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## SNO-ing at the Nociceptive Synapse?

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**Abstract**—Nitric oxide is generally considered a pro-nociceptive retrograde transmitter that, by activation of soluble guanylyl cyclase-mediated cGMP production and activation of cGMP-dependent protein kinase, drives nociceptive hypersensitivity. The duality of its functions, however, is increasingly recognized. This review summarizes nitric-oxide-mediated direct S-nitrosylation of target proteins that may modify nociceptive signaling, including glutamate receptors and G-protein-coupled receptors, transient receptor potential channels, voltage-gated channels, proinflammatory enzymes, transcription factors, and

redoxins. S-Nitrosylation events require close proximity of nitric oxide production and target proteins and a permissive redox state in the vicinity. Despite the diversity of potential targets and effects, three major schemes arise that may affect nociceptive signaling: 1) S-Nitrosylation-mediated changes of ion channel gating properties, 2) modulation of membrane fusion and fission, and thereby receptor and channel membrane insertion, and 3) modulation of ubiquitination, and thereby protein degradation or transcriptional activity. In addition, S-Nitrosylation may alter the production of nitric oxide itself.

### I. Introduction

It is increasingly recognized that reversible redox modifications of proteins constitute an important post-

translational regulatory mechanism (Lipton et al., 2002; Janssen-Heininger et al., 2008) that may contribute to the adaptation of pain signaling pathways to an extent similar to that of phosphorylation and dephosphorylation events. Redox proteomic approaches, mostly done in vitro, have revealed thiol nitrosylation (Lipton et al., 2002; Foster et al., 2009), glutathionylation (Viner et al., 1999), carbonylation (Smerjac and Bizzozero, 2008), and guanylation (Sawa et al., 2007). These post-transla-

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tional modifications modify protein-protein interactions and protein structure, hydrophobicity, and function. Under normal conditions, redox modifications of specific cysteine residues are reversible and important for redox adaptations of protein functions (Lipton et al., 1993; Haendeler et al., 2002; Uehara et al., 2006; Takahashi et al., 2007). However, aging and diseases are associated with irreversible sulfonic acid oxidation, preventing further participation in reversible redox signaling events, which may then further perpetuate aging or pathologic processes (Stadtman, 2001; Depuydt et al., 2009; Pérez et al., 2009).

S-Nitrosylation of the thiol group of cysteine residues mediated through nitric oxide (NO) has emerged as the prototype redox-based post-translational modification. Such S-nitroso-proteins are not produced by direct reaction between nitric oxide and thiol. Rather, nitrosothiol formation also requires oxygen, transition metals, or other relevant electron acceptors (Lindermayr et al., 2009). Alterations of pH and hypoxia or other disease-related changes of the local and subcellular environment may therefore crucially affect the occurrence and reversibility of such processes.

Crucial cysteine residues can also be involved in reduction-oxidation events, chelation of transition metals (mainly  $Zn^{2+}$ ,  $Mn^{2+}$  and  $Cu^{2+}$ ), or glutathionylation through reaction with reactive GSH intermediates (Shackelford et al., 2005; Sykes et al., 2007). Therefore, disparate reactions may compete for the same thiol group on a single cysteine residue, forming a molecular switch control system composed of various possible redox, nitric oxide, glutathione, or metal modifications to control protein function, stability, and ligand binding. Nitric oxide seems to play a predominant role in these modifications and has emerged as a major mediator for fast as well as long-lasting adaptations of pain signaling pathways (Schmidtke et al., 2009). The multifaceted functions of nitric oxide for endogenous pain control are not completely understood on the basis of soluble guanylyl cyclase (sGC<sup>1</sup>)-mediated

cGMP production and subsequent activation of cGMP-dependent protein kinase (sGC/cGMP/PKG pathway). This concept needs to be broadened in terms of post-translational compartmentalized NO-mediated modifications of target proteins; however, this is still hampered by the difficulty in visualizing specific SNO-modified proteins in vivo.

## II. Analysis and Detections of Protein S-Nitrosylation

The detection of in vivo S-nitrosylation (SNO) of proteins is challenging because of low concentrations of the modified proteins and the instability of the SNO-bond, so that current knowledge relies mostly upon in vitro cell culture experiments. The detection of S-nitrosylated proteins directly from complex mixtures by mass spectrometry is mostly inefficient. Chemiluminescent or colorimetric techniques, such as diaminofluorescein fluorometry, allow for quantification of the total content of SNO in tissue samples but do not identify modified proteins or cysteine residues. The "biotin switch technique" (Jaffrey and Snyder, 2001) has been developed to overcome these difficulties. S-Nitrosylated cysteines are first selectively reduced by ascorbic acid and subsequently tagged with biotin or histidine residues (6×His) allowing for immunoprecipitation and antibody-mediated identification or direct identification of SNO labeling sites by mass spectrometry (Hao et al., 2006). The biotin-switch is highly specific for SNO modifications. However, the method is technically challenging, and endogenous biotinylation may cause false-positive results. Instead of labeling with biotin, SNO-modified cysteine residues may be tagged with fluorescent dyes so that the "biotin-switch technique" can be used in combination with two-dimensional differential gel electrophoresis (2D-DIGE) (Hao et al., 2006) as a proteomic screening technique. The 2D-DIGE approach reduces false-positive results but tends to detect primarily SNO modifications in highly abundant proteins. To allow for high-throughput screening, SNO-site identification by mass spectrometry is an alternative. SNO-site identification employs the "biotin-switch method" to tag SNO-modified cysteine residues, which is followed by trypsin digestion of the protein, affinity purification, and enrichment of SNO-modified peptides and subsequent identification by mass spectrometry (Hao et al., 2006). Although highly specific, the method requires large sample sizes, limiting its usefulness for analysis of in vivo

<sup>1</sup>Abbreviations: 2D-DIGE, two-dimensional differential gel electrophoresis; AMPA,  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ASK1, apoptosis-related signaling kinase 1; CAPON, C-terminal PDZ domain ligand of neuronal nitric-oxide synthase; CICR,  $Ca^{2+}$ -induced  $Ca^{2+}$  release; CNG, cyclic nucleotide-gated ion channel; COX-2, cyclooxygenase 2; cPLA2, cytosolic phospholipase A<sub>2</sub>; dexas1, dexamethasone-activated Ras; DRG, dorsal root ganglia; eNOS, endothelial NOS; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GOSPEL, GAPDH's competitor of Siah protein enhances life; GPCR, G protein-coupled receptor; HCN, hyperpolarization-activated cyclic nucleotide-gated channel; HDM2, homolog of mouse double-minute-2; HIF, hypoxia-inducible factor; I $\kappa$ B, inhibitor of nuclear factor- $\kappa$ B; Ih, depolarizing current; IKK, I $\kappa$ B kinase complex; iNOS, inducible nitric-oxide synthase; KATP, ATP-sensitive potassium channel; LTP, long-term potentiation; MAP, mitogen-activated protein; MMP, matrix metalloproteinase; NF- $\kappa$ B, nuclear factor- $\kappa$ B; NGF, nerve growth factor; NMDA, N-methyl-D-aspartate; nNOS, neuronal nitric-oxide synthase; NOS, nitric-oxide synthase; NSF, N-ethylmaleimide-sensitive factor; PDI, protein disulfide

isomerase; PDZ, postsynaptic density 95/disc-large/zona occludens; PICK1, protein interacting with protein kinase C $\alpha$ ; PKG, cGMP-dependent protein kinase; RyR, ryanodine receptor; sGC, soluble guanylyl cyclase; SNARE, soluble NSF attachment protein receptor; SNO, S-nitrosylation; SUR, sulfonyleurea receptor; TrkA, neurotrophic tyrosine kinase receptor type 1; TRP, transient receptor potential; TRPC, transient receptor potential canonical; TRPV, transient receptor potential vanilloid; Trx, thioredoxin; TTX, tetrodotoxin; VGCC, voltage-gated calcium channel.

S-nitrosylation in some tissues or subcellular compartments. The functional analysis of SNO-modified cysteines requires knock-in of, for example, serine or alanine to dissect out the impact of S-nitrosylation for protein conformation, trafficking, interactions, or enzyme function. Hence, the development of highly sensitive techniques is likely to further the insight into S-nitrosylation as a functionally relevant post-translational modification of proteins.

### III. The Enigma of Nitric Oxide in Pain Signaling

NO is a very versatile, tightly controlled molecule (Fig. 1) that contributes to the functional adaptations of nociceptive synapses in the primary sensory neuron (Fig. 2), spinal cord (Figs. 3 and 4) and brain (Minami et al., 1995; Aley et al., 1998; Vetter et al., 2001; Ikeda et al., 2006; Miyamoto et al., 2009). Its production is tightly controlled at transcriptional and post-translational levels of nitric-oxide synthases (NOSs) and the availability of the enzyme cofactor tetrahydrobiopterin (Tegeger et al., 2006) (Fig. 1 and 5). Lack of balance of nitric-oxide synthases and tetrahydrobiopterin pose a constant source of reactive nitrogen species, which are produced instead of nitric oxide in case of tetrahydrobiopterin deficiency. Nitric oxide has been mainly implicated in the adaptations of nociceptive circuits toward ongoing stimuli because of its diffusible capabilities, which, in theory, allow a paracrine and retrograde targeting of presynaptic and neighboring neurons (Fig. 3) and glial cells (Fig. 4) that are localized

beyond the immediate vicinity of the NO-producing neuron (Ikeda et al., 2006). The current concept suggests that the nociceptive primary afferent neuron releases glutamate upon nociceptive stimulation at its central terminal in lamina I or II of the dorsal horn of the spinal cord (Schmidtko et al., 2009) (Fig. 3). Glutamate then acts at *N*-methyl-D-aspartate (NMDA) receptors of postsynaptic neurons, interneurons, and projection neurons and causes an influx of calcium ions, followed by stimulation of calcium calmodulin kinase and nNOS that is brought in close proximity to the NMDA receptor by the scaffolding protein CAPON and the postsynaptic density protein (PSD95) (Fig. 3) (Schmidtko et al., 2009). On the basis of studies in the hippocampus, it has been proposed that NO then diffuses back to the presynaptic primary afferent neuron to activate membrane bound soluble guanylyl cyclase (Schuman and Madison, 1991). The resulting increase of cGMP in the presynaptic neuron would then lead to a stimulation of cGMP-dependent protein kinase (PKG) localized in the presynaptic terminal (Lewin and Walters, 1999). PKG presumably further increases glutamate release by a multistep mechanism involving activation of myosin light chain, Rho kinase (Zulauf et al., 2009) and of enabled/vasodilator-stimulated phosphoprotein (Wang et al., 2005), which in concert enhance the recruitment and release of synaptic vesicles (Chen et al., 2008; Rentsendorj et al., 2008) (Fig. 3 and 5). The activation of the NO target sGC itself depends on redox modifications of cysteine residues within the catalytic center of sGC by

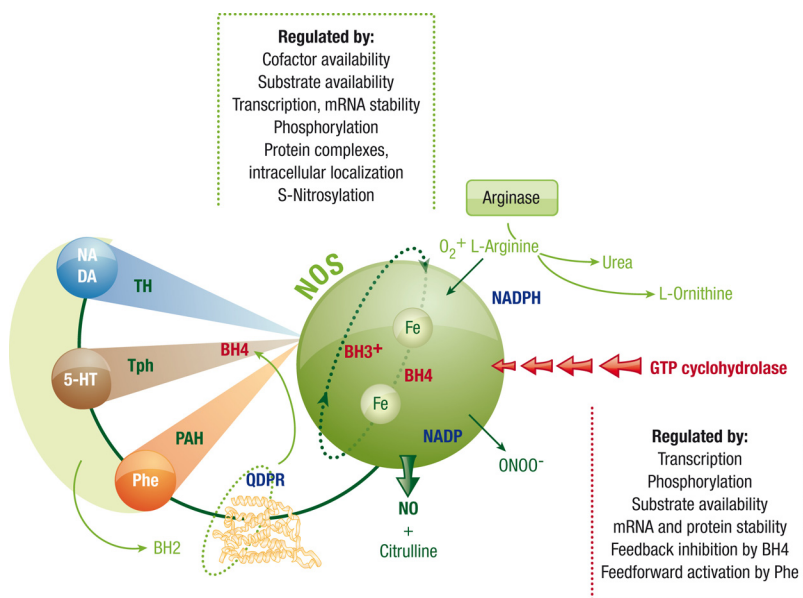


FIG. 1. Biosynthesis of nitric oxide enzymes and coupling of NOS enzymes activity to cofactor availability. NOS enzymes form dimeric complexes, each consisting of oxidation and reduction parts. The essential enzyme cofactor tetrahydrobiopterin (BH4) binds to the catalytic center, and its oxidation and reduction are coupled with the generation of NO. In case of BH4 deficiency, NOS oxidation-reduction coupling is less efficient, leading to enhanced production of reactive nitrogen species. NOS compete for BH4 with hydroxylases. These enzymes do not recycle BH4 within their catalytic center. Instead, BH4 is recycled from dihydrobiopterin (BH2) by quinoid dihydropteridine reductase (QDPR). NOS enzymes further compete with arginase for the substrate L-arginine that may be used either for NO synthesis or for the production of urea and L-ornithine and, subsequently, polyamines and proline. NOS expression and activity is tightly controlled at transcriptional and post-translational levels, including S-nitrosylation and cofactor availability, which in turn is tightly controlled by transcriptional and post-translational modification of the rate-limiting enzyme in BH4 synthesis, GTP cyclohydrolase 1 (GCH1).

