# Nitric oxide-mediated oxidative damage and the progressive demise of motor neurons in ALS

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ABSTRACT

Oxidative damage is a common and early feature of Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS) and other neurodegenerative disorders. Dr. Mark Smith and his colleagues have built the case for oxidative stress being a primary progenitor rather than a secondary end-stage epiphenomenon of neurodegeneration. They proposed that reactive oxygen species contribute to the "age-related cascade of neurodegeneration" whereby accumulative oxidative damage with age promotes other characteristic pathological changes in afflicted brain regions, including protein aggregation, metabolic deficiencies, and inflammation. Nitric oxide (NO) likely plays a critical role in this age-related cascade. NO is a major signaling molecule produced in the central nervous system (CNS) to modulate neurological activity through stimulating cyclic GMP synthesis. However, the same physiological concentrations of NO relevant in cellular signaling may also initiate and amplify oxidative damage by diffusion-limited reactions with superoxide ($O_2^{\cdot-}$) to produce peroxynitrite ($ONOO^-$). This is perhaps best illustrated in ALS where physiological levels of NO promote survival of motor neurons, but the same concentrations can stimulate motor neuron apoptosis and glial cell activation under pathological conditions. While these changes represent a complex mechanism involving multiple cell types in the pathogenesis of ALS, they also reveal general processes underlying neurodegeneration.

KEYWORDS: nitric oxide, peroxynitrite, amyotrophic lateral sclerosis, neurodegeneration, protein nitration

INTRODUCTION

Widespread oxidative damage to proteins, DNA and lipids is found in many neurodegenerative disorders including Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD). However, these biomarkers may only be tombstones that signal the end-stage of a chronic disease process. The work of Dr. Mark Smith and his colleagues over the past two decades has established that reactive oxygen and nitrogen species (ROS/RNS) are involved at the earliest stages of neuronal dysfunction, showing that oxidative stress may drive the disease process rather than just kill neurons outright. His hypotheses on the neurodegenerative cascade incorporate two major observations that other theories fail to address. First, oxidative damage occurs in neuronal populations prior to other pathological hallmarks, such as the presence of neurofibrillary fibers in AD (Nunomura *et al.*, 2001; Pratico *et al.*, 2001) For example, oxidized DNA and protein products are observed prior to the deposition of amyloid-beta protein and neuronal loss in patients with Down's syndrome (Nunomura *et al.*, 2000). Second, ageing is the primary risk factor of progressive neurodegenerative disorders (Katzman, 1986) and associated with a gradual decline in both the ability to scavenge reactive species and to repair tissue injury.

In 1994, our group developed the first antibodies that recognize nitrotyrosine, an indicator of peroxynitrite-mediated protein damage that persists in tissues (Beckman *et al.*, 1994). While we found extensive nitration in atherosclerosis, lung disease and acute inflammatory diseases, we were disappointed to find little evidence of nitration in advanced cases of AD. We provided the antibody to George Perry and Mark Smith, who identified the widespread presence of nitrated protein tyrosine residues in brain tissue surrounding soft plaques in early-stage AD brains (Smith *et al.*, 1997). Importantly, they observed that protein nitration diminishes in advanced stages of the disease. Since then, nitrated proteins have been identified in the early stages of many neurodegenerative diseases from human cases and animal models (Greenacre and Ischiropoulos, 2001; Casoni *et al.*, 2005; Pacher *et al.*, 2007).

The identification of nitrated proteins in AD brains provided the first evidence for the involvement of nitric oxide (NO)-mediated oxidative damage in the disease. NO itself is neither highly reactive nor particularly toxic under physiological or even pathological conditions. However, it can form peroxynitrite ($ONOO^-$) through a diffusion-limited reaction with superoxide, creating a more potent and specific oxidant (Beckman *et al.*, 1990). While levels of peroxynitrite normally remain low due to effective superoxide-scavenging by superoxide dismutase (SOD), even small simultaneous increases in NO and superoxide greatly increases peroxynitrite formation (Beckman and Koppenol, 1996). Peroxynitrite can oxidize biological molecules including lipids, DNA, and proteins through either direct reactions or via spontaneous homolysis to yield the two highly reactive free radicals: nitrogen dioxide ($^{\cdot}NO_2$) and carbonate radical ($CO_3^{\cdot-}$). In particular, these radicals derived from peroxynitrite are capable of modifying tyrosine residues in proteins to form nitrotyrosine, which can drastically effect protein structure and function (Ischiropoulos *et al.*, 1992). The toxic actions of peroxynitrite likely result from reactions with numerous cellular targets, but protein nitration represents a major cytotoxic pathway. Extensive discussion regarding the chemistry surrounding the formation of peroxynitrite, protein nitration, and reaction with biological molecules is beyond the

scope of this review but has been described in great detail elsewhere (Beckman, 1996; Pacher *et al.*, 2007; Calcerrada *et al.*, 2011).

Emerging evidence suggests that NO and its oxidation products play a central role in both triggering and amplifying oxidative damage in neurodegeneration. In the CNS, NO is produced as a major signaling molecule that participates in the regulation of blood flow, neurotransmission, memory, and synaptic plasticity. However, these same concentrations of NO that serve many pivotal and beneficial neurological functions also can also mediate cytotoxicity and neurodegeneration. Physiological levels of NO can trigger and amplify neurotoxicity under stressful conditions where superoxide is also being generated. These contrasting roles are perhaps best illustrated in models of ALS, where the same concentrations of NO that promote the survival of motor neurons under normal conditions can also induce apoptosis and glial cell activation following a variety of stressors (Estevez *et al.*, 1998a; Estevez *et al.*, 1998b; Barbeito *et al.*, 2004).

In this review, we describe how the levels of NO produced normally for cellular signaling within the CNS is sufficient to trigger neuronal oxidative stress to induce profound changes in the interactions between neurons and glial cells as well as the activation of neuronal death cascades. While the literature often considers the production from inducible nitric oxide synthase (iNOS) -- commonly associated with macrophages and microglia -- as the source of pathological levels of NO, we describe evidence here that physiological concentrations of NO mediating signaling may be subverted early in neurodegenerative processes through reaction with superoxide to initiate neurodegeneration.

