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Roles of Reactive Oxygen and Nitrogen Species in Pain

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Abstract

Peroxynitrite (PN, $ONOO^-$) and its reactive oxygen precursor superoxide (SO, $O_2\cdot^-$), are critically important in the development of pain of several etiologies including in the development of pain associated with chronic use of opiates such as morphine (also known as opiate-induced hyperalgesia and antinociceptive tolerance). This is now an emerging field in which considerable progress has been made in terms of understanding the relative contribution of SO, PN, and nitroxidative stress in pain signaling at the molecular and biochemical levels. Aggressive research in this area is poised to provide the pharmacological basis for development of novel non-narcotic analgesics that are based upon the unique ability to selectively eliminate SO and/or PN. As we have a better understanding of the role of SO and PN in pathophysiological settings, targeting PN may be a better therapeutic strategy than targeting SO. This is due to the fact that unlike PN, which has no currently known beneficial role, SO may play a significant role in learning and memory [1]. Thus, the best approach may be to spare SO while directly targeting its downstream product, PN. Over the last 15 years, our team has spearheaded research concerning the roles of SO/PN in pain and these results are currently leading to the development of solid therapeutic strategies in this important area.

Keywords

superoxide; peroxynitrite; pain; superoxide dismutase mimetics; peroxynitrite decomposition catalysts; rostral ventromedial medulla (RVM); central sensitization; glutamatergic neurotransmission; neuroimmune activation

Introduction

Pain that is refractory to the typical arsenal of analgesic drugs is a major health issue in the US [2]. While selective cyclooxygenase-2 (COX-2) inhibitors can be effective in certain types of chronic pain, their now well-document side-effects including increased risks of

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heart attack and stroke [3] limit their use. While opioid narcotics such as the mainstay morphine are the most effective treatments for acute and chronic severe pain, their clinical utility is nearly always hampered by the development of analgesic tolerance as well as painful hypersensitivity known now as morphine-induced hyperalgesia [4–6]. Typically the development of tolerance to morphine treatment necessitates escalating doses to achieve equivalent pain relief [7]. Unfortunately, with extended treatment, the onset of morphine-induced hypersensitivity counteracts the therapeutic impact of such dose increases [4–6]. Thus, in order to maintain the control of chronic severe pain in a growing number of older patients with a variety of complicating conditions, opioid treatment leads to debilitating side effects of oversedation, reduced physical activity, respiratory depression, constipation, and even potential for addiction [7]. Therefore, we have been keenly interested in developing new approaches that would maintain opiate efficacy during chronic dosing without engendering tolerance or unacceptable side effects. Considerable evidence implicates SO and PN [formed from the diffusion controlled reaction of SO with nitric oxide (NO)] in the development of chronic pain, the transition of acute to chronic pain, as well as opiate-induced hyperalgesia and antinociceptive tolerance. Thus, PN appears to be a key toxic mediator for target-based therapeutic strategies. To date, a number of approaches for the development of metal-based catalysts and non-metal scavenger systems [8–9] have been reported to effectively prevent the formation of PN through the dismutation of SO (superoxide dismutase mimetics, SODms) or to decompose PN once it is formed (PN-decomposition catalysts, PNDCs) [10–14]. The contributions of SO and PN to the development of peripheral and central sensitization associated with pain are also adding evidence that these species are novel targets for pain management (Figure 1). Importantly, numerous studies demonstrate that pharmacologic inhibition of SO and PN can prevent and reverse the characteristic pathologies associated with inflammatory pain, neuropathic pain, and morphine-induced hyperalgesia and tolerance.

Alterations in glutamatergic neurotransmission and neuroinflammation as well as modulation of ion channels such as the transient receptor potential cation channel, subfamily V, member 1 (TRPV1) underlie the development of central sensitization (a putative pathophysiologic state underlying persistent and chronic pain) associated with acute and chronic inflammatory and non-inflammatory neuropathic pain. These alterations take place in the periphery, in the spinal cord, and at supraspinal sites such as in the RVM. A comprehensive analysis in the scientific domain as it pertains to pain and non-pain related areas reveals that each of these signaling pathways can be affected by SO and PN. To fully appreciate the roles of SO and PN in pain, this review will discuss their involvement in pain signaling. It is not our intention to discuss the chemistry of SODm or PNDCs since this will be covered in this special issue of FRBM. In addition, it is not our goal to discuss the relative contribution of NO in pain signaling as this topic has been covered for over a decade. More importantly, it has become apparent that therapeutic strategies targeting this reactive oxygen species have not yet yielded successful outcomes. We will briefly discuss potential pharmacologic alternatives that target PN and will suggest future directions in nitroxidative pain research based upon collective scientific outcomes in the field.

Brief overview of existing therapeutic strategies targeting superoxide and peroxynitrite

Improvement in our knowledge of the mechanisms underlying the transition of acute to chronic pain, the formation and exacerbation of neuropathic pain and the development of opioid-induced tolerance and hyperalgesia, would have a major impact in the treatment of pain in general. Over the last decade, our research efforts have established the key role of SO and PN in the development of pain of several etiologies. This research has provided the foundation for improving our mechanistic knowledge regarding the formation and

maintenance of chronic pain states but has also illuminated a promising strategy for treatment of these conditions; namely the eradication of PN through prevention of formation or catalyzed decomposition. The most promising class of SO scavengers is comprised of compounds that are essentially functional mimetics of the SOD enzymes. To date, two classes have emerged as promising candidates for potential clinical agents and important pharmacological tools. These are iron (III) and manganese (III) complexes of the synthetic porphyrins (e.g. MnTE-2-PyP^{5+}, FeTM-4-PyP^{5+}), and functionalized manganese (II) polyazamacrocycles (e.g. SC-72325) [9, 15–16]. Both of these very distinct classes of metal complexes catalyze the disproportionation of SO to hydrogen peroxide and molecular oxygen. While most of the manganese (II) polyazamacrocycles have little reactivity toward PN, the metalloporphyrins highlighted above are potent PNDCs [9, 15–16]. Thus, most metalloporphyrins possess dual SODm and PN decomposing activities.