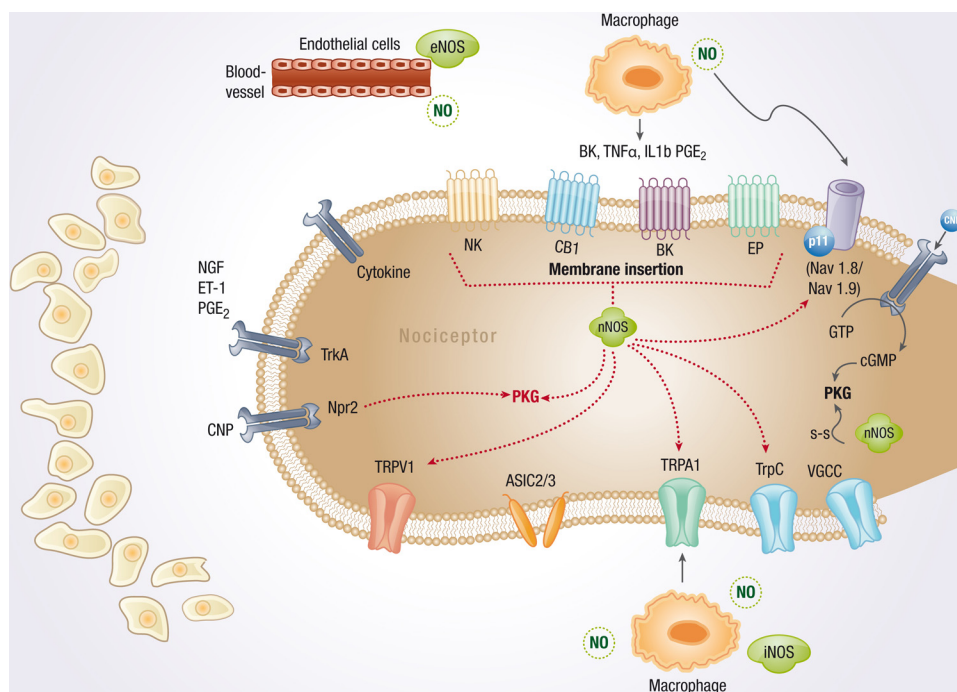


FIG. 2. NO-mediated modulation of nociceptor sensitivity. nNOS is localized in the peripheral nerve terminal of some nociceptive neurons, the number of which increases during tissue inflammation. Locally produced NO modulates by direct S-nitrosylation of channel cysteines the gating of TRP channels and VGCCs. The critical cysteine residues are accessible from the cytoplasm. By direct S-nitrosylation of NSF, nitric oxide may modify the membrane insertion of TRP channels. Stimulation of nociceptors through various GPCRs, including prostaglandin E<sub>2</sub> receptors, bradykinin receptors, endothelin receptors, and cannabinoid receptors (CB1), may be modified by GPCR cycling between membrane and clathrin-coated vesicles. S-Nitrosylation of the large GTPase dynamin and of  $\beta$ -arrestin increases the efficacy and velocity of the cycling process. Nitric oxide may also be produced by macrophages, which up-regulate iNOS during inflammation. NO produced in endothelial cells by eNOS may enhance blood flow and contribute to hyperemia and macrophage tissue invasion during inflammation. Activation of PKG in primary sensory neurons ensues after stimulation of natriuretic peptide receptor (Npr2) and subsequent cGMP production. NO may also directly activate PKG by facilitating the formation of disulfide bonds in PKG1- $\alpha$  dimers. ASIC, acid-sensing ion channel; BK, bradykinin receptor; CNP, ciliary natriuretic peptide; EP, prostaglandin E<sub>2</sub> receptor; ET, endothelin receptor; IL, interleukin; NK, neurokinin; Npr, natriuretic peptide receptor; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; TNF, tumor necrosis factor; TrpC, transient receptor potential cation.

binding of NO to nonheme sites showing the complicated fine tuning of this pathway (Sayed et al., 2007). The NO-cGMP-PKG-dependent pathway explains part of the wealth of experimental data that have been generated over the last 20 years. However, there are some caveats in this model.

A molecule as versatile as NO is unlikely to interact exclusively with its target sGC and, importantly, sGC is not localized in presynaptic endings of primary afferent neurons (Schmidtko et al., 2008a) (Figs. 2 and 3). In dorsal root ganglia (DRGs), sGC is instead found in satellite cells and vascular tissue (Schmidtko et al., 2008a) (Fig. 2) challenging the idea of a retrograde pre-synaptic NO-action that is mediated by sGC activation. It is more likely that cGMP production in DRG neurons is elicited by activation of particulate guanylyl cyclases (Figs. 2 and 3), which are known as receptors for natriuretic peptides (Kishimoto et al., 2008; Schmidt et al., 2009). Indeed, injection of ciliary natriuretic peptide produced hyperalgesia in mice (Schmidtko et al., 2008a), but the source of endogenous ciliary natriuretic peptide that may activate PKG in nociceptors is still unknown. nNOS is expressed in primary afferent DRG neurons and their central terminals in the spinal cord. It is also expressed in lamina III interneurons in the spinal cord

and few deep dorsal horn neurons (Maihöfner et al., 2000a). At all these sites, the expression increases upon peripheral inflammation or nerve injury, which is accompanied by an up-regulation of PKG in DRG neurons (Zhang et al., 1993; Vizzard et al., 1995; Maihöfner et al., 2000a; Tegeder et al., 2004a; Ruscheweyh et al., 2006; Schmidtko et al., 2008a). The localization and up-regulation raises the possibility that NO produced in DRG neurons directly activates PKG, through a mechanism independent of cGMP. Such a mechanism has been demonstrated for endothelial cells, where the PKG-I- $\alpha$  subunit upon exposure to exogenous hydrogen peroxide formed an interprotein disulfide, linking the two subunits of PKG and causing an increase of its catalytic activity independent of cGMP (Burgoyne et al., 2007). Although this mechanism has so far been demonstrated only for vascular tissue, it may represent a general mechanism by which PKG acts as a direct sensor for oxygen, NO, and the redox state.

Pharmacological experiments have revealed that pan-NOS inhibition unequivocally reduces nociceptive behavior in various models (Hao and Xu, 1996; Aley et al., 1998; Levy and Zochodne, 1998; Chen and Levine, 1999). However, intrathecal injection of an NO donor may either reduce or increase the sensitivity to nociceptive

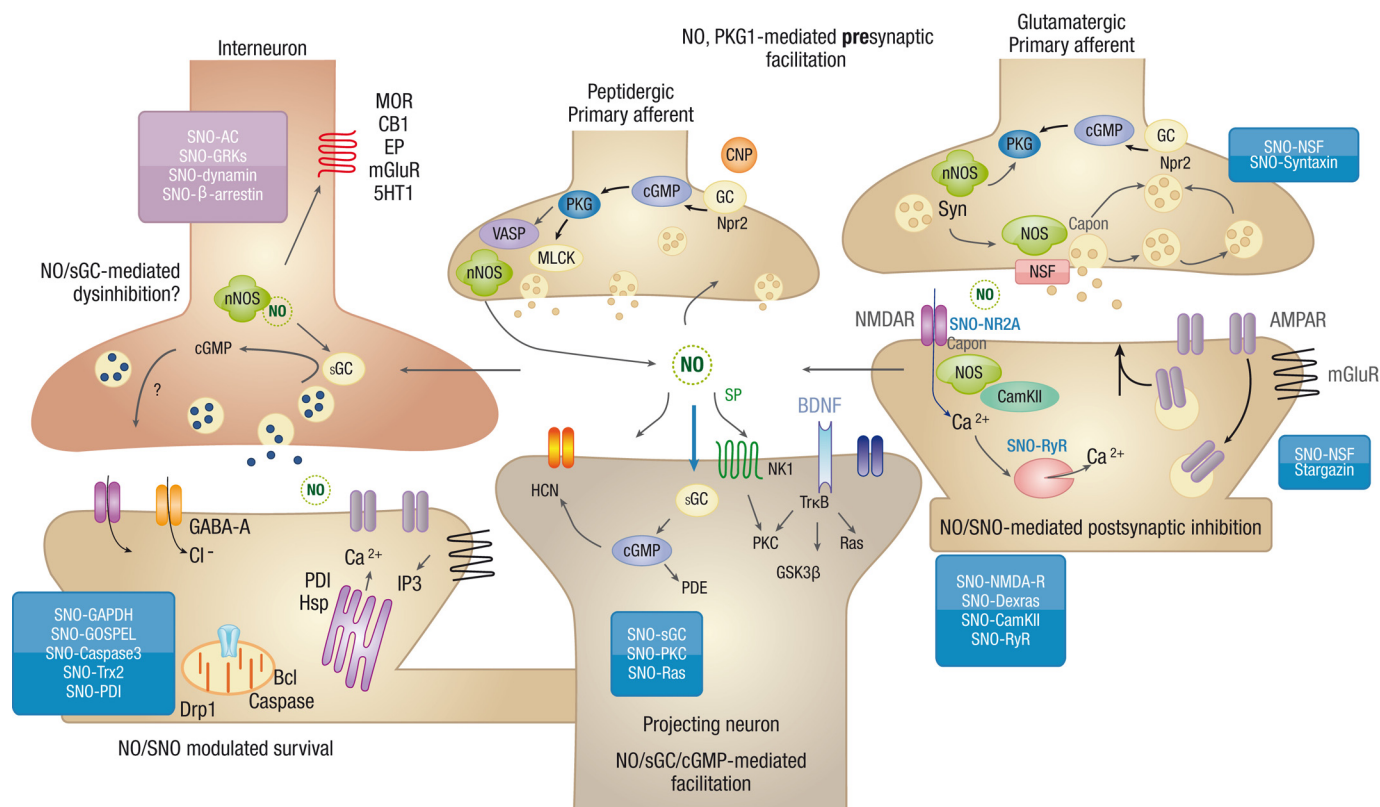


FIG. 3. Nitric oxide signaling at nociceptive synapses. nNOS is localized at the synaptic terminal of primary afferent neurons in the spinal cord and in interneurons. Ongoing nociceptive stimulation during inflammation or upon axonal injury causes an up-regulation of nNOS expression in sensory neurons and in the spinal cord. nNOS is activated upon NMDA receptor stimulation and in turn reduces NMDA receptor-mediated calcium influx by direct S-nitrosylation of NMDA receptor subunits. Direct S-nitrosylation of stargazin modifies membrane insertion of AMPA channels and thereby the signaling strength at nociceptive synapses. By S-nitrosylation of RyRs, NO can enhance intracellular CICR, causing amplification of the transmitter-evoked signal. NO produced at the presynaptic terminal may modify transmitter release by direct S-nitrosylation of NSF, synaptic vesicle proteins, or PKG. The function of interneurons receiving input from, for example, opioidergic neurons, may be modified by direct S-nitrosylation of  $\beta$ -arrestin and dynamin, which essentially control GPCR cycling between clathrin-coated pits and plasma membrane. S-Nitrosylation of mitochondrial proteins, redoxins, caspases, and GAPDH may enhance or reduce the vulnerability of neurons toward glutamate excitotoxicity. 5HT, 5-hydroxytryptamine (serotonin); AMPAR, AMPA receptor; BDNF, brain-derived neurotrophic factor; CamKII,  $Ca^{2+}$ /calmodulin-dependent protein kinase II; CB1, cannabinoid receptor 1; CNP, ciliary natriuretic peptide; EP, prostaglandin  $E_2$  receptor; GSK3 $\beta$ , glycogen synthase kinase 3 $\beta$ ; IP3, inositol trisphosphate; mGluR, metabotropic glutamate receptor; MLCK, myosin light-chain kinase; MOR,  $\mu$ -opioid receptor; NK1, neurokinin; Npr2, natriuretic peptide receptor B; PDE, phosphodiesterase; VASP, vasodilator-stimulated phosphoprotein.

stimuli (Tegeder et al., 2002; Schmidtke et al., 2009), suggesting that endogenously produced NO may act primarily at targets different from those affected by intrathecally injected NO-releasing agents. This apparent contradiction would be compatible with a compartmentalized action of NO and its dependence on the cellular and subcellular environment. Data from nodose ganglia support this idea, because in these experiments, exogenous and endogenous NO had opposite effects (Hsieh et al., 2010). Exogenous NO abolished glia-derived neurotrophic factor release, whereas endogenous NO mediated its up-regulation. The first was abolished by Tempol, a drug that prevents nitrosylation, whereas the latter depended on the PKG pathway (Hsieh et al., 2010).

