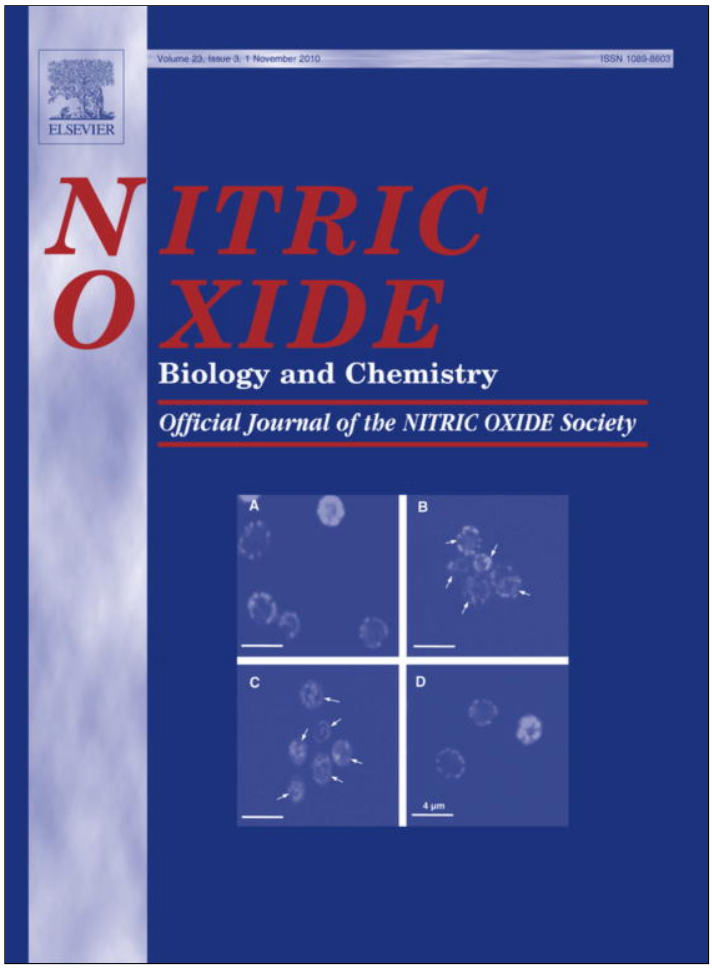

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Review

Nitric oxide and neuronal death

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ABSTRACT

NO and its derivatives can have multiple effects, which impact on neuronal death in different ways. High levels of NO induces energy depletion-induced necrosis, due to: (i) rapid inhibition of mitochondrial respiration, (ii) slow inhibition of glycolysis, (iii) induction of mitochondrial permeability transition, and/or (iv) activation of poly-ADP-ribose polymerase. Alternatively, if energy levels are maintained, NO can induce apoptosis, via oxidant activation of: p53, p38 MAPK pathway or endoplasmic reticulum stress. Low levels of NO can block cell death via cGMP-mediated: vasodilation, Akt activation or block of mitochondrial permeability transition. High NO may protect by killing pathogens, activating NF-κB or *S*-nitro(sy)lation of caspases and the NMDA receptor. GAPDH, Drp1, mitochondrial complex I, matrix metalloprotease-9, Parkin, XIAP and protein-disulphide isomerase can also be *S*-nitro(sy)lated, but the contribution of these reactions to neurodegeneration remains unclear. Neurons are sensitive to NO-induced excitotoxicity because NO rapidly induces both depolarization and glutamate release, which together activate the NMDA receptor. nNOS activation (as a result of NMDA receptor activation) may contribute to excitotoxicity, probably via peroxynitrite activation of poly-ADP-ribose polymerase and/or mitochondrial permeability transition. iNOS is induced in glia by inflammation, and may protect; however, if there is also hypoxia or the NADPH oxidase is active, it can induce neuronal death. Microglial phagocytosis may contribute actively to neuronal loss.

Contents

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Introduction

NO induces neuronal death; NO inhibits neuronal death; and NO does nothing to neuronal death. How this comes about, and why it matters, are the foci of this review. NO has been implicated in neuronal death in: brain ischemia (e.g. stroke), brain trauma (e.g. bang on head), brain infections (e.g. meningitis and AIDS dementia), brain aging and neurodegenerative diseases, such as Alzheimer's, Parkinson's, Multiple Sclerosis and motor neuron diseases. Given that NO may contribute both positively and negatively to these diseases, sometimes at the same time, it is important to disentangle these effects if rational intervention is to be attempted.

NO synthases

In the healthy brain, NO is produced mainly by nNOS found in a small subset of neurons, and eNOS in endothelium. It has also been reported that nNOS can be found at low levels in astrocytes, and that eNOS can be found in a subset of neurons and astrocytes. Within neurons, nNOS is found mainly in the cytoplasm, but is also concentrated in the post-synaptic membrane via its PDZ domain, which results in it being closely associated with the NMDA-type of glutamate receptor [1]. Activation of the NMDA receptor by glutamate in the synapse causes an influx of calcium, which transiently activates nNOS (via calcium–calmodulin binding) to produce relatively low levels of NO [2]. In the diseased brain iNOS is induced mainly in microglia and astrocytes by pathogens, damage or hypoxia, and once expressed produces moderate levels of NO chronically, without any requirement for further activation [3,4]. Some researchers refer to 'mitochondrial NOS' (mtNOS), a NOS activity found in mitochondria, but this remains controversial, and may be nNOS bound to/within mitochondria [5]. NO can also be produced by reduction of nitrite by (i) bacteria in gut or skin, (ii) acidification in stomach or ischemia, or (iii) myoglobin in relative hypoxia [6].

NO levels

The levels of NO that occur in vivo are still not entirely clear, but what evidence there is suggests that NO levels from nNOS and eNOS are normally 'low', in the range 0.2–2.0 nM, which is the range over which soluble guanylate cyclase is activated [2]. iNOS expression or gut bacteria may generate 'moderate' levels of NO, in the range 2–20 nM NO, and occasionally 'high' levels of NO, in the range 20–200 nM [7–9]. Anything above 200 nM NO we shall refer to here as 'pharmacological' levels of NO.

One difficulty with the literature on NO and neuronal death (which I am as guilty of as much as anyone else) is the use of pharmacological levels of NO to induce neuronal death. This has several problems: (i) it is difficult to know whether findings with such high levels of NO have any relevance to the situation in vivo with lower levels of NO, (ii) mitochondrial respiration will be strongly inhibited in such conditions which will have multiple cellular effects, and (iii) in the presence of oxygen, superoxide and/or thiols, such high levels of NO are mostly diverted to reactive nitrogen species (RNS, see below), and therefore any effects are likely to be due to RNS rather than NO, and will be very condition dependent. Having said that, it may be that mechanisms found with pharmacological levels of NO in vitro may give useful information relevant to lower levels in vivo. One way to test relevance of effects is to test whether they also occur when nNOS, eNOS or iNOS are expressed in cells in culture. However, it should be noted that NOS in cultured cells may well generate higher levels of NO than in vivo, because important NO degrading processes (such as haemoglobin and myoglobin) are missing.

A related problem in the literature is the use of NO donors. It used to be accepted practice in NO research to always test whether the effects of an NO donor are due to NO or the donor molecule (by for example testing whether an NO scavenger blocks the effect), but this important practice is becoming less common. Many NO donors (such as *S*-nitrosothiols) have biological effects independent of NO, which may or may not be biologically interesting in themselves. However, without testing whether the effect of an NO donor are due to NO or some other molecule, it is very difficult to come to useful conclusions. For example, we have sometimes used the NO donor DETA-NONOate on cells assuming any effects are due to NO. However, we recently found that high levels of DETA-NONOate could directly depolarise neurons, independent of NO [10]. Thus it is important to test whether the effect of an NO donor is blocked by an NO scavenger (such as haemoglobin or PTIO) and is replicated by authentic NO (at a reasonable concentration).

