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REVIEW ARTICLE

Reactive nitroxidative species and nociceptive processing: determining the roles for nitric oxide, superoxide, and peroxynitrite in pain

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Abstract Pain is a multidimensional perception and is modified at distinct regions of the neuroaxis. During enhanced pain, neuroplastic changes occur in the spinal and supraspinal nociceptive modulating centers and may result in a hypersensitive state termed central sensitization, which is thought to contribute to chronic pain states. Central sensitization culminates in hyperexcitability of dorsal horn nociceptive neurons resulting in increased nociceptive transmission and pain perception. This state is associated with enhanced nociceptive signaling, spinal glutamate-mediated *N*-methyl-D-aspartate receptor activation, neuroimmune activation, nitroxidative stress, and supraspinal descending facilitation. The nitroxidative species considered for their role in nociception and central sensitization include nitric oxide (NO), superoxide ($O_2^{\cdot -}$), and peroxynitrite ($ONOO^-$). Nitroxidative species are implicated during persistent but not normal nociceptive processing. This review examines the role of nitroxidative species in pain through a discussion of their contributions to central sensitization and the underlying mechanisms. Future directions for nitroxidative pain research are also addressed. As more selective pharmacologic agents are developed to target nitroxidative species, the exact role of nitroxidative species in pain states will be better characterized and should offer promising alternatives to available pain management options.

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Introduction

The socioeconomic burden of pain in the United States is substantial, with nearly 80 million pain-related cases per year and costs of approximately 100 billion dollars in medical bills, lost worker hours, and workers' compensation (Renfrey et al. 2003; National Centers for Health Statistics 2006). Those who suffer from pain also experience disability, reduced quality of life, and poor pain management (Renfrey et al. 2003; National Centers for Health Statistics 2006). Pain is "…an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (Merskey and Bogduk 1994)." Long considered a symptom of underlying pathology, chronic pain is now proposed as a disease state (Loeser 2006); thus, there is renewed interest in elucidating the mechanisms that cause chronic pain and discovering therapeutic targets to improve outcomes for pain patients.

Nitroxidative species are a group of molecules capable of oxidative, nitrosative, and nitrative activities (i.e. NO, $O_2^{\cdot -}$, $ONOO^-$-mediated nitroxidative stress) (Kirsch and De Groot 2001). Recently, unique perspectives on the contributions of nitroxidative species to pain are providing novel targets for pain management (Salvemini et al. 2002; Salvemini and Neumann 2009a, b; Salvemini and Timchenko 2009). Numerous studies demonstrate that pharmacologic inhibition of the synthesis of NO and

pharmacologic removal of $O_2^{\cdot -}$ and $ONOO^-$ can prevent and reverse the characteristic findings associated with inflammatory pain, neuropathic pain, and morphine antinociceptive tolerance states. These effects may occur in central nervous system (CNS) regions responsible for pain processing through the mediation of central sensitization.

Central sensitization is an excitatory state of spinal cord dorsal horn neurons that transmit nociception due to increased responsiveness to suprathreshold and/or a lowered threshold to nociceptive signals; this manifests behaviorally as hypersensitivity to noxious (hyperalgesia) and non–noxious (allodynia) stimuli (Woolf 1983; Sandkuhler 2009). This state is a result of physiologic, biochemical, and molecular changes within spinal and supraspinal nociceptive modulating centers in the CNS and is partly responsible for persistent pain pathology (Woolf and Thompson 1991; Sandkuhler 2009). Essential components of central sensitization appear to include enhanced nociceptive input to the spinal cord as well as local spinal and supraspinal (Porreca et al. 2002) modulatory influences. Two potential mechanisms that contribute to central sensitization and involve nitroxidative species include long-term potentiation (LTP) and neuroimmune activation.

Nociception

In spinal nociceptive signaling (Fig. 1a), input to the presynaptic nociceptive neuron triggers neurotransmitter release [e.g. glutamate, substance P, calcitonin gene-related peptide (CGRP), and adenosine triphosphate] into the synapse. During acute nociception, glutamate binds to postsynaptic neuronal α-amino-3-hydroxyl-5-methyl-4-isoxazole (AMPA) receptors initiating sodium influx and subsequent membrane depolarization (Woolf and Salter 2006). Enhanced and/or prolonged nociceptive signaling (Fig. 1b) further increases the release of presynaptic neurotransmitters (e.g. glutamate, substance P, CGRP), increasing AMPA and neurokinin receptor activation and augmenting membrane depolarization. Increased depolarization facilitates the removal of the magnesium (Mg^{2+}) block of *N*-methyl-D-aspartate receptor (NMDAR) (Ikeda et al. 2003; Woolf and Salter 2006), initiating an influx of intracellular calcium (Ca^{2+}) through NMDAR channels. Influx of Ca^{2+} upregulates AMPA surface presentation and strengthens the synapse via synaptic plasticity that may result in LTP and subsequent enhancement of the nociceptive signal.

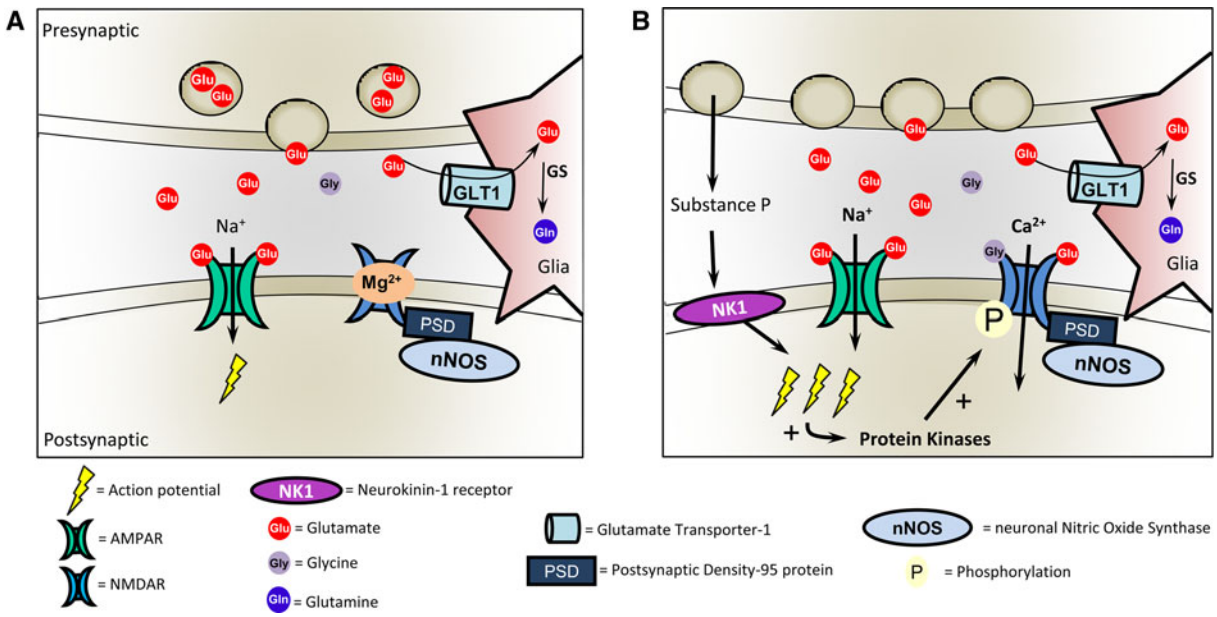

Fig. 1 The mechanisms of normal and enhanced nociceptive transmission at a typical spinal synapse. **a** In normal nociception, depolarization of the presynaptic nociceptive neuron causes a release of glutamate (*Glu*) and glycine (*Gly*). The relatively low synaptic glutamate levels bind to AMPAR allowing Na^+ influx, membrane depolarization, and perpetuation of the action potential through the post-synaptic neuron. Low levels of glutamate are readily taken up by glial cells via the glutamate transporter (*GLT-1*) and converted to non-toxic, non-excitatory glutamine (*Gln*) by glutamine synthase (*GS*). **b** In enhanced nociception, there is an increase in the release of neurotransmitters such as glutamate and substance P. Increased synaptic glutamate allows glutamate and glycine to bind NMDAR. This increases AMPA-dependent Na^+ influx and triggers protein kinase phosphorylation of NMDAR resulting in the removal of the Mg^{2+} block. Removal of the Mg^{2+} block facilitates an intercellular Ca^{2+} influx that stimulates enhanced AMPAR surface expression

Long-term potentiation

The role of LTP is well established in memory and learning; recent studies demonstrate that LTP also parallels synaptic plasticity in persistent pain states (Sandkuhler 2009). The development of LTP depends upon spinal neuronal NMDAR-enhanced intracellular Ca^{2+} influx and the production of nitroxidative species to increase post-synaptic surface AMPA receptor expression and enhance AMPA and NMDA sensitivity; thus stabilizing the synapse. Additionally, spinal LTP requires the activation of a subset of superficial dorsal horn nociceptive neurons that express the neurokinin-1 receptor, project to supraspinal modulatory centers, and mediate hyperalgesia (Mantyh et al. 1997; Todd et al. 2000). These projection neurons originate the spino-bulbo-spinal loop driving supraspinal descending facilitation of spinal nociception (Mantyh and Hunt 2004).