Recent concepts suggest that NO modifies signaling strength and synaptic functions by reversible S-nitrosylation of target proteins (Figs. 5 and 6), one of which is the NMDA receptor (Lipton et al., 2002). Activation of the NMDA receptor by glutamate increases the production of nitric oxide, which in turn suppresses calcium

currents through the NMDA receptor (Fig. 3). This feedback inhibition is mediated by direct S-nitrosylation of the NR2A subunit of the NMDA receptor or indirectly by S-nitrosylation of proteins that modify glutamate release or functions (Fig. 5 and 6) (Lipton et al., 2002). Although this mechanism has not been directly demonstrated in the context of nociception, it is likely that this NO-mediated feedback inhibition protects against excitotoxicity in nociceptive circuits, and malfunction may facilitate the development of hypersensitivity and pathological pain. Instead of thinking of NO-evoked nociceptive hypersensitivity in terms of a single signaling pathway consisting in NMDA receptor stimulation, nNOS activation, and subsequent NO-mediated enhancement of glutamate release, NO may be thought of more specifically in terms of a key post-translational redox regulator of compartmentalized cellular targets. The where and when of NO release may determine the outcome: either enhanced nociceptive sensitivity and neuronal death or recovery of homeostasis, neuron survival, and alleviation of pain. It will be

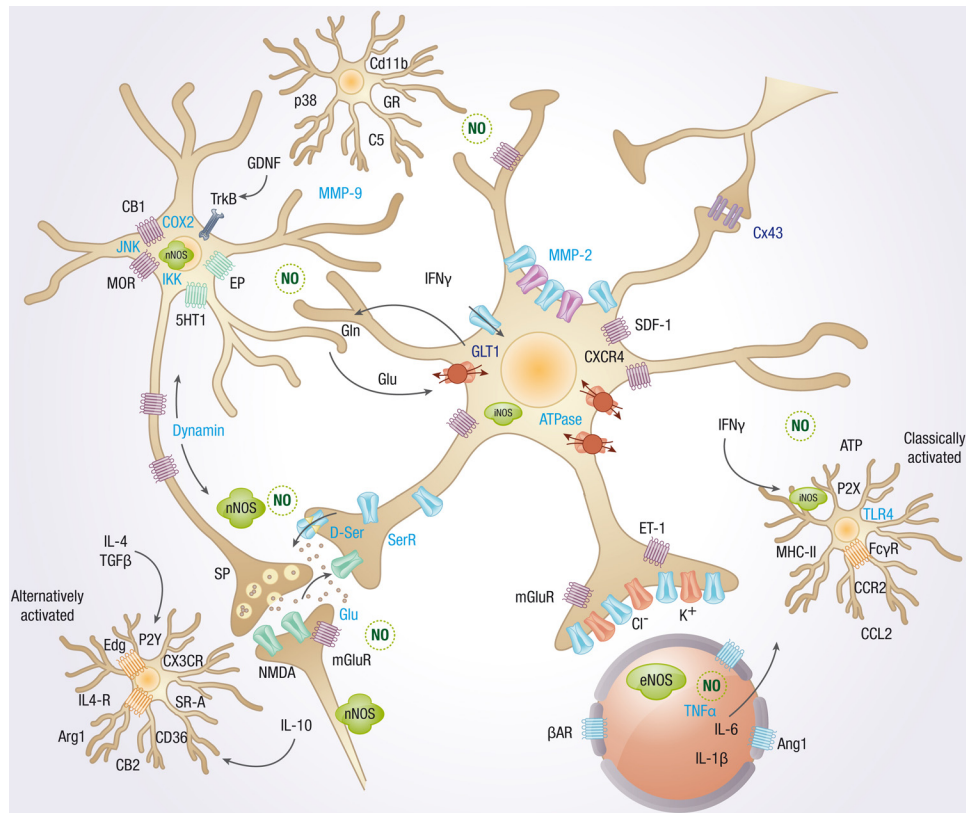


FIG. 4. Nitric oxide modulation of neuron-glia communication by direct S-nitrosylation. As a diffusible molecule, NO can be released by neurons or glial cells and may act on neighboring cells to modify their functions. Microglia and astrocytes are activated upon neuronal injury, ischemia, or inflammation and enhance NO production by iNOS. Up-regulation of iNOS occurs mainly in classically activated microglia upon interferon  $\gamma$  (IFN $\gamma$ ) or tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) stimulation. By direct S-nitrosylation of Toll-like receptor (TLR)-coupling proteins, MMPs, and stress enzymes, such as c-Jun N-terminal kinase (JNK), nitric oxide may promote inflammatory processes. Direct S-nitrosylation of COX-2 contributes to the NMDA receptor-mediated glutamate toxicity, which is further controlled by S-nitrosylation-mediated control of D-serine production in astrocytes and glutamate reuptake by glutamate transporters (GLT1). Nitric oxide produced in endothelial cells by eNOS modifies eNOS activity, functions of  $\beta$ -adrenoceptors ( $\beta$ -AR), and potassium channels by direct S-nitrosylation. The effect of NO depends on the site and time of its production and the redox status in its close proximity. Direct S-nitrosylation targets are in blue text. 5HT, 5-hydroxytryptamine (serotonin); CB1, cannabinoid receptor 1; CCL, chemokine ligand; CCR, chemokine receptor; EP, prostaglandin E $_2$  receptor; ET-1, endothelin receptor 1; GDNF, glia-derived neurotrophic factor; GR, glucocorticoid receptor; IL, interleukin; mGluR, metabotropic glutamate receptor; MHC, major histocompatibility complex; MOR,  $\mu$ -opioid receptor; TGF $\beta$ , transforming growth factor  $\beta$ ; TrkB, tyrosine kinase B receptor.

challenging in the future to dissect out the role of specific SNO targets and mechanisms in the context of and at different sites of nociceptive signaling, because it may require high-resolution mass spectrometry imaging of redox proteome networks and visualization of redox activation in vivo for example by use of transgenic mice expressing redox-sensitive green fluorescent protein.

#### A. Redox-Modification of Specific Target Proteins

Various proteins involved in pain processing, neuronal, and glial adaptations to ongoing nociceptive stimulation or injury are potential targets of redox modifications of specific cysteine residues. In particular, S-nitrosylation affects the functions of glutamate receptors (Lipton et al., 1993; Kim et al., 1999), receptor trafficking molecules (Huang et al., 2005; Selvakumar et al., 2009), transcription factors (Choi et al., 2000; Reynaert et al., 2004; Li et al., 2007; Tsang et al., 2009), proinflammatory enzymes (Gu et al., 2002), heat-shock proteins (Martínez-Ruiz et al., 2005), pro-

teases (Mannick et al., 1999, 2001), cytoskeletal (Lu et al., 2009) and synaptosomal (Palmer et al., 2008) proteins, and ion channels (Evans and Bielefeldt, 2000; Yoshida et al., 2006; Jian et al., 2007; Asada et al., 2009) (Figs. 5 and 6). Direct protein S-nitrosylation requires close proximity to nitric-oxide synthase and high metabolic activity of mitochondria. The likelihood increases in a hydrophobic environment, suggesting that proteins attached to membranes or localized within cellular microdomains that are enriched with mitochondria may be prone to this modification. This holds true for the presynaptic and the postsynaptic density. Although proteins targeted by S-nitrosylation seem to be quite diverse, three general schemes have evolved: 1) S-nitrosylation-mediated conformational changes of ion channels; 2) S-nitrosylation-modulated cycling of membrane-coated vesicles; and 3) modulation of ubiquitinylation and proteasomal degradation. SNO effects on kinases and proteases may in addition indirectly affect phosphorylation and dephosphorylation of target proteins. The

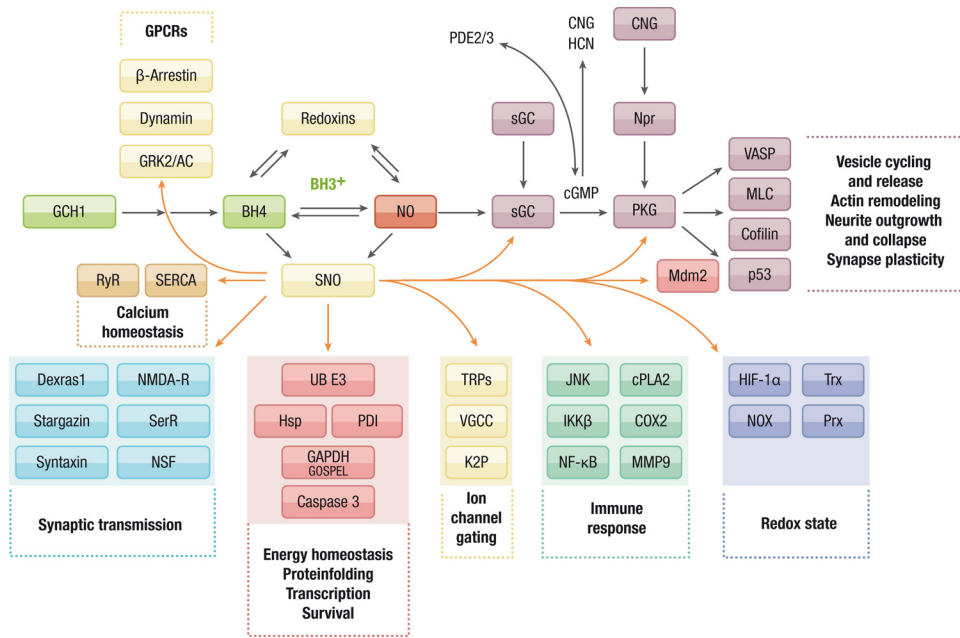


FIG. 5. Nitric oxide signaling pathways. NOS activity depends on the availability of its enzyme cofactor, tetrahydrobiopterin (BH4), which is produced in a multistep synthesis cascade initiated by the GTP cyclohydrolase 1 (GCH1). NO activates sGC, which produces cGMP, leading to activation of PKG, phosphodiesterases (PDE2 and PDE3), CNGs, and HCNs. In neurons, activation of PKG regulates neurite outgrowth and collapse and neurotransmitter release. In addition to this classic NO/sGC/PKG signaling pathway, NO modifies nociceptor sensitivity and synaptic functions by direct S-nitrosylation of glutamate receptors, scaffolding and motor proteins, ion channels, kinases, proteases, and redoxins. AC, adenylyl cyclase; Hsp, heat-shock protein; JNK, c-Jun N-terminal kinase; K2P, two-pore potassium channel; Mdm2, murine double minute 2; MLC, myosin light chain; NOX, NADPH oxidase; Npr, natriuretic peptide receptor; Prx, peroxiredoxin; SERCA, sarco/endoplasmic reticulum Ca<sup>2+</sup>-ATPase; SerR, serine racemase; UB E3, ubiquitin 3 ligase; VASP, vasodilator-stimulated phosphoprotein.

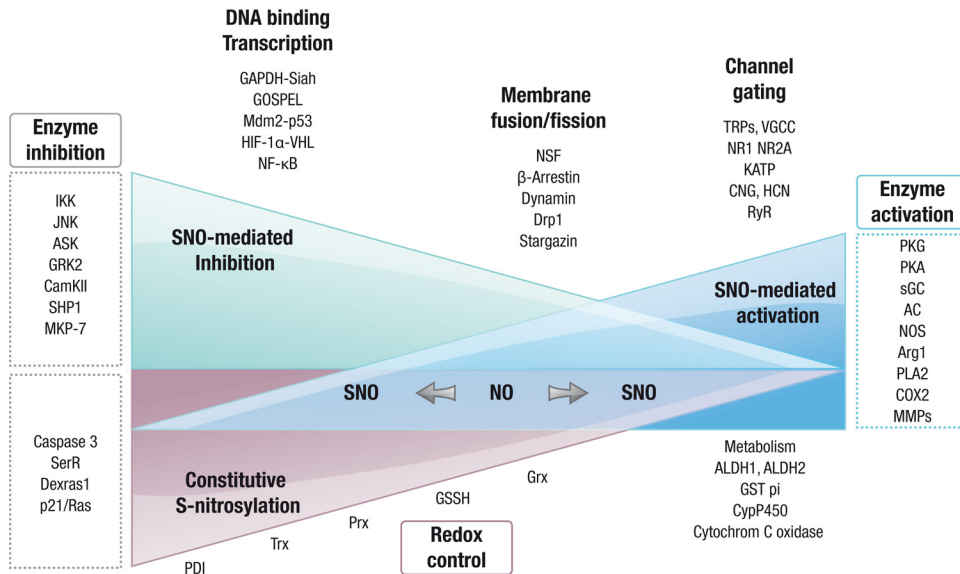


FIG. 6. S-Nitrosylation-mediated effects. AC, adenylyl cyclase; ALDH, aldehyde dehydrogenase; Arg, arginase; CamKII, calcium/calmodulin kinase II; cPLA2, cytosolic phospholipase A2; CypP450, cytochrome P450 oxidase; Drp1, dynamin-related protein 1; GRK2, G-protein-regulated kinase; Grx, glutaredoxin; GST, glutathione transferase; JNK, c-Jun N-terminal kinase; KATP, ATP-activated potassium channel, sulfonyleurea sensitive; Mdm2, murine double minute 2; MKP, MAP kinase phosphatase; PKA, cAMP-dependent protein kinase; Prx, peroxiredoxin; SerR, serine racemase; SHP, phosphotyrosine phosphatase.

first two mechanisms may account for nitric-oxide-mediated modifications of neuronal excitability, neurotransmitter release, and receptor sensitization/desensitization processes and thereby fast adaptations to nociceptive stimuli. Through the second mechanism, nitric oxide may modify transcription-factor activity and

thereby the delayed and long-lasting adaptations to ongoing nociceptor stimulation or nerve injury. For most of the S-nitrosylation targets discussed below, functional evidence has been provided in vitro in cell culture experiments. At present, there is mostly no direct proof of the functional relevance of a specific

SNO modification in terms of functional measures in animal models.

