Received: 28 May 2023 | Revised: 14 August 2023 | Accepted: 17 August 2023

DOI: 10.1111/ejn.16136

EJN European Journal of Neuroscience FENS WILEY

SPECIAL ISSUE REVIEW

DOPAMINE: From Release and Modulation to Brain Diseases

# Dopaminergic neuronal death via necroptosis in Parkinson's disease: A review of the literature

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**Funding information**
This research was funded by the Italian Ministry of Health grant number RF-2019-12369122, Telethon Foundation grant number GGP20048 and the Italian Ministry of University and Research PRIN 2017A9MK4R. This publication was produced with the co-funding European Union–Next Generation EU, in the context of The National Recovery and Resilience Plan, Investment Partenariato Esteso PE8 'Conseguenze e sfide dell'invecchiamento', Project Age-It (Ageing Well in an Ageing Society).

## Abstract

Parkinson's disease (PD) is a neurodegenerative disorder characterized by progressive dysfunction and loss of dopaminergic neurons of the substantia nigra pars compacta (SNc). Several pathways of programmed cell death are likely to play a role in dopaminergic neuron death, such as apoptosis, necrosis, pyroptosis and ferroptosis, as well as cell death associated with proteasomal and mitochondrial dysfunction. A better understanding of the molecular mechanisms underlying dopaminergic neuron death could inform the design of drugs that promote neuron survival.

Necroptosis is a recently characterized regulated cell death mechanism that exhibits morphological features common to both apoptosis and necrosis. It requires activation of an intracellular pathway involving receptor-interacting protein 1 kinase (RIP1 kinase, RIPK1), receptor-interacting protein 3 kinase (RIP3 kinase, RIPK3) and mixed lineage kinase domain-like pseudokinase (MLKL). The potential involvement of this programmed cell death pathway in the pathogenesis of PD has been studied by analysing biomarkers for necroptosis, such as the levels and oligomerization of phosphorylated RIPK3 (pRIPK3) and phosphorylated MLKL (pMLKL), in several PD preclinical models and in PD human tissue. Although there is evidence that other types of cell death also

**Abbreviations:** 6-OHDA, 6-hydroxydopamine; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; ATP, adenosine triphosphate; BBB, blood–brain barrier; cFLIP, cellular FLICE-like inhibitory protein; cIAPS, cellular inhibitors of apoptosis proteins; CNS, central nervous system; CSF, cerebrospinal fluid; CYLD, deubiquitinating enzymes cylindromatosis; DA, dopamine; DAMPs, damaged-associated molecular patterns; DAT, dopamine transporter; DISC, cytosolic death-inducing signalling complex; Drp1, dynamin-protein 1; DRs, death-domain receptors; FADD, FAS-associated protein with a death domain; hiPSC, human induced pluripotent stem cell; iDA, human induced pluripotent stem cell-derived DA neuron; IDO, indoleamine 2,3-dioxygenase; iNOS, inducible nitric oxide synthase; iPD, idiopathic PD; KD, kinase domain; KO, knockout; LPS, lipopolysaccharide; LRRK2, leucine-rich repeat kinase 2; LUBAC, linear ubiquitin chain assembly complex; MAPKs, mitogen-activated protein kinases; MLKL, mixed lineage kinase domain-like pseudokinase; MPP+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MTT, methyl-thiazol-tetrazolium; Nec-1, necrostatin-1; NF-κB, nuclear factor-κB; NOX4, mitochondrial NADPH oxidase-4; p55, tumour necrosis factor receptor type 1; PAMPs, pathogen-associated molecular patterns; PD, Parkinson's disease; PGAM5, phosphate glycerin mutase 5; pMLKL, phosphorylated MLKL; pRIPK3, phosphorylated RIPK3; RHIM, RIP homotypic interaction motifs; RIPK1, receptor-interacting protein kinase 1; RIPK3, receptor-interacting protein kinase 3; RNS, reactive nitrogen species; ROS, reactive oxygen species; SMAC, second mitochondria-derived activator of caspases; SMs, SMAC mimetics; SNc, substantia nigra pars compacta; TH, tyrosine hydroxilase; TLR, toll-like receptor; TNF, tumour necrosis factor; TNFR, tumour necrosis factor receptor; TRADD, TNFR-associated death domain protein; TRAF2, TNFR-associated factor 2; TRAILR, TNF-related apoptosis-inducing ligand receptor; WT, wild type; zVAD-fmk, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone.

Edited by: Louis-Eric Trudeau

have a role in DA neuron death, most studies support the hypothesis that this cell death mechanism is activated in PD tissues. Drugs that prevent or reduce necroptosis may provide neuroprotection for PD. In this review, we summarize the findings from these studies. We also discuss how manipulating necroptosis might open a novel therapeutic approach to reduce neuronal degeneration in PD.

**KEYWORDS**
dopamine, mixed lineage kinase domain-like pseudokinase-MLKL, movement disorders, neuroprotection, substantia nigra

## 1 | INTRODUCTION

Dopaminergic neurons of the substantia nigra pars compacta (SNc) are fusiform or multipolar cells with two to six thick dendrites that taper rapidly and extend for hundreds of microns (Grace & Onn, 1989). The dopaminergic axon arises from a dendritic site, tens to hundreds of microns from the soma, and projects to the dorsal striatum. The axon forms highly dense, widely spreading axonal bushes. A single dopaminergic neuron in the human SNc is estimated to give rise to between 1 and 2.4 million dopaminergic varicosities in the striatum (Matsuda et al., 2009). Devoid of synaptic membrane specialization and unassociated with postsynaptic density, these dopaminergic terminals release dopamine (DA) (Descarries et al., 1996). DA binds to metabotropic DA receptors of the D1 or D2 type located on medium spiny neurons, interneurons and corticostriatal terminals, where it regulates glutamate release, neuron excitability and synaptic plasticity. This nigro-striatal pathway is involved in the control of voluntary movement.

The total number of dopaminergic neurons in the SNc of a healthy human adult is about 550,000 (Pakkenberg et al., 1991). Progressive dopaminergic neuron dysfunction and death in the SNc lead to Parkinson's disease (PD) (prevalence between 100 and 300/100,000 people) (Pringsheim et al., 2014; Ray Dorsey et al., 2018), a movement disorder characterized by bradykinesia, rigidity, resting tremor and gait impairment. Despite efforts by research laboratories over the years, the exact mechanism(s) underlying DA neuron death in the brain of PD patients remains elusive. A better understanding of this mechanism could inform the design of drugs to prevent neuron loss.

Several programmed cell-death mechanisms may have a role in dopaminergic neuron death, such as apoptosis, necrosis, pyroptosis and ferroptosis, as well as cell death associated with proteasomal and mitochondrial dysfunction. Recent and previous reviews have been published on these topics (Fricker et al., 2018; Levy et al., 2009; Liu, Wang, et al., 2017; Mansour et al., 2023; Michel et al., 2016; Moujalled et al., 2021; Panicker et al., 2021; Venderova & Park, 2012). Necroptosis is a recently characterized regulated cell death mechanism that exhibits morphological features shared by apoptosis and necrosis. It requires activation of an intracellular pathway involving receptor-interacting protein 1 kinase (RIPK1), receptor-interacting protein 3 kinase (RIPK3) and mixed lineage kinase domain-like pseudokinase (MLKL), three crucial players in this pathway (Weinlich et al., 2017).

Recent studies suggest that necroptosis might have a role in dopaminergic neuron death in PD. Here, we summarize findings from studies on necroptosis in PD preclinical models and PD human tissue samples. We discuss how manipulating necroptosis may open a novel therapeutic approach to reduce neuronal degeneration in PD.

## 2 | MAIN CHARACTERISTICS OF PARKINSON'S DISEASE

PD is a movement disorder affecting over 6 million people worldwide (Ray Dorsey et al., 2018). It is characterized by motor symptoms (e.g., bradykinesia, rest tremor and rigidity) and nonmotor manifestations (e.g., olfactory dysfunction, constipation, sleep disorder and depression/anxiety). The age at onset can range from extreme old age to young adulthood, as seen in juvenile parkinsonism (Obeso et al., 2017). A combination of genetic changes and environmental factors is responsible for its development. Familial forms of PD account for 3%–5% of PD and stem from mutations in PD-linked genes (*SYNUCLEIN*, *PARKIN*, *LRRK2*, *PINK1* and others) (Funayama et al., 2023). Genetic and environmental factors (e.g., pesticides and infections) are believed to act synergistically in the pathogenesis of idiopathic PD (iPD).