## ALS AND SUPEROXIDE DISMUTASE

ALS is characterized by the progressive loss of motor neurons in the motor cortex, brain stem, and spinal cord, which results in increasing muscle weakness, paralysis and death within 3-5 years after symptom onset (Rowland and Shneider, 2001). The vast majority of ALS cases are sporadic without a genetic basis, while 2-3% are associated with mutations in the gene for copper, zinc-superoxide dismutase (SOD1) resulting in disease that is generally indistinguishable from sporadic ALS (Rosen *et al.*, 1993). A majority of the mutations to SOD1 result in the expression of an active protein with full superoxide scavenging activity. Furthermore, SOD1 knockout mice do not develop neuron disease (Reaume *et al.*, 1996), demonstrating that loss of SOD activity is not responsible for disease. However, overexpression of many mutant SOD1 forms in transgenic rats and mice results in development of motor neuron disease, which supports a toxic gain of function in mutant SOD1 (Gurney *et al.*, 1994). SOD1 was previously shown to catalyze peroxynitrite-mediated tyrosine nitration (Ischiropoulos *et al.*, 1992), so it was proposed that SOD1 mutations might enhance nitration (Beckman *et al.*, 1993). This claim is supported by observations of increased tyrosine nitration in both human ALS patients and transgenic ALS animal models (Beal *et al.*, 1997; Ferrante *et al.*, 1997), but the initial concept proved overly simplistic.

Despite nearly two decades of work, the basis for SOD1-induced motor neuron death in ALS remains controversial. However, a general consensus exists that the mutations structurally weaken SOD1 to form a toxic partially unfolded intermediate. One of the key characteristics of SOD1 that is frequently overlooked in ALS is the binding of the two metal cofactors, copper and zinc, which are critical for the functioning of the

enzyme (Rhoads *et al.*, 2011). Multiple laboratories have now established that the first step in unfolding of SOD1 is the most likely the loss of the structural zinc atom (Trumbull and Beckman, 2009).

We found that many mutant SOD1 proteins can have identical activities as wild type SOD1 when care was taken to ensure that copper and zinc were both present (Crow *et al.*, 1997a). Furthermore, delivery of mutant SOD1, carefully purified to contain both copper and zinc, were equally protective as wild type Cu,Zn-SOD1 to motor neurons deprived of trophic factors (Estevez *et al.*, 1999; Sahawneh *et al.*, 2010). However, the mutant SOD1 proteins were challenging to prepare replete with both metals. Much of the mutant SOD1 protein lacked zinc when expressed in *E. coli* (Crow *et al.*, 1997a). This led us to investigate the properties of copper-containing, zinc-deficient SOD1, which proved to be extremely toxic to motor neurons in culture (Estevez *et al.*, 1999). The toxicity required both copper to be bound to the protein as well as NO synthesis by the motor neurons. Of particular importance, the loss of zinc from wild type SOD1 was equally toxic as loss of zinc from mutant SOD1. Competitive blockade of tyrosine nitration also prevented the toxicity of zinc-deficient SOD1 in motor neurons. These cell-based data argue that the loss of zinc from SOD1 is sufficient to instigate the same cell death cascades as trophic factor deprivation and Fas-mediated apoptosis discussed later in this review (Sahawneh *et al.*, 2010). Despite the controversy surrounding the role of SOD1 in promoting oxidative damage, extensive work in primary cultures and in vivo shows that copper in SOD1 interacts with NO and peroxynitrite to initiate the death of motor neurons. The role of copper in zinc-deficient SOD1 toxicity in ALS was recently more fully reviewed than here (Trumbull and Beckman, 2009).

## MOTOR NEURON APOPTOSIS MEDIATED BY PEROXYNITRITE

The two faces of NO in motor neuron biology are evidenced through support for survival as well as induction of apoptosis. The survival of motor neurons in culture requires appropriate of several trophic factors (Hughes *et al.*, 1993; Oppenheim, 1996). In the presence of brain-derived neurotrophic factor (BDNF), motor neurons cultured from embryonic rat spinal cord tissue constitutively express endothelial NOS (eNOS), which supports motor neuron survival through binding of NO to the heme group of soluble guanylate cyclase thereby stimulating cyclic GMP (cGMP) synthesis (Estevez *et al.*, 1998b) (Figure 1A). Inhibition of NO synthesis under these same conditions decreases motor neuron survival, while protection is afforded by either by generating steady-state concentrations of <100 nM exogenous NO or by the addition of cell-permeant cGMP analogs.

In contrast, deprivation of trophic factors increases NO production via up-regulation of neuronal NOS (nNOS) in cultured motor neurons. Trophic factor deprivation also stimulates co-generation of superoxide, which promotes the endogenous production of peroxynitrite. This led to increased nitrotyrosine immunoreactivity and induction of apoptosis within 24 hours in greater than 60% of cultured neurons (Estevez *et al.*, 1998a). Interestingly, NO-dependent activation of cGMP was later shown to prevent apoptosis in motor neurons in part by blocking the expression of nNOS through sequestration of free intracellular calcium (Estevez *et al.*, 2002) (Figure 1A). Inhibition of NO production in trophic factor-deprived motor neurons prevented increases in nitrotyrosine immunoreactivity and cell death (Estevez *et al.*, 1998a). This protection was

lost by the addition of an NO donor maintaining a steady-state concentration of <100 nM, the same concentration that shown protection in models described above. Lower steady-state NO concentrations (5-10 nM) in culture media induce less neuron death at 24 h but are still toxic. After 3 days, lower NO levels can produce cell death to the same extent compared to higher concentrations at 24 hr (Estevez *et al.*, 1998a; Estevez *et al.*, 1998b). Importantly, these rates of NO production showed no toxicity to motor neurons adequately supplied with trophic factors and do not potentiate trophic factor-deprived motor neuron death. Furthermore, intracellular scavenging of superoxide was also protective and prevented the toxicity of exogenous nitric oxide (Estevez *et al.*, 1998b). Together, these results suggest that NO itself is neither sufficient nor limiting to produce motor neuron apoptosis.