NO chemistry

In order to trace the mechanisms by which NO affects cell death it is useful to distinguish between the direct and indirect effects of NO. The direct affects of NO include: (i) activation of soluble guanylate cyclase to produce cGMP, (ii) binding to haemoglobin, myoglobin and cytoglobin resulting in NO breakdown, (iii) inhibition of cytochrome oxidase and hence mitochondrial oxygen consumption, and (iv) inhibition of catalase and hence H_2O_2 breakdown. All of these rapid and direct actions result from NO binding to the haem of these proteins. But the first two of these actions occur at the low physiological levels of NO produced by eNOS or nNOS, while the latter two actions require high levels of NO [11]: possibly the levels of NO produced by iNOS. Other direct actions of NO are: (v) reaction with superoxide to produce peroxynitrite ($ONOO^-$), and (vi) reaction with oxygen to produce NO_2 and N_2O_3 [12]. The reaction with superoxide occurs at the diffusion limited rate, so can occur at any level of NO (physiological, pathophysiological, or pharmacological), but is usually limited by the low rate of superoxide production in cells [13]. Peroxynitrite can spontaneously isomerise to nitrate with a halftime of seconds, but is also

a strong oxidant, causing oxidation of thiols e.g. glutathione or protein thiols. It can also nitrate (add a NO_2^+ group to) protein tyrosine residues resulting in nitrotyrosine residues. However, if CO_2 is present, most peroxynitrite will react with CO_2 to produce the two radicals NO_2 and $CO_3^{\cdot -}$, which are both strong oxidants [13]. The reaction of NO with oxygen has a second order dependence on NO concentration, and so only tends to occur rapidly at high NO concentrations (and preferentially within membranes where NO and O_2 concentrations are higher) [14]. The reaction produces NO_2, which may react with another NO to produce N_2O_3. N_2O_3 can nitrosate (i.e. add a NO^+ group to) thiols such as glutathione or protein cysteine residues to produce *S*-nitrosothiols. Although quantitatively most *S*-nitrothiols may result from NO reacting with the thiyl radical ($RS^{\cdot}$), which is generated by oxidation of reduced thiols (RSH) by NO_2, $CO_3^{\cdot -}$ or other oxidants [12]. Because *S*-nitrosothiols may arise from addition to thiols of an NO group (nitrosylation) or addition of an NO^+ group (nitrosation) this reaction is sometimes referred to as *S*-nitro(sy)lation. Excessive *S*-nitro(sy)lation is often referred to as nitrosative stress. $ONOO^-$, NO_2 and N_2O_3 are collectively known as 'reactive nitrogen species' (RNS)[1]. Excessive nitration (due to $ONOO^-$ or NO_2) is sometimes referred to as nitrative stress. However, high levels of NO generally lead to a mixture of nitrosative, nitrative and oxidative stress, which has been referred to as nitroxidative stress [12].

The actions of NO are in general dependent on: (a) the concentration of NO, (b) the time course of exposure to NO, (c) the presence/absence of particular ROS at relevant levels, and (d) the presence/absence of particular pathways in particular cells. In general this tends to mean that NO is cytoprotective at low levels and toxic at high levels [15,16].

NO and oxygen metabolism

NO inhibits mitochondrial respiration by different means: (A) NO itself causes rapid, selective, potent, but reversible inhibition of cytochrome oxidase [17], and (B) RNS cause slow, non-selective, weak, but irreversible inhibition of many mitochondrial components, including complex I [18,19]. These and other effects result in NO have multiple interactions with oxygen and reactive oxygen species (ROS) metabolism [20]. NO inhibition of mitochondrial oxygen consumption, together with NO-induced vasodilation, may increase tissue oxygen levels. And oxygen is a limiting substrate for the production of NO (by nNOS and iNOS), of ROS (by the mitochondrial respiratory chain and NADPH oxidase) and of RNS (NO_2 and N_2O_3 by reaction of NO and O_2). At limiting arginine or tetrahydrobiopterin levels, NOS can also produce superoxide [21]. NO/RNS inhibition of the mitochondrial respiratory chain stimulates the leakage of superoxide and hydrogen peroxide production from mitochondria [22]. And this superoxide may react with NO to produce peroxynitrite, which further inhibits the respiratory chain, potentially causing a vicious circle [13]. Note however that there are also negative feedbacks on this system as superoxide and peroxynitrite potently inactivate aconitase [23] and other enzymes responsible for feeding electrons into the mitochondrial respiratory chain, hence limiting ROS production. NO stimulates H_2O_2 production from the mitochondrial respiratory chain, and NO directly inhibits catalase [24], while peroxynitrite oxidizes glutathione and inactivates glutathione reductase [25]. Thus NO can raise H_2O_2 levels in cells. H_2O_2 and NO can react on superoxide dismutase to produce peroxynitrite [26]. Thus high NO levels in tissue can, in the presence of oxygen, give rise to a potent mixture of ROS and RNS, sometimes collectively referred to as RONS (reactive oxygen and nitrogen species). And perhaps the most significant result of this is oxidation of protein cysteine residues, although protein nitration and nitrosation, lipid oxidation and DNA mutation may be important in particular cases. However, NO can also act as an anti-oxidant (e.g. by reacting with superoxide ($O_2^{\cdot -}$), thiyl ($RS^{\cdot}$) and lipid peroxyl ($ROO^{\cdot}$) radicals), and has been reported to also protect cells from oxidant-induced death [16].

NO and RONS-induced necrosis and apoptosis

In non-neuronal cells, high levels of NO induce cell death by two different means: (i) energy depletion mediated necrosis, or (ii) oxidative/nitrosative stress mediated apoptosis [27]. We will briefly discuss these mechanisms, and point out where they are relevant to neurons, although additional mechanisms are involved NO-induced death of neurons. Necrosis involves rupture of the plasma membrane, due to cell swelling, phospholipases or proteases, often as a consequence of energy depletion and/or calcium overload. Apoptosis involves chromatin condensation and fragmentation, phosphatidylserine exposure, and cellular disassembly and fragmentation without plasma membrane rupture. Apoptosis generally results from caspase activation, induced by either death receptor activation or cytochrome *c* release from mitochondria permeabilized by Bcl-2 homologous proteins.

NO-induced necrosis

NO can induce cellular energy depletion by multiple mechanisms: (i) NO inhibits mitochondrial cytochrome oxidase and thus ATP production, (ii) NO, *S*-nitrosothiols and peroxynitrite inhibit multiple other components of the mitochondrial respiratory chain, (iii) peroxynitrite and *S*-nitrosothiols activate the mitochondrial permeability transition, which results in hydrolysis of all cellular ATP, (iv) peroxynitrite and *S*-nitrosothiols inhibit GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and hence glycolytic ATP production, and (v) RONS cause DNA strand breaks which activate PARP, which consumes cytosolic NAD^+. All of these processes deplete the cell of ATP (Fig. 1), which in general causes necrosis via failure of the ATP-driven calcium pump and sodium pumps, which lead to flooding of the cell with calcium (activating proteases, phospholipases and, as discussed below, the mitochondrial permeability transition) and with sodium, resulting in osmotic rupture of the plasma membrane. ATP depletion also prevents apoptosis, because caspase activation requires ATP, so although apoptosis sometimes causes secondary necrosis, necrosis prevents apoptosis. Since high NO inactivates mitochondrial ATP production, whether neurons or astrocytes die by necrosis in these conditions depends on whether glycolytic ATP production can continue [28], and this in turn depends on whether cellular glutathione levels can be maintained [29,30]. But if glycolysis continues in the presence of high NO, then apoptosis is usually induced [31].

