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Molecular basis of programmed cell death involved in neurodegeneration

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Rapid progress in understanding the molecular basis of neurodegeneration has been tightly linked with recent discoveries in the field of programmed cell death (PCD). Analysis of PCD in neuronal demise has led to identification of several associated phenomena, such as re-initiation of the cell cycle and the key role of oxidative stress, although putative causal relationships between these events are still debatable. These issues are reviewed here in the context of acute and chronic neurodegenerative processes. In addition, newly emerging concepts concerning cell-cycle re-initiation are discussed in terms of their potential impact on the development of more effective therapeutic strategies.

Introduction

Neurons in the adult nervous system are terminally differentiated, postmitotic cells. This state implies two major physiological features: downregulation of mechanisms controlling cell division [1] and upregulation of those offering protection from programmed cell death (PCD), especially apoptosis [2]. Under pathological conditions such as acute and chronic cytotoxic insults associated with oxidative stress, these adaptations are lost, leading to re-entry into the cell cycle before death [3]. Neurons are highly sensitive to mediators of oxidative stress such as reactive oxygen species (ROS), which are well-known triggers of PCD (reviewed in [4]). It is also well established that several human neurodegenerative pathologies, including stroke, Alzheimer's disease (AD) and Parkinson's disease (PD) are accompanied by elevated oxidative stress and neuronal loss, including PCD (reviewed in [5]).

Programmed cell death

PCD is generally defined as a series of stereotypical biochemical and morphological alterations leading to cell demise. These characteristics are often employed to distinguish the 'active' character of PCD, by which dying cells are removed in a safe, non-inflammatory manner, from 'passive' cell death by necrosis [2]. Under physiological conditions, PCD is tightly controlled and regulates the balance between proliferation and differentiation both in the course of development and during the optimization of adult cell and tissue functions [6]. Alterations in the regulation of PCD have been implicated in several pathologies including cancer and neurodegeneration [2].

There is currently no consensus on the classification of different types of PCD. One of the more restraining (but currently considered one of the most accurate [6]) classifications is based on the criterion of nuclear morphology. This approach divides PCD into classical apoptosis, apoptosis-like PCD and necrosis-like PCD, respectively characterized by nuclear chromatin condensation that is 'crescent-like' (type 2), partial or peripheral (type 1), or absent [2,6,7] (Figure 1). In this regard, the concept of 'apoptosis' has enabled tremendous progress in our understanding of PCD, but its widespread use as synonymous to PCD has turned out to be confusing and thus counter-productive [2,6].

Classical apoptosis, the best-known phenotypic expression of PCD, consists of at least two phases: initiation and execution. These ultimately lead to a series of stereotypic morphological and biochemical events resulting from the activation of cystein-dependent, aspartate-directed proteases called caspases. The canonical pathways of caspase activation during initiation include the 'death-receptor-mediated' recruitment of procaspase-2, procaspase-8 and procaspase-10, and a 'mitochondrial' pathway through which caspase-9 is activated via release of cytochrome *c*. The two pathways converge, leading to activation of procaspase-3 and, further downstream, to activation of caspase-6 and caspase-7. All these pathways are associated with activation of caspase-activated DNase (CAD), and so also with 'typical' inter-nucleosomal DNA fragmentation (reviewed in [8]) (Figure 1).

Apoptosis-like PCD is broader than classical apoptosis and includes caspase-independent mitochondrial pathways. In this context, the apoptosis-inducing factor (AIF) has attracted much interest because it is the best-known caspase-independent cell death effector [9,10]. Upon mitochondrial outer-membrane permeabilization, AIF is released from the inter-membrane mitochondrial space and translocates to the nucleus where it is associated with large-scale DNA fragmentation [9]. Given the absence of intrinsic AIF-endonuclease activity, the DNA-degrading