Symptomatic pharmacotherapy for PD motor symptoms is primarily DA based and comprises preparations of the DA precursor Levodopa, DA receptor agonists, monoamine oxidase-B inhibitors and catechol-O-methyl transferase inhibitors. Despite decades of research, no disease-modifying pharmacologic treatment has been found. Indeed, the lack of mechanistic insights into dopaminergic neuron death has hindered the development of effective drugs for neuronal protection in PD: current therapies are merely symptomatic, lose their efficacy with time and produce difficult-to-treat side effects.

## 3 | MECHANISM OF NECROPTOSIS

The term *necroptosis* was first introduced in 2005 by Yuan's research team (Degterev et al., 2005), who described regulated necrotic cell death occurring in the absence of caspase signalling and that it could be blocked by necrostatin-1 ((Nec-1; 5-((1H-indol-3-yl)methyl)-3-methyl-2-thioxoimidazolidin-4-one), a potent small-molecule inhibitor. Necroptosis is the best-understood form of regulated necrosis (Berghe et al., 2014). It exhibits morphological features of necrosis (disruption of plasma membrane, cell and organelle swelling, gain in cell volume). Considered an active form of cell death, it differs both morphologically and functionally from apoptosis (Degterev et al., 2005) (Table 1).

The necroptotic intracellular pathway involves RIPK1, RIPK3 and MLKL (Weinlich et al., 2017). RIP kinases constitute a family of seven members, all of which contain an N-terminal kinase domain and are crucial regulators of cell death (Festjens et al., 2007). RIPK1 and RIPK3 were found to collaborate with death receptor proteins; their function can be metaphorically described as a crossroads of apoptosis, necroptosis and cell survival (Declercq et al., 2009). Although the protein RIPK7, also known as leucine-rich repeat kinase 2 (LRRK2), is not strictly involved in necroptosis, it is associated with PD. Mutation in this gene is one of the most common causes of inherited PD (*PARK8*; OMIM 607060).

Necroptosis can be initiated by various stimuli upon activation of death-domain receptors (DRs), including tumour necrosis factor (TNF) receptors 1 and 2 (TNFR1 and TNFR2), Fas, TNF-related apoptosis-inducing ligand receptor 1 and 2 (TRAILR1 and TRAILR2) (Choi et al., 2019; Grootjans et al., 2017). Several other stimuli also result in necroptosis, such as toll-like receptor (TLR) signalling, interferon, pathogen-associated molecular patterns (PAMPs), mitochondrial antiviral signalling protein, activation of the T-cell receptor, treatment with anticancer drugs (Obatoclax, Shikonin), cytomegalovirus infection and DNA damage (de Almagro & Vucic, 2015; Grootjans et al., 2017; Berghe et al., 2014).

TNF-induced necroptosis is the best-characterized necroptotic intracellular pathway. TNFR1 stimulation leads to the formation at the plasma membrane of a multiprotein complex (complex I) comprising the receptor itself, TNFR-associated death domain protein (TRADD), TNFR-associated factor 2 (TRAF2) and RIPK1 (Micheau & Tschopp, 2003). Depending on its ubiquitination status, RIPK1 can function as either a pro-survival molecule or a kinase protein that promotes cell death through apoptosis or necroptosis (Declercq et al., 2009). The recruitment of cellular inhibitors of apoptosis proteins (cIAPs) and the linear ubiquitin chain assembly complex (LUBAC) leads to the Lys63-linked polyubiquitination of RIPK1, triggering cell survival through activation of the nuclear factor-κB (NF-κB) signalling

**TABLE 1** Morphological aspects of cell death pathways.

| | Apoptosis | Necrosis | Necroptosis | References |
|---|---|---|---|---|
| Activation | Regulated | Unregulated | Regulated | (Belizário et al., 2015; Bertheloot et al., 2021; D. Chen et al., 2016; Chen, Kos, et al., 2019; D'Arcy, 2019; Dunai et al., 2011; Galluzzi et al., 2018; Grootjans et al., 2017; Häcker, 2000; Kerr et al., 1972; Liu, Zhang, et al., 2017; Ziegler & Groscurth, 2004) |
| Cell size | Shrinkage | Swelling | Swelling | |
| Plasma membrane | Blebbing<br>No loss of integrity | Loss of integrity<br>Increased permeability | Loss of integrity<br>Increased permeability | |
| Nucleus | Fragmentation<br>Chromatin condensation | Intact | No nuclear fragmentation | |
| Organelles | Disintegrated | Swelling | Swelling | |
| Specific features | Apoptotic bodies<br>Membrane blebbing<br>No inflammatory response | Translucent cytoplasm<br>Release of intracellular contents<br>Inflammatory response<br>No apoptotic bodies | Translucent cytoplasm<br>Release of intracellular contents<br>Inflammatory response<br>No apoptotic bodies | |

pathway and mitogen-activated protein kinases (MAPKs) (Wegner et al., 2017). Conversely, deubiquitination of RIPK1 by deubiquitinating enzymes cylindromatosis (CYLD) and A20 promotes the dissociation of TRADD and RIPK1 from TNFR1 and the formation of a cytosolic death-inducing signalling complex (DISC), better known as complex II (Micheau et al., 2003).

Different types of complex II (IIa and IIb) can be distinguished, depending on the composition of complex II and the activity of the proteins therein (Grootjans et al., 2017). Complex IIa consists of TRADD, RIPK1, FAS-associated protein with a death domain (FADD) and caspase-8, which is recruited to the complex in its pro-caspase-8 form (Chen, Kos, et al., 2019). The subsequent activation of pro-caspase-8 induces apoptosis independent of the kinase activity of RIPK1 (Wang et al., 2008). When cIAPs are absent or inactivated by second mitochondria-derived activator of caspases (SMAC) mimetics (SMs), complex IIb is formed, which consists of RIPK1, FADD and caspase-8. SMs are small-molecule antagonists that mimic the N-terminal four residues (Ala-Val-Pro-Ile) of endogenous mitochondrially processed SMAC (Jensen et al., 2020). Specifically, SM compounds promote rapid autoubiquitylation and proteasomal degradation of the cIAPs (Feltham et al., 2011). In the absence of TRADD, RIPK1 activity is needed for the activation of caspase-8, leading to RIPK1-dependent apoptosis (Chen, Kos, et al., 2019; Wang et al., 2008). In contrast, when caspase-8 is inhibited, for example, by the pan-caspase pharmacological inhibitor zVAD-fmk, necroptosis is induced: RIPK3 is recruited and interacts with RIPK1 through RIP homotypic interaction motifs (RHIM) present in both proteins (Festjens et al., 2007). This interaction leads to the formation of the necroptosis-inducing complex, known as necrosome (Cho et al., 2009; He et al., 2009; Humphries et al., 2015; Zhang et al., 2009).

RIPK1 kinase activity is a key step in the necroptosis pathway, because its inhibition by Nec-1 prevents RIPK1/RIPK3 interaction and blocks necroptosis (Degterev et al., 2005, 2008). In the necrosome, RIPK1/RIPK3 heterodimers recruit other RIPK3 molecules and promote homodimerization of RIPK3, which triggers its autophosphorylation (X.-N. Wu et al., 2014). Phosphorylated RIPK3 then recruits and phosphorylates MLKL, which has been identified as a key RIPK3 downstream mediator in necroptosis signalling (Sun et al., 2012; Zhao et al., 2012). MLKL functions as the executor of necroptosis: Upon phosphorylation, MLKL undergoes oligomerization and forms an octamer comprising two tetramers, which is then released and translocated to the plasma membrane. At the plasma membrane, MLKL induces the formation of pores, resulting in membrane permeabilization and rupture (Chen et al., 2014; Huang et al., 2017; Quarato et al., 2016).

The loss of cell membrane integrity causes the release of immunogenic endogenous molecules, known as damaged-associated molecular patterns (DAMPs), which induce an inflammatory response (Kaczmarek et al., 2013). The induction of necroptosis and the release of DAMPs in the central nervous system (CNS) can promote neuroinflammation, which then activates microglia and exacerbates neuronal death (Lima et al., 2021; Yuan et al., 2019) (Figure 1).

There is a mutual interplay between necroptosis and other types of cell death. Both the extrinsic apoptotic pathway and necroptosis can be elicited by activation of DRs such as TNFR, in which the key player is the activation of caspase-8 and its binding to the pseudo-caspase cellular FLICE-like inhibitory protein (cFLIP). When FLIP levels are low, active caspase-8 homodimers form, are released from complex II and induce apoptosis. Under conditions of FLIP upregulation, caspase-8/FLIP heterodimers inhibit local caspase-8 activity, allowing RIPK1/3-mediated necroptosis (Tsuchiya et al., 2015). In addition, intracellular adenosine triphosphate (ATP) levels have a central role in the interplay between apoptosis and regulated forms of necrosis: High ATP levels enable a cell to undergo apoptosis, whereas low ATP levels enable necrosis (Miyoshi et al., 2006). Several death initiator and effector molecules, signalling pathways and subcellular sites have been identified as key mediators in both processes: They can either constitute common modules or work as a switch that directs cells which route to take.