### *B. Redox Modulation of Glutamate Signaling in Nociceptive Pathways*

The NMDA type glutamate receptor is one of the best studied redox targets in the nervous system. It is modified by direct S-nitrosylation of its NR2A subunit at a critical cysteine residue (Figs. 5 and 6), resulting in a change of the channel gating properties. Site-directed mutagenesis identified Cys399 as the critical cysteine (Choi et al., 2000) (Table 1) targeted by endogenous nitrogen species and various redox modulators, including reducing and oxidizing agents (Tang and Aizenman, 1993; Kim et al., 1999; Sanchez et al., 2000; Herin et al., 2001). They all modify the NMDA receptor at this specific cysteine (Lipton et al., 1993; Choi et al., 2000) and thereby modify glutamate-evoked calcium fluxes through the channel. S-Nitrosylation of Cys399 curtailed excessive calcium influx in neurons and thus provided neuroprotection from excitotoxic insults (Kim et al., 1999). Glutamate is one of the most important primary excitatory neurotransmitters of afferent nociceptive neurons in the spinal cord dorsal horn (Fig. 3), and pharmacological experiments demonstrate that NMDA-receptor activation elicits nociceptive behavior in models of acute and chronic pain in rodents (Laughlin et al., 1999). Blocking the NMDA receptor not only reduces nociception but also prevents nerve injury-evoked death of nociceptive neurons in the dorsal horn (Scholz et al., 2005), suggesting that glutamate excitotoxicity and NMDA receptor hyperexcitability are involved in this trans-synaptic neuronal death and the development of neuropathic pain (Moore et al., 2002; Scholz et al., 2005). Such hyperexcitability of the NMDA receptor can be triggered by post-translational phosphorylation of the NR2b subunit by Src nonreceptor tyrosine kinases (Kawasaki et al., 2004; Liu et al., 2008) and results in excessive calcium influx through the receptor-associated ion channel. The subsequent calcium overload then leads to protein misfolding and mitochondrial dysfunctions and increases the likelihood of redox modifications in the postsynaptic density (Sinor et al., 1997; Nakamura and Lipton, 2008) (Fig. 3). Neuronal NOS is tethered via its PDZ domain to the scaffolding protein CAPON and thereby to the NMDA receptor and calcium calmodulin kinase II (Cheah et al., 2006) (Fig. 3) and, hence, is brought near the NMDA receptor. NMDA receptor channel opening directly controls nNOS activity and production of NO, which in turn by direct S-nitrosylation may slow down the calcium influx. In chronic pain, this physiological NO-mediated feedback control of the NMDA receptor may be insufficient or offset by S-nitrosylation of other targets with opposing effects (Fig. 6) or by NMDA receptor phosphorylation. In addition, S-nitrosylation in the presence of low oxygen levels may alter the physiological nitrosylation events at the NR2A subunit by a mechanism involving the NR1 subunit at Cys744 and Cys798 residues (Table 1), which constitute NO-reactive oxygen

sensor motifs that, upon S-nitrosylation, change the agonist binding properties of the receptor (Takahashi et al., 2007). These NR1 thiol groups trigger S-nitrosylation of other sites on the NMDA receptor and thereby dictate the pathological effects of hypoxia (McLaughlin et al., 2003).

### *C. S-Nitrosylation-Mediated Indirect Control of the N-Methyl-D-aspartate Receptor*

Dexas1, dexamethasone-activated Ras, is a 30-kDa G-protein in the Ras subfamily. It binds to nNOS via the adaptor protein CAPON (Fang et al., 2000), and this binding then elicits the S-nitrosylation and activation of dexas1 (Jaffrey et al., 2002; Cheah et al., 2006) (Figs. 5 and 6). Dexas1, by binding to peripheral benzodiazepine receptor-associated protein PAP7, facilitates iron import by modification of the function of a neuronal iron import transporter (Cheah et al., 2006). Overstimulation of the NMDA receptor and permanent dexas1 S-nitrosylation may thus cause an iron overload and neurotoxicity. After sciatic nerve injury, mRNAs for CAPON and dexas1 increased in DRGs and spinal cord in parallel to nNOS (Shen et al., 2008) and may be involved in nerve injury-evoked adaptations of nNOS activity.

NMDA-receptor signaling is further modified indirectly by S-nitrosylation of astroglial serine racemase (Fig. 4), which generates D-serine, a coagonist of glutamate at NMDA receptors (Mustafa et al., 2007). Serine racemase is physiologically S-nitrosylated at cysteine 113 (Table 1). The enzyme activity of this constitutively SNO-modified racemase is low so that the physiological D-serine supply at postsynaptic NMDA receptors is limited. NMDA receptor activation further enhances the S-nitrosylation by activating nNOS and releasing NO in the postsynaptic neuron (Mustafa et al., 2007). Although neurons themselves are able to release D-serine upon depolarization (Rosenberg et al., 2010) development of long-term potentiation (LTP) depends on the release of D-serine from astrocytes (Henneberger et al., 2010), which thereby control NMDA-receptor-dependent plasticity in nearby excitatory synapses. NMDA receptor-dependent LTP at the nociceptive synapse in the spinal cord presumably depends on neuron-to-glia and glia-to-neuron communication via nitric oxide (Chiang et al., 2007) (Fig. 4).

Proteomic screens additionally detected potential S-nitrosylation sites in the excitatory amino acid transporter 2 and glutamine synthase (Butterfield et al., 2006; Görg et al., 2007), which are two proteins that regulate the extraneuronal levels of glutamate by reuptake and glutamate metabolism to glutamine, respectively. EAAT2 and glutamine synthase are localized in astroglial cells. S-Nitrosylation of these enzymes may be relevant for glutamate homeostasis, but the functional consequences of S-nitrosylation have not yet been evaluated.



TABLE 1  
*Functional consequences of target protein S-nitrosylation in nociceptive signaling pathway*

Target	Modification Site	Effect of SNO on Target Function	Presumed Consequence in Terms of Nociception	Reference
NMDA-R subunit NR2A	Cys399	Reduction of calcium influx	Prevention of glutamate excitotoxicity and control of hyperexcitability	Lipton et al., 1993; Choi et al., 2000
NMDA-R subunit NR1	Cys744 and Cys798 Subsequent facilitation of S-nitrosylation at further sites	Reduction of calcium influx, sensitization of other cysteines for NO-mediated modification	Prevention of glutamate excitotoxicity during ischemia and hypoxia	Takahashi et al., 2007
Serine racemase	Cys113	Restriction of catalytic activity and production of D-serine, which acts as coactivator at NMDA-R	Modulation of NMDA-R dependent LTP	Mustafa et al., 2007
Glutamine synthase	N.D.	N.D.	Prevention of glutamate excitotoxicity by conversion to nontoxic glutamine	Görg et al., 2007
Excitatory glutamate transporter EAAT2 (GLT1)	N.D.	N.D.	Glutamate reuptake in astrocytes	Butterfield et al., 2006
Dexas1	Cys11	Increase of the proportion of GTP-bound Ras leading to increase of neuronal iron import	Regulation of iron supply for heme-dependent enzymes	Cheah et al., 2006
p21/Ras	Cys118	Increase of the proportion of GTP-bound Ras leading to increased activation of PI3K $\gamma$ and PKB/Akt kinase	Prosurvival effect after nerve injury	Lander et al., 1995
NSF	Cysteines 11, 21, 334, 568, and 582	Inhibition of SNARE complex disassembly, reduction of synaptic vesicle exocytosis, increase of membrane insertion of AMPA-R GluR2	Decrease of nociceptive neuron sensitivity to noxious stimuli, decrease of LTP at nociceptive synapses	Matsushita et al., 2003; Huang et al., 2005
Syntaxin	Cys145	Regulation of synaptic vesicle exocytosis	Prevention of excess glutamate or neuropeptide release	Palmer et al., 2008
Stargazin	Cys302 intracellular in the C-terminal tail	Increase of AMPA-R membrane insertion	Increase of nociceptive neuron sensitivity	Selvakumar et al., 2009
Caspase 3	Cys163, catalytic site	Constitutively nitrosylated mitochondrial caspase 3, denitrosylation through Trx caused activation and apoptosis	Prevention of glutamate excitotoxicity and neuron death after axonal injury	Mannick et al., 1999; Mannick et al., 2001
TRPC5	C553+C558 N-terminal cytoplasmic tail, pore forming region	Increase of channel gating for calcium entry	Increase of nociceptive neuron sensitivity	Yoshida et al., 2006
TRPC5	Extracellularly accessible cysteines sensitive to reduced extracellular thioredoxin	Reduced extracellular rTRX evoked increase of channel gating for calcium entry	Increase of nociceptive neuron sensitivity	Xu et al., 2008b
TRPA1	N-terminal cytoplasmic side, pore-forming region, Cys415, 422, 622	Covalent binding of endogenous and chemical reactive electrophilic compounds, increase of calcium influx	Increase of nociceptive neuron sensitivity, pain sensation evoked by chemical irritants	Macpherson et al., 2007
TRPV1	N-terminal cytoplasmic side, pore-forming region	Covalent binding of endogenous and chemical reactive electrophilic compounds, increase of calcium influx	Increase of nociceptive neuron sensitivity, pain sensation evoked by chemical irritants	Salazar et al., 2008
VGCCs	N.D.	Increase of calcium influx	Increase of nociceptive neuron sensitivity	Jian et al., 2007
Membrane K-ATP, Kir6.2/Sur1	N.D.	Increase of potassium influx	Hyperpolarization of primary sensory neurons, antinociceptive effects of NO, peripheral antinociceptive effects of morphine	Kawano et al., 2009a; Cunha et al., 2010

TABLE 1.—Continued.

Target	Modification Site	Effect of SNO on Target Function	Presumed Consequence in Terms of Nociception	Reference
Ryanodine channel	RyR1 C3635 muscle	Increase of CICR	Modulation of EPSCs in lamina II nociceptive neurons in spinal cord slices	Sun et al., 2001; Aracena-Parks et al., 2006
Ryanodine channel	RyR2, RyR3 brain	Increase of CICR, neuropeptide release sensory neurons	Modulation of EPSCs in lamina II nociceptive neurons in spinal cord slices	Bull et al., 2003, 2008
sGC	Cysteines in vicinity to heme group C243 in alpha and C122 in beta subunit	Desensitization of sGC, decrease of cGMP production	Desensitization of sGC positive projecting neurons	Sayed et al., 2007
CNG2, rat	Cys460 within C-linker region	Increase of cation entry into cell	Enhancement of nociceptive neuron sensitivity	Broillet, 2000
HCN	N.D.	Setting of the excitation threshold in sensory neurons	Hyperexcitability and spontaneous discharge after nerve injury	Jaffrey et al., 2001
c-Jun N-terminal kinase (JNK1)	Cys116	Inhibition of catalytic activity	Reduction of microglia activation after nerve injury	Park et al., 2000
SHP-1 phosphotyrosine phosphatase	Cys453, active site	Inhibition of phosphatase activity	Increase of NGF mediated hyperalgesia due to enhanced phosphorylation of TrkA	Barrett et al., 2005
MAP kinase phosphatase (MKP7)	N.D.	Inhibition of phosphatase activity, leading to JNK activation	Proinflammatory, activation of microglia and neuronal stress response	Pi et al., 2009
MMP-9		Increase of catalytic activity	Enhancement of glia activation after nerve injury, increase of hyperalgesia	Gu et al., 2002; McCarthy et al., 2008
cPLA2	Cys152	Increase of catalytic activity and release of arachidonic acid from membrane lipids	Facilitation of the production of proinflammatory lipid mediators such as prostaglandins	Xu et al., 2008a
COX-2	N.D.	Increase of catalytic activity and prostaglandin E2 production	Direct activation of nociceptive neurons and disinhibition, promoting glutamate NMDA-R-dependent excitotoxicity	Tian et al., 2008
Myeloid differentiation primary response gene (MyD88)	N.D.	Control of Toll-like receptor-mediated signaling	Anti-inflammatory effects	Into et al., 2008
IKK $\beta$	Cys179 located between critical phosphorylation site at Ser177 and Ser181, conserved in IKK $\alpha$	Inhibition of IKK-mediated I $\kappa$ B $\alpha$ phosphorylation resulting in inhibition of NF- $\kappa$ B activation	Anti-inflammatory effects	Reynaert et al., 2004
NF- $\kappa$ B p50	Cys62	Inhibition of DNA binding capacity	Anti-inflammatory effects	Marshall and Stamler, 2001
NF- $\kappa$ B p65	N.D.	Inhibition of DNA binding capacity	Anti-inflammatory effects	Kelleher et al., 2007
HIF1- $\alpha$	Cys533, oxygen-dependent degradation domain	Stabilization of HIF-1 $\alpha$ and increase of its transcriptional activity	Increase of nociceptive neuron sensitivity, pro-inflammatory	Palmer et al., 2000; Li et al., 2007
Adenylyl cyclase (AC6)	N.D.	Inhibition of cAMP production	Antinociceptive effects (e.g. reduction of PACAP-signaling)	Ostrom et al., 2004
GRK2	Cys340	Prevention of agonist-stimulated receptor desensitization	Modulation of GPCR signaling	Whalen et al., 2007
Dynamin	Cys607 conserved in dynamin 1 and 2	Facilitation of GPCR endocytosis and desensitization	Modulation of GPCR signaling, synaptic vesicle exo- and endocytosis, axonal transport, general membrane fission	Wang et al., 2006

TABLE 1.—Continued.