Mitochondrial permeability transition (MPT)

Mitochondria can undergo something called 'permeability transition', which is induced by high calcium and/or RONS, and

[1] *Abbreviations used:* AIF, apoptosis inducing factor; ALS, amyotrophic lateral sclerosis; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; Drp1, dynamin-related protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; cGMP, guanosine 3′,5′-cyclic monophosphate; GSH, glutathione; GSNO, *S*-nitrosoglutathione; L-NAME, N^G-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MAP kinase, mitogen-activated protein kinase; MPT, mitochondrial permeability transition; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NMDA, *N*-methyl-*D*-aspartate; NO, nitric oxide; NOS, nitric oxide synthase; eNOS, endothelial NOS; iNOS, inducible NOS; nNOS, neuronal NOS; PARP, poly-ADP-ribose polymerase; PDI, protein-disulphide isomerise; PHOX, phagocytic NADPH oxidase; PKG, protein kinase G; PMA, phorbol 12-myristate 13-acetate; RNS, reactive nitrogen species; RONS, reactive oxygen and nitrogen species; ROS, reactive oxygen species; SNOC, *S*-nitrosocysteine; SOD, superoxide dismutase.

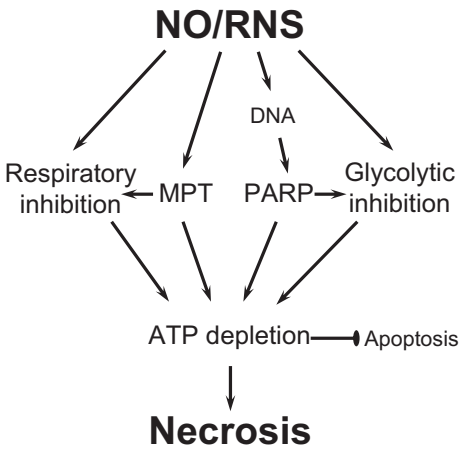

Fig. 1. Mechanisms by which NO/RNS cause energy-depletion-induced necrosis. NO and RNS (reactive nitrogen species) directly inhibit mitochondrial respiration. Activation of MPT (mitochondrial permeability transition) by RNS can inhibit respiration due to loss of mitochondrial cytochrome *c* and NADH, and can more directly cause ATP depletion by allowing protons and ATP free access across the inner membrane, resulting in reversal of the ATP synthase. RNS damage to DNA can cause PARP (poly ADP-ribose polymerase) activation, leading to depletion of its substrate NAD^+, which may deplete ATP either because ATP is required to resynthesis NAD^+ or because NAD^+ is required for glycolysis. RNS directly inhibits (or uncouples) glycolysis at glyceraldehydes-3-phosphate dehydrogenase when glutathione is depleted. ATP depletion inhibits apoptosis, and causes necrosis, usually via failure of the sodium and/or calcium pumps.

involves a dramatic increase in the permeability of the inner mitochondrial membrane to all molecules <1500 Da [32]. Sustained opening of the permeability transition pore (PTP) generally leads to necrotic cell death, as in this state the mitochondria can rapidly hydrolyse mitochondrial and cytosolic ATP. However, transient permeability transition can lead to apoptosis, as the mitochondria swell, rupturing the outer membrane, leading to release of cytochrome *c*, which triggers caspase activation, if ATP is maintained. Peroxynitrite, *S*-nitrosothiols and ROS can trigger mitochondrial permeability transition, and cytochrome *c* release, and can induce cell death by this means [33–35]. And excitotoxic death of neurons may be by similar means: glutamate activation of NMDA receptors, activating nNOS to produce peroxynitrite, which activates mitochondrial permeability transition (see below).

Poly ADP-ribose polymerase (PARP)

PARP is a DNA-repair enzyme, which can also mediate cell death induced by NO. Of the multiple forms of the enzyme, PARP1 is predominant and localized exclusively to the nucleus where it is activated by DNA strand breaks [36]. PARP1 attaches long stretches of ADP-ribose to nuclear targets such as histones and PARP1 itself, utilizing NAD^+ as the substrate, which triggers chromatin-structure relaxation and increases the access of DNA-repair enzymes to the DNA strand break. However, overactivation of PARP by excess DNA damage depletes NAD^+ (PARP's substrate), and thereby depletes ATP, because ATP is used to remake NAD^+, and because NAD^+ depletion inhibits glycolysis. ATP depletion in turn causes necrotic cell death. Neurotoxicity of cultured neurons is markedly diminished by PARP inhibitors [37], and stroke damage following middle cerebral artery occlusion is reduced up to 80% in $PARP1^{-/-}$ mice [38]. In rodents, PARP inhibitors substantially reduce stroke damage [39]. Peroxynitrite causes damage to DNA, which results in PARP activation [36]. PARP activation by brain ischemia is decreased in nNOS-knockout mice, suggesting that ischemia activates nNOS to produce peroxynitrite, which activates PARP, causing neuronal death via energy depletion [40].

How PARP1 activation causes neuronal death remains somewhat mysterious, but appears to involve NAD^+ depletion, followed by mitochondrial permeabilization (possibly by activation of the permeability transition pore), followed by AIF (apoptosis inducing factor) release from mitochondria, going to the nucleus where AIF causes DNA fragmentation. AIF appears to contribute to neuronal death induced by ischemia [41], consistent with AIF being downstream of PARP activation. PARP1 activation within glia may also mediate inflammation via aiding NF-κB function, so that PARP1 inhibition may protect partly by blocking microglial inflammation [42,43].

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

S-nitros(yl)ation or oxidation of GAPDH has been linked to neuronal death either via inhibition of glycolysis in the cytosol or activation of ubiquitination and acetylation in the nucleus [44–46]). Peroxynitrite and other RONS oxidizes the active-site cysteine residue of GAPDH, inhibiting its dehydrogenase activity and inducing an acyl phosphatase activity in the enzyme [47]. This results in uncoupling of glycolytic flux from ATP synthesis by substrate level phosphorylation. NO-producing macrophages or cells exposed to NO donors exhibit reduced GAPDH activity, increased glycolysis and decreased ATP content and turnover, potentially leading to ATP-depletion-induced necrosis.

NO from iNOS or nNOS may lead to *S*-nitros(yl)ation of GAPDH at its catalytic cysteine (C152 for human GAPDH), abolishing catalytic activity and thus inhibiting glycolysis, but conferring upon GAPDH the ability to bind to Siah1, an E3-ubiquitin-ligase. Siah1, which possesses a nuclear localization signal, escorts GAPDH to the nucleus. Within the nucleus, GAPDH stabilizes the rapidly turning over Siah1, enabling its E3-ubiquitin-ligase activity to degrade diverse nuclear proteins that lead to cell death. Nuclear GAPDH is acetylated at Lys 160 by the acetyltransferase p300/CREB binding protein (CBP), which in turn stimulates the acetylation and catalytic activity of p300/CBP. Consequently, downstream targets of p300/CBP, such as p53 are activated and cause cell death [48]. Deprenyl is a drug that potently antagonizes *S*-nitros(yl)ation of GAPDH, prevents NO-induced cell death and is neuroprotective [49]. The neurotoxicity seen in Huntington's disease may involve the NO-GAPDH-Siah1 cascade [50–52]. The neurotoxicity of mutant Huntingtin, which accounts for cytotoxicity, requires nuclear translocation of its N-terminal fragment, which lacks a nuclear localization signal [53]. Mutant Huntingtin occurs in a ternary complex with GAPDH and Siah1, which mediates its nuclear translocation and cytotoxicity [52].