Meanwhile, activation of Fas death receptors was identified in motor neuron death during trophic factor deprivation (Raoul *et al.*, 1999). Further investigation showed that Fas-triggered death occurred via two parallel pathways involving induction of nNOS in association with established FADD and caspase-8-mediated mitochondrial cascades of apoptosis (Chinnaiyan *et al.*, 1995; Muzio *et al.*, 1996; Scaffidi *et al.*, 1998; Raoul *et al.*, 2002). This pathway was activated in the presence of low-level extracellular steady-state NO (as low as 10-20 nM) in mutant SOD1-expressing motor neurons with adequate trophic support, but not in nontransgenic or wild-type SOD1-expressing cultures (Raoul *et al.*, 2006), suggesting a unique sensitivity of ALS-associated SOD mutations. Exogenous NO triggered upregulation of membrane-bound Fas ligand (FasL) with subsequent activation of Fas receptor-mediated death cascades in addition to upregulation of nNOS and further NO synthesis (Figure 1B). Antagonistic anti-Fas antibodies and expression of a Daxx-dominant negative form were protective, providing further evidence that NO produced from nNOS upregulation alone was insufficient to induce neuronal death. The amplification of this Fas/NO positive feedback loop was necessary to induce cell death suggesting that its chronic activation may contribute to the slow, progressive motor neuron loss in ALS. Additionally, motor neurons with positive immunoreactivity for several elements of this pathway including FasL, Daxx, and p38 kinase have also been observed in presymptomatic transgenic animals and sporadic human ALS (Bendotti *et al.*, 2004; Raoul *et al.*, 2006), suggesting a common molecular pathway between familial ALS and ALS patients without SOD1 mutations.

The involvement of NO, nNOS, and Fas pathways also extend to motor neuron death *in vivo*. Upregulation of nNOS occurs in motor neurons prior to iNOS in both ALS patients and pre-symptomatic transgenic animal models (Sasaki *et al.*, 2001a; Chen *et al.*, 2010; Moreno-Lopez *et al.*, 2011), consistent with nNOS having the potential to contribute to motor neuron loss. Motor neuron death following axonal injury is also associated with induction of nNOS and increased nitrotyrosine immunoreactivity (Wu, 1993; Wu *et al.*, 1994a; Wu *et al.*, 1994b; Martin *et al.*, 1999; Martin *et al.*, 2005). Inhibition of NOS offers protection to motor neurons against ventral root aversion, while SOD1 deficiency increases motor neuron vulnerability to the same injury (Wu and Li, 1993; Reaume *et al.*, 1996). Axotomy-induced motor neuron apoptosis was also prevented in Fas-knockout mice and transgenic mice expressing dominant negative form of FADD (Ugolini *et al.*, 2003; Martin *et al.*, 2005). Together, these results suggest that NO/Fas-mediated pathways involved in the induction of apoptosis in cultured motor neurons are also active *in vivo* and play a role in the degeneration of adult motor neurons.

NO-dependent oxidative damage can also promote apoptosis in motor neurons via mechanisms involving non-neuronal cells. Astrogliosis is associated with increased expression and release of several growth factors and cytokines, including nerve growth factor (NGF) (Vargas *et al.*, 2004). NGF plays an essential role in differentiation and survival of some neuronal populations, but also serves to eliminate damaged neurons and glia (Chao, 2003). NGF mediates apoptosis through the p75 neurotrophin receptor ($p75^{NTR}$), which induces ceramide-dependent mitochondrial superoxide production and nitrotyrosine immunoreactivity in affected neurons (Barker, 2004; Nykjaer *et al.*, 2005; Pehar *et al.*, 2007). Although $p75^{NTR}$ is presumed lacking in adult motor neurons, expression has been observed following nerve injury and in cases of ALS (Seeburger *et al.*, 1993; Lowry *et al.*, 2001). The re-expression of $p75^{NTR}$ may act as a mechanism to signal or increase susceptibility of damaged motor neurons to undergo apoptosis. Cultured motor neurons expressing $p75^{NTR}$ showed sensitivity to NGF-induced apoptosis with significant immunoreactivity for nitrotyrosine when exposed to low-level (<50 nM) exogenous NO (Pehar *et al.*, 2004). Follow up studies showed that peroxynitrite-mediated nitration of NGF also induced motor neuron apoptosis (Pehar *et al.*, 2006b). Additionally, motor neurons overexpressing ALS-linked SOD1 mutation showed increased susceptibility to NGF-dependent apoptosis occurring in the absence of exogenous NO. This sensitivity was associated with decreased expression of key anti-oxidant elements including Nrf2 expression and glutathione biosynthesis (Pehar *et al.*, 2007).

Importantly, these studies demonstrate that NO-mediated apoptosis occurs in motor neurons via initial induction of nNOS. iNOS is also upregulated in motor neurons from ALS patients and symptomatic transgenic mice, although generally occurs later than increases in nNOS (Moreno-Lopez *et al.*, 2011). NO alone is insufficient to initiate apoptosis in motor neurons, but simultaneous production of superoxide appears to be a critical step in triggering cell death pathways. The above cases demonstrate that trophic factor deprivation, depletion of antioxidant reserves, expression of mutant SOD1, activation of p75 receptor, or other stressors arising from aging or environmental and genetic factors can directly or indirectly increase production of superoxide within motor neurons. Subsequent reaction with NO produced constitutively for physiological purposes can promote oxidative stress and apoptosis via two mechanisms: 1) preventing NO-dependent stimulation of cyclic GMP synthesis and 2) increasing peroxynitrite formation (Estevez *et al.*, 2002).

Although peroxynitrite is known to exert cytotoxicity, among the earliest evidence that it could induce apoptosis was demonstrated in PC12 rat pheochromocytoma cells and cortical cell cultures (Bonfoco *et al.*, 1995; Estevez *et al.*, 1995). Later, we showed that tyrosine-containing peptides could be used to identify the critical role of peroxynitrite-mediated nitration of tyrosine residues in apoptosis of these same cells as well as cultured motor neurons (Ye *et al.*, 2007). These peptides significantly reduced nitrotyrosine immunoreactivity to a similar degree as scavengers of superoxide and peroxynitrite. However, these peptides did not scavenge peroxynitrite, but instead served as competitive inhibitors for tyrosine nitration. These same peptides offered no protection against cell death by hydrogen peroxide or staurosporin (Ye *et al.*, 2007), suggesting that endogenous production of peroxynitrite in motor neurons induces apoptosis through nitration of critical proteins.