Roles of superoxide and peroxynitrite in pain

Superoxide and PN have emerged as powerful pronociceptive reactive oxygen and nitrogen species [17–18]. This realization was achieved by use of SODm such as SC-72325 or PNDCs including FeTM-4-PyP^{5+} and MnTE-2-PyP^{5+} as pharmacological tools to identify them and to dissect signaling pathways. Direct contribution of SO and PN was demonstrated by showing that intraplantar injection of SO or PN leads to the development of hyperalgesia [19–20]. Subsequent studies revealed that increased formation of SO/PN is critically important in the development of thermal hyperalgesia associated with acute and chronic inflammation [19–24], in response to spinal activation of the N-methyl-D-aspartate receptor (NMDAR) [25], in the development of orofacial pain [24] and in the development of opiate-induced hyperalgesia and antinociceptive tolerance [26–28]. An imbalance between oxidant/antioxidant activities has also been observed in other models of inflammatory nociception. For example, increased levels of hydrogen peroxide (the dismutated product of SO) [29] and decreased levels of SOD activity in the spinal trigeminal nucleus coincided with facial hyperalgesia induced by a formalin injection into the lip [30]. Superoxide is also increased in dorsal horn neurons during neuropathic pain induced by spinal nerve ligation [31] and neurogenic-induced hyperalgesia via capsaicin administration[32]. Importantly, the PNDCs evaluated to date, synergize with non-selective COX-1/COX-2 inhibitors, selective COX-2 inhibitors [20], and opiates (Salvemini, manuscript in preparation). This has an enormous added advantage, as it would allow for the possibility of increasing the efficacy of these drugs at much lower doses thereby reducing their well-documented side-effect profile [33–34]. These findings led us to put forth the hypothesis that targeting SO and PN should lead to development of novel analgesics for the management of pain [17–18]. Importantly, it should be noted that SO and PN have no role in acute and thus beneficial physiological nociception [19–20]. A role for nitroxidative stress (herein defined as stress induced in the presence of SO, PN and related species) was supported using a variety of non-selective agents such as phenyl N-tert-butylnitrone (PBN) and 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL) [15, 17, 35]. These agents showed efficacy in inflammatory [22] and neurogenic pain [32, 36–38], visceral pain [39], neuropathic pain [31, 37, 40–41], and chemotherapy-induced pain [42]. As we emphasized [9, 35, 43], non-selective agents such as TEMPOL or PBN cannot be used to delineate the contribution of a specific nitroxidative species (i.e. SO vs. PN), since these agents will remove many reactive oxygen and nitrogen species including but not limited to NO, SO, PN and hydroxyl radicals [9, 35, 43]. When using such non-selective probes (for example TEMPOL or PBN) it is important to discuss results in terms of “nitroxidative stress and nitroxidative species” rather than implicating a specific species such as SO or PN. This would lead to inappropriate implication of one species versus another in a particular setting- for instance results obtained with TEMPOL implicate nitroxidative species and not SO (SO would only be one component) and results should be discussed accordingly to avoid misinterpretations.

Enzymatic pathways leading to overt formation of superoxide and peroxynitrite in pain

Unraveling the enzymatic sources that produce SO and PN and understanding the signaling pathways engaged by these species in nociceptive processing is of paramount importance [17–18]. Inactivation of mitochondrial manganese superoxide dismutase (MnSOD), the enzyme that normally keeps SO under tight control, [29] is a central source for SO-derived PN in several diseases driven by overt production of PN [44]. Such enzymatic inactivation results from nitration of Tyr-34 by PN in a manganese-catalyzed process [45]. In a series of studies, our group revealed that spinal nitration and inactivation of MnSOD provides a critical “feed-forward” mechanism that allows for the accumulation of SO and PN during the development and maintenance of central sensitization [19, 25–28]. These findings were confirmed and subsequently extended by others [32, 38]. Thus, inactivation of mitochondrial MnSOD is a central site for the increased production of SO and PN in nociceptive signaling [17–18, 46]. Superoxide and PN can also be generated from the mitochondrial electron transport chain; to this end it was reported that production of mitochondrial SO by intrathecal injection of inhibitors of the electron transport complex (i.e. antimycin A or rotenone) in mice leads to mechanical hyperalgesia [47]. Another important SO-generating enzyme system is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [48] which was recently implicated in the development of central sensitization associated with inflammatory hyperalgesia [49] and peripheral nerve injury-induced neuropathic pain [50]. This SO-generating enzyme is dormant in resting cells and produces SO only upon activation. The principal regulation of NADPH oxidase is post-translational and depends on the assembly of several membrane-bound and cytosolic components to form an active enzyme complex [51]. In resting cells, the enzyme consists of two membrane-bound components, gp91*phox* and p22*phox*, and several cytosolic components, including p47*phox*, p40*phox*, p67*phox*, and rac1/2 [51]. Gp91*phox* is a flavocytochrome and the catalytic core of the enzyme. Upon activation, the cytosolic components translocate to the membrane and associate with membrane components to form an assembled, activated, and SO-producing enzyme complex [51]. Although this enzyme is best characterized in immune cells and leukocytes for its involvement in SO production, it is now known that various protein components of NADPH oxidase are expressed in neurons, astrocytes, and microglia [52–54]. Importantly, SO auto-augments its formation by up-regulating the expression of the Rac1 and gp91*phox* subunits of the holoenzyme and creates a self-perpetuating cascade [55–56]. Furthermore, protein kinase C (PKC), a kinase activated in peripheral [57–60] and central sensitization [61–65], regulates many of the NADPH oxidase subunits directly [66–67] or through the activation of extracellular signal-regulated kinases (ERK)1/2 or mitogen-activated protein kinase (MAPK) pathways [67–69]. Serine phosphorylation of the p47*phox* subunit by PKC α [67, 70–72], βII [66–67, 70], δ [67, 70, 73], ε [67], or ζ [70, 74] initiates the translocation of the cytosolic NADPH-oxidase regulatory complex and stimulates SO production. PKC-induced phosphorylation of other NADPH oxidase subunits (p67*phox* [75], p40*phox* [76], and gp91*phox* [77]) that induce or enhance SO production have also been described. PKC has also been demonstrated to be a key regulator in growth factor receptor-induced NOX1 expression [78], an isoform equivalent to catalytic gp91*phox* subunit. Therefore, post-translational nitration and inactivation of MnSOD and activation of NADPH oxidase may represent two pathways that operate in synchrony to maintain central sensitization (Figure 2). We have reported this to be the case in the development of morphine-induced hyperalgesia and antinociceptive tolerance [79].

Potential signaling pathways engaged by superoxide and peroxynitrite in nociceptive signaling

Modulation of protein kinases, alterations in glutamatergic neurotransmission, neuroinflammation, and modulation of ion channels such as TRPV1 underlie the development of central sensitization associated with acute and chronic inflammatory and non-inflammatory neuropathic pain. These alterations take place in the periphery, in the spinal cord and at supraspinal sites such as in the RVM. A comprehensive analysis in the scientific domain as it pertains to pain and non-pain related areas reveals that each of these signaling pathways can be affected by SO and PN. Additionally, nitroxidative species may be involved more subtly in central sensitization at least in part by sensitizing wide dynamic range neurons in the dorsal horn [36]. We will briefly review some of these signaling pathways.