Phylogenetic analysis of genes associated with necroptosis has shown the absence of RIP-like kinases and MLKL in lower order organisms such as *Drosophila* or *C. elegans*. These genes are relatively novel products of evolution, and the earliest example of RIPK1-like kinases was found in bony fish. This means that necroptosis is not conserved in the whole animal kingdom and that in lower organisms alternative mechanisms regulating necroptosis or necroptosis may apparently be absent (Chan et al., 2015; Dondelinger et al., 2016).

### 3.1 | Possible mechanisms associated with necroptosis activation that can lead to impaired function of dopaminergic neurons

One of the critical factors in the selective degeneration of dopaminergic neurons in PD may be the association between necrosome signalling and mitochondrial dysfunction (Marshall & Baines, 2014; Sivagurunathan

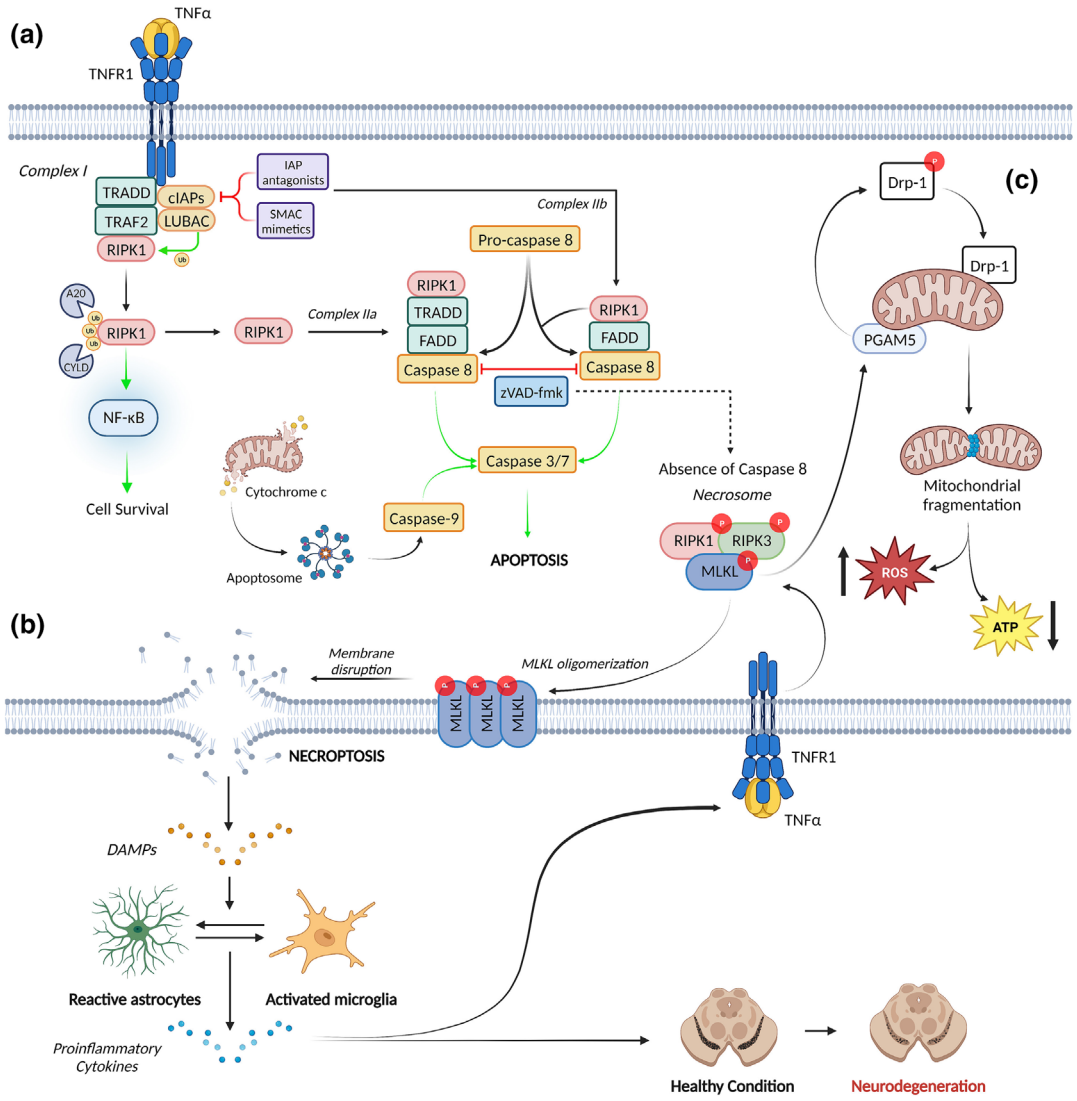


**FIGURE 1** Molecular mechanism of necroptosis and its involvement in neurodegeneration. (a) Stimulation of the cells with TNFα allows TRADD, TRAF2 and RIPK1 to be recruited to TNFR1. The recruitment of cIAPs and LUBAC leads to ubiquitination of RIPK1. When RIPK1 is ubiquitinated, complex I is formed by activation of NF-κB, thus triggering cell survival. Complex I is a crucial checkpoint for cell survival and necroptosis. Deubiquitination of RIPK1 results in complex II, together with FADD and procaspase 8. Complex II can exist in two different forms depending on protein composition and activity. Recruitment of deubiquitinating enzymes (CYLD or A20) leads to the formation of complex IIa, which is made up of TRADD, RIPK1, FADD and caspase-8. Conversely, when cIAPS are inhibited by SMAC mimetics or IAP antagonists, complex IIb is formed, which consists of RIPK1, FADD and caspase-8. Caspase-8 is recruited to both complexes in its pro-caspase-8 form. When caspase-8 activity is blocked by zVAD-fmk, the pan-caspase inhibitor, the caspase cascade is inactivated and RIPK1 interacts with RIPK3. RIPK3 is then phosphorylated and forms a filamentous amyloid structure known as the necrosome. RIPK3 interacts with MLKL, resulting in MLKL oligomerization. (b) After oligomerization, MLKL translocates to the plasma membrane, where it induces pore formation, resulting in membrane disruption and cell death. The release of DAMPs activates microglia and astrocytes, which then induce an inflammatory response that exacerbates neuronal death. The release of inflammatory mediators also contributes to activate the necroptosis pathway. (c) The RIPK1/RIPK3/MLKL necrosome can also be transported to mitochondrial membranes where it interacts with and activates phosphate glycerin mutase 5 (PGAM5) located on the outer membrane of mitochondria. PGAM5 further activates dynamin-related protein 1 (Drp1), resulting in mitochondrial fragmentation, reduced energy production and increased ROS generation. Created with Biorender.com. TNFα, tumour necrosis factor α; TNFR1, tumour necrosis factor receptor 1; TRAF2, TNF receptor-associated factor 2; RIPK1, receptor-interacting serine/threonine kinase 1; RIPK3, receptor-interacting serine/threonine kinase 3; cIAPs, cellular inhibitor of apoptosis proteins; LUBAC, linear ubiquitin assembly complex; NF-κB, nuclear factor-κB; CYLD, cylindromatosis; TRADD, tumour necrosis factor receptor type 1-associated death domain protein; FADD, Fas-associated via death domain; SMAC, second mitochondria-derived activator of caspase; zVAD-fmk, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone; MLKL, mixed lineage kinase domain-like pseudokinase; DAMPs, damage-associated molecular patterns; PGAM, phosphate glycerin mutase 5; Drp-1, dynamin-related protein; ROS, reactive oxygen species; ATP, adenosine triphosphate.

et al., 2023). Mitochondrial dysfunction is mainly characterized by the generation of reactive oxygen species (ROS), a decrease in mitochondrial complex I enzyme activity, cytochrome-c release, ATP depletion and caspase 3 activation (Moon & Paek, 2015). It was demonstrated that RIPK3 acts upstream of mitochondrial dysfunction via upregulation of mitochondrial NADPH oxidase-4 (NOX4). Because NOX4 is a ROS-generating enzyme, its activation results in increased mitochondrial ROS production and extracellular release (Uni & Choi, 2022). Moreover, the RIPK1/RIPK3/MLKL necrosome can be transported to mitochondrial membranes where it interacts with and activates the phosphate glycerin mutase 5 (PGAM5) located on the outer membrane of mitochondria. PGAM5 further activates dynamin-protein 1 (Drp1), resulting in mitochondrial fragmentation, reduced energy production and increased ROS generation (Wang, Jiang, et al., 2012). A recent study suggested that MLKL oligomerizes in the outer and the inner mitochondrial membranes, leading to a loss in mitochondrial membrane potential (Deragon et al., 2023).