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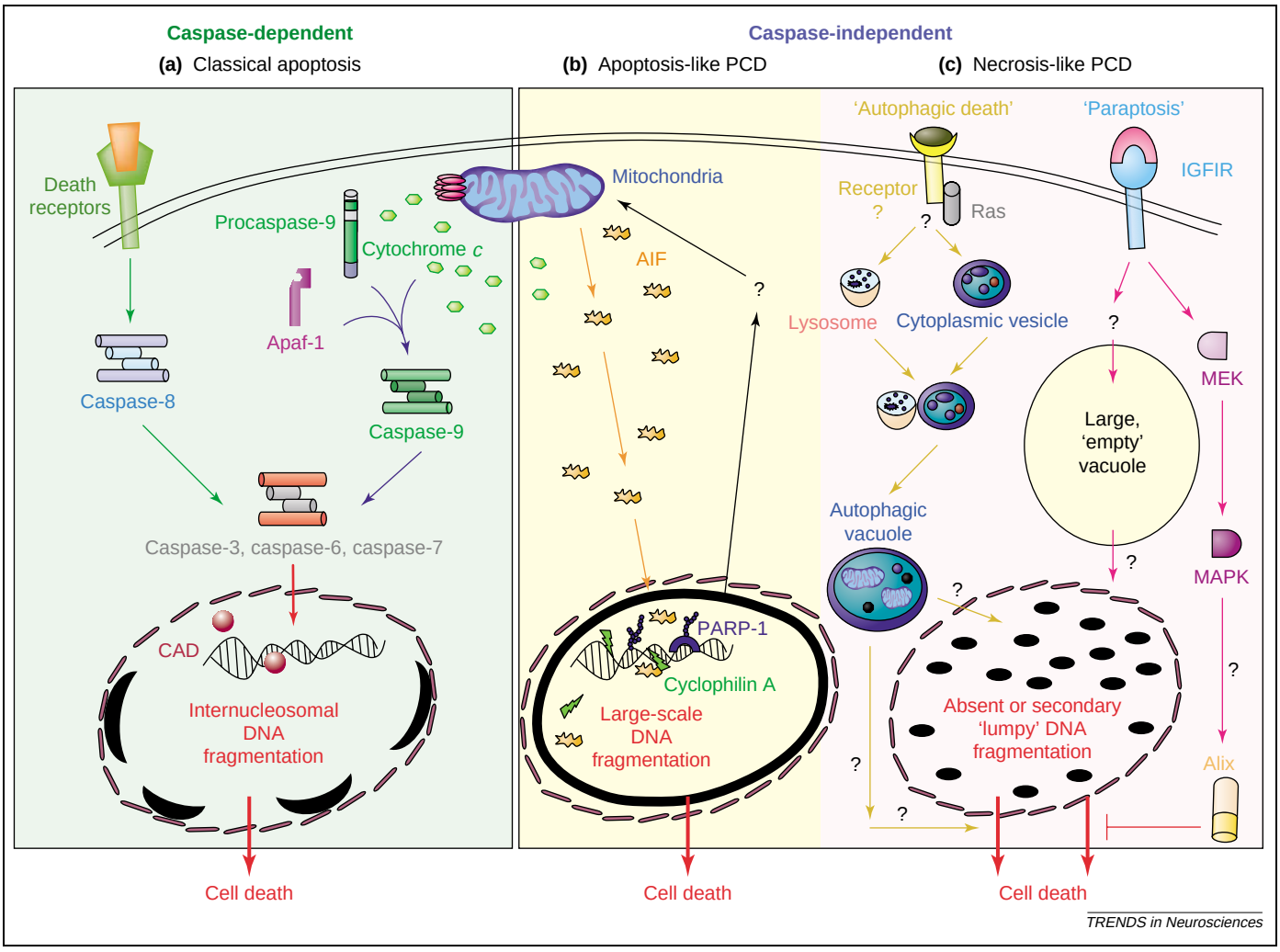

