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Nitroxidative signaling mechanisms in pathological pain

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Abstract

Tissue injury can initiate bidirectional signaling between neurons, glia and immune cells that creates and amplifies pain. While the ability for neurotransmitters, neuropeptides, and cytokines to initiate and maintain pain has been extensively studied, recent work has identified a key role for reactive oxygen and nitrogen species (nitroxidative species), including superoxide, peroxynitrite, and hydrogen peroxide. In this review, we describe how nitroxidative species are generated after tissue injury, and the mechanisms by which they enhance neuroexcitability in pain pathways. Finally, we discuss potential therapeutic strategies for normalizing nitroxidative signaling, which may also enhance opioid analgesia, to help to alleviate the enormous burden of pathological pain.

Keywords

NADPH oxidase; mitochondria; sensitization; TRP channels; neuroinflammation; exercise

The link between nitroxidative signaling and pain

Investigation of oxidative processes, such as rusting, began with the “phlogiston theory”, developed by Georg Ernest Stahl during the scientific revolution, which postulated that a fire-like element (phlogiston) is released during combustion. Oxidation was formally linked to biology during the early 20th Century, when it was found to underpin cellular metabolism [1–3]. The connection between reactive oxygen species (ROS) and altered sensory processing was empirically identified around the same time [4]. Since then, research has shown that prolonged, unchecked increases in reactive oxygen and nitrogen (nitroxidative) species after infection or tissue damage can promote cytotoxicity and inflammation. These

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processes can cause peripheral and central sensitization, which underlie pathological pain (see Glossary) [5,6]. Thus, restoring nitroxidative balance in peripheral and central nervous systems (PNS, CNS) is a possible therapeutic approach for ameliorating neuropathology [6–10].

In this review, we summarize recent research on how nitroxidative species participate in neuroimmune signaling throughout the neuraxis to drive pathological pain. We additionally discuss potential therapeutic strategies for normalizing nitroxidative signaling by activating endogenous antioxidant systems, which may also enhance opioid analgesia. As pathological pain is often intractable to current therapies, new strategies to normalize nitroxidative signaling may help to alleviate the enormous burden of pain [11].

Production of nitroxidative species by neurons, glia, and immune cells

The role of nitroxidative signaling in pain has been studied using rodent experimental models of inflammatory pain (e.g. intraplantar complete Freund's adjuvant (CFA), formalin) and neuropathic pain (e.g. peripheral nerve injury (PNI), chemotherapy-induced peripheral neuropathy (CIPN), diabetic neuropathy (DN), spinal cord injury (SCI), experimental autoimmune encephalomyelitis (EAE)), which have recently been reviewed elsewhere [12]. There are numerous endogenous sources of ROS and nitric oxide (NO) that are engaged during pain processing [13]. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, NO synthases and mitochondrial respiration are among the best characterized ROS/NO producers, and will be discussed here (Figs. 1 and 2).

NADPH oxidases

NADPH oxidases (NOX) are membrane-bound enzyme complexes. They transport electrons donated from cytosolic NADPH to generate extracellular or luminal superoxide anions or hydrogen peroxide, that can be transported into the cytosol via aquaporin channels [13,14]. In contrast to other sources of ROS that are generated as a byproduct of catabolism, ROS generation is the primary function of NOX. There are seven members in the NOX family; NOX1, 2, and 4 have been implicated in pathological inflammatory and neuropathic pain models [13,15,16]. NOX1 and 2 are expressed at the cellular membrane, and produce superoxide anions following phosphorylation of cytosolic subunits [17]. NOX4 is expressed on organelles, such as the endoplasmic reticulum, and constitutively produces hydrogen peroxide [17].

NOX1 is inducibly expressed by microglia, neurons, astrocytes, and macrophages in the dorsal root ganglion (DRG) and CNS [17–19]. Nociceptive hypersensitivity induced by the inflammatory stimuli formalin and carrageenan is attenuated in *Nox1* deficient mice [18]. NOX1-derived ROS induce translocation of PKCε to the membrane to enhance Transient Receptor Potential (TRP) V1 activity in DRG neurons [18], a change consistent with pain amplification (Fig. 2). In contrast, another study showed that NOX1 mRNA failed to upregulate in the DRG following peripheral nerve injury (PNI) [20]. These results indicate that DRG NOX1 may have a preferential role in inflammatory versus neuropathic pain.

NOX2 is predominantly expressed by phagocytic cells—peripheral macrophages and CNS microglia [13]. PNI induces a rapid upregulation of NOX2 mRNA by DRG macrophages and spinal microglia, which is correlated with increased intracellular superoxide [20,21]. PNI-induced nociceptive hypersensitivity was attenuated in *Nox2* deficient mice [20,21]. *Nox2* deficiency attenuated TNF, but not IL-1β, mRNA expression, as well as expression of the neuronal injury marker ATF3 in DRG (Fig. 2) [20]. However, *Nox2* deficiency did not influence macrophage recruitment to the injured DRG, suggesting a role for NOX2 in macrophage function rather than chemotaxis [20]. *Nox2* deficiency attenuated PNI-induced Iba1 expression and the attendant expression of pro-inflammatory cytokines TNF and IL-1β in the spinal dorsal horn [21]. As these studies were performed in global knockouts, it is still unclear whether alterations in the DRG and dorsal horn are subject to NOX-dependent changes in macrophage function at the injury site. In contrast to NOX1, NOX2 activity in monocytes appears to play no role in inflammatory pain [22].

NOX4 is expressed by DRG neurons—both myelinated (A-fibers) and unmyelinated (C-fibers) DRG neurons—and by microglia, astrocytes and macrophages [13,23,24]. Nociceptive hypersensitivity following PNI is attenuated in *Nox4* deficient mice, with attenuation of hydrogen peroxide at the sciatic nerve injury site [23]. These results are supported by the absence of NOX4 upregulation in the DRG after PNI [20]. The myelin proteins MPZ and PMP22 are decreased at the sciatic nerve injury site over time in an NOX4-dependent fashion, suggesting that myelin degeneration by hydrogen peroxide may maintain neuropathic pain (Fig. 2). However, attenuated damage at the injury site did not alter expression of the nitroxidative stress and neuroinflammation indices at the spinal dorsal horn or DRG (microglia proliferation, hydrogen peroxide levels) [23]. This contrasts with other studies showing that such processes are dependent on manipulations at the sciatic nerve [25–27]. Finally, a role for NOX4 may be limited to neuropathic, rather than inflammatory pain [23].

Together, these data suggest that NOX1, 2, and 4 isoforms contribute to pathological pain. Future studies could expand the role of various NOX isoforms to other sites in the neuraxis, and well as identifying a role for other NOX isoforms in pain. These data may help to guide development of therapeutics that target the activity of specific NOX isoforms to reduce nitroxidative stress and pain.