Another critical factor in the degeneration of dopaminergic neurons in PD is the association between necroptosis and axonal dysfunction. A close link between necroptosis and axonal degeneration was first observed in mouse models of amyotrophic lateral sclerosis (Y. Ito et al., 2016). A more recent study tested whether necroptosis is involved in axonal degeneration of the sciatic nerve and the optic nerve (Arrázola et al., 2019). The study reported that pharmacological inhibition of RIPK1 using Nec-1 protected in vitro sensory axons from degeneration after mechanical and toxic insult and strongly delayed axonal degeneration in the peripheral nervous system and the CNS of wild-type (WT) mice. Similar effects were also observed after genetic knockdown of RIPK3 and the downstream effector MLKL. Electrophysiological analysis demonstrated that inhibition of necroptosis delays not only the morphological degeneration of axons but also the loss of their electrophysiological function after nerve injury. These results demonstrate that axonal degeneration proceeds by necroptosis (Arrázola et al., 2019). More recently, Oñate and coauthors demonstrated activation of necroptosis in postmortem brain tissue from PD patients and in a toxin-based mouse model of PD. Inhibition of key components of the necroptotic pathway resulted in a significant delay of axonal degeneration in vitro and in preclinical models of PD (Oñate et al., 2020).

Overall, changes in mitochondrial function and changes associated with axonal degeneration are two possible cellular modifications associated with necroptosis activation that can lead to impaired function of dopaminergic neurons.

## 3.2 | Studies on necroptosis in PD preclinical models

### 3.2.1 | In vitro studies

Ideally, in vitro studies should be performed on dopaminergic neuron cultures prepared from SNc to identify the mechanisms that lead to the death of dopaminergic neurons in PD patients. Although there are established procedures for the preparation of cultures from mouse and rat embryos, the in vitro yield is low and less than 4% of the neurons express dopaminergic markers (Gaven et al., 2014). This complicates the interpretation of the results, increases the number of animals necessary and incurs added expense. In addition, the low yield in dopaminergic neurons precludes the use of whole-cell pellets for biochemical analysis, such as Western blotting.

Tumour cell lines (e.g., human neuroblastoma cell line SH-SY5Y and rat pheochromocytoma cell line PC12) that produce DA are used as an alternative to primary neurons. Although studies have identified several potential molecular mechanisms for cell death, tumour cell lines cannot be considered the best model for studying neuronal cell death mechanisms because of their uncontrolled proliferation rate and intrinsic capacity to inhibit mechanisms of programmed cell death. Furthermore, in vitro studies use toxins such as 1-methyl-4-phenylpyridinium ($MPP^+$) and 6-hydroxydopamine (6-OHDA) to induce cell death. Although these toxins have been widely used to induce dopaminergic neuron death in vitro and in vivo, there is no certainty that they kill dopaminergic neurons by the same mechanism as in PD. With these caveats in mind, we summarize recent findings on necroptosis in PD (Table 2).

$MPP^+$ is the final neurotoxic agent formed by metabolism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is widely used in PD research because it induces severe damage to the nigro-striatal dopaminergic system and selectively kills dopaminergic cells (Arai et al., 1990; Przedborski & Vila, 2003; Sundström et al., 1990). MPTP can rapidly cross the blood–brain barrier (BBB) and, once in the brain, it enters glial cells, where it is oxidized into $MPP^+$. $MPP^+$ is then released and actively and selectively transported by the dopamine transporter (DAT) into DA neuronal terminals and soma, where it can accumulate in the mitochondria and synaptic vesicles. Inside the dopaminergic neuron, in the mitochondria, $MPP^+$ interferes with mitochondrial complex I activity of the respiratory chain. The subsequent severe deficit in ATP formation and the increase in ROS production leads to an energy crisis, oxidative stress and ultimately cell death (Blesa et al., 2012). Ito et al. (2017) studied the effect of $MPP^+$ on the human neuroblastoma

**TABLE 2** Studies on necroptosis: in vitro studies.

| Cell type | Treatment | Phenotype | Necroptosis aspects | References |
|---|---|---|---|---|
| SH-SY5Y cells | $MPP^+$ and rotenone | Death of neuronally differentiated SH-SY5Y cells | RIPK3 is not expressed in SH-SY5Y cells<br>Nec-1 prevents $MPP^+$-induced death independent of RIPK1<br>RIPK1 silencing does not affect either the rate of $MPP^+$-induced cell death or the inhibitory effect of Nec-1 | (Ito et al., 2017) |
| PC12 cells | 6-OHDA | Death of PC12 cells<br>Increase in number of autophagy vacuoles 6 and 12 h after treatment<br>Reduction of mitochondrial membrane potential<br>Increase of LC3-II autophagy marker | Nec-1 increases cell viability, attenuates mitochondrial membrane potential reduction, reduces activation of LC3-II | (Wu et al., 2015) |
| Rat primary cultures of mesencephalic neurons | $MPP^+$, 6-OHDA, rotenone | DA neuron death | Increase in RIPK3 signal after exposure to $MPP^+$ for 48 h<br>Increase in RIPK3 signal after exposure to rotenone for 24 h<br>No increase in RIPK3 signal after exposure to 6-OHDA | (Callizot et al., 2019) |
| Mouse mesencephalic neurons | 6-OHDA | Neurite degeneration with fragmentation and beading | Punctate staining pattern of pMLKL in the neurites<br>Increase in pMLKL levels by threefold after 6 h of 6-OHDA treatment<br>Nec-1s and GW806742x prevent neurite degeneration | (Oñate et al., 2020) |

Abbreviations: 6-OHDA, 6-hydroxydopamine; DA, dopaminergic; $MPP^+$, 1-methyl-4-phenylpyridinium; Nec-1, Necrostatin-1; pMLKL, phosphorylated mixed lineage kinase domain-like protein; RIPK1, receptor-interacting protein kinase 1; RIPK3, receptor-interacting protein kinase 3.

cell line SH-SY5Y and found that $MPP^+$ predominantly induces non-apoptotic death of neuronally differentiated SH-SY5Y cells. Treatment with Nec-1, an allosteric inhibitor of RIPK1, strongly inhibits cell death (K. Ito et al., 2017). RIPK1 silencing with specific shRNA does not affect either the rate of $MPP^+$-induced cell death or the inhibitory effect of Nec-1, suggesting that $MPP^+$-induced death is not necroptosis and that it involves Nec-1-targeting molecule(s) other than RIPK1. In addition, RIPK3 expression was not detected in SH-SY5Y cells. Overall, these results do not support a role for necroptosis in the $MPP^+$-induced death of SH-SY5Y cells but rather suggest that Nec-1 prevents $MPP^+$-induced death independently of RIPK1 (K. Ito et al., 2017). In another study, Nec-1 was reported to inhibit other enzymes, such as indoleamine 2,3-dioxygenase (Takahashi et al., 2012).

6-OHDA is another neurotoxic compound widely used to damage the nigro-striatal dopaminergic pathway. It is a hydroxylated analogue of DA with a high affinity for DAT, which transports the toxin inside dopaminergic neurons. Because 6-OHDA does not cross the BBB, it has to be injected into the SNc, the medial forebrain bundle or the striatum (Blandini & Armentero, 2012). Once inside the dopaminergic cells, 6-OHDA induces cell death through oxidative stress which increases ROS production (e.g., superoxide radicals, hydroxyl radicals, hydrogen peroxide). Also, 6-OHDA accumulates in the mitochondria, where it inhibits mitochondrial complex I activity (Glinka et al., 1997; Schober, 2004).

Wu et al. (2015) treated PC12 cells with 6-OHDA and analysed cell viability, mitochondrial membrane potential and expression patterns of apoptotic and necroptotic death signalling proteins. Cell viability, as measured by the methyl-thiazol-tetrazolium (MTT) assay, showed that 6-OHDA induces PC12 cell death and that treatment with Nec-1 has a protective effect on cell viability. 6-OHDA also induces changes in autophagic vacuoles: large quantities of autophagy vacuoles were labelled with

monodansyl cadaverine and observed at 6 and 12 h after 6-OHDA treatment. Staining with JC-1, a membrane-permeant dye used as an indicator of mitochondrial membrane potential, showed that 6-OHDA reduces membrane potential, an effect that was attenuated by Nec-1. 6-OHDA treatment increases the level of autophagy marker LC3-II, an effect that was also downregulated in the cells pretreated with Nec-1. These findings suggest that necroptosis occurs in PC12 cells treated with 6-OHDA and that crosstalk is likely between necroptosis and other cell death pathways like apoptosis and autophagy (J. Wu et al., 2015).