Figure 1. Classification and molecular events leading to major types of PCD. The presented classification is based on nuclear morphology, as proposed by Jäättelä and Tschopp [7]. PCD can be divided in three types: **(a)** classical apoptosis (featuring 'crescent-like' chromatin condensation), **(b)** apoptosis-like PCD (featuring partial and peripheral chromatin condensation) and **(c)** necrosis-like PCD (featuring no primary chromatin condensation). (a) Classical apoptosis encompasses two pathways, both coupled to a caspase cascade ultimately leading to activation of executive caspases including caspase-3, caspase-6 and caspase-7. These bring about internucleosomal DNA lysis by the endonuclease caspase-activated DNase (CAD), yielding fragments that are multiples of 180–200 bp. Depending on the stimulus or cells involved, the apoptotic response is mediated by an 'extrinsic' pathway following activation of caspase-8 by a death receptor, or an 'intrinsic' pathway involving apoptosis-inducing mitochondrial proteins such as cytochrome *c*. Cytochrome *c* is released in the cytosol after permeabilization of the outer mitochondrial membrane and subsequently associates with procaspase-9 and the adaptor protein apoptotic activating factor-1 (Apaf-1) to form a complex called the apoptosome. This complex gives rise to active caspase-9, which has been implicated in stimulation of executive caspases. (b) Apoptosis-like PCD is mediated by mitochondrial effectors such as the apoptosis-inducing factor (AIF). Nuclear DNA damage is detected by poly-ADP-ribose polymerase-1 (PARP-1) and signalled directly or indirectly to mitochondria via an unknown mechanism. This leads to AIF release and its translocation to the nucleus, which is then associated with chromatin condensation and large-scale DNA fragmentation (>50 kbp). AIF, by itself, does not have endonuclease catalytic activity. Hence, its DNA-degrading capacity requires association with endonucleases such as cyclophilin A in mammalian cells. (c) Necrosis-like PCD is not as well understood. Despite this, the existence of at least two forms of such PCD has recently been proposed. Both forms display cytoplasmic vacuoles, albeit with distinct morphologies. Paraptosis, which can be triggered by the activation of the insulin-like growth factor 1 receptor (IGFIR) via the mitogen-activated protein kinase (MAPK) pathway [including extracellular-signal-regulated kinase (ERK)1, ERK2 and ERK kinase (MEK)2, and probably MEK4, MEK7 and c-Jun N-terminal kinase (JNK)1], is accompanied by the formation of large, apparently empty vacuoles and can be selectively inhibited by the ALG-2-linked protein X (Alix) [74]. The other form of necrotic cell death, 'autophagic degeneration', is mediated by the activation of mutated Ras. This death is associated with the formation of cytoplasmic, lysosome-derived vacuoles with their characteristic double-membrane appearance [75].

capacity of AIF relies on recruitment of downstream nucleases, such as cyclophilin A [11] and, at least in *Caenorhabditis elegans*, endonuclease G [12] (Figure 1).

In necrosis-like PCD, the cell-death program is triggered by organelles other than mitochondria, such as lysosomes, endoplasmic reticulum and the nucleus, and by proteases other than caspases, such as cathepsins and calpains originating from lysosomes and the endoplasmic reticulum, respectively (reviewed in [2,6,7]) (Figure 1). The molecular mechanisms underlying such PCD are less well understood, although it is generally believed that they represent 'alternative' death pathways when caspases are inhibited (reviewed in [2,7]). Caspase inhibition can result from genetic factors such as mutation of apoptotic activating factor 1 (Apaf-1) [13], blockade by other proteases activated simultaneously [14], silencing by viral proteins in the course of infection (reviewed in [15]), energy depletion [16], and nitrative and/or oxidative stress [17]. Neurons are highly sensitive to the nitrative and oxidative stress because they depend entirely on the aerobic metabolism of glucose, which generates ROS as by-products of incomplete oxygen reduction to water during oxidative phosphorylation (reviewed in [4,5]).

PCD in neurons

Classical apoptosis is the most prevalent form of PCD during developmental neurogenesis. In the adult brain, it remains the most studied form, although it does not account for all death phenotypes. The involvement of classical apoptosis remains controversial for normal brain aging [18,19], in contrast to acute injuries [20] and chronic diseases [21]. In PD, classical apoptosis is associated with either mutation [22] or overexpression [23] of α-synuclein. Similarly, the occurrence of classical apoptosis has been reported in AD [24] and might be related to maturation of amyloid-precursor protein (APP). Indeed, different types of amyloid-β (Aβ) peptides and activated caspase-3 accumulate in the hippocampus and frontal cortex in AD [19]. It has also been reported that Aβ peptides can trigger the classical apoptotic program through a p53-dependent mechanism [25], and direct transcriptional control of the p53 promoter by Aβ peptides has recently been demonstrated [26].