Target	Modification Site	Effect of SNO on Target Function	Presumed Consequence in Terms of Nociception	Reference
Dynamamin-related protein 1 (Drp1)	Cys644	Promotion of mitochondrial fission, control of neuronal cell death	Enhancement of neuronal death after axonal injury	Cho et al., 2009; Westermann, 2009
$\beta$ -Arrestin	Cys410	Promotion of GPCR endocytosis and recycling	Function of cannabinoid and opioid receptors, implications for opioid and cannabinoid tolerance	Ozawa et al., 2008
GAPDH		Increase of GAPDH binding to and signaling of Siah	Increase of glutamate excitotoxicity	Molina y Vedia et al., 1992; Hara et al., 2005
GOSPEL	Cys47	Facilitation of GAPDH-GOSPEL binding, and neuroprotection by GOSPEL.	Decrease of glutamate excitotoxicity	Sen et al., 2009
PDI		Inhibition of catalytic activity, accumulation of polyubiquitinated proteins, and activation of the unfolded protein response	Increase of ER stress	Uehara et al., 2006
Trx 1	Cys69 and Cys73, upon oxidative stress active site Cys32 and Cys35	Reduction of catalytic activity, transnitrosylation of caspase 3	Modulation of apoptosis, modulation of constitutively nitrosylated proteins	Mitchell and Marletta, 2005; Hashemy and Holmgren, 2008
Peroxiredoxin (Prx2)	Cys51 and Cys172, catalytic site	Inhibition of catalytic activity, loss of antioxidant capacity	May increase neuronal damage after injury	Forrester et al., 2009
Glutaredoxin, (Grx1)	Cys-8 and either Cys-79 or Cys-83	Partial inhibition of catalytic activity, loss of antioxidant capacity	May increase neuronal damage after injury	Hashemy et al., 2007
Glutathione transferase (GST) pi	Cys47 and Cys101	NO-carrier function		Lo Bello et al., 2001; Townsend et al., 2006

EPSC, excitatory postsynaptic current; PACAP, pituitary adenylate cyclase-activating polypeptide; PI3K, phosphatidylinositol 3-kinase; -R, receptor.

#### D. Receptor Trafficking and Synaptic Vesicle Exocytosis

A positive shift of the postsynaptic membrane potential mediated by  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor activation and calcium influx enhances the open probability of the NMDA receptor by expelling the  $Mg^{2+}$  ion that blocks the channel from the outside. Blocking or deletion of AMPA receptor subunits therefore reduces glutamate-dependent signaling and alleviates hyperalgesia and allodynia in rodent models of inflammatory or neuropathic pain (Garry et al., 2003; Larsson and Broman, 2008; Luo et al., 2008). Synaptic plasticity at glutamatergic synapses is mediated by changes in the surface expression of AMPA receptors (Fig. 3), and this is controlled by transmembrane auxiliary proteins that have been identified as potential S-nitrosylation targets.

Stargazin is a prototype of such auxiliary proteins of AMPA receptors. It is a small tetraspanning membrane protein and interacts with a large proportion of AMPA receptor subunits (Chen et al., 2000; Tomita et al., 2005). Therefore, it seems to be a principal determinant of AMPA receptor surface expression. Stargazin is physiologically S-nitrosylated at a cysteine in the cytoplasmic tail (Table 1). This part determines AMPA receptor trafficking. NMDA receptor-mediated activation of nNOS resulted in increased S-nitrosylation of stargazin (Figs.

3, 5, and 6) and, subsequently, increased binding and surface expression of the AMPA receptor subunit GluR1 (Selvakumar et al., 2009) suggesting a nitric-oxide-dependent feed-forward activation that may contribute to the development of hyperalgesia. Inflammatory hyperalgesia is associated with an increase of the membrane density of GluR1-containing AMPA receptors in nociceptive neurons of the dorsal horn of the spinal cord and a relative decrease of GluR2/3 subunits (Larsson and Broman, 2008). Deletion models revealed that a lack of GluR2 intensifies long-term potentiation upon tetanic stimulation (Jia et al., 1996; Youn et al., 2008). Hence, the NO-facilitated exchange of GluR2/3 in favor of GluR1 by membrane insertion of the GluR1 subunit and endocytosis of GluR2/3 is likely to fortify nociceptive glutamatergic signal transduction. Indeed, rats treated with intrathecally delivered stargazin antisense oligonucleotides and thus malfunctioning of GluR1 insertion into the membrane showed reduced nociceptive responses in the formalin test (Tao et al., 2006).

The NMDA receptor activation is further modified by physiological NO-dependent S-nitrosylation of the ATPase *N*-ethylmaleimide-sensitive factor (NSF) (Matsushita et al., 2003; Söllner and Sequeira, 2003), which is a crucial protein for membrane trafficking in many cells, including neurons (Figs. 5 and 6). NSF functions as a soluble NSF attachment protein receptor (SNARE)

chaperone that binds to SNARE complexes and uses the energy of ATP hydrolysis to disassemble the complex, thus facilitating SNARE recycling. Post-translational modifications of NSF by S-nitrosylation or phosphorylation determine the efficiency of vesicle recycling and may be particularly important in case of high neuronal activity (Huang et al., 2005) (Fig. 3). Upon S-nitrosylation of NSF, vesicle exocytosis was reduced because SNO-NSF lost the ability to disassemble the SNARE complex, whereas it maintained its ATPase activity. Nitric oxide also inhibited the dissociation of NSF from the synaptic vesicle protein syntaxin (Matsushita et al., 2003), which itself is modified by S-nitrosylation (Pongrac et al., 2007; Palmer et al., 2008) and is involved in the regulation of neurotransmitter release (Fig. 3). Syntaxin 1a-deficient mice developed enhanced allodynia after peripheral nerve injury and dorsal horn excitatory postsynaptic currents were enhanced in both frequency and amplitude in these mice (Takasusuki et al., 2007). Via CAPON, nNOS also forms a ternary complex with the synaptic-vesicle protein synapsin II, which anchors synaptic vesicles to the actin-based cytoskeleton to maintain a reserve pool of vesicles (Ceccaldi et al., 1995). Upon activation, synapsins dissociate from vesicles and the actin cytoskeleton (Bähler and Greengard, 1987; Benfenati et al., 1992; Greengard et al., 1993; Chi et al., 2003) and allow the vesicle transport to the plasma membrane and release of stored neurotransmitters (Humeau et al., 2001). Mice with a targeted deletion of synapsin II develop less inflammatory and neuropathic pain because they release less glutamate (Schmidtko et al., 2005, 2008b). The close association of nNOS with synapsin II suggests that S-nitrosylation controls the functions of synapsin II as a guardian of the synaptic vesicle pool. SNO-mediated effects on synaptic vesicle transport and release fit well into the concept of SNO-control of membrane fusion and fission. So far, however, there is no direct *in vivo* evidence for nociception-evoked functional S-nitrosylation at the presynaptic site.

NSF also interacts with the AMPA receptor GluR2 subunit in the postsynaptic neuron (Fig. 3). Nitrosylation of NSF at this site increased NSF-GluR2 associations and unclustering of GluR2 from the scaffolding protein PICK1 (Huang et al., 2005; Sossa et al., 2007). Subsequently, membrane insertion of GluR2-containing AMPA-receptors increased relative to GluR1 subunits. As a result, inflammatory hyperalgesia was reduced (Katano et al., 2008). This is due in part to a switch in subunit composition of AMPA receptors (Katano et al., 2008). Hence, NMDA receptor activation can drive the surface delivery of GluR2-AMPA receptors from a pool of intracellular AMPA receptors retained by PICK1 through an NO-dependent modification of NSF (Huang et al., 2005; Sossa et al., 2007) to reduce nociceptive signaling. S-Nitrosylation of NSF at the central nociceptive synapse would therefore presumably reduce hyperexcitability and oppose the effects brought about by direct S-nitrosylation of stargazin. It is

impossible at present to predict which S-nitrosylation event might be relevant for *in vivo* nociception, and SNO events at the peripheral nerve terminal may further complicate the picture (Fig. 2). At the peripheral nerve terminal, S-nitrosylation events also involve NSF-regulated membrane insertion of ion channels and receptors (Zhang et al., 2005). From the discussion above emerges the scheme that nitric oxide, by direct S-nitrosylation of one crucial ATPase, may modify nociceptive signal transduction at multiple sites with variable outcome, depending on the stimulus and the site of NO release and being mediated mainly by adaptation of membrane fusion and fission and receptor compartmentalization.

### *E. Redox Modulation of Ion Channels in Pain Signaling*

**1. Transient Receptor Potential Channels.** Transient receptor potential (TRP) channels of primary neurons mediate sensation of various nociceptive stimuli including noxious heat, cold, acid, and pressure (Clapham, 2003; Voets et al., 2005; Christensen and Corey, 2007) (Fig. 2). Potentiation of receptor sensitivity is mediated by a variety of proinflammatory factors and neuropeptides. Two complementary mechanisms of TRP channel sensitization have been proposed: 1) a decrease of the activation threshold by post-translational phosphorylation and 2) an increment of the surface expression in nociceptors mediated by S-nitrosylation of NSF (Bezzardes et al., 2004; Camprubi-Robles et al., 2009) (Fig. 2). Sensitization of the heat-sensitive TRP channel TRPV1 by pronociceptive factors, including nerve growth factor (NGF), ATP, and insulin growth factor, enhanced the TRPV1 channel exposure at the neuronal plasma membrane (Zhang et al., 2005). Blocking the formation of the SNARE prevented this TRPV1 membrane insertion (Camprubi-Robles et al., 2009). NGF-mediated fast insertion of TRPV1 into the membrane is additionally modulated by tyrosine phosphorylation of TRPV1 (Zhang et al., 2005) and of the NGF receptor, TrkA. The latter is negatively controlled by the SHP-1 phosphotyrosine phosphatase (Marsh et al., 2003), which dephosphorylates TrkA and is itself negatively regulated through NO by a mechanism involving direct S-nitrosylation of the active site Cys453 of SHP-1 (Barrett et al., 2005) (Fig. 6; Table 1). One might infer from these data that NO-mediated S-nitrosylation events at the peripheral nerve ending would particularly sustain NGF-evoked thermal hypersensitivity.

A third mechanism of TRP channel regulation may involve direct S-nitrosylation of critical cysteine residues that modify channel gating properties, as exemplified by the TRPC channels (Yoshida et al., 2006). The predominant TRPC channels in the mammalian brain are TRPC4, -5, and -1. They are activated by stimulation of metabotropic glutamate receptor 5, and, in general, TRPC channels can be activated by phospholipase C

stimulation and by diacylglycerol (Hofmann et al., 1999). For TRPC5, labeling and functional assays using cysteine mutants revealed that two cysteines, Cys553 and the nearby Cys558, represent the nitrosylation sites that determine the NO sensitivity of the channel (Yoshida et al., 2006) (Table 1). NO-responsive TRP proteins all have conserved cysteines on the N-terminal tail, and S-nitrosylation mediates an increase of calcium influx through the channel. For example, nitrosylation of native TRPC5 upon stimulation of a G-protein-coupled ATP receptor elicited the entry of calcium into endothelial cells. The studies revealed a structural motif for the NO-sensitive activation gate in TRP channels of different TRP families, suggesting that other TRP channels are also directly regulated by S-nitrosylation. Supporting this idea, NO elicited a calcium influx in primary sensory neurons, which was abolished when TRPV1 and TRPA1 were both deleted (Miyamoto et al., 2009). However, whether exchange of the critical cysteine in either channel would similarly abolish the gating effect of nitric oxide was not evaluated.

Cysteines Cys553 and Cys558 of TRPC5 are also sensitive to reduced extracellular thioredoxin (Trx), thereby directly coupling the redox state to cell activity (Xu et al., 2008b). Thioredoxin is an intracellular redox protein (Holmgren and Lu, 2010) that is also secreted and reaches particularly high extracellular concentrations at sites of inflammation (Maurice et al., 1999). In its reduced form, thioredoxin has the capability to break disulfide bridges and may act as a novel type of ion channel agonist that breaks a restraining intrasubunit disulfide bridge between cysteine residues in TRPC5, stimulating the channel. This mechanism may have particular relevance in conditions such as rheumatoid arthritis in which extracellular Trx concentrations are strongly elevated (Maurice et al., 1999), but the broad distributions of Trx and the TRP channels suggest that many cells may use this mechanism (Xu et al., 2008b).

Although NO-mediated S-nitrosylation of TRP channels is a reversible physiological process, oxidative stress may result in long-lasting modifications of critical cysteines, particularly of the transient receptor potential A1 channel (Fig. 2). TRPA1 acts as a sensory receptor for noxious cold and for various chemical and endogenous irritants, such as cinnamaldehyde and formalin (Bautista et al., 2005, 2006; Macpherson et al., 2007; McNamara et al., 2007), bradykinin (Bandell et al., 2004), and lipid peroxidation products, such as 4-hydroxynonenal (Trevisani et al., 2007). Environmental irritants activate TRPA1 by covalent modification of specific cysteine residues located within the putative cytoplasmic N-terminal domain of the channel (Macpherson et al., 2007). Pungent extracts from onion and garlic similarly activate TRPV1 by covalent modification of a single critical cysteine residue located within the N-terminal region (Salazar et al., 2008). The resulting small hydrophobic augmentations of the channel protein at the identified

nucleophilic sites then trigger robust channel opening (Hinman et al., 2006). The cysteines in these channels are also targets of electrophilic products produced under conditions of oxidative stress such as hydrogen peroxide ( $H_2O_2$ ), lipid peroxidation products, and 15-deoxy- $\Delta^{12,14}$ -prostaglandin  $J_2$  (Andersson et al., 2008; Maher et al., 2008; Taylor-Clark et al., 2008). These products evoked depolarizing inward currents in primary DRG neurons, resulting in increases in intracellular calcium that were lacking in TRPA1-deficient mice (Andersson et al., 2008; Cruz-Orengo et al., 2008). It is therefore likely that critical cysteines of these TRP channels act as sensors for pain evoked by oxidative stress.