Callizot et al. (2019) prepared rat primary cultures of mesencephalic neurons; treated the cells with $MPP^+$, 6-OHDA, or rotenone, a mitochondrial complex I inhibitor; and investigated by immunohistochemistry the RIPK3 level. An increase in the RIPK3 cytoplasmic area was observed after exposure to $MPP^+$ for 48 h, suggesting that the necroptosis pathway is strongly activated by $MPP^+$ treatment. A modest increase in the RIPK3 signal was observed after exposure to rotenone for 24 h. After exposure to 6-OHDA for 48 h, no increase in the RIPK3 signal was observed in tyrosine hydroxylase-positive (TH) neurons. These results suggest that dopaminergic toxins can induce dopaminergic cell death via diverse pathways (Callizot et al., 2019).

In their study on necroptosis in mouse mesencephalic neuronal cultures, Oñate et al. (2020) used 6-OHDA to evaluate the expression and activation of the necroptotic component MLKL. Investigating the phosphorylation of MLKL by immunofluorescence, the authors found a clear difference in the pMLKL staining pattern in neurons treated with 6-OHDA: diffuse in the control neurites and punctate in the neurites of cells treated with 6-OHDA. This result suggests the formation of MLKL oligomers in degenerating neurons. Western blot analysis of pMLKL showed low basal expression of pMLKL in the controls and a threefold increase after exposure to 6-OHDA. Mesencephalic neuronal cultures were exposed to 6-OHDA in the presence of Nec-1s, a derivative of Nec-1 (Iannielli et al., 2018; Takahashi et al., 2012). 6-OHDA induces neurite degeneration with fragmentation and beading, which were inhibited by Nec-1s treatment. Pharmacological inhibition of MLKL using GW806742x, which binds to MLKL and blocks its translocation to the plasma membrane, prevented neurite degeneration (Oñate et al., 2020).

In summary, these findings suggest that necroptosis can occur in in vitro dopaminergic neurons prepared from rodent brain treated with PD toxins. What remains to be demonstrated is whether the same phenomena occur in the human PD brain. A future area of focus could be the extension of these experiments to dopaminergic neurons from induced pluripotent stem cells obtained from human subjects carrying PD-associated mutations.

### 3.2.2 | In vivo studies

*Phenotypic and mechanistic models of PD*

In vivo PD preclinical models can be divided into phenotypic and mechanistic models (Bakshi et al., 2019). Phenotypic models are obtained by administering toxins that selectively kill dopaminergic neurons such as MPTP and 6-OHDA. MPTP and 6-OHDA models reproduce features of the disease process (e.g., loss of dopaminergic neurons in the SNc and depletion of DA in the striatum); however, the 6-OHDA and MPTP models are acute toxicological models that differ considerably from human PD in neuropathology and neurological symptoms. Nonetheless, because the procedure is relatively simple, inexpensive and highly reproducible, these models are the most widely used to induce a nigro-striatal lesion in experimental animals and to study dopaminergic neuron death.

Differently, mechanistic models are based on a disease-causing gene mutation and are expected to reproduce the same molecular dysfunction occurring in PD patients. Because they carry PD-associated gene mutations, these models are critical for providing information on the causative mechanisms of disease. Unfortunately, no mechanistic model devised so far perfectly mimics the neuropathology and the clinical syndrome of PD. The literature on mechanistic models has been extensively reviewed elsewhere (Aniszewska et al., 2022; Bastioli et al., 2021; Blesa & Przedborski, 2014; Gamber, 2016).

*Necroptosis in PD phenotypic models*

Iannielli et al. (2018) investigated necroptosis in the subchronic MPTP mouse model and found a 50% reduction in dopaminergic fibres in the striatum after exposure to the toxin; in contrast, necroptosis inhibition by Nec-1 treatment (1 μg of Nec-1/day for 21 days) leads to a 34% decline in fibre density, accounting for about 15% of recovery. Furthermore, MPTP treatment caused a 60% loss of dopaminergic nigral neurons, which was reduced to 27% with Nec-1 co-administration. Similar results were achieved by treating mice with Nec-1s (10 mg/kg i.p.), the derivative of Nec-1 (Iannielli et al., 2018; Takahashi et al., 2012). The data suggest that RIPK1 inhibition by Nec-1 or Nec-1s effectively reduces DA neuronal loss caused by MPTP and that necroptosis has a role in in vivo dopaminergic neuron death after exposure to MPTP. In another study, however, Nec-1s treatment (10 mg/kg i.p.) did not induce neuroprotection in MPTP-treated mice (Dionísio, Oliveira, Amaral, &

Rodrigues, 2019). This result contrasted with the data obtained by Iannielli et al. (2018).

Lin et al. further explored the involvement of necroptosis in mice treated with MPTP. In the midbrain of the MPTP-treated mice, there was a strong reduction in the striatal level of DA and in the number of dopaminergic neurons in the SNc, with a concomitant increase in the protein levels of RIPK1, RIPK3 and MLKL. The authors assessed the role of the RIPK3/MLKL pathway in neuroinflammation in the SNc and found marked activation of astrocytes and microglia after MPTP induction. Pretreatment with Nec-1 (1.65 mg/kg i.p.) or knockout of the *RIPK3/MLKL* genes prevented dopaminergic neuron loss and reduced inflammatory cytokine levels (Lin et al., 2020).

Hu et al. (2019) analysed RIPK1, RIPK3 and MLKL levels and markers of neuroinflammation in MPTP-treated mice and found that MPTP treatment increases the immunofluorescence signal for RIPK1 and RIPK3 in dopaminergic neurons in the nigra. In addition, the necroptotic marker pMLKL often colocalized with TH-positive neurons in the brain of the MPTP-treated mice. Activation of microglia and astroglia and increased TNFα release were observed in the SNc. Gene profiles of the SNc obtained using an mRNA microarray indicated an increase in RIPK1 mRNA after MPTP treatment. In addition, MPTP toxicity resulted in upregulation of genes linked to TNFα response and regulation, neuronal death and neuroinflammatory response. miR-425 was found to correlate with necroptosis and dopaminergic neuron loss. Taken together, these data demonstrate that necroptosis and inflammatory response occur in dopaminergic neurons in the MPTP mouse model (Hu et al., 2019).

Oliveira et al. performed a cell-based phenotypic screening assay and identified Oxa12 as a new compound that can strongly inhibit necroptotic cell death in zVAD-fmk-treated murine BV2 microglia cells (Oliveira et al., 2018, 2021). The efficacy of Oxa12 was also determined in the sub-acute MPTP mouse model: Oxa12 or Nec-1s partially protected dopaminergic neuronal cells against MTPT toxicity. Oxa12 was identified as a new chemotype that can tackle necroptosis (Oliveira et al., 2021).

Dionisio and coauthors evaluated the role of RIPK3 in the sub-acute MPTP mouse model by using WT and RIPK3 knockout (*RIPK3* KO) mice. They found that the deletion of RIPK3 can protect against dopaminergic neuron death in the SNc, suggesting the involvement of necroptosis. However, no markers of necroptosis were detected at 4, 6 or 30 days after MPTP exposure (Dionísio, Oliveira, Gaspar, et al., 2019).

To establish whether the necroptotic pathway contributes to dopaminergic neuron degeneration in another PD phenotypic model, Oñate et al. (2020) analysed the levels of activation of critical molecular mediators of necroptosis in mice injected with 6-OHDA. Western blot analysis indicated a transient upregulation of total MLKL and pMLKL in the striatum. Analysis of the distribution of pMLKL and TH by double immunofluorescence revealed a marked increase in pMLKL in dopaminergic fibres in the striatum, the nigro-striatal pathway and in dopaminergic neurons in the SNc. Similar results in the same regions were obtained when phosphorylated RIPK3 (pRIPK3) was analysed. Formation of the necrosome was evaluated in the striatum by immunoprecipitation: Pull down of RIPK1 revealed an increase in pMLKL-RIPK1 interaction in the 6-OHDA injected hemisphere compared to the contralateral side.

In the same study, *MLKL* KO and *RIPK3* KO mice were exposed to 6-OHDA to better understand whether the necroptosis machinery contributed to axonal degeneration. TH labelling in serial coronal sections of the entire nigro-striatal circuit showed that ablation of MLKL expression protects DA axonal integrity in animals challenged with 6-OHDA. In the SNc, TH-positive neurons in the *MLKL* KO mice were partially protected against 6-OHDA, with a 21% of loss compared with a loss of 34% in the *MLKL* WT mice. To validate these results, the experiments were repeated with *RIPK3* KO mice. Ablation of RIPK3 expression was found to protect the dopaminergic axonal tracks after exposure to 6-OHDA. Given that MLKL and RIPK3 deficiency reduced the axonal neurodegeneration induced by 6-OHDA in vivo, akinesia was measured using the cylinder test and motor coordination using the rotarod test. A slight trend with no significant differences was found in the *RIPK3* KO mice, whereas marked improvement in forepaw akinesia was observed in the *MLKL* KO mice. The rotarod test showed reduced impairment in motor function in both the *RIPK3* KO and the *MLKL* KO mice after 6-OHDA injection, as compared with the *RIPK3* WT and the *MLKL* WT mice. Intraperitoneal administration of Nec-1s daily (8 mg/kg i.p.) for 3 days before and after exposure to the 6-OHDA challenge protected the nigro-striatal tract from denervation and increased motor performance in all genotypes. Taken together, these results demonstrate the activation of necroptosis machinery in the rodent 6-OHDA experimental model of PD (Oñate et al., 2020).