As already discussed, it is now clear that forms of cell death different from classical apoptosis commonly occur in postmitotic neurons. These include canonical, 'passive' necrosis in addition to caspase-independent, non-apoptotic PCD. For example, the relevance of apoptosis-like PCD in PD has recently been reported on the basis of the capacity of MPP+ to activate calpain I in a caspase-independent manner, subsequently triggering AIF translocation to the nucleus [27]. MPP+ is a metabolically active product of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrimidine (MPTP), a toxin commonly used to induce PD-like neurodegeneration. In AD, the involvement of PCD distinct from classical apoptosis has been suggested on the basis of morphological studies of AD brains [28], animal models [29] and cell cultures treated with Aβ peptides [30]. The first direct evidence for involvement of AIF-mediated apoptosis-like PCD in AD came from a recent study demonstrating that Aβ peptides trigger nuclear AIF translocation in cultured embryonic rat cerebral cortex [31]. Finally, some dying neurons display a wide range of 'atypical' death morphologies [32,33] considered as necrosis-like types of PCD according to the classification of Jäättelä and Tschopp [7].

Oxidative stress as a trigger of neuronal PCD

ROS production inherent to oxidative stress is generally associated with neuronal PCD, irrespective of the death phenotype involved. ROS are harmful to proteins, lipids and DNA because of their ability to oxidize these cellular components and induce their structural and functional alteration. Under physiological conditions, ROS are rapidly cleared in scavenging reactions by antioxidant enzymes such as superoxide dismutase (SOD) [34], catalase [35], glutathione-reductase and glutathione-peroxidase [36] and the recently discovered peroxiredoxins [37]. Dysregulation of ROS scavenging capacity following glutamate excitotoxicity and Aβ-induced cytotoxicity have been implicated in acute brain injuries and chronic neurodegeneration, respectively [5,38].

Glutamate excitotoxicity is considered as an initial trigger for apoptosis-like PCD in stroke, epileptic seizures, and traumatic brain and spinal cord injuries (reviewed in [38]). Indeed, superoxide-anion scavenging by exogenous glutathione peroxidase, which decreases oxidative stress, can attenuate glutamatergic injury [39]. Glutamate excitotoxicity is mediated mainly through NMDA receptors, and the NMDA-receptor antagonist MK-801 substantially decreases cell death both *in vivo* [40] and *in vitro* [41]. Activation of NMDA receptors leads to increased intracellular Ca^{2+} concentration and subsequent activation of enzymes such as neuronal nitric oxide synthase (nNOS). Nitric oxide is a substrate for the production of ROS (peroxynitrites), which are responsible for protein nitration (reviewed in [42]). Increased intracellular Ca^{2+} concentration also triggers depolarization of the mitochondrial membrane and the subsequent loss of membrane potential ($\Delta\phi_m$), yielding to additional ROS generation [43]. DNA damage resulting from ROS production triggers over-stimulation of the DNA damage-sensing enzyme poly-ADP-ribose polymerase-1 (PARP-1), followed by AIF translocation from mitochondria to the nucleus [44]. This translocation is PARP-1-dependent because it is abolished in PARP-1-knockout mice, and it precedes caspase-independent neuronal demise that has characteristics of apoptosis-like PCD [45].

Some studies have reported that the initial release of AIF from mitochondria requires caspase activation, suggesting a parallel induction of both caspase-dependent (classical apoptosis) and caspase-independent (AIF-mediated) cell death, at least in non-neuronal cell types [46]. However, the relevance of classical apoptosis in acute neuronal injuries has been seriously challenged by the recent demonstration that glutamate-induced excitotoxicity is caspase-independent [47]. Indeed, reduced AIF expression enables cortical neurons *in vitro* to resist glutamate-induced excitotoxic death, and protects the hippocampus *in vivo* from damage associated with kainic-acid-induced seizures [47]. Taken together, these data strongly suggest that AIF is the key effector of cell death occurring in acute neuronal injuries. However, it is also apparent that multiple death phenotypes can be triggered by the same stimulus in a timely ordered manner within a given brain region. For example, Young *et al*. have shown that the initial wave of cell loss observed in acute injuries involves 'excitotoxic cell death' [48] – an expression used by the authors in the sense of necrosis-like PCD, as defined in [7]. The second wave of neuronal demise involves classical apoptosis of neurons that have lost their synaptic targets in the first wave [48].

The relevance of oxidative stress and the role of excitotoxicity in chronic neurodegenerative processes are now broadly recognized. The predominant view is that in chronic neurodegeneration, glutamate excitotoxicity represents an important proximal regulator of neuronal death, although the original triggers of ROS accumulation are probably additional pathogenic factors [49]. These include a mutated form of SOD1 in amyotrophic lateral sclerosis (ALS) that increases the sensitivity of motoneurons to glutamate excitotoxicity by a mechanism involving oxidative stress [50]. Furthermore, oxidative-stress-related protein modifications such as lipid peroxidation and protein nitration have been reported in ALS patients and in relevant animal models [51].