Protein kinases, superoxide and peroxynitrite

Activity of protein kinases such as PKC, protein kinase A (PKA) and calcium/calmodulin-dependent protein kinase II (CaMKII) are central to many of the neuronal and glia signal transduction pathways leading to the development of peripheral and central sensitization. Activation of PKC, in particular, is necessary for the development of peripheral [57, 59–60, 80] and central sensitization [61–65] of several pain etiologies. Peripheral activation of PKC occurs within many of the G protein-coupled receptor signaling pathways that include protease- [81] and interleukin (IL)-6-induced [82] TRPV1 sensitization, prostaglandin (PG) E_2-mediated sensory neuron sensitization [83–84], paclitaxel-induced hyperalgesia [85], calcitonin gene related peptide (CGRP) production in dorsal root ganglia through sensory neuron specific receptor (Mrg) stimulation [86], and TRPV4-induced mechanical hypersensitivity [87]. Activation of PKC in the central nervous system (CNS) occurs in neurons in response to peptide neurotransmitters [61, 88] and following glutamatergic receptor stimulation [89–92] that enhance synaptic plasticity. In astrocytes, PKC activation modulates various processes in central sensitization that include: cytokine/PGE_2-mediated increases in inducible NOS (iNOS) [93], bradykinin-induced phospholipase A2 (PLA2) activation and COX2 production[94–95], IL-1β-induced reduction in gap junction formation [96], morphine-induced ERK activation [97], sphingosine 1-phosphate-induced nerve growth factor (NGF) expression [98], and glutamate-induced glial activation and subsequent reduction in the glutamate transporter (GT) GLT-1 surface expression [99–100]. The importance of PKA activity is demonstrated in tumor necrosis factor (TNF)-α [101] or PGE_2-mediated [83–84, 102–103] sensitization of peripheral sensory neurons, TRPV1 [104–105] and TRPV4 [87] sensitization, paclitaxel-induced hyperalgesia [85], and phosphorylation and activation of NMDA receptors [106]. CAMKII activity facilitates the development of inflammatory- [107–109] and capsaicin-mediated hyperalgesia [110], morphine tolerance [111–113], and central sensitization [114].

The activation of protein kinases can be modulated, in part, by nitroxidative species. As described earlier, PKC is a recognized regulator of NADPH oxidase activity [89, 115] in a wide variety of pathologies and tissues. However, SO can activate PKC as demonstrated by increased autophosphorylation and activation of PKC by endogenous SO in hippocampal tissue [116] and by enhanced PKC-modulated persistent sodium currents [117] in the presence of SO, whereas removal of SO by antioxidants or SODm attenuates high frequency-stimulated PKC activation in neurons [116]. In addition to SO, PN facilitates PKC activation and translocation to the membrane [118] by direct nitration of PKCα, γ, and ε isoforms [118–120] or by stimulating proteolytic activation of PKC [121]. Ibi et al [49] demonstrated that accelerated PKCε translocation, possibly resulting from cysteine oxidation in the catalytic 1A site of PKCε [49] in response to NADPH oxidase-derived SO

production in DRG neurons, was essential for the development of thermal and mechanical hyperalgesia. However, the effects of PN on PKC activity can be 1) dose-dependent as low PN concentrations enhance co-factor dependant PKC activity and higher PN concentrations irreversibly inhibit PKC activity through nitrotyrosine formation [120] and 2) cell-type specific as PN can be lethal in neurons [122], whereas PN-mediated PKC activity is cytoprotective for astrocytes [123–124], endothelial cells [125], and monocyte/macrophage through activation of cPLA2 and arachidonic acid formation [126].

In addition to PKC, nitroxidative species may play a role in PKA-mediated and CAMKII-mediated hyperalgesia, though the mechanisms are less well understood. Evidence for nitroxidative activation of PKA comes from studies where the antioxidant, PBN, reduced the levels of PKA-specific NMDA receptor phosphorylation and attenuated capsaicin-induced hyperalgesia [37] and where PN decomposition prevented PGE_2-mediated thermal hyperalgesia and potential downstream PKA activity following intraplantar SO administration [20]. Although there is little evidence for direct nitroxidative activation of CAMKII, CAMKII is activated in the presence of mitochondrial SO-stimulated calcium influx following electrical stimulation [116] and PN-mediated p38 activation in PC12 cells requires, in part, the activation of CAMKII [127]. One possible nitroxidative-mediated CAMKII regulatory mechanism may occur through inactivation of the protein phosphatases responsible for downregulating CaMKII autophosphorylation and activity [128–129], since a reduction in SOD activity or increased SO reduces the protein phosphatase levels and dephosphorylation of CAMKII-activated cAMP response element-binding (CREB) [130] by calcineurin, respectively.

Alterations of glutamatergic neurotransmission by nitration

Dysfunction of the glutamatergic pathway is a key component of nociception [4, 131–134]. A key property of PN lies is its ability to post-translationally nitrate tyrosine and consequently modify protein function [12, 135–137]. Protein nitration is increasingly recognized as an important occurrence during cell signaling and regulation of protein activity [138]. The advent of proteomics and the development of immunological and analytical methodologies have revealed that tyrosine nitration is limited to specific proteins though the basis for this selectivity is not fully understood [12, 135–137]. Although there is no defined mechanism of removal of this modification, there is evidence that such a signal could be turned off by protein degradation or denitration by a “denitrase” [139]. Of the known proteins that are post-translationally modified by PN, the following are of particular significance: neuronal NMDARs [140–142], PKC [118–120], glia-derived GTs such as GLT-1 and GLAST [143–144] and glutamine synthetase (GS) [145–150]. These proteins are involved in ensuring optimal glutamatergic neurotransmission and thus optimal neuronal activation whereas dysregulation of their biological properties, such as would occur by nitration, will have critical consequences in events underlying central sensitization (findings summarized in Figure 3). [18, 46]