During enhanced nociception and the development of LTP, the NMDAR-enhanced Ca^{2+} influx activates several protein kinases and phosphatases (Chan and Sucher 2001) that modulate the phosphorylation state, and thus the activity, of NMDAR (Fig. 2a). Protein kinase C (PKC) and protein kinase A (PKA) are threonine/seronine kinases (Caudle et al. 2005; Velazquez et al. 2007) activated in response to high frequency stimulation (HFS) (Hongpaisan et al. 2004) and NMDA (Brennan et al. 2009) or in dorsal horn neurons following chemical (Yashpal et al. 1995), thermal (Yashpal et al. 1995), and morphine treatments (Mayer et al. 1995). In response to NMDAR-mediated Ca^{2+} influx, phosphorylated PKC translocates to the membrane surface where it can phosphorylate NMDAR (Chen and Huang 1992; Zheng et al. 1997) and NMDAR NR1 subunit (Leonard and Hell 1997; Tingley et al. 1997; Zou et al. 2002; Yang et al. 2009), AMPA GluR2 subunit (Park et al. 2009), P2X2 channels (Boue-Grabot et al. 2000), and induce extracellular signal-regulated kinase (ERK)-mediated cAMP response element binding (CREB) phosphorylation (Kawasaki et al. 2004); all of which can enhance nociceptive signaling. The Ca^{2+} influx also enhances cAMP-binding of the PKA regulatory subunits; whereby, the catalytic subunits of PKA are released and phosphorylate NMDAR NR1 subunits at a separate serine from PKC-enhancing NR1 surface expression (Leonard and Hell 1997; Tingley et al. 1997; Zou et al. 2002; Yang et al. 2009) and ERK-activation of CREB (Kawasaki et al. 2004; Wu et al. 2005). A third kinase, Ca^{2+}/calmodulin-dependent protein

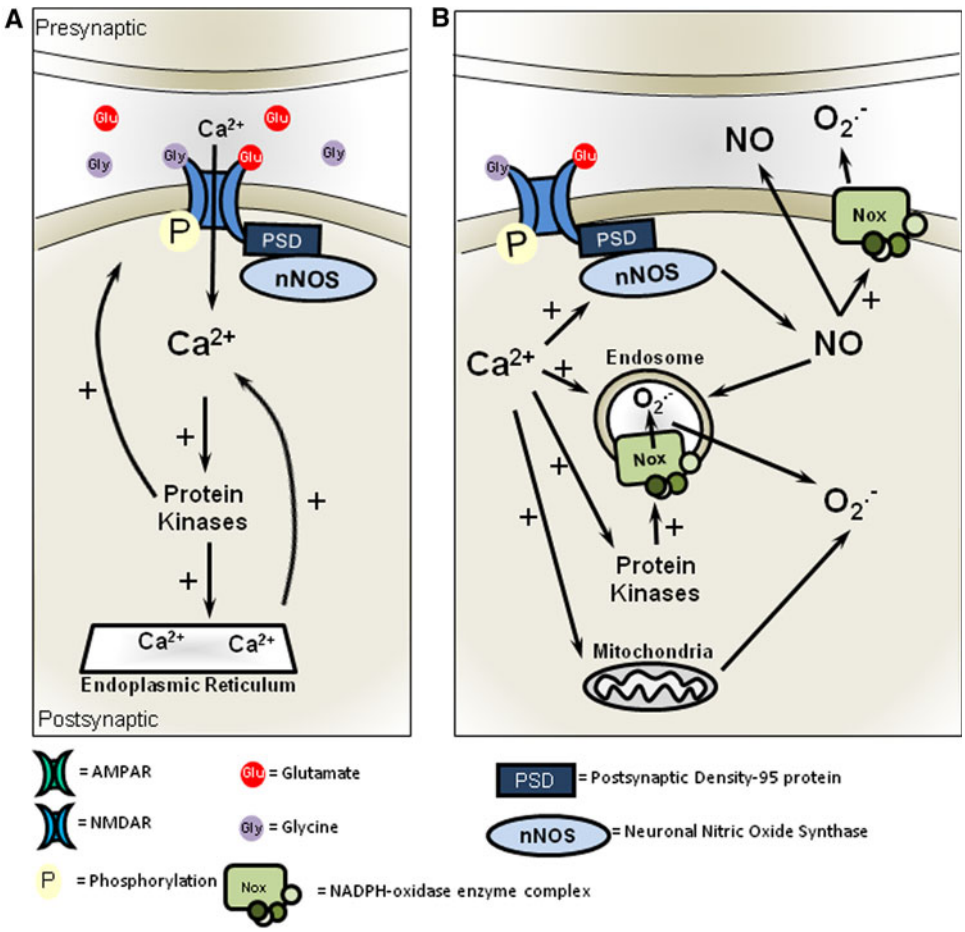

Fig. 2 Intracellular calcium influx enhances post-synaptic membrane responsiveness and produces nitroxidative species in the development of long-term potentiation. **a** The synaptic glutamate/glycine binding of NMDAR triggers a Ca^{2+} influx that activates several protein kinases. These kinases, in addition to NMDAR phosphorylation, reinforce the intracellular Ca^{2+} by facilitating Ca^{2+} release from endoplasmic reticulum. **b** Increased intracellular Ca^{2+} stimulates the production of nitroxidative species. NO can be stimulated from NMDAR-associated nNOS; whereas $O_2^{\cdot-}$ can be produced from the disruption of the mitochondrial respiratory chain or from NADPH-oxidase stimulated by Ca^{2+} and Ca^{2+}-activated protein kinases

kinase II (CaMKII), is located within NMDAR complexes (Strack et al. 1997; Garry et al. 2003) and involved in synaptic plasticity (Garry et al. 2003; Yang et al. 2004; Ikeda et al. 2006). Following a nociceptive stimulus, Ca^{2+} binds CaMKII (pCaMKII) within nonpeptidergic C-fibers of the dorsal horn (Larsson and Broman 2006, 2008) and initiates autophosphorylation of Thr286 proximal to its catalytic site (Lisman et al. 2002). Furthermore, CaMKII-inhibition or the loss of the protein that links CaMKII with NMDAR (PSD95) prevents the development of thermal hyperalgesia and mechanical allodynia (Garry et al. 2003).

Neuroimmune activation

Classically, central sensitization has focused on neuronal properties as defined by the mechanisms underlying synaptic plasticity and nociceptive modulation. However, recent evidence indicates that glial cells, thought to serve as an inert support system for neurons, are active in the development and maintenance of central sensitization (Watkins and Maier 2005). Glial cells (e.g. astrocytes and microglia) surrounding or adjacent to nociceptive synapses can be activated by neuroactive substances, NO, and other proinflammatory stimuli (Milligan and Watkins 2009). Activated glial cells can release pro-inflammatory cytokines, excitatory amino acids, and nitroxidative species that can sensitize dorsal horn neurons (Milligan and Watkins 2009). The role for neuroimmune activation in central sensitization is supported by numerous studies. For example, astrocytes produce tumor necrosis factor (TNF)-α and interleukin (IL)-1β and microglia produce IL-6 following CGRP receptor activation (Wang et al. 2009). Further, inhibition of glial cells with minocycline prevents the development of neuropathic hyperalgesia and allodynia by blocking glial cell activation and production of proinflammatory cytokines (Raghavendra et al. 2003). The contribution of neuroimmune activation to central sensitization may occur through Toll-like receptor-4 (TLR4) signaling in glial cells.

Recent investigations have identified TLR4 as an important contributor to central sensitization (Watkins et al. 2009). Inhibition of TLR4 signaling through CD14 knockout (KO) (Cao et al. 2009), TLR4 KO (Tanga et al. 2005; Bettoni et al. 2008), TLR4 point mutation (Tanga et al. 2005), TLR4 antagonists (Hutchinson et al. 2010; Bettoni et al. 2008), or TLR4 knockdown (Tanga et al. 2005) reduces mechanical allodynia and thermal hyperalgesia. Furthermore, TLR4 antagonists enhance morphine analgesia (Hutchinson et al. 2010), while morphine-3-glucuronide, a morphine metabolite, enhances hyperalgesia, allodynia, and microglia production of IL-1β through the activation of TLR-4 (Lewis et al. 2009). Stimulation of TLR-4 can result in the production of the proinflammatory cytokines TNF-α and IL-1β that are important contributors to central sensitization.

Nitroxidative species and nociception

Nitroxidative species (NO, $O_2^{\cdot -}$, and $ONOO^-$) are only implicated in persistent pain states, having no role in acute physiological nociception (Meller and Gebhart 1993; Wang et al. 2004). Levels of nitroxidative species can significantly increase during times of stress, resulting in nitroxidative stress, and often implicated in aging and many diseases involving multiple systems including persistent pain (Pacher et al. 2007; Salvemini and Neumann 2009a). Nitroxidative species are also intimately involved in inflammation and modulate the arachidonic acid inflammatory cascade during central sensitization (Mollace et al. 2005; Salvemini et al. 2006; Cuzzocrea and Salvemini 2007; Ndengele et al. 2008); this appears to occur through the activation of transcription factors [e.g. activator protein 1 (AP-1) and nuclear factor kappa B (NF-*k*B)] and mitogen-activated protein kinases (MAPK) (e.g. p38) to activate cyclooxygenase (COX) enzymes (Gius et al. 1999; Matata and Galinanes 2002; Ndengele et al. 2005; Tsatsanis et al. 2006) and increase production of prostaglandins (Salvemini et al. 1994, 1995a, b; Mollace et al. 2005). Although the mechanisms of nitroxidative species in pain states are presently incompletely understood, it is thought that they may play an important role in the development and maintenance of central sensitization (Salvemini et al. 1996b; Tal 1996; Wang et al. 2004; Salvemini and Neumann 2009a).