Nitric oxide synthases

NO is a diffusible gas mediator that is synthesized from L-arginine by one of three nitric oxide synthase (NOS) isoforms: NOS1 (neuronal), 2 (inducible), and 3 (endothelial). NO and all three NOS isoforms have a well-established role in nociception (Fig. 2) [28]. It easily passes through membranes to directly impact nearby cells.

NOS1 is constitutively expressed in the cytosolic compartment of postsynaptic terminals of neurons, and of stressed Schwann cells, and requires calcium for its activation [29–31]. In abnormal pain states, N-methyl-D-aspartate (NMDA) receptors are activated, resulting in calcium influx and activation of NOS1 [28]. Nociceptive hypersensitivity induced by PNI and CIPN is attenuated by genetic ablation and pharmacological inhibition of NOS1 [32–35].

NOS2 is a cytosolic isoform that is widely expressed in many immune cells and in glia. Transcription of NOS2 is initiated by Toll like receptors (TLRs) and, once translated, is constitutively active—that is, unlike NOS1 and 3, its activity is independent of calcium [28]. NOS2 inhibition attenuates nociceptive hypersensitivity associated with inflammatory and neuropathic pain models [15,36,37].

NOS3 is best known for its expression in the cardiovascular system as a regulator of vascular tone. NOS3 is a membrane-bound enzyme that is constitutively expressed; however, it requires the interaction of calcium and calmodulin for its activation [28]. NOS3 expression is increased in the DRG after subcutaneous administration of CFA, and is correlated with allodynia, suggestive of increased NOS3 activity [38]. CFA-induced inflammatory pain is attenuated by NOS3 inhibition [38].

Cellular respiration

One critical function of mitochondria is energy metabolism. The mitochondrial electron transport chain (mETC) is a series of five molecular complexes through which electrons are transported to synthesize ATP from ADP. Premature electron leakage can occur during cellular respiration, particularly at Complexes I and III, resulting in superoxide production (Fig. 2) [39].

Mitochondrial ROS are elevated in spinal neurons, microglia and astrocytes in neuropathic pain models [21,40,41]. Furthermore, blocking the mETC attenuates hyperalgesia associated with a range of inflammatory and neuropathic pain models [42–45]. However, a direct link between mETC-dependent pain and mitochondrial ROS has yet to be shown. These results suggest that cellular respiration is increased, but is inefficient due to enhanced ROS-generating electron leakage from the mETC, as ATP production by sciatic nerves is impaired during CIPN [46].

Mechanisms of nitroxidative signaling in neuronal hyperexcitability

Injury or disease can provoke intense, repeated, and sustained activity of primary afferent (sensory) neurons. This activity, together with the release of mediators from reactive glia and immune cells, elicits well-characterized changes in neuronal and biochemical processing at peripheral terminals and central synapses [5,47–50]. This is termed ‘sensitization’, and results in nociceptive hypersensitivity. Here, we discuss how nitroxidative signaling engages neurons in pain pathways, leading to peripheral and central sensitization (Figs. 1 and 3).

Nitroxidative species as neuromodulators in pain pathways

Nitroxidative species can directly increase the excitability of nociceptive neurons. Intraplantar administration of superoxide, peroxynitrite, or intrathecal delivery of the ROS donor *tert*-butyl hydroperoxide (tBOOH) is sufficient to induce nociceptive hypersensitivity in naïve rats [51–54]. These studies demonstrated that ROS activates calcium calmodulin-dependent protein kinase II (CamKII) in glutamatergic spinal neurons, and induced presynaptic inhibition of GABAergic interneurons (disinhibition). Furthermore, hydrogen peroxide enhanced the frequency and amplitude of action potentials of DRG neurons from neuropathic rats (Fig. 3) [55].

In neuropathic pain models, administration of the non-selective ROS scavenger phenyl-N-tert-butylnitrone (PBN), selective small molecule superoxide and peroxynitrite decomposition catalysts such as M40403, $FeTMPyP^{5+}$ and $MnTE\text{-}2\text{-}PyP^{5+}$, or selective peroxynitrite decomposition catalysts such as SRI6 and SRI110 attenuated nociceptive hypersensitivity [15,51,53,54,56–58]. Accordingly, PBN attenuated injury-induced hyperexcitability of spinal dorsal (sensory) horn "pain" responsive neurons and phosphorylation of CamKII [51,57], an effect consistent with pain normalization. Several mechanisms of enhanced excitatory signaling have been identified. Hydrogen peroxide can activate cGKIα, resulting in increased neurotransmitter release from the terminals of primary afferent neurons in the dorsal horn [59,60]. Peroxynitrite and ROS disrupt glutamate homeostasis leading to potentiation of synaptic currents and calcium influx, and ultimately excitotoxicity [56,61]. Mechanisms include nitration and phosphorylation of several NMDA receptor subunits, as well as inhibition of glutamine synthetase and the glutamate transporter GLT-1 that limit the synaptic half-life of glutamate [15,56,62,63]. Nitroxidative products also induce disinhibition after PNI, as PBN normalized the decrease in $GAD\text{-}67^{+}$ GABAergic dorsal horn neurons, and increased GABA release (Fig. 3) [53,64]. Together, these data suggest that nitroxidative species directly enhance neuroexcitability in pain pathways.

Nitroxidative species activate TRP channels

The TRP family of nonselective cation channels plays a vital role in the molecular integration of multiple endogenous and exogenous sensory stimuli [65]. Several of these channels, expressed at the peripheral and central terminals and cells bodies of primary afferent neurons, are activated by nitroxidative species and products. TRP channel activation by nitroxidative species can also initiate neurogenic inflammation— recruitment and activation of immune cells following release of neuropeptides by neurons—which is a key process underlying pathological pain (Fig. 3) [5,66]. Here, we focus on known roles of TRPA1, TRPM2, and TRPV1.

TRPA1 is a chemoreceptor expressed exclusively by peptidergic C-fibers [65]. Nitroxidative species induce protein carbonylation, and membrane phospholipid peroxidation and nitration, and subsequent production of reactive aldehydes such as acrolein (Fig. 1). These products all share the ability to induce nociceptive hypersensitivity by directly activating TRPA1 [67–72]. Acrolein is elevated in the DRG and spinal cord after SCI, and blockade with hydralazine or phenelzine partially attenuated allodynia [73,74]. Moreover, nociceptive hypersensitivity induced by CIPN was abolished in *Trpa1* deficient mice, or with a TRPA1 antagonist [75]. In this model, the chemotherapeutic bortezamib did not directly activate TRPA1, suggesting that ROS may act as an intermediate [75].