In conclusion, available evidence argues for the occurrence of necroptosis in the dopaminergic neurons of experimental toxin-induced PD. No claim can be made that toxin-induced cell death in rodent brain recapitulates what occurs in the human brain in PD; nonetheless, these studies demonstrate that dopaminergic neurons express proteins necessary for necroptosis and that this type of cell death can be activated in dopaminergic neurons (Table 3).

**TABLE 3** Studies on necroptosis: in vivo studies in PD phenotypic models.

| Model | Phenotype | Necroptosis aspects | References |
|---|---|---|---|
| MPTP mouse model | 50% reduction in TH-positive fibres in the striatum<br>60% loss of DA nigral neurons | RIPK1 inhibition by Nec-1 and Nec-1s reduces DA neuronal loss in the SNc and fibre density decline in the striatum | (Iannielli et al., 2018) |
| MPTP mouse model | DA neuron loss in the SNc<br>Decrease in striatal level of dopamine<br>Activation of astrocytes and microglia | Increase in protein levels of RIPK1, RIPK3 and MLKL in the midbrain of MPTP-treated mice<br>Pretreatment with Nec-1 or knockout of *RIPK3/MLKL* genes prevents DA neuron loss and reduces inflammatory response | (Lin et al., 2020) |
| MPTP mouse model | Neuronal death<br>Activation of microglia and astroglia<br>Neuroinflammatory response<br>Increase in TNFα release in the SNc | Increase in RIPK1 and RIPK3 levels in TH-positive neurons in the SNc<br>Presence of pMLKL in TH-positive neurons<br>Increase in RIPK1 mRNA in the SNc | (Hu et al., 2019) |
| MPTP mouse model | Reduction of TH-positive staining in the SNc and the striatum | Oxa12 and Nec-1s treatments protect cells against MPTP toxicity | (Oliveira et al., 2021) |
| WT and RIPK3 KO mice treated with MPTP | Neuronal loss in WT mice treated with MPTP | Deletion of RIPK3 protects against DA neuron death<br>No MLKL phosphorylation<br>Stable RIPK1 and MLKL protein level<br>Nec-1s treatment does not induce neuroprotection | (Dionísio, Oliveira, Gaspar, et al., 2019) |
| 6-OHDA mouse model | Striatal denervation 3 and 7 days after 6-OHDA treatment<br>Axonal degeneration 3 and 7 days after 6-OHDA treatment<br>DA neuron loss in the SNc 7 days after 6-OHDA treatment | Transient upregulation of total MLKL and pMLKL in the striatum<br>Increase in pMLKL and pRIPK3 in DA fibres in the striatum and in the DA neurons of the SNc<br>Increase in pMLKL-RIPK1 interaction in the 6-OHDA treated hemisphere<br>Nec-1s treatment protects the nigrostriatal tract against denervation and increases motor performance | (Oñate et al., 2020) |
| MLKL KO mice treated with 6-OHDA | No striatal denervation<br>Partial protection against TH-positive neurons loss in the SNc<br>The cylinder test showed marked improvement in forepaw akinesia<br>Improved motor function and coordination with rotarod test | N.D. | (Oñate et al., 2020) |

TABLE 3 (Continued)

| Model | Phenotype | Necroptosis aspects | References |
|---|---|---|---|
| RIP3 KO mice treated with 6-OHDA | No striatal denervation<br>Protection from TH-positive neuron loss in the SNc<br>The cylinder test showed a slight tendency albeit no difference in forepaw akinesia<br>Improved motor function and coordination with rotarod test | N.D. | (Oñate et al., 2020) |

Abbreviations: 6-OHDA, 6-hydroxydopamine; DA, dopaminergic; KO, knockout; MLKL, mixed lineage kinase domain-like protein; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; N.D., no date; Nec-1, Necrostatin-1; pMLKL, phosphorylated mixed lineage kinase domain-like protein; pRIPK3, phosphorylated receptor-interacting protein kinase 3; RIPK1, receptor-interacting protein kinase 1; RIPK3, receptor-interacting protein kinase 3; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase; TNFα, tumour necrosis factor α; WT, wild type.

*Necroptosis in PD mechanistic models*

Loss of function mutations in the *PARKIN* gene are associated with autosomal recessive juvenile parkinsonism (ARJP) (Kitada et al., 1998); the loss of PARKIN function or the expression of PARKIN variants induces dopaminergic neuron death. Because somatic *PARKIN* gene alterations have been found in a variety of tumour biopsies and tumour cell lines, *PARKIN* is also considered a tumour suppressor gene (Cesari et al., 2003; Mehdi et al., 2011; Wahabi et al., 2018). How the loss of PARKIN induces neurodegeneration and tumour formation is poorly understood. Regulation of necroptosis and inflammation are two potential mechanisms underlying these phenomena.

Lee and coauthors investigated whether PARKIN regulates necroptosis and inflammation in the intestine of *PARKIN* KO mice. Elevated RIPK1/RIPK3 and MLKL phosphorylation in the small intestine correlated with increased inflammation, as assessed by measuring the mRNA levels of TNFα, IL-1β and IL-6, and with spontaneous tumour formation. Knockdown of *PARKIN* in various cell lines also increased RIPK3 and MLKL phosphorylation after treatment with TNFα, cycloheximide, Smac mimetic and the caspase inhibitor zVAD-fmk. They then investigated by co-immunoprecipitation the molecular mechanism by which PARKIN regulates necroptosis and found that PARKIN interacts with RIPK3 and promotes its polyubiquitination. The findings suggest that the loss of PARKIN function promotes inflammation and necroptosis in the intestine and in cancer cell lines (Lee et al., 2019).

Dionísio, Oliveira, Amaral and Rodrigues (2019) tested the hypothesis that PARKIN modulates necroptosis and inflammation in microglia. They induced necroptosis in BV-2 microglial cells by treating them with the caspase inhibitor zVAD-fmk; they observed necrosome assembly, sequestration of RIPK1/RIPK3 in insoluble fractions, MLKL phosphorylation and TNFα secretion. They then performed siRNA-mediated knockdown of PARKIN. PARKIN knockdown in BV-2 cells treated with zVAD-fmk was found to reduce TNFα secretion and mRNA levels of pro-inflammatory mediators such as TNFα, interleukin IL-1β, IL-6 and inducible nitric oxide synthase (iNOS). PARKIN knockdown also reduced MLKL phosphorylation and attenuated necroptosis progression. These results suggest that PARKIN silencing mitigated zVAD-fmk-induced necroptosis in BV-2 cells. Furthermore, because RIPK1 polyubiquitination may attenuate RIPK1-dependent necroptosis, RIPK1 ubiquitination was assessed by immunoprecipitation of RIPK1 from lysates of PARKIN-silenced cells treated with zVAD-fmk. PARKIN silencing markedly increased RIPK1-linked ubiquitin moiety levels after zVAD-fmk exposure, thus implicating RIPK1 polyubiquitination status in the mitigation of zVAD-fmk-induced necroptosis. The authors speculated that PARKIN may influence protein levels of one or more mediators that stabilize RIP1 ubiquitination. To better understand the role of PARKIN during inflammation, Dionisio and coauthors performed siRNA-mediated knockdown of PARKIN in BV-2 cells and then exposed them to lipopolysaccharide (LPS). PARKIN silencing led to a marked increase in TNFα secretion and mRNA levels of TNFα, IL-1β, IL-6 and iNOS. The study findings suggest that loss of PARKIN may promote microglial survival by inhibiting necroptosis in this cell type. It is therefore possible that PARKIN promotes microglial-mediated pro-inflammatory activation in PD and contributes to PD pathogenesis through chronic neuroinflammation (Dionísio, Oliveira, Amaral, & Rodrigues, 2019).

In summary, studies suggest that PARKIN is linked to necroptosis. What remains to be determined, however, is whether *PARKIN* mutations induce dopaminergic neuron death by necroptosis. The same question applies to other

genes linked to genetic forms of PD. To the best of our knowledge, no studies to date have investigated these aspects (Table 4).