NO-induced apoptosis

As noted above high levels of NO induce RONS, which cause oxidative/nitrosative stress to cells. This stress can in turn induce apoptosis by diverse mechanisms, including: (i) induction of p53, (ii) ER stress, (iii) activation of mitochondrial permeability transition to cause cytochrome *c* release (see section above), and (iv) activation of the p38 or other MAP kinase pathways (Fig. 2).

p53

Pharmacological levels of NO (>200 nM) can induce p53 [15], via RONS-induced DNA damage or GAPDH/Siah1, and p53 may then induce apoptosis via PUMA expression or possible direct action on mitochondria to cause cytochrome *c* release. However, nitrosation of p53 may prevent its translocation to mitochondria [54]. Pharmacological levels of NO cause death of astrocytes via p53 phosphorylation and Bax translocation to mitochondria [55],

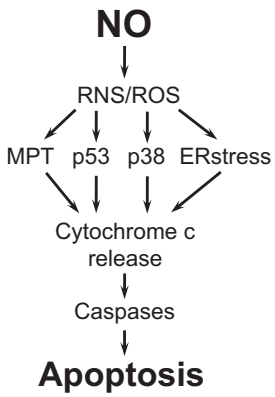

Fig. 2. Mechanisms by which NO/RNS cause oxidative apoptosis. NO can induce oxidative and/or nitrosative stress, which activates the mitochondrial pathway of apoptosis by several different pathways, including: (i) stimulation of MPT, (ii) upregulation of p53, (iii) activation of the p38 MAP kinase pathway, and (iv) induction of endoplasmic reticulum stress.

but the caspases become inactivated probably by the RONS (see below).

ER stress

Endoplasmic reticulum (ER) stress is a cellular stress condition, involving endoplasmic reticulum dysfunction, induced by unfolded proteins, viral infection or calcium overload. ER stress induces expression of proteins such as chaperones, and may induce apoptosis. ER stress has been implicated in neurodegeneration. High NO levels, peroxynitrite or iNOS expression can induce expression of CHOP/GADD153, a C/EBP family transcription factor mediating ER stress-induced apoptosis in a range of cell types including microglia [56]. In cerebellar granule neurons, there is limited evidence that high levels (1 mM) of *S*-nitrosoglutathione (GSNO) induce ER stress (measured by ER calcium release and CHOP expression) and cell death [57]. But there was no evidence in this paper that the effect of GSNO was mediated by NO, or that the induced death was due to ER stress. It is possible that GSNO induced ER stress in these neurons by *S*-nitros(yl)ation and inhibition of protein-disulphide isomerase (PDI), as *S*-nitrosocysteine (SNOC) has been shown to *S*-nitros(yl)ate purified PDI and thence inhibits it [58]. PDI is mainly located in the ER, where it catalyses protein thiol-disulphide exchange, which helps proteins fold. In primary cultures of cortical neurons it was found that excitotoxicity induced by NMDA (see below) caused *S*-nitros(yl)ation of PDI, accumulation of polyubiquitinated proteins, CHOP expression and apoptosis, all of which were prevented by inhibition of nNOS, and the last three effects were prevented by overexpression of PDI [58]. The authors concluded that NMDA activated nNOS via the NMDA receptor, resulting in *S*-nitros(yl)ation and hence inhibition of PDI, which resulted in accumulation of misfolded proteins, that induced neuronal dysfunction and death. However, there was no quantification of the % of PDI *S*-nitros(yl)ated or direct evidence that *S*-nitros(yl)ation was responsible for cell death, although they did show that overexpressing wild-type (but not dominant negative) PDI prevented cell death.

p38

Peroxynitrite or high NO can induce apoptosis via activation of the p38 MAP kinase pathway [59] And NO-induced activation of p38 MAPK appears to mediate neuronal loss in some models of ischaemic brain damage [60]. However, in some cases activation of p38 may be downstream of peroxynitrite-induced intracellular zinc release causing mitochondrial permeability transition in neurons [61].

NO inhibition of apoptosis

Although NO can induce apoptosis via RONS, NO can also inhibit apoptosis induced by other agents in neurons [62]. Several mechanisms have been suggested (Fig. 3), including: (a) cGMP/PKG-mediated inhibition mitochondrial permeability transition [63]), (b) cGMP/PKG-mediated activation of the PI-3K/Akt survival pathway, (d) *S*-nitros(yl)ation of caspases, (f) activation of MAP kinase pathways, (g) activation of transcription factors NF-kB and/or AP-1 [64], (h) increased expression of heat shock proteins and Bcl-2 [65], and (i) mitochondrial hyperpolarization [66].

Low levels of NO have been shown to partially block MPT via a cGMP/PKG pathway, in heart and liver, and ischemic preconditioning of heart is thought to be mediated by this pathway [67]. In astrocytes, this pathway mediates an inhibition of apoptosis by low levels of NO [63,68]. If this protective pathway was operational in neurons, it might protect against excitotoxicity, but there is no evidence for or against NO blocking MPT in neurons.

The active-site cysteine residue of caspases can be inactivated by *S*-nitros(yl)ation [69] or oxidation e.g. by H_2O_2 [70]. Thus if NO exposure is chronic e.g. from iNOS, then the caspases may be activated then inactivated by RONS. However, inhibition of caspases by NO does not generally prevent neuronal death [71], because cells usually commit to cell death before caspase activation. And it is unclear that *S*-nitros(yl)ation of caspases could ever be quantitatively sufficient to significantly inhibit cellular caspase activity, given that the percentage of a given protein species *S*-nitros(yl)ated in a cell is generally low, and pro-caspases have buried active-site cysteine residues that are unlikely to be susceptible to *S*-nitros(yl)ation.

Cerebellar granule neurons expressing nNOS in culture appear to be dependent on this NO for survival via cGMP-dependent activation of: (i) the Akt survival pathway [72], and/or (ii) CREB (cGMP-dependent cAMP-responsive element binding protein) and Bcl-2 expression [73]. NO can also prevent 6-hydroxydopamine-induced apoptosis in PC12 cells through a cGMP-dependent activation of PI3 kinase and thence Akt [74].

In vivo NO can also protective neurons against local ischemia in the brain via the vasodilatory action of NO, and this can be induced by either eNOS or iNOS [75].

How can NO both induce apoptosis and inhibit apoptosis?

Part of the answer is simply that these actions of NO are mediated by different pathways, and are activated by different concentrations of NO. Wink et al. [76] distinguished five distinct

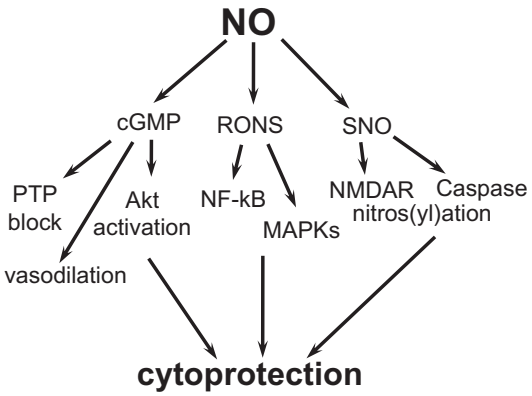

Fig. 3. Mechanisms by which NO/RNS cause cytoprotection. Low levels of NO can induce cytoprotection via cGMP-mediated: vasodilation, Akt activation and MPT (mitochondrial permeability transition) inhibition. High levels of NO may protect by RONS-mediated activation of the NF-κB and MAP kinase pathways, or via *S*-nitros(yl)ation of caspases or the NMDA receptor (NMDAR).

concentration levels of NO activity: cGMP-mediated processes ([NO] < 1–30 nM), Akt phosphorylation ([NO] = 30–100 nM), stabilization of HIF-1α ([NO] = 100–300 nM), phosphorylation of p53 ([NO] > 400 nM), and nitrosative stress (1 μM). This distinction is certainly simplistic, but illustrates that the anti-apoptotic actions of NO are at lower concentrations than the pro-apoptotic actions. That high concentrations of NO can induce apoptosis, when low concentrations inhibit, must mean either that the particular anti-apoptotic pathways activated by NO do not block the particular pro-apoptotic pathways activated by NO, or that they are not sufficiently strong to fully block the pro-apoptotic pathways. The latter is presumably the case in SH-SY5Y neuronal cells where NO activates Akt signalling, which partially but not fully protects against the NO-induced apoptosis [77,78]. The anti-apoptotic action of NO may also have a limited timescale in neurons [79].