TRPM2 is expressed by neurons, and abundantly by immune cells, including monocytes/macrophages, neutrophils and T cells, and microglia. This channel is directly activated by hydrogen peroxide, and cytosolic ADP-ribose that is generated after nitroxidative damage to mitochondria [76–81]. Furthermore, TRPM2 activation is critical for activation of spinal microglia and for macrophage infiltration into the spinal cord after PNI [82]. TRPM2 also activates ERK MAPK and induces nuclear translocation of NFκB, resulting in production of

proinflammatory cytokines and chemokines [76,77,81,83,84]. Consequently, pharmacological and genetic studies have demonstrated that TRPM2 contributes to inflammatory and neuropathic nociceptive hypersensitivity [77–79,82,85].

TRPV1 is found on unmyelinated, slowly conducting neuronal C-fibers, and is an essential component underlying injury-elicited thermal hyperalgesia and nociceptive hypersensitivity [65]. TRPV1 expression is upregulated by an exogenous ROS donor (tBOOH), and is a target of oxidation and nitration events that increase responsiveness of the channel [18,86–88]. Moreover, linoleic acid metabolites, created during production of eicosanoids, are endogenous TRPV1 agonists when oxidized, and contribute to nociceptive signaling [89,90].

Nitroxidative species induce mitochondrial dysfunction

Mitochondria have pivotal roles in a variety of cellular functions, including energy metabolism, calcium homeostasis, lipid synthesis, and apoptosis. As noted above, cellular respiration can be elevated under neuropathic pain conditions, with an attendant elevation of ROS derived from neuronal and microglial mitochondria [21,40,41]. Together with nitroxidative species derived from NOX and NOS enzymes, these species disrupt mitochondrial homeostasis via several mechanisms, leading to bioenergetic crisis (due to impaired mETC efficiency) and degeneration of primary afferents (Fig. 3) [91].

Mitochondrial DNA is a target of oxidation and nitration, while peroxidated lipid end-products, such as reactive aldehydes, can form covalent modifications (adducts) with an array of mitochondrial proteins, including antioxidants [92,93]. Together, these changes impair the structural integrity and function of mitochondria. Nitroxidative species can also trigger release of pro-apoptotic factors from mitochondria. For example, NO can disrupt mitochondrial dynamics (fission and fusion; responsible for maintaining metabolic homeostasis) that results in translocation of Bcl-2-associated X protein from the cytosol to the organelle membrane, where it activates apoptosis pathways [94–96]. Activation of apoptosis pathways contributes to neuropathic pain, as inhibition of several caspase enzymes attenuates vincristine- and dideoxycytidine-induced nociceptive hypersensitivity [97]. Neuropathic pain is associated with impaired mitochondrial function, and nociceptive hypersensitivity is accordingly attenuated by pharmacologically normalizing mitochondrial dynamics or preventing mitotoxicity [46,98–100].

Nitroxidative species induce neuroinflammatory signaling

Pro-inflammatory mediators released by glial and immune cells increase neuroexcitability in pain pathways after injury (e.g. TNF, IL-1β, BDNF) [5,50,101–103]. Several mechanisms include enhanced glutamate release, increased AMPA receptor expression, phosphorylated NMDA receptor subunits, and downregulated astrocyte glutamate transporters [5]. These proinflammatory mediators can also induce disinhibition of neuronal excitability by attenuating GABA and glycine release from interneurons and inhibitory descending projections, and downregulating KCC2 on postsynaptic terminals [5].

Nitroxidative species regulate the production of proinflammatory mediators during pathological pain. For example, NFκB and p38 MAPK are responsible for the production of a wide array of proinflammatory mediators in immune cells. Nitroxidative products degrade/

inhibit IκB and MAPK phosphatases, resulting in activation of NFκB and p38 that both mediate inflammatory and neuropathic pain [52,104–107]. Furthermore, nitroxidative species may promote release of neuron-to-glia signals, such as matrix metalloproteases (MMPs) (Fig. 3) [108].

Nitroxidative species also elicit proinflammatory responses via toll-like receptor (TLR) signaling. TLRs bind a variety of endogenous ligands (danger associated molecular patterns: DAMPs), including DNA and N-formyl peptides from nitroxidatively damaged mitochondria, to trigger innate immune responses that contribute to pathological pain [5,109]. ROS serve a vital role as second messengers for TLR signaling. A rapid (minutes) respiratory burst occurs upon activation of TLR2 and 4, which is mediated by a direct interaction with the intracellular domains of NOX1, 2, and 4 enzymes. This NOX activity is essential for downstream NFκB- and p38 MAPK-dependent cytokine production [110–114]. Furthermore, activation of NOX enzymes by TLR signaling induces transcription of TLRs, and promotes membrane expression in lipid rafts, which is necessary for efficient signaling [111,115,116]. In concert with disruption of blood-brain barrier tight junctions by nitroxidative species, the TLR2-NOX1 interaction also upregulates adhesion molecules via CCL3 to facilitate transendothelial cell migration, which contributes to nociceptive hypersensitivity after PNI (Fig. 3) [102,110,117].

ROS have been implicated in the activation of NLRP3 inflammasomes [118]—protein complexes responsible for the proteolytic activation of IL-1β, a pro-inflammatory cytokine with a well-established role in pathological pain [5,101,119]. Among the various sensor molecules that trigger formation of inflammasomes, NLRP3 has been most widely investigated, and has a recently described role in neuropathic pain [120]. The relative contributions of ROS to the activation versus priming of NLRP3 inflammasomes remains to be elucidated [119]. Mitochondria are key participants in the activation of NLRP3 inflammasomes; they are a source of ROS that can directly activate NLRP3, as well as oxidized mitochondrial DNA that can also activate NLRP3 (Fig. 3) [118,121–123]. Furthermore, TRPM2 activation by nitroxidative species induces a calcium flux that activates the NLRP3 inflammasome [124].

Finally, there is a reciprocal relationship between nitroxidative species and inflammatory signaling. For example, the transcription of NOX and NOS enzymes is upregulated by TLR4 and 9 signaling, and by NFκB and p38 activation [19,125–129]. The purinergic receptor P2X7, which has a documented role in pathological pain, also induces ROS production [5,120,130]. ATP signaling through P2X7R activates NOX2 in a calcium and p38-dependent fashion [131–133].

Endogenous regulators of nitroxidative signaling

Under healthy conditions, nitroxidative species and antioxidants exist in a balanced state, as nitroxidative products play a vital physiological role in cellular processes (e.g. signal transduction, pathogen defense [134–136]). In response to increased production of nitroxidative species during injury or infection, antioxidant and regulatory systems are activated in an attempt to recover homeostasis (Fig. 1) [14].