Nitric oxide (NO)

Nitric oxide has been extensively studied in pain models (for review: Callsen-Cencic et al. 1999; Luo and Cizkova 2000; Calabrese et al. 2007). It functions within a synapse as a neuromediator (Bredt and Snyder 1992; Garthwaite and Boulton 1995) and acts as a retrograde messenger from post-synaptic to presynaptic neuron terminals (O'dell et al. 1991; Pacher et al. 2007) exerting its effects through soluble guanyl cyclase-mediated (Ignarro 1990) increases in second messenger cyclic GMP (cGMP) (Ignarro 1991). Moreover, NO exhibits proinflammatory properties through its ability to modulate and activate COX enzymes to produce prostaglandins (Salvemini et al. 1993; Mollace et al. 2005). Administration of non-steroidal anti-inflammatory pharmacologic agents prevents NO donor-induced hyperalgesia (Tassorelli et al. 2006). Though NO is recognized as a contributor to central sensitization, the exact role of NO remains unclear.

The properties of NO appear to be both antinociceptive (Goettl and Larson 1996) and pronociceptive (Malmberg and Yaksh 1993). Some authors believe that this may be due to the activities of the different isoforms of NOS, each with varying effects upon nociception (Gonzalez-Hernandez and

Rustioni 1999). There are three NOS isoforms: two Ca^{2+}-dependent constitutive forms, neuronal (nNOS) and endothelial (eNOS), and a Ca^{2+}-independent inducible (iNOS) form. Although synthesis of NO in the CNS is predominantly due to nNOS (Downen et al. 1999), all three isoforms are possible sources. In the CNS, the nNOS isoform is expressed primarily within neurons (Cork et al. 1998), eNOS within endothelial cells (Pollock et al. 1993), and iNOS within glial cells (i.e. astrocytes and microglia) (Simmons and Murphy 1992) following appropriate stimuli (e.g. immunological and inflammatory) (Murphy 2000).

The constitutive nNOS expression in spinal and supraspinal nociceptive modulation centers suggests that NO is capable of contributing to nociception (Valtschanoff et al. 1992; Vincent and Kimura 1992; Rodrigo et al. 1994). During central sensitization in animal models of inflammatory and neuropathic pain, the expressions of nNOS and iNOS isoforms are reported to increase in the spinal nociceptive modulating center (i.e. superficial dorsal horn) (Gordh et al. 1998; Callsen-Cencic et al. 1999; Martucci et al. 2008); although there are also reports of no change or a decrease in these expressions throughout the experimental time course (Goff et al. 1998; Callsen-Cencic et al. 1999). The NOS regulatory proteins also increase in expression within the dorsal root ganglion and dorsal horn during inflammatory and neuropathic pain (Dreyer et al. 2003; Martucci et al. 2008).

Pharmacologic manipulations of NO through various routes of administration have confirmed the functional involvement of NO in pain states at the injury site, in the periphery, and within the CNS; all contribute to central sensitization. Local administration of non-selective NOS inhibitors [e.g. L-*Nω*-nitro-L-arginine methyl ester (L-NAME); NG-monomethyl-L-arginine (L-NMMA)] and selective iNOS inhibitors [e.g. aminoguanidine (AG); *N*-iminoethyl-L-lysine (L-NIL)] reduces markers of inflammatory pain (Nakamura et al. 1996; Lawand et al. 1997; Omote et al. 2001). Injury site and intravenous injections of nNOS inhibitors block the early phase while iNOS inhibitors block the late phase of hindpaw edema in an inflammatory model of pain (Salvemini et al. 1996b; Handy and Moore 1998). Systemic administration of non-selective NOS inhibitors [e.g. L-NAME; NG-nitro-L-arginine (L-NOArg)] prevents inflammatory (Semos and Headley 1994; Handy and Moore 1998) and neuropathic (Yamamoto and Shimoyama 1995; Hao and Xu 1996) pain behaviors. More specifically, systemic administration of nNOS inhibitors [e.g. 7-nitro indazole (7-NI)] attenuates inflammatory pain (Lawand et al. 1997; Handy and Moore 1998), blocks inflammatory hyperalgesia (Tao and Johns 2002) and prevents vincristine-induced neuropathic pain (Bujalska and Makulska-Nowak 2009). Further, systemic administration of an iNOS inhibitor (L-NIL) prevents streptazocin-induced peripheral neuropathic hyperalgesia (Bujalska and Makulska-Nowak 2009) and (GW274150) reverses inflammatory and neuropathic hyperalgesia (De Alba et al. 2006).

Within the CNS, intrathecal administration of non-selective NOS inhibitors attenuate pain behaviors from a number of causes including: NMDA (Kitto et al. 1992; Malmberg and Yaksh 1993), mas gene related (Chang et al. 2009), inflammation (Moore et al. 1991; Malmberg and Yaksh 1993), and neuropathic (Meller et al. 1992; Lui and Lee 2004) pain. Intrathecal administration of selective nNOS and iNOS inhibitors also blocks or attenuates inflammation and peripheral nerve injury-induced hyperalgesia (Osborne and Coderre 1999; Chu et al. 2005; Tanabe et al. 2009). Additionally, inhibition of the downstream targets of NO, soluble guanyl cyclase and cGMP, provide similar effects (Meller et al. 1992; Tanabe et al. 2009). Consistent with these findings, intrathecal injection of NO donors induces transient, dose-dependent hyperalgesia and intensifies inflammatory and neuropathic hyperalgesia (Inoue et al. 1997; Tassorelli et al. 2003).

Although non-selective NOS inhibition confirms a role for NO, the exact role of each NOS isoform is less certain because of variable results between investigations using selective pharmacologic inhibition of NOS isoforms. For example, an intrathecal injection of a nNOS inhibitor was reported to decrease inflammatory hyperalgesia (see above); however, others report that selective iNOS [N-(3-(Aminomethyl)benzyl)acetamidine (1400W)] but not nNOS inhibitors suppress formalin and carrageenan-induced hyperalgesia (Tang et al. 2007). Further, intraperitoneal injection of a selective iNOS inhibitor, but not a nNOS inhibitor, reduces diabetic neuropathy-induced hyperalgesia (Bujalska et al. 2008; Bujalska and Makulska-Nowak 2009) while others report that both selective iNOS and nNOS inhibitors block neuropathic hyperalgesia (Bujalska and Gumulka 2008). Moreover, recent findings demonstrate that selective inhibition of each of the three isoforms is able to block intramuscular capsaicin-induced hyperalgesia (Lee et al. 2009).

The specific role of NOS isoforms in pain has also been investigated through genetic manipulation with conflicting results. Animals with nNOS deletion demonstrate attenuation of delayed, but not early inflammatory hyperalgesia (Tao et al. 2004; Chu et al. 2005). Deletion of iNOS appears to have no effect on carrageenan-induced hyperalgesia (Tao et al. 2003); whereas, the lack of iNOS reduces early zymosan-induced hyperalgesia (Guhring et al. 2000). Further, Boettger et al. (2007) reported that only nNOS KO mice have significant reductions of thermal hyperalgesia and absence of mechanical hyperalgesia in response to complete Freund's adjuvant (CFA) in an inflammatory pain model. Another study using CFA

demonstrated that iNOS KO mice have reduced thermal hyperalgesia and paw edema, while nNOS KO mice have reduced paw edema and mechanical allodynia, as well as a modest rapid recovery from thermal hyperalgesia in comparison to wild type mice (Leanez et al. 2009).

The variability of the reported roles of NOS isoforms may reflect dissimilar animal model pain mechanisms, the low selectivity of NOS inhibitors (Alderton et al. 2001), and the reported compensatory upregulation of remaining NOS isoforms in genetically deleted NOS isoform animals (Tao et al. 2003). Recent investigations are also suggesting that during central sensitization, these variable findings may reflect the interaction of NO and $O_2^{\cdot-}$ to form $ONOO^-$; this contributes to sensitization through nitroxidative stress.

Inhibition of NOS appears to be an advantageous approach for the treatment of numerous disease states (e.g. pain, septic shock, asthma, atherosclerosis, and chronic tension type headache); however, clinical trials with non-selective (Alexander et al. 2007) or iNOS selective inhibitors (Singh et al. 2007; Van Der Schueren et al. 2009) have performed poorly and the reasons are multifactorial (Salvemini and Timchenko 2009).