RONS-induced phagocytosis

How apoptosis causes cell death is, surprisingly, not always clear, but in vivo the main means is by triggering exposure of 'eat-me' signals (such as phosphatidylserine) on the cell surface, which induce phagocytes to eat the cell. RONS can induce PS exposure directly via activation of the phospholipid scramblase and inactivation of aminophospholipid translocase (flippase) [80] or indirectly via induction of apoptosis [2,77,78], see Fig. 4. However, it is important to realise that phosphatidylserine exposure can be reversible [81] including NO-induced phosphatidylserine exposure of neurons [82]. And RONS-induced PS exposure of neurons may induce inflammatory activation of microglia [2,77,78], which may then phagocytose the neurons. Thus we find that at low levels RONS-induced neuronal loss can be prevented simply by blocking phagocytosis (unpublished).

NO, excitotoxicity and ischemia

NO-induced excitotoxicity

Neurons in culture are sensitive to cell death induced by NO, NO donors or NO-producing cells. A major cause of this sensitivity is that NO causes mitochondrial inhibition, glutamate release and subsequent excitotoxic death of neurons [83], see Fig. 5. Thus NO-induced death of cultured neurons is strongly blocked by inhibitors of the NMDA receptor [84,85]. High concentrations of extracellular glutamate (the main excitatory neurotransmitter in the brain) can kill neurons via activation of glutamate receptors (either the NMDA receptor or the AMPA receptor), and such neuronal death is known as excitotoxic death [86]. Activation of the NMDA receptor to form a cation channel (allowing both Na^+ and Ca^{2+} into the neuron) requires both an excitatory neurotransmitters, such as glutamate or aspartate (or agonist NMDA, *N*-methyl-D-aspartate), and depolarization of the plasma membrane. Thus respiratory inhibitors strongly potentiate glutamate-induced death by depolarising the plasma membrane, greatly sensitising neurons to extracellular glutamate [87]. Because NO acutely inhibits mitochondrial respiration and causes glutamate release, it induces excitotoxicity.

NO-induced glutamate release

High levels of NO causes acute (within seconds) glutamate release from neurons, [83,88,89], which has been attributed either to inhibition of mitochondrial respiration followed by reversal of glutamate uptake [83,88,90], or to a direct (calcium-independent) activation of vesicular exocytosis by modifying protein thiols [91,92]. The latter effect requires high levels of *S*-nitro(sy)lating NO donors, and is blocked by Botulinum neurotoxins that inactivate the exocytosis machinery, but it is unclear whether NO itself at reasonable levels could cause exocytosis by this means. Peroxynitrite can also activate vesicular exocytosis, apparently by nitration of exocytotic proteins [93], but again it unclear whether this could occur at reasonable levels of peroxynitrite. High NO induces acute glutamate release also from astrocytes, but in this case by causing intracellular calcium release followed by calcium activation of vesicular exocytosis [94], see Fig. 7.

NO-induced excitotoxicity mediated by caspases and calpains

NO- or peroxynitrite-induced excitotoxicity of cultured neurons is not blocked by PARP1 knockout, but is blocked by caspase inhibitors or calpain inhibitors [84,85,95,96].

Calpain inhibitors can block glutamate, NMDA or NO-induced excitotoxicity, but the mechanism by which calpain mediates neuronal death is not entirely clear. Calpains are a family of cysteine proteases of which the best characterized are: (i) calpain I (calpain

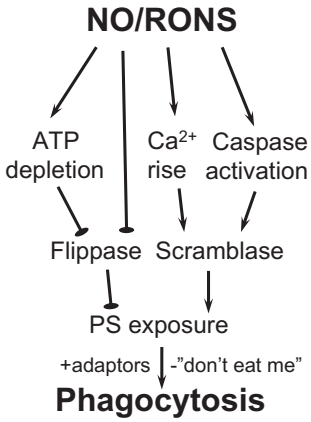

Fig. 4. Mechanisms by which NO/RNS cause phagocytosis. NO/RONS can activate phagocytosis by causing phosphatidylserine (PS) exposure on the outside of a cell, via either inhibiting a PS flippase (aminophospholipid translocase, catalyses ATP dependent PS transport in) or stimulating a scramblase (randomises phospholipids in the membrane). Inhibition of the flippase is either direct (by thiol modification) or via ATP depletion. Stimulation of the scamblase is either via elevating cytosolic calcium or via activating apoptosis.

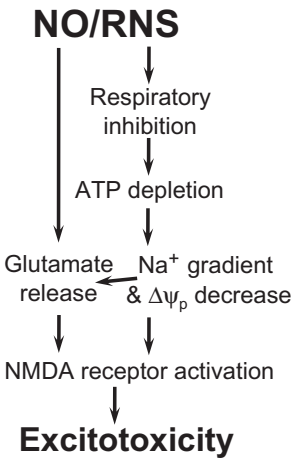

Fig. 5. Mechanisms by which NO/RNS cause excitotoxicity. NO/RNS-induced respiratory inhibition causes neuronal ATP depletion, decreasing the plasma membrane sodium gradient and membrane potential, resulting in glutamate release, which together with the decrease in membrane potential ($\Delta\psi_P$) activates the NMDA (*N*-methyl-D-aspartate) receptor, causing excitotoxicity. NO/RNS may also cause glutamate release by stimulating exocytosis of glutamate-containing vesicles in neurons and astrocytes.

μ) activated by micromolar calcium, and (ii) calpain II (calpain m) activated by millimolar calcium. Calpain I is located primarily in the neuronal cytoplasm and is activated by excessive calcium entering the cell via the NMDA receptor (or other routes). Chronic activation of this protease causes it to degrade the cytoskeleton, neurofilaments and ion channels, leading to necrotic cell death. However, more recently it has been shown that excitotoxicity is mediated by calpain I truncation of mitochondrial AIF (apoptosis inducing factor), leading to AIF translocation from mitochondria to nucleus, where AIF causes DNA fragmentation [97,98]. Calpain I can also truncate Bid (to tBid), which can then directly cause AIF and cytochrome *c* release from mitochondria [99]. AIF is emerging as a key mediator of excitotoxic neuronal death in ischemia and traumatic brain injury, see Fig. 6.

nNOS-mediated excitotoxicity

nNOS in neurons is partly bound close to the NMDA receptor, and is activated by calcium entering via the receptor-gated ion channel. There is evidence that this nNOS activation contributes to excitotoxicity, i.e. glutamate killing of neurons. In culture, NMDA-induced neurotoxicity is decreased by NOS inhibitors [100,58] and is decreased in neurons isolated from $nNOS^{-/-}$ mice [101]. In vivo damage following middle cerebral artery occlusion (a model of brain ischemia) is diminished by treatment with NOS inhibitors [102] and in $nNOS^{-/-}$ mice [103].

However, if glutamate kills neurons via NO from nNOS (this section), and NO kills neurons via glutamate release and activation of NMDA receptors (previous section), we have a bit of a paradox. This paradox may be resolved if: glutamate kills neurons also independently of NO, and/or NO also kills neurons independently of glutamate. Now both of these get-out clauses clearly apply in some conditions, i.e. many researchers find NOS inhibitors only partially protect neurons from glutamate or NMDA (or do not protect at all), and NO can kill neurons independently of the glutamate and NMDA receptor (particularly when the NO level is high or sustained).

Garthwaite and colleagues [104] have suggested that during excitotoxicity NO can become artifactually toxic in cell culture conditions. They found that components of cell culture media (such as riboflavin) cause rapid breakdown of NO into peroxynitrite [105], and these reactions may contribute artifactually to excitotoxicity in culture.

However, it is clear from in vivo experiments that nNOS inhibitors or inactivation of the nNOS gene can partially prevent ischemia-induced brain damage in rats and mice [103,104,106]. One possible mechanism for this role of nNOS in ischaemic damage, is the NO inhibition of cytochrome oxidase in competition with oxygen. Ischemia kills neurons primarily by reducing the oxygen supply to cytochrome oxidase. But NO can dramatically increase the apparent K_M of cytochrome oxidase for oxygen, because it directly competes with oxygen for binding to cytochrome oxidase. Thus the K_M for cytochrome oxidase and neuronal respiration for oxygen is about 0.5 μM in the absence of NO, but in the presence of 60 nM NO the K_M increases to 30 μM O_2 [107], which is the median level of oxygen in the healthy brain. We have shown that NO from nNOS in cultured neurons can increase the K_M for oxygen in this way, and thereby sensitise neurons to hypoxia-induced death, which is thus partially prevented by nNOS inhibitors [108]. Whether this occurs in vivo is unknown.