Antioxidant defense

Transcription of antioxidant genes is a critical step in controlling nitroxidative signaling. One key transcription factor is nuclear factor E2-related factor 2 (Nrf2). Nrf2 is expressed in CNS and PNS neurons, macrophages, Schwann cells, astrocytes, and microglia [137–139]. Under homeostatic conditions, cytosolic Nrf2 is sequestered by the protein Keap1 and ubiquinated for degradation. However, in the presence of oxidants and electrophiles Nrf2 is released from Keap1 and translocates to the nucleus [140]. Nrf2 binds to the antioxidant response element (ARE) promoter region to elicit expression of 200+ antioxidant genes, including superoxide dismutases (SOD1: cytosolic; SOD2: mitochondrial), catalase, glutathione, and heme-oxygenases [140]. Another transcription factor, forkhead box, class O (FoxO), is also responsible for the production of SOD2 and catalase [141]. Many of these antioxidants are ubiquitously expressed, and their catabolic function is summarized in Figure 1 [142].

These endogenous antioxidant systems collaborate to detoxify reactive nitroxidative species (Fig. 1). Evidence is mixed whether neuroinflammatory or traumatic events increase nervous system antioxidant levels [143–152]. This likely reflects a temporally-and injury-specific antioxidant response, and the fact that injury-induced nitroxidative species can negatively regulate antioxidant production [15,76]. Antioxidant system activation can limit pathological pain: deletion of SOD1 exacerbates neuropathic pain, while exogenous antioxidants attenuate nociceptive hypersensitivity in a range of inflammatory and neuropathic pain models [37,108,153–155]. Similarly, hemeoxgenases, which elicit expression of various antioxidants, protects cells and could improve inflammation and neuropathic pain [21]. Therefore, therapies that increase antioxidant systems could resolve neuroinflammation and pain symptoms.

Anti-inflammatory cytokine and adenosine signaling

Cytokines such as IL-10 and TGFβ counter-regulate proinflammatory signaling and contribute to the resolution of neuropathic pain hypersensitivity [5,156,157]. One mechanism of action is regulation of nitroxidative signaling. For example, IL-10 and TGFβ inhibit NOX2 activity and promote antioxidant production [158–160]. This is a reciprocal relationship, as antioxidants can also drive production of anti-inflammatory cytokines [161,162]. Adenosine signaling is also anti-nociceptive in pathological pain models [163–165]. Signaling through A_{2A} and A_3 receptors inhibits NOX activity, and drives production of anti-inflammatory cytokines and antioxidants [163,166,167].

Opposition of opioid analgesia by nitroxidative species

Opioid analgesics remain the cornerstone of management of moderate-to-severe pain. However, the clinical utility of opioids is limited by tolerance, which is characterized by dose escalation due to reduced sensitivity to an opioid agonist, as well as hyperalgesia, a paradoxical increase in pain sensitivity due to opioid exposure [168,169]. Recent evidence has identified a role for nitroxidative signaling in these phenomena [6,170].

NOX activity is elevated by morphine, and genetic or pharmacological disruption of these enzymes attenuates tolerance and hyperalgesia [171–173]. Superoxide and peroxynitrite have been implicated as downstream mediators, as decomposition catalysts also attenuate tolerance and hyperalgesia [174–176]. It remains unclear how morphine engages these enzymes, but it may be mediated by classical μ-opioid receptors and/or TLR4 [168]. The pro-nociceptive mechanisms of nitroxidative species, described above, may act as an opponent process of neuronally-mediated opioid analgesia to create tolerance, or may overshadow analgesia to induce hyperalgesia. Therefore, correcting nitroxidative imbalance may improve the clinical profile of opioids [170].

Nitroxidative signaling also disrupts endogenous opioid analgesia in supraspinal sites that is engaged to inhibit spinal nociception via descending projections. For example, induction of peroxynitrite during inflammatory pain results in nitration of met-enkephalin in the rostral ventromedial medulla (RVM), which reduces opioid receptor binding affinity [177]. This may be normalized by intra-RVM microinjections of $FeTMPyP^{5+}$, which was antinociceptive in inflammatory and neuropathic pain models [177].

Nitroxidative signaling as a therapeutic target for pathological pain

Under pathological conditions, endogenous antioxidant responses can be insufficient, leading to an accumulation of toxic nitroxidative species. As mentioned above, unchecked increases in nitroxidative species can promote cytotoxicity and inflammation via cascading pronociceptive signaling. Therefore, discovering therapeutic treatments that enhance cellular antioxidant capacity could help achieve nitroxidative balance to recover homeostasis.

Initial efforts to combat increases in nitroxidative species in a wide range of neurological disorders used direct antioxidant compounds (e.g. vitamins C and E, co-enzyme Q). The consensus view is that the possible beneficial effects are outweighed by unfavorable pharmacokinetic and pharmacodynamic profiles [13,178,179]. A variety of redox-active therapeutics are being developed to overcome these issues and are effective in in treating cancer-induced bone pain, inflammatory, and neuropathic pain, and can also potentiate opioid analgesia [9,10,180].

Newer approaches have instead aimed to inhibit sources of nitroxidative species, stimulate endogenous antioxidants, and prevent nitroxidative damage [13,178]. To this end, inhibitors of specific NOX and NOS isoforms, and ROS toxifiers such as MPO, are being developed and may prove effective for pain treatment [13,181]. As noted above, A_{2A} and A_3 adenosine receptor agonists attenuate spinal NOX activity and promote antioxidant production, with a concomitant decrease in neuropathic pain [163–165]. Another promising approach is the development of small molecules that catalyze the clearance of reactive aldehydes [182].

Indirect antioxidants augment the redox response without being antioxidants themselves. For example, sulforaphane, resveratrol, and curcumin induce nuclear translocation of Nrf2, a transcription factor responsible for the production of a wide array of antioxidants, and attenuate nociceptive hypersensitivity in neuropathic pain models [21,183–186]. Non-pharmacological approaches may also function in this capacity. For example, exercise

increases Nrf2 expression and promotes the expression of antioxidants in the CNS as well as peripherally [187–189]. Consequently, voluntary wheel running has been shown to both prevent and reverse neuropathic pain [187,190].

Finally, ROS have a role in normal physiological processes [134–136], and there is some evidence that ROS may have protective effects after injury. For example, inflammation induced by endotoxin is exacerbated in NADPH-impaired mice, relative to their wild-type counterparts [191]. In another study, yeast survival to hydrogen peroxide stress was dependent on superoxide [192]. Further work is required to determine whether reactive oxygen species may also have a protective role after sterile nervous system injury. However, agents have been developed to spare superoxide (e.g. peroxynitrite decomposition catalysts SRI110 and SRI6 [15]), and such approaches may prove to be important for restoring homeostasis after nervous system injury.