**2. Voltage-Gated Sodium and Calcium Channels.** DRG neurons express three types of voltage-dependent  $Na^+$  currents: fast tetrodotoxin (TTX)-sensitive, slow TTX-resistant, and persistent TTX-resistant. In particular, TTX-resistant  $Na^+$  currents mediated by Nav1.8 and Nav1.9 in small- to medium-sized nociceptive neurons are involved in pain sensation and development of pathological nociceptive hypersensitivity after nerve injury (Benn et al., 2001; Lai et al., 2002; Roza et al., 2003; Rush et al., 2006; Jarvis et al., 2007) (Fig. 2). Increases in TTX-resistant and TTX-sensitive  $Na^+$  currents coincided with nerve injury-induced increases in excitability and alterations in spike shape across the whole population of sensory DRG neurons (Roza et al., 2003), and it was observed that nitric oxide inhibits all three types of  $Na^+$  currents.  $Na^+$  currents were restored during washout of NO donors and upon application of the NO scavenger hemoglobin. Effects of NO donors were not mimicked by cGMP analogs or blocked by PKG inhibitors, whereas alkylation of free thiols with *N*-ethylmaleimide prevented the actions of the NO donor, suggesting that NO, or a related reactive nitrogen species, modifies sulfhydryl groups on  $Na^+$  channels (Renganathan et al., 2002). Likewise, exogenous NO donors inhibited both TTX-sensitive and TTX-insensitive  $Na^+$  currents in baroreceptor neurons, again independent of cGMP (Li et al., 1998). It is unclear whether NO produced by endogenous NOS may cause equivalent effects in vivo.

Noxious stimulation of peripheral nociceptors also leads to activation and up-regulation of voltage-gated calcium channels (VGCC) in primary and secondary nociceptive neurons (Saegusa et al., 2001) (Fig. 2). After peripheral nerve injury, subunits of VGCCs are increasingly transported to the site of injury and inserted into the membrane, giving rise to ectopic discharge upon minimal changes in the membrane potential. The antinociceptive effects of gabapentin are partially mediated by blocking the  $\alpha 2\text{-}\delta 1$  subunit of VGCCs (Xiao et al., 2007); recently, ziconotide, a specific inhibitor of neuronal *N*-type VGCC, has been launched for intrathecal treatment of serious drug-resistant pain (Schmidtke et al., 2010). In primary neurons, NO-releasing agents stimulated the calcium currents of VGCCs and increased the channel sensitivity to depolarizing voltages (Jian et al., 2007). Pretreatment with a thiol-specific

alkylating agent or with  $\omega$ -conotoxin blocked the neuronal current response evoked by NO, suggesting an involvement of S-nitrosylation of N/Q-type VGCCs (Chen et al., 2002). Specific cysteine residues have not been identified, and the molecular mechanisms for these NO-mediated effects may involve fast adaptations of membrane insertion of these ion channels or direct changes of channel gating properties.

**3. Potassium Channels.** Nitric oxide reduces insulin release from pancreatic  $\beta$  cells through a mechanism involving direct or indirect activation of ATP-sensitive potassium channels, KATP. In neurons, KATP channels regulate excitability, neurotransmitter release, and cell death. The NO-mediated activation of KATP channels in nociceptive neurons involves direct S-nitrosylation of cysteine residues in the SUR1 subunit and is suppressed after peripheral sciatic nerve injury (Kawano et al., 2009a,b) (Figs. 5 and 6). NO donors elicited potassium currents in cells expressing recombinant sulfonylurea receptors SUR1 or SUR2 and Kir6.2 channels (Lin et al., 2004; Kawano et al., 2009a). Channels formed only from Kir6.2 subunits were insensitive to NO (Kawano et al., 2009a), and KATP activation was prevented with an NO scavenger but not by inhibition of guanylyl cyclase (Lin et al., 2004). In hippocampal neurons, NO-mediated KATP activation required S-nitrosylation of Ras rather than direct S-nitrosylation of the channel subunits (Lin et al., 2004). In these experiments, blockade of Ras activation or expression of an S-nitrosylation-site mutant Ras protein significantly abrogated the effects of NO (Lin et al., 2004). Because the protective effects of ischemic preconditioning in neurons depend on KATP and NO synthase activity during preconditioning, it may be suggested that NO-mediated neuroprotection involves S-nitrosylation of either Ras or KATP and subsequent KATP-mediated hyperpolarization. It should be noted that hydrogen sulfide initiates a similar signaling cascade by S-sulfhydration of critical KATP cysteines, thereby exerting a protective effect against neuronal cell death in cerebral hypoxia (Tay et al., 2010). KATP stimulation in sensory neurons contributes to antinociceptive effects of morphine or dipyrone in models of inflammatory pain (Sachs et al., 2004). Morphine has been observed to evoke the production of nitric oxide in primary nociceptive neurons, and its peripheral antinociceptive effects were lost in nNOS knockout mice (Cunha et al., 2010). The activation of the NO pathway by morphine was dependent on an initial stimulation of phosphatidylinositol-3-kinase  $\gamma$  and protein kinase B/Akt, and culminated in the activation of KATP channels. NO-mediated effects therefore seem to be crucial for the peripheral antinociceptive effect of morphine (Cunha et al., 2010).

**4. Cyclic Nucleotide-Gated Ion Channels.** Cyclic nucleotide-gated ion channels (CNGs) constitute the critical final elements for visual and olfactory transduction in sensory-receptor cells, and NO donors directly acti-

vate olfactory CNGs by S-nitrosylation of Cys460 of the  $\alpha$ -subunit (Broillet, 2000). Cys460 is located immediately adjacent to the cyclic-nucleotide-binding domain so that S-nitrosylation at this site might allosterically regulate channel activity through changes in tertiary or quaternary structure (Broillet, 2000). The role of CNGs in cGMP-mediated nociceptive signaling is not clear. CNG  $\alpha$  and  $\beta$  subunits are expressed at the mRNA levels in neurons of the dorsal root ganglia and in the spinal cord (Tegeger et al., 2002). However, functional analyses are hampered by the lack of specific inhibitors and the considerable pathologic conditions generated in conventional deletion models. Members of the closely related family of hyperpolarization-activated cyclic-nucleotide-modulated cation channels are additional neural substrates for endogenous S-nitrosylation (Jaffrey et al., 2001) (Figs. 5 and 6). Activation of HCN channels occurs at negative membrane potentials and upon repolarization. The resulting depolarizing current (I<sub>h</sub>) influences the threshold for subsequent action potential generation. Consequently, HCN channels play a critical role in regulating neuronal excitability. HCN-mediated I<sub>h</sub> currents are prominent in many peripheral sensory nerves, with highest current density typically found in large-diameter mechanosensitive neurons in which the I<sub>h</sub> current seems to support abnormal spontaneous firing after nerve injury (Chaplan et al., 2003; Takasu et al., 2010).

**5. Intracellular Calcium Stores.** The release of Ca<sup>2+</sup> from intracellular stores contributes to diverse neuronal functions, including synaptic plasticity. Two different Ca<sup>2+</sup> channels mediate the fast release of Ca<sup>2+</sup> from intracellular stores, the ryanodine receptors (RyR) and the inositol trisphosphate receptors (Fig. 3). In neurons, both Ca<sup>2+</sup>-release channels can increase their activity in response to calcium influx. This response generates "Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release" (CICR), a universal cellular mechanism that allows amplification and propagation of the Ca<sup>2+</sup> signals initially created by Ca<sup>2+</sup> influx. In primary sensory DRG neurons, CICR arose mainly from type 3 RyR channels on subsurface cisternae of the endoplasmic reticulum. CICR essentially contributed to the release of calcitonin gene-related peptide and substance P (Solovyova et al., 2002; Ouyang et al., 2005a,b), which are two of the best studied pronociceptive neuropeptides.

Activation of RyRs depends on the redox state (Bull et al., 2008) (Figs. 5 and 6, Table 1). Highly reduced channels with mostly free thiol groups respond poorly to Ca<sup>2+</sup> activation, whereas increasing cysteine oxidation or alkylation increases the channel response to micromolar [Ca<sup>2+</sup>]<sub>i</sub> and decreases the inhibitory effect of higher [Ca<sup>2+</sup>]<sub>i</sub>. For example, ATP (Bull et al., 2007) and glycosphingolipids (Lloyd-Evans et al., 2003) differentially activate RyR channels depending on their redox state, so that more oxidized channels require less ATP or glycosyl ceramide to attain maximal activation (Marengo et al., 1998; Bull et al., 2003). Oxidative stress

and alterations in  $\text{Ca}^{2+}$  homeostasis are likely to enhance RyR-mediated CICR in neurons and may contribute to neuronal adaptations to ongoing nociceptive activation (Ohsawa and Kamei, 1999). In hippocampal neurons, induction of LTP entails a combined increase of  $[\text{Ca}^{2+}]_i$  and reactive oxygen and nitrogen species with subsequent modification of RyR-mediated  $\text{Ca}^{2+}$  release from the endoplasmic reticulum. Recordings from spinal cord slice preparations revealed that the release of NO contributes to long-term potentiation of C-fiber-evoked field potentials elicited by tetanic stimulation of the sciatic nerve. RyR antagonists reduced the LTP of C-fiber-evoked responses in the spinal cord, whereas inositol trisphosphate receptor antagonists had no significant effect (Cheng et al., 2010), suggesting that spinal cord CICR is due mainly to RyR-mediated  $\text{Ca}^{2+}$  release (Fig. 3). Endogenous activation of RyR channels by cellular reactive oxygen or nitrogen species may represent a physiological mechanism of cross-talk between  $\text{Ca}^{2+}$  and redox signaling pathways. Nociceptive neurons may use redox-modulated and RyR-mediated  $\text{Ca}^{2+}$  release mechanisms to either amplify or inhibit  $\text{Ca}^{2+}$  signals as needed for a specific response (Kyrozis et al., 1996). Similar mechanisms may also contribute to the stimulated adaptations in glial cells, because NO-releasing substances can initiate intercellular  $\text{Ca}^{2+}$  waves in glia as a result of combined influx and intracellular release (Aracena-Parks et al., 2006).

#### F. Proinflammatory Mediators

Cyclooxygenase-derived prostaglandin E<sub>2</sub> is released in the dorsal horn of the spinal cord after peripheral nociceptive stimulation and contributes to inflammatory hyperalgesia by two distinct mechanisms: 1) direct activation of nociceptive neurons (Baba et al., 2001) and 2) dysinhibition of inhibitory glycinergic synapses (Ahmadi et al., 2002). Peripheral inflammation causes up-regulation of cyclooxygenase 2 and nNOS in nociceptive neurons in the spinal cord (Maihöfner et al., 2000b) and promotes interaction of both enzymes (Fig. 4). nNOS, via its PDZ domain, binds COX-2 and the NO generated by nNOS then S-nitrosylates and activates cyclooxygenase 2 with subsequent production of prostaglandin E<sub>2</sub> (Tian et al., 2008). Selective disruption of nNOS-COX-2 binding prevents NMDA neurotoxicity, suggesting that glutamate-mediated neurotoxicity involves S-nitrosylation of cyclooxygenase 2 (Tian et al., 2008). In addition, cytosolic phospholipase A<sub>2</sub>  $\alpha$  (cPLA<sub>2</sub> $\alpha$ ), which is the rate-limiting key enzyme that cleaves arachidonic acid from membrane phospholipids for the biosynthesis of eicosanoids, is a target of SNO-mediated potentiation (Xu et al., 2008a) (Fig. 6). cPLA<sub>2</sub> $\alpha$  is S-nitrosylated by NO-releasing compounds, and its activity and arachidonic acid release were enhanced after stimulation of inducible NOS expression (Xu et al., 2008a). COX-2 enhanced the iNOS-mediated cPLA<sub>2</sub> $\alpha$  S-nitrosylation by facilitating the formation of a ternary complex of COX-2, iNOS

and cPLA<sub>2</sub> $\alpha$ . Maximal prostaglandin synthesis was achieved only by synergistic interaction among the three enzymes and depended on the SNO-mediated augmentation of cPLA<sub>2</sub> $\alpha$  activity (Xu et al., 2008a).