## ALS AND PROTEIN NITRATION

While peroxynitrite can cause oxidative damage to multiple biological macromolecules, protein nitration represents a major cytotoxic pathway contributing to neurodegenerative disease. The reactions, conditions, and specificity driving peroxynitrite-mediated nitration of protein tyrosine residues has been detailed elsewhere (Koppenol *et al.*, 1992; Beckman, 1996; Radi, 1998; Souza *et al.*, 1999; Ischiropoulos, 2003). Protein nitration is a highly selective process that is limited to specific tyrosine residues on a small number of proteins. Nitration of a single tyrosine residue can induce significant changes in protein structure and function. In the context of neurodegenerative diseases, nitrated proteins have been identified with altered enzyme activity, increased propensity to form aggregates, and ability to elicit immunogenic response (Reynolds *et al.*, 2007). The consequences of protein nitration are particularly relevant in ALS (Crow *et al.*, 1997a; Estevez *et al.*, 1999). Here, we discuss how some of the nitrated proteins found in ALS might effect the development of motor neuron disease.

Structural proteins are major targets for nitration given their high abundance in cells and high proportion of tyrosine residues. Neurofilament proteins are among the most abundant structural proteins found within motor neurons and proper assembly is critical for axon integrity and structure (Hoffman *et al.*, 1987). Mutations in highly conserved regions of neurofilament subunits leads to development of motor neuron disease in humans and transgenic mice, providing intriguing genetic evidence that neurofilament dysfunction alone could be adequate to cause ALS (Figlewicz *et al.*, 1994; Lee *et al.*, 1994). Aggregation of neurofilaments within motor neurons is an early sign of degeneration and a pathological hallmark of ALS. Immunoreactivity for nNOS and SOD1 are found in regions of neurofilament accumulation implicating their involvement in the nitration of neurofilaments in motor neurons (Chou *et al.*, 1996a; Chou *et al.*, 1996b). More specifically, neurofilament light subunit (NF-L) is selectively nitrated by SOD1 *in vitro* in the head and rod domains, which are essential in intersubunit contact and polymerization of neurofilaments (Crow *et al.*, 1997b). As a consequence of nitration in these regions, nitrated NF-L monomers could inhibit proper assembly of NF-L, ultimately leading to aggregation of the protein. Nitration of less than 10% of total NF-L was sufficient to disrupt neurofilament assembly.

NF-L may also play a critical role in promoting SOD1-catalyzed protein nitration. Neurofilament proteins show a high affinity and binding capacity for zinc (Pierson and Evenson, 1988), and NF-L can specifically extract zinc from wild-type and mutant forms of SOD1. NF-L may form a high affinity and abundant site favoring the accumulation of zinc-deficient SOD1 (Crow *et al.*, 1997a). Zinc-deficient SOD1 shows reduced scavenging activity for superoxide and greater efficiency of peroxynitrite-mediated protein nitration. This process can create a positive feedback loop whereby NF-L removes zinc from SOD, which promotes nitration of proteins, including NF-L itself and increased oxidant levels. Nitrated NF-L inhibits proper subunit assembly leading to increased levels of NF-L monomers, which may further chelate zinc from SOD1 or form protein aggregates. Ultimately, motor neuron death may occur as a result of detrimental effects caused by zinc-deficient SOD1 (Estevez *et al.*, 1999). Therefore, initial nitration of NF-L may serve to propagate motor neuron injury.

Mitochondrial dysfunction associated with oxidative damage is commonly observed in nearly all cases of neurodegeneration. Mitochondrial-localized SOD (SOD2)

is responsible for scavenging superoxide in the mitochondrial matrix. While motor neurons develop normally in SOD1-deficient mice (Reaume *et al.*, 1996), SOD2 is essential for survival. SOD2-deficient mice exhibit neonatal lethality with severe neurological deficits and oxidative injury (Lebovitz *et al.*, 1996). Partial knockdown of SOD2 accelerates motor neuron loss and disease progression in transgenic mice expressing mutant SOD1 (Andreassen *et al.*, 2000). SOD2 is quite distinct from SOD1, using manganese to catalyze superoxide dismutation rather than copper in the active site and with a tyrosine at position 34 at the entrance to the active site.

SOD2 was among the first proteins to be identified as an endogenous and specific target of nitration (MacMillan-Crow *et al.*, 1996). Nitration of a single tyrosine residue in close proximity to manganese in the active site leads to complete enzyme inactivation (MacMillan-Crow *et al.*, 1998). Recent studies have demonstrated that peroxynitrite is the responsible agent for SOD2 nitration and subsequent inactivation *in vivo* (Surmeli *et al.*, 2010). Impaired scavenging of superoxide upon inactivation of SOD2 has significant implications on the formation of peroxynitrite and resulting oxidative damage in mitochondria. Importantly, nitrated SOD2 has been detected PD, ALS, AD, and traumatic brain injury (Aoyama *et al.*, 2000; Bayir *et al.*, 2007) suggesting that peroxynitrite-induced mitochondrial damage is a commonly shared mechanism in various cases of neurodegeneration.

NO serves a physiological role in mitochondria by reversibly inhibiting cytochrome *c* oxidase (complex IV) of the respiratory chain (Palacios-Callender *et al.*, 2004). This inhibition of electron flow increases electron leakage at upstream complexes, resulting in superoxide production in the matrix and formation of peroxynitrite. Subsequently, peroxynitrite can nitrate and inactivate SOD2, inhibiting scavenging of superoxide and thereby accelerating peroxynitrite formation. Hence, NO production can rapidly amplify mitochondrial dysfunction when mitochondria produce a significant amount of superoxide.