Concluding remarks

Nitroxidative species are generated by mitochondria and by NOX and NOS enzymes. They enhance neuroexcitability in pain pathways through direct neuronal interactions, and indirectly by impairing mitochondria and inducing neuroinflammation. Normalizing nitroxidative signaling may be an alternative strategy to help to alleviate the enormous burden of pathological pain, which affects ~20% of the population, and is poorly treated [11,193,194]. There are several areas of basic science research that may move us towards that goal (see Outstanding Questions).

Despite the extensive research implicating nitroxidative species in pathological pain states, no studies to date have quantified the critical relationships between real-time local cellular creation of nitroxidative species, their concentration at the effect site, or the distribution of their direct effect. This challenge has not been overcome owing to the volatility of these nitroxidative species and hence the very short life-time *in vivo* and *ex vivo*. Several new technologies are being developed to address these issues, and are discussed in Box 1.

Lessons from the failure of direct antioxidants to improve clinical disease need to be recognized within the pain field; the effects of direct antioxidants on preclinical pain models continue to be reported, despite the strong probability that the results will not translate clinically. Several studies suggest that more robustly engaging antioxidant systems after injury can help alleviate pain: for instance, in animal pain models, increasing action of master antioxidant transcription factors Nrf2 or FoxO, or activating the heme-oxygenase system show promising pain-relieving effects. Future studies could explore whether combinatorial strategies – aimed at boosting multiple antioxidants or targeting both antioxidant and nitroxidative systems simultaneously – dampen inflammation and pain. Nitroxidant dysregulation clearly contributes to neuropathology; thus, discovering new targets and therapies that restore nitroxidative balance could help relieve pathological pain.

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Glossary

Neuroimmune signaling

bidirectional communication between leukocytes, glia and neurons

Pathological pain

maladaptive pain that serves no useful purpose

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Box 1

New and emerging tools to study nitroxidative species

Colorimetric and fluorescent methods for detecting the “shadow” of the presence of nitroxidative species production is well established by the quantification of attendant cellular events (e.g. oxidative stress such as lipid peroxidation (TBARS) [195]; and DNA damage (8-Oxoguanine: 8-OxoG) [196,197]) or the quantification of more stable metabolites (e.g. nitrite/nitrate using Griess reaction [198]). These methods are not only limited in their temporal and spatial resolution, but also due to their insufficient ability to define concentrations and time courses of specific nitroxidative species. Establishing differential regulation of distinct nitroxidative species would be useful, as specific oxygen or nitrogen species have unique outcomes in the neuroinflammatory responses. A recent example demonstrated that specifically targeting peroxynitrite reduced inflammatory progression via NLRP3 inflammasome-dependent IL-1β/IL-18 release following ICH induced inflammatory injury [199]. Thus, new biosensors are required to improve our mechanistic understanding of how nitroxidative species affect the nervous system.

The chemistry of fluorescent probes for specific detection of both *ex vivo* and *in vivo* production of nitroxidative species has grown rapidly. A range of approaches and hence biosensors have been created that exploit platform sensing modalities, such as photoinduced electron transfer (PET) and Förster resonance energy transfer (FRET) signalling. Additionally, composite biosensors that incorporate a sensor functionalised to a nanoparticle (gold particles, UCNP and QDots) are used to detect and/or measure ROS/RNS species (detailed in Table 1). Such ROS species probes can quantify hypochlorite [200,201], hydroxyl [202,203], superoxide [204], hydrogen peroxide [205] and singlet oxygen [206]. Biosensors for nitric oxide [207,208], nitroxyl [209–211], peroxynitrite [212] are also being developed.

These probes detect targeted species either in cell-lines, in *ex vivo* tissue, or in *in vivo* models of inflammation. However, these biosensor tools require further optimization. Further refining biosensors will help improve the stability of the probe; the brightness of the fluorescing molecule; the specificity to defined species; the sensitivity of detection; and the consumption of the probe in the sensing process. Thus real-time continued visualisation and/or quantification of nitroxidative species within the CNS of a behaving preclinical rodent model of pathological pain remains an elusive goal.

The ultimate nitroxidative species biosensor would have real-time sensing capacity, with signal brightness that detected subcellular localisation of the nitroxidative species; ideally, this probe would not be consumed/bleached in the sensing process allowing for repeated measurements *in vivo*. Next generation probes will address some of these limitations. For instance, a redox sensitive fluorescent protein (rxRFP1), whose fluorescence intensity is positively related to the extent of oxidation of the probe, can detect varying amounts of oxidative stress within separate cellular compartments [213]. Further refining these tools will enable an improved understanding of how certain species

contribute to oxidative or nitrosative stress, and will allow researchers to define how spatiotemporal regulation of nitroxidative activity contributes to pathological pain.

Outstanding Questions

- How ubiquitous are nitroxidative signaling mechanisms within the neuraxis, beyond the classical sites already tested (peripheral nerve injury site, DRG, spinal cord)?
- Are nitroxidative signaling mechanisms common or different between different preclinical pain models?
- What is the relationship between the antioxidant and anti-inflammatory cytokine systems?
- Do indirect antioxidants have improved translational potential for treatment of pathological pain?

Trends

- Nitroxidative species (reactive nitrogen and oxygen species, and their products) contribute to peripheral and central sensitization after tissue injury, which leads to pathological pain.
- There is a reciprocal relationship between nitroxidative and inflammatory signaling that drives peripheral and central sensitization.
- New approaches to restoring nitroxidative balance may reveal effective strategies to treat pathological pain
- The development of new tools may enhance our understanding of the critical relationships between real-time local creation of nitroxidative species, their concentration at the effect site, and the distribution of their direct effects.