Almeida and Bolanos [109] found that nNOS activation in cultured neurons caused a transient inhibition of neuronal mitochondria leading to apoptosis, even in normoxia. However, Keynes et al. [105] found that nNOS activation in slice cultures only produced sufficient NO to activate cGMP production, but not enough to inhibit mitochondrial respiration or induce neuronal death. And this led them to conclude that NO, at least low levels from nNOS, was largely neuroprotective in ischemic brain pathology [110].

NO or nNOS may also induce neuronal death via peroxynitrite. NO may react with superoxide produced elsewhere in the cell (e.g. mitochondria or the NADPH oxidase), or superoxide may be produced by nNOS itself particularly in conditions of low arginine or low BH4 or high oxygen. For example, in the absence of

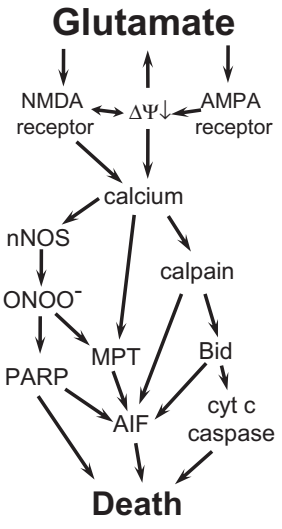

Fig. 6. Mechanisms of glutamate-induced neuronal death. Extracellular glutamate activates neuronal NMDA receptors (NMDAR) and AMPA receptors (AMPAR), which decrease plasma membrane potential ($\Delta\psi_p$), which increases cytosolic calcium and activates NMDAR, which also increases calcium. Calcium elevation may: (i) stimulate nNOS production of NO and peroxynitrite, which activates mitochondrial permeability transition (MPT) and activates poly ADP-ribose polymerase (PARP), which induces death by energy depletion and induces AIF release from mitochondria; (ii) activates MPT, which induces death via energy depletion or AIF release, which induces DNA strand breaks; and (iii) activates calpain, which induces AIF release and Bid cleavage, which may itself induce AIF release and cytochrome *c* release (also caused by calcium activation of calcineurin to dephosphorylate Bad, resulting in Bax activation), which activates caspases.

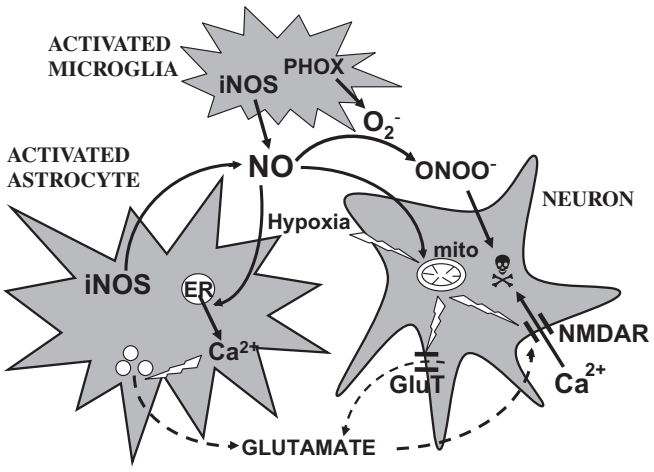

Fig. 7. Mechanisms by which activated glia kill neurons. Activated glia express iNOS and produce NO that (a) causes calcium mobilization from the endoplasmic reticulum (ER), which activates release of vesicular glutamate from astrocytes, and (b) inhibits mitochondrial (mito) respiration in neurons synergistically with hypoxia. This mitochondrial inhibition depolarises the neuron, causing both release of glutamate via the glutamate transporter (GluT) and sensitization of the NMDA receptor (NMDA), resulting in neuronal death. Activated microglia may also release: (i) glutamate that contributes to the activation of the NMDA receptor, and (b) superoxide from NADPH oxidase, which reacts with superoxide to produce peroxynitrite, which induces neuronal death.

L-arginine, NMDA caused superoxide production and cell death in cultured neurons, which was prevented by L-arginine or by nNOS inhibitors, and the death was also blocked by peroxynitrite or superoxide scavengers [111]. They concluded that at low arginine levels, nNOS produces superoxide and thence peroxynitrite, which kills the neurons by activating mitochondrial permeability transition. They further showed that glia can produce extracellular L-arginine sufficient to protect neurons from NMDA-induced death in culture.

Peroxynitrite produced as a result of nNOS activation may also inactivate the mitochondrial superoxide dismutase (Mn-SOD) via nitration, and this has been shown to occur in traumatic and ischaemic brain injury [112]. Inactivation of Mn-SOD will promote further superoxide and peroxynitrite production, but is unclear whether this inactivation contributes to the brain damage.

The three isoforms of NOS play different roles in ischaemic brain damage: eNOS protects (probably by maintaining vasodilation and promoting angiogenesis), nNOS mediates neuronal damage (probably by leading to peroxynitrite production), and iNOS can play protective or damaging roles or both [110]. Similarly in an animal model of excitotoxicity, nNOS mediated the neuronal loss, eNOS mediated the blood–brain barrier breakdown, and iNOS had no detectable role [113].

Mitochondrial fragmentation/fission induced by NO

Mitochondria in cells undergo slow continuous fission and fusion (with other mitochondria). If fusion predominates, then mitochondria form long, branched threads, and eventually a continuous network/reticulum in cells. If fission predominates, then the number of small, discrete, roundish mitochondria greatly increases in cells. Mitochondrial fission in mammals is mediated in part by dynamin-related protein 1 (Drp1), which is normally located in cytoplasm, but translocates to mitochondria and oligomerizes on the outer membrane to mediate fission. During apoptosis, mitochondria often fission/fragment mediated by Drp1, and this also appears to occur in neurons during brain ischemia [114]. Bossy-Wetzel and colleagues showed that NMDA can induce rapid mitochondrial fission in cortical neurons, and this can be partially blocked by NOS inhibitors [114]. *S*-nitrosocysteine (SNOC, 100 μM, generating 3 μM NO) caused rapid (3 h) mitochondrial fission in cortical neurons, which was partially blocked by expression of a dominant negative version of Drp1, or by overexpression of Mfn1 (which mediated mitochondrial fusion). And these treatments also blocked SNOC-induced neuronal death, indicating that SNOC-induced neuronal death via activating Drp1-mediated mitochondrial fission. Note, however, that NMDA-induced neuronal death was not blocked by expression of Mfn1 or mutant Drp1. And the unphysiologically high levels of SNOC and NO used are likely to have caused unphysiological *S*-nitros(yl)ation [115], inhibition of mitochondrial respiration and induced permeability transition. Mitochondrial complex I inhibitors: rotenone and 1-methyl-4-phenylpyridinium (MPP^+), which induce a Parkinson's like syndrome, and β-amyloid (associated with Alzheimer's disease), also induced mitochondrial fission in neurons that was blocked by expression of Mfn1 or mutant Drp1 [114]. Bossy-Wetzel and colleagues subsequently showed that the SNOC-induced mitochondrial fission in neurons was associated with Bax translocation to the fission sites on mitochondria, and this translocation and the subsequent neuronal death (but not the fission) could be blocked by overexpression of Bcl-xL [116]. Lipton and colleagues then showed that SNOC could induce *S*-nitros(yl)ation of Drp1 in neurons, and this triggered mitochondrial fission, which was necessary but not sufficient for neuronal death [117]. They also showed that β-amyloid induced mitochondrial fission in neurons and subsequent neuronal death via *S*-nitros(yl)ation of Drp1 (however, see section on *S*-nitros(yl)ation and neurodegenerative disease below). In relation to this literature it should be remembered that mitochondrial fission is a physiological process, and fission alone does not induce cell death, rather cell death may in certain circumstances require fission or be enhanced by fission.