Superoxide ($O_2^{\cdot-}$)

Although less characterized than NO in pain models, $O_2^{\cdot-}$ also contributes to central sensitization. The production of $O_2^{\cdot-}$ can occur from a number of sources including mitochondrial oxidative phosphorylation and the activation or upregulation of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, xanthine oxidase, NOS, and COX enzymes (Pacher et al. 2007). Interestingly, NADPH-oxidase can be activated by NO-mediated activities (Girouard et al. 2009). Mitochondrial $O_2^{\cdot-}$ production increases in both the superficial and deep dorsal horn in models of neuropathic (Park et al. 2006) and inflammation-induced hyperalgesia (Lee et al. 2007; Schwartz et al. 2008, 2009). Following the appropriate stimulus, neurons, astrocytes, and microglia can synthesize $O_2^{\cdot-}$ and NO (Colton and Gilbert 1987; Boje and Arora 1992; Pacher et al. 2007; Schwartz et al. 2008; Milligan and Watkins 2009). Levels of $O_2^{\cdot-}$ are kept under control by the manganese superoxide dismutase (MnSOD) isoform in the mitochondrion and copper, zinc-SOD (Cu, ZnSOD) in the cytoplasm (Politzer et al. 1971; Paschen and Weser 1973). The SOD functions as a catalyst in the dismutation of $O_2^{\cdot-}$ into oxygen and hydrogen peroxide (Fridovich 1995).

Expression of SOD is noted throughout the neuroaxis with the highest regional specific activity in the brainstem and hypothalamus (Thomas et al. 1976). The distribution pattern of SOD may overlap with nNOS in regions dependent upon NO activity (Okabe et al. 1998; Lindenau et al. 2000). At the cellular level in the CNS, MnSOD immunoreactivity occurs strongly in neurons and in glia surrounding blood vessels; while the expression of Cu, ZnSOD is found predominantly in astrocytes (Noack et al. 1998; Lindenau et al. 2000).

The contribution of $O_2^{\cdot-}$ to central sensitization in peripheral and spinal regions was demonstrated for the first time by the use of selective SOD mimetics (SODm) such as M40403 (Salvemini 2001, 2002; Wang et al. 2004; Muscoli et al. 2004). Studies using antioxidants [phenyl *N*-tert-butylnitrone (PBN) and 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL) and Mn(III) tetrakis (4-benzoic acid) porphyrin (MnTBAP)] support the roles of nitroxidative species in pain of several etiologies including, neuropathic (Tal 1996; Kim et al. 2004; Tanabe et al. 2009), inflammatory (Wang et al. 2004; Khattab 2006), neurogenic (Lee et al. 2007; Schwartz et al. 2008, 2009), opiate-induced hyperalgesia (Muscoli et al. 2007), and visceral models (Wang et al. 2008). At the site of injury, administration of TEMPOL produces a reduction in carrageenan-induced hyperalgesia (Wang et al. 2004; Khattab 2006). Intrathecal free radical scavengers also significantly reduce capsaicin-induced (Lee et al. 2007) and nerve injury-induced (Kim et al. 2004; Tanabe et al. 2009) behavioral hypersensitivities. Further, systemic, spinal (Lee et al. 2007; Schwartz et al. 2008, 2009), and supraspinal (Lee et al. 2007) administration of TEMPOL and PBN significantly reduce capsaicin-induced secondary mechanical hyperalgesia and the expression of spinal neuronal mitochondrial $O_2^{\cdot-}$. These results demonstrate that the administration of TEMPOL and PBN are analgesic in pain states, however, these drugs cannot be used to assess the specific contribution of $O_2^{\cdot-}$ or any other reactive nitroxidative species because they are non-selective scavengers of nitroxidative species; this is a critical concept addressed in previous reviews (Muscoli et al. 2003; Salvemini and Timchenko 2009).

Among the potential sources for $O_2^{\cdot-}$ in central sensitization, MnSOD nitration or MnSOD-2 inactivation is critical for the development of inflammatory, morphine, and NMDA-induced hyperalgesias (Wang et al. 2004; Muscoli et al. 2004, 2007; Schwartz et al. 2009). Additionally, capsaicin-induced thermal and mechanical hyperalgesia requires a catalytic subunit for NADPH-oxidase, NOX-1 (Ibi et al. 2008), and is associated with dorsal horn neuronal mitochondrial production of $O_2^{\cdot-}$ (Schwartz et al. 2008). Further, a role for xanthine oxidase-mediated $O_2^{\cdot-}$ production was recently reported as xanthine oxidase inhibition with allopurinol significantly reduces chronic post-ischemic pain (Kwak et al. 2009).

Peroxynitrite ($ONOO^-$)

Many of the detrimental effects of $O_2^{\cdot-}$ and NO are attributed to $ONOO^-$ formation and its subsequent activities (Beckman et al. 1990; Salvemini et al. 2006; Pacher et al. 2007). The formation of $ONOO^-$, a known oxidant and nitrating agent, occurs through the interaction of $O_2^{\cdot-}$ and NO (Beckman et al. 1990; Radi et al. 1991). Upon enhanced $O_2^{\cdot-}$ and NO production, $ONOO^-$ is preferentially formed as NO has a greater reactivity with $O_2^{\cdot-}$ than SOD (Huie and Padmaja 1993). The formation of $ONOO^-$ is spatially approximated to the cellular location of $O_2^{\cdot-}$ due to the properties of $O_2^{\cdot-}$ (e.g. short lifespan and restricted membrane diffusion) (Gryglewski et al. 1986; Szabo et al. 2007). Within the CNS, both glial cells and neurons are capable of forming $ONOO^-$.

The proposed contribution of $ONOO^-$ to central sensitization is through proapoptotic, proinflammatory, and nitrosative processes (Salvemini and Neumann 2009b; Salvemini and Timchenko 2009). $ONOO^-$ has proinflammatory properties through its ability to increase microvascular permeability (Ridger et al. 1997), alter blood brain barrier integrity (Hooper et al. 2000; Knepler et al. 2001), and activate redox-sensitive transcription factors (e.g. NF-κB and AP-1) and MAPK kinases (e.g. p38 kinase) (Matata and Galinanes 2002; Ndengele et al. 2005). Further, $ONOO^-$ can oxidatively inactivate or modify amino acid residues in COX enzymes (Markey et al. 1987; Landino et al. 1996) and, along with NO, increases COX-2 levels to produce prostaglandins through $O_2^{\cdot-}$ and the p38 pathway (Habib et al. 1997; Eligini et al. 2001; Yang et al. 2006; Ndengele et al. 2008). This nitroxidative species is also capable of modifying cell-signaling molecules (Zhang et al. 2003) and is implicated in neurotoxic states (Calabrese et al. 2007).

The ability of $ONOO^-$ to post-translationally nitrate proteins results in modification of protein activity (Radi 2004). Although myeloperoxidase can facilitate tyrosine nitration (Kettle et al. 1997), carefully controlled research has implicated $ONOO^-$ as the source of nitration in many pathologic states (Szabo et al. 2007). Proteins important to the maintenance of normal nociceptive processing such as MnSOD (Radi 2004), glutamate transporter-1 (GLT-1), glutamine synthase (GS) (Trotti et al. 1996; Gorg et al. 2005), and NMDAR subunits (Zanelli et al. 2000) are nitrated by $ONOO^-$. The $ONOO^-$-mediated nitration of MnSOD inactivates the enzyme, resulting in increased $O_2^{\cdot-}$ levels (Yamakura et al. 1998; Macmillan-Crow and Thompson 1999; Macmillan-Crow et al. 2001); whereas, nitration of GLT-1 and GS disrupts glutamate homeostasis, increases glutamate neurotransmission and results in excitotoxicity (Trotti et al. 1996), neurotoxicity (Mennerick et al. 1999; Lievens et al. 2000), and cytotoxicity (Muscoli et al. 2005). Furthermore, nitration of NMDAR subunits results in constant potentiation of synaptic currents, Ca^{2+} influx, and neuronal excitotoxicity (Zanelli et al. 2000, 2002).

Recent evidence supports the contribution of $ONOO^-$ to pain as peripheral administration of $ONOO^-$ or $ONOO^-$ precursors can induce inflammatory hyperalgesia (Ndengele et al. 2008). Additionally, nitrotyrosine expression, a marker for $ONOO^-$-mediated nitration, increases at the injury site following both neuropathic and inflammatory pain (Salvemini et al. 1996a; Khalil et al. 1999). In the CNS, nitrotyrosine immunoreactivity significantly increases in the dorsal horn of the spinal cord (including superficial laminae) following carrageenan (Wang et al. 2004), NMDA (Muscoli et al. 2004), and morphine-induced (Muscoli et al. 2007; Ndengele et al. 2008) hyperalgesic states. Inflammation-induced hyperalgesia is associated with spinal cord MnSOD, GLT-1, and GS $ONOO^-$-mediated nitration (Chen et al. 2010). Further, Muscoli et al. (2004) reported that during NMDA-induced hyperalgesia, MnSOD nitration mirrors the severity of thermal hyperalgesia. More recent studies suggest that $ONOO^-$ is involved in higher centers of the CNS as MnSOD is inactivated supraspinally during morphine-induced hyperalgesia (Doyle et al. 2009).