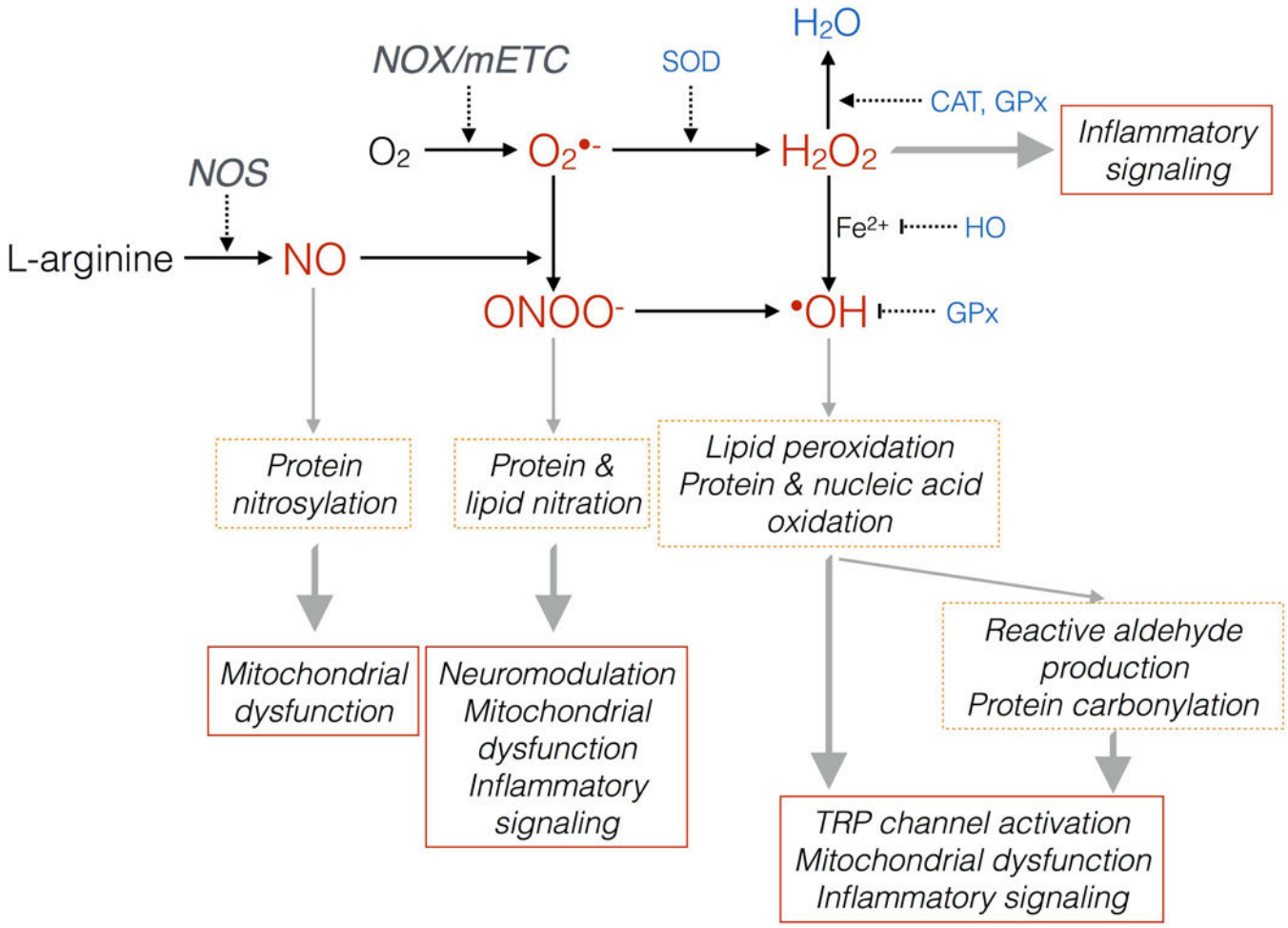

Figure 1. Induction of nitroxidative species after tissue injury

Nitroxidative species can induce posttranslational modifications of proteins and lipids, which subsequently drive pathological pain by modulating nociceptive neurotransmission, activating TRP channels, inducing mitochondrial dysfunction, and induce inflammatory signaling. In healthy cells, endogenous antioxidant systems prevent nitroxidative damage. Cell damage/pathology can perturb this balance, driving accumulation of potentially damaging nitroxidative species. O_2: oxygen; NO: nitric oxide; $O_2^{\bullet-}$: superoxide; $ONOO^-$: peroxynitrite; H_2O_2: hydrogen peroxide; $^{\bullet}OH$: hydroxyl radical; H_2O: water; NOX: NADPH oxidase; NOS: nitric oxide synathse; mETC: mitochondrial electron transport chain; SOD: superoxide dismutase; CAT: catalase; GPx: glutathione; HO: heme oxygenase.