Peroxynitrite formed in the mitochondria can also nitrate other susceptible proteins including cytochrome *c*, respiratory chain complexes, and enzymes of the tricarboxylic acid cycle, all of which can significantly impact cellular metabolism and redox biology (Radi *et al.*, 2002). For example, nitration of cytochrome *c* yields a more acidic protein species with peroxidatic activity, leading to increased production of hydrogen peroxide and oxidation of membrane phospholipids in the mitochondrial membrane (Cassina *et al.*, 2000). Most components of the respiratory chain can also be inhibited by peroxynitrite through a variety of oxidative mechanisms including nitration. Complex I (NADH dehydrogenase) is particularly sensitive to nitration and has been implicated in the pathology of PD (Chinta and Andersen, 2011). Damage to the respiratory chain promotes electron leak and increases superoxide production in the mitochondria, amplifying the formation of peroxynitrite and creating a positive feedback loop that further escalates oxidant production and mitochondrial dysfunction.

Mitochondrial dysfunction, along with increased production of superoxide, may have significant implications on the survival of neurons and support by glial cells. Mitochondrial dysfunction in mutant SOD1-expressing astrocytes promotes a reactive phenotype associated with increased superoxide production and mitochondrial protein nitration, which in turn promotes motor neuron degeneration (Cassina *et al.*, 2008). Furthermore, increased superoxide resulting from decreased scavenging by SOD2 could

divert NO from stimulating cGMP synthesis and promote oxidative stress and apoptosis through formation of peroxynitrite.

## PEROXYNITRITE PROMOTES TOXIC GLIAL PHENOTYPES

Neurodegenerative diseases, including ALS, have long been viewed as diseases mainly affecting vulnerable neuron populations. Increasing evidence suggests that interactions between motor neurons and glial cells, including astrocytes and microglia, play a crucial role in determining the selective vulnerability characteristic of ALS. The involvement of diffusible molecules such as NO and activation of receptor-mediated apoptosis via FasL and NGF suggests that neighboring cells and local environment strongly influence motor neuron survival (Raoul *et al.*, 2000).

Although exuberant astrogliosis is not observed in the spinal cord of ALS patients, reactive astrocytes expressing either GFAP or S100β are hallmarks in ALS (Hirano, 1991; Schiffer *et al.*, 1996; Migheli *et al.*, 1999). The same glial populations have also been shown to increase expression of MnSOD, suggesting a compensatory response to increased oxidative stress (Blaauwgeers *et al.*, 1996). Some degree of gliosis is also found in the lateral descending corticospinal tracts and in the entering points of the tracts into the gray matter (Schiffer *et al.*, 1996), thus forming a continuum along the damaged regions. Microglia also proliferate and become activated in these regions, and invading T cells can be found around the capillaries (McGeer and McGeer, 2002).

Compared to ALS patients, astrocytosis and neuroinflammation is more dramatic in mice and rats carrying SOD1 mutations (Kawamata *et al.*, 1992; Bruijn *et al.*, 1997; Bruijn *et al.*, 1998; Hall *et al.*, 1998; Levine *et al.*, 1999; Alexianu *et al.*, 2001; Olsen *et al.*, 2001; Howland *et al.*, 2002; Henkel *et al.*, 2004). This may be reflective of the extraordinarily rapid development of disease in the SOD transgenic animal models. Reactive astrocytes found in ALS show expression of inflammatory mediators such as iNOS and immunoreactivity for nitrotyrosine (Almer *et al.*, 1999; Sasaki *et al.*, 2000) and down-regulate glutamate transporters (Rothstein *et al.*, 1992; Sasaki *et al.*, 2001b; Howland *et al.*, 2002), suggesting that astrocytes might play a pathogenic role in the disease by both peroxynitrite-mediated and excitotoxic mechanisms.

In co-cultures, astrocytes support the survival and growth of motor neurons. However, upon activation by LPS or cytokines, astrocytes can become neurotoxic by mechanisms involving iNOS expression (Dawson *et al.*, 1994). Production of NO by astrocytes damages mitochondrial complexes in co-cultured neurons and enhances NMDA-induced excitotoxicity (Hewett *et al.*, 1994; Bolanos *et al.*, 1995; Stewart *et al.*, 2000). We have shown that brief exposure to peroxynitrite, but not hydrogen peroxide, was sufficient to elicit an inflammatory-like phenotype in isolated spinal cord astrocytes, and would initiate apoptosis of subsequently co-cultured motor neurons (Cassina *et al.*, 2002). Antioxidant scavengers of peroxynitrite and NOS inhibitors significantly reduced motor neuron loss, suggesting that the neurotoxic effect was dependent on NO production from astrocytes and mediated via peroxynitrite. Similar dependence of motor neuron death upon NO production via iNOS was also observed in activated microglia (Zhao *et al.*, 2004). Both NO and superoxide are produced by motor neurons *in vitro* in response to trophic factor deprivation, Fas pathway activation, or zinc-deficient SOD1 (Estevez *et al.*, 1998a; Estevez *et al.*, 1999; Raoul *et al.*, 2002). The resultant peroxynitrite formation constitutes a potential mechanism for astrocyte activation in ALS. Peroxynitrite can also

hinder communications via gap junctions and inhibit glutamate transporters in astrocytes, preventing their regulatory role on neuronal excitability and neurotransmission (Bolanos and Medina, 1996; Trotti *et al.*, 1996).

Following injury, motor neurons may also secrete inflammatory mediators and cytokines that can induce astrocytes to adopt a reactive phenotype. One such example is fibroblast growth factor 1 (FGF1). FGF1 is expressed at high concentrations in motor neurons (Elde *et al.*, 1991; Kresse *et al.*, 1995). Oxidation of critical sulfhydryl groups leads to the secretion of FGF1, which is mediated is mediated by a copper-dependent assembly of a multi-protein aggregate (Landriscina *et al.*, 2001). Thus, oxidative stress or possibly copper from zinc-deficient SOD in ALS may facilitate the release of FGF1 from motor neurons. Transgenic mice expressing mutant SOD1 show more widely distributed expression of FGF1 than nontransgenic littermates, consistent with FGF1 being released from motor neurons following oxidative stress (Cassina *et al.*, 2005). Although FGF1 shows neurotrophic activity *in vitro* and is protective in models of spinal cord injury (Walicke, 1988; Cuevas *et al.*, 1995; Teng *et al.*, 1998; Teng *et al.*, 1999), FGF1 effectively transforms astrocytes into a reactive phenotype that induces motor neuron death in co-cultures via a NGF/NO-dependent pathway (Cassina *et al.*, 2005).