The positive outcomes following pharmacologic removal of $ONOO^-$ in animal models of pain suggest a role for $ONOO^-$ in enhanced pain states. In a neuropathic pain model, administration of daily uric acid decreases $ONOO^-$-mediated nitration in peripheral nerves and alleviates thermal hyperalgesia and Wallerian degeneration (Liu et al. 2000). The administration of a $ONOO^-$ decomposition catalyst [i.e. Fe(III)5,10,15,20-tetrakis(N-methylpyridinium-4-yl)porphyrin (FeTMPyP)] alone or with a poly (ADP-ribose) polymerse (PARP) inhibitor significantly reduces thermal hyperalgesia and mechanical allodynia in models of diabetic neuropathy (Arora et al. 2008; Negi et al. 2009). Additionally, FeTMPyP (Ndengele et al. 2008; Chen et al. 2010) or 5,10,15,20-Tetrakis (4-sulfonatophenyl) porphyrinato iron (III), chloride) (FeTPPS) (Salvemini et al. 1998b; Yeo et al. 2008) prevents the development of carrageenan-induced behavioral hypersensitivities. In the CNS, a role for spinal $ONOO^-$ in central sensitization was elucidated by preventing the formation of $ONOO^-$ and removal of $ONOO^-$ via administration of $ONOO^-$ decomposition catalysts. Indeed, systemic and intrathecal administration of SODm prevents dorsal horn MnSOD nitration and blocks hyperalgesia in various animal pain models (Muscoli et al. 2004, 2007; Wang et al. 2004), while FeTMPyP also prevents hyperalgesia and spinal cord protein nitration (i.e. GLT-1, GS, and MnSOD) in opioid-induced antinociceptive tolerance (Muscoli et al. 2007). In agreement

with these findings, manganese porphyrins, Mn(III) *meso*-tetrakis(*N*-n-hexylpyridinium-2-yl)porphyrin (MnTnHex-2-PyP^{5+}) and Mn(III) *meso*-tetrakis(*N*-ethylpyridinium-2-yl) porphyrin (MnTE-2-PyP^{5+}), pharmacologic compounds with greater bioavailability and $ONOO^-$ scavenging rate constants than iron porphyrins, were also able to block the development of morphine antinociceptive tolerance (Batinic-Haberle et al. 2009). An important trigger in the formation of $ONOO^-$ in response to chronic administration of morphine is the sphingolipid ceramide (Bryant et al. 2009; Ndengele et al. 2009).

Metalloporphyrins are reported as $ONOO^-$ decomposition catalysts; however, it is important to note that these drugs also have $O_2^{\cdot-}$ scavenging properties. For example, FeTMPyP, MnTnHex-2-PyP^{5+}, and MnTE-2-PyP^{5+} scavenge both $O_2^{\cdot-}$ and $ONOO^-$ with similar efficacy (Pasternack et al. 1981; Salvemini et al. 1996a, 1998a; Batinic-Haberle et al. 1999; Jensen and Riley 2002; Batinic-Haberle et al. 2010). This dual property contributes, at least in part, to their potent pharmacological effects.

Pharmacologic investigations of nitroxidative species are critical to advance the body of knowledge concerning the contribution of these species to pain; however, conclusions based on results from such studies must carefully consider the properties of pharmacologic agents. As we emphasized, the use of non-selective agents such as TEMPOL or PBN cannot be used to make claims about the contribution of a specific nitroxidative species (Salvemini et al. 2002, 2006; Muscoli et al. 2003; Salvemini and Neumann 2009b; Salvemini and Timchenko 2009). Ignoring this critical component of the chemistry of such agents leads to the interpretation of data that confounds our understanding of the roles of distinct nitroxidative species in disease states. For example, a recent study using a murine model of nerve injury reported that $O_2^{\cdot-}$ and NO may operate through two independent pathways without converging to $ONOO^-$ (Kim et al. 2009). This conclusion was based on the findings that, TEMPOL (which the authors describe as a $O_2^{\cdot-}$ scavenger) and L-NAME, but not FeTMPyP, block the development of central sensitization (Kim et al. 2009). The conclusion based upon these findings is misleading for two main reasons: (1) FeTMPyP scavenges $O_2^{\cdot-}$ and $ONOO^-$ with similar efficacy (Pasternack et al. 1981; Salvemini et al. 1996a, 1998a; Batinic-Haberle et al. 1999, 2010; Jensen and Riley 2002), thus it should have exerted protective effects similar to TEMPOL, if the latter is a $O_2^{\cdot-}$ scavenger (Goldstein et al. 2003, 2006). (2) TEMPOL cannot be used to argue that $O_2^{\cdot-}$ is or is not included in the mechanism of action since, as discussed; it is not selective for $O_2^{\cdot-}$. In order for any $O_2^{\cdot-}$ to be scavenged, TEMPOL first needs to be oxidized to oxoammonium cation by the $ONOO^-$ reaction manifold. Therefore, the use of TEMPOL may actually show that $ONOO^-$ is in fact involved. Future identification of selective probes and their use in combination with in vivo detection of the specific nitroxidative species in question is critical in defining and implicating the role of one species versus another. Until then, caution needs to be exerted when reaching conclusions derived by the use of non-selective pharmacological tools.

Although research supports the contribution of nitroxidative species to central sensitization, the underlying mechanisms are still unclear. The following sections discuss the peripheral, spinal, and supraspinal mechanisms involving nitroxidative species that contribute to central sensitization.

Peripheral contributions of nitroxidative species to central sensitization

Following tissue damage and skin inflammation, inflammatory mediators (e.g. bradykinin, glutamate, histamine, IL-1, IL-6, nerve growth factor, platelet-activating factor, prostaglandin E_2, protons, serotonin, substance P, and TNF-α) are released from cells surrounding the injury site and act upon the peripheral endings of the nociceptor (Johanek et al. 2006). These signals sensitize the nociceptive neurons, causing hypersensitivity to noxious stimulus at the injury site (i.e. primary hyperalgesia) (Johanek et al. 2006), and contributing to central sensitization through enhanced nociceptive signaling at the dorsal horn of the spinal cord. Nitroxidative species have a well-characterized crucial contribution to peripheral sensitization (Salvemini et al. 1996a, b; Wang et al. 2004; Ndengele et al. 2008). For example, nitroxidative species sensitize peripheral afferent fibers (Salvemini et al. 1996a, b), enhance the formation of pro-inflammatory/pronociceptive cytokines and prostaglandins (Salvemini et al. 1996a, b; Ndengele et al. 2008), activate PARP (Wang et al. 2004), act as signaling molecules to induce TNF receptor 1 expression following transient receptor potential cation channel, subfamily V, member 1 (TRPV1) activation (Ma et al. 2009), and increase the sensitivity of TRPV1 (Ibi et al. 2008; Chuang and Lin 2009; Keeble et al. 2009).

Spinal contributions of nitroxidative species to central sensitization

Central sensitization depends upon glutamate-mediated NMDAR activation, Ca^{2+} influx, and subsequent downstream production of nitroxidative species (Haley et al. 1990; Coderre and Melzack 1991, 1992; Woolf and

Thompson 1991; Wang et al. 2004) (Fig. 2b). The resultant NO and $O_2^{\cdot-}$ are integral to the development and maintenance of two proposed mechanisms of central sensitization, LTP (O'dell et al. 1991; Schuman and Madison 1991; Nowicky and Bindman 1993) and neuroimmune activation (Watkins and Maier 2005).

LTP and nitroxidative species

The contribution of nitroxidative species to central sensitization is closely associated with NMDAR activity. Activation of NMDAR is associated with increases in the levels of $O_2^{\cdot-}$ (Lafon-Cazal et al. 1993) and NO (Garthwaite et al. 1989; Gunasekar et al. 1995) and this is required for LTP (Schuman and Madison 1991; Klann 1998; Klann et al. 1998), a proposed positive feedback mechanism of synaptic plasticity in central sensitization (Sandkuhler 2009). Nitroxidative species alter NMDAR activation by mediating the activation of PKA and PKC phosphorylation of NMDAR subunit NR1 (Gao et al. 2007). Indeed, free radical scavengers significantly reduce spinal NMDAR NR1 subunit phosphorylation in neuropathic and inflammatory pain (Gao et al. 2007).

The increases in NMDAR-mediated NO levels contribute to central sensitization, in part, through numerous cellular and molecular mechanisms at the nociceptive synapse. Meller and Gebhart (1993) proposed that NO contributes to dorsal horn nociceptive neuron sensitization through the development of a monosynaptic neuronal mechanism of LTP at the nociceptive synapse. Central to this proposal is glutamate-mediated activation of postsynaptic NMDAR and Ca^{2+} influx, leading to Ca^{2+}-dependent NO synthesis (Fig. 3a). The NO diffuses back into the nociceptive synapse, where it retrogradely activates presynaptic soluble guanyl cyclase to form the intracellular second messenger cGMP. Increased cGMP potentiates synaptic transmission through the enhanced release of presynaptic neuroactive substances into the synapse, facilitating additional postsynaptic neuronal release of NO and its retrograde activities; thus contributing to sensitization of dorsal horn neurons. Numerous studies support the role of spinal NO in central sensitization, as previously discussed. Zhang and colleagues (2005) confirmed the contribution of NO to LTP by demonstrating that L-NAME and hemoglobin block the induction of spinal LTP following tetanic stimulation of the sciatic nerve and this is reversible with the administration of a NOS substrate, L-arginine.