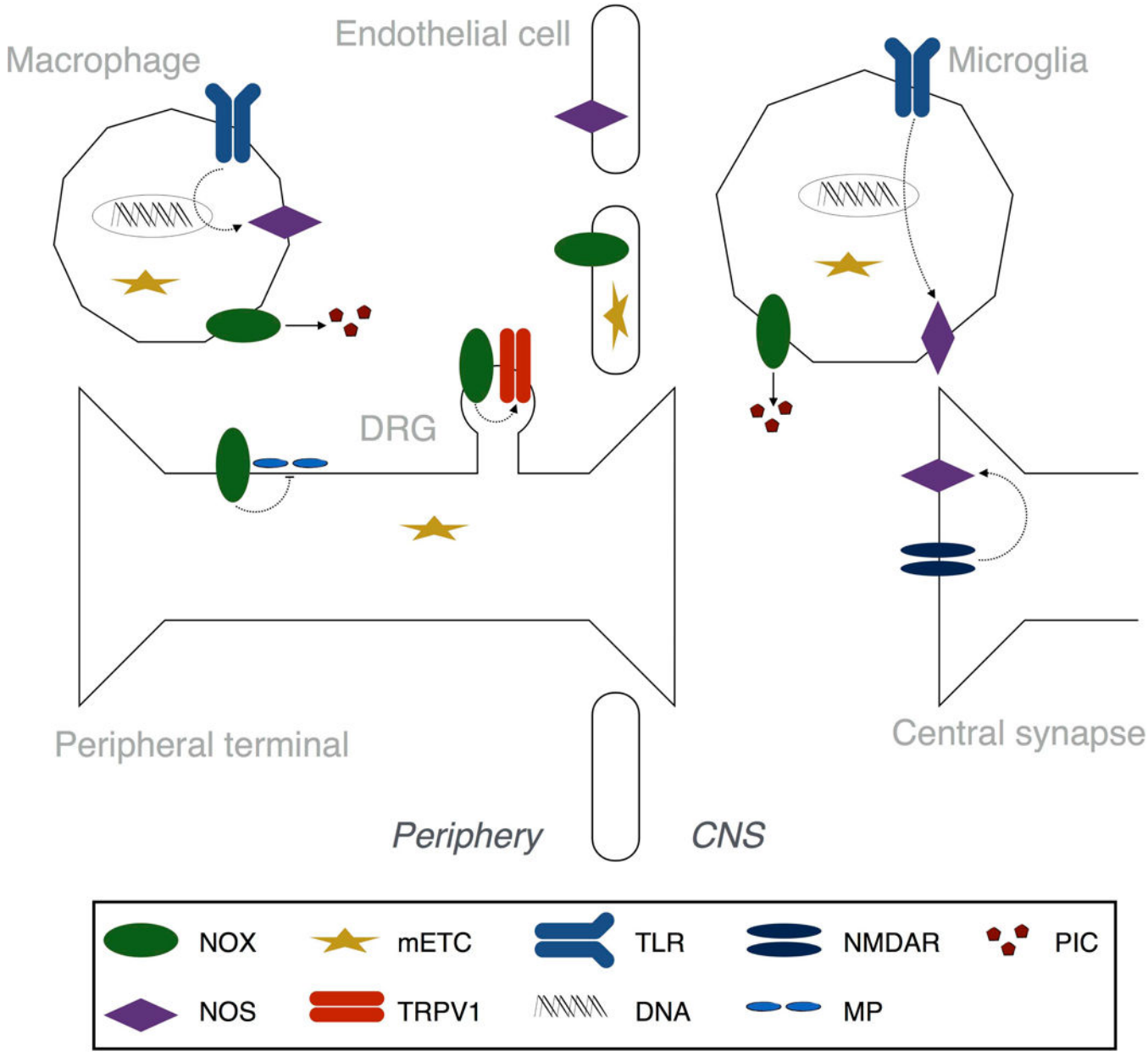

Figure 2. Sources of nitroxidative species after tissue injury

Principal sources of nitroxidative species include NADPH oxidase (NOX), nitric oxide synthase (NOS), and electron leakage from the mitochondrial electron transport chain (mETC). The NOX1, 2, and 4 isoforms are differentially expressed across cell types and tissues after injury. NOX1-derived reactive oxygen species induce enhance Transient Receptor Potential (TRP) V1 activity in dorsal root ganglia (DRG) neurons. NOX2 activity in macrophages and microglia drives mRNA expression of proinflammatory cytokines (PIC) in DRG the spinal dorsal horn. NOX4 expression at the site of peripheral nerve injury decreases expression of myelin proteins (MP). The three NOS isoforms—NOS1 (neuronal), 2 (inducible), and 3 (endothelial)—are also differentially expressed by cell type. In abnormal pain states, N-methyl-D-aspartate receptors (NMDARs) are activated, resulting in calcium

influx and activation of NOS1. Transcription of NOS2 is initiated by Toll like receptors (TLRs). These enzymes and processes have a well-established role in pathological pain.

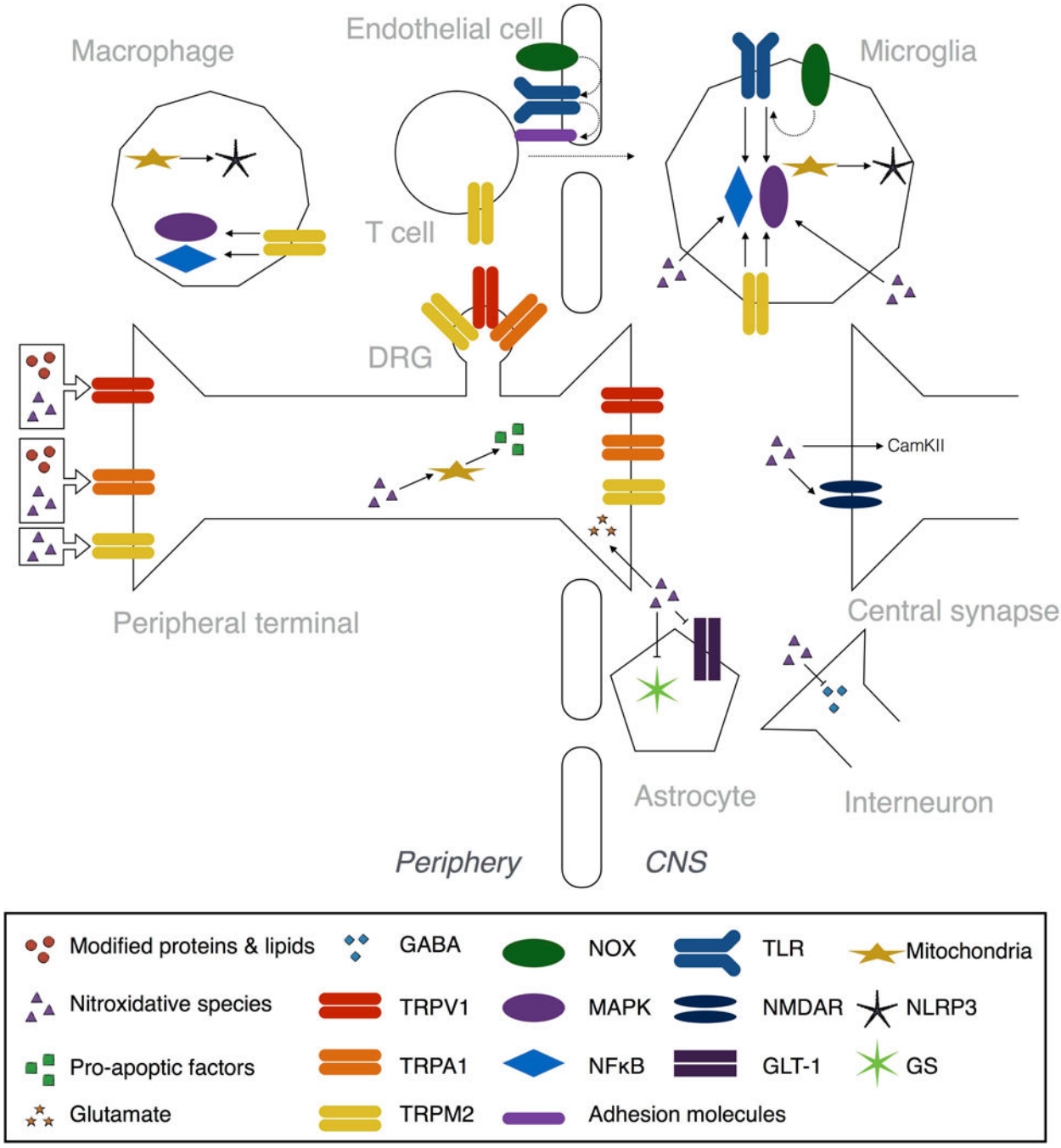

Figure 3. Nitroxidative mechanisms of neuroexcitability after tissue injury

Reactive nitroxidative species, such as hydrogen peroxide and peroxynitrite, and modified proteins and lipids, like carbonylated proteins, peroxidated and nitrated lipids, and reactive aldehydes, all contribute to peripheral and central sensitization after tissue injury. These processes drive pathological pain. Several of the Transient Receptor Potential (TRP) family of nonselective cation channels are activated by nitroxidative species and modified proteins and lipids (see *Nitroxidative species activate TRP channels*). TRPA1 is expressed by peptidergic C-fibers, and is activated by modified proteins and lipids. TRPM2, which is