*Studies on necroptosis in tissues from PD patients*

In theory, human brain tissues from PD patients provide the best model for the investigation of experimental hypotheses in PD pathogenesis. However, studying autopsy tissue can present significant confounds due to premortem and postmortem conditions that may influence tissue quality and its ability to yield accurate results. Recognized confounds that reduce tissue quality are agonal factors (e.g., coma, hypoxia and hyperpyrexia at the time of death) and long postmortem interval (Krassner et al., 2023; Kretzschmar, 2009; Nagy et al., 2015; Samarasekera et al., 2013; Stan et al., 2006). With this caveat in mind, we revise the main findings from studies on programmed cell death mechanisms and necroptosis in human brain tissues.

The pathology seen in autopsy tissue of the SNc in human PD shows atrophy, degeneration and loss of large, multipolar melanin-containing neurons (Martin, 2010). Early studies based on in situ DNA-end labelling reported that classical apoptosis contributes to neurodegeneration in human PD, whereas other studies reported that nigral neurons in PD do not degenerate with the morphology consistent with the process of classical apoptosis (Jellinger, 1999). Biochemical studies on postmortem brains revealed increased levels of pro-inflammatory cytokines (e.g., TNFα and IL-6), increased levels of apoptosis-related factors (e.g., TNFα receptor R1 (p 55), soluble Fas and bcl-2) and increased activity of caspases 1 and 3 (Nagatsu & Sawada, 2007).

Oñate and coauthors assessed the activation of necroptosis markers by immunohistochemical analysis of the phosphorylation levels of MLKL in postmortem brain samples from PD patients and age-matched healthy controls (three PD patients and three healthy controls). Analysis of pMLKL in the SNc indicated extensive MLKL phosphorylation in the PD brain samples. Colocalization analysis revealed that around 50% of the pMLKL signal was localized in TH-positive neurons, 20% in astrocytes, and about 5% in microglia, suggesting that necroptosis occurs in neuronal and non-neuronal cells in the brain of PD patients (Oñate et al., 2020).

Chou et al. analysed gene expression of the SNc of PD patients by microarray profiling (12 PD patients and eight controls) and noted marked upregulation of RIPK3 in the PD patients compared with the controls, whereas the comparison of upregulation of RIPK1 and MLKL did not reach statistical significance (Chou et al., 2021).

Alegre-Cortés et al. characterized the necroptosis pathway in primary fibroblasts from PD patients harbouring the G2019S *LRRK2* mutation, a common cause of autosomal dominant PD (Bonifati, 2006). The study included data from three control cell lines, three cell lines from iPD and three cell lines from PD patients bearing the G2019S *LRRK2* mutation (Alegre-Cortés et al., 2020).

**TABLE 4** Studies on necroptosis: in vivo studies in PD mechanistic models.

| Model | Phenotype | Necroptosis aspects | References |
|---|---|---|---|
| *PARKIN* KO mice | Increased inflammation<br>Spontaneous tumour formation | Increase in RIPK1/RIPK3 and MLKL phosphorylation in the small intestine<br>PARKIN interacts with RIPK3 and promotes its polyubiquitination | (Lee et al., 2019) |
| BV-2 microglial cells treated with zVAD-fmk | TNFα secretion | Necrosome assembly, sequestration of RIPK1/RIPK3 in insoluble fractions, MLKL phosphorylation | (Dionísio, Oliveira, Amaral, & Rodrigues, 2019) |
| PARKIN knockdown in BV-2 microglial cells treated with zVAD-fmk | Reduction in TNFα secretion and in mRNA levels of pro-inflammatory mediators | Reduction of MLKL phosphorylation<br>Attenuation of necroptosis progression<br>Increase in RIPK1-linked ubiquitin moiety levels | (Dionísio, Oliveira, Amaral, & Rodrigues, 2019) |
| PARKIN knockdown in BV-2 microglial cells treated with LPS | Increase in TNFα secretion and mRNA levels of TNFα, IL-1β, IL-6 and iNOS | N.D. | (Dionísio, Oliveira, Amaral, & Rodrigues, 2019) |

Abbreviations: IL, interleukin; iNOS, inducible nitric oxide synthase; KO, knockout; LPS, lipopolysaccharide; MLKL, mixed lineage kinase domain-like protein; N.D., no date; RIPK1, receptor-interacting protein kinase 1; RIPK3, receptor-interacting protein kinase 3; TNFα, tumour necrosis factor α.

Western blot analysis showed that both RIPK1 and RIPK3, and their phosphorylated forms, are expressed in human fibroblasts. Although there were no differences in pRIPK1 levels between the groups, pRIPK3 was significantly decreased in the iPD cells. The cell lines were then treated with rotenone, a mitochondrial complex I inhibitor. Rotenone treatment increased the level of pMLKL in the PD cells, and co-treatment with Nec-1 reduced it. The data suggest that rotenone treatment made the cells from the PD patients more susceptible to activation of necroptosis. However, Nec-1 affected mitochondrial morphology and failed to protect the mitochondria against rotenone toxicity (Alegre-Cortés et al., 2020). Although rotenone is an inhibitor of mitochondrial complex I and an efficient ROS generator, it also induces mitophagy. Because Nec-1 was shown to downregulate autophagy (Wang, Wang, et al., 2012) and prevent mitophagy (Alegre-Cortés et al., 2020), Nec-1 may have a dual role in PD: On the one hand, it inhibits necroptosis, and on the other, it may directly affect mitochondrial morphology and clearance (Alegre-Cortes et al., 2021).

Taken together, these studies suggest that human PD brain cells and tissues may be prone to activation of necroptosis. Table 5 summarizes the study results.

Recent advances in human induced pluripotent stem cell (hiPSC) technology and differentiation into hiPSC-derived dopaminergic neuron (iDA) has provided an opportunity to study cell death mechanisms in vitro in humans. iDA carrying the PD-associated mutation A53T in the synuclein gene exhibited diminished mitochondrial spare-respiratory capacity and increased basal levels of ROS and reactive nitrogen specie (RNS) compared with the isogenic corrected controls. Brief exposure to mitochondrial toxins or commonly used pesticides is sufficient to further increase ROS/RNS, contributing to apoptosis, as assessed by the TUNEL assay (Ryan et al., 2013). Similar results have been obtained in iDA-carrying mutations in the *PARKIN* gene. These neurons demonstrate abnormal α-synuclein accumulation and downregulation of the proteasome and anti-oxidative pathways. Environmental triggers such as proteasome inhibitor MG132 and $H_2O_2$ markedly induce cell death (Chang et al., 2016). Unfortunately, none of these studies has explored necroptosis features; therefore, future studies on these cells are warranted to elucidate the activation of necroptosis in PD.

### *Efficacy, safety and potential application of necroptosis inhibitors in preclinical studies and clinical trials*

The first RIPK1 inhibitor Nec-1 was identified in 2005 upon screening of a chemical library of 15,000 compounds for chemical inhibitors of the death of human monocytic U937 cells induced by TNFα and zVAD-fmk. Necroptosis was also identified as a key mechanism of ischemic brain injury; Nec-1 and the analogous 7-Cl-Nec-1 prevented ischemic brain injury in in vitro and in vivo preclinical models (Degterev et al., 2005). The study disclosed a novel mechanism of cell death and identified a novel class of compounds with potentially broad relevance to human disease. Following the identification of Nec-1, necroptosis inhibition is attracting increasing research interest from multiple disciplines. To date, many active compounds have been identified by targeting the key component of necroptosis RIPK1.

Nec-1 and its derivatives named necrostatins were shown to inhibit RIPK1 in an ATP-competitive manner, thus preventing its catalytic activity and rescuing cells from necroptosis. Unfortunately, Nec-1 is unstable in vivo ($t1/2 < 5$ min in mouse liver microsomes) and toxic at concentrations >100 μM (Gardner et al., 2023; Teng et al., 2005). The derivative Nec-1s is more potent and more stable in vivo ($t1/2 \sim 60$ min in mouse liver microsomes) (Gardner et al., 2023). Other necrostatins did not achieve the potency and subsequent widespread use of Nec-1.

**TABLE 5** Studies on necroptosis: studies on tissues from PD patients.

| Model | Necroptosis aspects | References |
|---|---|---|
| Postmortem brain samples from PD patients | Extensive MLKL phosphorylation in the SNc | (Oñate et al., 2020) |
| SNc from PD patients | Upregulation of RIPK3<br>No statistically significant increase in RIPK1 and MLKL | (Chou et al., 2021) |
| Primary fibroblasts from iPD and PD patients harbouring the G2019S *LRRK2* mutation | No differences in pRIPK1 levels<br>Reduction of pRIPK3 levels in iPD cells<br>Increase in pMLKL level after rotenone treatment; co-treatment with Nec-1 reduces pMLKL level | (Alegre-Cortés et al., 2020) |

Abbreviations: iPD, idiopathic Parkinson's Disease; MLKL, mixed lineage kinase domain-like protein; Nec-1, Necrostatin-1; PD, Parkinson's Disease; pMLKL, phosphorylated mixed lineage kinase domain-like protein; pRIPK1, phosphorilated receptor-interacting protein kinase 1; pRIPK3, phosphorilated receptor-interacting protein kinase 3; RIPK;1, receptor-interacting protein kinase 1; RIPK3, receptor-interacting protein kinase 3; SNc, substantia nigra pars compacta.