Numerous studies have suggested a common link between ALS-associated SOD1 mutations and the involvement of non-neuronal cell populations in motor neuron death. Restricting expression of mutant SOD1 in neurons or astrocytes alone may not be sufficient to induce motor neuron disease (Gong *et al.*, 2000; Pramatarova *et al.*, 2001). Further evidence emerged in chimeric mice possessing mixtures of mutant SOD1-expressing and normal cells, which indicated that toxicity to motor neurons required damage from mutant SOD1 acting within non-neuronal cells. More intriguing, normal non-neuronal cells extended the survival of mutant SOD1-expressing motor neurons (Clement *et al.*, 2003). Reduced expression of mutant SOD1 in astrocytes or microglia in transgenic mice did not affect disease onset but significantly slowed disease progression (Boillee *et al.*, 2006; Yamanaka *et al.*, 2008). These animal models demonstrate that mutant SOD1 expression within non-neuronal cells can be a determinant of disease progression, while the mutant enzyme's presence in motor neurons contributes to disease onset. However, a recent study showed that such models utilizing conditional transgene expression based on a Cre-loxP strategy was associated with microencephaly, which may cause the loss of SOD1-expressing cells during development leading to observed phenotype (Qiu *et al.*, 2011)

Astrocytes expressing mutant SOD1 are also more prone to adopting an activated, inflammatory phenotype (Hensley *et al.*, 2006), display mitochondrial dysfunction, and increased NO plus superoxide production (Cassina *et al.*, 2008). Interestingly, astrocytes expressing mutant SOD1 decrease the survival of motor neurons *in vitro* through secretion of soluble neurotoxic factors (Vargas *et al.*, 2006; Nagai *et al.*, 2007). Recently, astrocytes from postmortem tissue of human sporadic and familial ALS cases showed similar toxicity to motor neurons (Haidet-Phillips *et al.*, 2011). Most importantly, knockdown of SOD1 in sporadic ALS cases, with no mutations to the protein, significantly attenuated astrocyte-linked motor neuron death. These results suggest a common link for SOD1 and astrocyte-mediated toxicity leading to motor neuron death in both sporadic and familial ALS.

In addition to NO produced from NOS up-regulation and enhanced mitochondrial superoxide production, reactive glial cells also release NGF and various cytokines that promote inflammatory response and motor neuron death (Vargas *et al.*, 2006; Di Giorgio *et al.*, 2007; Nagai *et al.*, 2007; Di Giorgio *et al.*, 2008; Marchetto *et al.*, 2008). Elevated levels of NGF have been reported in multiple sclerosis, AD, and muscle from ALS patients (Crutcher *et al.*, 1993; Fahnestock *et al.*, 1996; Stuerenburg and Kunze, 1998). As described previously, NGF plays an essential role for survival and differentiation of neuronal populations, but can also stimulate death through activation of $p75^{NTR}$. Recent evidence shows that reactive spinal cord astrocytes secrete an oxidized form of immature precursor NGF, suggesting a novel mechanism for astrocytes to contribute to loss of $p75^{NTR}$-expressing motor neurons in ALS (Pehar *et al.*, 2004; Pehar *et al.*, 2006a). The interplay between motor neurons and astrocytes is summarized in Figure 2.

## MODEL FOR THE PROGRESSIVE DEATH OF MOTOR NEURONS IN ALS

Given the results reviewed here, we hypothesize that NO-mediated oxidative stress serves key roles in the initiation, amplification, and spread of neurodegeneration in ALS (Figure 3). Various environmental insults or genetic defects are capable of inducing damage in motor neurons and astrocytes. In most cases, effective repair mechanisms exist to restore cells to their normal function. However, physiological responses associated with aging or chronic stressors can lead to adaptations in motor neurons including upregulation of nNOS, $p75^{NTR}$, FasL/Fas, and cytokines, decreased antioxidant capacities and mitochondrial dysfunction. Under normal circumstances, these changes may serve to determine which neurons survive or undergo apoptosis following damage.

Neighboring astrocytes adopt a hypertrophic morphology in response to mediators, including peroxynitrite and FGF-1, in an effort to isolate the site of injury, promote the growth and survival of healthy motor neurons, as well as initiate the death of those that are critically damaged. However, physiological adaptations or genetic susceptibilities such as mutant SOD1 expression may cause further damage to neuronal cells through a combination of factors including mitochondrial dysfunction, increased excitatory inputs, enhanced NO production, and nitrative and oxidative stress. Astrocytes become further activated, adopting a reactive phenotype with upregulated NOS activity, and themselves suffer non-lethal peroxynitrite-mediated oxidative stress. Astrocytes further amplify damage to motor neurons through excessive NO production and release of proapoptotic factors such as NGF and FasL. During this process, both motor neurons and astrocytes accumulate general markers of pathology including increased protein nitration, oxidation and aggregation. Functional and structural changes to proteins such as MnSOD and NF-L resulting from nitration may be pathogenic by further propagating oxidative damage and cellular dysfunction. These cumulative changes are sufficient to initiate apoptosis in the initially damaged cells, but diffusion of NO and secretion of soluble inflammatory and proapoptotic factors spreads damage to surrounding cells. Susceptible motor neurons in the vicinity can be damaged directly or activation of neighboring glial cells can render additional motor neurons vulnerable to damage, thus propagating the disease as increasing number of motor neurons are affected.

## CONCLUSIONS

Among Dr. Mark Smith's most significant contributions were his novel views towards origin of neurological disease including the recognition that physiological responses and ageing are fundamental aspects of degenerative processes. In particular, Dr. Smith and colleagues recently proposed an "age-induced cascade of neurodegeneration" whereby chronic and self-propagating oxidative stress plays a key role in the age-related progression of disease (Bonda *et al.*, 2010). A major contribution to this cascade is likely to involve NO because 1) NO promotes oxidative damage through its reaction with superoxide to form peroxynitrite and 2) the production of NO is a key step in many feed-forward loops driving injury and activating death cascades.