Similar oxidative-stress-mediated lipid and protein modifications have been reported to occur in the substantia nigra in PD [52]. The molecular mechanisms involved have recently been investigated in the MPTP-treated mouse model. In wild-type mice, but not in PARP-1-knockout MPTP-treated mice, AIF translocates to the nucleus of tyrosine-hydroxylase-positive neurons, demonstrating that the cytotoxic effects of MPTP involve PARP-1 [53]. Wang *et al*. postulated that MPP+ blocks complex I of the mitochondrial respiratory chain, consequently inhibiting oxidative phosphorylation and leading to a decrease in intracellular ATP levels. This event is indirectly involved in neuronal depolarization, release of glutamate and over-stimulation of NMDA receptors, resulting in increased intracellular Ca^{2+} levels. The following cascade of events is analogous to that reported in acute injuries [53]. However, in light of the recent finding that AIF is a key factor in regulation of complex I assembly and/or maintenance [54], inhibition of this complex by MPP+ could perturb oxidoreduction by affecting the capacity of AIF to regulate the complex. It remains to be established whether, by itself, this signal is sufficient to trigger the release of AIF from mitochondria.

Further clues concerning the relevance of AIF in oxidative stress and chronic neurodegeneration came from study of Harlequin mutant mice, which present a dramatic reduction (~80%) in AIF expression owing to ectopic proviral insertion in the AIF gene. These mice develop age-related ataxia and blindness from degeneration of neurons in the cerebellum and retina [55]. Moreover, this AIF deficiency inhibits oxidative phosphorylation by disrupting respiratory-chain complex I function both *in vitro* and *in vivo*, and is accompanied by severe losses of this complex in retina and brain [54]. In addition, cerebellar neurons derived from Harlequin mutant mice display particular vulnerability to oxidative stress induced by endogenous or exogenous peroxides, leading to the hypothesis that AIF might also act as a peroxide scavenger [55].

Cell-cycle re-initiation and neuronal PCD

From a molecular perspective, neuronal PCD consistently displays the unique property of 'pathological' re-initiation of the cell cycle. Entry of neurons into the cell cycle from quiescence (G0 phase) is, as in other cell types, controlled by a family of cyclin-dependent kinases (CDKs). Activation of CDKs relies on their association with regulatory units called cyclins in a cell-cycle phase-specific manner. The cyclin A family is involved in both G1–S and G2–M transitions through its association with CDK4 or CDK6 (for G1–S) and CDK2 (for G2–M), whereas cyclin B1 and cyclin B2 interact with CDK1 and are required for initiation and progression through M phase. Four mammalian G1-phase cyclins have been identified: the D-type cyclins D1, D2 and D3, and cyclin E. Extracellular signals impinge on the cell cycle mainly during a limited time window in the G1 phase. Thus, at least in non-neuronal cells, the induction of cyclin D expression depends on the presence of growth factors in the extracellular environment and is therefore considered as the growth factor sensor (reviewed in [56]). By contrast, in neurons, the induction of cyclin D (and other cyclins) is segregated from cell division and is generally considered as a prelude to cell death [1].

Expression of different cell-cycle molecules has been found to precede excitotoxic death in various experimental models. For example, cyclin D1 induction has been reported to occur in focal [57] and mild [58] cerebral ischemia, and in epilepsy [59]. Moreover, decreased expression of CDK inhibitor p16INK4 has also been found to precede neuronal death [58,60]. Despite these findings, the involvement of cell-cycle molecules in neuronal PCD is still debatable. For instance, the induction of cyclin D1 was not observed in a model of combined hypoxia–ischemia [60]. Apparent discrepancies might relate to how 're-initiation' of the cell cycle is defined. In many studies, re-initiation of cell division is assessed using incorporation of bromodeoxyuridine (BrdU; an S-phase marker) or expression of cell proliferation antigens (e.g. PCNA or Ki67), thus enabling discrimination between quiescent (G0) cells (e.g. postmitotic neurons) and cells in any other phases of the cycle (i.e. G1, S, G2 or M). It has recently been demonstrated that expression of these parameters in postmitotic neurons is not only related to re-initiation of cell division, but also can reflect DNA-repair activity [60].