Despite the widespread use of Nec-1, by analysing the specificity and toxicity of Nec-1 and Nec-1s, a study raised some critical issues concerning Nec-1 in vivo use (Takahashi et al., 2012). The authors reported that Nec-1 is identical to methyl-thiohydantoin-tryptophan, an inhibitor of the immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO). As IDO is upregulated in inflammation and has a major immunomodulatory role, dual activity on RIPK1 and IDO may have important in vivo implications. According to the study by Takahashi et al., Nec-1 inhibited human IDO, but Nec-1s did not. Therefore, Nec-1s is a more specific RIPK1 inhibitor lacking the IDO-targeting effect. Along the same line, in vivo, high doses of Nec-1 (6 mg/kg) prevented TNF-induced mortality. However, paradoxically, low doses of Nec-1 (0.6 mg/kg) sensitized mice to TNF-induced mortality. This paradoxical finding has major implications for the interpretation of dose-dependent effects of Nec-1 in murine experimental disease models and may also explain some controversies in the literature. Nec-1s did not exhibit this low-dose toxicity (Takahashi et al., 2012).

Concerning other RIPK1 inhibitors in the chemical class of dihydropyrazoles, GSK′963 was shown to be highly specific for RIPK1, efficacious in murine cells stimulated with TNF and zVAD-fmk and able to protect mice against TNF + zVAD-fmk-induced lethal shock in vivo. However, poor oral absorption and its short half-life in vivo limit the utility of this molecule to study acute models of necroptotic disease (Berger et al., 2015).

The RIPK1 inhibitor GSK′547 was generated with the intent to improve potency and pharmacokinetic profile and provide a tool for in vivo use. GSK′547 was tested in a mouse model of human multiple sclerosis and a mouse model of human retinitis pigmentosa. A delay in disease onset and reduced clinical severity were demonstrated in the multiple sclerosis model, and protection of retinal cell function and survival were observed in the retinitis pigmentosa model (Harris et al., 2019).

Another valuable RIPK1 inhibitor is DNL747, which was tested in healthy volunteers and patients with Alzheimer's disease (AD) or amyotrophic lateral sclerosis (ALS). Double-blind phase I/Ib studies found that RIPK1 inhibition by DNL747 was safe and well-tolerated for up to 28 days in patients with AD or ALS. DNL747 was distributed into the cerebrospinal fluid (CSF) after oral administration and demonstrated RIPK1 inhibition in peripheral blood mononuclear cells (Vissers et al., 2022). Unfortunately, DNL747 development was suspended after the report of toxicity issues in preclinical studies. However, studies on the analogous inhibitors DNL788, DNL758 and DNL104 are in progress. Clinical trials investigating DNL104 in healthy human volunteers showed CNS safety, but concerns arose about liver toxicity. A study with DNL104 showed that CNS-penetrant inhibition of RIPK1 phosphorylation may prevent brain inflammation and necroptosis in vivo (Grievink et al., 2020). Another RIPK1 inhibitor named R552 completed a phase I clinical trial and will enter phase II studies in autoimmune and inflammatory diseases (Gardner et al., 2023).

In conclusion, potential RIPK1 inhibitors are under investigation in clinical trials for the treatment of neurodegenerative diseases, autoimmune, ischemic conditions and chronic inflammatory diseases such as ulcerative colitis, psoriasis and rheumatoid arthritis (Mifflin et al., 2020). Drawing on the evidence for activation of necroptosis in preclinical models of PD, it would be interesting to test these molecules on selected cohorts of PD patients.

Another set of compounds were developed against RIPK3 and MLKL, both located downstream of RIPK1. This pharmacological approach can potentially circumvent the issues associated with other cellular functions of RIPK1. However, no clinical trials are currently investigating RIPK3- or MLKL-inhibitors (Mansour et al., 2023). Interestingly, many anti-cancer drugs, which are all multi-targeting kinase inhibitors (e.g., vemurafenib, ponatinib, pazopanib, TAK-632, sorafenib and dabrafenib), displayed anti-necroptotic effects by targeting RIPK1, RIPK3 and MLKL (Chen, Zhuang, et al., 2019; Fulda, 2018). Although these inhibitors lack selectivity, some of these anti-cancer drugs may be repurposed for neurodegenerative diseases such as PD, ALS and AD.

## 4 | CONCLUSION AND PERSPECTIVES

The majority of studies on necroptosis and PD are consistent with the hypothesis that this cell death mechanism is activated in PD tissues; however, the coexistence of other types of cell death is very likely. Indeed, a variety of programmed cell death mechanisms are likely to occur in degenerating dopaminergic neurons (e.g., apoptosis, necroptosis, pyroptosis and ferroptosis), as well as cell death associated with proteasomal and mitochondrial dysfunction. This evidence is derived from in vitro PD models and studies on murine models and *C. elegans* models of PD, including transgenic worms that express α-synuclein or LRRK2 and worms with deletions in *PARKIN*, *PINK1*, *DJ-1* and *ATP13A2* (Cooper & Van Raamsdonk, 2018). Which of these pathways plays a causal role in the death of dopaminergic neurons is difficult to discern, however.

PD is very heterogeneous and often arises from a combination of genetic and environmental factors. At

least 15 different genes with high penetrance are associated with PD. Many more genes, if inherited in certain haplotypes, can increase the risk of developing the disease (Blauwendraat et al., 2020). In addition, numerous environmental toxins have been associated with elevated risk for PD (Goldman, 2014). Because the mechanisms underlying dopaminergic neuron death may differ by patient category, PD may be better envisaged as a syndrome rather than as a single disease. Necroptosis can occur in some categories but not in others. Preclinical models of advantageous use are mouse models expressing mutant forms of *synuclein*, *LRRK2*, *PARKIN* or other PD genes. It would be interesting to see whether necroptosis can be developed to marked levels in certain genetic PD models and whether it can be blocked with pharmacological or genetic techniques to inhibit neurodegeneration. Such studies could open the way to therapeutic approaches based on precision medicine, in which inhibitors of the necroptosis pathway could be tested on various categories of PD patients. Given the progress in clinical trials of some anti-necroptosis molecules for other pathologies and the possibility of repurposing various kinase inhibitors (see previous chapter), it would be interesting to extend these studies to PD patients.

Another important yet understudied aspect is the relationship between necroptosis and ageing. Ageing is the main risk factor for PD, but how, from a molecular perspective, ageing induces dopaminergic neuron dysfunction and death remains to be elucidated. There is evidence for an association between ageing and increased necroptosis and inflammation. Thadathil and coauthors found an increase in the levels of phosphorylated MLKL and neuroinflammation markers with age in mouse brain; the necroptosis markers were mainly localized to the neurons (Thadathil et al., 2021). Arrazola and coauthors showed that the genetic deletion of MLKL delayed age-associated axonal degeneration and neuroinflammation; aged MLKL mice were protected against decreased synaptic transmission and memory decline. Moreover, treatment with the RIPK3 inhibitor GSK'872 reversed structural and functional hippocampal impairment. Finally, necroptosis inhibition leads to an overall improvement of the aged hippocampal proteome, including a subclass of molecular biofunctions associated with brain rejuvenation. The study concluded that necroptosis inhibition constitutes a potential geroprotective strategy to treat brain age-related disabilities (Arrázola et al., 2023). In this context, we may hypothesize that ageing is a co-factor in inducing necroptosis in dopaminergic neurons. Finally, the necroptosis pathway is a potential target for PD; drugs that prevent or reduce necroptosis may provide neuroprotection against this life-changing disease.

## AUTHOR CONTRIBUTIONS

**Maria Regoni:** Writing—original draft; writing—review and editing. **Flavia Valtorta:** Writing—review and editing. **Jenny Sassone:** Conceptualization; writing—original draft; writing—review and editing.

## ACKNOWLEDGEMENTS

Open access funding provided by BIBLIOSAN.

## CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/ejn.16136.

## DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

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**How to cite this article:** Regoni, M., Valtorta, F., & Sassone, J. (2024). Dopaminergic neuronal death via necroptosis in Parkinson's disease: A review of the literature. *European Journal of Neuroscience*, *59*(6), 1079–1098. https://doi.org/10.1111/ejn.16136