Glutamate (a primary endogenous ligand for the NMDAR) is a major neurotransmitter mediating the fast excitatory transmission at central synapses, and it plays critical roles in synaptic plasticity and development. It is now well established that glutamate and glutamate neurotransmission plays a critical role in the development of acute and chronic inflammatory pain (i.e. arthritis), neurogenic pain [104, 151], neuropathic pain [152–154] and in the development of opioid hyperalgesia/tolerance [155–156] associated, in part, with increased neuronal NMDA receptor activity in the spinal cord as reflected by increased phosphorylation of its NR1 subunit [152–153, 155–183]. The increased expression [184–186] and PKC-mediated phosphorylation [184, 187] of NR1, a NMDAR subunit essential for central sensitization, [188] increases the activation of NMDARs and thus neuronal activity [189]. For example, and in agreement with these findings, spinal administration of

an antisense oligonucleotide to NR1 attenuates hyperalgesia/tolerance [190–191]. In addition, a free radical scavenger reduces spinal NR1 phosphorylation in neuropathic and inflammatory hyperalgesia, underscoring the contribution of nitroxidative species to NMDAR activity in central sensitization [37]. PN interacts with NMDARs leading to nitration of the tyrosine residues present on NR1 subunits [140–142]. This event is an irreversible reaction that leads to constant potentiation of the synaptic currents and calcium influx, overt activation of NMDARs and ultimately excitotoxicity [140–142]. In addition to its direct effects on NMDARs, PN nitration and activation of PKC provides a potential mechanism for enhancing NR1 phosphorylation. Collectively, these processes lead to neuronal activation which has been extensively studied following noxious stimulation by quantifying neurons expressing the proto-oncogene c-fos protein in regions of the dorsal horn of the spinal cord that are responsible for nociceptive signaling (i.e. laminae I, II, and V) and have been corroborated with electrophysiologic patterns of activity [192–198]. Importantly, c-fos expression increases in the superficial dorsal horn in morphine tolerant rats [199–200] and inhibition of NMDAR activation [201] reduces c-fos expression. Phosphorylation of neuronal NR1 increases neuronal excitation, as evidenced by increased c-fos expression in the spinal cord [189]. This phenomenon has been linked to the development of central sensitization associated with pain of several etiologies as well as in the development of morphine-induced hyperalgesia and antinociceptive tolerance [189, 202–204]. In support, administration of a NR1 antisense oligonucleotide reduces c-fos expression [191].

Extracellular glutamate concentration has to be kept low enough to terminate glutamate receptor activation and to protect neurons from glutamate excitotoxicity [205–207]. Glutamate is not metabolized by extracellular enzymes, but rather has to be removed from the synaptic cleft by cellular uptake: increased [glutamate] will affect both neuronal and glial cell function with dysregulation of this pathway impacting optimal neuronal activity and neurotransmission. The homeostasis of extracellular glutamate is tightly regulated by sodium-dependent high-affinity GTs in the plasma membranes of both neurons and glia [208–211]. At least five membrane GT subtypes have been cloned including GLAST (EAAT1), GLT-1 (EAAT2), EAAC1 (EAAT3), EAAT4, and EAAT5. Three GT subtypes isolated in the spinal cord [GLAST and GLT-1 associated with glial cells [212–213] (mainly in astrocytes but also found in microglia), and EAAC1 associated with neurons [214–219]] are considered essential to maintain low resting levels of glutamate (<1 μM) and to prevent over-stimulation of NMDARs [210, 220–223]. The glia-derived GLAST and GLT-1 concentrated in the superficial dorsal horn of the spinal cord are responsible for >90% of total glutamate transport [224]. If GLAST/GLT-1 function is compromised (i.e. reduced or eliminated) such as would occur when these GTs are nitrated, [glutamate] increases in the CSF contributing to rapid alterations in synaptic transmission [225–228]. Besides regulating synaptic levels of glutamate, these GTs play a crucial role in the uptake of cysteine, and thus contribute to the overall thiol redox state of cells normally regulated by intracellular levels of glutathione (GSH). GSH plays a critical role in protecting cells from oxidative stress as well as maintaining the thiol redox state. The depletion of GSH enhances oxidative stress leading to neuronal degeneration as shown in several studies [229–230]. Glutathione is a tripeptide composed of glutamate, cysteine and glycine residues with an unusual peptide bond between the α-amine of cysteine and the side chain carboxylate of glutamate. In neurons, cysteine is the rate-limiting substrate for GSH synthesis [231] and in neurons approximately 90% of total cysteine uptake is mediated by GTs and in particular EAAC1[232–234]. Thus, EAAC1 transports cysteine at a rate comparable to that of glutamate, with an affinity 10- to 20-fold higher than that of GLAST or GLT-1 [235]. Recent studies have shown that PN-mediated nitration of EAAC1 in neurons reduces the uptake capacity of cysteine leading to a depletion of intracellular GSH and neuronal cell death [236]. Integrating these findings, central sensitization could also develop due to

excitotoxicity from increased synaptic concentrations of glutamate and a decrease in neuronal thiol redox state due to decreased intracellular levels of cysteine and thus GSH (Figure 3).

In contradistinction to the central role of GTs in regulating the homeostasis of extracellular glutamate and cysteine, GS another key enzyme in the regulation of glutamate neurotransmission in the CNS plays a pivotal role in its intracellular metabolic fate [237]. GS catalyzes the synthesis of glutamine from glutamate, and is responsible for the detoxification of ammonia in the brain.[237] GS is of critical importance in the CNS as although glutamate has a number of metabolic enzymes, the only known pathway for the synthesis of glutamine is via GS. GS is a major control point for nitrogen metabolism and since glutamine is the amino group donor in many important biosynthetic reactions, reducing its concentration in astrocytes could lead to defects in nitrogen homeostasis and hinder key biosynthetic pathways In the CNS, GS is located mainly in astrocytes and one of the primary roles of these cells is to protect neurons against excitotoxicity by taking up excess ammonia and glutamate, converting them into glutamine [237–238]. Indeed neurons depend on astrocytes for protection against glutamate toxicity [239] since it has been reported that glutamate is highly toxic to neurons in the absence of astrocytes [240]. Glutamine is then transported out of the astrocyte via glutamine transporter into neurons, where it serves as a precursor for the formation of glutamate and γ-aminobutyric acid (GABA) [241–242]. Nitration of GS (on Tyr 160) is intimately linked to inactivation of its biological function triggering loss of enzyme activity [148]. GS inactivation through nitration has been shown to occur after *in vitro* treatment with PN [147], in ammonia-intoxicated rat brain [149], endotoxin-treated rat liver [148], brains of epilepsia [150], hepatic encephalopathy [146] and as shown by our group pain of various etiologies [27, 243]. Nitration of GS will facilitate neuronal excitation [237, 244]. Inhibition of GS will also lead to [ammonia] with ensuing additional detrimental effects. One of the earliest events associated with increased [ammonia] is astrocyte swelling. This phenomenon could be very important in contributing to increased synaptic [glutamate] and overall excitotoxicity since astrocyte swelling has been linked to increased release of glutamate due to the opening of channels activated by swelling [245–246]. Other astrocytic activities such as neurotransmitter uptake, GS synthesis, blood-brain barrier transport and so forth can also be disrupted in response to astrocyte swelling [237]. Furthermore, astrocyte swelling leads to the formation of nitroxidative species and in turn, nitroxidative species trigger astrocyte swelling providing a self-amplifying signaling loop, which may enhance pathways leading to central sensitization [149, 247]. Furthermore, through feedback regulation, a decrease in the GS activity, as would occur when this enzyme is nitrated, reduces the activity of GTs [237] and in turn, reduced GT activity (following direct nitration or in response to reduced GS) can directly increase NMDAR activity [184, 226] underscoring the reciprocal feed-forward impact of post-translational nitration on these pathways. Increased levels of glutamate can be decreased by reducing the production of cytokines such as TNF-α and IL-6 that have been shown to inhibit glutamate uptake [248]. Since PN increases cytokine production (*vide infra*), it is likely that PN modulates glutamate homeostasis via the cytokine signaling pathway.