In addition to acute neuronal injuries, cell-cycle re-initiation, and specifically the G1–S transition associated with increased cyclin D1 expression, has been implicated in different forms of chronic neurodegeneration. However, although increased cyclin D1 expression has been reported in ALS [61], death of SOD1-depleted sympathetic neurons could not be inhibited by CDK inhibitors [62], as would be expected from effective cyclin D1-mediated CDK activation.

G1–S transition associated with increased cyclin D1 expression has also been reported in a model of PD (injection of 6-hydroxydopamine into the mouse striatum) [63]. Moreover, the PD-associated toxicity of oxidized dopamine metabolites is accompanied by upregulation of cyclin B2 expression, suggesting the occurrence of G2–M transition in this model [64]. However, in MPP+-treated PC12 cells, induction of the CDK inhibitor p21WAF1/Cip1 was reported to prevent both entry into the cell cycle and apoptosis, leading to a non-apoptotic death due to energy depletion [65].

Finally, re-entry into the cell cycle has been proposed to be an inherent part of neuronal PCD in AD. Indeed, Aβ peptides trigger a G1–S transition in neurons [66], and increased cyclin D1 expression has been shown in AD brains [67]. Moreover, aberrant expression of CDK1–cyclin B1, the hallmark of G2–M transition, has been documented in degenerating neurons of AD brains [67]. As in other neurodegenerative diseases, upregulated expression of the INK4 family of CDK inhibitors has been observed in AD [68].

Relationship between oxidative stress and cell-cycle re-initiation

Many questions remain unanswered in the exciting field of neuronal PCD. For instance, although the relevance of cell-cycle re-initiation and oxidative stress in

neurodegenerative processes are now generally recognized, it is not clear which event determines the type of cyclin involved and the precise stage of the cell cycle when neuronal death occurs. Why does cell-cycle re-initiation in acute neuronal injuries apparently not proceed beyond G1–S transition, whereas an attempt is made toward the G2–M transition under more chronic conditions? Moreover, the mechanisms underlying the selective vulnerability of certain neuronal populations to oxidative stress have not been elucidated beyond possible variations in the level of SOD expression [69].