The implication of NO in neurodegeneration provides an intriguing case whereby a molecule involved in numerous beneficial physiological processes in the CNS can also be transformed to promote tissue damage and disease progression. Evidence for peroxynitrite-mediated oxidative stress extends beyond ALS to include nearly all neurodegenerative diseases including PD, AD, multiple sclerosis, Huntington's disease, cerebral ischemia and even the genesis of central chronic pain (Pacher *et al.*, 2007; Calabrese *et al.*, 2009; Salvemini *et al.*, 2011).

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FIGURE LEGENDS

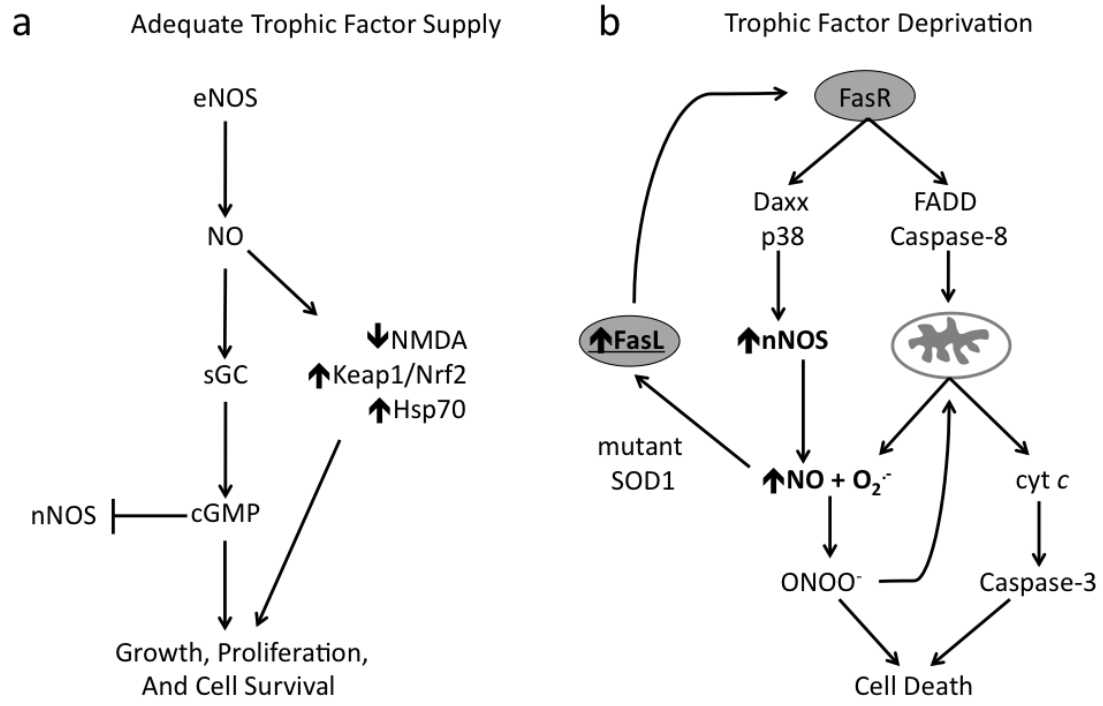


**Figure 1. The neuroprotective and neurotoxic effects of NO in motor neurons.** (A) Motor neurons supported by trophic factors produce NO through constitutive expression of eNOS. NO exerts neuroprotective effects via two mechanisms: stimulation of sGC/cGMP system and induced expression of antioxidant and pro-survival pathways. nNOS expression is blocked in part by sequestration of free intracellular calcium via cGMP and inhibition of calcium influx through NMDA receptor. (B) Trophic factor deprivation in motor neurons leads to activation of the Fas pathway associated with induction of nNOS and increased NO production. Membrane-bound or soluble FasL binding to Fas receptor activates both Daxx and FADD components of the pathway. Downstream of Daxx, NO produced from p38-induced nNOS expression reacts with superoxide ($O_2^{\cdot-}$) to form of peroxynitrite ($ONOO^-$). FADD activates caspase- and mitochondrial-dependent mechanisms of apoptosis. Fas pathway activation can also occur in mutant SOD1-expressing motor neurons exposed to exogenous NO with adequate trophic factor support, suggesting unique susceptibility to Fas- or NO-triggered cell death in familial ALS. Feed-forward amplification of the Fas/NO loop may contribute to the slow, progressive demise of motor neurons.