In summary, post-translational modifications of proteins involved in the tight regulation of glutamate homeostasis may provide a unifying link in signaling events underlying central sensitization.

Neuroinflammation, superoxide and peroxynitrite

Neuroinflammation following the activation of glial cells is important to the development and maintenance of central sensitization [249]. Numerous stimuli such as neurotransmitters and proinflammatory mediators can activate glial cells (e.g. astrocytes and microglia), which

results in glial cell release of pro-inflammatory cytokines, excitatory amino acids, and nitroxidative species that contribute to central sensitization [250]. For example, the neuropeptide CGRP causes the release of proinflammatory cytokines from astrocytes (TNF-α and IL-1β) and microglia (IL-6) [251]. Additionally, prevention of neuroinflammation with minocycline blocks neuropathic hyperalgesia and allodynia [252]. Neuroinflammation may occur through Toll-like receptor-4 (TLR4) signaling in glial cells; this signaling contributes to both hyperalgesia and allodynia [253–257]. A series of studies by Watkins and colleagues has established that TLR4 is necessary for the actions of morphine including TLR4 activation for morphine metabolite (morphine-3-glucuonide)-induced hyperalgesia, allodynia, and neuroimmune activation [257–258]. Stimulation of TLR-4 can result in the production of the proinflammatory cytokines TNF-α and IL-1β that contribute to central sensitization through the downstream initiation of inhibitor of κBα (IκBα), c-Jun N-terminal kinase (JNK), and p38 pathways that promote proinflammatory cytokine transcription through nuclear factor κB (NFκB) and activator protein (AP-1) [253, 259]. It is well known that PN also activates the redox-sensitive transcription factor NFκB and several MAPKs, including p38 and ERK1/2, that regulate the production of many proinflammatory mediators and cytokines [131–133, 260–265]; to this end we have previously reported that spinal PN contributes to the development of central sensitization associated with inflammation and chronic administration of morphine via increased formation of glia-derived cytokines [17–19, 26–27, 79, 266]. Thus, systemic or intrathecal delivery of SODm blocks increased formation of cytokines in spinal cord and attenuates hyperalgesia [19, 25, 27]. Inhibition of cytokine formation also prevents the development of peripheral sensitization and hyperalgesia associated with inflammation [19, 26].

Superoxide, peroxynitrite and cyclooxygenase

One potential molecular pathway by which SO and/or PN may influence inflammatory events associated with the development of altered pain sensitivity is through modulation of COX enzymes [267–268]. As originally reported by our group [269] and subsequently extended by several other investigators [268, 270–271], the COX enzymes (constitutive COX-1 and inducible COX-2) are “receptor targets” for the multifaceted action of NO and as such are regulated in its presence. Although the mechanisms by which NO activates COX enzymes remain undefined, we now know that PN is involved in this activation through the oxidative inactivation and/or modification of key amino acids residues in the COX polypeptide backbone [272–273]. In addition to effects on COX-2 enzyme activity, PN (and NO) increase the production of PGs from macrophages by acting post-transcriptionally or translationally to increase COX-2 protein levels or to increase its mRNA stability, at least in part through SO and the p38 MAPK pathway [270–271, 274–277]. Furthermore, iNOS binds COX-2, and iNOS-derived NO increases the catalytic activity of COX-2 through S-nitrosylation in a macrophage cell line [278]. Other possibilities in this complex reaction biochemistry have been raised and discussed in detail [279]. We have reported that activation of COX-1 and activation/induction of COX-2 by SO and PN and subsequent increase in PGE_2 contributes to the development of peripheral sensitization associated with inflammation [20].

Superoxide, peroxynitrite and TRPV1

The TRPV1 integrates multiple endogenous and exogenous pain stimuli and its activation results in intracellular sodium and calcium influx [280–283]. These receptors are found in the periphery (small and medium primary afferent neurons) and CNS within areas responsible for nociceptive signaling [284–286] and are essential to inflammatory thermal hyperalgesia [287–289]. Indeed, TRPV1 activation is also associated with enhanced glutamatergic signaling [290–294] and facilitation of long-term potentiation [295], which

are important contributors to central sensitization. The unique ability of TRPV1 to modulate pain resulted in therapeutic targeting of this receptor for pain management [296–298].

Sensitization of TRPV1 during inflammatory pain depends upon numerous mechanisms. For example, phospholipase C activation (via growth factors, neurotransmitters, and inflammatory mediators), PGE_2 (via cAMP activation of PKA), and prostacyclin all enhance TRPV1 sensitization [105, 298–299]. Phosphorylation of serine and/or threonine residues also results in TRPV1 sensitization. Sources of TRPV1 phosphorylation include PKA, PKC and CAMKII [105, 300–304]. The sensitization of TRPV1 may be regulated by nitroxidative species through the modulation of PKC and PKA (see above). Nitroxidative species are involved in both the downstream actions of TRPV1 activation and modulating TRPV1 expression and sensitization.

Numerous studies suggest that TRPV1 sensitization results in the production of nitroxidative species [305]. Production of reactive oxygen species can be TRPV1 dependent such as following TRPV1-mediated substance P release [306] and during reactive oxygen species-mediated afferent nerve fiber stimulation [307]. Direct activation of TRPV1 *in vitro* enhances the production of unstable hydroxyl radicals which is blocked with administration of a free radical scavenger and the TRPV1 antagonist, capsaizepine, in a joint inflammation model [308]. In addition, the NADPH oxidase-mediated production of reactive oxygen species in activated microglia is dependent upon TRPV1 receptor activity [309]. The NADPH/gp91*phox* production of SO also contributes to neurogenic vasodilation following TRPV1 activation and its mediated release of the neuropeptides substance P and CGRP [310]. Activation of TRPV1 in retinal explants was recently shown to enhance a biomarker of PN-mediated protein nitration, 3-nitrotyrosine [311]. Capsaicin administration and low pH solutions activate TRPV1 receptors and results in calcium influx with subsequent SO production in synoviocytes from arthritic rats [312]. This TRPV1-mediated calcium influx and nitroxidative species production is associated with reduced SOD levels and implicated in the mechanisms of cell death in numerous cell types [313–316]. Cell death via TRPV1 activation may result from the activities of nitroxidative species through p38 activation [317] and PN-mediated oxidative stress [316]. Recent evidence suggests that TRPV1 activation contributes to the maintenance of inflammation through the production of reactive oxygen species which act as signaling molecules to increase the expression of the TNF receptor in dorsal root ganglion neurons [318].