Building upon the proposal from Meller and Gebhart, Wang et al. (2004) first demonstrated that spinal $O_2^{\cdot-}$ and $ONOO^-$ also mediate the development of central sensitization associated with inflammatory pain. Following a peripheral insult, spinal formation of $ONOO^-$ from excessive production of NO and $O_2^{\cdot-}$, leads to $ONOO^-$-mediated activities (i.e. nitration and inactivation of MnSOD) (Fig. 3b) that sustain and enhance $O_2^{\cdot-}$ and $ONOO^-$ levels creating an additional positive feedback mechanism that contributes to LTP. Support for this hypothesis was provided through a number of studies. Application of a glutamate agonist to neurons induces $O_2^{\cdot-}$ production (Bindokas et al. 1996), NMDAR antagonism blocks $O_2^{\cdot-}$ generation (Li et al. 2001), and SODm block NMDA-mediated hyperalgesia (Muscoli et al. 2004). In addition, MnSOD nitration deactivates MnSOD and results in maintenance of high levels of $O_2^{\cdot-}$ (Yamakura et al. 1998; Macmillan-Crow and Thompson 1999; Macmillan-Crow and Cruthirds 2001; Schwartz et al. 2009), while SODm block and significantly reduce inflammation-induced hyperalgesia (Wang et al. 2004). Moreover, $ONOO^-$ may contribute to LTP through the nitration and modification of other proteins important for the maintenance of normal nociception (e.g. GLT-1 and GS) (Fig. 3c), thereby creating a pro-LTP environment. Importantly, hyperalgesia and nitration of spinal cord GLT-1, GS, and MnSOD were blocked with NMDAR antagonism (dizocilpine hydrogen maleate (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d]cyclohepten-5,10-imine hydrogen maleate [MK-801]) and a $ONOO^-$ decomposition catalyst (FeTMPyP), demonstrating the importance of NMDAR-dependent $ONOO^-$ formation and activity during pain (Chen et al. 2010).

Another potential role of nitroxidative species in LTP is their ability to modulate PKC and PKA activity. Nitric oxide can enhance PKC activity through the release of zinc from metallothioneins during the development of morphine tolerance (Rodriguez-Munoz et al. 2008). Endogenous $O_2^{\cdot-}$ enhances neuronal NMDA-stimulated PKC autophosphorylation and repetitive HFS-stimulated PKA activity in the presence of a SOD inhibitor (Hongpaisan et al. 2004). However, the effects of $ONOO^-$ upon PKC and PKA activity are less certain. For example, exogenous $ONOO^-$ potentially activates and inhibits PKC activity in hippocampal homogenates and purified rat brain PKC (Knapp et al. 2001). Low-dose $ONOO^-$ enhances co-factor dependant PKC activity via $ONOO^-$-derived oxidative radicals, possibly hydroxyl radicals (Knapp et al. 2001); whereas, higher $ONOO^-$ concentrations irreversibly inhibit PKC activity through nitrotyrosine formation. Indirect $ONOO^-$ activation of PKA may occur in response peripheral activation of the COX2/prostaglandin E_2 pathway (Ndengele et al. 2008). Despite the established role of $ONOO^-$ in hyperalgesia, there remains a paucity of data on its in vivo modulatory effects on PKC and PKA activity.

Nitroxidative species may also contribute to LTP by regulation of CaMKII. While there is little evidence of direct regulation of CaMKII by nitroxidative species, the

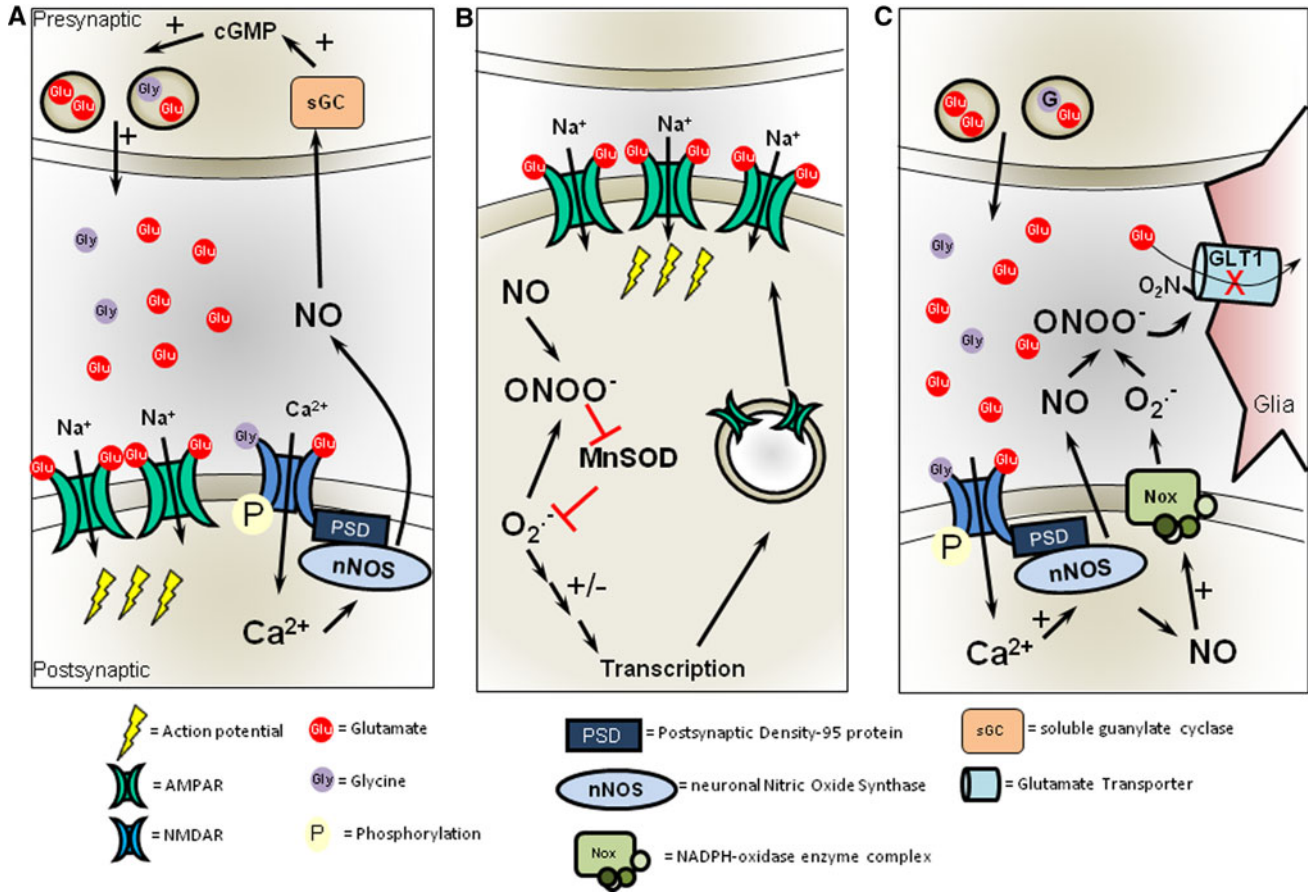

Fig. 3 Nitroxidative species contribute to long-term potentiation by enhancing glutamate signaling. **a** NO readily diffuses across the membranes and acts on pre-synaptic soluble guanidine cyclase (sGC) to enhance glutamate release via cGMP. **b** Increased levels of $O_2^{\cdot-}$ and NO can form intracellular $ONOO^-$ that is capable of nitroxidatively inactivating mitochondrial MnSOD, thus, reinforcing mitochondrial superoxide production. The enhanced intracellular $O_2^{\cdot-}$ can modulate the de novo production of receptors and regulatory proteins leading to enhanced postsynaptic neuronal responsiveness, in part, from increased surface AMPAR expression. **c** Increased synthesis of NO can activate plasma membrane NADPH-oxidase-derived superoxide production to raise $O_2^{\cdot-}$ levels. The rise in $O_2^{\cdot-}$ and NO levels within the synapse can form $ONOO^-$ capable of nitrating GLT-1. Nitration of GLT-1 prevents removal of glutamate from the synapse, thus, enhancing glutamate signaling

protein phosphatases that downregulate CaMKII autophosphorylation and signal cascade are susceptible to nitroxidative inhibition (Sommer et al. 2002; Namgaladze et al. 2005). Mice overexpressing G93A mutant SOD, a model of amyotrophic lateral sclerosis, decreases most spinal cord protein phosphatases subunit levels (Hu et al. 2003). Superoxide also inhibits calcineurin-induced dephosphorylation of CaMKII-mediated pCREB (Bito et al. 1996) whereas inhibition of protein phosphatases increased CaMKII phosphorylation in HFS-treated hippocampal neurons (Hongpaisan et al. 2004). Furthermore, CaMKII can be activated by the NO/cGMP pathway in the presynaptic membrane (Liu et al. 2007) and CaMKII activation promotes the phosphorylative inhibition of nNOS in the post-synaptic membrane (Komeima et al. 2000; Osuka et al. 2002; Watanabe et al. 2003; Rameau et al. 2004; Yan et al. 2004).