S-nitros(yl)ation of the NMDA receptor and PDI

NO from nNOS may alternatively protect against excitoxicity by *S*-nitros(yl)ating the NMDA receptor, rendering it inactive [118,119]. So according to these authors (Lipton and Stamler and colleagues) whether NO induces neuronal death or protection depends on whether NO is converted to peroxynitrite (by superoxide) which induces death, or converted to *S*-nitrosothiols which induces protection. However, it is not clear how the NMDA receptor is *S*-nitros(yl)ated by NO. Garthwaite and colleagues have suggested that this *S*-nitro(syl)ation of the NMDA is a cell culture artefact requiring UV light [120]. Lipton and colleagues have also provided evidence that excitotoxic activation of nNOS causes *S*-nitros(yl)ation and thence inhibition of protein-disulphide isomerase (PDI), which results in ER stress-mediated dysfunction or death of neurons [58] (see section above on ER stress).

NO in inflammatory neurodegeneration

Inflammatory neurodegeneration is neuronal degeneration induced by inflammation. Astrocytes and microglia (brain macrophages) become activated in most CNS pathologies, including during infection, trauma, aging, after ischemia or stroke, and most inflammatory and neurodegenerative diseases. We and others [121,122] have identified various modes and mechanism by which inflammatory-activated glia kill neurons in culture (Figs. 7 and 8). The first mode is mediated by high levels of iNOS expression in glia [83,121]. We showed that the resulting high levels of NO induce neuronal death by causing inhibition of mitochondrial cytochrome oxidase in neurons [83,107]. NO inhibition of neuronal respiration caused neuronal depolarization and glutamate release, followed by excitotoxicity via the NMDA receptor [83,88,123,124,108]. This excitotoxicity may be potentiated by a second mechanism, as NO from iNOS results in glutamate release from astrocytes via calcium release from intracellular stores stimulating exocytosis of vesicular glutamate [94]. Thus inflammatory-activated astrocytes maintained a higher extracellular glutamate level [94], which is probably not sufficient to induce excitotoxicity alone, but may well be sufficient if in addition neuronal respiration is inhibited so that

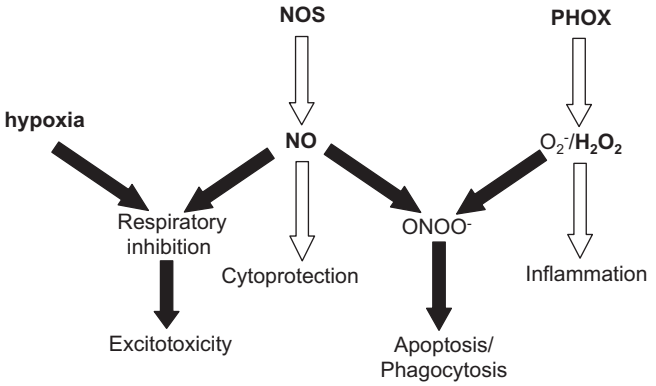

Fig. 8. Hypoxia or PHOX synergise with NOS to induce neuronal death. NOS (NO synthase) activation alone may by neuroprotective, but when activated at the same time as hypoxia or PHOX (phagocyte NADPH oxidase) may result in neuronal death. PHOX activation alone (or hypoxia alone) may result in activation of microglia without neuronal death.

NMDA receptor is activated by both depolarization and glutamate [125].

NO and hypoxia

However, the above mechanism require relatively high levels of NO or iNOS expression, and iNOS can be expressed in vitro [126,127] or in vivo [128] apparently with little or no neuronal death. Indeed NO from iNOS may be protective by blocking brain cell death [63,129,130]. On the other hand, low levels of iNOS expression may synergize with other conditions to induce cell death [131]. For example, hypoxia strongly synergizes with NO or iNOS expression to induce neuronal death via respiratory inhibition [126]. This is because NO inhibits cytochrome oxidase in competition with oxygen, so that NO greatly increases the apparent K_M of neuronal respiration for oxygen [126]. This sensitization to hypoxia is potentially importance in stroke, trauma, vascular dementia, Alzheimer's and brain aging, where both inflammation and hypoxia may coexist.

NADPH oxidase

A second mode that we identified was the dual-key (iNOS and PHOX) mechanism of inflammatory neurodegeneration [127]. We found that activation of iNOS or the NADPH oxidase (NOX/PHOX) alone caused little or no neuronal death, but when both were activated together, this caused considerable neuronal death mediated by peroxynitrite. We showed that inflammatory neurodegeneration induced by TNFα, IL-1β, prion peptide, LPS, IFNγ, arachidonate, ATP and/or PMA was mediated by this mechanism in particular conditions [127]. Simultaneous activation of PHOX and iNOS in microglia resulted in the disappearance of NO [132], appearance of peroxynitrite, and massive death of co-cultured neurons that was prevented by inhibitors of iNOS or PHOX, or by scavengers of superoxide or peroxynitrite [127].

Importantly however, activation of PHOX alone caused no neuronal death [126], but did activate the microglia to proliferate [133] and release TNFα and IL-1β in response to fibrillar β-amyloid [134]. PHOX has been shown by many other labs to be a key regulator of inflammatory activation of microglia [122], and thus potentially a target for anti-inflammatory strategies.

Mitochondrial electron transport

NO and NO-producing astrocytes cause reversible and irreversible damage to respiratory complexes of neuronal and astrocyte mitochondria [135]. This irreversible damage may be mediated by: (i) oxidation or nitration of the respiratory complexes by peroxynitrite, (ii) NO-induced glutamate release and excitotoxicity, as it is blocked by NMDA receptor antagonists [122], or (iii) NO or peroxynitrite inhibition of cytochrome oxidase causing ROS production resulting in secondary damage of other respiratory complexes (possibly by peroxynitrite) [136].

Anti-inflammatory NO

Low levels of NO from nNOS in neurons and astrocytes may be anti-inflammatory by blocking activation of astrocytes and microglia [137]. Activation of soluble guanylate cyclase blocks inflammatory activation of microglia in culture [138]. Hence chronic nNOS inhibition may promote inflammatory neurodegeneration in vivo [139].

Peroxynitrite and ALS

Amyotrophic lateral sclerosis (ALS) is a subtype of motor neuron disease, and a proportion of ALS is due to inheritance of mutants of Cu, Zn-superoxide dismutase (SOD1). The SOD1 mutants are toxic to motor neurons, but the mechanism of this toxicity is unclear and controversial. However, one hypothesis is that the mutant SODs lose zinc, making their active-site copper more accessible to reductants, and this reduced copper reduces oxygen to superoxide, which may in turn react with NO to produce peroxynitrite. This peroxynitrite may then contribute to neuronal death directly, or be used by mutant SOD to nitrate other proteins [140]. This type of mechanism may also be relevant in non-genetic ALS, as wild-type SOD1 can lose Zn in a variety of conditions, resulting in a SOD which is equally toxic to motor neurons. We showed a related reaction on wild-type SOD1: SOD1 catalyses rapid peroxynitrite production and nitration in the presence of H_2O_2 and NO, and this is sufficient to induce cell death in the presence of NO and H_2O_2 [26]. In addition there is an inflammatory reaction in ALS which appears to contribute to the pathology [141], thus neuronal loss may be inflammatory neurodegeneration.