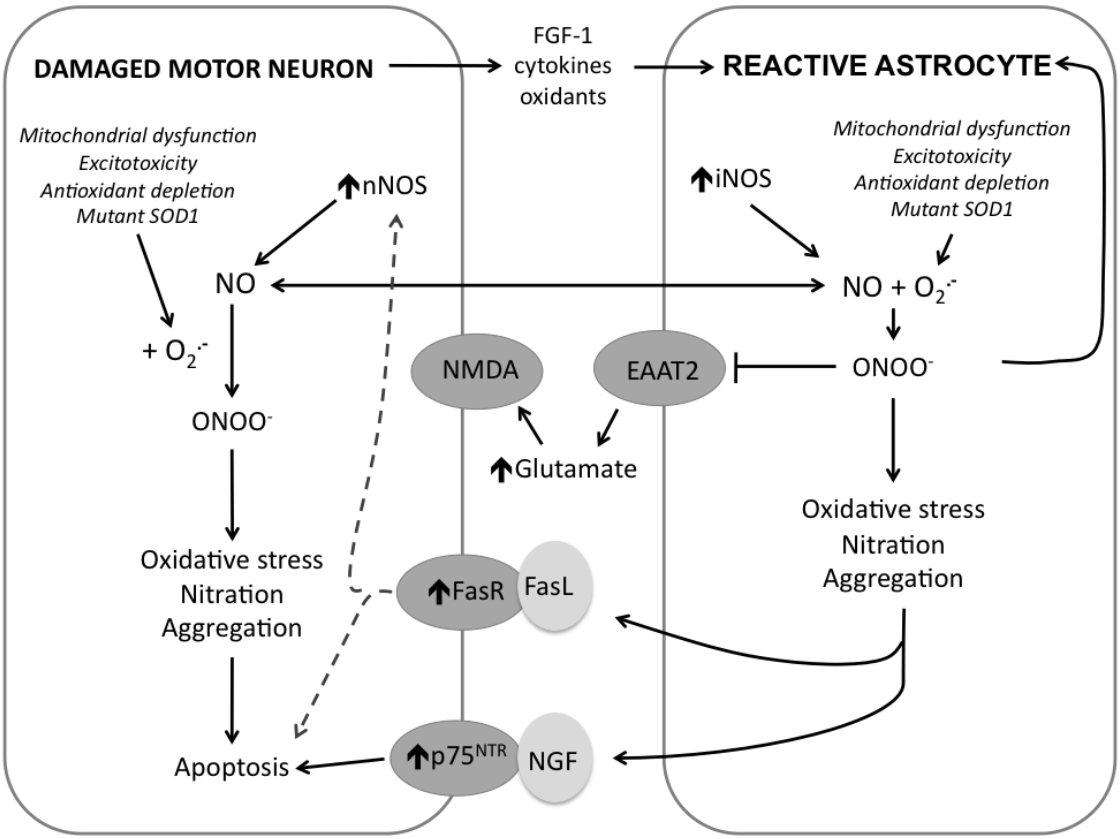


**Figure 2. Interplay between motor neurons and astrocytes in ALS.** In response to injury or chronic oxidative stress, damaged motor neurons upregulate expression of critical genes involved in their survival/death including nNOS, Fas, and $p75^{NTR}$, increase superoxide production via numerous sources, and release inflammatory mediators such as FGF-1, cytokines, NO, and other oxidants. These soluble factors promote astrocytes to adopt a reactive phenotype associated with induction of iNOS and mitochondrial dysfunction. Increased production of NO and superoxide favors the formation of peroxynitrite ($ONOO^-$). Peroxynitrite has multiple effects: 1) further promoting reactive astrocyte phenotype, 2) inhibiting glutamate transporters (EAAT2), leading to increased extracellular glutamate concentrations and NMDA-induced excitotoxic injury in motor neurons, and 3) causing oxidative damage to cellular macromolecules leading to increase in general markers of pathology including protein nitration and aggregation. Reactive astrocytes release excessive NO and soluble proapoptotic factors (FasL and NGF) that activate upregulated receptors to amplify damage in affected motor neurons, ultimately leading to apoptosis.

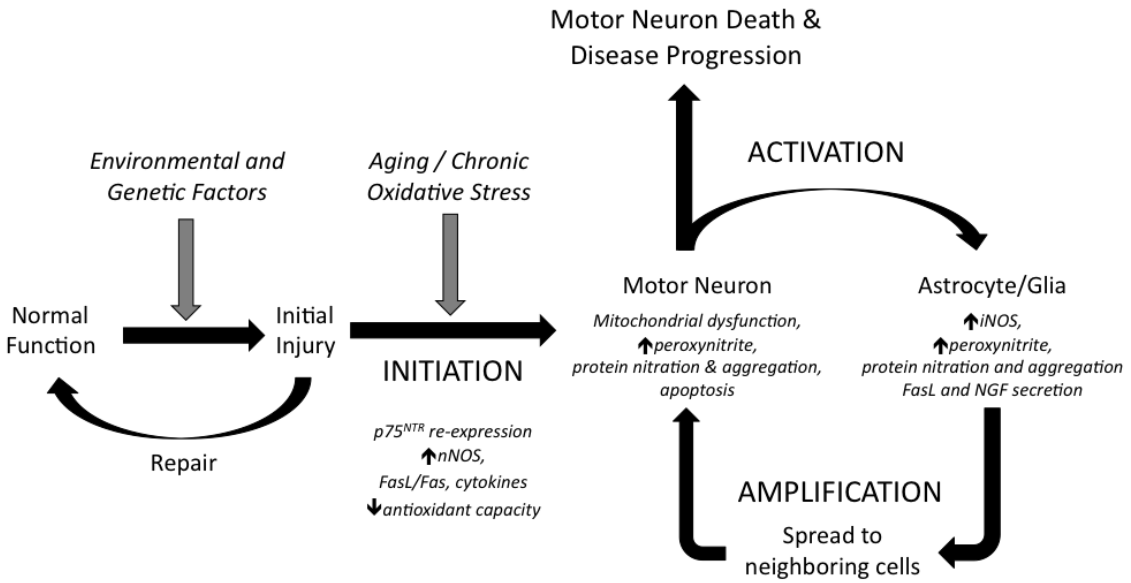


**Figure 3. Model of progressive neurodegeneration in ALS.** The proposed mechanism leading to motor neuron death in ALS includes three key steps: initiation, activation, and amplification. **INITIATION:** Cells and tissue possess effective means to repair injury, but aging or chronic stressors including oxidative stress can lead to physiological adaptations aimed to determine cellular survival. Changes including upregulation of nNOS, $p75^{NTR}$, Fas, and cytokines coupled with decreased anti-oxidant capacity leave damaged motor neurons susceptible to oxidative stress resulting from increased ROS/RNS production. **ACTIVATION:** Neighboring astrocytes and other glial cells become hyperactive in response to mediators (FGF1, cytokines) released by motor neurons and enact an inflammatory response with induction of iNOS. Pathological changes cause motor neurons to become further damaged as the result of oxidative stress mediated by enhanced production of NO and peroxynitrite. Astrocytes become further activated, adopt a reactive phenotype, and suffer non-lethal peroxynitrite-mediated oxidative damage. **AMPLIFICATION**: Astrocytes amplify motor neuron damage through production of NO, cytokines, and proapoptotic factors such as NGF and FasL. Both motor neurons and astrocytes display increasing markers of pathology including protein nitration and aggregation and mitochondrial dysfunction. Feed-forward reinforcement of the activation/amplification loop via NO-mediated oxidative damage spreads damage to neighboring cells within tissue via diffusion of NO and secretion of soluble inflammatory and pro-apoptotic factors. Increasing numbers of motor neurons and glia become affected which leads to disease progression.