Conversely, nitroxidative species also modulate the activity, expression, and sensitivity of TRPV1 receptors. For example, SO stimulates TRPV1 activity in inflammatory states [307, 319] and this may occur through PKC phosphorylation of TRPV1 [320–321]. More specifically, peripheral SO-derived hydrogen peroxide requires TRPV1 activity to maintain thermal hyperalgesia [322]. Further, *in vitro* studies demonstrate that NADPH oxidase activity induces TRPV1 channel activity [323] and t-BOOH, a reactive oxygen species donor, increases TRPV1 protein expression [308]. Oxidative modification of TRPV1 cysteine residues (e.g. the formation of inter-cysteine disulfide bonds within its cytoplasmic termini) results in resistance to desensitization as well as sensitization of normal and reactivation of desensitized TRPV1 [324–325]. This direct oxidative modification and subsequent sensitization of TRPV1 may result in long-lasting pain signaling in nociceptive neurons [324].

Nitroxidative species may regulate TRPV1 expression through growth factors, transcription factors, and MAPK kinases. Nerve growth factor, a well described regulator of TRPV1 expression, acts through the tyrosine kinase receptor A (TrkA) and depends upon the Rac1/NADPH oxidase pathway-mediated activation of p38 MAPK to increase TRPV1 expression in PC12 and dorsal root ganglion cells [56, 326]. Puntambekar and colleagues [56] proposed

that the generation of reactive oxygen species (e.g. SO) and their subsequent activity as signaling molecules causes a positive feedback regulation to induce TRPV1 expression which helps to maintain peripheral neuron integrity, inflammation, and pain perception.

Interestingly, TRPV1 receptors also contribute to the development of hyperalgesia and allodynia through activation by endogenous lipids, the oxidized linoleic acid metabolites (9- and 13-hydroxyoctadecadienoic acid), which act as TRPV1 agonists in the periphery and spinal cord [327–328]. Because of the well-documented nitroxidative stress that occurs in the periphery and spinal cord during inflammatory hyperalgesia [19, 25, 329–330], it is possible that SO and PN also regulate TRPV1 activity through oxidation of linoleic acid and the increased formation of oxidized metabolites.

Nitroxidative species in supraspinal descending facilitation of nociception

A recent wealth of evidence has demonstrated that supraspinal descending modulation of spinal nociception is essential to pathologic pain states [331–337]. The role of nitroxidative species in supraspinal descending facilitation of nociception during pathologic pain is unclear; however, there is increasing evidence that suggests their activities in the brain may be critical to pain of several etiologies. We reported that PN-mediated activity, as evidenced by expression of the nuclear enzyme poly (ADP-ribose) polymerase (PARP) and decreased MnSOD activity, occurs in the brain during hyperalgesia and is prevented with PNDCs (MnTnHex-2-PyP^{5+} and MnTE-2-PyP^{5+}) [266]. Further, intracerebroventricular injections of free radical scavengers (i.e. PBN) attenuate inflammation and nerve injury-induced hypersensitivity to noxious and innocuous stimuli [36, 338]. More specifically, in the amygdala, facilitation of somatosensory and visceral nociception following type 1 metabotropic glutamate receptor activation is mediated by reactive oxygen species [339].

As previously described by our laboratory [46], one supraspinal nociceptive modulating center, the RVM, is a promising locus for nitroxidative species activities that substantially contribute to pain. The RVM facilitates nociception and drives central sensitization through well-described glutamatergic signaling pathways that depend upon the synthesis of the PN precursor NO [331]. During central sensitization, time-dependent cellular, biochemical, and molecular changes occur in the RVM that are comparable to those that occur in the spinal cord (e.g. enhanced glutamatergic signaling, NMDAR activation, neuroimmune activation with release of IL-1, IL-6, and TNF-α, and protein kinase activation) [335, 340–347]. As nitroxidative species are intimately involved with each of these changes in the spinal cord, it strongly suggests their potential role within the RVM. These findings have directed our current investigations and support our hypothesis that nitroxidative species activity within the RVM contributes to central sensitization [46].

Apoptosis, superoxide and peroxynitrite

Neuronal apoptosis is emerging as a significant contributor to the development of hyperalgesia and sensitization particularly in neuropathic pain and morphine-induced antinociceptive tolerance [157, 226, 348]. Nitroxidative species are potent inducers of apoptosis in neurodegenerative disease such as Alzheimer’s disease [349–350]. In pain, the role for nitroxidative stress-induced apoptosis is suggested by evidence that administration of a PNDC attenuates spinal apoptosis as marked by the reduction in spinal DNA damage and PARP activation in addition to preventing the development of morphine-induce hypersensitivity [27].

The specific mechanisms through which nitroxidative species initiate apoptosis in pain are poorly understood. However, PN administration leads to the nitration of mitochondrial proteins associated with the respiratory chain [351] and regulation of the mitochondrial

permeability transition pore complex (MPTPC) [352]. Protein nitration inactivates mitochondrial respiratory chain proteins [351] that, in turn, alter the mitochondrial membrane potential and reduce adenosine triphosphate (ATP) production [353]. The alterations in mitochondrial membrane potential and cellular energy status from mitochondrial protein nitration lead to the release apoptogenic protein such as caspases [354–358] and apoptosis inducing factor [359–360] through the MPTPC. Additionally, PN can nitrate the adenosine nucleotide translocator [352] protein that regulates the MPTPC activity [361], thus providing additional mechanism of nitroxidative stress-induced apoptosis. Peroxynitrite also induces DNA strand breaks and activates the PARP enzyme [362–363]. High PARP activity in response to high concentrations of PN reduces nicotinamide adenine dinucleotide (NAD+) concentrations that, in turn, reduces glycolysis and electron transport [364]. Excessive PARP activation and reduction in ATP leads to necrosis [365–366], however, caspases released from by the MPTPC cleave PARP reducing the necrosis and favoring apoptosis [367].