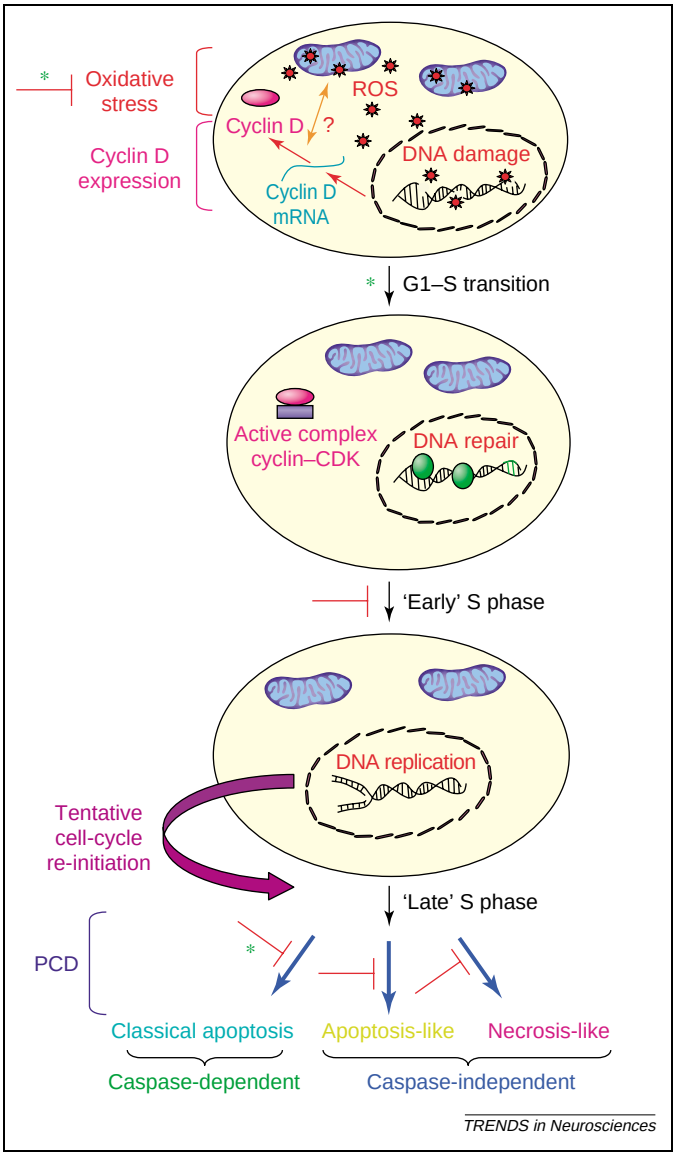

Figure 2. An emerging working hypothesis for links between oxidative stress and cell-cycle re-initiation, featuring novel intervention strategies to regulate neuronal cell death. Insults such as cell over-stimulation by excess glutamate or the accumulation of cytotoxic proteins (e.g. Aβ, synuclein, parkin or mutated SOD1) generate ROS which, if not compensated for, lead to oxidative stress. One consequence of oxidative stress is accumulation of damaged DNA, subsequently triggering AIF-mediated apoptosis-like PCD through PARP-1 activation (not shown here for clarity; see Figure 1 for details). In parallel, cyclins (mostly D-type) are induced. The G1–S transition resulting from the formation of active complexes of cyclin D with CDK4 or CDK6 might be required for DNA repair [71]. The G1–S transition could thus be part of a physiological defence response mechanism to the presence of damaged DNA rather than a first step in 'pathological' cell-cycle re-initiation leading to cell death. According to this hypothesis, blocking the transition form 'early' to 'late' S-phase would prevent further progression of the cell cycle, leaving sufficient time to repair damaged DNA. Of course, this approach should be combined with the upstream inhibition of oxidative stress to avoid further DNA damage. It could also be combined with strategies to block not only classical apoptosis but also the two other major forms of PCD, to protect neurons escaping the therapeutic inhibition of 'early' to 'late' S-phase transition. The double-ended orange arrow in the top cell indicates that the precise nature of the relationship between ROS production and cyclin D induction is not known. Blunt-ended red lines point to the stages considered for neuroprotection. Green asterisks indicate the targets of current prevention strategies.

In our view, however, the main issue that remains to be clarified concerns the precise relationship between oxidative stress and cell-cycle re-initiation. It is still unknown whether oxidative-stress-induced neuronal PCD requires re-initiation of the cell cycle (reviewed in [70]). According to an interesting alternative concept that is currently emerging, cell-cycle re-entry in neurons is a prerequisite for DNA repair, as shown *in vitro* for neurons exposed to DNA-damaging agents [71]. Accordingly, signs of DNA damage such as the accumulation of 8-hydroxydeoxyguanosine (8–0HdG) are frequently seen in postmortem human brains and in cellular and animal models of oxidative-stress-associated neurodegeneration (reviewed in [5]). Re-initiation of the cell cycle in dying neurons could be a tentative attempt to repair oxidative-stress-induced DNA damage, and might thus be considered as a physiological 'defence' mechanism in the presence of damaged DNA (Figure 2).

This hypothesis is conceptually at odds with the current view that unscheduled re-entry of neurons into the cell cycle is pathological, leading to 'abortive mitosis' and cell death. However, in accordance with the hypothesis proposed here is that DNA repair is less efficient in terminally differentiated postmitotic neurons than in actively proliferating neuronal precursors [72]. The hypothesis that re-initiation of the cell cycle in dying neurons is an attempt at repair should now be tested in different models of acute and chronic neurodegeneration. If confirmed, it could have a tremendous impact on the concept of neuroprotection because the current strategies based on CDK inhibitors are designed to prevent 'pathological' cell-cycle re-initiation. Strategies that decrease oxidative stress (by uncoupling mitochondrial oxidation from phosphorylation), control cell-cycle re-entry (by inhibition beyond the G1–S transition checkpoint, thus enabling DNA repair but inhibiting DNA replication) and/or block non-classical apoptotic forms of PCD could prove more efficient than current approaches (Figure 2). Interestingly, some caspases (caspase-2) and a cyclin D (D3) seem to have a role in DNA repair (reviewed in [73]); their presence in dying neurons is therefore not obviously linked to classical apoptosis and cell-cycle re-initiation, respectively. Hence, the inhibition of caspase-independent PCD, which requires better knowledge of the relevant underlying mechanisms, could provide targets for the development of more effective neuroprotective therapies.

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