The contribution of nitroxidative species to the development and maintenance of central sensitization stems from persistent strong Ca^{2+} influx following nociception. The NMDAR-mediated Ca^{2+} influx can activate the nNOS; the AMPA- and NMDA-associated PKC, PKA, and CaMKII; as well as stimulate $O_2^{\cdot-}$ production from activated NADPH-oxidase and disrupted mitochondrial respiration. Collectively, these contribute to sensitization of dorsal horn neurons and assist in developing persistent pain.

Neuroimmune activation and nitroxidative species

Nitroxidative species are associated with neuroimmune activation, a state known to contribute to central sensitization (Muscoli et al. 2007; Batinic-Haberle et al. 2009; Ndengele et al. 2009; Salvemini and Neumann 2009a).

Minocycline, anti-TNFα, anti-IL-1β, and IL-10 therapies are also capable of reducing hyperalgesia and allodynia. These studies indicate that in addition to LTP, neuroinflammatory activation of astrocytes and microglia contributes to the development and maintenance of persistent pain. Though the neuroinflammatory mechanisms are still incompletely understood, several well-known nitroxidatively regulated innate immune responses have been identified (Fig. 4).

Activated glia produce and release NO and $O_2^{\cdot -}$ (Milligan and Watkins 2009) possibly through c-Jun N-terminal kinase (JNK), p38 MAPK, and ERK-activation via NO (Kawasaki et al. 2007) and could lead to the formation of $ONOO^-$. Inhibition of NOS is associated with attenuation of both spinal neuroimmune activation and cytokine (pro-inflammatory and pro-nociceptive) release by blocking redox-sensitive transcription factors and MAPK (e.g. p38) (Watkins and Maier 2005; Cui et al. 2006; Liu et al. 2006; Guo et al. 2007; Muscoli et al. 2007). Further, inhibition of glial cell metabolism with intrathecal administration of fluorocitrate decreased the number of NOS expressing cells following formalin injection (Sun et al. 2009). Moreover, $ONOO^-$ was implicated as a neuroimmune activator and proposed as a cell-signaling molecule (through activation of NF-κB, AP-1 and MAPK [p38]) that mediates the release of proinflammatory cytokines in the spinal cord (Muscoli et al. 2007). Increased primary afferent input, NMDAR activation, and increased NO and $O_2^{\cdot -}$ levels can trigger $ONOO^-$ formation; this may result in $ONOO^-$-mediated glial activation with pro-inflammatory cytokine release and further production of nitroxidative species potentially contributing to the induction and maintenance of central sensitization.

Nitroxidative species are produced through and moderate TLR4 pathways that result in the production of

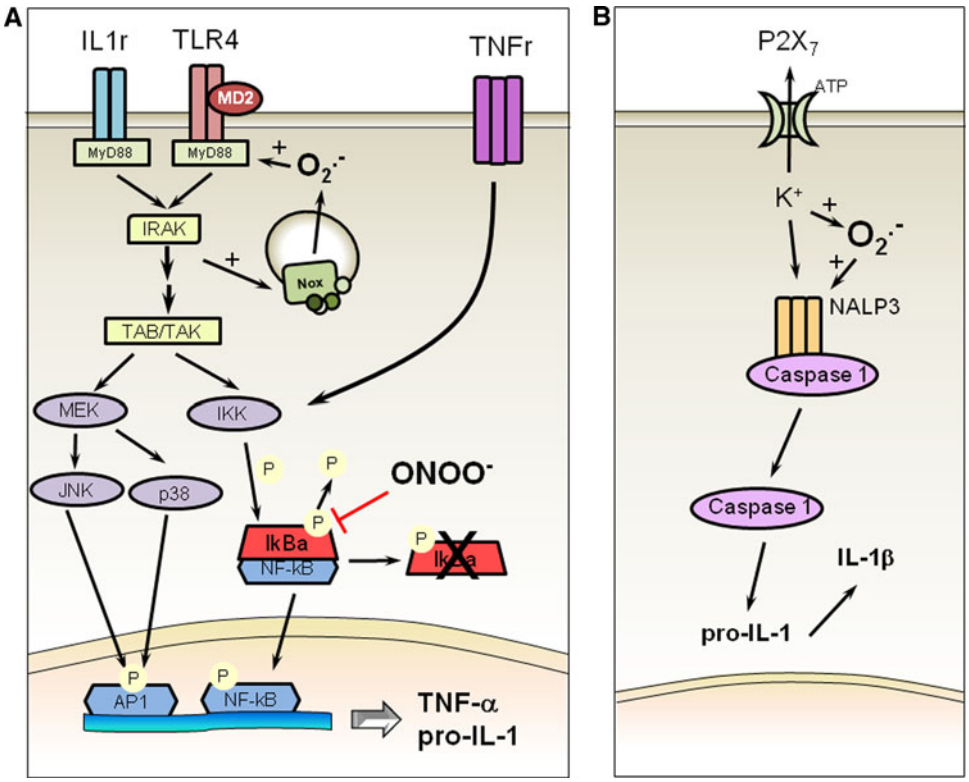

Fig. 4 The roles of nitroxidative species in neuroimmune activation. **a** TLR4 and IL1r stimulation triggers similar phosphorylative cascades leading to the transcription of inflammatory mediators. These pathways transduce their signals through IRAK-4 protein; whereby, IRAK-4 activates TAB/TAK complex. Additionally, IRAK4 can directly interact with NADPH-oxidase to produce $O_2^{\cdot -}$ that, in turn, leads to increased accumulation of TLR4 within lipid rafts. TAB/TAK activates the MEK pathways leading to JNK and p38 activation of AP-1 and the IKK pathways leading to NFkB activation. To activate NFkB, IkBa is phosphorylated by IKK, removed, and degraded to allow NFkB translocation. IkBa removal and degradation is prevented if there is a phosphate group at Tyr42. However, nitration by $ONOO^-$ prevents Tyr42 phosphorylation, thereby, enhancing the removal of IkBa and the translocation of NFkB. Furthermore, TNFr signaling can enhance IKK activation. **b** IL1b requires the activation of caspase-1 to form active protein. Caspase-1 is activated through activation of inflammasomes. One potential inflammasome stimulus in pain may be the activation of the $P2X_7$ receptor. Upon activation with ATP, the $P2X_7$ receptor allows for an outward K^+-current that stimulates $O_2^{\cdot -}$ production and activates the NALP3 inflammasome. Finally, the production of TNF allows for TNFR signaling that enhances $O_2^{\cdot -}$ production and apoptosis or enhances the activation of IKK-regulated transcription. The net effect of neuroimmune activation is the release of pro-inflammatory cytokines that enhance the production of other inflammatory mediators and augment neuronal sensitivity to stimuli

proinflammatory cytokines that may contribute to central sensitization (Fig. 4a). The signaling following TLR4 stimulation leading to TNF-α and IL-1β production is facilitated by the recruitment of MyDD88 Toll-like/Interleukin-1 receptor (TIR) domains of the TLR. Through MyD88, IL-1 receptor kinases (IRAK-4 and IRAK-1) initiate cascades leading to IκBα kinase, JNK, and p38 kinase pathways responsible for NF-κB and AP-1 promoted pro-inflammatory TNF-α, IL-1β, and IL-6 transcription (Palsson-Mcdermott and O'neill 2004; Watkins et al. 2009). Following TLR4 stimulation, the pro-IL-1β peptide also requires caspase-1 cleavage to form the mature IL-1β (Bryant and Fitzgerald 2009) (Fig. 4b). Caspase-1 is activated by the inflammasome complex composed of NOD-like receptor (NLR) proteins, which are activated by pathogen-associated molecular patterns and danger-associated molecular patterns (Bryant and Fitzgerald 2009) and may contribute to hyperalgesic states (Li et al. 2009).

Activation of the TLR4 pathways by lipopolysaccharide (LPS), the canonical TLR4 agonist, induces production of $O_2^{\cdot-}$, NO, and $ONOO^-$. LPS elicits NADPH-oxidase production of $O_2^{\cdot-}$ in microglia (Qin et al. 2004, 2005; Qian et al. 2007; Cheret et al. 2008); whereas, co-treatment with LPS and interferon (IFN)-γ induces astrocytic $O_2^{\cdot-}$ (Pawate et al. 2004). Furthermore, microglia treated with NADPH-oxidase inhibitors demonstrate lower $O_2^{\cdot-}$ production and a subsequent 50% reduction in TNF-α production (Qin et al. 2004). Co-treating microglia with LPS and IFN-γ induces iNOS transcription and increases NO synthesis (Pawate et al. 2004). However, when NADPH-oxidase is stimulated with phorbol 12-myristate 13-acetate (PMA), NO reacts with the $O_2^{\cdot-}$ to form $ONOO^-$ switching a beneficial nitroxidative species to a destructive species (Possel et al. 2000).