Conclusions and future outlook

Pharmacologic investigations of SO and PN and nitroxidative stress are critical to advance the body of knowledge concerning the contribution of these species to pain. As we move forward, it will be of paramount importance for researchers to fully understand the dominant in vivo pharmacological activities and selectivities of compounds capable of attenuating nitroxidative stress. To sort out the mechanistic details accurately, researchers need better pharmacological tools than are currently available (e.g. compounds with documented selectivity toward either SO or PN but not both). Until these types of selective scavengers are developed, we need to use what we have appropriately. It is important to note that retrospective analysis in clinical trials with recombinant bovine Cu/Zn SOD (Orgotein®) [9, 368–369] reveals that in humans, removal of SO (and thus PN) may have some analgesic effects. Interestingly, the first clinical pilot studies with the native enzyme were done as early as 1970's in rheumatoid arthritis (RA) and osteoarthritis (OA) with preliminary results demonstrating efficacy. Further studies showed that Orgotein® given by intraarticular injection, attenuated inflammation and pain of RA and OA [370–377] and led to a 60% decrease in the consumption of analgesics. Furthermore, Orgotein® was effective when given by intraarticular injection to patients with temporomandibular joint dysfunction and associated pain who had failed to respond to standard therapy [378] and reduced pain in patients with duodenal ulcer pain [379]. Other clinical settings in which SOD (whether recombinant or native bovine) was used included patients with Crohn's disease and various forms of periarticular inflammation. Eighteen patients with Peyronie's disease (exhibiting severe symptoms) who received Orgotein injected monthly into indurated areas of the penis, showed marked improvement, notably the loss of pain on erection [380–383]. Additional beneficial effects were seen in trigeminal pain, fibromyalgia and temporomandibular joint dysfunction [30, 384], chronic pancreatitis [385], and post-irradiation of breast cancer fibrosis [386]. Whether PN is the culprit or not remains to be proven, but it will be interesting and exciting to see whether levels of nitrated proteins correlate with pain in humans. We believe that continued research in this field will soon provide a valid pharmacological basis for developing PN-targeted therapeutic agents as novel non-narcotic analgesics in the management of pain and in particular chronic pain. The metalloporphyrin-based PN scavengers are exciting lead candidates in strategies that target PN alone or in synergistic combination with opioids or COX inhibitors. As metalloporphyrin systems have evolved in nature to be encased in protein (e.g. the cytochromes), small molecule porphyrin-based PNDCs will require peripheral synthetic modification to impart human pharmaceutical properties (i.e. membrane solubility, reduced charge, reduced non-target binding, reduced toxicity, optimal pharmacokinetics, etc). As the metal center in these systems is site for antioxidant action, the periphery of the porphyrin macrocycle is wide-

open for synthetic manipulations to control *in vivo* performance without negative perturbation of the catalytic apparatus. In the field of catalytic antioxidants, structure activity studies regarding the *in vivo* biodistribution of metalloporphyrin PNDCs of varying functionality has been largely unexplored. We believe that through medicinal chemistry modification of the ligand periphery, true drug candidates with tuned selectivities can be engineered.

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List of Abbreviations

TEMPOL	4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl
AP-1	Activator protein 1
ATP	Adenosine triphosphate
CREB	cAMP response element-binding
CGRP	Calcitonin gene related peptide
CaMKII	Calcium/calmodulin-dependent protein kinase II
JNK	c-Jun N-terminal kinase
cAMP	Cyclic adenosine monophosphate
COX	Cyclooxygenase
Cys	Cysteine
NO	Nitric oxide
EAAC	Excitatory amino acid channel
EAAT	Excitatory amino acid transporter
ERK	Extracellular signal-regulated kinases
FeTM-4-PyP^{5+}	Fe(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachlorideporphyrin
GABA	γ-Aminobutyric acid
Glu	Glutamate
GT	Glutamate transporters
GLAST	Glutamate-aspartate transporter
GLT-1	Glutamate transporter 1
Gln	Glutamine
GS	Glutamine synthetase
GlnT	Glutamine Transporter
GSH	Glutathione
Gly	Glycine
IκBα	Inhibitor of κBα
IL	Interleukin
MnSOD	Manganese Superoxide Dismutase

MPTPC	Mitochondrial permeability transition pore complex
MAPK	Mitogen-activated protein kinase
MnTE-2-PyP^{5+}	Mn(III) 5,10,15,20-tetrakis(N-n-hexylpyridinium-2-yl)porphyrin
NGF	Nerve growth factor
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase
nNOS	Neuronal nitric oxide synthase
NMDAR	N-methyl-D-aspartate receptor
NFκB	Nuclear factor κB
OA	Osteoarthritis
PN	$ONOO^-$, Peroxynitrite
PNDCs	Peroxynitrite-decomposition catalysts
PBN	Phenyl N-tert-butylnitrone
PLA2	Phospholipase A2
PARP	Poly (ADP-ribose) polymerase
PG	Prostaglandin
PKA	Protein kinase A
PKC	Protein kinase C
RVM	Rostral ventromedial medulla
Mrg	Sensory neuron specific receptor
SO	$O_2{\cdot}^-$, Superoxide
SODms	Superoxide dismutase mimetics
TLR4	Toll-like receptor-4
TRPV1	Transient receptor potential cation channel, subfamily V, member 1
TNF	Tumor necrosis factor
Tyr	Tyrosine
TrkA	Tyrosine kinase receptor A

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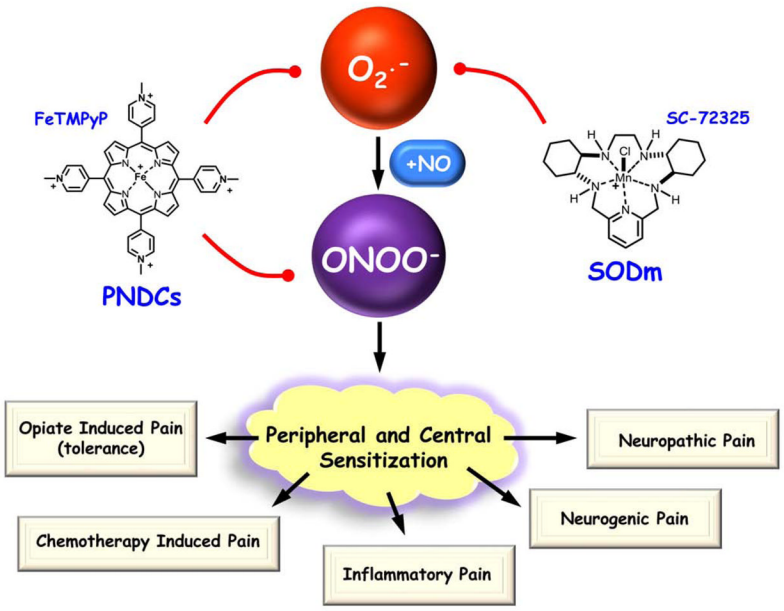

Fig. 1. Superoxide and peroxynitrite are targets for novel pain therapy

Superoxide ($O_2\cdot^-$) and peroxynitrite ($ONOO^-$) are key mediators in the development of peripheral and central sensitization of the various pain etiologies. The use of superoxide-dismutase mimetics (SODm, i.e. SC-72325) and peroxynitrite decomposition catalysts (PNDCs, i.e. FeTMPyP) reduce nitroxidative stress and attenuate the development of peripheral and central sensitization; providing promising novel therapy for chronic pain management.