1. *Nuclear Factor  $\kappa\text{B}$  and Mitogen-Activated Kinases.* Up-regulation of cyclooxygenase-2 in spinal cord neurons upon ongoing nociceptive stimulation requires activation of the transcription factor nuclear factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) and presumably involves primarily the classic inhibitor  $\kappa\text{B}$  kinase (IKK) pathway (Lee et al., 2004; Tegeder et al., 2004b). Activation of IKK through phosphorylation of the catalytic subunits  $\alpha$  and  $\beta$  (Delhase et al., 1999) promotes the ubiquitination and proteasomal targeting of inhibitor  $\kappa\text{B}$  (I $\kappa\text{B}$ ), which allows NF- $\kappa\text{B}$  to translocate from the cytoplasm to the nucleus and regulate transcription. In neurons, spontaneous calcium transients maintain active NF- $\kappa\text{B}$  in the nucleus (Kaltschmidt et al., 1994; Lilienbaum and Israël, 2003), presumably mediated by constitutively active phosphorylated I $\kappa\text{B}$ - $\alpha$  and IKK in axon initial segment (Schultz et al., 2006) and at nodes of Ranvier (Politi et al., 2008). Depolarization or stimulation with glutamate leads to a redistribution of NF- $\kappa\text{B}$  from neurites to the nucleus (Mikenberg et al., 2007) so that NF- $\kappa\text{B}$  acts as a signal transducer, transmitting transient glutamatergic signals from distant synaptic sites to the nucleus (Kaltschmidt et al., 1994). In sensory neurons, constitutively active IKK regulates the activation threshold of TRP channels and dampens responses to acute nociceptive stimulation (Bockhart et al., 2009). Oxidative stress leads to activation of IKK and subsequent NF- $\kappa\text{B}$ -mediated gene expression and NOS up-regulation. The subsequent increase of nitric oxide production promotes S-nitrosylation of the critical cysteine residue Cys179 within the  $\beta$ -subunit of IKK, which results in inhibition of both I $\kappa\text{B}$  phosphorylation and degradation (Reynaert et al., 2004) (Fig. 6, Table 1). Cys179 is located between the two critical phosphorylation sites of IKK $\beta$ , Ser177 and Ser181, raising the intriguing possibility that phosphorylation modulates the likelihood of S-nitrosylation or vice versa. The SNO-dependent feedback control is strengthened by S-nitrosylation of a single cysteine, Cys62 within the conserved Rel-homology domain of the p50 subunit of NF- $\kappa\text{B}$  (Matthews et al., 1996; Marshall and Stamler, 2001). The respective cysteine is conserved in other Rel-homology domain-containing proteins that can serve as NF- $\kappa\text{B}$  subunits, including p65, p52, p100, p105, and c-Rel. S-Nitrosylated NF- $\kappa\text{B}$  dimers partially lose their DNA binding capacity and transcriptional activity (Marshall et al., 2004). Hence, nitric oxide may enhance COX-2 activity, but it may also stop its further transcription and up-regulation.

S-Nitrosylation-mediated adaptations to stress responses extend beyond the NF- $\kappa\text{B}$  pathway. In particular, modifications of mitogen-activated protein (MAP) kinase pathways act in concert with NF- $\kappa\text{B}$  and may be particularly important for adaptations in nociceptive

neurons and glial cells evoked by axonal injury (Zhuang et al., 2006) (Fig. 4). Apoptosis-related signaling kinase 1 (ASK1), a mitogen-activated protein kinase kinase, can activate NF- $\kappa$ B by phosphorylation of I $\kappa$ B (Zhao and Lee, 1999) and also stimulates the MAP signaling cascade, which involves the sequential phosphorylation of MAP kinase kinase, c-Jun N-terminal kinase, and activator protein 1 and culminates in c-Jun/c-Fos mediated gene transcription. Activation of MAP kinase pathways in nociceptive neurons in the DRGs and spinal cord mediates fast adaptation to noxious stimulation (Ma and Quirion, 2002; Obata et al., 2004) and is essential for the activation of microglial cells after nerve injury (Jin et al., 2003; Scholz et al., 2008). Nitric oxide controls the MAP kinase pathway by S-nitrosylation-mediated inhibition of ASK1 (Park et al., 2004) and of c-Jun N-terminal kinase 1 (Park et al., 2006) (Fig. 6). In microglial cells, interferon- $\gamma$  treatment caused nitric oxide production, which in turn suppressed JNK1 activation by S-nitrosylation of Cys116 (Table 1). The inhibitory effects of NO were abolished after treatment with the reducing agent dithiothreitol or when Cys116 was substituted with serine, suggesting that endogenous NO acted by direct S-nitrosylation of JNK1.

The activation of spinal cord glial cells upon peripheral nerve injury contributes to the development of neuropathic pain (Fig. 4). The activation process involves multiple mechanisms, including purine receptors (Tsuda et al., 2003), chemokines (Zhang et al., 2007), growth factors (Coull et al., 2005), and Toll-like receptors (Tanga et al., 2005; Kim et al., 2007). The latter are subject to nitric-oxide-mediated control through S-nitrosylation of the Toll-like receptor adaptor protein MyD88 (Into et al., 2008). This mechanism may be particularly relevant for glia activation in response to pathogens (Aravalli et al., 2005; Tanga et al., 2005). Once activated, glial cells secrete various proteases that after nerve injury initiate the degradation of myelin proteins.

**2. Matrix Metalloproteinases.** Matrix metalloproteinases (MMPs) are of central importance in the proteolytic remodeling of the extracellular matrix. MMPs have a conserved catalytic domain containing a zinc ion as well as a prodomain that regulates enzyme activation by modulation of a cysteine residue within that domain. Recent evidence suggests that the quiescent state of the pro-enzyme results from formation of an intramolecular complex between the single cysteine residue in its propeptide domain and the essential zinc atom in the catalytic domain, a complex that blocks the active site. MMPs can be activated by multiple means, all of which effect the dissociation of the cysteine residue from the complex, referred to as "cysteine-switch," which is probably applicable to all members of this gene family. Nitric oxide and NO-derived reactive nitrogen species target both the zinc ions and cysteine thiols. Exposure of purified pro-MMP-9 to exogenous NO caused a concentration-dependent bell-shaped activation and inactivation

of the enzyme (Gu et al., 2002; Ridnour et al., 2007). Further *in vitro* experiments revealed that pro-MMP-9 is S-nitrosylated and activated by exposure to nitroso-cysteine (McCarthy et al., 2008), and the active derivative of MMP-9 was identified by mass spectrometry as a stable sulfinic or sulfonic acid of the zinc-coordinating cysteine residue, whose formation was triggered by S-nitrosylation (Gu et al., 2002).

In the rodent brain and spinal cord, MMP-9 colocalizes with neuronal nitric-oxide synthase and is activated by ischemia/reperfusion or after peripheral axonal injury (Kawasaki et al., 2008) (Fig. 4). In neuronal NOS knockout mice, this activation does not occur, supporting an NO-dependent stimulation (Kawasaki et al., 2008). After spinal nerve ligation, MMP-9 is up-regulated rapidly in injured DRG neurons, followed by a delayed up-regulation of MMP-2 in DRG satellite cells and spinal astrocytes. Inhibition of either MMP-9 or MMP-2 resulted in a reduction of nociceptive responses after sciatic nerve injury, partially mediated by blocking interleukin-1 $\beta$  cleavage and glia activation (Kawasaki et al., 2008). Short- and long-term therapy with a broad-spectrum MMP inhibitor also protected from injury-induced myelin basic protein degradation, caspase-mediated apoptosis, macrophage infiltration, and astrocyte activation in the spinal cord (Kobayashi et al., 2008). This suggests that the activation of metalloproteinases by S-nitrosylation may constitute an essential step in the reorganization and adaptive processes that, after nerve injury, pioneer the restitution of function, possibly at the expense of temporary enhanced nociceptive sensitivity.

### *G. Regulation of G-Proteins Involved in Inhibitory Pain Control through Redox Pathways*

Recent studies indicate that the G protein-coupled receptor (GPCR) signaling machinery can serve as a direct target of reactive oxygen species, including nitric oxide and S-nitrosothiols. GPCRs comprise the largest family of ligand-activated plasma membrane receptors and transduce signals through the activation of heterotrimeric G-proteins. Transduction through GPCRs is regulated through agonist-induced desensitization, in which receptors are functionally uncoupled from G-protein activation, and by internalization, in which receptors undergo endocytosis followed by recycling or degradation (Gainetdinov et al., 2004; Reiter and Lefkowitz, 2006). The scaffolding protein  $\beta$ -arrestin is an essential element in those processes.  $\beta$ -Arrestin is recruited to and binds the activated conformation of GPCRs, where it sterically inhibits coupling of GPCRs and G-proteins and serves as an adaptor to link GPCRs to nascent clathrin-based endocytotic vesicles (Pierce and Lefkowitz, 2001; Reiter and Lefkowitz, 2006), the partition of which from the plasma membrane is controlled by the large GTPase dynamin (Sweitzer and Hinshaw, 1998). S-Nitrosylation that follows stimulation of G-protein-coupled receptors either enhances or inhibits cellular responses, both by targeting G-protein signaling mole-



cules and by receptor internalization processes (Figs. 2, 3, and 6). As exemplified for the  $\beta$ -adrenoceptor stimulation, it was found that adenylyl cyclase type VI (Ostrom et al., 2004), protein kinase A (Burgoyne and Eaton, 2009), G-protein receptor kinase 2 (Whalen et al., 2007), the ATPase dynamin (Wang et al., 2006), and the scaffolding protein  $\beta$ -arrestin (Ozawa et al., 2008) can all be modified by S-nitrosylation.

Stimulation of  $\beta$ -adrenoceptors in vascular endothelial cells resulted in interaction of endothelial NOS (eNOS) with  $\beta$ -arrestin 2 and subsequent S-nitrosylation at a single cysteine of  $\beta$ -arrestin 2. Subsequently, eNOS dissociated from nitrosylated  $\beta$ -arrestin 2 and promoted binding of SNO- $\beta$ -arrestin 2 to clathrin heavy chain/ $\beta$ -adaptin, thereby accelerating receptor internalization, which was followed by denitrosylation and recycling of  $\beta$ -arrestin 2 (Ozawa et al., 2008). The agonist- and NO-dependent shift of  $\beta$ -arrestin 2 associations presumably serves as a general mechanism for GPCR-trafficking and hence modifies the sensitivity to a number of endogenous signaling molecules involved in nociception, including opioid peptides (Bohn et al., 1999, 2000, 2002; Gainetdinov et al., 2004) and cannabinoids (Jin et al., 1999; Rubino et al., 2006; Bakshi et al., 2007), which constitute essential pain defense systems (Figs. 2, 3 and 6). Guanosine 5'-O-(3-[<sup>35</sup>S]thio)triphosphate autoradiography studies in brain cryostat sections revealed that cannabinoid CB1 receptors were inhibited after exposure to nitrosylating agents, whereas  $\mu$ -opioid receptors were less affected (Kokkola et al., 2005). Potent SNO-mediated inhibition was also observed for lysophosphatidic acid receptors, whereas S-nitrosylation facilitated signaling of muscarinic receptors (Kokkola et al., 2005). By S-nitrosylation of  $\beta$ -arrestin, nitric oxide may modify opioid (Bohn et al., 1999, 2000, 2002; Gainetdinov et al., 2004) and cannabinoid (Rubino et al., 2006; Wu et al., 2008) tolerance and dependence. Although not directly demonstrated in the context of S-nitrosylation, it is well accepted that inhibition of neuronal NOS reduces morphine tolerance (Kolesnikov et al., 1993; Elliott et al., 1994), which reflects the dynamics of endocytotic cycling of the receptor between membrane and clathrin coated pits.

The velocity of receptor internalization is also modulated by receptor phosphorylation through GPCR kinases, which are inhibited upon S-nitrosylation (Whalen et al., 2007). The residence time of clathrin-coated pits at the membrane is further regulated locally by the cargo itself via a PDZ-dependent linkage to the actin cytoskeleton and the recruitment of the ATPase dynamin (Puthenveedu and von Zastrow, 2006), which upon S-nitrosylation promotes endocytosis (Kang-Decker et al., 2007). The S-nitrosylation-mediated regulation of the GTPase dynamin affects cellular functions that extend beyond cycling of GPCRs. Particularly in neurons, S-nitrosylation of dynamin and dynamin-related protein Drp1 might promote mitochondrial dysfunction and neuronal death (Fig. 3). Dynamins are large GTPases mainly involved in membrane trafficking. They can form

collars around the neck of clathrin-coated buds and are required for pinching off vesicles from the plasma membrane (McNiven et al., 2000). This function is lost in dynamin mutants, which are defective in GTP binding or hydrolysis. Dynamins also mediate intracellular membrane trafficking from endosomes and Golgi apparatus, axonal transport (Fig. 4), synaptic vesicle exocytosis, and regulation of actin and microtubule networks, the latter independent of membrane-trafficking processes (McNiven et al., 2000). Hence, S-nitrosylation of dynamin 1 or 2, although so far investigated only in the context of GPCR internalization, may modulate axonal transport and synaptic structure.