expressed by neurons, monocytes/macrophages, microglia, and T cells, is directly activated by nitroxidative species. TRPM2 also activates intracellular signaling pathways, including mitogen activated protein kinase (MAPK) and nuclear translocation of nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) pathways. TRPV1 is found on C-fibers and is directly activated by some modified proteins and lipids, as well as being a target of oxidation and nitration events by nitroxidative species that increase responsiveness of the channel. Reactive nitroxidative species can directly modulate neuroexcitibility in central synapses by promoting glutamate release from primary afferent terminals, by activating calcium calmodulin-dependent protein kinase II (CamKII) in glutamatergic spinal neurons, and by inhibiting GABAergic interneurons (see *Nitroxidative species as neuromodulators in pain pathways*). Nitroxidative species also disrupt glutamate homeostasis by nitration and phosphorylation of NMDA receptor (NMDAR) subunits, as well as inhibiting glutamine synthetase (GS) and the glutamate transporter GLT-1. Mitochondrial DNA is a target of oxidation and nitration, while some nitroxidative species can form adducts with many mitochondrial proteins, which together impairs the structural integrity and function of mitochondria (see *Nitroxidative species induce mitochondrial dysfunction*). Nitroxidative species can also trigger release of pro-apoptotic factors from mitochondria by disrupting organelle dynamics. Nitroxidative species induce production of proinflammatory mediators, and can activate NFκB and MAPK intracellular signaling pathways (see *Nitroxidative species induce neuroinflammatory signaling*). Toll like receptors (TLRs) bind a variety of endogenous danger signals, including those released from nitroxidative-damaged mitochondria, to activate NFκB and MAPKs. NOX-derived ROS are second messengers for NFκB- and p38 MAPK-dependent TLR signaling, and TLR expression. The TLR2-NOX1 interaction also upregulates adhesion molecules via CCL3, which facilitates transendothelial cell migration into the CNS. Mitochondria-derived ROS also activate NLRP3 inflammasomes, which are protein complexes responsible for the proteolytic activation of IL-1β.

Table 1

Probes able to detect specific ROS and RNS species, *in vitro, ex vivo or in vivo*

ROS Species	Probe type	Imaging platform used	Tested in vitro	Tested ex vivo/in vivo	ROS/RNS stimulation	Ref
Hypochlorite	Iridium (III) complex-based two-photon phosphorescent probe.	Two-photon laser scanning fluorescence microscope & Confocal laser microscope	HeLa cells/RAW 264.7 cells	Zebrafish	10mM NaClO (*HeLa*) 1mg/mL LPS (*RAW 264.7 & Zebrafish*)	[201]
Hypochlorite	Rhodamine-based hydrazide protein fluorescent probe	Fluorescence microscope	HeLa cells		50μM OCl−	[200]
Hydroxyl	Ratiometric fluorescence biosensor (gold particles conjugated with organic fluorophore)	Confocal laser microscope	HeLa cells		10μg/mL LPS	[203]
Hydroxyl	Ratiometric fluorescence biosensor (upconversion nanaoparticles conjugated with organic fluorophore)	Fluorescence microscope equipped with 980nm laser.	HeLa cells	Mouse liver	500ng/mL PMA (*in vitro*) 1–4mg LPS/100g body weight (*in vivo*)	[202]
Superoxide	Fluorescein protein based fluorescent probe	Confocal laser microscope	HCT116/BV-2/RAW 264.7 cells	Zebrafish	500ng/mL LPS & 50ng/mL IFN-γ (*in vitro*) PMA 200 ng/mL or antimycin A 500 nM (*in vivo*)	[204]
Hydrogen Peroxide	Chemo-selective fluorescent naphthylimide peroxide probe	Two-photon laser scanning fluorescence microscope	RAW 264.7 cells	Mouse lung & skin	1μg/ml LPS *(in vitro*) 20μg LPS (*in vivo*)	[205]
Singlet Oxygen	Far-red silicon-rhodamine based chemical fluorescent probe	Fluorescence microscope with 640nm laser	HeLa cells/RAW 264.7 cells		Photosensitizers: 150μg/mL 5-ALA 5μM TMPyP4	[206]
RNS Species						
Nitric oxide	Chemo-selective copper(II) based fluorescence probe	Confocal laser microscope	HeLa cells/RAW 264.7 cells		50–200μM DEA-NONOate (HeLa) 200ng/mL LPS (RAW 264.7)	[208]
Nitric oxide	Far-red two-photon chemical fluorescent probe	Two-photon laser scanning fluorescence microscope	HeLa cells/RAW 264.7 cells	Mouse liver	25μM NOC-9 (HeLa) 20μg/mL LPS, 200U/mL IFN-γ and 0.5mg/mL L- arginine (RAW 264.7) 1–4mg/ml LPS *(in vivo)*	[207]
Nitroxyl	FRET-based ratiometric chemical fluorescent probe	Confocal laser microscope	HeLa cells		100μM AS	[211]
Nitroxyl	Near infra-red chemical fluorescent probe	Confocal laser microscope & In Vivo Imaging System	RAW 264.7 cells	Mouse *(in vivo)*	100μM AS *(RAW 264.7)* 500μM AS *(i.p. Mouse)*	[210]
Nitroxyl	Lysosome-targetable near infra-red chemical fluorescent probe	Confocal laser microscope & In Vivo Imaging System	RAW 264.7 cells	Mouse *(in vivo)*	200μM AS *(RAW 264.7)* 1mM AS *(i.p. Mouse)*	[209]

ROS Species	Probe type	Imaging platform used	Tested in vitro	Tested ex vivo/in vivo	ROS/RNS stimulation	Ref
Peroxynitrite	Boronate-based chemical fluorescent probe	Confocal laser microscope	HeLa cells/RAW 264.7 cells		5 & 20μM Peroxynitrite solution *(HeLa)* 1μg/mL LPS, 50ng/ml IFN-γ, 2.5ng/ml PMA *(RAW 264.7)*	[212]

LPS: Lipopolysaccharide (produces endogenous ROS/RNS); **PMA**: phorbol 12-myristate-13-acetate (activates protein kinase C *in vivo* and *in vitro*); **IFN-γ**: Interferon gamma (produces endogenous ROS/RNS); **Antimycin A**: Produces endogenous ROS/RNS by driving apoptosis; **5-ALA**: 5-Aminolevulinic acid (drug used in photodynamic therapy, known to produce singlet oxygen); **TMPyP4:** 5, 10, 15, 20-tetra-(N-methyl-4-pyridyl)porphyrin (drug used in photodynamic therapy, known to produce singlet oxygen); **DEA-NONOate**: 2-(N,N-Diethylamino)-diazenolate 2-oxide (Nitric Oxide donor); **NOC-9**: 6-(2-Hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-hexanamine (Nitric Oxide donor); **AS**: Angeli's salt (Nitroxyl donor)