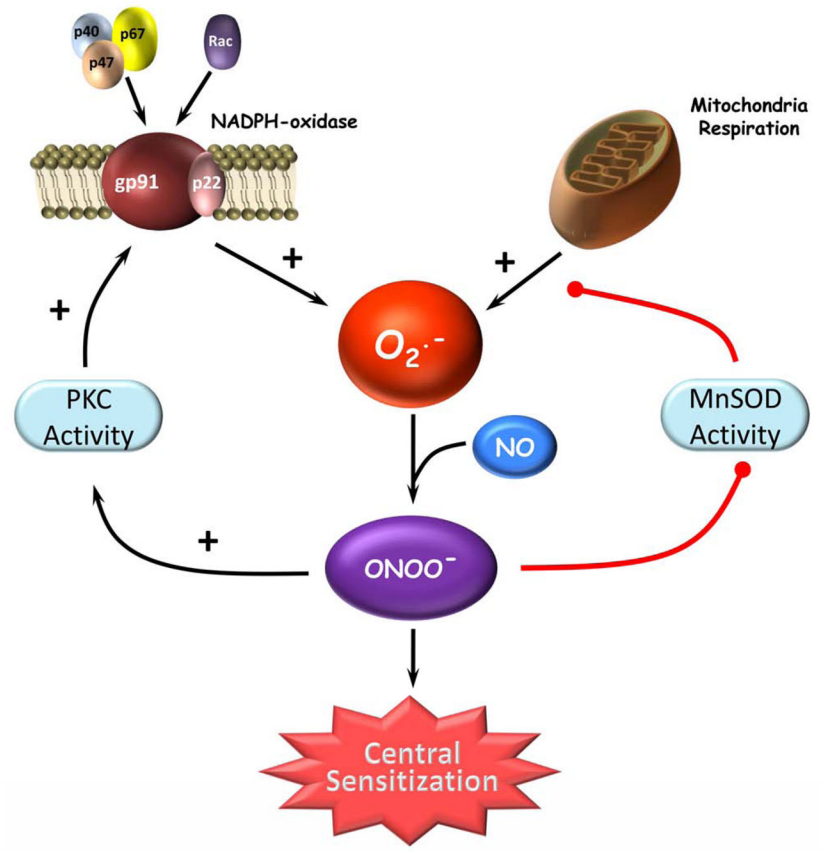

Fig. 2. Peroxynitrite-reinforced superoxide production in central sensitization: two feed forward mechanisms

Two major sites of superoxide ($O_2\cdot^-$) production, NADPH oxidase and mitochondrial respiration, are active in the development of central sensitization. Peroxynitrite ($ONOO^-$) formed from NADPH oxidase- and mitochondrial-derived superoxide nitrates and inactivates the manganese SOD (MnSOD) enzyme preventing the removal of mitochondrial-derived superoxide. Peroxynitrite enhances protein kinase C (PKC) activity and, in turn, enhances translocation of NADPH oxidase regulatory subunits to the membrane to increase the NADPH oxidase-derived superoxide production. Combined, these two mechanisms amplify superoxide-derived peroxynitrite formation leading to the development of central sensitization.

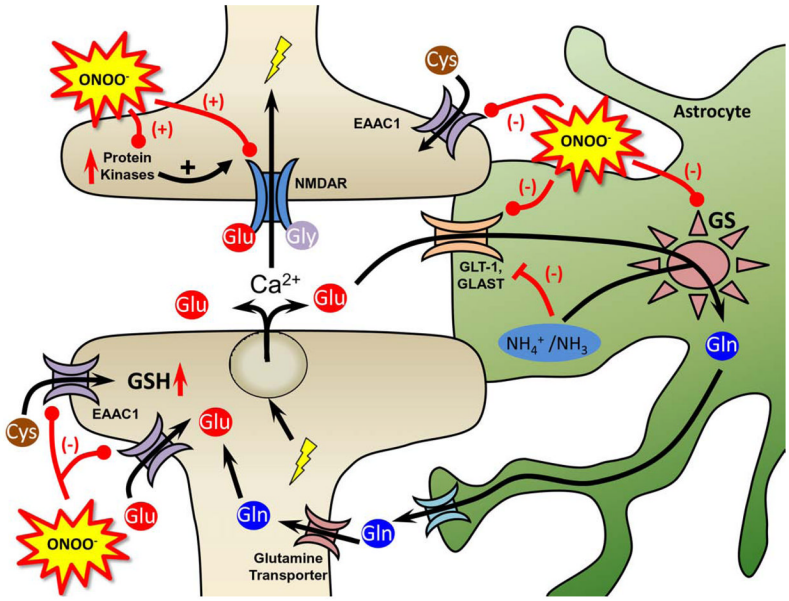

Fig. 3. The role of peroxynitrite in glutamatergic homeostasis and signaling

Peroxynitrite ($ONOO^-$) enhances glutamatergic signaling through nitration and activation of NMDARs and the protein kinases responsible for NMDAR activation. Peroxynitrite further enhances glutamatergic signaling by nitrating and inactivating the glutamate transporters (GLT-1, GLAST, and EAAC1) that remove glutamate (Glu) from the synapse and extrasynaptic regions and glutamine synthetase (GS) that converts glutamate, ammonia and ATP to glutamine, which is then taken back up by the neurons via the glutamine transporter. Nitration and inactivation of these enzymes results in toxic levels of glutamate and activation of neuroimmune responses. Inactivation of GS may also lead to increase ammonia levels that can inhibit glutamate transport. In addition to glutamate uptake, EAAC1 transports cysteine (Cys) into the neuron a process that is key in the biosynthesis of glutathione (GSH), a major cellular antioxidant. Compromised Cys transport by nitrated EAAC1 could lead to increased neuronal nitroxidative stress.