegenerative disorders that translate into each disease phenotype. Thus, specifically targeting degenerative mechanisms of the neuron appears as a direct strategy that might cover a wide range of different diseases. In this context,
the participation of necroptosis as a common mediator of neuron demise emerges as a promising target.

As discussed in this review, different molecular mechanisms underlie soma and axonal destruction. Axonal degeneration, a largely neglected target for neuroprotection, corresponds to an early event during the progression of NDs, which therefore underscore axons as cellular targets for therapeutic intervention. Thus, the concept of axon degeneration being an initial, and potentially reversible, pathological phenomenon during the course of NDs has been the focus of increasing interest as represents a promising target for therapeutic intervention. Indeed, axonal degeneration was long ago proposed as a target for neurological conditions. However, it was not until recently, that the necroptotic machinery was revealed as a mediator of axonal loss after diverse pro-degenerative stimuli. Hence, necroaxoptosis emerges as a potential mechanism for axonal loss, representing a novel and druggable target for rescuing axons that are at a potential risk for degeneration, providing a new strategy for therapeutic intervention to prevent functional impairment in NDs. As it seems reasonable to propose that axon degeneration is a common feature of neurodegenerative conditions, targeting necroptosis-mediated axonal degeneration might be beneficial for a wide range of NDs.

The signaling cascade downstream RIPK1 activation presents a variety of candidate molecules as potential therapeutic targets. The necroptotic pathway was not long ago discovered and since nec-1 was the first identified RIPK1 kinase activity inhibitor, a good amount of work has been carried out using this small molecule to target necroptosis. However, it was later found that nec-1 has off-target effects, and therefore the experiments that have rely on this drug should be confirmed using other molecules that more specifically target the necroptosis pathway. We consider this as a weakness in this field; however, as research on necroptosis progresses, additional small molecules have been identified that target other key players of the pathway with demonstrated specificity. Also, there are many published works that only demonstrate the upregulation of necroptotic markers under certain experimental conditions, but lack functional studies to demonstrate that necroptosis activation can modify disease progression. Thus, it becomes imperative that researchers complement in vitro mechanistic studies with functional in vivo studies, as well as with descriptive, qualitative work. Moreover, it has to be considered that the necroptosis pathway is not only involved in the etiology of diseases, but it also participates in important physiological functions. For example, RIPK1 is an important player in inflammatory processes by mediating proinflammatory cytokine production [164]. Additionally, although RIPK3 is a protein player in the necroptosis cascade, it also regulates other pathways independent of cell death, such as inflammasome activation and neuroinflammation [42]. Notably, both RIPK1 and RIPK3 have roles in apoptosis. Thus, considering the pleiotropic nature of these necroptotic mediators, chronic inhibition could affect essential physiological functions, therefore focusing on more specific targets becomes crucial. One such target might be the assembly of the necrosome, as it seems that this process constitutes a necroptotic cell death-specific step. That being said, the small molecule research field constitutes an important area that must be exploited in the search for new drugs that could help to move forward in the study of the exact mechanisms that lead to necroptosis activation and execution, as well as the role of necroptosis in the pathology of diseases.

The necroptosis signaling pathway was recently identified; nonetheless, increasing evidence has shed light on the role of necroptosis as a mediator of the pathophysiology of neurodegeneration. Although there is undoubtedly a long research way to go before the successful translation of this approach into the clinic as a treatment for age-associated NDs, clinical trials targeting necroptosis are already currently being undertaken for inflammatory diseases, and notably for ALS and AD [80]. We think that the outcome of these clinical trials is critical, as they will give us important information as to whether necroptosis constitutes a potential target for neurodegeneration. Although some debate still exists associated with the activation of this pathway in the nervous system, considerable research has been performed in the last 5 years demonstrating activation of key players of necroptosis in human tissue as well as in laboratory models of NDs. As any research field in its early stages, we still need more confirmatory results from different research groups and more mechanistic insight related to necroptosis activation in the brain. Nonetheless, the evidences discussed in this review indicate that targeting the necroptosis pathway will indeed open up new therapeutic avenues in the next decade.

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In this work, RIPK3 was found to be required for RIPK1-dependent reactive oxygen species production. Cell Death Differ. 2011;18(4):656–665.

In this report MLKL was identified as a key mediator of necroptosis signaling downstream of RIP3K.

In this study demonstrates that necroptosis activation is necessary for the inflammatory response against viral infection.

This study evidences for necroptosome translocation to the mitochondria is presented.

In this work, RIPK3 was found to be required for RIPK1-mediated necrosis and was identified to be an essential component for necroptosis activation.

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In this article, the protective effect of nec-1 was for the first time demonstrated in an in vivo model of ischemic brain injury. Here, the term necroptosis was put forward.


This manuscript shows that necroptosis activation correlates with the stage of AD and that inhibiting the pathway reduces neuronal loss in mice.


This is the first report demonstrating the involvement of the necroptotic pathway in the pathology of PD.


**In this work, necroptosis was for the first time shown to be involved in the mechanism of axonal degeneration.**


**The contribution of necroptosis in ALS is demonstrated for the first time in *in vitro* models of the disease.**