S-nitros(yl)ation and neurodegenerative disease

NO production can (probably via RNS) result in *S*-nitros(yl)ation of many proteins [142,143]), including MMP-9, parkin, and GAPDH, which has been linked to neuronal death [144]. However, research on *S*-nitros(yl)ation remains controversial [145]. Partly this is because it is unclear how proteins become *S*-nitros(yl)ated. Also levels of *S*-nitros(yl)ation are rarely quantified, but when quantified are usually low, suggesting the modification may not be functionally significant and/or *S*-nitros(yl)ation is unstable. Evidence for *S*-nitro(sy)lation of proteins is mainly based on the biotin-switch assay [146], which does not always reliably distinguish between *S*-nitros(yl)ated, oxidised or reduced cysteine residues [147]. And in cellular conditions, *S*-nitros(yl)ated thiols are unstable and tend to react relatively rapidly to leave oxidised thiols (dithiols, sulfinic or sulfonic acid derivatives). Using the biotin-switch assay, several proteins have been identified as *S*-nitros(yl)ated in healthy brain (or rather freshly isolated homogenate of rat cerebellum), including tubulin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the sodium pump ATPase, and NMDA receptors [145]. Basal *S*-nitros(yl)ation of these proteins was abolished in the brains of $nNOS^{-/-}$ mice, supporting a physiological role for *S*-nitros(yl)ation. However, it is not entirely clear that this assay was detecting *S*-nitros(yl)ation, and the level of endogenous modification was very low compared to that induced by added *S*-nitros(yl)ating agents. The point here is NOT that the biotin-switch method is not a good and sensitive method for detecting *S*-nitros(yl)ation of proteins, but rather that if the user is not very careful, it can artifactually over or under detect *S*-nitros(yl)ation for a whole variety of reasons reviewed in [146], and it is rarely used quantitatively to give the % of a particular protein *S*-nitros(yl)ated.

There is evidence that neurotoxicity may involve excess *S*-nitros(yl)ation. For instance, cerebral ischemia augments *S*-nitros(yl)ation of matrix metalloprotease-9 (MMP-9), stimulating its activity and leading to the formation of stable sulfinic or sulfonic acid derivatives whose irreversible activation may trigger neuronal apoptosis [148]. Parkin is an E3-ubiquitin-ligase whose mutation is associated with some forms of genetically determined Parkinson's disease [149]. In Parkinson's disease, *S*-nitros(yl)ation of parkin inhibits its E3-ubiquitin-ligase activity and protective function, promoting cytotoxicity [150,151]. Also in Parkinson's disease, there is evidence of *S*-nitros(yl)ation of XIAP, blocking its ability to inhibit caspases, and thereby promoting apoptosis ([152]). In Parkinson's and Alzheimer's disease there is evidence suggesting *S*-nitros(yl)ation of

protein disulfide isomerase (PDI), which leads to disruption of protein folding in the endoplasmic reticulum, which may cause ER stress and contribute to the protein aggregation that characterizes these and other diseases [58] (see section above on ER stress). But again it was not demonstrated that the level of *S*-nitros(yl)ation in diseased brains was sufficient to have a functional effect – this would require quantification of the % of PDI *S*-nitros(yl)ated.

There is evidence from a variety of sources that inhibition of mitochondrial complex I is involved in Parkinson's disease [153]. For example, the substantia nigra from Parkinson's disease patients has decreased complex I activity, and inhibitors of complex I (rotenone or MPTP) induce Parkinson's syndromes in humans and animals. Incubation of cells with NO results in inactivation of complex I, which is partly mediated by *S*-nitros(yl)ation, and which is reversed by light of reduced thiols [154–157]. Inhibition is probably mediated by *S*-nitrosothiols or peroxynitrite, and is dependent on a reversible conformational change of complex I [158]. There is also an irreversible inhibition of complex I by NO/RNS probably mediated by oxidation, nitration and/or release of iron from iron-sulphur centres [159,160,18]. *S*-nitros(yl)ation of complex I inhibits electron transport, but stimulates ROS production by mitochondria [22,157], which might contribute to cell dysfunction or death. Whether this *S*-nitros(yl)ation of complex I occurs in Parkinson's or other brain diseases in unknown. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, or its active metabolite MPP^+) is a complex I inhibitor, which induces a Parkinson's syndrome in humans and animals, and surprisingly this appears to be mediated by nNOS and iNOS, in that iNOS or nNOS-knockout mice are protected from the dopaminergic neuronal loss (and nNOS inhibitors prevent such loss) [161–164]. Neuronal loss in Parkinson's appears to involve inflammatory neurodegeneration (see section above).

In relation to Alzheimer's disease, it has recently been found that β-amyloid can induce mitochondrial fission and neuronal death via promoting *S*-nitros(yl)ation of Drp1, a key mediator of mitochondrial fission [117] (see Mitochondrial fission section above), and that *S*-nitros(yl)ated Drp1 is increased in brain samples from people dying with Alzheimer's disease. However, more recently it was reported that *S*-nitros(yl)ation of Drp-1 had no effect on its function, and that levels of *S*-nitros(yl)ated Drp-1 were unchanged in Alzheimer's and Parkinson's brains [165]. Rather NO triggered phosphorylation of Drp-1, which mediated its recruitment to mitochondria [165]. Neuronal loss in Alzheimer's disease involves inflammation and may progress by inflammatory neurodegeneration [166]. However, there is some evidence that NO from iNOS may be protective: iNOS-knockout mice crossed with amyloid precursor protein (APP) mutant knockin mice, have increased neuronal loss and pathology [167].

A variety of neuronal stressors have been found to cause *S*-nitrosylation of GAPDH that binds to Siah and translocates to the nucleus, where the complex augments p300/CBP-associated acetylation of nuclear proteins, including p53, which mediate neuronal death (see section on GAPDH above). Recently is was found that a cytosolic protein, GOSPEL, prevented excitotoxicity by binding GAPDH, in competition with Siah, retaining GAPDH in the cytosol. Furthermore, *S*-nitrosylation of GOSPEL enhanced GAPDH-GOSPEL binding and the neuroprotective actions of GOSPEL in culture and in vivo [168]. The neurotoxicity of Huntingtin in Huntington's disease may involve the NO-GAPDH-Siah1 cascade [50–53] (see section on GAPDH above).

Conclusions

Things that are clear, unclear or needing clarification

It is clear that at high concentrations NO can kill neurons in culture, whereas low concentrations of NO can protect some neurons against some stimuli. In vivo, it is clear that nNOS-knockout mice are partially protected against ischemia- and excitotoxin induced neuronal death, indicating that nNOS-derived NO is damaging in this context. It is less clear how NO- from nNOS is toxic to neurons in vivo, although there is no shortage of mechanisms worked out in culture, involving either NO, peroxynitrite or *S*-nitros(yl)ation. Extrapolation of these in vitro studies to in vivo is complicated because it is still unclear what the levels of NO, peroxynitrite and *S*-nitrosothiols are in vivo. Roles for *S*-nitros(yl)ation in both protection (via the NMDA receptor) and damage (via PDI, Drp-1 or mitochondrial complex I) remain controversial, because of the lack of specificity, stability and quantification of protein *S*-nitros(yl)ation, and lack of understanding as to how such *S*-nitros(yl)ation occurs. However, similar criticisms could be levelled at the general presumption that peroxynitrite is responsible for pathology, which is often based on the finding that peroxynitrite scavengers block pathology, when the specificity of these scavengers is unclear, and peroxynitrite-induced protein nitration or oxidation is rarely quantified. The theory that neuronal death is mediated by NO inhibition of cytochrome oxidase in competition with oxygen remains untested in vivo.

The role of iNOS-derived NO in neuropathology is less clear (than nNOS), probably because it has both protective and damaging effects. For example, iNOS-derived peroxynitrite, if it does not kill neurons, can induce protection against subsequent insults in vivo [169]. A recent study of three different strains of iNOS-knockout found no effect of iNOS on infarct size after transient focal brain ischemia, and there was no induction of iNOS expression by ischemia [170]. However, there are in vivo experiments showing that iNOS-knockout mice are protected against neuronal damage in models of Alzheimer's disease [171], Parkinson's disease [172], motor neuron disease [173], delayed brain damage [174] and peritheral nerve damage in diabetes [175]. This may suggest that iNOS is more likely to be toxic during chronic, rather than acute, inflammation.

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