Particularly under conditions of neuronal stress, S-nitrosylation of dynamin related protein, Drp1 may promote neuronal death. Drp1 plays a role similar to that of dynamin in vesicle formation. However, it is not involved in secretory or endocytotic pathways or vesicular transport (Smirnova et al., 2001). Instead, Drp1 specifically affects mitochondrial morphology (Smirnova et al., 2001). In primary cortical neurons, nitric oxide caused mitochondrial fragmentation and neuronal cell death via S-nitrosylation of Drp1, forming SNO-Drp1, which was increased in the brains of human Alzheimer patients and may contribute to the pathogenesis of neurodegeneration (Cho et al., 2009). Nitric oxide-evoked mitochondrial fission comes along with ultrastructural damage of mitochondria, autophagy, declining ATP production, and generation of free radicals. Expression of a dominant-negative Drp1 inhibited mitochondrial fission induced by NO, rotenone, and amyloid- $\beta$  peptide; conversely, overexpression of native Drp1 increased neuronal loss. Thus, persistent nitrosylation of Drp1 may play a causal role in NO-mediated neurotoxicity (Barsoum et al., 2006). Although mitochondrial fission in the context of chronic pain is unexplored, there is emerging evidence that neuronal loss after axonal injury contributes to the development and maintenance of nerve injury-evoked neuropathic pain (Joseph and Levine, 2004; Scholz et al., 2005; Ilytska et al., 2006; Meisner et al., 2010).

#### H. Ubiquitin-Mediated Proteasomal Protein Degradation and Protein Processing

Redox reactions triggered by excessive levels of NO can entail protein misfolding, the hallmark of a number of neurodegenerative disorders and stress responses. S-Nitrosylation-mediated targeting of protein ubiquitination and proteasomal degradation apparently serves as a general mechanism by which nitric oxide interferes with the proteasomal degradation of misfolded proteins and the stability of transcription factors and hence long-term adaptations to neuronal stress. For example, S-nitrosylation of protein disulfide isomerase (PDI) and various chaperones links protein misfolding to neuronal death (Uehara et al., 2006). Mild neuronal stress, including stress due to axonal injury, causes up-regulation of heat-shock proteins, such as the 27-kDa heat-shock pro-

teins (Lewis et al., 1999; Benn et al., 2002) and protein disulfide isomerase (Walker et al., 2010), which facilitate protein maturation and transport of unfolded secretory proteins. This adaptation helps the neuron to survive the injury, and the capacity for secreting locally synthesized proteins in axons seems to increase upon axonal injury (Zheng et al., 2001; Merianda et al., 2009). When PDI is S-nitrosylated, its catalytic chaperone and thiol-disulfide oxidoreductase function is compromised, leading to accumulation of misfolded proteins and induction of autophagy and cell death (Figs. 3, 5, and 6). Hence, excessive NO levels may interfere with an adaptive response to protect neurons (Uehara et al., 2006).

Secreted PDI or PDI exposed at the cell surface maintains the reduced state of extracellular proteins. In particular, cell surface PDI may enable the transfer of NO from extracellular S-nitroso proteins to intracellular thiols to transfer NO bioactivity from the extracellular environment into the cytosol (Shah et al., 2007). In addition, cell surface PDI maintains the sheddase ADAM17/TACE (tumor necrosis factor  $\alpha$ -converting enzyme) in an inactive closed state. Upon redox modulation of the environment, and consequent PDI inactivation, ADAM17 adopts an active conformation which is accompanied by changes in disulfide bonds in the ADAM17 ectodomain (Willems et al., 2010). Consequently, activated ADAM17 processes pro-tumor necrosis factor  $\alpha$  and cleaves diverse cell-surface receptors and adhesion molecules, including the p75 neurotrophin receptor (Weskamp et al., 2004), which is involved in the development of nociceptive hypersensitivity (Obata et al., 2006).

An additional theme that has emerged from recent studies shows that effects of S-nitrosylation may involve the control of protein stability via modulation of ubiquitination and proteasomal degradation. In this context, S-nitrosylation has been found to regulate the activity of hypoxia-inducible factor (Li et al., 2007), tumor suppressor p53, iron response proteins, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and I $\kappa$ B/NF- $\kappa$ B by regulating the degradation of the S-nitrosylated protein, a regulatory partner, or the ubiquitin E3 ligase (Fig. 6). Thus, although the mechanism and localization of S-nitrosylation may differ and depend on the close association of NOS and the respective targeted protein, S-nitrosylation ultimately affects proteasomal targeting. Ubiquitination and proteasomal targeting of p53 is mediated by its interaction with human homolog of mouse double-minute-2 (HDM2), which is disrupted by S-nitrosylation of a single critical cysteine within HDM2, resulting in increased p53 stability (Schonhoff et al., 2002). The NO-dependent transcriptional activity of hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) is mediated, in part, by S-nitrosylation of a single cysteine residue within HIF-1 $\alpha$ , which facilitates binding of the transactivator cAMP response element-binding protein (p300/CBP) (Cho et al., 2007). In addition, an NO-dependent

modification prevented binding of hydroxylated HIF-1 $\alpha$  to the E3 ubiquitin ligase, von Hippel Lindau protein, that ligates ubiquitin to HIF-1 $\alpha$ , thereby priming it to degradation (Park et al., 2008). As a result, NO also prevents the ubiquitination-dependent routing of HIF-1 $\alpha$  to the proteasome. Although HIF-1 $\alpha$  has been widely studied in the context of brain ischemia, its functions in nociception or axonal injury-evoked adaptations are still unknown.

*1. Glyceraldehyde-3-phosphate Dehydrogenase.* GAPDH is generally regarded as a housekeeping glycolytic enzyme. However, several studies implicate GAPDH in cell signaling, and proteomic analyses revealed its up-regulation in DRG neurons after sciatic nerve injury (Zhang et al., 2008). Nitric oxide S-nitrosylates GAPDH and thereby abolishes the catalytic activity of the enzyme, conferring upon it the ability to bind to Siah, an E3 ubiquitin ligase (Figs. 5 and 6). The nuclear localization signal of Siah elicits the nuclear translocation of the GAPDH-Siah complex. In the nucleus, GAPDH binds to and activates the transcriptional coactivator p300/CBP, which, because of its histone acetyltransferase activity, can acetylate various targets including p53. This ultimately results in an augmentation of cytotoxicity (Hara et al., 2005). The outcome is further modified by a protein interactor of GAPDH, designated GOSPEL (GAPDH's competitor of Siah protein enhances life). Cell stress elicits S-nitrosylation of GOSPEL, enabling it to bind GAPDH in competition with Siah. The binding of GOSPEL to GAPDH prevents nuclear translocation of GAPDH and prevents neurotoxicity. In naive mice, GOSPEL overexpression in the cerebral cortex reduced NMDA neurotoxicity, whereas mutant GOSPEL that cannot bind GAPDH provided no protection (Sen et al., 2009).

Except for the giant E3 ligase known as "protein associated with myc" (Ehnert et al., 2004), the functions of ubiquitin ligases in the context of nociception are largely unexplored, although proteasomal degradation and de novo protein synthesis in the dendrite and synapse are requirements for the plasticity of synapses (DiAntonio and Hicke, 2004; Bingol and Schuman, 2005; Sutton and Schuman, 2006) and may be particularly important in the case of axonal injury or ongoing nociceptive stimulation (Moss et al., 2002) to allow for temporary or persistent presynaptic silencing (Jiang et al., 2010).

### *I. Denitrosylation by Redoxins*

Nitric-oxide-mediated protein S-nitrosylation seems to be a mechanism by which desensitization and resensitization of nociceptive neurons is fine-tuned and subtly modulated. It is not surprising, therefore, that nitric oxide also controls the velocity of denitrosylation by S-nitrosylation of redoxins (Fig. 6), exemplified by thioredoxin-1, which, together with thioredoxin reductase, composes one of the major denitrosylation systems (Benhar et al., 2008, 2009). Nitrosylation occurs very rapidly, and denitrosylation,

whether spontaneous or via denitrosylating enzymes, can be comparably rapid. In most instances, the time course of these processes might not be rate limiting for the overall events that make up nociceptive activation (i.e., stimulation of nNOS to generate NO, nitrosylation of target proteins, and subsequent conformational changes of ion channels), membrane fusion or fission, translocation, and transcriptional processes. In some cases, however, both basal and stimulated denitrosylation may affect the continuance of nitrosylation-dependent cascades at the synapse (Benhar et al., 2008, 2009), and denitrosylation may constitute an important regulatory mechanism for constitutively nitrosylated proteins that are turned on or off primarily by denitrosylation.

Two cellular enzyme systems in particular have emerged as physiologically relevant denitrosylases: the thioredoxin system and the (S)-nitrosoglutathione reductase system. The latter constitutes a system together with GSH that governs the reversible S-nitrosylation of multiple SNO proteins, including G-protein receptor kinase,  $\beta$ -arrestin, and NF- $\kappa$ B (Benhar et al., 2009). In vitro experiments with spinal cord slices revealed that SNO proteins are primarily denitrosylated via rapid transnitrosylation with GSH, suggesting that this system may be particularly important for SNO-mediated signaling in the nociceptive system (Romero and Bizozero, 2009).

Thioredoxin-1 and -2 are small oxidoreductase enzymes containing a dithiol-disulfide active site. They are kept in the reduced state by the flavoenzyme thioredoxin reductase in an NADPH-dependent reaction. Thioredoxins exert antiapoptotic effects by binding and inactivating apoptosis signaling kinase-1 (Fujino et al., 2007; Matsuzawa and Ichijo, 2008) and caspase 3 (Mitchell and Marletta, 2005) and by maintaining the cellular redox equilibrium (Benhar et al., 2008). In addition, thioredoxins can denitrosylate and thereby activate auto-S-nitrosylated nitric-oxide synthases, thus helping to maintain the cellular SNO content. Nitric-oxide synthases, in turn, maintain the activity of thioredoxin by S-nitrosylation of Cys69 and Cys73 (Sumbayev, 2003) (Fig. 6; Table 1). Upon oxidative stress, thioredoxin may undergo S-nitrosylation of further active-site vicinal cysteines of the CXXC motif (Cys32 or Cys35 in human Trx1), which may affect its capability to denitrosylate target proteins such as ASK1 (Fujino et al., 2007; Matsuzawa and Ichijo, 2008) and caspases. Stimulus-coupled denitrosylation may precisely modulate the function of proteins that are constitutively S-nitrosylated (Fig. 6). In particular, a subpopulation of caspase-3 that is associated with mitochondria is constitutively S-nitrosylated at the active-site cysteines (Mannick et al., 2001), and stimulation of death receptors of the tumor necrosis factor superfamily promotes denitrosylation by thioredoxin (Benhar et al., 2008) and disinhibition of these caspases (Mannick et al., 1999). Inhibition of caspase 3 can prevent the apoptosis of lamina II neurons

in the spinal cord after sciatic nerve injury and is associated with attenuation of nerve injury-evoked neuropathic pain (Scholz et al., 2005) suggesting that the S-nitrosylation status of caspases in dorsal horn neurons modulates mitochondrial functions and, eventually, nociceptive hypersensitivity after axonal injury.

Peroxiredoxins reduce intracellular peroxides with help of the thioredoxin system as the electron donor. Under conditions of oxidative stress, peroxiredoxins may become overoxidized, causing inactivation of peroxidase activity. This is reversible because of the catalytic activity of two ATP-dependent reductases, sulfiredoxin and sestrin (Jeong et al., 2006; Soriano et al., 2008; Noh et al., 2009). In stimulated macrophages, endogenous NO prevented the overoxidation-dependent inhibition of 2-Cys peroxiredoxins as a result of NO-mediated up-regulation of sulfiredoxin and accelerated recovery of 2-Cys Prxs (Diet et al., 2007; Essler et al., 2009). In neurons, however, nitric oxide might also promote overoxidation of peroxiredoxin-2 by S-nitrosylation of the two critical cysteines Cys51 and Cys172 (Fang et al., 2007) (Table 1). It is unknown whether neurons exhibit the capacity to reduce overoxidized Prxs, and the specific role and control of this system in nociceptive circuits is unknown.

#### IV. Concluding Remarks

Nitric oxide-mediated dynamic S-nitrosylation and denitrosylation of multiple inter-related elements that may serve the propagation of nociceptive signals is highly reminiscent of the regulation by phosphorylation and dephosphorylation and may be equally important to shape the nociceptive response and adaptation to long-lasting insults such as axonal injury. Three major schemes have emerged that may be modulated by S-nitrosylation of target proteins: 1) ion channel gating, 2) membrane fusion and fission and hence compartmentalization, and 3) proteasomal or protease-mediated protein degradation. Direct targets and outcome may differ as the case arises and although endogenous nitric oxide is generally thought of a pronociceptive molecule, there is also growing appreciation of its neuroprotective effects in normal synaptic activity and physiological compartmentalized protein functions at pre- and postsynaptic neurons. S-Nitrosylation modifies the catalytic activity of some kinases and phosphatases, raising the intriguing possibility that S-nitrosylation modulates the likelihood of phosphorylation or vice versa. Hence, one may think of NO as a subtle, multifaceted modulator of nociception with dual effects in the context of chronic pain. The growing understanding of the neuroprotective effects of physiological synaptic NMDA receptor activity and NO production, coupled with the established role of NO in synaptic plasticity, mediated in part by direct S-nitrosylation, suggests that global NOS inhibition may not be appropriate as an antinociceptive or antiexcitotoxic therapeutic strategy. Excessive NMDA receptor activity,

however, may trigger calcium overload, oxidative stress, and overproduction of nitric oxide that, in combination with a shift of the redox equilibrium, may cause neuronal damage. It may be therapeutically advantageous to modulate the redox environment to restore protective effects to NO rather than to block NOS activity completely.

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#### Authorship Contributions

Performed data analysis: Tegeder, Scheving, and Wittig

Wrote or contributed to the writing of the manuscript: Tegeder and Geisslinger.

Other: Tegeder designed the graphics and illustrations.

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