The mechanisms by which TLR4 stimulate NADPH-oxidase are incompletely understood; and though NADPH-oxidase is regulated by PKC phosphorylation (Lassegue and Clempus 2003), evidence suggests a more direct regulatory link of NADPH-oxidase to TLR4. In neutrophils, there is evidence that IRAK-4 can phosphorylate the p47-phox subunit; whereas IkBα kinase-γ (IKK-γ/NEMO), a regulatory subunit of the kinase complex responsible for NF-κB activation (Yamaoka et al. 1998), can phosphorylate both p47-phox and p67-phox (Singh et al. 2009). Additionally, NADPH-oxidase 4, the constitutive apocynin-insensitive NOX isoform expressed in the microglia (Harrigan et al. 2008) and neurons (Vallet et al. 2005), can directly associate with TIR domain of TLR4 and produce $O_2^{\cdot-}$ in response to LPS stimulation (Park et al. 2004). Finally, TLR4 may regulate its own transcription (Lin et al. 2006) and trafficking (Nakahira et al. 2006) through activation of NADPH-oxidase.

The role of nitroxidative species in supraspinal descending facilitation of central sensitization

The spinal cord is the primary, but not exclusive, site involved in the development and maintenance of central sensitization (Vanegas 2004). While little is known about the contribution of supraspinal nitroxidative species to hyperalgesic states (e.g. temporospatial expression or mechanisms), previous studies suggest that nitroxidative species within the rostral ventromedial medulla (RVM), a supraspinal nociceptive modulating center, may have a role in central sensitization. The RVM facilitates nociception and drives central sensitization through descending axonal projections to the dorsal horn of the spinal cord (Fields et al. 1991; Porreca et al. 2002). Some authors suggest that descending facilitation requires sensitization of the RVM neurons in a similar fashion to dorsal horn nociceptive neurons (Carlson et al. 2007) and this may occur through LTP (Ren and Dubner 2007).

Well-described changes in spinal nociceptive modulation centers that contribute to central sensitization such as NMDAR–NO cascades, PKC activation and translocation, and neuroimmune activation also occur in the RVM (Terayama et al. 2000; Carlson et al. 2007; Ren and Dubner 2007). For example, during hyperalgesic states there is an upregulation of mRNA encoding NMDAR subunits in the RVM (Miki et al. 2002; Terayama et al. 2002) and activation of the NMDAR NR1 subunit in a subpopulation of RVM cells that drive central sensitization at the dorsal horn (Budai et al. 2007). Hyperalgesia is also associated with phosphorylation and activation of the NR2 subunit of the NMDAR within the RVM in response to brain-derived neurotrophic factor released from the periaquaductal gray axons (Guo et al. 2006; Ren and Dubner 2007). Moreover, intra-RVM administration of NMDAR antagonists [2-amino-5-phosphonovaleric acid (APV) and MK801] significantly reduces inflammatory hyperalgesia (Coutinho et al. 1998, 2001; Urban et al. 1999), while administration of NMDA enhances inflammatory hyperalgesia (Urban et al. 1999).

Similar to mechanisms involved in spinal central sensitization, NO also contributes to RVM descending facilitation. Inflammation-induced nociception significantly increases the number of nNOS and NADPH-diaphorase positive cells in the RVM (Coutinho et al. 1998, 2001; Urban et al. 1999); this temporospatial expression parallels the duration and severity of hyperalgesia (Urban et al. 1999). Further, investigations using intracerebroventricular injections of non-selective NOS inhibitors (L-NAME, NOArg) (Moore et al. 1991; Kolesnikov et al. 2009) and a guanyl cyclase inhibitor (l–H-[1,2,4]oxadiazalo[4,3-a]quinoxalin- 1-one) (Salter et al. 1996) were successful in reducing behavioral manifestations of central sensitization

in various animal models. More specifically, RVM microinjections of L-NAME (Coutinho et al. 1998, 2001; Urban et al. 1999) and the nNOS inhibitor (ARL17477) (Coutinho et al. 2001) were able to significantly reduce secondary hyperalgesia following peripheral and visceral inflammation. Conversely, a RVM microinjection of a NO donor (GEA 5024) enhances inflammatory hyperalgesia (Urban et al. 1999).

Recent studies also demonstrate that neuroimmune activation occurs in the RVM and contributes to central sensitization during neuropathic and inflammatory pain (Wei et al. 2008; Roberts et al. 2009). Wei et al. (2008), found that during nerve injury-induced hyperalgesia there is time-dependent glial hyperactivation, increased levels of pro-inflammatory cytokines (i.e. IL-1β, IL-6, and TNF-α), and neuronal NMDAR NR1 subunit phosphorylation within the RVM. In addition, intra-RVM injections of glial inhibitors attenuate thermal and mechanical hyperalgesia and allodynia (Wei et al. 2008; Roberts et al. 2009).

The RVM drives central sensitization through NMDAR activation-, NO-, and neuroimmune activation-mediated descending facilitation, however, the contribution of $O_2^{\cdot-}$-derived $ONOO^-$ to descending facilitation is unknown. There is evidence for other supraspinal nitroxidative species contributing to pain as intracerebroventricular injections of free radical scavengers (PBN) attenuate neuropathic and inflammatory hyperalgesia (Kim et al. 2004; Lee et al. 2007). Furthermore, $ONOO^-$ -mediated activities occur in the brain during morphine-induced hyperalgesia as evidenced by the expression of the nuclear enzyme PARP and decreased MnSOD activity (Doyle et al. 2009). Administration of $ONOO^-$ decomposition catalysts ($MnTnHex\text{-}2\text{-}PyP^{5+}$ and $MnTE\text{-}2\text{-}PyP^{5+}$) prevents hyperalgesia and markers of $ONOO^-$-mediated stress in the brain, indicating that supraspinal $ONOO^-$ may contribute to descending facilitation of central sensitization (Doyle et al. 2009). Thus, we propose that RVM descending facilitation is also mediated by $O_2^{\cdot-}$ derived $ONOO^-$ activities using mechanisms similar to those described in the spinal cord during central sensitization as discussed above and reviewed elsewhere (Salvemini and Neumann 2009a, b).

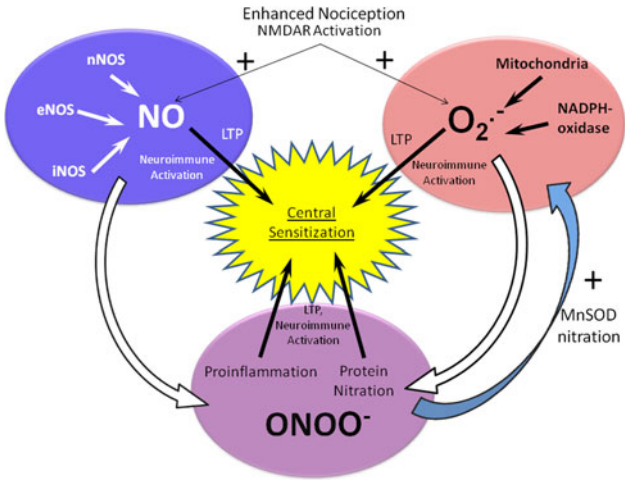

Fig. 5 Summary of the contributions of nitroxidative species to central sensitization and pain. Enhanced nociception and the activation of NMDAR stimulates the synthesis of NO via NOS enzymes and the production of $O_2^{\cdot-}$ from mitochondria and NADPH-oxidase. Individually, NO and $O_2^{\cdot-}$ contribute to the development of long-term potentiation and neuroimmune activation. Together, $O_2^{\cdot-}$ and NO can produce $ONOO^-$ that can nitrate and inactivate the glutamate transporter, glutamate synthase and MnSOD. Nitration of these key proteins enhances synaptic plasticity and production of additional nitroxidative species. Combined, processes mediated by nitroxidative species facilitate the development of central sensitization and persistent pain

Conclusion

Nitroxidative species are critical components of pain as a result of peripheral, spinal, and supraspinal contributions to central sensitization. Figure 5 summarizes the proposed contributions of nitroxidative species through LTP and neuroimmune activation in the spinal cord. Although numerous studies support the role of nitroxidative species in the periphery and spinal cord, little is known about the supraspinal contributions of $O_2^{\cdot-}$ and $ONOO^-$; further investigations are needed to determine this role. Important starting points are investigations of temporospatial and cellular expressions of specific markers of nitroxidative species during central sensitization. Our laboratory is currently undertaking these studies.

The understanding of pathologic mechanisms that result in central sensitization is crucial to the development of therapeutic interventions that can effectively manage pain states. The development and experimental application of pharmacologic agents such as SODm (Salvemini et al. 2002) and $ONOO^-$ decomposition catalysts (e.g. metalloporphyrins) (Salvemini et al. 1998a; Batinic-Haberle et al. 2002; Szabo et al. 2007) have helped to support the role of nitroxidative species in central sensitization. Because of this, nitroxidative species, especially $O_2^{\cdot-}$ and $ONOO^-$, are intriguing targets and pharmacologic options for improved pain management as synergists, adjuncts, and alternatives to currently available treatments (Salvemini 2009; Salvemini and Neumann 2009a, b). Further development and characterization of more selective SODm and $ONOO^-$ decomposition catalysts should provide the necessary tools to better characterize the contributions of $O_2^{\cdot-}$ and $ONOO^-$ to pathologic pain states. Our laboratory is currently pursuing such advances in pharmacologic agents.

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